Bazooka and atypical Protein Kinase C Regulate Dynamic Actomyosin Networks During Drosophila Amnioserosa Apical Constriction

by

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University of Toronto
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Abstract

Cell shape changes drive tissue remodeling. Polarized activity of actin and myosin drive apical constriction, as seen for example in Drosophila embryonic amnioserosa cells during dorsal closure (DC). My first aim was to characterize interactions between apical actomyosin networks and the cell polarity regulators Bazooka (Baz, the Drosophila Par-3), Par-6, and atypical Protein Kinase C (aPKC) (the PAR complex) during Drosophila DC. I found that both actomyosin networks and the PAR complex are enriched at the apical surfaces of amnioserosa cells and that actomyosin contractility is driven by cyclical assembly and disassembly of actomyosin networks. The pulsatile actomyosin networks translocate across persistent apical surface PAR complex puncta. To assess whether the PAR complex interacts with actomyosin, I characterized myosin dynamics with PAR loss- and gain-of-function perturbations. Baz enhances, whereas Par6/aPKC inhibits actomyosin. These studies suggest that PAR proteins regulate pulsatile apical
actomyosin networks mediating constriction of amnioserosa cells. My next aim was to characterize actomyosin networks during a shift in their dynamics. Amnioserosa constriction transitions from pulsatile to persistent from early to late DC. Since oscillatory networks result from delayed negative feedback, I examined whether such regulation exists in these actomyosin networks. The actomyosin inhibitor aPKC is recruited to the apical surface by actomyosin and, in turn, aPKC recruits Baz. To examine the significance of Baz – aPKC dynamic interactions, I ectopically stabilized their interactions. This served to inhibit the antagonism of actomyosin networks by aPKC, suggesting that Baz can act as a competitive inhibitor of aPKC. I found that interactions between Par-6 and Baz increase during DC, suggesting an increase in aPKC inhibition during DC progression. To examine whether decreased inhibition can tune oscillatory actomyosin networks, we collaborated with Qiming Wang and Dr. James Feng (University of British Columbia) to test this oscillation in silico. Computer modeling suggests that decreasing delayed negative feedback can transition actomyosin oscillations towards stabilized constriction. Together, these results demonstrate the requirement of Baz – aPKC interactions for their localization and for dynamic regulation of aPKC by a competitive inhibitor. Finally, my research reveals a regulatory circuit to tune actomyosin behaviour during development.
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I dedicate this to my many wonderful nieces and nephew. I have tried my best to be a great role model for you, and I hope that I have inspired you as much as you have all inspired me to persevere and be the best that I can be. I am very proud of all you and I cannot wait to see you all grow up.
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List of Abbreviations

14-3-3 – Protein eluted in the 14th fraction of bovine brain homogenate, position 3.3 of subsequent electrophoresis

ACK – Activated Cdc42-associated kinase

Actomyosin – actin and myosin

ADF – actin depolymerizing factor

AJ – adherens junction

aPKC – atypical Protein Kinase C (PKC-3 in C. elegans)

Arm – Armadillo, the Drosophila β-catenin

attP – phage attachment site

Baz – Bazooka, the Drosophila Par-3

CA-MLCK – constitutively-active myosin light chain kinase

CAAX – cysteine, aliphatic amino acid, aliphatic amino acid, any amino acid

Cdc42 – Cell division cycle 42

CR3 – Conserved region 3

CRIB – Cdc42/Rac interactive binding

CytoD – Cytochalasin-D

D-V – dorsoventral

Da-GAL4 – daughterless>GAL4

DC – dorsal closure
DE-cad – *Drosophila* epithelial cadherin

Dpp – decapentaplegic, the *Drosophila* TGFβ

Dlg – Discs large

DMSO – dimethyl sulfoxide

DSHB – Developmental Studies Hybridoma Bank

Ed – Echinoid

EGFR – Epidermal growth factor receptor

Ena – Enabled

F-actin – filamentous actin

FDB – FERM-domain binding region

FERM – Band 4.1, ezrin, radixin, moesin

FRAP – fluorescence recovery after photobleaching

GAP – GTPase activating protein

GEF – guanine nucleotide exchange factor

GFP – green fluorescent protein

GMC – ganglion mother cell

GTP – guanosine triphosphate

GTPase – guanosine triphosphatase

JAM – Junctional adhesion molecule

JNK – c-Jun N-terminal Kinase
LE – leading edge

Lgl – Lethal giant larvae

LIM domain – Lin-1, Isl-1, and Mec-3 domain

mCh – monomeric Cherry fluorescent protein

MDCK – Madin-Darby canine kidney

MLCK – myosin light chain kinase

MoeABD – Moesin actin binding domain

NA – numerical aperture

OD – oligomerization domain

PAK – p21-activated kinase

PALS-1 – Protein associated with Lin-7 protein 1 (known in Drosophila as Stardust, Sdt)

PAR – partition-defective

PAR-1 – Partition-defective protein 1

PAR-2 – Partition-defective protein 2

PAR-3 – Partition-defective protein 3

PAR-6 – Partition-defective protein 6

PATJ – Pals1-associated tight junction protein

PB1 – Phox and Bem 1

PDZ – Postsynaptic density-95, Discs-large, Zonula-occludens-1

PKC-3 – Protein Kinase C-3, the C. elegans homolog of Drosophila aPKC
PTEN – Phosphatase and tensin homolog

Rac1 – Ras-related C3 botulinum toxin substrate 1

Rho1 – Ras homolog protein 1

ROCK – Rho-associated, coiled-coil containing kinase

Sdt – Stardust, the *Drosophila* homolog of PALS-1

SIF – Still life, the *Drosophila* homolog of Tiam1

SJ – septate junction

Sqh – Spaghetti-squash, the *Drosophila* non-muscle myosin regulatory light chain

TGFβ – Transforming growth factor β

Tiam1 – T-cell lymphoma invasion and metastasis-inducing protein 1

TJ – tight junction

UAS – Upstream activating sequence

WASP - Wiskott–Aldrich syndrome protein

WT – wild type

ZA – Zonula adherens

Zip – Zipper, the *Drosophila* non-muscle myosin heavy chain
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Chapter 1: Introduction

1.1 Epithelia are essential for physiology and development

Epithelia are the foundation of many tissues and are composed of adherent, polarized cells. Epithelial sheets form boundaries between distinct bodily components, demarcate organs, and separate the body from the external environment. For example, epithelial skin cells provide a robust and resilient boundary between our bodies and the outside world. Epithelia are also polarized, with distinct apical and basolateral surfaces that allow for directional diffusion and active transport of ions, nutrients, and signaling molecules. For instance, intestinal epithelial cells directionally transport nutrients from the apical lumen into the bloodstream. While epithelia confer the aforementioned benefits to adult organisms, developing embryos also use epithelia as the foundation from which to construct and reshape their tissues. Since epithelia are essential for both fully-developed and developing organisms, examining epithelial formation and the remarkable array of morphogenetic changes during development is critical for understanding fundamental biology and how this is misregulated during disease.

Epithelial cells are adherent and have distinct apical and basolateral surfaces. Cell-cell adhesion is facilitated by an adhesion belt known as the zonula adherens (ZA). Ultrastructural studies have revealed the ZA as electron-dense plaques of tight association between adjacent cell membranes and are closely linked to the underlying cellular cytoskeleton (reviewed by Harris and Tepass, 2010). The ZA also demarcates the boundary between apical and basolateral cell membranes (reviewed by Baum and Georgiou, 2011; Tepass, 2012). Tight junctions (TJs) in vertebrates and the analogous septate junctions (SJs) in *Drosophila melanogaster* block transepithelial diffusion and thus play a critical role in epithelial barrier function (Fig. 1.1A) (reviewed by Baum and Georgiou, 2011; Tepass and Tanentzapf, 2001). Apicobasal polarity is important for proper positioning of the ZA (Tepass, 2012). In addition, polarized surfaces of cells in a sheet result in distinct faces of epithelia that carry out fundamental physiological roles. For example, transport of glucose from the intestinal lumen into the bloodstream is
Figure 1.1: General structure of epithelia

(A) General structure of vertebrate and invertebrate epithelia. Epithelial cells are polarized into distinct apical and basolateral surfaces and adhere to one another with adherens junctions (AJs). Tight junctions (TJs) in vertebrates or analogous septate junctions (SJs) in invertebrates form the epithelial barrier.

(B) Adherens junctions mediate cell-cell contacts and link to the underlying cytoskeleton.

(C) Apical constriction can mediate initial steps of morphogenesis.
mediated by sodium-glucose transmembrane symporters on the apical surface of intestinal epithelia, and glucose transporters along the basal surface (reviewed by Kellett et al., 2008; Thorens, 1993; Wright, 1993).

Extensive research has elucidated the molecular players involved in epithelial cell adhesion and polarity. Cell-cell contacts at the ZA are mediated by adherens junctions (AJs) composed primarily of transmembrane E-cadherin that forms homophilic interactions in cis and in trans. The cytoplasmic tail of cadherin interacts with β-catenin (Drosophila Armadillo, Arm), which in turn interacts with α-catenin. This so-called cadherin-catenin complex binds to the cortical actin cytoskeleton and is thus a likely link between cell-cell adhesion and the underlying cytoskeleton (Fig. 1.1B) (Desai et al., 2013; reviewed by Harris and Tepass, 2010; Harris, 2012; St Johnston and Sanson, 2011). The microtubule cytoskeleton also plays an important role in trafficking cadherin-containing vesicles and serving as a polarity landmark (reviewed by Harris and Tepass, 2010; Harris, 2012; Laprise and Tepass, 2011). Thus, the AJs are connected to the underlying cytoskeleton. Polarity is another key aspect of epithelial cells and will be discussed in detail below. Briefly, polarity is regulated by the evolutionarily-conserved PAR complex composed of the scaffolding protein Par-3 (Drosophila Bazooka, Baz), atypical Protein Kinase C (aPKC), and its binding partner Par-6. The apical surface is also characterized by the transmembrane protein Crumbs (Crb), which in turn recruits the scaffolding protein PALS-1 (Drosophila Stardust, Sdt), PATJ (Pals-1-associated tight junction protein), and Par-6. These apical regulators are mutually antagonistic with basolateral proteins including the Scribble (Scrib) complex, the Yurt/Cora complex, and the kinase Par-1 (reviewed by St Johnston and Sanson, 2011; Tepass, 2012).

Epithelia are not static structures. As organisms grow and develop, cells are lost and gained, cells exchange neighbours, and tissues change size and shape. Experiments in different model systems have revealed coordination of cell shape changes and movements during tissue morphogenesis. Formation of multiple epithelial layers can be driven by delamination or extrusion of cells from the epithelial sheet or polarized cell division orthogonal to the epithelial plane. Tissue elongation can be achieved by cell intercalation and elongation. Further, tissue invagination or tube formation can be accomplished by
coordinated apical constriction of a subset of cells (reviewed by Baum and Georgiou, 2011; Kasza and Zallen, 2011). Despite this wide variety of dramatic morphogenetic movements, tissue integrity is maintained. Therefore, changes to cell shape and cell-cell contacts must be coupled with regulation of adhesion and polarity.

My research has focused on tissue morphogenesis driven by apical constriction. To mediate tissue invagination, epithelial cells undergo constriction along their apical surfaces, changing from columnar to conical in shape (Fig. 1.1C). Apical constriction has been modeled in tissue culture with Madin-Darby canine kidney (MDCK) cells. *In vivo*, apical constriction drives vertebrate neural tube formation in mouse, *Xenopus laevis*, and chick embryos (reviewed by Davidson, 2012; Martin, 2004; Suzuki et al., 2012). Using model organisms more amenable to live imaging has yielded information about the dynamic nature of apical constriction during development. Apical constriction is fundamental during *Caenorhabditis elegans* gastrulation and during multiple stages of *Drosophila* embryonic morphogenesis, including ventral furrow formation (Dawes-Hoang et al., 2005), dorsal fold formation (Wang, Y.C. et al., 2012), and dorsal closure (reviewed by Davidson, 2012; Sawyer et al., 2010). Cell shape changes must be coupled to polarity to direct changes only within a specific cellular region, and coupled to cell adhesion to allow for both deformation of the cell cortex and maintenance of tissue integrity. Finally, cell shape change must integrate forces generated on molecular, cellular, and tissue-wide scales. This raises the question of how these constriction events are regulated and coordinated with mechanical forces, protein regulators, and cell polarity.

1.2 Actomyosin structures are seen in diverse configurations

Cell contractility is mainly mediated by the coordinated action of actin and myosin (together, actomyosin). Non-muscle myosin II (hereafter myosin) is an actin-based motor protein that is directed towards the “plus” (barbed) ends of actin (Fig. 1.2). Myosin oligomerizes into bipolar filaments and can exert contractile forces by pulling actin networks (Fig. 1.2B). When sufficiently cross-linked and linked to the cell cortex, actomyosin contraction can lead to localized cellular deformation. Actomyosin-based
Figure 1.2: Actomyosin networks can have diverse configurations

(A) Actin filaments preferentially elongate at their “plus” (+) or “barbed” ends while preferentially disassembling at their “minus” (-) or “pointed” ends.

(B) Non-muscle myosin II assembles first into a double-headed complex, then assembles into bipolar filaments.

(C) The ordered, paracrystalline arrangement of actin and muscle myosin in myofibres allowed for easy observation of actin and myosin interactions that mediate contraction. In the “sliding filament” model, F-actin arrays are pulled by arrays of myosin filaments that translocate towards actin (+) ends.

(D) Apical constriction was first thought to proceed via sarcomeric-like constriction of a circumferential actomyosin belt associated with adherens junctions. (Box) The dynamics of this circumferential belt, as well as its method of assembly and disassembly required for progression and shrinking of the actomyosin belt are currently unknown (?).

(E) Apical constriction of certain cells proceeds via pulsatile contraction of apically-localized actomyosin networks. (Box) The actomyosin networks are thought to constrict with repeated rounds of assembly, contraction, disassembly, and reassembly.
contraction was first modeled in sarcomeres of skeletal muscle cells, wherein the paracrystalline arrangement of parallel and antiparallel actin and myosin into respective “thin” and “thick” filaments arrays allowed for easy observation of actomyosin networks (Fig. 1.2C) (reviewed by Huxley, 1969; Koubassova and Tsaturyan, 2011; Ono, 2010). However, as discussed in this section, actomyosin networks in non-muscle cells are found in a diverse arrangement of network architectures and dynamics, and the molecular mechanisms governing this diversity remain unclear.

Actomyosin networks are often dynamic structures that assemble and disassemble, and whose properties differ depending on the network architecture. At the molecular scale, F-actin networks display polarized dynamics. Actin filaments preferentially assemble and disassemble at their “plus” (barbed) and “minus” (pointed) ends respectively, leading to treadmilling of filaments (Fig. 1.2A). Tuning both the balance of assembly and disassembly and myosin contractility (Fig. 1.2B) takes place in a variety of contexts on a cellular and tissue-wide scale.

Actomyosin networks acquire different configurations in multiple contexts. A classic model of actomyosin networks is the contractile ring during cell division (reviewed by Fededa and Gerlich, 2012; Lee et al., 2012; Pollard, 2010). Here, a mitotic cell assembles an actomyosin-based contractile ring at the equator of the dividing cell. This zone of active actomyosin then assembles into a relatively well-defined tight zone at the equator. Contraction of this equatorial actomyosin structure leads to cleavage between the cytoplasm of the two nascent cells. Although constriction of the cytokinetic contractile ring was originally thought to be mediated by sarcomeric-like arrangements of contractile actomyosin, recent work suggests that dynamic assembly and disassembly and crosslinking may be important. During cytokinesis, the contractile ring can arise from both de novo assembly and equatorial cortical flows of pre-existing filaments (reviewed by Fededa and Gerlich, 2012; Lee et al., 2012; Pollard, 2010). Mathematical modeling suggests that, in yeast, contractile rings can assemble from rounds of actin polymerization, cross-linking by myosin to pull actin foci together, then a release to repeat the process (a “search-capture-pull-release” mechanism (Coffman et al., 2009; reviewed by Green et al., 2012; Lee et al., 2012; Pollard and Wu, 2010). Rather than
persistent contraction of the actomyosin belt as expected from a sarcomeric-like structure, depolymerization of F-actin is thought to drive contraction of the actomyosin ring during cytokinesis in *C. elegans* (Carvalho et al., 2009) and yeast (Mendes Pinto et al., 2012). Even the role traditionally ascribed to myosin during cytokinesis as an actin-based motor protein has recently been questioned. Though myosin is thought to translocate over actin filaments to mediate contractility, recent work suggests that its role as a motor protein may not be as important as its role as an actin cross-linker in cytokinesis of animal cells (Ma et al., 2012) and yeast (Mendes Pinto et al., 2012). Thus, the dynamics of the actomyosin network and the role of myosin contractility during cytokinesis remain to be precisely defined.

Another well-studied model of actomyosin structures are those found in migratory cells. The leading edge of cultured migratory cells is characterized by membranous protrusions at the lamellipodia, filled with dendritic F-actin networks (reviewed by Pollard and Borisy, 2003; Rottner and Stradal, 2011). Immediately behind the lamellipodia is the lamella, characterized by bundles of F-actin roughly parallel to the cell edge, enriched with myosin and associated with focal adhesions (Burnette et al., 2011; Koestler et al., 2008; Ponti et al., 2004; Rouiller et al., 2008). F-actin protrusions in the lamellipodia are thought to drive leading edge advancement, whereas focal adhesion-associated actomyosin bundles in the lamella are thought to stabilize attachment to the substratum. The actin network of lamellipodia is a highly dynamic structure with constant assembly and disassembly at the leading edge (Ponti et al., 2004) resulting in repeated protrusion and retraction of the cell membrane. Recently, it was shown that lamellipodial protrusions can be coupled with myosin-based contraction during the retraction phase (Burnette et al., 2011). The dendritic actin networks become cross-linked and pulled by myosin. The actin networks then evolve into actin arcs and then into actin bundles of the lamella, showing that lamellipodial networks transition into lamellar networks in a myosin-dependent manner. Thus, the highly dynamic and meshwork-like actin structures of the lamellipodia can be modified by myosin contractility into a highly bundled actin network.
Actomyosin structures are also seen as as stress fibres; long arrays of contractile actomyosin bundles spanning a large proportion of a cell. Since stress fibres bind at one or both ends to focal adhesions, they connect the actin cytoskeleton to the extracellular substratum in a tension-dependent manner (reviewed by Burridge and Wittchen, 2013; Tojkander et al., 2012). Periodic and alternating distribution of myosin and actin cross-linkers across stress fibres is reminiscent of sarcomeres in striated muscle cells. Primarily seen in cells cultured in 2D systems, stress fibres have been considered artifacts of cell culture (reviewed by Burridge and Wittchen, 2013; Tojkander et al., 2012). However, arrays of parallel actomyosin bundles resembling stress fibres are seen in endothelial cells lining arteries (Wong et al., 1983; Millan et al., 2010), though these actomyosin bundles are connected to adherens junctions. Parallel bundles of actin connected to an underlying substratum form on basal surfaces of Drosophila follicular epithelial cells (He et al., 2010). Both endothelial tissues and Drosophila follicular epithelial cells are subject to mechanical stress, suggesting stress-fibre-like networks can arise in tissues exposed to constant mechanical stress. Much like sarcomeres of muscle cells, the large size of stress fibres in cultured cells has made it an ideal system for studying actomyosin arrays. In nonmigratory cells, bundling of pre-existing actin filaments by myosin may form stress fibres (Chrzanowska-Wodnicka and Burridge, 1996; reviewed by Burridge and Wittchen, 2013). In contrast, in migratory cells, de novo actin polymerization, cross-linking, and bundling by actin cross-linkers and myosin may be required for formation of a class of stress fibres (Hotulainen and Lappaleinen, 2006; reviewed by Burridge and Wittchen, 2013; Tojkander et al., 2012). Intriguingly, to generate contractile force, stress fibres must consist of alternating antiparallel F-actin bundles (Hotulainen and Lappaleinen, 2006; reviewed by Burridge and Wittchen, 2013; Tojkander et al., 2012). Antiparallel bundles in stress fibres may form as a result of severing, capping, and de novo polymerization of F-actin, resulting in the creation of repeating sarcomeric-like arrays along the length of the stress fibre (reviewed by Burridge and Wittchen, 2013) though the molecular mechanisms are unknown. Furthermore, stress-fibre-like actin bundles along basal surfaces of Drosophila follicular epithelial cells are relatively stable, in contrast to highly pulsatile myosin that assembles and disassembles over the actin bundles (He et al., 2010). Thus, the precise molecular
architecture and dynamics of stress fibres both in cultured cells and in vivo still remain to be fully elucidated.

Contractile actomyosin bundles are seen in vivo on a tissue-wide scale. For example, a circumferential “belt” of contractile actomyosin is closely associated with adherens junctions of epithelial cells (Fig. 1.2D). Both actin and myosin are thought to somehow cluster the AJs together, as seen both in cell culture (Shewan et al., 2005; Suzuki et al., 2001) and in vivo (Cavey et al., 2008; Ebrahim et al., 2013; reviewed by Harris, 2012). At cell adhesions, the actomyosin directly associates with the AJ complex via the catenins. Myosin contractility is important for AJ clustering, and recent work has shown actomyosin can be arranged into sarcomeric-like structures in mammalian epithelial cells (Ebrahim et al., 2013). However, it is unknown if sarcomeric-like actomyosin belts maintain their arrangement during dynamic tissue remodelling and during events like apical constriction (Fig. 1.2D).

Parallel bundles of actomyosin are also present during wound repair as a “supra-cellular purse-string” of actomyosin. After wounding of tissue, parallel bands of actomyosin form at the leading edges of cells at the wound boundary. The actomyosin networks attached to AJs form a purse-string connected between cells of the leading edge. Constriction of this purse-string aids in wound closure (reviewed by Abreu-Blanco et al., 2012b; Sonnemann and Bement, 2011). Here, it is thought that bundled arrays of actin are what mediate the constriction, though recent evidence suggests actin-based protrusions can also play a role (Abreu-Blanco et al., 2012a).

Actomyosin networks are also configured as web-like networks. For example, the C. elegans one-cell embryo undergoes a partitioning of the cell cortex into anterior and posterior halves; a process requiring cortical flow of actomyosin networks (Munro et al., 2004). Prior to fertilization, cortical actomyosin networks appear as dynamic, interconnected foci distributed uniformly over the C. elegans oocyte. Upon fertilization, sperm delivers an unknown signal, probably a RhoGAP (Jenkins et al., 2006), to locally inhibit myosin. The ensuing polarized contraction of actomyosin interconnected networks then flows towards pole of the embryo opposite the site of sperm entry, helping
to define the anterior end (Munro et al., 2004). The area depleted of actomyosin contractility then becomes the posterior half of the embryo. The actomyosin flows appear to carry along other proteins, notably the anterior PAR proteins (Munro et al., 2004), possibly through advection (the bulk flow of material, in this case as being swept along by cortical actomyosin flows) (Goehring et al., 2011). The first mitotic division occurs along the established anterior-posterior axis, and is asymmetric to define the first two cell lineages of the developing embryo. At later stages of C. elegans embryogenesis, contractile web-like actin networks are also found over the apical (apicomedial) cell surface. At the eight-cell stage, actomyosin foci appear at the apical surface of endodermal precursor cells just prior to the onset of apical constriction (Nance and Priess, 2002; Roh-Johnson et al., 2012). Eventually the actomyosin networks engage the adherens junctions to apically constrict (Roh-Johnson et al., 2012). Together, these results suggest that web-like actomyosin networks can drive both polarization and apical constriction.

Actomyosin networks can also assemble and disassemble as cortical meshworks that flow. Anterior-ward cortical flow of the C. elegans one-cell embryo is dependent on contractile myosin and localized inactivation and disassembly of actomyosin at the posterior pole and is thus dependent on localized activation and inactivation of actomyosin contractility. The actomyosin foci are also dynamic. The foci have limited but regular lifetimes, suggesting periodic assembly and disassembly (Munro et al., 2004). During later stages of C. elegans embryogenesis at gastrulation, the apically enriched actomyosin foci along the apical surfaces of endodermal precursor cells assemble and disassemble in a regular and periodic fashion (Roh-Johnson et al., 2012). The foci flow and coalesce towards the centre of the apical surface, suggesting network contraction, disassembly, and reassembly in a cyclical manner. At later stages, the actomyosin network engages the adherens junctions to mediate productive apical constriction. Thus, the web-like actomyosin networks during early stages of C. elegans development are dynamic and can impact apical constriction.

Apical constriction driven by apically-enriched medial actomyosin meshworks also occurs during Drosophila ventral furrow formation. During early Drosophila

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embryogenesis, in the process known as ventral furrow formation, a thin band of ventral-most epithelia of the embryo undergoes apical constriction. Apical constriction of these cells leads to the initial bending of the epithelium to form what is known as the ventral furrow. After ventral furrow formation, these cells then ingress to eventually become the mesoderm. Myosin is generally apically enriched just prior to, and during, apical constriction of the presumptive ventral furrow cells (Dawes-Hoang et al., 2005). It was thought that the apical actomyosin associated with an actomyosin circumferential belt connected to the ZA (Fig. 1.2D). However, recent live imaging of the *Drosophila* embryo has revealed that apical constriction of the presumptive ventral furrow cells is a dynamic process. High-speed live imaging revealed that actomyosin mesh-like networks span the apicomedial surface of presumptive ventral furrow cells (Fig. 1.2E) (Martin et al., 2009). These apical actomyosin networks undergo periodic and pulsatile assembly and disassembly (Martin et al., 2009). The actomyosin pulses coincide with periodic constriction and relaxation of the cell cortex. Importantly, each constriction and relaxation cycle results in the cell relaxing into progressively smaller states, eventually “ratcheting” towards constriction. The apical meshwork configuration of actomyosin requires association with adherens junctions (Dawes-Hoang et al., 2005; Martin et al., 2010) though the exact mechanisms mediating this connection remain unclear.

Actomyosin networks are therefore highly dynamic structures that assemble and disassemble depending on the context. It is interesting to consider whether different actomyosin configurations have distinct assembly and disassembly mechanisms. To address this question, actomyosin regulation must be considered.

### 1.3 Chemical and mechanical regulation of actomyosin networks

The variety of network configurations and the dynamic nature of actomyosin networks suggest a high degree of regulation. Actomyosin networks are subject to control by protein regulators that affect assembly and contractility. Furthermore, as contractile, relatively macroscopic assemblies, actomyosin networks are subject to regulation by
mechanical forces. In this section, I describe some examples of chemical and mechanical regulators of actomyosin.

Actomyosin network assembly is regulated by chemical modifiers (Fig. 1.3A). Actin alone polymerizes very slowly, with nucleation being the rate-limiting step. F-actin assembly can thus be catalyzed via nucleation by protein regulators such as Arp2/3, spire-like proteins, and formins. Actin polymerization is further enhanced by profilin, formins, and Ena (reviewed by Campellone and Welch, 2010). Myosin can also be activated by chemical modifiers (Fig. 1.3B). The most common form of myosin activation is by phosphorylation of its regulatory light chain by Rho-activated Kinase (ROCK) and Myosin Light Chain Kinase (MLCK) (reviewed by Lecuit et al., 2011).

The Rho-family GTPases, RhoA, Rac1, and Cdc42, are also well-known regulators of both actin and myosin networks. In cell culture, overexpression of constitutively-active RhoA, Rac1, and Cdc42 can respectively form ectopic stress fibres, lamellipodia, and filopodia (Ridley and Hall, 1992; Ridley et al., 1992; Kozma et al., 1995; Nobes and Hall, 1995). When activated (GTP-bound), a lipid motif of Rho GTPases is exposed, thus localizing active Rho GTPases to the cell cortex (Bokoch et al., 1994; reviewed by Cherfils and Zeghouf, 2013). RhoA-GTP can stimulate formins to catalyze actin polymerization. Furthermore, ROCK enhances myosin contractility through direct phosphorylation of the myosin regulatory light chain and by inhibiting myosin phosphatase (reviewed by Campellone and Welch, 2010; Lecuit et al., 2011; Rottner and Stradal, 2011). ROCK also enhances actin polymerization by phosphorylating and activating LIM-kinase that in turn phosphorylates and deactivates the actin depolymerizing factor coflin (Maekawa et al, 1999; Ohashi et al., 2000; reviewed by Bernard, 2007). GTP-bound Rac1 and Cdc42 both bind to WASP-family proteins that in turn activate Arp2/3 (reviewed by Campellone and Welch, 2010; Lecuit et al., 2011). Furthermore, Rac1 and Cdc42 activate p21-activated kinase (Pak) that in turn phosphorylates and activates LIM-kinase (Edwards et al., 1999); thus both Rac1 and Cdc42 catalyze actin polymerization through multiple pathways. Guanine nucleotide exchange factors (GEFs) catalyze exchange of GDP for GTP in Rho GTPases, whereas GTPase activating proteins (GAPs) enhance Rho GTPase hydrolysis of GTP into GDP;
Figure 1.3: Chemical and mechanical modifiers of actomyosin assembly and disassembly.

(A-B) Chemical modifiers can affect assembly and disassembly of F-actin (A) and myosin (B).

(C-D) Tension can lead to both assembly and disassembly of F-actin (C) and myosin (D) directly and by regulating chemical modifiers (not shown). Tension can also be exerted by contractile actomyosin networks themselves.
A  Assembly  Disassembly

Polymerization  e.g., formin, Ena
Nucleation  e.g., Arp2/3

Actin

Severing  e.g., coflin, gelsolin
Capping  e.g., capping protein

B

Phosphorylation  e.g., ROCK, MLCK

Myosin

Dephosphorylation  e.g., myosin phosphatase

C

Actin

Tension

D

Myosin
thus both GEFs and GAPs are often considered when discussing actomyosin regulators. In addition, phosphoinositides have also been studied as regulators of actin polymerization. For example, phosphoinositides can bind to RacGEFs, which activate Rac1 (Ceccarelli et al., 2007; Crompton et al., 2000) and can bind to WASP-family proteins that stimulate Arp2/3 (Oikawa et al., 2004; Suetsugu et al., 2006; reviewed by Brill et al., 2011; Cain and Ridley, 2009; Padrick and Rosen, 2010).

Protein regulators can also lead to actomyosin disassembly (Fig. 1.3A,B). For example, actin networks can be disassembled by intrinsic depolymerization of older filaments (Fig. 1.2A), or actively severed by gelsolin and coflin/actin depolymerizing factor (ADF). Furthermore, de novo actin polymerization at filament plus ends is inhibited by Capping Protein (CP) (reviewed by Campellone and Welch, 2010; Levayer and Lecuit, 2012; Mullins and Hansen, 2013). In addition, myosin can be inactivated when its regulatory light chain is dephosphorylated by myosin phosphatase. GTPase activating proteins (GAPs) inactivate the Rho GTPases and are thus also considered when discussing actomyosin inhibition (reviewed by Cherfils and Zeghouf, 2013). Thus, multiple layers of protein regulators exist to tune actomyosin assembly.

To transmit contractile forces, actomyosin networks must be effectively cross-linked and anchored to the plasma membrane. To form an interconnected network, individual actin filaments are cross-linked together to form a more intertwined meshwork. Actomyosin networks must also be linked to the cell cortex to mediate productive contraction of cells. In presumptive Drosophila ventral furrow cells, upon disruption of adherens junction integrity, contracted actomyosin detach from the cell junctions, forming contracted aggregates in the middle of the cells (Dawes-Hoang et al., 2005; Martin et al., 2010).

As large mesoscale networks, assembly and disassembly of actomyosin networks are subject to regulation by mechanical forces. Contractility can be determined by the architecture of the underlying actin network: whether it is heavily crosslinked, branched or unbranched, and parallel or antiparallel (Reymann et al., 2012; reviewed by Goehring and Grill, 2013; Lecuit et al., 2011; Levayer and Lecuit, 2012). Furthermore, tension can activate actomyosin networks (Fig. 1.3C,D). For example, in studies with in vitro
reconstituted actomyosin, tension both increases the activity of myosin (Kovacs et al., 2007) and reduces the propensity of F-actin to be disassembled (Hayakawa et al., 2011; Ren et al., 2009). Mechanosensory activation of actomyosin has been seen in vivo; cortical myosin builds in response to outside pulling forces in both Dictyostelium cells (Ren et al., 2009) and Drosophila embryos (Fernandez-Gonzalez et al., 2009). However, mechanical forces can also inhibit actomyosin. For instance, excess tension on F-actin favours disassembly at filament ends (Lee et al., 2013). Furthermore, contracted networks can buckle actin filaments, leading to disassembly either through passive compaction (Reymann et al., 2012) or through increased accessibility to actin depolymerizing factors (Fig. 1.3C,D) (Murrell and Gardel, 2012; Soares e Silva et al., 2011).

Recent work suggests that mechanical forces can also affect protein regulators of actomyosin. For example, at adherens junctions, α-catenin can function as a mechanosensor; a force-induced conformational change on α-catenin exposes a vinculin-binding site (Yonemura et al., 2010). Vinculin is also an F-actin binding protein, thus serving to further reinforce F-actin at the adherens junctions. The ability for α-catenin to sense tension requires its actin-binding site, thus implicating actomyosin contractility for the development of adherens junctions (Yonemura et al., 2010). Furthermore, tension can activate protein regulators of actomyosin contractility. For example, mechanosensitive calcium channels and the resultant Ca$^{2+}$ influx activate MLCK (Kapustina et al., 2008). In addition, mechanical stress can activate formins (Higashida et al., 2013), and contracted F-actin networks are more accessible to ADF (Murrell and Gardel, 2012; Soares e Silva et al., 2011). Thus, assembly and disassembly of actomyosin networks likely integrate both mechanical and chemical inputs.

1.4 Actomyosin networks can be oscillatory

Whereas actomyosin networks were thought to alternate between steady states favouring either assembly or disassembly, recent work suggests that dynamic oscillations are a key aspect of their regulation. In vitro, actomyosin networks can self-assemble into contractile bundles (Stachowiak et al., 2012; Thoresen et al., 2011) that progress towards
complete contraction and then disassembly (Murrell and Gardel, 2012; Soares e Silva et al., 2011; Stachowiak et al., 2012). However, actin networks in *Xenopus* egg extracts can undergo pulsatile contractions (Clark and Merriam, 1978; Field et al., 2011; Valentine et al., 2005), suggesting that pulsatility may be an intrinsic property of cytoplasmic actomyosin networks.

Oscillations of actomyosin networks are also seen in diverse cellular contexts. Recent work has revealed actomyosin oscillations within individual cells. For example, the leading edge of cultured migratory vertebrate cells has oscillatory protrusions and retractions that depend on actin polymerization and myosin contraction (Burnette et al., 2011), and on interactions between Rho GTPases (Machacek et al., 2009). Motile *Dictyostelium* cells also display periodic accumulations of cortical F-actin upon cAMP stimulation (Westendorf et al., 2013). Another example of oscillatory networks is during cytokinesis, where rounds of actin assembly and disassembly and myosin crosslinking may be required for efficient cytokinetic ring contraction (Coffman et al., 2009; Chen and Pollard, 2011; Ma et al., 2012). Oscillatory actomyosin networks also arise in developing tissues such as during *Drosophila* follicular epithelial morphogenesis (He et al., 2010) and *Drosophila* embryonic ventral furrow formation, (Martin et al., 2009), germ band extension (Rauzi et al., 2010; Sawyer et al., 2011), and dorsal closure (Blanchard et al., 2010; David et al., 2010; Solon et al., 2009). Oscillatory apical constriction is also seen in *C. elegans* embryos (Roh-Johnson et al., 2012). Oscillatory constriction is thought to mediate productive constriction by “ratcheting” towards more contracted states (Fig. 1.2E). Interestingly, oscillations of actomyosin are usually on the timescale of minutes, as seen in multiple tissues of *Drosophila* (Blanchard et al., 2010; David et al., 2010; He et al., 2010; Martin et al., 2009) and *C. elegans* (Roh-Johnson et al., 2012) embryos, but can be as short as tens of seconds, as seen in *Dictyostelium* (Westendorf et al., 2013), suggesting different regulatory schemes may be at play. To better understand actomyosin oscillations, we can turn to studies of other well-known oscillatory networks.
1.5 Oscillatory networks can arise from delayed negative feedback loops

Oscillatory networks are seen in diverse biological systems with varying degrees of cycle lengths. The time scale of biological networks can range from roughly one day of circadian rhythms, to hours of cell cycle progression, to seconds of actomyosin oscillations. For example, circadian rhythms are controlled by the activity of transcription factors CLOCK and BMAL1 that induce the expression of their own repressors PER and CRY. During the night, high levels of CLOCK and BMAL1 slowly induce transcription of target proteins, among them PER and CRY. During the day, high levels of PER and CRY protein repress CLOCK and BMAL1 transcription. Degradation of PER and CRY allow once again for CLOCK and BMAL1 transcription, restarting the circadian cycle (Smolen et al., 2002; reviewed by Masri et al., 2012). Another well-studied example of biological oscillatory networks is during cell cycle progression. Cell cycle progression is driven either into or out of mitosis by the respective activities of cyclin-dependent kinase (CDK1) and anaphase-promoting complex (APC). The activity levels of CDK1 and APC fluctuate, with the peak of APC lagging behind that of CDK1 (reviewed by Ferrell et al., 2011). Thus, oscillatory networks are characterized by a periodic rise and fall of effectors and their regulators. In this section, I describe the general regulatory circuitry for oscillatory networks and describe how actomyosin regulators may also serve this role.

Mathematical modeling suggests that oscillations can arise from negative feedback systems if certain requirements are satisfied (reviewed by Ferrell et al., 2011; O’Brien et al., 2012). One such requirement is that circuits have a delayed negative feedback loop (Fig. 1.4A). Time delays are common in biological systems since signals such as chemicals take time to propagate through a cell or an entire organism. Networks will also oscillate if there are ultra-sensitive switches. Multiple layers of regulators will promote ultra-sensitivity as well as introduce further time delay. Finally, oscillation is promoted if the circuits are bistable and hysteretic (which is to say networks can exist in one of two states and resist changes to their states) (reviewed by Ferrell et al., 2011). If the
**Figure 1.4: Oscillatory networks arise from delayed negative feedback.**

(A) Schematic of a generic delayed negative feedback loop. The levels of output C oscillate with respect to time.

(B) Schematic for the “repressilator” gene circuit where a series of genes repress one another in tandem. A product for one of the genes tetR in turn represses GFP transcription. The levels of GFP oscillate with respect to time, but with poor oscillatory behaviour (varying amplitude and period). LacI from *E. coli* tetR, tetracycline-resistance gene from transposon Tn10; cI from λ phage.

(C) Schematic for the “tunable repressilator” where genes in a circuit both repress and activate one another’s expression. Here, two genes, *ara* and *LacI* also positively-feedback to activate their own transcription. It is tunable because the magnitude of oscillation can be modulated by degree of input (*ara* and *lacI*). The levels of GFP fluctuate with respect to time.

(D) Possible delayed negative feedback circuits for actomyosin networks. ROCK activates actomyosin, that recruits its depolymerizing factor cofilin. Alternatively, actomyosin contraction causes tension that both activates then suppresses actomyosin networks. The levels of activated actomyosin networks can oscillate with respect to time.
aforementioned conditions are not met, networks relying on delayed negative feedback loops will have dampened and unsustained oscillations.

Experimentally, delayed negative feedback circuits have been studied in synthetic gene regulatory networks. Here, a circuit of repressive genes in a loop is inserted into an organism and the resultant gene and reporter genes levels are seen to sequentially oscillate (Fig. 1.4B) (Elowitz and Leibier, 2000; Tigges et al., 2009). In this case, the time delay is thought to result from the time required to transcribe and translate the resulting genes (O’Brien et al., 2012). Furthermore, addition of positive feedback in a network with delayed negative feedback can add a degree of tunability and robustness of the circuit (Fig. 1.4C) (Stricker et al., 2008; Tigges et al., 2009). In other words, in this type of network, oscillatory frequency can correlate with the magnitude of input, and circuits can oscillate within a wider range of input levels. Thus, much of the experimental analysis of biological oscillations has arisen from the study of synthetic gene networks.

Actomyosin network contraction has also been observed as highly oscillatory and subject to positive and negative feedback (Fig. 1.4D). For example, formation of an F-actin network into a contractile ring leads to local enrichment of the actin depolymerizing factor cofilin (Chen and Pollard, 2011; Nakano and Mabuchi, 2006). Other results have suggested that RhoA can mediate oscillations of actomyosin in tissue culture (Costigliola et al., 2010). In addition, mechanical regulation may also play a role in negative feedback (Fig. 1.3C,D and Fig. 1.4D). For example, the Ca\(^{2+}\) influx from stretch-activated calcium channels can activate MLCK, but the resultant myosin-induced tension is thought to close calcium channels (Kapustina et al., 2008). As another example, mechanical tension created by constriction of actomyosin networks can activate myosin (Fernandez-Gonzalez et al., 2009; Kovacs et al., 2007; Ren et al., 2009) but also lead to network disassembly (Lee et al., 2013; Soares e Silva et al., 2011). Indeed, mathematical modeling of the oscillatory constriction of amnioserosa cells during Drosophila dorsal closure suggests that actomyosin oscillation depends on some form of mechanical coupling between cells (Wang, Q. et al., 2012). Ablation of an amnioserosa cell by laser wounding results in cessation of pulsing in cells both adjacent to and further from the
wound, suggesting a role for intercellular tension in oscillations (Solon et al., 2009). Therefore, actomyosin network oscillations are subject to both positive and negative feedback loops. Whether regulatory circuits for actomyosin networks are configured for delayed negative feedback optimal for oscillatory behaviour may be context-dependent. Furthermore, it is important to understand how actomyosin regulators interact with regulators of cell adhesion and cell polarity.

1.6 Interactions between protein complexes regulate cell polarity

Since actomyosin network architectures are context-dependent and, in the case of apical constriction, polarized towards the apical surface, understanding cell polarity is also critical. Cell polarity is regulated by evolutionarily conserved protein complexes and their interactions with one another (Fig. 1.5). Several main polarity complexes have been characterized: the PAR, Crumbs (Crb), and Scribble (Scrib) complexes, and the Yurt/Coracle group. Mathematical models suggest that cell polarity can arise if the networks contain both cooperative recruitment within complexes and mutual antagonism between different complexes (Fletcher et al., 2012; Goehring et al., 2011). In this section, I discuss the interactions within and between these complexes.

The PAR complex components Baz, Par-6, and aPKC can directly bind to one another (Fig. 1.6A). The adaptor protein Par-6 binds to GTP-loaded Cdc42 via its semi-CRIB (Cdc42/Rac interactive binding) domain and part of its PDZ (Postsynaptic density-95, Discs-large, Zonula-occludens-1) domain (Atwood et al., 2007; Garrard et al., 2003; Rolls et al., 2003). Interestingly, Cdc42-GTP binding to Par-6 can increase the affinity for the Par-6 PDZ domain for putative C-terminal peptide ligands (Peterson et al., 2004), but the exact biological consequences of this remain to be explored further. The Par-6 PB1 (Phox and Bem1) domain binds to the PB1 domain of aPKC (Lin et al., 2000; Noda et al., 2003). It was initially thought that Par-6 suppresses aPKC kinase activity, while binding of Cdc42-GTP to Par-6 relieves Par-6-mediated inhibition of aPKC (Atwood et al., 2007; Yamanaka et al., 2001). However, genetic evidence suggests Par-6 activates aPKC; par-6 loss-of-function mimics apkc loss-of-function in both Drosophila
Figure 1.5: Interactions between protein complexes regulate cell polarity.

The apical polarity complexes, the Crb and PAR complexes, positively regulate one another. Mutual antagonism exists between the apical polarity regulators and the basolateral polarity regulators (the Scrib and Yurt/Cora complexes, and Par-1)
Figure 1.6: Dynamic interactions between PAR complex proteins.

(A) PAR complex components can bind directly with one another. The Par-6 PB1 domain binds the aPKC PB1 domain. The Par-6 PDZ domain binds to the Baz PDZ1 domain. The Baz OD mediates homo-oligomerization. aPKC binds Baz in a region encompassing PDZ2-3 and a region containing its phosphorylation site, CR3.

(B) aPKC phosphorylation of Ser980 on Baz (P) disrupts the binding of Par-6/aPKC to Baz.
(Petronczki and Knoblich, 2001; Wodarz et al., 2000) and *C. elegans* (Munro et al., 2004; Tabuse et al., 1998), whereas co-overexpression of Par-6 and aPKC enhances aPKC gain-of-function effects (David et al., 2010). Furthermore, Par-6 can activate aPKC *in vitro* (Graybill et al., 2012). Par-6 can also bind to the PAR complex member Baz via the Par-6 PDZ and the first PDZ domain of Baz (Joberty et al., 2000; Lin et al., 2000; Morais-de-Sa et al., 2010). Baz in turn interacts with a wide variety of factors (reviewed by Tepass, 2012), including those involved in regulation of cell adhesion (Wei et al., 2005), cell polarity (Krahn et al., 2010), phosphoinositides (Pickering et al., 2013), and actomyosin (Georgiou and Baum, 2010). Baz can homo-oligomerize via a conserved N-terminal oligomerization domain (OD) (Benton and St Johnston, 2003) and this is somehow important for membrane association. Baz recruitment to the apical cortex also employs multiple and redundant interactions on separate sites to promote interaction with the cell cortex (Krahn et al., 2010) and to promote localization to AJs (McKinley et al., 2012). Baz is also an aPKC substrate and they can bind in two distinct regions: the Conserved Region 3 (CR3) and a region encompassing PDZ2-PDZ3 of Baz. The Baz CR3 contains an aPKC phosphorylation site on a conserved serine (S980 in *Drosophila*) (Nagai-Tamai et al., 2002; Wodarz et al., 2000). Mutation of Baz S980 to the phosphomimetic glutamic acid abolishes aPKC binding, whereas mutation of S980 to the nonphosphorylatable alanine greatly enhances aPKC binding (Morais-de-Sa et al., 2010). These results suggest that aPKC binds to Baz on this site, and that upon phosphorylation, the aPKC – Baz interaction is greatly decreased. aPKC binding to Baz can also be affected by regions outside its phosphorylation site; deletion of a region encompassing PDZ2 – PDZ3 of Baz greatly diminishes Baz – aPKC binding (Wodarz et al., 2000). Furthermore, mutation of an additional serine (S982, also found within the Baz CR3) to alanine somehow greatly reduces aPKC – Baz binding (Nagai-Tamai et al., 2002; Wodarz et al., 2000). Thus, the components of the PAR complex have multiple modes of binding to one another and for localizing to the apical cortex.

The Crb complex is composed of the transmembrane protein Crb, Stardust (Sdt, the *Drosophila* homolog of PALS1), and PATJ. Crb is an apical determinant (Pellikka et al., 2002; Tepass et al., 1990; Wodarz et al., 1995). The large Crb extracellular domain may
mediate cis and trans homophilic binding (Fletcher et al., 2012; Hafezi et al., 2012; Roper, 2012; Zou et al., 2012). The Crb cytoplasmic tail contains two known regions: a FERM-domain binding region (FDB) and a PDZ-domain binding region. The FDB can bind to FERM-domain proteins Expanded, Yurt, Moesin, and β-heavy spectrin (Ling et al., 2010; Laprise et al., 2006; Médina et al., 2002; Pellikka et al., 2002). The Crb PDZ-binding domain binds to Sdt (Bachmann et al., 2001; Hong et al., 2001; Krahn et al., 2010; Roh et al., 2002). The Crb complex also cooperatively binds to PAR complex components. The Crb PDZ-binding domain binds to the PDZ domain of Par-6 (Kempkens et al., 2006; Lemmers et al., 2004; Morais-de-Sa et al., 2010), and Crb localization at the apical cortex is stabilized by aPKC phosphorylation (Sotillos et al., 2004). Sdt can also bind to Baz (Krahn et al., 2010). Therefore, the Crb and PAR complexes cooperatively interact to specify the apical surface.

The Scribble complex is found along the basolateral cortex and is composed of the scaffolding proteins Scribble (Scrib), Discs-large (Dlg), and lethal giant larvae (Lgl). These proteins localize to the basolateral cortex and are found in the same genetic pathway, although evidence for their interaction as a physical complex is lacking. For example, Scrib and Dlg may bind to one another through an adaptor protein known as GUK holder (Mathew et al., 2002). Moreover, Lgl co-immunoprecipitates with Scrib in mammalian cell culture, but this may or may not be direct (Kallay et al., 2006). In addition to the Scrib complex proteins, the kinase Par-1, and the scaffolding protein 14-3-3 are found along the basolateral surfaces of epithelial cells. The Yurt group, composed of Yurt, Coracle, Neurexin IV, and the sodium-potassium ATPase, are also found on the basolateral surface and are recently-discovered basolateral polarity regulators in Drosophila (Laprise et al., 2009). The Yurt group components function in similar pathways but have not been isolated as a single complex. Although the basolateral regulators are commonly referred to as complexes, evidence of their interactions is genetic and does not necessarily suggest they bind as biochemical complexes.

Apical polarity complex proteins intermingle and their interactions are dynamic in nature. Crb and Baz compete for binding to the Par-6 PDZ domain (Morais-de-Sa et al., 2010). Furthermore, although aPKC and Baz can bind, aPKC phosphorylation on a conserved
serine (S980 on Baz) greatly diminishes the affinity of Baz for aPKC (Fig. 1.6B) (Morais-de-Sa et al., 2010) and for Sdt (Krahn et al., 2010). Baz binding to Par-6 and aPKC can change the substrate preference of aPKC from Lgl to Numb in *Drosophila* neuroblasts (Wirtz-Peitz et al., 2008). Whereas Baz and Par-6/aPKC colocalize in *Drosophila* neuroblasts and the *C. elegans* one cell embryo, Par-6 and aPKC often localize more apically than Baz in other epithelia (Harris and Peifer, 2005).

Mutual antagonism exists between apical and basolateral regulators (Fig. 1.5). Genetic evidence suggests that Crb and Scrib complexes are antagonistic (Bilder et al., 2003; Tanentzapf and Tepass, 2003). aPKC excludes Lgl from the apical cortex by phosphorylating it and thus dissociating it from the membrane. In turn, Lgl binds to aPKC and inhibits its kinase activity, presumably through competitive inhibition (Atwood and Prehoda, 2009; Betschinger et al., 2003) and promotes removal of Crb from the basolateral membrane (Tanentzapf and Tepass, 2003). Furthermore, aPKC phosphorylates Par-1 to dissociate it from the membrane (Hurov et al., 2004; Suzuki et al., 2004). Likewise, Par-1 phosphorylates Baz to enhance its interaction with 14-3-3, leading to Baz dissociation from the membrane in *Drosophila* (Benton and St Johnston, 2003; McKinley et al., 2012). In addition, Yurt can directly bind Crb and inhibit its cortical localization (Laprise et al., 2006) though the molecular mechanisms are unclear. Therefore polarity complexes interact in cooperative and mutually antagonistic relationships to maintain polarity in the cell.

### 1.7 Functions of the PAR complex in regulating cellular processes

The PAR complex regulates a wide variety of cellular processes including epithelial cell polarity, epithelial morphogenesis and migration, asymmetric cell division, and axon outgrowth (reviewed by St Johnston and Ahringer, 2010; Tepass, 2012). The PAR complex and cell polarity are also subverted in diseased states such as as pathogen infection and cancer progression (reviewed by Engel and Eran, 2011; Nikitas and Cossart, 2012; Tepass, 2012). Thus, understanding the activity of the PAR complex is important for understanding an array of biological processes, and give clues as to how the
PAR complex may impact actomyosin networks and morphogenesis. Below, I describe two of the best understood examples of PAR complex activity in vivo: adherens junction formation and asymmetric cell division.

Research in Drosophila has uncovered the role of the PAR complex for proper formation of cell-cell junctions. During early Drosophila embryogenesis, the syncytial embryo forms individual cells in a process known as cellularization. During cellularization, the embryonic syncytium begins to demarcate cells by ingressing membranes in “furrows” around each nucleus (reviewed by Harris, 2012). The nascent cells are polarized prior to formation of AJs, suggesting a role for polarity in proper recruitment of AJs (Harris and Peifer, 2004; Harris and Peifer, 2005). Indeed, during cellularization, Baz is enriched at the apicolateral membrane, and this somehow requires an apically-localized actin scaffold, microtubule-based dynein delivery (Choi et al., 2013; Harris and Peifer, 2005), and removal of mislocalized Baz from the basolateral cortex (McKinley and Harris, 2012). Baz then recruits Par-6 and aPKC to the apicolateral membrane (Harris and Peifer, 2005) but these three proteins do not precisely colocalize. Instead, Par-6 and aPKC localize just apically to Baz (Harris and Peifer, 2005), presumably because aPKC phosphorylation of Baz results in reduced aPKC – Baz affinity (Morais-de-Sa et al., 2010). Baz localizes to the nascent AJs, helping to recruit E-Cad and β-catenin (Harris and Peifer, 2004; McGill et al., 2009). Thus, Baz is critical for the establishment of polarity and proper positioning of AJs. While Par-6 and aPKC are dispensable for establishment of polarity, they are required for the maintenance of polarity during later stages of embryogenesis. Immediately after cellularization, Drosophila epithelial cells undergo dramatic rearrangements during gastrulation, a process that requires maintenance of polarity and adhesion in order to maintain tissue integrity. In embryos lacking aPKC, AJs form normally, but at gastrulation tissue integrity breaks down (Harris and Peifer, 2007). Perturbations of Baz, Par-6, and aPKC also perturb the integrity of the Drosophila epithelial neuroectoderm, a site from which neuroblasts delaminate (Harris and Tepass, 2008). Together, these results suggest that Par-6 and aPKC are required for maintenance of polarity during dynamic cell rearrangements. Thus, Baz and Par-6/aPKC play distinct roles for the establishment and maintenance of polarity.
In contrast to *Drosophila*, external cues such as sites of cell adhesion are thought to serve as the primary landmarks for cell polarity in mammalian cells. In MDCK cells, it has been shown that nascent adherens junctions, independent of the PAR complex, are a polarization cue (Capaldo and Macara, 2007; reviewed by Nelson et al., 2013). In 2D cell culture, MDCK cells form junctions and become an epithelial monolayer. As MDCK cells re-establish E-cad-based cell-cell junctions upon calcium addition, nascent spot AJs form contact spots that mature into an adherens belt. Junctional adhesion molecule (JAM) binds to and recruits Par-3 to junctions (Ebnet et al., 2001), which is then thought to recruit Par-6 and aPKC (Hirose et al., 2002; Suzuki et al., 2001). aPKC kinase activity is important for the formation of TJs (Iden et al., 2012; Suzuki et al., 2001). Par-3 is also believed to control TJ assembly by interacting with the RacGEF Tiam1 (Chen and Macara, 2005). However, there is evidence that MDCK cells cultured in 3D differ slightly in their mechanisms of polarization. In 3D MDCK cell culture, a cell mass forms a cyst composed of a polarized epithelial monolayer, with the apical surfaces facing inwards into a lumenal space, and basal surfaces exposed to extracellular matrix. In contrast to 2D monolayers wherein AJ formation serves as the primary polarizing cue, in 3D cell culture, the initial orientation of the axis of apical-basal polarity is regulated by interaction of the basal surface with surrounding extracellular matrix (Yu et al., 2005). In 3D cell culture, the PAR complex is essential for targeting vesicular compartments towards the apical domain (Horikoshi et al., 2009) and polarizing phosphoinositide gradients (Martin-Belmonte et al., 2007). Furthermore, in the formation of MDCK cell cysts, Par-3 is essential for correct orientation of mitotic spindles to maintain polarity of the epithelial monolayer by recruiting aPKC to the apical surface, possibly independently of Rac1 (Hao et al., 2010). Thus, the PAR complex plays an important role in TJ formation and maintenance of epithelial polarity through polarized cell division, though the mechanisms of polarization may differ depending on the 2D or 3D cellular context.

The PAR proteins also play an important role during asymmetric cell division. During this process, a mitotic cell differentially partitions fate determinants between two nascent daughter cells and can thus be used to specify different cell fates. Often, a polarized cortex serves as a cue for appropriate segregation of fate determinants. One such
example is during the first stages of *C. elegans* embryogenesis, wherein the one cell embryo is partitioned into distinct anterior and posterior halves (Fig. 1.7A). Mutual antagonism between the anterior and posterior proteins establishes and maintains their respective domains. As mentioned above, the anterior-ward flow of an actomyosin network brings with it the anterior PAR proteins PAR-3, PAR-6, and PKC-3 (the *C. elegans* aPKC) (Munro et al., 2004). This results not only in enrichment of these PAR proteins at the anterior cortex, but also depletion of these proteins from the posterior cortex. Since PKC-3 phosphorylates PAR-2 to dissociate from the membrane in *C. elegans* (Hao et al., 2006), posterior depletion of PKC-3 allows PAR-2 to stably localize to the posterior cortex, though PAR-2 can also localize to the posterior cortex via microtubule-based delivery (Motegi et al., 2011). PAR-2 then serves as a cue for PAR-1 localization (Munro et al., 2004). PAR-1 phosphorylates PAR-3 to destabilize its cortical localization (Motegi et al., 2011). In turn, PKC-3 phosphorylates PAR-1 to destabilize its cortical localization (Hurov et al., 2004). LGL-1, the *C. elegans* homolog of Lgl, also functions redundantly with PAR-1 to inhibit cortical localization of anterior PARs (Beatty et al., 2010; Beatty et al., 2013; Hoege et al., 2010). Thus, the mutual antagonism between the apical and basolateral PAR proteins seen in *Drosophila* appears also conserved in *C. elegans* to establish and maintain polarity, though there is no known PAR-2 homolog in *Drosophila* (reviewed by Nance and Zallen, 2011). Once the anterior and posterior domains are established and maintained, the embryo then undergoes cell division along the anterior-posterior axis, with the two daughter cells enriched for either the anterior or posterior PAR proteins, as well as different cell fate determinants. Thus, the first mitotic division of *C. elegans* is an asymmetric cell division that is dependent on proper polarization by the PAR proteins.

Another well-studied example of asymmetric cell division is the *Drosophila* neuroblast. Fate determinants for neuroblast self-renewal are found along the apical cortex whereas determinants for the differentiated ganglion mother cell (GMC) are found along the basal cortex. After division along the apicobasal axis, the apical-most cell inherits neuroblast determinants whereas the basal-most cell inherits determinants for GMC fate, further differentiating into neural descendants. The PAR complex is required for polarization of
Figure 1.7: Localization of PAR complex proteins and actomyosin networks in vivo.

(A) In the *C. elegans* one cell embryo, dynamic, interconnected actomyosin foci and networks (green) colocalize with PAR complex puncta (orange).

(B) In *Drosophila* extending germ band during embryogenesis, Par-3 (orange) is enriched in domains reciprocal to domains of actomyosin enrichment (green) in intercalating epidermal cells.

(C) In the amnioserosa (AS) cells during *Drosophila* dorsal closure, apical PAR complex puncta (orange) colocalize dynamically with apical actomyosin networks (green). In the dorsal-most epidermal (Epi) cells, the PAR complex is depleted at the leading edge, where there is an enrichment of an actomyosin cable.
cell fate determinants and correct spindle orientation. aPKC is found in a crescent along
the apical cortex and phosphorylates Miranda to exclude it from the apical membrane
(Rolls et al., 2003). Miranda then localizes to the basal cortex where it recruits the cell
fate determinant Prospero and Brat to specify GMC fate (Atwood and Prehoda, 2009). In
addition, an apical crescent of Baz binds to Insuteable, which in turn binds to Pins, Gi, and Mud for proper orientation of the mitotic spindle along the apicobasal axis (Bowman
et al., 2006; Izumi et al., 2006; Schober et al., 1999; Siller et al., 2006). Thus, the PAR
complex regulates asymmetric cell division by polarizing the cell cortex and properly
aligning the mitotic spindle.

1.8 The PAR complex and actomyosin networks

In addition to regulating apicobasal polarity, proper positioning of junctions, and
asymmetric cell division, the PAR complex has also been recently implicated in
regulating actomyosin networks. Below, I describe examples of interactions between
actomyosin and the PAR complex.

A well-studied example of PAR interaction with actomyosin networks is the C. elegans
one-cell embryo (Fig. 1.7A). Here, anterior and posterior PARs are required for
establishment or maintenance of partitioning of the embryo cortex along the anterior-posterior axis (Munro et al., 2004; reviewed by Nance and Zallen, 2011). The anterior
PAR proteins colocalize with actomyosin cortical nodes and move at similar speeds,
suggesting that, as discussed above, they are swept anteriorly by advection (Goehring et
al., 2011; Munro et al., 2004). In addition, PAR puncta and actomyosin networks
colocalize to apical surfaces at later stages of C. elegans embryogenesis prior to the onset
of apical constriction (Nance et al., 2003). Despite the correlation between the PAR
complex and actomyosin networks, the molecular mechanisms underlying their
interactions remain unclear.

Research in other model systems has suggested a regulatory feedback between Par-3 and
actomyosin networks. Par-3 is phosphorylated by ROCK in mammalian cell culture and
in vitro, and this disrupts Par-3 association with aPKC (Nakayama et al., 2008). During
*Drosophila* embryonic germ band extension, Baz and ROCK (and with ROCK, actomyosin) are enriched in complementary cortical regions along the AJs of the ectoderm (Fig. 1.7B) (Simoes Sde et al., 2010; Zallen and Wieschaus, 2004). This suggests that an antagonistic relationship between ROCK and Par-3 may be conserved, though the ROCK phosphorylation site of mammalian Par-3 is not conserved in *Drosophila* Baz. Par-3 also interacts with Rac1, but different results from mammalian cell culture and *Drosophila* mean that the relationship between Par-3 and Rac1 remains unclear. In mammalian cell culture, Par-3 interacts with the Rac GEF Tiam1, evidently to target and activate Tiam1 and thus localize Rac1 activation (Nishimura et al., 2005; Wang, S. et al., 2012). However, other results from mammalian cell culture suggest that Par-3 inhibits Tiam1 presumably through sequestration (Chen and Macara, 2005). Furthermore, genetic evidence in *Drosophila* suggests Baz inhibits Still Life (SIF, the *Drosophila* homolog of Tiam1) (Georgiou and Baum, 2010). Thus, the relationship between Par-3/Baz and Rac1 still remains to be clarified. In addition, Baz can also interact with PATJ (Krahn et al., 2010), and PATJ has recently been shown to activate myosin by sequestering myosin phosphatase (Sen et al., 2012). Baz can also modulate phosphoinositide signalling to promote actin-based protrusions during *Drosophila* dorsal closure (Pickering et al., 2013).

Evidence also suggests that aPKC can antagonize actomyosin networks. In mammalian cell culture, Par-6 and aPKC localize to actin protrusions and recruit Smurf1, an E3 ubiquitin ligase, to ubiquitinate and thus target RhoA for degradation (Wang et al., 2003). aPKC can also phosphorylate ROCK, displacing it from the cell cortex in mammalian cell culture (Ishiuchi and Takeichi, 2011). Recent results suggest this antagonistic relationship between ROCK and aPKC may also be conserved in *Drosophila*. In the *Drosophila* embryonic salivary gland placode, a planar polarized depletion of Crb and aPKC from the placode boundary is coincident with an enrichment of ROCK and a band of actomyosin (Roper, 2012). Overexpression of membrane-targeted aPKC that uniformly localizes along the cell cortex abolishes ROCK and myosin enrichment at the placode cell boundary (Roper, 2012). This suggests that *Drosophila* aPKC can also inhibit ROCK cortical localization, presumably by phosphorylating ROCK. In addition,
aPKC can phosphorylate mammalian p190RhoGAP, perturbing its GAP preference from Rac towards RhoA, thereby leading to inactivation of RhoA (Levay et al., 2009; Zhang and Macara, 2008). Thus, investigations in mammalian cell culture suggest that aPKC antagonizes actomyosin networks. However, the molecular mechanisms behind this antagonism, and the conservation of such mechanisms from C. elegans, Drosophila, and mammals remain to be investigated further.

As discussed below, my research has focused on Drosophila dorsal closure, which harbours dramatic apical actomyosin web-like networks associated with nearby apical surface PAR complex puncta (Fig. 1.7C)

1.9 Dorsal closure is a model of apical constriction

My research has focused on Drosophila dorsal closure as a model to study apical constriction. The Drosophila embryo offers many advantages for study. A wide array of genetic tools is available in Drosophila for loss- and gain-of-function experiments. Furthermore, Drosophila lines with stably-integrated transgenic constructs are easily created with well-established protocols and are widely available to the research community. In addition, the embryo is highly amenable to live imaging due to its external development and its envelopment by the transparent vitelline membrane. The amnioserosa tissue is a thin, squamous epithelial monolayer found at the surface of the embryo and is thus amenable to high-speed live imaging and physical perturbation. The combination of powerful genetics and ease of imaging of Drosophila embryos has been instrumental to my study of Drosophila dorsal closure. Below, I describe the dramatic morphogenetic event known as dorsal closure, the regulatory networks known to be involved, the role of actomyosin in driving this process, and recent questions raised about the oscillatory nature of actomyosin networks.

A remarkable array of morphogenetic changes takes place during Drosophila embryogenesis. Almost immediately after embryonic cells are formed, the beginning of gastrulation is marked by the formation ventral presumptive mesodermal cells. These cells apically constrict to cause an initial inward bend of the tissue and eventually ingress.
The ectoderm of the embryo proper (the “germ band”) elongates roughly two-fold along the anterior-posterior axis and shortens along the dorsal-ventral axis in a process known as germ band elongation (reviewed by Blanchard and Adams, 2011; Kasza and Zallen, 2011; Lecuit et al., 2011). During this time, the extraembryonic amnioserosa cells flatten along their apicobasal axis whilst elongating within the plane of the epithelium (Pope and Harris, 2008), transforming from columnar to squamous in shape. During germ band retraction, the extended germ band then shifts such that the caudal end of the embryo is at the posterior pole. This exposes a “gap” in the embryo proper along the dorsal surface, a space occupied by the extraembryonic squamous epithelial monolayer known as the amnioserosa. Dorsal closure, which occurs at ~11 – 15 hours after egg laying concurrently with head involution, serves to seal this gap.

Dorsal closure requires the coordination and integration of multiple forces from multiple tissues. During dorsal closure, the entire embryonic epidermis moves dorsally, closing over the amnioserosa (Fig. 1.8A). Eventually the two sides of the epidermis meet and fuse into one sheet (reviewed by Harden, 2002; Jacinto et al., 2002). As dorsal closure proceeds, approximately 10% of amnioserosa cells undergo apoptosis (Toyama et al., 2008). Upon dorsal closure completion, the rest of the amnioserosa cells undergo apoptosis (Reed et al., 2004).

Genetic and laser ablation experiments have uncovered the contributions of forces from multiple tissues. By observing the initial recoil velocity of a tissue in response to wounding, laser ablation can give an idea of the proportionality of forces present in a substance (reviewed by Colombelli and Solon, 2013). A supracellular actomyosin “purse string” forms along the leading edge (LE) of the dorsal-most epidermal cells and is thought to pull the epidermal sheet dorsally (Kiehart et al., 2000). Laser ablation of the LE actomyosin cable results in immediate recoil of the epidermis ventrally and away from the cut site (Kiehart et al., 2000), suggesting that the LE cable is under tension and pulls the sheet dorsally. However, genetically perturbing regulators of the actomyosin cable in a subset of cells leads to increased LE cellular protrusions and dorsal-ward movement relative to their unperturbed neighbouring cells (Jacinto et al., 2002; Laplante and Nilson, 2011), suggesting the actomyosin cable may also form a restraining barrier to
Figure 1.8: *Drosophila* dorsal closure (DC) is a model to study oscillatory apical constriction.

(A) During dorsal closure (DC), the epidermis closes over the amnioserosa cells. The amnioserosa apically constricts, pulling on the epidermis. Anterior is left, posterior is right. This is a view of the dorsal surface of the embryo. Red boxes are enlarged in (B). DC, dorsal closure.

(B) Actomyosin networks are pulsatile and periodic in the amnioserosa cells during early dorsal closure. By later stages of dorsal closure, actomyosin networks are more persistent and cover the apical surface of the amnioserosa. AS, amnioserosa; Epi, epidermis.

(C) The oscillatory constriction of amnioserosa cells occurs during early dorsal closure. By later stages of dorsal closure, the amplitude of surface area oscillations is dampened, concomitant with a persistent and sustained reduction in apical surface area.
ensure even movement of the epidermal cells. Endogenous actin-based protrusions from the dorsal-most epidermal LE ensure “zippering” or sealing of the epidermal sheets as they meet at the vertices of the dorsal opening (the “canthi”) (Jacinto et al., 2000), and ensure proper matching of body segments prior to sealing (Millard and Martin, 2008). In addition, the epidermal cells also elongate along the dorsal-ventral axis and appear to migrate collectively as a tissue over the amnioserosa (Jacinto et al., 2002; Laplante and Nilson, 2011). The dorsal-most epidermal cells are the first to elongate dorsally in an active process driven by signaling (Zahedi et al., 2008). The remaining ventrolateral epidermis is thought to largely resist the progression of dorsal closure; upon laser ablation, the ventrolateral epidermis initially recoils away from the injury site and the LE moves dorsally, indicating that not only is the ventrolateral epidermis under tension but that this tension hinders the dorsalward progression of the LE (Hutson et al., 2003; Kiehart et al., 2000). The amnioserosa cells also generate force through apical constriction, pulling on the neighbouring epidermis (Franke et al., 2005; Hutson et al., 2003; Kiehart et al., 2000). In addition, delamination and apoptosis of approximately 10% of amnioserosa cells also aids dorsal closure (Toyama et al., 2008). The individual forces generated by these processes are several orders of magnitude larger than the net force present during dorsal closure (Hutson et al., 2003; Kiehart et al., 2000); thus dorsal closure involves an integration of several forces at play.

Two main signal transduction pathways play major roles during dorsal closure, the c-Jun N-terminal Kinase (JNK) and transforming growth factor β (TGFβ) pathways. Much research has uncovered the signaling pathways mediating transcriptional control during dorsal closure. At first, JNK signalling occurs in the LE cells prior to germ band retraction (Fernández et al., 2007). JNK then activates transcription of decapentaplegic (Dpp, the *Drosophila* homolog of TGFβ), a diffusible ligand that mediates both autocrine and paracrine signalling (reviewed by Harden, 2002). Both JNK and Dpp increase actomyosin networks on a transcriptional level; JNK upregulates expression of profilin in the epidermis (Jasper et al., 2001) and Dpp upregulates expression of myosin heavy chain (*Drosophila* zipper) in both the amnioserosa and the epidermis, though the exact pathway is unknown (Zahedi et al., 2008). In addition, signalling from activated Cdc42-associated
kinase (ACK) increases zipper transcription (Zahedi et al., 2008), possibly by inhibiting epidermal growth factor receptor (EGFR) signalling (Shen et al., 2013). EGFR may impede progression of dorsal closure by transcriptional repression of zipper in the amnioserosa and dorsal most epidermis, and by suppression of apoptosis in the amnioserosa (Shen et al., 2013). In addition, a Dpp downstream target, the transcription factor U-shaped, must be expressed in both the epidermis and the amnioserosa to promote LE actomyosin cable formation, though the molecular mechanisms are unclear (Lada et al., 2012). Thus, during dorsal closure, multiple modes of transcriptional regulation are at play.

In addition to uncovering some of the signalling pathways upregulating transcription during dorsal closure, research has revealed some of the molecular players that have a more direct role. LE cells have upregulated expression of actomyosin components myosin (Zahedi et al., 2008) and profilin (Jasper et al., 2001) presumably to form the LE actomyosin cable during later dorsal closure (Fig. 1.8B). In addition to upregulation of myosin and actin regulators, formation of the LE cable is also thought to arise from interactions between the amnioserosa and the LE. Depletion of Echinoid (Ed, a member of the nectin and nectin-like immunoglobulin superfamily of adhesion molecules) and also Baz from the LE leads to formation of the LE cable (Laplante and Nilson, 2011), though the molecular mechanisms remain unclear. Rho-family GTPases also play a role during dorsal closure. Overexpression of dominant negative or constitutively-active RhoA, Rac1, or Cdc42 in the epidermis disrupts the LE actomyosin cable (Harden et al., 1999; Jacinto et al., 2000; Jacinto et al., 2002). Loss of function of Cdc42 or RhoA cause defects in overall dorsal closure (Genova et al., 2000; Jacinto et al., 2002; Magie et al., 1999).

In the amnioserosa, several proteins are known to be upregulated during dorsal closure. Zipper is transcriptionally upregulated in amnioserosa cells (Zahedi et al., 2008), and levels of activated phospho-myosin accumulate along the apical surface of amnioserosa cells during dorsal closure (Fig. 1.8B) (Blanchard et al., 2010). Interestingly, Crb has a role in amnioserosa contraction; it is present along the apical surfaces of amnioserosa cells (David et al., 2010) and Crb overexpression in the amnioserosa induces ectopic
contraction (Harden et al., 2002). Overexpression of Rac1 in the amnioserosa causes ectopic tissue contraction (Harden et al., 2002) suggesting a possible role for Rac1 in amnioserosa morphogenesis. In contrast, overexpression of either Cdc42 or RhoA in amnioserosa cells has no effect on amnioserosa constriction (Harden et al., 2002), but this may be due to different levels of expression of the various constructs. Indeed, the RhoA activator DRhoGEF2 has been recently implicated in regulating constriction of amnioserosa cells during dorsal closure (Azevedo et al., 2011), suggesting RhoA may be involved in amnioserosa apical constriction.

Recent studies have shown that the amnioserosa facilitates apical constriction with oscillatory contractions that develop into a more persistent constriction over time (Fig. 1.8C). Apical constriction of the amnioserosa is driven at first by pulsatile actomyosin networks (Blanchard et al., 2010; David et al., 2010; Solon et al., 2009). During later dorsal closure, amnioserosa cell oscillations are dampened in amplitude, coincident with fast apical constriction and increased apical surface phosphorylated myosin (Blanchard et al., 2010; David et al., 2010; Sokolow et al., 2012; Solon et al., 2009; Wang, Q. et al., 2012). Mathematical modeling has suggested that the actin cable “purse string” can act as an external, tissue-scale ratchet to stabilize local dorsal-ward migration of the epidermis and decrease amnioserosa cell oscillations (Solon et al., 2009). However, others have suggested that actomyosin within amnioserosa cells can act as internal, cellular-scale ratchets to stabilize cell contractions and eventually dampen oscillations (Blanchard et al., 2010; Wang, Q. et al., 2012). Recent mathematical modeling has also led to the hypothesis that the oscillations of actomyosin are modulated by a cytoskeletal regulator that itself oscillates with actomyosin-dependent oscillations (Wang, Q. et al., 2012). Thus, models have attempted to address the question of what mediates the transition of amnioserosa cell behaviour from oscillatory to more persistent, sustained constriction, though this remains unclear.
1.10 Outline and objectives

My thesis research has focused on pulsatile actomyosin networks found in the amnioserosa during Drosophila dorsal closure, and their interactions with the cell polarity regulators the PAR complex.

The first aim of my thesis has been to characterize the interactions between pulsatile actomyosin networks and the PAR complex found within the apical surfaces of amnioserosa cells during Drosophila amnioserosa apical constriction. It was previously thought that apical constriction is mediated by a contractile actomyosin circumferential belt-like structure associated with the zonula adherens, with constriction proceeding in a sarcomeric-like manner. However, recent results implicated apically-localized actomyosin meshworks as mediating apical constriction of Drosophila ventral furrow cells (Martin et al., 2009). During my research, I analyzed the relationship between actomyosin networks and the PAR complex during amnioserosa apical constriction during dorsal closure. I, along with Alisa Tishkina (a Thesis Honours Student), characterized the accumulation of actomyosin networks in the apical surfaces of amnioserosa cells. We found that actomyosin contractility in the amnioserosa is driven by cyclical assembly and disassembly of actomyosin networks. I also found that the PAR complex accumulated at the apical surfaces of amnioserosa cells during dorsal closure, in stark contrast to their colocalization with AJs in the bordering epidermis. Using dual live imaging, we noted that as the actomyosin networks assemble and disassemble, they translocate across persistent apical surface PAR complex puncta. To assess whether the apical PAR proteins interact with pulsatile apical actomyosin networks, I characterized myosin dynamics with PAR complex loss- and gain-of-function perturbations. I found that the PAR proteins separately regulate distinct aspects of actomyosin network dynamics – Baz regulates network duration whereas Par-6/aPKC inhibits network formation. My results, discussed in Chapter 2, characterize pulsatile apical actomyosin networks that mediate contraction of amnioserosa cells, and show that these networks are regulated by apically-localized PAR proteins.
To investigate the actomyosin networks further, the next aim of my thesis has been to characterize their shift in dynamics, and to identify cues mediating the shift of the PAR complex towards the apical surface. Recent investigations have shown that amnioserosa apical constriction transitions from highly pulsatile to more persistent, stabilized constriction during the transition from early to late dorsal closure (Solon et al., 2009; Blanchard et al., 2010; Sokolow et al., 2012). Since oscillatory networks are typically the result of delayed negative feedback, I sought to assess whether such regulation exists in the actomyosin networks of the amnioserosa during dorsal closure. I found that the actomyosin network inhibitors Par-6 and aPKC are recruited to the apical surface of amnioserosa cells during dorsal closure, and that aPKC recruits Baz to the apical surface. Baz is phosphorylated by aPKC, suggesting a dynamic interaction between these two proteins. To examine the significance of their dynamic interactions, I overexpressed a nonphosphorylatable Baz that has stabilized interactions with aPKC. This served to inhibit the antagonism of actomyosin networks by aPKC, suggesting that Baz can act as a competitive inhibitor of aPKC, though this would be a delayed inhibitor. I found that interactions between Par-6 and Baz increase during dorsal closure, suggesting an increase in the inhibition of aPKC during dorsal closure progression. To test whether tuning levels of delayed negative feedback can modulate actomyosin networks, we collaborated with Qiming Wang and Dr. James Feng (University of British Columbia) to construct a mathematical model of amnioserosa cell oscillation. Extending their previously-published model of cell behaviour during dorsal closure (Wang, Q. et al., 2012), we showed that decreasing the amount of delayed negative feedback can tune actomyosin networks towards stabilized and persistent networks. Together, these results, as discussed in Chapter 3, describe the interactions between apical actomyosin networks and the PAR proteins in the apical surface of the amnioserosa. My results also underscore the requirement of Baz – aPKC interactions for their localization and for dynamic regulation of aPKC activity utilizing the ability of Baz to act as a competitive inhibitor. Collectively, my research describes regulatory circuits involved in tuning actomyosin behaviour during development.
Chapter 2:  
The PAR complex regulates pulsed actomyosin contractions during amnioserosa apical constriction in *Drosophila*


**Contributions:**
I performed all experiments and quantifications, with the exception of the following contributions:

Alisa Tishkina quantified the dynamics parameters of actin and myosin probes during germ band retraction and mid-dorsal closure (Fig. 2.3 H-K, L-O, and contributed to Table 2.2). Alisa also performed dual-live imaging of moeABD::GFP and Arm::CFP, quantified their impact on cell diameter, and measured the directionality of actin network flows (Fig. 2.5).

Tony Harris calculated the Pearson correlation coefficients for protein colocalization (Table 2.1). Tony performed the genetic crosses to examine genetic interactions between myosin and the PAR complex (Fig. 2.2A). Tony also performed dual-live imaging of Baz::mCh with PAR-6::GFP, and of Baz::GFP with Sqh::mCh (Fig. 2.6)
2.1 Abstract

Apical constriction is a major mechanism underlying tissue internalization during development. This cell constriction typically requires actomyosin contractility. Thus, understanding apical constriction requires characterization of the mechanics and regulation of actomyosin assemblies. We have analyzed the relationship between myosin and the polarity regulators Par-6, aPKC and Bazooka (Par-3) (the PAR complex) during amnioserosa apical constriction at Drosophila dorsal closure. The PAR complex and myosin accumulate at the apical surface domain of amnioserosa cells at dorsal closure, the PAR complex forming a patch of puncta and myosin forming an associated network. Genetic interactions indicate that the PAR complex supports myosin activity during dorsal closure, as well as during other steps of embryogenesis. We find that actomyosin contractility in amnioserosa cells is based on the repeated assembly and disassembly of apical actomyosin networks, with each assembly event driving constriction of the apical domain. As the networks assemble they translocate across the apical patch of PAR proteins, which persist at the apical domain. Through loss- and gain-of-function studies, we find that different PAR complex components regulate distinct phases of the actomyosin assembly/disassembly cycle: Bazooka promotes the duration of actomyosin pulses and Par-6/aPKC promotes the lull time between pulses. These results identify the mechanics of actomyosin contractility that drive amnioserosa apical constriction and how specific steps of the contractile mechanism are regulated by the PAR complex.

2.2 Introduction

Epithelial morphogenesis is driven by various cellular processes, including changes to cell shape, cell-cell interactions and cell numbers. Polarized changes to cell shape and cell-cell interactions are often driven by actomyosin contractility generated by actin and non-muscle myosin II (henceforth myosin). Actomyosin contractility drives apical constriction and invagination of the vertebrate neural tube and the Drosophila ventral furrow (reviewed by Lecuit and Lenne, 2007) and cell ingression in C. elegans (reviewed by Cowan and Hyman, 2007). It also drives cell intercalation and convergent extension in the Drosophila germband (reviewed by Lecuit and Lenne, 2007; Zallen, 2007) and in
Xenopus (Skoglund et al., 2008). Additionally, supracellular actomyosin cables function in *Drosophila* dorsal closure (DC) (reviewed by Jacinto et al., 2002) and in *C. elegans* embryo elongation (reviewed by Simske and Hardin, 2001). To understand such morphological changes, we must define the mechanics of actomyosin assemblies and their regulation.

Par-6, aPKC and Bazooka (Baz; Par-3) are major regulators of apical polarity. They can function as a complex and also separately, with Par-6 and aPKC typically acting together and the scaffold protein Baz functioning apart (reviewed by Goldstein and Macara, 2007; Suzuki and Ohno, 2006). They regulate asymmetric cell divisions, the positioning of cellular junctions, cell migration and axon outgrowth (reviewed by Goldstein and Macara, 2007; Munro, 2006; Wiggin et al., 2005; Wodarz and Näthke, 2007), but few links to actomyosin contractility are known. In *C. elegans*, the PAR complex regulates apical myosin accumulation during cell ingression (Nance et al., 2003) and actomyosin flows in the one-cell embryo (Munro et al., 2004). In *Drosophila*, the PAR complex promotes apical myosin accumulation in egg chamber follicle cells (Wang and Riechmann, 2007). In these examples, PAR proteins and myosin localize to the same regions of the cell. By contrast, Baz and myosin have mutually exclusive planar polarized distributions in the *Drosophila* germband (Zallen and Wieschaus, 2004).

The *Drosophila* amnioserosa provides an excellent model of tissue morphogenesis. It undergoes two types of morphogenesis during embryogenesis. First, the squamous tissue forms from a columnar epithelium at gastrulation. During this process, Baz and myosin display the same, reciprocal, planar polarized pattern as in the germband, but are gradually lost from the cortex as the amnioserosa flattens (Pope and Harris, 2008). The loss of apical contractility appears to allow microtubules and other factors to extend and flatten the apical domain, generating a squamous epithelium on the dorsal surface of the embryo during gastrulation (Pope and Harris, 2008). The second major change to the amnioserosa occurs at DC, when the surrounding epidermis closes over the amnioserosa, which is internalized and degraded. The amnioserosa functions with the epidermis to drive DC (Kiehart et al., 2000). As the process begins, amnioserosa cells undergo rounds of constriction and relaxation (Solon et al., 2009). The cells display apical myosin, and
expressing myosin solely in the amnioserosa of myosin mutants is sufficient to rescue amnioserosa cell constriction and overall DC (Franke et al., 2005). The epidermis normally also plays a role, forming a supracellular ring of actomyosin that surrounds the amnioserosa and promotes closure (Franke et al., 2005; Kiehart et al., 2000; Rodriguez-Diaz et al., 2008; Solon et al., 2009). The leading edge actomyosin ring is well studied (reviewed by Harden, 2002; Jacinto et al., 2002), but the mechanics and regulation of amnioserosa actomyosin contractility are ill defined.

We analyzed associations between the PAR complex and myosin during amnioserosa morphogenesis at DC. We find that amnioserosa apical constriction is based on the repeated assembly and disassembly of apical actomyosin networks. As the networks form, they interact transiently with an apical PAR protein patch. Remarkably, different PAR proteins regulate distinct phases of the actomyosin assembly/disassembly cycle.

2.3 Materials and Methods

2.3.1 Fly stocks

zip::GFP and baz::GFP were GFP gene traps into endogenous loci (FlyTrap, flytrap.med.yale.edu). MoeABD-GFP (Kiehart et al., 2000), sqh-GFP in a null sqh mutant background (Royou et al., 2002), sqh::mCherry (Martin et al., 2009), UAS-par-6 (Pinheiro and Montell, 2004), UASaPKC::CAAX (Sotillos et al., 2004) and UAS-baz (M. Pellikka and U. Tepass, University of Toronto, Canada) were gifts. UAS-par-6::GFP was generated with standard molecular methods and inserted into the genome by P-element insertion (Genetic Services). arm::CFP and UAS-baz::mCherry were generated previously (McGill et al., 2009; Pope and Harris, 2008). For visualization, UAS constructs were expressed zygotically using Actin-5CGAL4 [Bloomington Drosophila Stock Center (BDSC) #3954]. For perturbation studies, UAS constructs were specifically expressed in the amnioserosa using 332.3-GAL4 (BDSC). bac^XII06 mutants (A. Wodarz, University of Göttingen, Germany) and par-6^A226 and apkc^X06403 mutants (C. Doe, University of Oregon, USA) were gifts. zip^1 and zip^2 mutants were from the BDSC. yellow white was used as wild type.
2.3.2 Cuticle preparations

Embryos were collected for 24 hours (25°C), removed from adults and allowed to develop for another 48 hours. Unhatched embryos were washed and dechorionated with 50% bleach, mounted on slides with Hoyer’s mountant:lactic acid (1:1) and baked overnight (60°C).

2.3.3 Embryo staining

Embryos were fixed for 10 minutes in 1:1 10% formaldehyde in PBS:heptane, devitellinized by hand peeling and stained with phalloidin. For other stainings, embryos were fixed in 1:1 3.7% formaldehyde in PBS:heptane for 20 minutes and devitellinized in methanol. Blocking and staining were in PBS containing 1% goat serum and 0.1% Triton X-100. Primary antibodies used were: mouse anti-Arm [1:500; Developmental Studies Hybridoma Bank (DSHB)] and anti-Crb (1:500; DSHB); rabbit anti-Baz (1:3500); and anti-aPKC (1:1000; Santa Cruz Biotechnology); and rat anti-DE-Cad (1:100; DSHB) and anti-Par-6 (1:100; C. Doe). F-actin was stained with Alexa Fluor 568-conjugated phalloidin (1:200; Invitrogen). Secondary antibodies were conjugated with Alexa Fluor 488, 546 and 647 (Invitrogen).

2.3.4 Imaging

Fixed embryos were mounted in Aqua-Poly/Mount (Polysciences) and imaged with a LSM 510 confocal microscope (Carl Zeiss) at room temperature with 40× (Plan-NeoFluor, NA 1.3) and 63× (Plan-Apochromat, NA 1.4) objectives and LSM 510 AIM software. z-stacks had a 0.3 μm step size.

For live imaging, dechorionated embryos were mounted in halocarbon oil (series 700; Halocarbon Products) on petriPERM dishes (Sigma) and imaged with a Quorum spinning disk confocal system (Quorum Technologies) at room temperature with a 63× (Plan-Apochromat, NA 1.4) objective, a piezo top plate, a Hamamatsu EM CCD camera and using Volocity software (Improvision). z-stacks had a 0.3 μm step size. Autofluorescent egg shell vitelline membrane served as a marker for the apical surface of the cells lying just beneath. For quantifying DC rates, embryos were glued to coverslips, covered with
halocarbon oil and imaged as above but with halocarbon oil exposed to air and with a 20× (Plan-Apochromat, NA 0.8) objective and 2 μm step sizes.

For post-acquisition image analysis and manipulation, maximum intensity projections were performed with Volocity software. Three-dimensional reconstructions were with Imaris software (Bitplane). To quantify colocalization, the apical domain was cropped to the region where proteins were enriched, and the minimum and maximum intensity values were adjusted for each channel to include only the top half of the original values (ImageJ) and the Colocalization Test plug-in was applied (ImageJ). For figure preparation, input levels were adjusted in Adobe Photoshop so that the main signal range spanned the full output grayscale, and image resizing was by bicubic interpolation (minimal changes occurred at normal magnifications).

2.3.5 Statistics
Comparisons were made using unpaired two-tailed t-tests in Excel (Microsoft).

2.4 Results
2.4.1 A PAR protein patch forms at the apical surface of amnioserosa cells at dorsal closure
To characterize Par-6, aPKC and Baz localization in the amnioserosa after gastrulation, we immunostained wild-type Drosophila embryos at full germband extension, germband retraction and DC. As seen previously (Pope and Harris, 2008; Wodarz et al., 2000), Par-6, aPKC and Baz were present at lower levels at the apical circumference of amnioserosa versus epidermal cells at full germband extension (stages 9-11) (Fig. 2.1A shows aPKC), in contrast to adherens junctions (AJs), which had comparable apical circumferential levels in each tissue [Fig. 2.1A shows Armadillo (Arm)]. From germband retraction into DC, Par-6, aPKC and Baz accumulated in a patch at the central apical surface of amnioserosa cells (Fig. 2.1A, arrows) and also localized around the apical circumference. AJ proteins Arm and DE-Cadherin (DE-Cad; the gene product of shotgun) were absent from these apical surface patches (Fig. 2.1A). By late DC, Par-6, aPKC and Baz covered a large proportion of the remaining apical surface (Fig. 2.1A, brackets). Thus, Par-6,
Figure 2.1: The PAR complex at the apical surface of amnioserosa cells.

(A) aPKC and Arm immunostaining of Drosophila embryos from stage 11 to dorsal closure (DC). Amnioserosa at top, epidermis below. Note the localization of aPKC at the apical surface (arrows/brackets).

(B-D) Single amnioserosa cells at DC stained for PAR proteins (B, Par-6; C, aPKC; D, Baz) and Crb (B-D) in Sqh::GFP embryos. Colocalization of PAR protein with Crb is bracketed. Note the lack of local colocalization of PAR protein and myosin (Sqh, arrows).
aPKC and Baz relocalize to a specific patch at the apical surface of amnioserosa cells at germband retraction and DC.

2.4.2 The apical PAR protein patch associates with Crumbs and a myosin network

To assess what complexes Par-6, aPKC and Baz might form within the apical domain, we localized each protein along with the apical transmembrane protein Crumbs (Crb) (by immunostaining) and Spaghetti squash (Sqh) (by Sqh::GFP fluorescence) (Sqh encodes non-muscle myosin II regulatory light chain). High-magnification imaging revealed that the PAR protein patches include small puncta (Fig. 2.1B-D, brackets). Par-6 and Crb often displayed a high degree of colocalization at these puncta (Fig. 2.1B). aPKC and Baz only occasionally showed this high degree of colocalization with Crb (Fig. 2.1C,D), suggesting fixation artifacts or more dynamic interactions. Comparisons with Sqh::GFP showed that the patches of Crb, Par-6, aPKC and Baz overlapped with apical surface myosin networks, but there was little colocalization between specific puncta and nodes of the myosin networks (Fig. 2.1B-D, arrows). The PAR protein patches and myosin networks also had different overall shapes (Fig. 2.1B-D). Quantification of where the PAR protein patches and myosin networks overlapped showed that each PAR protein distribution had a significantly higher correlation with Crb than with the distribution of Sqh::GFP, although colocalization with Crb varied (Table 2.1). Thus, the PAR protein patches are closely linked with Crb and appear less directly associated with myosin networks.

2.4.3 Components of the PAR complex support myosin activity

Since amnioserosa myosin activity contributes to DC (Franke et al., 2005), we hypothesized that the PAR proteins support this activity. To test functional interactions with myosin and a role for PAR proteins in DC, we examined the terminal embryonic phenotypes of mutants using cuticle preparations. Zygotic single mutants for zip¹ (zipper encodes non-muscle myosin II heavy chain) are embryonic lethal and display a prominent dorsal hole due to failed DC (Fig. 2.2A, outlined). By contrast, zygotic single mutants for apkc¹⁰⁶⁴⁰³ and par-6¹²²⁶ complete embryogenesis due to maternally inherited gene
Table 2.1: Pearson correlation coefficients for protein colocalization at the apical surface.

<table>
<thead>
<tr>
<th></th>
<th>Crb</th>
<th>Sqh::GFP</th>
<th>P-value</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Par-6</td>
<td>0.64±0.13</td>
<td>0.42±0.18</td>
<td>4.24×10⁻⁸</td>
<td>35</td>
</tr>
<tr>
<td>Crb</td>
<td>0.39±0.15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aPKC</td>
<td>0.53±0.15</td>
<td>0.45±0.17</td>
<td>0.0423</td>
<td>29</td>
</tr>
<tr>
<td>Crb</td>
<td>0.36±0.14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baz</td>
<td>0.36±0.18</td>
<td>0.22±0.16</td>
<td>0.00882</td>
<td>18</td>
</tr>
<tr>
<td>Crb</td>
<td></td>
<td>0.27±0.14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actin (phalloidin)</td>
<td>−</td>
<td>0.70±0.10</td>
<td>−</td>
<td>30</td>
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</table>

<table>
<thead>
<tr>
<th></th>
<th>Sqh::mCherry</th>
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</thead>
<tbody>
<tr>
<td>Baz::GFP (live)</td>
<td>−</td>
</tr>
<tr>
<td>Par-6::GFP</td>
<td>−</td>
</tr>
</tbody>
</table>

<table>
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<tr>
<th></th>
<th>Baz::mCherry</th>
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</thead>
<tbody>
<tr>
<td>Baz::GFP (live)</td>
<td>0.42±0.13</td>
</tr>
<tr>
<td>Par-6::GFP (live)</td>
<td>−</td>
</tr>
</tbody>
</table>

Columns 2 and 3 show mean ± s.d.
Figure 2.2: Genetic interactions between PAR complex and myosin mutants.

(A) *Drosophila zip*\(^1\) single-mutant cuticle with large dorsal hole (yellow dotted outline). Double and triple mutants with *par-6* and *aPKC* alleles showing dorsal holes merged with head holes (arrows).

(B) *zip*\(^2\) single-mutant cuticle compared with *par-6; zip*\(^2\) double-mutant cuticle with combined dorsal and head holes (outlined).

(C) *baz* single-mutant cuticle compared with *baz* single-mutant with half the dosage of *zip*, showing a combined dorsal and head hole (outlined) and ventral hole (arrow).

(A’-C’) Quantification of the embryonic lethality associated with the hole phenotypes for each genotype examined.
product. However, \textit{apkc}^{606403}, \textit{zip}^1 and \textit{par-6}^{44226}; \textit{zip}^1 double mutants displayed a prominent dorsal hole merged with a large head hole (Fig. 2.2A, overall holes outlined, arrows mark head holes), indicating enhancement of the \textit{zip}^1 phenotype. \textit{par-6}^{44226}; \textit{apkc}^{606403}, \textit{zip}^1 triple mutants showed similar enhancement (Fig. 2.2A). All of the dead embryos from heterozygous parents were analyzed and scored (Fig. 2.2A'). Thus, Par-6 and aPKC support myosin activity, but specific effects on DC could not be assessed because of the severity of the \textit{zip}^1 DC phenotype.

To assess effects on DC, we tested interactions between single mutants that lack DC phenotypes on their own. \textit{zip}^2 zygotic single mutants lacked dorsal holes (Fig. 2.2B). By contrast, \textit{par-6}^{A226}; \textit{zip}^2 double mutants displayed merged dorsal and head holes (Fig. 2.2B, outlined). All of the dead embryos from doubly heterozygous parents were analyzed and scored (Fig. 2.2B'). We also tested \textit{baz}^{Xi106} zygotic single mutants, most of which lacked dorsal holes (Fig. 2.2C). Reducing the dosage of \textit{zip} by half in these mutants enhanced the phenotype, generating merged dorsal and head holes (Fig. 2.2C, outlined) and ventral holes (Fig. 2.2C, arrow; quantified in Fig. 2.2C'). Thus, genetic interactions between PAR complex components and myosin affect DC and the morphogenesis of other tissues.

### 2.4.4 Apical actin and myosin networks undergo repeated assembly/disassembly cycles in amnioserosa cells

We pursued how the PAR complex interacts with myosin in the amnioserosa at DC. First, we needed to characterize the development and activity of the myosin networks. We analyzed their development by live imaging at full germband extension, germband retraction and DC. A gene-trap line with GFP inserted into the \textit{zip} locus (Zip::GFP) revealed little cortical myosin in the amnioserosa at full germband extension (Fig. 2.3A), consistent with previous observations (Pope and Harris, 2008). However, myosin networks began assembling and disassembling sporadically across the amnioserosa during germband retraction (Fig. 2.3B, arrows), both apically and basally. By DC, all cells displayed repeated cycles of myosin network assembly and disassembly (Fig. 2.3C, arrows; see Movie 2.1 in Appendix 1) that were restricted to the apical domain. Sqh::GFP displayed similar developmental changes and network behavior (data
Figure 2.3: Pulsing actin and myosin networks develop at the apical surface of amnioserosa cells

(A-F) Live imaging of *Drosophila* embryos from germband extension to DC. Arrows mark networks. (A-C) Zip::GFP. (D-F) MoeABD::GFP. (G) Fixed amnioserosa cells at mid-DC. The colocalization of Sqh::GFP with phalloidin (F-actin) at networks is indicated by arrows.

(H,I) Zip::GFP (H, arrow) and MoeABD::GFP (I, arrow) network dynamics at germband retraction. Note the cell protrusions from the MoeABD::GFP network (yellow arrows). s, seconds.

(J,K) Zip::GFP (J, arrow) and MoeABD::GFP (K, arrow) network dynamics at mid-DC.

(L-O) Quantification of MoeABD::GFP, Zip::GFP and Sqh::GFP network durations (L,N) and lull times between pulses (M,O) at germband retraction (L,M) and mid-DC (N,O).

(P-Q) Quantification of Sqh::GFP network dynamics (P,Q) at late DC in central versus canthi cells, as illustrated in P'.

61
Germ Band Extended  Germ Band Retraction  Mid-Dorsal Closure

A  B  C
Zip::GFP  Zip::GFP  Zip::GFP

D  E  F
MoeABD::GFP  MoeABD::GFP  MoeABD::GFP

G
Sphx::GFP  Phalloidin

H  I  J
Zip::GFP  245s  252s
MoeABD::GFP

K
Zip::GFP  176s  129s
MoeABD::GFP

L  M
Germ Band Retraction
MoeABD::GFP  Zip::GFP  Sphx::GFP

N  O
Mid-Dorsal Closure
MoeABD::GFP  Zip::GFP  Sphx::GFP

P  Q
Late Dorsal Closure
Sphx::GFP in Control Cells

P'  Q'
Sphx::GFP in Control Cells

Percentage of Cells

Pulse Duration (s)

Time Between Pulses (s)
not shown). To probe for F-actin, we imaged the F-actin-binding domain of Moesin (Moe) fused to GFP (MoeABD::GFP). MoeABD::GFP localized to circumferential cell protrusions at each developmental stage, but only displayed pulsing apical surface networks at germband retraction and DC (Fig. 2.3D-F, arrows). Thus, apical actin and myosin networks form with the same developmental timing as the apical PAR protein patch, and display a pulsing behavior.

To assess the link between the actin and myosin networks, we first compared them in fixed tissues. Dual imaging of Sqh::GFP and phalloidin staining (for F-actin) revealed colocalization in amnioserosa apical surface networks at DC (Fig. 2.3G, arrows; Table 2.1). Next, we compared their behavior using live imaging. At germband retraction, Zip::GFP networks were relatively small but moved along the long axis of the cell (Fig. 2.3H, arrow). They had durations of 121.3±18.0 (mean ± s.d.) seconds (5 embryos, 3-9 events from 2-5 cells, averaged per embryo), with highly variable lull times between pulses (258.1±160.1 seconds; 5 embryos, 4-6 events from 2-5 cells) (Fig. 2.3M; Table 2.2). Sqh::GFP networks had statistically indistinguishable durations (113.4±32.1 seconds; 5 embryos, 3-9 events from 3-4 cells) (Fig. 2.3L) and lull times (287.1±158.0 seconds; 5 embryos, 7-12 events from 2-4 cells) (Fig. 2.3M). F-actin networks appeared larger (Fig. 2.3I, white arrow), and as they moved along the length of the cell they often formed lamellipodia-like protrusions that extended over neighboring cells (Fig. 2.3I, yellow arrows). Moreover, the actin network durations were significantly longer than both the Zip::GFP and Sqh::GFP networks at this stage (237.4±44.2 seconds; 6 embryos, 4-5 events, each from different cells; P<0.01) (Fig. 2.3L), but lull times were statistically indistinguishable from the myosin networks and highly variable (200.0±228.3 seconds; 6 embryos, 4-5 events, each from different cells) (Fig. 2.3M). Thus, the actin and myosin networks have distinct properties as they first form at germband retraction; the myosin networks are presumably linked to actin, but perhaps only for a portion of an actin network lifetime.

Next, we compared the actin and myosin networks at DC. At mid-DC (with the epidermal leading edge taut, but before epidermal zippering), each network typically traversed the apical cell surface as they assembled and disassembled, but none converted into cell

63
Table 2.2: Actin and myosin network dynamics at germband retraction and mid-dorsal closure.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Germband retraction</th>
<th></th>
<th>Mid-dorsal closure</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Durations</td>
<td>Lull times</td>
<td>Durations</td>
<td>Lull times</td>
</tr>
<tr>
<td>Zip::GFP</td>
<td>121.3±18.0</td>
<td>258.1±160.1</td>
<td>174.0±64.4</td>
<td>81.1±39.7</td>
</tr>
<tr>
<td>Sqh::GFP</td>
<td>113.4±32.1</td>
<td>287.1±158.0</td>
<td>123.7±32.5</td>
<td>113.8±49.4</td>
</tr>
<tr>
<td>MoeABD::GFP</td>
<td>237.4±44.2</td>
<td>200.0±228.3</td>
<td>145.8±23.1</td>
<td>102.0±53.9</td>
</tr>
</tbody>
</table>

Values are in seconds (mean ± s.d.).
protrusions (Fig. 2.3J,K). The Zip::GFP and Sqh::GFP networks became larger (Fig. 2.3J), and the duration of the Zip::GFP networks increased compared with that at germband retraction: 174.0±64.4 seconds (9 embryos, 4-5 events, each from different cells) at DC versus 121.3±18.0 seconds at germband retraction (Fig. 2.3N versus 2.3L; Table 2.2) (P=0.044). By contrast, the MoeABD::GFP network durations decreased compared with those at germband retraction: 145.8±23.1 seconds (9 embryos, 4-5 events, each from different cells) at DC versus 237.4±44.2 seconds at germband retraction (Fig. 2.3N versus 2.3L) (P<0.01) and, as a result, overlapped with the Zip::GFP network durations (Fig. 2.3N). The Sqh::GFP duration times only displayed a slight and statistically insignificant increase at DC (123.7±32.5 seconds; 8 embryos, 4-6 events, each from different cells) versus germband retraction (113.4±32.1 seconds). Nonetheless, the Sqh::GFP, Zip::GFP and MoeABD::GFP duration times were statistically indistinguishable at DC. The lull times between pulses were also statistically indistinguishable among MoeABD::GFP (102.0±53.9 seconds; 5 embryos, 3-5 events, each from different cells), Zip::GFP (81.1±39.7 seconds; 6 embryos, 4-5 events, each from different cells) and Sqh::GFP (113.8±49.4 seconds; 8 embryos, 3-6 events, each from different cells), and these times were less variable than at germband retraction (Fig. 2.3O versus 2.3M). Thus, the actin and myosin networks (hereafter referred to as actomyosin networks) become closely entrained into repeated assembly/disassembly cycles at the apical surface of amnioserosa cells at DC.

To complete the developmental analysis, we analyzed Sqh::GFP at the zippering stage of DC, when cells at the canthi (the corners of the eye-shaped amnioserosa) have a much smaller apical circumference than central amnioserosa cells (Fig. 2.3P'). Remarkably, network durations and lull times between pulses were indistinguishable between the cells (Fig. 2.3P,Q). However, relative to mid-DC, network durations were slightly shorter: 114.4±14.4 seconds for central cells (6 embryos, 7-24 events from 4-5 cells) (P=0.14) and 100.1±16.7 seconds for canthi cells (5 embryos, 8-23 events from 3-5 cells) (P=0.03); and lull times between pulses were substantially shorter: 33.4±30.6 seconds for central cells (6 embryos, 10-27 events from 4-5 cells) (P<0.01) and 30.4±18.0 seconds for canthi cells (5 embryos, 8-21 events from 3-5 cells) (P<0.01). Thus, network dynamics change with
development, but at any given developmental stage network dynamics are apparently independent of apical area. All analyses below are at mid-DC.

To confirm where the networks form in the cell, we analyzed three-dimensional reconstructions of single cells expressing Sqh::GFP. Top views revealed network formation and side views revealed formation specifically in the apical domain (Fig. 2.4A, arrows). To understand how the networks assemble, we analyzed kymographs of single Sqh::GFP networks. This showed individual nodes of the networks growing in intensity at different times (Fig. 2.4B, arrows). These nodes could form by the local rearrangement of a diffuse pre-existing network, the recruitment of diffusible subunits, or both. To test whether local changes could solely account for node growth, we photobleached the centers of Sqh::GFP networks as they neared full constriction. We detected recovery of fluorescent nodes evenly across the photobleached regions (Fig. 2.4C, arrows), indicating Sqh::GFP recruitment from other parts of the cell. Thus, assembling myosin networks are likely to incorporate diffusible subunits, although some incorporation of pre-existing networks cannot be excluded.

2.4.5 The pulsing actomyosin networks drive pulsed apical constriction

To assess whether the apical surface actomyosin networks drive amnioserosa apical constriction, we tested how network assembly and disassembly affect the apical cell circumference. Dual live imaging of MoeABD::GFP and Arm::CFP showed that apical actin network assembly coincides with cell constriction, and that network disassembly coincides with cortical relaxation (Fig. 2.5A; see Movie 2.2 in the supplementary material). Constriction and relaxation occurred specifically at the level of AJs (Fig. 2.5A, white arrows) and were significantly reduced 1.5 μm below (Fig. 2.5A, yellow arrows; Fig. 2.5B shows quantification of cortical constriction and relaxation at both levels). Thus, actomyosin network assembly and disassembly are linked to apical constriction and relaxation, respectively. However, for a single cycle, the degree of apical constriction (a 21.7±7.2% change in cell diameter; 23 events, one cell each from 5 embryos) was followed by an indistinguishable degree of apical relaxation (a 19.7±9.2% change in cell diameter). Overall tissue constriction must therefore be gradual.
Figure 2.4: Assembly of apical myosin networks.

(A) Three-dimensional reconstructions of Sqh::GFP in a single *Drosophila* amnioserosa cell during network assembly and disassembly. Note network assembly at the apical surface (arrows).

(B) Kymograph of Sqh::GFP network showing individual node growth (arrows). Grayscale images, left; RGB rainbow, right.

(C) Photobleached center of a Sqh::GFP network. Note the relatively uniform recovery of node fluorescence (arrows).
**Figure 2.5: The networks mediate apical constriction.**

(A) Live dual imaging of *Drosophila* amnioserosa cells. Top and middle rows show the same apical focal plane. Note cell constriction at adherens junctions (AJs) (Arm::CFP; arrows) with actin network (MoeABD::GFP). Bottom row shows a focal plane 1.5 μm below, where the cortex, as marked by MoeABD::GFP, is less responsive (yellow arrows).

(B) Quantification of the change in cell diameter in response to actin networks at AJs versus the same position 1.5 μm below (n=23 events, each from different cells, from 5 embryos). Cell diameters were measured along the most-affected cell axis. Asterisks indicate significant differences (P<0.01).

(C) Live imaging of MoeABD::GFP network disassembly in one cell (white arrows) followed by network assembly in a neighbor (yellow arrows).

(D,E) Scheme for (D), and results of (E), quantifying which neighboring cell assembles a network after network disassembly at the indicated cell contact (D, arrow) (n=28 events from 8 embryos).

(F) Network translocation angles relative to the anteroposterior embryo axis (n=92 events from 5 embryos for anterior amnioserosa; n=98 events from 5 embryos for posterior amnioserosa).
Intriguingly, the assembly and disassembly of a network in one cell was often followed by the assembly of a network in a neighboring cell (Fig. 2.5C, white and yellow arrows): 30/66 networks analyzed were followed by a neighboring cell network at the next time point collected (8 MoeABD::GFP embryos scored; time points were separated by 4.84-20.21 seconds). To test whether this was non-random, we quantified which neighboring cell formed a network relative to the cell-cell contact where the original network terminated. Analyzing cell arrangements with the original network in a central cell surrounded by six neighbors, the neighbor at the contact where the network terminated was termed ‘Cell 1’, and neighbors further away were termed ‘Cells 2-4’ (Fig. 2.5D). The neighboring networks preferentially formed next to where the original network terminated (Cell 1), and less frequently in more distant neighbors (Fig. 2.5E). Since this network propagation might coordinate networks across the amnioserosa, we examined the orientation of network movements relative to the embryonic axes. We analyzed cells at both the anterior and posterior regions of the amnioserosa because they have different cell shapes (Gorfinkiel et al., 2009). For both regions, the orientation of network movement was biased towards the dorsoventral (D-V) axis of the embryo (Fig. 2.5F). Thus, actomyosin activity may propagate between cells to coordinate amnioserosa tissue constriction.

2.4.6 The actomyosin networks interact transiently with the apical PAR protein patch

To assess how PAR proteins could affect myosin, we used live imaging to evaluate PAR protein dynamics in amnioserosa cell apical domains. For Baz, we imaged a gene-trap line with GFP inserted at the baz locus (Baz::GFP). For Par-6, we generated a fly line with inducible Par-6::GFP. Both Baz::GFP and Par-6::GFP formed a central patch of puncta at the apical surface of amnioserosa cells at DC (Fig. 2.6A,B, bracketed) plus circumferential localization. In each case, although the patch showed some movement, it persisted at the apical surface (Fig. 2.6A,B). Dual live imaging of overexpressed Par-6::GFP and Baz::mCherry confirmed colocalization of these proteins in puncta at the apical surface (Fig. 2.6C, arrows), although it was only partial (Table 2.1). Dual live
Figure 2.6: Comparison of PAR complex and myosin dynamics.

(A) *Drosophila* amnioserosa cells showing localization of Baz::GFP. Apical surface puncta are bracketed.

(B) Par-6::GFP. Apical surface puncta are bracketed.

(C) Dual live imaging of Baz::mCherry and Par-6::GFP. Some apical surface puncta colocalize (arrows).

(D) Dual live imaging showing how the Sqh::mCherry network translocates across the Baz::GFP patch (arrows). The Baz::GFP patch is present before and after the network assembles (brackets).
imaging of Baz::GFP and Sqh::mCherry showed the presence of apical Baz::GFP before and after myosin networks formed (Fig. 2.6D, brackets). Network assembly sites did not correlate with the Baz::GFP patch, but the networks inevitably traversed the patches because they covered much of the apical surface (Fig. 2.6D, arrow marks myosin network; see Movie 2.3 in the supplementary material). Dual live imaging of Par-6::GFP and Sqh::mCherry showed a similar relationship. Quantification revealed low local colocalization of Baz::GFP or Par-6::GFP with Sqh::mCherry when the PAR protein patches and myosin networks overlapped in the apical domain (Table 2.1). Thus, the apical PAR complexes are not core components of the actomyosin networks. Instead, they persist at the apical domain and associate transiently with the pulsing networks.

2.4.7 Baz and Par-6/aPKC affect different phases of the actomyosin assembly/disassembly cycle

To test how PAR complex components affect the actomyosin networks, we first imaged Zip::GFP in zygotic homozygous mutants for bazXi106, par-64226 or apkcK06403 at mid-DC. Networks formed of apparently normal size and structure in each case (Fig. 2.7A,B,D,E). Since Baz protein is undetectable in bazXi106 zygotic mutants at this stage (Tanentzapf and Tepass, 2003) (our unpublished observations), network formation might be independent of Baz. We cannot conclude the same for Par-6 or aPKC because of the maternally inherited gene product present in par-6 and aPKC mutants (Rolls et al., 2003 and our unpublished results). We noted 8/21 baz mutant embryos and 5/16 par-6 mutant embryos with amnioserosa morphological defects in Zip::GFP live imaging (Fig. 2.7C,F, yellow arrows). Myosin networks still formed (Fig. 2.7C,F, white arrows) and pulsed, but to discount possible non-specific effects of tissue disruption we excluded these embryos from the analyses below. No aPKC mutants displayed amnioserosa morphological defects.

To test whether the networks pulse normally in the mutants we quantified pulse durations, lull times between pulses and overall pulse frequencies. Duration times in par-6 and aPKC mutants were indistinguishable from that of controls with equal dosage of
Figure 2.7: Effects of PAR complex loss of function on myosin.

(A-F) Zip::GFP localization in *Drosophila par-6* (B,C), *aPKC* (D) and *baz* (E,F) single zygotic mutants compared with control with equal Zip::GFP dosage (A). White arrows indicate myosin networks. Yellow arrows indicate myosin fibers associated with tissue holes (outlined).

(G-I) Comparison of Zip::GFP network durations (G), lull time between pulses (H) and pulses per 10 minutes (I) between *par-6*, *aPKC* and *baz* single zygotic mutants and control. For each bar chart, each color describes the distribution of one embryo and the black line is the average distribution of all embryos. Asterisks indicate significant differences (P<0.05).

(J-L) DE-Cad and Dlg in *aPKC* (K) and *baz* (L) single zygotic mutants and control (J).
Zip::GFP, but baz mutants had significantly shorter duration times (117.9±11.4 seconds; 8 embryos, 11-30 events from 6-10 cells) than controls (153.0±19.7 seconds; 7 embryos, 13-28 events from 5-12 cells) (P<0.01) (Fig. 2.7G). By contrast, par-6 and baz mutant lull times were indistinguishable from those of controls, but aPKC mutant lull times were significantly shorter (39.0±31.4 seconds; 7 embryos, 7-30 events from 3-9 cells) than in controls (110.3±48.2 seconds; 7 embryos, 11-33 events from 5-12 cells) (P<0.01) (Fig. 2.7H). Pulses per 10 minutes for par-6 and baz mutants were also indistinguishable from those of controls, but aPKC mutants had significantly more frequent pulses (3.98±1.09 pulses per cell per 10 minutes; 7 embryos, 3-9 cells) than controls (2.65±0.55 pulses per cell per 10 minutes; 6 embryos, 7-14 cells) (P=0.0196) (Fig. 2.7I). These results suggest a separation of PAR protein function, with Baz promoting pulse durations and aPKC promoting lull times between pulses. Importantly, aPKC mutants had normal apicobasal polarity and AJs [Discs large (Dlg) and DE-Cad staining; 12/12 embryos analyzed; Fig. 2.7K] as in controls (6/6 embryos analyzed; Fig. 2.7J). Consistent with our Zip::GFP live imaging, 4/13 baz mutants showed amnioserosa morphology defects with DE-Cad and Dlg staining, but baz mutants with normal morphology had normal apicobasal polarity and AJs (Fig. 2.7L). Thus, the effects on myosin occur without general epithelial defects.

Separation of function suggests separate PAR protein localization mechanisms. Indeed, in baz zygotic mutants, we detected aPKC apically in both epidermal and amnioserosa cells despite undetectable Baz. Thus, aPKC might be able to regulate lull times without Baz. We detected both Baz and aPKC in aPKC mutants, preventing conclusions about Baz function in the absence of aPKC. Quantification of these proteins is described further in Chapter 3.

Next, we evaluated Zip::GFP with PAR gene overexpression targeted to the amnioserosa. Overexpressing untagged Par-6 or membrane-targeted aPKC (aPKC-CAAX) had no significant effects alone (Fig. 2.8A-C). However, co-overexpressing Par-6 plus aPKC-CAAX significantly reduced network pulse frequency (2.05±0.52 pulses per cell per 20 minutes; 5 embryos, 7-11 cells) versus controls with equal dosage of Zip::GFP (5.81±2.11 pulses per cell per 20 minutes; 5 embryos, 5-11 cells) (P=0.014) (Fig. 2.8C).
Figure 2.8: Effects of PAR complex gain of function on myosin.

(A-C) Zip::GFP network durations (A), lull times between pulses (B) and pulses per 10 minutes (C) in *Drosophila* embryos overexpressing Par-6, aPKC-CAAX, Par-6 plus aPKC-CAAX, or Baz, compared with control with equal Zip::GFP dosage. For each bar chart, each color describes the distribution of one embryo and the black line is the average distribution of all embryos. Asterisks indicate significant differences (P<0.05).

(D-F) Zip::GFP localization with Par-6 plus aPKC-CAAX overexpression (E) or Baz overexpression (F) compared with control (D). White arrows indicate myosin networks. Cells with reduced myosin levels are outlined.

(G-I) DE-Cad (green) and Dlg (red) with Par-6 plus aPKC-CAAX overexpression (H) or Baz overexpression (I) compared with control (G).
Lull times were also significantly higher than in controls (P=0.04), and had a wide variation (Fig. 2.8B). However, pulse durations were statistically indistinguishable from those of controls (Fig. 2.8A).

Next, we overexpressed untagged Baz. This significantly increased duration times (189.8±17.1 seconds; 6 embryos, 22-60 events from 6-13 cells) versus controls (108.3±31.2 seconds; 5 embryos, 9-45 events from 5-12 cells) (P<0.01) (Fig. 2.8A) and decreased lull times between pulses (28.7±14.2 seconds; 6 embryos, 22-60 events from 6-13 cells) versus controls (108.3±31.2 seconds; 5 embryos, 9-45 events from 5-12 cells) (P=0.04) (Fig. 2.8B), and, as a result, had no statistically significant effect on pulse frequency versus controls (Fig. 2.8C). Myosin networks were of apparently normal size and structure with Par-6 plus aPKC-CAAX overexpression or Baz overexpression as compared with controls (Fig. 2.8D-F, arrows), although some cells with Par-6 plus aPKC-CAAX overexpression never formed networks (Fig. 2.8C) and had lower overall Zip::GFP levels (Fig. 2.8E, outlined). Dlg and DE-Cad staining showed normal apicobasal polarity and AJs in controls (10/10 embryos) and in most embryos overexpressing Par-6 plus aPKC-CAAX (12/19) or overexpressing Baz (14/16) (Fig. 2.8G-I). Although a portion of Par-6 plus aPKC-CAAX-overexpressing embryos and Baz-overexpressing embryos displayed AJ fragmentation, all embryos analyzed had effects on Zip::GFP dynamics. Thus, defects in myosin dynamics were more prevalent than defects in epithelial structure, arguing in favor of more direct effects on myosin.

These data further indicate that Par-6/aPKC and Baz have distinct effects on myosin pulses, with Par-6/aPKC promoting lull times and Baz promoting network durations. Baz overexpression also decreased lull times, which might result from the increase in pulse durations or more direct effects on the lull phase.

To test whether amnioserosa-targeted PAR protein overexpression affects DC, we measured the rate of change in amnioserosa diameter at the center of the tissue, from one epidermal leading edge to the other, over DC in embryos with the same dosage of Zip::GFP (see Fig. 2.9A). Control, Par-6 plus aPKC-CAAX-overexpressing and Baz-overexpressing embryos had relatively linear DC rates (Fig. 2.9B-D). For quantification, we generated lines of best fit for each embryo centered at 50 μm diameter and extending
Figure 2.9: PAR protein overexpression in the amnioserosa modifies overall dorsal closure (DC) rates.

(A) DC in a control embryo expressing Zip::GFP. Amnioserosa is outlined. White line indicates the amnioserosa diameter (d) measured at the center of the tissue along the anterior-posterior axis (yellow line).

(B-G) DC rates shown as the change in diameter over time; 50 µm of closure was positioned at 0 minutes for each.

(B) DC rates for control embryos expressing Zip::GFP. Each line represents the closure of one embryo.

(C) DC rates for Par-6 plus aPKC-CAAX-overexpressing embryos expressing Zip::GFP. Each line represents the closure of one embryo.

(D) DC rates for Baz-overexpressing embryos expressing Zip::GFP. Each line represents the closure of one embryo.

(E) The DC rates for Par-6 plus aPKC-CAAX-overexpressing embryos versus those for controls.

(F) The DC rates for Baz-overexpressing embryos versus those for controls.

(G) The DC rates for Baz-overexpressing embryos versus those for embryos overexpressing Par-6 plus aPKC-CAAX.
±60 minutes. Control DC rates (5.3±1.5 nm/second; 5 embryos) were statistically indistinguishable from those of PAR protein-overexpressing embryos, but DC was faster with Baz overexpression (7.0±1.2 nm/second; 6 embryos) than with Par-6 plus aPKC-CAAX overexpression (4.0±2.5 nm/second; 7 embryos) (P=0.028).

2.5 Discussion

2.5.1 Pulsing actomyosin networks and amnioserosa apical constriction

The repeated assembly and disassembly of apical actomyosin networks is an integral part of amnioserosa tissue morphogenesis during DC. Restricting myosin to the amnioserosa alone is sufficient for amnioserosa apical constriction and overall DC (Franke et al., 2005). Franke et al. also described ‘dynamic’ apical myosin in the amnioserosa (Franke et al., 2005). We defined these dynamics as repeated assembly and disassembly cycles of actomyosin networks. Moreover, assembly and disassembly are linked to apical constriction and relaxation, respectively. This is consistent with laser ablation studies showing that the apical surfaces of amnioserosa cells maintain tension across the tissue (Ma et al., 2009). Moreover, AJ live imaging has revealed general pulsing of amnioserosa cells from germband retraction through DC (Solon et al., 2009). The pulsing actomyosin networks arise with this same developmental timing. Solon et al. (Solon et al., 2009) observed a 230±76 second periodicity of cortical pulsing at DC, similar to that of the pulsing actomyosin networks. We find that increased network durations and decreased lull times with amnioserosa-targeted Baz overexpression coincide with faster DC, as compared with amnioserosa-targeted Par-6 plus aPKC-CAAX overexpression, which increases lull times. We conclude that the pulsing actomyosin networks mediate the constriction of individual amnioserosa cells and that this contributes to DC.

Remarkably, a single amnioserosa apical constriction event is followed by an almost equal relaxation (Fig. 2.3). However, over many constrictions the cells progressively reduce their apical surface area (Solon et al., 2009). This suggests that ratcheting mechanisms incrementally harness the constrictions for overall tissue change.
Intracellular and extracellular ratchets are possible. Cells of the *Drosophila* ventral furrow also display pulsed contractions of apical actomyosin networks as they apically constrict (Martin et al., 2009). However, there is minimal relaxation after each constriction. Instead, residual myosin filaments are retained between pulses, and may act as intracellular ratchets to harness the pulsed contractions (Martin et al., 2009). By contrast, we rarely observed residual myosin filaments between actomyosin pulses in amnioserosa cells, possibly explaining their relaxation after each cell constriction. Solon et al. proposed that the leading edge actomyosin cable of the surrounding epidermis acts as an extracellular ratchet to harness amnioserosa contractility (Solon et al., 2009). However, the ability of myosin expression in the amnioserosa alone to drive DC (Franke et al., 2005) suggests that other mechanisms contribute. Indeed, DC is a robust process with redundant contributions from both amnioserosa and epidermis (Franke et al., 2005; Kiehart et al., 2000). At later stages, filopodia-based epidermal zippering at the canthi could provide another extracellular ratchet (Gorfinkiel et al., 2009). In addition, each amnioserosa cell has a persistent circumferential actin belt that might act as an intracellular ratchet, and other uncharacterized processes, such as membrane trafficking or basal activities, could also contribute.

Actomyosin activity also appears to be linked between cells. The networks display preferential D-V movement, and a network in one cell appears to promote network formation in neighbors. Overall amnioserosa cell shape changes are also coordinated between neighbors (Solon et al., 2009). Moreover, myosin activity in isolated amnioserosa cells can elicit cortical myosin accumulation in neighboring epidermal cells (Franke et al., 2005). We speculate that feedback from epidermal cells might orient the D-V movement of amnioserosa actomyosin networks. Interestingly, amnioserosa cells also preferentially contract along the D-V axis (Gorfinkiel et al., 2009). Although the actomyosin networks move in this direction, it is unlikely that they are solely responsible for the directional cell shape changes – the networks affect the cell circumference both along the axis of their trajectory and perpendicular to it, and, as discussed, both effects are transient. Thus, forces from the epidermis might be needed for the biased D-V
amnioserosa cell contraction, and they might also direct the D-V movement of amnioserosa actomyosin networks to facilitate DC.

2.5.2 Regulation of the actomyosin networks by the PAR complex

As the actomyosin networks assemble and disassemble, they translocate across a persistent PAR protein patch. These transient associations and lack of specific colocalization between the actomyosin networks and the PAR proteins argue against PAR proteins being integral parts of the actomyosin networks. However, our results show that the PAR proteins regulate the networks. Our genetic interaction tests indicate that Baz, Par-6 and aPKC support myosin activity for proper DC. Strikingly, the live imaging revealed that Baz and Par-6/aPKC regulate distinct phases of the myosin assembly/disassembly cycle. Together, our loss-of-function and gain-of-function studies show that Baz promotes network durations, whereas Par-6 and aPKC promote lull times between pulses. Baz overexpression also decreased lull times, which could result indirectly from increased network durations or from more direct inhibition of the lull phase. Importantly, our overexpression experiments indicate that the effects occur specifically in amnioserosa cells, and analyses of cell polarity and AJs indicate that the PAR proteins have relatively direct effects on the actomyosin networks. However, it remains possible that the PAR proteins have additional functions in the amnioserosa.

A number of molecular interactions must control PAR protein activity in the apical domain of amnioserosa cells. The PAR proteins often, but not exclusively, colocalize in amnioserosa cells, suggesting a dynamic relationship consistent with separate Baz and Par-6/aPKC functions. They also show colocalization with Crb, an apical transmembrane protein at the core of the Crb polarity complex (reviewed by Tepass and Tanentzapf, 2001). Interestingly, Crb is known to regulate DC (Harden et al., 2002), and Par-6 and aPKC can bind Crb complex components (Nam and Choi, 2003; Sotillos et al., 2004). Thus, Crb might be one anchor for PAR proteins at the apical surface of amnioserosa cells.

Molecular mechanisms connecting PAR proteins to myosin and actin have been implicated in a number of studies. For example, aPKC phosphorylates and inhibits
mammalian myosin IIB (Even-Faitelson and Ravid, 2006), although these sites are not present in *Drosophila* Myosin II (Zipper). Par-6/aPKC also inhibits Rho by activating the ubiquitin ligase Smurf1 in mammalian cells (Ozdamar et al., 2005; Wang et al., 2003). Additionally, Baz and aPKC immunoprecipitate with Sqh from *Drosophila* egg chambers (Wang and Riechmann, 2007). Analogous to amnioserosa morphogenesis, mammalian Par-3 and Par-6/aPKC regulate distinct aspects of cell shape change through different cytoskeletal regulators during dendritic spine morphogenesis: Par-3 inhibits cell protrusions by inhibiting Rac through sequestering the RacGEF Tiam1 (Chen and Macara, 2005; Zhang and Macara, 2006), whereas Par-6/aPKC promotes protrusions by inhibiting Rho via p190 RhoGAP (Zhang and Macara, 2008).

Amnioserosa cell apical constriction has similarities to endoderm precursor cell apical constriction during *C. elegans* gastrulation. Here, myosin activity drives cell ingression (Lee and Goldstein, 2003). Similar to in amnioserosa cells, the PAR complex and myosin accumulate at the center of the apical surface of these cells (Nance and Priess, 2002) and of earlier cells as well (Munro et al., 2004). However, these *C. elegans* actomyosin networks do not appear to undergo full assembly/disassembly cycles and instead progressively accumulate (Nance et al., 2003) or display continual network flows (Munro et al., 2004). Interestingly, apical myosin enrichment requires PAR-3 in *C. elegans* endodermal precursor cells (Nance et al., 2003). Apical myosin enrichment also requires Baz, Par-6 and aPKC in *Drosophila* egg chamber follicle cells (Wang and Riechmann, 2007). These results suggest that the PAR complex initiates actomyosin network assembly, contrasting with the amnioserosa, in which networks can assemble without detectable Baz and are inhibited by Par-6/aPKC. Perhaps, actomyosin networks with full assembly/disassembly cycles are regulated distinctly. In the one-cell *C. elegans* embryo, PAR protein puncta move with a multifaceted cortical myosin network to the embryo anterior (Munro et al., 2004). Each facet of the network assembles and disassembles with durations similar to those of the amnioserosa actomyosin networks. The network can also form without the PAR proteins, but the overall flow of the network fails with loss of PAR-3, PAR-6 or aPKC (Munro et al., 2004). It would be interesting to test whether PAR-3, PAR-6 and aPKC have distinct effects on the individual facets of these networks.
2.5.3 Other regulators of the actomyosin assembly/disassembly cycle

What triggers actomyosin network assembly in amnioserosa cells? It appears to be independent of Baz, and must overcome Par-6/aPKC inhibition. The Rho pathway triggers actomyosin contractility in many contexts (reviewed by Lecuit and Lenne, 2007; Matsumura, 2005; Raftopoulou and Hall, 2004). However, amnioserosa-targeted expression of dominant-negative Rho does not appear to block DC (Harden et al., 2002). Alternatively, actin assembly might trigger the networks. Actin networks appear larger and last longer than myosin networks as both start forming during germband retraction. This suggests that actin might organize these networks during germband retraction and possibly DC. Intriguingly, Rac inhibition disrupts DC and reduces amnioserosa actin levels (Harden et al., 2002). The trigger might also involve intercellular forces from networks in neighboring cells.

How is the actomyosin assembly/disassembly periodicity regulated? Since we rarely observed more than one network per cell, network assembly might require disassembly of the existing network. Disassembly might begin a cascade that ultimately triggers formation of the next network. For cycling, assembly might likewise elicit disassembly. Our data indicate that the PAR proteins are important elements of the regulatory network that is involved. Once a network is triggered, Baz prolongs it, but as the network persists, trigger and maintenance signals must be overcome for network disassembly. With disassembly, Par-6/aPKC activity appears to inhibit new assembly, promoting lull times. With time, this Par-6/aPKC activity must diminish and/or be overwhelmed by the trigger mechanism for new network assembly to occur. Identifying trigger and feedback mechanisms within this cycle will be key for understanding how pulsed actomyosin contractions are regulated in the amnioserosa.

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Chapter 3: Bazooka inhibits aPKC to limit antagonism of actomyosin networks during amnioserosa apical constriction

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Contributions:

I performed all experiments and analysis, with the exception of the following contributions:

Qiming Wang and James Feng created the in silico model of amnioserosa pulsatile apical constriction in response to decreasing negative feedback (Fig. 3.10 and Fig. 3.11).

Tony Harris quantified colocalization of pS980-Baz to total Baz::GFP (Fig. 3.6B).
3.1 Abstract

Cell shape changes drive tissue morphogenesis during animal development. An important example is apical cell constriction that initiates tissue internalization. Apical constriction can occur through a phase of cyclic assembly and disassembly of apicomedial actomyosin networks, followed by stabilization of these networks. Delayed negative feedback mechanisms typically underlie cyclic behavior, but the mechanisms regulating cyclic actomyosin networks remain obscure, as do mechanisms that transform overall network behaviour. Here, we show that a known inhibitor of apicomedial actomyosin networks in *Drosophila* amnioserosa cells, the Par-6-aPKC complex, is recruited to the apicomedial domain by actomyosin networks during dorsal closure of the embryo. This finding establishes an actomyosin-aPKC negative feedback loop in the system. Additionally, we find that aPKC recruits Bazooka/Par-3 to the apicomedial domain, and phosphorylates Bazooka for a dynamic interaction. Remarkably, stabilizing aPKC-Bazooka interactions can inhibit the antagonism of actomyosin by aPKC, suggesting Bazooka acts as an aPKC inhibitor, and providing a possible mechanism for delaying the actomyosin-aPKC negative feedback loop. Our data also implicate an increasing degree of Par-6-aPKC-Bazooka interactions as dorsal closure progresses, potentially explaining a developmental transition in actomyosin behavior from cyclic to persistent networks. This later impact of aPKC inhibition is supported by mathematical modeling of the system. Overall, this work illustrates how shifting chemical signals can tune actomyosin network behavior during development.

3.2 Introduction

For animal development and tissue morphogenesis, cells must change shape. For example, the invagination of epithelial sheets is initiated by apical cell constriction (Harris, 2012; Martin, 2010; Sawyer et al., 2010; Suzuki et al., 2012). Non-muscle myosin II (hereafter myosin) pulling on filamentous actin (F-actin) provides the major contractile forces in such cells. With linkage to plasma membrane complexes, such
contractility can change cell shape, and with linkage to adherens junctions (AJs), the contractility also pulls on neighboring cells for coordinated tissue morphogenesis.

In many models of apical constriction, such as the *Drosophila* ventral furrow (Martin et al., 2009), the *Drosophila* amnioserosa at dorsal closure (DC) (David et al., 2010) and *C. elegans* mesodermal precursor cells (Munro et al., 2004; Roh-Johnson et al., 2012), apical actomyosin networks form webs across the apical domain. Additionally, the networks display a range of dynamic properties, with pulsatile, flowing and/or persistent elements. These network properties lead to distinctive apical constriction mechanisms in the different cell types, and the networks can change their properties over development. However, mechanisms regulating these networks, and their changes, are poorly understood.

Cyclic assembly and disassembly of actomyosin networks should obey general principles governing cyclic signaling (Ferrell et al., 2011; Lim et al., 2013). From studies of the cell cycle, circadian rhythms and other systems, the principle of delayed negative feedback has emerged. With an activating input signal, there are two responses: (1) the output response and (2) a delayed inhibition of the input signal. After signaling from the input, the output occurs followed by the delayed input inhibition that leads to loss of both the output and the negative feedback. If the input signal is continually available, the cycle will repeat ad infinitum.

For actomyosin networks many activating inputs are known (e.g., Rho family small GTPases and downstream actin nucleation promoting factors, actin elongation factors, and myosin activating kinases (Chhabra and Higgs, 2007; Pollard, 2007), but we are just beginning to understand mechanisms of delayed negative feedback that could promote network assembly-disassembly cycles. Delayed negative feedback could arise physically or chemically (Kruse and Riveline, 2011; Levayer and Lecuit, 2012). For example, myosin contractility can depolymerize F-actin in vitro, suggesting a mechanism of delayed negative feedback in which actomyosin network assembly leads to myosin contractility and subsequent network disassembly. Alternately, cyclic actomyosin activity has been shown to be entrained by cyclic calcium signaling in cell culture.
During *Drosophila* DC, amnioserosa cells provide an excellent model of apical constriction (reviewed by Gorfinkiel and Blanchard, 2011; Harris, 2012). Midway through embryogenesis, the squamous epithelium formed by these cells becomes covered by the surrounding epidermis. One way the amnioserosa contributes to this internalization is by undergoing apical constriction. Indeed, apical constriction of amnioserosa cells is sufficient to drive DC (Franke et al., 2005). During early DC, the apical constriction of amnioserosa cells is oscillatory and driven by the cyclic assembly and disassembly of apical actomyosin networks (Solon et al., 2009; Blanchard et al., 2010; David et al., 2010). During later DC, amnioserosa cell oscillations are dampened in magnitude (Solon et al., 2009; Blanchard et al., 2010; Sokolow et al., 2012), and this change coincides with increased levels of activated (phosphorylated) apical myosin (Blanchard et al., 2010). Thus, the amnioserosa provides a model for studying cyclic actomyosin networks and potentially for studying the dampening of such cycling. Recent mathematical modelling showed that amnioserosa cells can undergo sustained oscillations through (1) cell autonomous feedback loops in which a hypothetical signal activates myosin and is then depleted with myosin activity, and (2) mechanical coupling between neighbouring cells (Wang, Q. et al., 2012). However, the cell autonomous signalling mechanisms remain obscure, and the model was not able to explain the dampening of cell oscillations at late DC.

Proteins of the Partitioning defective (Par) complex regulate the cycling of amnioserosa actomyosin networks (David et al., 2010). The PAR complex controls various aspects of cell polarity across a wide range of cell types from nematodes to flies to humans. It is composed of Par-3 (Bazooka, Baz, in *Drosophila*), Par-6, and atypical Protein Kinase C (aPKC). However, the complex is dynamic, with Par-6-aPKC complexes often acting separately from Par-3/Baz (Goldstein and Macara, 2007; St Johnston and Ahringer, 2010; Tepass, 2012). Indeed, during amnioserosa apical constriction, Baz promotes actomyosin networks whereas Par-6-aPKC complexes inhibit the networks (David et al., 2010). Here, we show that actomyosin networks recruit Par-6-aPKC complexes to the apical domain, suggesting a negative feedback loop. aPKC acts in turn to recruit Baz, and Baz promotes apical constriction by inhibiting Par-6-aPKC activity. Our data suggest that this
inhibition increases during DC, providing a mechanism that could induce the transition from cyclic to persistent actomyosin networks by late DC, an idea supported by mathematical modelling.

3.3 Materials and Methods

3.3.1 Fly Stocks

yellow white and histone::GFP (a gift from A. Wilde, University of Toronto, Canada) flies were used as wild-type and internal fixation controls. GFP gene traps into endogenous loci for zipper::GFP (CC01626) and Baz::GFP (CC01941) were from FlyTrap (Buszczak et al., 2007; http://flytrap.med.yale.edu/). GAL4 drivers used were c381-Gal-4 (Bloomington Drosophila Stock Center (BDSC) 3734), daughterless-Gal-4 (BDSC 5460), paired-Gal-4 (BDSC 1947), and a $\text{baz}^{\text{Xi106}}$, maternal-α4-tubulin-Gal-4-VP16 recombinant chromosome (McKinley et al., 2012). UAS-constructs were UAS-CA-MLCK (Kim et al., 2002), UAS-Ed (Laplante and Nilson, 2011), UAS-Baz::GFP and UAS-Baz$^{\text{AaPKC}}$::GFP inserted into the attp2 integration site (McKinley et al., 2012), and Baz::GFP, Baz$^{S980A}$::GFP and Baz$^{S980E}$::GFP P-element insertions (Morais-de-Sa et al., 2010). Other fly stocks were: moeABD::GFP (Edwards et al., 1997), $\text{baz}^{\text{Xi106}}$ (a gift from Andreas Wodarz, University of Gottingen, Germany), and a $\text{par-6}^{\Delta226}$, Par-6::GFP genomic rescue line (Wirtz-Peitz et al., 2008).

3.3.2 Embryo staining

Embryos were dechorionated in 50% bleach for 5 minutes, washed in 0.1% Triton X-100, then fixed in 1:1 3.7% formaldehyde in PBS:heptane for 20 minutes and devitellinized in methanol. Embryos stained with phalloidin were fixed in 1:1 10% formaldehyde in PBS:heptane for 10 minutes and manually devitellinized. Blocking and staining were in PBS containing 1% goat serum, 1% sodium azide, and 0.1% Triton X-100.

Primary antibodies used were: mouse antibodies against Arm [1:350; Developmental Studies Hybridoma Bank (DSHB) N27A1]; rabbit antibodies against Baz (1:3500; McKinley et al., 2012); aPKC (1:1000; Santa Cruz Biotechnology C20), Baz-pS980
(1:350; Morais-de-Sa et al., 2010); cleaved caspase-3 (1:200, Cell Signaling #9661); rat antibodies against DE-cad (1:100; DSHB DCAD2) and Echinoid (1:100; Laplante and Nilson, 2011). F-actin was stained with Alexa Fluor 568-conjugated phalloidin (1:200; Invitrogen). Secondary antibodies were conjugated with Alexa Fluor 488, 546 and 647 (Invitrogen).

3.3.3 Pharmacological treatments

Embryos were dechorionated as above then rinsed twice in 0.9% NaCl, nutated in 1:1 octane:0.9% NaCl with DMSO or 10 µg/mL of Cytochalasin D (Sigma-Aldrich) for 30 minutes at RT in the dark. Following treatments, embryos were fixed and hand-peeled as above.

3.3.4 Imaging

Fixed samples were mounted in Aqua-Poly/Mount (Polysciences) and imaged with a Quorum spinning disk confocal system (Quorum Technologies) at room temperature with a 40x (Plan-Neofluor, NA 1.3) or 63x (Plan-Apochromat, NA 1.4) objective, a piezo top plate, a Hamamatsu EM CCD camera and using Volocity software (Improvision). z-stacks had a 0.3 μm step size. For live imaging, dechorionated embryos were glued to coverslips, covered with halocarbon oil (series 700; Halocarbon Products), and imaged with the Quorum spinning disk confocal system as above. Autofluorescent egg shell vitelline membrane served as a marker for the apical surface of the underlying cells. Imaging for quantitative comparisons performed on the same day with the same settings.

3.3.5 Post-acquisition analyses

Kymographs were created with Image J 1.46r (NIH).

To quantify puncta in the Baz::GFP Trap and Par-6::GFP rescue lines, individual puncta intensity sums were obtained with Imaris (Bitplane) in 3.3 μm$^3$ cubes. Per embryo, 2-3 puncta measurements were taken in the amnioserosa, and 3 measurements were taken outside the embryo, averaged, and used for background correction. To measure apical surface puncta densities, 3.3–4.9 μm deep maximum intensity projections were made.
(Volocity) and regions of interest encompassing the amnioserosa were thresholded (Image J) to select prominent apical circumferential and apicominal puncta at early DC. The same thresholding was then applied to early and mid-late DC, and the percentages of the amnioserosa areas occupied by puncta were calculated.

To compare apical surface distributions of Baz::GFP and Baz^\Delta\alphaPKC::GFP in the baz^{Xi106} mutant background, line scans were obtained from 4 neighbouring rows of pixels across a single confocal section of a single amnioserosa cell apical domain. For each cell, the highest fluorescence intensity value was normalized to one, and the normalized intensities were averaged along the 4 lines, and the averaged line scan values were plotted.

To quantify aPKC apical surface enrichment in zip^1 mutants, mean fluorescence intensities within a box encompassing 2-3 amnioserosa cells were measured in Image J per z-section starting just above the apical domain and moving into the cell. For Baz, separate regions of interest were measured and averaged for each of four cells per embryo to avoid the higher apical circumferential signal of this protein. For both proteins, measurements were normalized to the mean fluorescence intensity of the z-section 1.8 μm below the highest intensity apical surface section.

To quantify phospho-S980-Baz::GFP colocalization, brightness and contrast of single confocal sections were adjusted to match epidermal signal and background levels across the samples. Then, non-amnioserosa portions of the images were deleted and the two channels compared using the Colocalization Test in Image J. To focus on apical surface distribution, circumferential localization was excluded by thresholding and subtracting DE-cad staining from the phospho-S980 and Baz::GFP images.

Apical surface areas were measured following manual traces (Image J) of maximum intensity projections created with Volocity. Highest and lowest expressing determined by thresholding GFP intensities (Image J).

To quantify expression levels of Baz::GFP constructs (WT, phosphomimetic, and nonphosphorylatable), a 39.05 x 39.05 x 4.8 μm box was created using Imaris (Bitplane)
such that the cropped volume encompasses roughly 50% amnioserosa and 50% epidermis. Surfaces encompassing voxels above a brightness threshold were created, and the brightness intensity was measured per volume (in arbitrary units per $\mu m^3$).

For figure preparation, input levels were adjusted in Adobe Photoshop while maintaining signal range over full output grayscale. Images were resized by bicubic interpolation with minimal changes at normal magnifications.

Statistical comparisons were done with unpaired two-tailed $t$-tests in Excel (Microsoft).

3.4 Results

3.4.1 Amnioserosa apicomedial actomyosin networks become more persistent at later dorsal closure

Amnioserosa apical constriction occurs in two main phases. During earlier DC, apical domains are oscillatory due to the pulsatile assembly and disassembly of actomyosin networks (Solon et al., 2009; Blanchard et al., 2010; David et al., 2010). During later DC, amnioserosa cell oscillations are strongly dampened (Solon et al., 2009; Blanchard et al., 2010; Sokolow et al., 2012). This altered behavior coincides with increased levels of phosphorylated myosin regulatory light chain over the apical domain (Blanchard et al., 2010), suggesting that more persistent actomyosin networks account for the dampened cell dynamics. However, apical pulses of GFP-tagged myosin regulatory light chain were previously observed after substantial apical constriction and reduced cell oscillation (David et al., 2010). These later pulses were accompanied by a separate increase in the GFP-tagged protein around the apical circumference of amnioserosa cells (David et al., 2010). Since this apical circumferential distribution was not detected when the phosphorylated form of the endogenous protein was probed (Blanchard et al., 2010), we suspected that the GFP-tagged protein may have abnormal properties. Thus, we used two different probes to assess actomyosin network dynamics at late DC.

To further test if cytoskeletal stabilization could account for the reduced cell oscillation at DC, we live imaged the GFP-tagged actin-binding domain of moesin (moeABD::GFP),
and a GFP-tagged form of non-muscle myosin II heavy chain (Zipper, Zip) expressed in a
gene trap line with GFP inserted into the endogenous zip locus. At early DC, both probes
revealed pulsatile networks over the apicomerial cortex of amnioserosa cells (Fig. 3.1A-
B). moeABD::GFP also localized to protrusions around the apical circumference and to
cell-cell junctions. In contrast, by late DC, both probes revealed more persistent
actomyosin networks over the apicomerial cortex of amnioserosa cells (Fig. 3.1A-B).
Thus, amnioserosa apicomerial actomyosin networks become more persistent at later
DC.

3.4.2 Par proteins progressively shift apicomerially over dorsal closure

Since Baz and Par-6-aPKC help coordinate the pulsing of early actomyosin networks, we
hypothesized that they might also affect the transition to more persistent networks. Thus,
we compared PAR protein localization between early and late DC in fixed and live
samples. During early DC, fixation and staining for Baz, aPKC and a Par-6::GFP
construct (expressed under the control of the par-6 promoter in a par-6 mutant
background) revealed a punctate distribution over the apical surface of amnioserosa cells,
in addition to circumferential staining, for each protein (Fig. 3.2A, C, E), as seen
previously (David et al., 2010). The circumferential staining was greater for Baz in the
fixed samples, but live imaging of the Par-6::GFP construct and Baz::GFP (expressed in a
gene trap line with GFP inserted into the endogenous baz locus) revealed similar patterns
of apicomerial and apical circumferential puncta (Fig. 3.2G-H). Furthermore, Baz::GFP
live imaging looked similar to fixed staining against endogenous Baz, suggesting that
detection of circumferential Par-6-aPKC is altered by fixation at this stage. In contrast,
by late DC, all probes, fixed or live, revealed a loss of the PAR proteins from the apical
circumference and a concentration of PAR protein puncta over the apical surface of the
cells (Fig. 3.2B, D, F, I-J). Of note, we observed no region-specific changes, suggesting
the re-localizations occur uniformly across the tissue. Thus, amnioserosa PAR proteins
are progressively lost from the apical circumference and accumulate apicomerially as DC
proceeds.
Figure 3.1: A transition from pulsatile to persistent amnioserosa actomyosin networks over DC.

(A) MoeABD::GFP and (B) Zip::GFP live at early and late DC. Red boxes outline the regions of the apicomedial networks depicted in kymographs at right. Turquoise edges of red boxes are at the bottoms of kymographs (network durations bracketed; cell edges dot-outlined).
Figure 3.2: An increase in apicomedial PAR protein puncta over DC.

(A-F) Fixed imaging of Baz (A, B), aPKC (C, D) and a Par-6::GFP rescue construct (E, F) at early and late DC. Boxes outline magnified regions at right (epidermis at the base). Arrowheads indicate apical surface puncta. DE-cadherin/Arm show cell circumferences.

(G-J) Live imaging of a Baz::GFP trap line (G, I) and the Par-6::GFP rescue line (H, J) at early (G, H) and late (I, J) DC. Amnioserosa is central.

(K) Quantification of individual Baz::GFP and Par-6::GFP puncta intensities in the amnioserosa apicomedial domain in live embryos at early and mid-late DC (means ± SD; N=4-6 embryos per genotype per stage).

(L) Percentage of apical domain area covered by Baz::GFP and Par-6::GFP puncta in live embryos at early and mid-late DC (means ± SD; N=4-6 embryos per genotype per stage).
Compared to apical protein levels in the surrounding epidermis, the apicomedial PAR protein levels in the amnioserosa were relatively low at early DC (Fig. 3.2A, C, E, G-H), but their total apicomedial levels increased substantially by late DC to generally match levels around the apical circumference of epidermal cells (Fig. 3.2B, D, F, I-J). However, quantification of individual puncta intensities in live images of the Par-6::GFP rescue line and the Baz::GFP trap line revealed that, for each protein, prominent amnioserosa apical surface puncta had similar total intensities between early and late DC (although a slight increase in the intensity of apicomedial amnioserosa Par-6::GFP puncta occurs at late DC) (Fig. 3.2K). Notably, the prominent Baz::GFP puncta were ~3-fold less intense than the prominent Par-6::GFP puncta (Fig. 3.2K), suggesting Baz is present at sub-stoichiometric levels compared to Par-6-aPKC complexes. Previous live imaging of co-over-expressed Par-6-GFP and Baz-mCherry revealed a mixture of overlapping and non-overlapping apicomedial puncta (David et al., 2010). To evaluate the potential for colocalization of the Par-6::GFP and Baz::GFP constructs expressed at endogenous levels, we quantified the apical area occupied by puncta in live images taken with the same settings. Large increases in the apical surface covered by Par-6::GFP and Baz::GFP puncta occurred from early to mid-late DC (Fig. 3.2L). Thus, the apicomedial PAR protein accumulation may be due to clustering and addition of puncta (in addition to diffuse protein), and the PAR proteins have an increasing potential to interact as DC proceeds.

3.4.3 Amnioserosa actomyosin networks preferentially recruit aPKC

Patches of PAR protein puncta continually persist at the apicomedial domain of amnioserosa cells during both assembly and disassembly of actomyosin networks (David et al., 2010). These observations suggested that PAR protein localization does not continually rely on the actomyosin networks, but it remained possible that the networks influence the PAR proteins.

To probe for immediate effects of actomyosin network assembly on the PAR complex patches, we monitored patches of Baz::GFP and Par-6::GFP as amnioserosa cells constricted. During the pulsing phase of early DC, PAR protein patches condensed as
constriction occurred (Fig. 3.3A), suggesting a local effect of actomyosin network contraction on the apical PAR protein puncta. At later DC, when pulsing subsided, the PAR protein patches became less dynamic (Fig. 3.3A). Thus, the PAR protein patches displayed dynamic properties mirroring those of the actomyosin networks from early to late DC.

To test if the pulsing actomyosin networks are important for PAR protein apical accumulation, we sought a mutant that lacks apicomedial network assembly. We hypothesized that the networks would depend on myosin, and thus analyzed MoeABD::GFP in zygotic zip1 mutants. The pulsing actin networks seen in WT did not develop in the mutants (Fig. 3.4). To evaluate differences in aPKC and Baz levels, we co-fixed, stained and mounted the mutants with WT embryos expressing histone::GFP. The mutants displayed a marked reduction in the apical surface enrichment of aPKC and Baz (Fig. 3.3B). Thus, over developmental time, the repeated assembly of actomyosin networks appears to contribute to the full recruitment of apicomedial PAR proteins.

To examine how closely the PAR proteins are linked to the actomyosin networks, we analyzed two ectopically induced apicomedial actin networks in amnioserosa cells. First, amnioserosa expression of constitutively active chicken myosin light chain kinase (CA-MLCK) is known to increase apical surface myosin networks (Blanchard et al., 2010). CA-MLCK overexpression also induced abnormally compacted actin networks and cables over the apical surface of amnioserosa cells. Co-staining for aPKC revealed striking co-localization with the ectopic actin assemblies (Fig. 3.3C, arrows). In contrast, Baz displayed less direct colocalization with the actin assemblies, displaying instead a more disperse accumulation in proximity to the actin structures (Fig. 3.3C, brackets). In the second approach, we treated embryos with cytochalasin D with the expectation of inhibiting F-actin. Such inhibition was apparent with the loss of F-actin staining from the actomyosin belt at the leading edge of epidermal cells (Fig. 3.3D-G). However, the treatment also produced ectopic F-actin structures both in the epidermis and in the amnioserosa, consistent with reports of cytochalasin D-induced actin networks, which are presumed to occur from de novo polymerization though the mechanism of their formation.
Figure 3.3: Actomyosin activity promotes apicominal PAR protein accumulation.

(A) Kymographs of Baz::GFP and Par-6::GFP show transient clustering of persistent PAR protein puncta as amnioserosa cells constrict at early DC (red arrows), and less puncta movement at late DC (cell edges dot-outlined).

(B) aPKC and Baz amnioserosa apical surface enrichment in histone::GFP control embryos (arrows) is lost in zip1 zygotic mutants fixed, stained, mounted and imaged together. For quantification, data normalized to cytoplasmic signal below apical surface (means ± SD; N=4-7 embryos per protein per genotype).

(C) Imaging of aPKC, Baz and F-actin in amnioserosa cells over-expressing CA-MLCK at mid-DC. Boxes outline magnified regions at right. Ectopic F-actin structures specifically recruit aPKC (arrows) and more diffusely recruit Baz (brackets).

(D-G) Effects of Cytochalasin D on F-actin, aPKC and Baz at mid-late DC in the Zip::GFP trap line. (D-F) DMSO carrier controls. (E-G) Cytochalasin D treatments. Purple boxes outline magnified epidermal regions (E’, G’). Turquoise boxes outline magnified amnioserosa regions (E”, G”). Ectopic F-actin structures recruit aPKC and Baz but not Zip::GFP (arrows), specifically in amnioserosa cells.
Figure 3.4: Myosin mutants have decreased apical actomyosin pulses.

(A) Live imaging of control embryos heterozygous for zip^1 reveals apical actin pulses in amnioserosa cells. Arrowheads, apical F-actin pulses.

(B) Live imaging of zip^1 homozygous mutant embryos reveals severely decreased incidences of apical actin pulses in amnioserosa cells. Arrowheads, apical F-actin pulses; dotted line outlines a tear in the embryo.
is unclear (Mortensen and Larsson, 2003; Schliwa, 1982). In the epidermis, the circumferential localization of aPKC and Baz were not obviously affected by the treatment, and no recruitment to the ectopic actin networks was apparent (Fig. 3.3E’, G’). In contrast, both aPKC and Baz were recruited to the ectopic actin networks in amnioserosa cells (Fig. 3.3E”, G”, arrows; aPKC, 17/18 embryos; Baz, 8/8 embryos). However, imaging of Zip::GFP revealed exclusion from the actin structures (Fig. 3.3E”, G”, arrows), suggesting not all actin-associated proteins are recruited. Overall, these results reveal that the PAR proteins can be recruited by actin networks in the amnioserosa, and that this connection may be closer for aPKC than for Baz.

3.4.4 aPKC recruits Baz to the apicomedial domain

Since the PAR proteins affect each others’ localization in other contexts (Goldstein and Macara, 2007; St Johnston and Ahringer, 2010; Tepass, 2012), we hypothesized that their interactions would also affect their localization in amnioserosa cells. Par-6 and aPKC directly bind and act as a unit downstream of Cdc42 activation (Goldstein and Macara, 2007; St Johnston and Ahringer, 2010; Tepass, 2012). Additionally, Par-6 and aPKC interact with Baz: Par-6 binds to Baz PDZ1 (Morais-de-Sa et al., 2010); aPKC binds to Baz PDZ2-3 (Wodarz et al., 2000) and to a conserved C-terminal aPKC binding region (Morais-de-Sa et al., 2010). aPKC phosphorylation of the Baz C-terminal aPKC binding region causes their dissociation from one another (Morais-de-Sa et al., 2010).

To assess how Baz affects the localization of aPKC in amnioserosa cells, we examined baz\textsuperscript{xi106} zygotic mutants in which maternally supplied Baz is undetectable during DC (Tanentzapf and Tepass, 2003; McKinley et al., 2012). To evaluate differences in aPKC levels, we co-fixed, stained and mounted the mutants with WT embryos expressing histone::GFP. In contrast to the WT controls, aPKC levels were markedly reduced at the apical surface of baz\textsuperscript{xi106} mutant amnioserosa cells but also in the epidermis (Fig. 3.5A), as reported previously (Wodarz et al., 2000). Levels of the AJ marker Armadillo were unaffected, except where epithelial structure was lost in the mutants. Thus, Baz is needed for full amnioserosa apicomedial localization of aPKC, but this effect appears to reflect
Figure 3.5: The aPKC binding region of Baz functions in apicomedial Baz recruitment.

(A) Reduced aPKC levels in baz<sup>Xi106</sup> zygotic mutant amnioserosa and epidermal cells. Top images collected with same settings. Bottom image enhanced to show aPKC. Armadillo marks circumferences and shows region of amnioserosa breakdown in the baz mutant.

(B) Comparison of Baz::GFP and Baz<sup>ΔaPKC</sup>::GFP expressed with the Gal-4-UAS system in the baz<sup>Xi106</sup> zygotic mutant background. Amnioserosa Baz::GFP is apicomedial (arrows) but Baz<sup>ΔaPKC</sup>::GFP is circumferential. Armadillo marks circumferences. Quantified below with line scans across single amnioserosa cell apical domains (N=4 embryos each; see Methods).
the general role of Baz in maintaining apical aPKC in all epithelial cells. Of note, Baz::GFP rescued the general loss of aPKC in baz Xi106 zygotic mutants, but a Baz construct in which the C-terminal aPKC binding region was deleted, Baz∆aPKC::GFP, did not (data not shown; and McKinley et al., 2012).

To test how aPKC binding affects the localization of Baz in amnioserosa cells, we analyzed Baz∆aPKC::GFP. We chose this strategy to assess the effects of aPKC on Baz at DC because zygotic mutants of apkc k06403 (a null allele) have sufficient maternally supplied aPKC to complete embryogenesis and they die as larvae (Rolls et al., 2003) whereas maternal-zygotic mutants have early embryonic defects (Harris and Peifer, 2007). To test the role of the aPKC binding region in Baz, we expressed the deletion construct and full-length Baz at near endogenous levels in the baz Xi106 zygotic mutant background (McKinley et al., 2012). In the amnioserosa at early-mid DC, Baz::GFP localized to both the apical circumference and apicomedially (Fig. 3.5B; arrows show apicomedial localization), as seen for endogenous Baz (Fig. 3.2, David et al., 2010). In contrast, Baz∆aPKC::GFP failed to localize apicomedially, and displayed greater enrichment around the apical circumference of amnioserosa cells (Fig. 3.5B). Baz::GFP and Baz∆aPKC::GFP were indistinguishable in the epidermis, as reported previously (McKinley et al., 2012), and similar overall effects were observed in live embryos (data not shown). Thus, the C-terminal aPKC binding region in Baz is specifically required for recruiting Baz apicomedially in amnioserosa cells.

3.4.5 aPKC phosphorylation of Baz destabilizes apicomedial aPKC-Baz complexes

The C-terminal aPKC binding region in Baz contains a conserved serine (S980) that is phosphorylated by aPKC (Wodarz et al., 2000; Morais-de-Sa et al., 2010). Phosphorylation of S980 by aPKC weakens aPKC-Baz binding (Morais-de-Sa et al., 2010). Since our data implicated the aPKC binding region in the apical localization of Baz, we wondered how aPKC phosphorylation of Baz affects PAR protein localization and function during DC.
To assess the phosphorylation of Baz by aPKC, we immunostained embryos with an antibody specific for Baz phospho-S980 (Morais-de-Sa et al., 2010). During early DC, circumferential and apicomedial phospho-S980 was detected in amnioserosa cells (Fig. 3.6A). During later DC, amnioserosa apicomedial phospho-pS980 staining increased to levels comparable to that seen around the circumference of epidermal cells (Fig. 3.6A). To compare the detection of phospho-S980 with total Baz, we stained the Baz::GFP trap line (Fig. 3.6B). At early DC, there was substantial colocalization between the two over the amnioserosa, but the degree of colocalization decreased significantly when regions overlapping with circumferential DE-cad staining were excluded from the analysis. At later DC, the overall degree of amnioserosa colocalization was indistinguishable from early DC, but when only the apicomedial distributions were compared, there was a significant increase from early to late DC. Thus, we detect Baz-aPKC interactions in the form of Baz phosphorylation in the amnioserosa and, notably, these interactions shift from the apical circumference to the apicomedial domain as DC proceeds.

To test how the aPKC phosphorylation site affects aPKC-Baz interactions in amnioserosa cells, we overexpressed either WT or mutant forms of Baz that abolished or mimicked S980 phosphorylation (S980A and S980E, respectively). In other cell types, non-phosphorylatable Baz$^{S980A}$ is known to have increased affinity for aPKC compared to Baz$^{S980E}$ or Baz$^{WT}$ (Morais-de-Sa et al., 2010). In amnioserosa cells at DC, both Baz::GFP and Baz$^{S980E}$::GFP localized to the apical circumference and apical surface of amnioserosa cells (Fig. 3.6C, 3.7). In contrast, Baz$^{S980A}$::GFP localized predominantly to the apical surface of amnioserosa cells, where it accumulated in intense puncta (Fig. 3.6C, 3.7). Staining for aPKC revealed a dramatic and dose-dependent recruitment of aPKC to the Baz$^{S980A}$::GFP puncta since aPKC was noticeably increased in cells expressing highest levels of Baz$^{S980A}$::GFP as compared to neighbouring cells expressing lower levels of the construct (Fig. 3.5C). The general ability of Baz$^{S980A}$::GFP to recruit and accumulate aPKC was confirmed by expressing the construct in a striped pattern in the epidermis, whereas minimal or no effects were seen with Baz::GFP and Baz$^{S980E}$::GFP, respectively (Fig. 3.7). To test if these effects of Baz$^{S980A}$::GFP were
Figure 3.6: Phosphorylation of the aPKC binding region in Baz inhibits Baz-aPKC interactions in amnioserosa apicomedial domains.

(A) Phosphorylation of the aPKC binding region in Baz detected at early and late DC with a phospho-specific antibody in a WT embryo.

(B) Comparisons of phospho-specific antibody staining with total Baz in the Baz::GFP trap line at early and late DC. Similar overall amnioserosa colocalization occurs at both stages, but the colocalization shifts from circumferential pools to apicomedial pools over development. Quantified at right (N=8 embryos for each stage).

(C) Expression of Baz^{S980A}::GFP recruits aPKC and forms high intensity apicomedial puncta in amnioserosa cells. Expression of Baz^{S980E}::GFP has much milder effects on aPKC and its own accumulation. Turquoise boxes outline magnified regions in middle. White box outlines highly magnified region at far right. Blue and green brackets show cells with low and high Baz^{S980A}::GFP expression, respectively.
Figure 3.7: Assessments of Baz construct expression levels and effects on aPKC in the epidermis.

(A) Comparisons of Baz constructs imaged with the same setting after amnioserosa expression (c381-Gal-4) or after ubiquitous expression (daughterless (da)-Gal-4).

(B) Quantification of Baz constructs after ubiquitous expression (da-Gal-4) (N=5 embryos for Baz::GFP, N=5 embryos for BazS980E::GFP and N=6 embryos for BazS980A::GFP).

(C) Effects of constructs on aPKC after expression in stripes in the epidermis and amnioserosa (paired (prd)-Gal-4). Epidermis at bottom. DE-cadherin shows cell circumferences.
based on higher expression levels alone, we compared the brightness intensity of Baz::GFP, Baz\textsuperscript{S980A}::GFP and Baz\textsuperscript{S980E}::GFP in puncta in the epidermis using the ubiquitous daGAL4 driver, and saw that Baz\textsuperscript{S980A}::GFP had the lowest expression levels of the three constructs (Fig. 3.7). Thus, the effects of Baz\textsuperscript{S980A}::GFP appear to be due to its non-phosphorylatable state. These data further implicate the aPKC binding region in Baz in the recruitment of both Baz and aPKC to the apical surface of amnioserosa cells. More significantly, the phosphorylation of Baz by aPKC is critical for reversing complex formation in the apicominal domain of amnioserosa cells.

3.4.6 Stabilization of apicominal Baz–aPKC complexes leads to apical constriction

Since Baz and aPKC have opposing effects on the pulsing of apicominal actomyosin networks (David et al., 2010), increasing Baz-aPKC interactions, and the levels of each protein at the apicominal domain, allowed us to test if their effects on apical constriction are somehow linked. Thus, we compared the apical surface areas of amnioserosa cells overexpressing Baz\textsuperscript{S980A}::GFP, Baz\textsuperscript{S980E}::GFP, Baz::GFP or GFP. Amnioserosa cells expressing the highest levels of Baz\textsuperscript{S980A}::GFP were consistently more constricted than cells in the same tissue with the lowest construct expression, and this induced constriction was only observed for this construct (Fig. 3.8A-D). We confirmed the specific effect of Baz\textsuperscript{S980A}::GFP by measuring the apical surface areas of the eight highest expressing cells and the eight lowest expressing cells per embryo for each of the constructs. Only Baz\textsuperscript{S980A}::GFP lead to a significant difference in the apical surfaces areas between the two groups of cells (Fig. 3.8E; p<0.05, n=10 embryos). We considered the possibility that Baz\textsuperscript{S980A}::GFP overexpression might be inducing apoptosis and apical constriction associated with delamination (Toyama et al., 2008), but we detected no cleaved caspase-3 in the constricted cells (Fig. 3.9). Also, apoptotic cells undergo a relatively smooth (non-pulsatile) constriction process (Sokolow et al., 2012), whereas the cells induced to constrict by Baz\textsuperscript{S980A}::GFP continually expanded and contracted (we could not distinguish if these movements were due to cell autonomous contractile pulsations or due to the contractions of neighboring cells with lower construct expression—e.g.,
Figure 3.8: Stabilized Baz-aPKC interactions promote apical constriction

(A-D) Amnioserosa expression of GFP alone (A), Baz^{S980A}::GFP (B), Baz^{S980E}::GFP (C), and Baz::GFP (D). Armadillo shows circumferences. Amnioserosa cells top. Blue and green shows eight cells with lowest and highest construct expression, respectively.

(E) Quantification of apical surface areas of the eight highest expressing cells and eight lowest expressing cells for GFP alone, Baz^{S980A}::GFP, Baz^{S980E}::GFP, and Baz::GFP (means ± SD; N=7-10 embryos each).
Figure 3.9: Overexpression of Baz\textsuperscript{S980A}::GFP does not induce caspase-dependent apoptosis.

Cells overexpressing higher levels of Baz\textsuperscript{S980A}::GFP have no noticeable increase in cleaved caspase-3 as compared cells expressing lower levels of the construct.
actomyosin behavior was not clearly revealed by co-imaging with Sqh-mCherry because of interference from intense cytoplasmic protein aggregates). Overall, these data indicate that stabilized Baz-aPKC interactions can induce apical constriction of amnioserosa cells. Since aPKC is known to inhibit the actomyosin networks responsible for apical constriction, it appears that in cells expressing Baz$^{S980A}$::GFP, the elevated apicominal aPKC recruited by Baz$^{S980A}$::GFP is inhibited from antagonizing actomyosin, presumably because of its stabilized interactions with the Baz construct.

3.4.7 Gradual reductions in myosin inhibition dampen amnioserosa cell oscillations \textit{in silico}

Since apicominal Baz-aPKC interactions appear to increase over DC, we hypothesized that a gradual increase in the inhibition of aPKC by Baz would lead to a gradual decrease in the antagonism of actomyosin networks by aPKC and thus a stabilization of the networks and a dampening of cell oscillations. To test if gradual reductions of myosin inhibitory factors would dampen cell oscillations, we turned to a recently developed mathematical model of DC (Wang, Q. et al., 2012). This model mechanically couples amnioserosa cells through passively elastic circumferential edges and apicominal spokes. In addition, kinetic equations describe myosin and signaling dynamics that control the assembly and action of myosin on the apicominal spokes (summarized in Fig. 3.10B). With these components alone, amnioserosa cells continually oscillate (Fig. 3.10C; before 0min). Mid-DC can be simulated by applying two ratchets: (1) an elastic, and continually shortening, cable around the perimeter of the entire tissue (an external ratchet), and (2) the continual shortening of edges and spokes inside each cell (an internal ratchet) (Wang, Q. et al., 2012) (Fig. 3.10C; simulation (0.5,0,0) after 0min).

In the model, there are two parameters that inhibit myosin assembly onto spokes: (1) $k_0$ depletes an activating signal for myosin assembly, and (2) $k_1$ directly affects the myosin-spoke Dissociation constant (Fig. 3.10B). Thus, we tested if gradual reductions of $k_0$ or $k_1$, or both, would dampen cell oscillations after mid-DC in the model. Starting at 0min, the resting lengths of edges and spokes were decreased by 0.5\% per average oscillation cycle, and additionally $k_0$ and $k_1$ were decreased from 0-1\% per cycle. The most striking
Figure 3.10: Conceptual and mathematical models of PAR proteins effects on each other, actomyosin networks and cell oscillations.

(A) Outline of proposed regulatory circuit connecting PAR proteins and actomyosin networks. During DC, pulsatile actomyosin networks recruit the PAR complex, preferentially recruiting aPKC. aPKC inhibits actomyosin networks by phosphorylating an unknown cytoskeletal inhibitor (green arrow). aPKC also recruits and phosphorylates Baz (red arrow). Baz may act as a competitive inhibitor of aPKC to diminish the ability of aPKC to phosphorylate its cytoskeletal regulator. As DC proceeds, a gradual increase in Baz – aPKC interaction may increase aPKC inhibition, decreasing the ability for aPKC to phosphorylate its other target and thus diminish the ability of aPKC to inhibit actomyosin network formation.

(B) Summary of mathematical model (see Wang, Q. et al., 2012 for details) and explanation of two parameters that inhibit myosin ($k_0$ and $k_1$).

(C) Simulations of DC with internal and external ratchets activated at 0min, along with no or various reductions in $k_0$ and $k_1$ per average oscillation cycle [(X,Y,Z):percentage reductions in spoke and edge resting lengths (X), $k_0$ (Y) and $k_1$ (Z) per average oscillation cycle]. The three curves are the normalized cell areas of the three cells marked in Fig. 7B. Note abrupt dampening of cell oscillation following incremental $k_0$ and $k_1$ reductions (arrows). Unnatural instabilities occurred with greater $k_1$ reductions (asterisks).
A. Proposed circuit of cytoskeletal and Par proteins

B. Mathematical model of DC

Myosin changes at edges and spokes defined by:
\[
\frac{dm_{ij}}{dt} = k^+ s_i h_j - k^- m_{ij}
\]
\( k^+ \) Association constant
\( h_j \) Activating signal distributor

Myosin inhibited by two parameters \((k_0\) and \(k_1)\):
Activating signal changes:
\[
\frac{ds_k}{dt} = \text{production} - k_0 M_k, M_k = \sum m_{ij}
\]
Dissociation constant:
\( k^- = k_1 e^{-k_0 \text{[force on spoke or edge]}} \)

C. Simulations of DC with or without repeated dampenings of Myosin II inhibitions

At \( t=0 \) minutes, one to three changes are initiated (X,Y,Z):
- X: Cell edge and spoke lengths decreased at each cycle (% change)
- Y: \( k_0 \) decreased at each cycle (% change)
- Z: \( k_1 \) decreased at each cycle (% change)
dampening of cell oscillations occurred when both $k_0$ and $k_1$ were reduced (Fig. 3.10C; compare simulation (0.5,0,0) with (0.5,0.5,0.5), (0.5,0.5,1) and (0.5,1,1)). Following 0min, oscillations were initially similar to the control (which had no changes to $k_0$ or $k_1$), but abrupt dampening or loss of oscillations occurred between 50-100min, in contrast to the control in which oscillations continued. Similar trends were observed when the same changes to $k_0$ and $k_1$ were applied to simulations undergoing 1.0% decreases of edge and spoke resting lengths per cycle, although these length changes alone dampened oscillations (Fig. 3.11), and 2.0% length changes alone eliminated oscillations. Of note, the reductions to myosin inhibition in the simulations did not lead to great reduction in cell area nor to complete closure of the amnioserosa tissue, suggesting elements of the model may be unnatural (e.g., the resistance of edges and spokes to compression Wang, Q. et al., 2012) or that elements are missing from the model (e.g., the effects of filopodial zipper (Millard and Martin, 2008)). Nonetheless, these simulations indicate that incremental and small reductions to myosin inhibition can lead to abrupt dampening of cell oscillations during DC.

3.5 Discussion

Our data outline a regulatory circuit for guiding amnioserosa apical constriction (Fig. 3.10A). The circuit controls both the localization and activity of its components. In terms of protein localization, we find that amnioserosa actomyosin networks recruit the PAR proteins to the apicomedial domain. Although PAR protein puncta are not continually dependent on the actomyosin networks, their numbers build over developmental time, apparently due to the cumulative effect of multiple rounds of actomyosin network assembly. The networks appear to impact aPKC most directly and, in turn, aPKC recruits Baz to the apical domain. This recruitment depends on the C-terminal aPKC-binding region of Baz, which aPKC phosphorylates for a dynamic relationship with Baz in the apical domain of amnioserosa cells.

Par-6-aPKC activity inhibits amnioserosa actomyosin networks (David et al., 2010), and the recruitment of aPKC by the networks implicates a negative feedback loop. Since delayed negative feedback tied to a continual input signal can produce an oscillatory
Figure 3.11: Simulations of DC with 1% reductions in edge and spoke resting lengths per average oscillation cycle.

Internal and external ratchets activated at 0min, along with no or various reductions in $k_0$ and $k_1$ per average oscillation cycle [(X,Y,Z): percentage reductions in spoke and edge resting lengths (X), $k_0$ (Y) and $k_1$ (Z) per average oscillation cycle]. Note that abrupt dampening of cell oscillation often occurs following incremental $k_0$ and $k_1$ reductions (arrows). Also note that unnatural instabilities occurred at higher levels of $k_1$ (asterisks).
output (Ferrell et al., 2011; Lim et al., 2013), the actomyosin-aPKC negative feedback loop may explain how aPKC regulates actomyosin network assembly-disassembly cycles (David et al., 2010). However, apical populations of Par-6-aPKC puncta are not fully recruited and fully removed with each actomyosin cycle, suggesting an additional mechanism. Importantly, Par-6-aPKC activity can be tempered by Baz. Thus, aPKC inhibition by Baz may delay the actomyosin-aPKC negative feedback loop during early DC, promoting the actomyosin assembly-disassembly cycles. We further propose that a gradual increase in apicomedial aPKC-Baz interactions leads to stabilization of actomyosin networks in late stages of DC. Our simulations indicate that this transition in network behaviour can occur abruptly following incremental reductions in myosin inhibition during earlier DC.

We propose that Baz acts as a competitive inhibitor to reduce aPKC phosphorylation of cytoskeletal regulators. This idea is consistent with reports of Par-3 inhibiting aPKC in kinase assays in vitro (Lin et al., 2000; Graybill et al., 2012). However, Baz is also known to promote aPKC localization in the epidermis (Wodarz et al., 2000; Harris and Peifer, 2005) and amnioserosa (Fig. 3.5A). Thus, Baz appears to both promote and inhibit aPKC activity, potentially forming a paradoxical circuit (or incoherent feedforward loop) (Hart and Alon, 2013; Lim et al., 2013) in which Baz and aPKC promote each other’s recruitment, and in which Baz competitively inhibits aPKC activity. Significantly, Baz has multiple binding sites for the Par-6-aPKC complex (Par-6 binds Baz PDZ1 (Morais-de-Sa et al., 2010); aPKC binds BazPDZ2-3 (Wodarz et al., 2000); aPKC binds the Baz C-terminal aPKC-binding region (Morais-de-Sa et al., 2010), suggesting cooperative binding and that Baz interactions with the Par-6-aPKC complex are stronger than those of the Par-6-aPKC complex with its cytoskeleton targets. Notably, we find that Baz apical surface levels are ~3-fold lower than those of Par-6, suggesting the inhibitory effect of Baz must be dynamic—Baz cannot simply sequester all Par-6-aPKC complexes by outnumbering them. The inhibitory effect must also depend on phosphatases since aPKC interactions with Baz are weakened following phosphorylation (Morais-de-Sa et al., 2010). Baz/Par-3 is known to be regulated by Protein phosphatase 1 (Traweger et al., 2008) and Protein phosphatase 2A (Krahn et al.,
2009) with Protein phosphatase 1 dephosphorylating the aPKC phosphorylation site of Par-3 (Traweger et al., 2008). Thus, Baz may act as a strong and dynamic inhibitor of Par-6-aPKC to buffer and eventually overcome the actomyosin-aPKC negative feedback loop.

A critical unknown is the identity of the cytoskeletal target(s) of aPKC. Cytoskeletal targets of aPKC have not been examined during amnioserosa apical constriction. In mammalian cells, Par-6-aPKC can phosphorylate Smurf1, an E3 ubiquitin ligase, in turn leading to RhoA degradation in cellular protrusions (Wang et al., 2003). During dendritic spine morphogenesis, Par-6-aPKC acts though p190RhoGAP to inhibit RhoA (Zhang and Macara, 2008). As well, aPKC phosphorylation of Rho-kinase leads to its cortical dissociation in mammalian cell culture (Ishiuchi and Takeichi, 2011), and apparently during salivary gland tubulogenesis in Drosophila (Roper, 2012). It will also be important to determine how actomyosin networks recruit aPKC. The recruitment of PAR proteins by actomyosin networks has been documented during Drosophila cellularization (Harris and Peifer, 2005) and C. elegans one-cell polarization (Munro et al., 2004), and Baz and aPKC can co-immunoprecipitate with myosin (Wang and Riechmann, 2007), but specific linkages have not been identified in these contexts. Defining further components of the actomyosin-aPKC negative feedback loop will be critical for understanding its regulation and its effects on actomyosin network dynamics. In particular, despite identifying a potential delay mechanism for the loop, it is unclear how the loop and the delay mechanism could translate into oscillatory network behaviour. Perhaps the cytoskeletal target(s) of aPKC are co-recruited with the assembling networks, which, in combination with Baz buffering effect, could delay their phosphorylation by aPKC. It is also possible that the clustering of PAR protein puncta with each network assembly event could somehow modify the Baz buffering effect.

Another unanswered question is the influence of circumferential anchors for Baz or Par-6-aPKC. Weakening of these anchors could contribute to apicominal PAR protein accumulation over DC. Intriguingly, Echinoid (Ed), a transmembrane protein associated with AJs that can bind directly to Baz (Wei et al., 2005), is normally lost from the amnioserosa during DC (Laplante and Nilson, 2006; Laplante and Nilson, 2011). We
hypothesized that this loss of Ed might promote the loss of Baz from AJs and its apicomedial accumulation. However, ectopic expression of Ed in the amnioserosa, leading to circumference Ed levels higher than seen in the epidermis, had no apparent effect on apicomedial Baz localization (Fig. 3.12). Thus, differences in Ed expression alone cannot account for the differential localization of PAR proteins between the amnioserosa and epidermis. It is possible that the effects of actomyosin can overpower ectopic Ed, or that other changes to the apical circumference of amnioserosa cells are involved. More generally, other PAR protein interaction partners should be considered. For example, Baz also interacts with Stardust (Krahn et al., 2010a), which, together with Crumbs and PATJ, forms the apical Crumbs complex (reviewed by Tepass, 2012). Recent results suggest PATJ can activate myosin by suppressing myosin light chain phosphatase (Sen et al., 2012). Intriguingly, amnioserosa Baz$^{S980A}$ apical surface puncta also recruit PATJ (Fig. 3.13), suggesting this pathway might contribute to regulating myosin activity as well.

In summary, our data argue that the differential regulation of amnioserosa actomyosin networks by Baz and Par-6-aPKC seen previously (David et al., 2010) can be explained by a single pathway in which Baz inhibits Par-6-aPKC antagonism of the cytoskeletal networks. We also find that the actomyosin networks recruit aPKC, forming a negative feedback loop. We propose that the inhibition of aPKC by Baz delays the negative feedback at earlier DC for cycling actomyosin networks, and with increased inhibition of aPKC by later DC, the actomyosin networks persist. These findings provide an example of how chemical signaling, and changes to this signaling, can modify the behavior of actomyosin networks during embryo development.
Figure 3.12: Ectopic expression of Echinoid in the amnioserosa does not shift PAR proteins towards the apical circumference.

(A) Control embryos lack Echinoid (Ed) expression in the amnioserosa and are demarcated with the presence of GFP.

(B) Embryos ubiquitously-overexpressing Ed. Ectopic Ed in amnioserosa cells localizes to adherens junctions. There is no noticeable effect on Baz localization in annioserosa cells.
da-Gal4; UAS-GFP (on balancer chromosome)

Baz
Ed
GFP and Arm

Baz
Ed
Arm

da-Gal4; UAS-Ed

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Figure 3.13: Overexpressed Baz$^{S980A}$::GFP colocalizes with apical PATJ.

Apicomeral puncta of Baz$^{S980A}$::GFP colocalize with PATJ. Baz$^{S980A}$::GFP is overexpressed with the amnioserosa GAL4 driver. Cyan box is enlarged to the right. Arrowheads, apical surface puncta of Baz SA::GFP and PATJ.
3.6 Acknowledgements

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Chapter 4: General Discussion

At the start of my thesis research, apical constriction was first presumed to proceed via sarcomeric-like contraction of an actin belt associated with adherens junctions. During my thesis work I discovered that dynamic actomyosin networks function in such constriction. I further documented an actomyosin-PAR protein regulatory circuit important for network regulation.

4.1 Different configurations of actomyosin networks to mediate apical constriction

Apical constriction of epithelial cells was previously thought to proceed via contraction of sarcomeric-like actomyosin belt. This model stemmed largely from results in MDCK cell culture. Here, circumferential actin belts are observed (Nakajima and Tanoue, 2011; Nishimura and Takeichi, 2008), actomyosin activators accumulate at roughly the apical domains, and apical constriction is presumed to follow via sarcomeric-like contraction (Nakajima and Tanoue, 2011; Nishimura and Takeichi, 2008). Apical constriction has also been studied extensively in the Drosophila ventral furrow. Again, earlier results showed accumulation of actomyosin activators at the apical regions of the presumptive ventral furrow cells (Dawes-Hoang et al., 2005).

However, recent results from model systems amenable to live imaging have suggested that apical constriction in vivo can proceed via apically-enriched actomyosin web-like networks. Evidence for contractile actomyosin networks has been seen extensively during C. elegans embryogenesis (Munro et al., 2004; Nance et al., 2003; Roh-Johnson et al., 2012). These networks have also been observed in Drosophila embryonic ventral furrow formation (Martin et al., 2009) and dorsal closure (Blanchard et al., 2010; David et al., 2010).
It is still unclear whether meshwork-like actomyosin networks are a conserved mode of contractility mediating cell shape changes. Recent work has shown that apical constriction may proceed without obvious actomyosin differences. In *Drosophila*, dorsal fold formation proceeds by basal-ward shifting of AJJs, without obvious modifications of apical myosin networks (Wang, Y.C. et al., 2012). Conversely, apical constriction can be induced by an apparent apical accumulation of AJ proteins in *Drosophila* follicular epithelial cells (Morais-de-Sa et al., 2010), though absence or presence of pulsatile apical actomyosin networks was not explicitly investigated in this case. Actomyosin network configurations have also been observed in other cases requiring contractility but not apical constriction. For example, interconnected actomyosin foci are present during *Xenopus* convergent extension (Skoglund et al., 2008). Apical actomyosin meshwork flows may reinforce planar polarized contractility during *Drosophila* embryonic germ band extension (Rauzi et al., 2010) and egg chamber elongation (He et al., 2010). However, recent higher-resolution live imaging of mammalian cells both from cell culture and from organs has revealed a sarcomeric-like arrangement of actomyosin at adherens junctions (Ebrahim et al., 2013). Since the preceding example involved cells not undergoing morphogenesis (Ebrahim et al., 2013), it will be interesting to see whether pulsatile apical actomyosin networks or AJ-associated actomyosin belts mediate apical constriction in vertebrate morphogenetic events such as neural tube formation. Furthermore, it will be interesting to investigate whether pulsatile actomyosin networks are also seen in other cases of *Drosophila* apical constriction such as the formation of the cephalic furrow, posterior midgut invagination, and tracheal pits.

Another interesting actin configuration that exists in amnioserosa cells during dorsal closure is that of actin protrusions. Actin-based protrusions have been studied in the leading edge of epidermal cells during dorsal closure, where they ensure proper sealing of the incoming epidermal sheets (Millard and Martin, 2008). However, it is quite clear that amnioserosa cells themselves harbour dramatic actin-based protrusions extending from the apical circumference (David et al., 2010; Stevens et al., 2008). Although the protrusions may not be well preserved during fixation (David et al., 2010), my preliminary results suggest that these protrusions are not an artefact of the actin probe
used during live imaging, and that these protrusions cross over their neighbouring cells. On the basal surface of amnioserosa cells, actin-based protrusions facilitate integrin-based cell adhesion to the underlying yolk membrane (Reed et al., 2004). The function of apical actin protrusions in amnioserosa cells is currently unknown, although globally perturbing them by inhibiting Ena results in amnioserosa cell lamellipodia, in place of filopodia, and disrupted dorsal closure (Gates et al., 2007; Stevens et al., 2008). The effects of actin-based protrusions in the amnioserosa can be tested by expressing inhibitors of Ena that are under UAS control within the amnioserosa. For example, expression of a peptide bearing a strong Ena-binding motif fused to a mitochondrial-localizing sequence under UAS control can sequester Ena towards mitochondria and away from the cell cortex (Gates et al., 2007). Likewise, overexpression of Abelson kinase will phosphorylate and inhibit Ena (Stevens et al., 2008). These constructs could be expressed in the amnioserosa with the GAL4/UAS system and the effects on apical actomyosin networks, the PAR complex, and overall dorsal closure can be studied. As yet another actin configuration in the amnioserosa during dorsal closure, these actin-based protrusions should be investigated if they enhance or inhibit pulsatile apical actomyosin networks, affect apical surface PAR complex localization, and affect dorsal closure.

4.2 Investigating mechanisms of PAR complex localization to the cell cortex

Previous research has outlined possible mechanisms for PAR complex localization to the plasma membrane. Since the PAR complex components are not integral membrane proteins, other mechanisms must be involved in their cortical localization. Baz itself can localize directly to the cell cortex. Baz requires its N-terminal oligomerization domain for membrane localization (Benton and St Johnston, 2003), and its C-terminal region has phosphoinositide binding sites (Krahn et al., 2010). Baz can also interact with transmembrane proteins; it can bind directly to Echinoid/nectin (Wei et al., 2005), to E-Cadherin via β-catenin (Wei et al., 2005), and to Crbs via Sdt (Krahn et al., 2010). Although mammalian aPKC can bind to phosphatidic acid in vitro (Limatola et al., 1994),
other interactions between Par-6/aPKC and the plasma membrane are unclear. However, Par-6 binds activated Cdc42 (Atwood et al., 2007; Garrard et al., 2003; Rolls et al., 2003). Since activated Cdc42 is a peripheral membrane protein like other Rho GTPases (Bokoch et al., 1994; reviewed by Cherfils and Zeghouf, 2013), this could be a possible link between Par-6/aPKC and the plasma membrane. Par-6 can also bind to the transmembrane protein Crb both directly and via Sdt (Kempkens et al., 2006; Lemmers et al., 2004; Morais-de-Sa et al., 2010).

The PAR complex has been primarily characterized in the context of localizing with cell junctions, though PAR complex components can localize apically to the junctions. In 2D cultured MDCK cells, Par-3 localizes to sites of cell-cell junctions, with Par-6/aPKC localizing just apically (Hiroshe et al., 2002; Joberty et al., 2000). However, in 3D MDCK cysts, Par-3 localizes to cell-cell junctions whereas aPKC localizes to the apical surface (Hao et al., 2010). In the C. elegans one-cell embryo, PAR complex proteins are associated with the cell cortex even in the absence of sites of cell adhesion (Munro et al., 2004). During later stages of embryogenesis, PAR complex proteins are also localized to “non-contact” surfaces (Nance et al., 2003). Together, these results suggest that the PAR complex can localize to the cell cortex independently of AJs.

The actomyosin network could also be another possible link between the PAR complex and the cell cortex. Indeed, work from others has previously shown that actomyosin networks and the PAR complex are somehow linked. For example, in the C. elegans one-cell embryo, the anterior PAR complex proteins appear along the same cortical domain as contractile actomyosin networks (Munro et al., 2004). As the actomyosin network contracts, PAR complex puncta move at the same speed as the networks (Goehring et al., 2011; Munro et al., 2004), suggesting that the actomyosin networks and PAR complex are linked. However, it is unknown if and how the PAR complex links directly to the actomyosin cytoskeleton.

Part of my thesis work has characterized the localization of the PAR complex at the apical surface, away from the adherens junctions. My first publication noted that in the amnioserosa cells, the PAR complex is localized to the apical surface as well as in a
punctate pattern along the cell junctions (David et al., 2010). This is in stark contrast to the bordering epidermal cells, wherein the PAR complex is localized along the apical circumference. Afterwards, however, I performed a more thorough analysis of the PAR complex localization of the amnioserosa and noted a difference in their localization from early to late stages of dorsal closure. During early dorsal closure, Baz, Par-6, and aPKC are localized both to the adherens junctions and to the apical surface in punctate structures. However, by later dorsal closure, Baz, Par-6, and aPKC are enriched at the apical surface of amnioserosa cells. These results suggest apical surface cues can recruit the PAR complex during dorsal closure. When endogenous Baz is depleted, a Baz construct that is unable to bind to aPKC no longer localizes to the apical surface of amnioserosa cells. Instead, Baz localizes to amnioserosa cell junctions, suggesting that binding to aPKC is required for apical surface localization of Baz.

Since Ed is lost from the amnioserosa during dorsal closure (Laplante and Nilson, 2006), its absence may be a cue to localize Baz and aPKC away from cell-cell junctions. Indeed, the absence of Ed from the LE of epidermal cells is proposed to cause depletion of Baz from the LE (Laplante and Nilson, 2011). However, my results show that ectopic expression of Ed in amnioserosa cells during dorsal closure has no appreciable effect on the localization of the PAR complex in amnioserosa cells. Thus, it is not the absence of Ed alone that can account for the junctional to apicominal shift of the PAR complex in the amnioserosa. The cues localizing the PAR complex away from junctions and towards the apical surface have yet to be identified.

To test apical cues further, I then perturbed the actomyosin networks and assessed PAR complex localization. In amnioserosa cells, loss of apical actomyosin networks leads to a decreased apical PAR complex proteins. In contrast, ectopic apical actin networks strongly recruit aPKC, and to a lesser extent, Baz to the apical surface. My results suggest that apical surface actomyosin networks are somehow important for apical surface PAR complex localization, but the molecular mechanisms linking actomyosin to the PAR complex have yet to be detailed.
The Rho family GTPases are a possible mechanism of localizing PAR complex proteins to the apical surface. Since activated Cdc42 can bind to Par-6/aPKC (Atwood et al., 2007; Garrard et al., 2003; Rolls et al., 2003), it will be interesting to evaluate Par-6/aPKC localization with perturbations to Par-6 – Cdc42 interaction. For example, expression of a Par-6 construct with mutation to its Cdc42-binding site disrupts apicobasal polarity in Drosophila neuroblasts (Atwood et al., 2007). It will also be useful to examine PAR complex localization in the presence of Cdc42 perturbations. Cdc42 perturbations lead to defective dorsal closure (Genova et al., 2000), but this has been attributed primarily to the actin-based protrusions at the leading edge (Harden et al., 1999; Jacinto et al., 2000). Thus, overexpressing dominant-negative and constitutively-active Cdc42 constructs in the amnioserosa and assessing PAR complex localization may be useful in testing the requirement of Cdc42 for the apical recruitment of the PAR complex, namely through Par-6. However, several caveats exist; absence or presence of effects may result from differences in expression level of the constructs, Cdc42 can affect overall actomyosin networks, and there are interactions with other Rho GTPases (reviewed by Cherfils and Zeghouf, 2013; Guilluy et al., 2011). Thus, in addition to the preceding perturbations, it will be useful to evaluate the degree of endogenous Cdc42 activation in the amnioserosa and examine colocalization with apical surface PAR complex puncta. Since active Rho GTPases are membrane-bound, Cdc42 localization has been probed with expression of epitope-tagged Cdc42 in Drosophila neuroblasts and found to polarize to the apical cortex (Atwood et al., 2007). However, since the main mechanism of Rho GTPase regulation is their activity and not localization per se, biosensors for activated Cdc42 may be of use to better visualize zones of activation.

Indeed, earlier work has implicated the Rho GTPases in dorsal closure, though mostly in the context of the leading edge epidermal cells (Harden et al., 1999; Jacinto et al., 2000; Jacinto et al., 2002), and there has been some success with biosensors in assessing Rho1 activity in amnioserosa cells during dorsal closure (Azevedo et al., 2011), and of Cdc42 activity during Drosophila wound healing (Abreu-Blanco et al., 2012).

Another possible mode of PAR complex interaction with the apical surface is through the apical transmembrane protein Crb. Par-6 can bind to Crb directly and indirectly.
aPKC can phosphorylate and thus interact transiently with Crb (Sotillos et al., 2004). Indeed, recent work has shown that in the *Drosophila* embryonic placode boundary, the planar-polarized absence of Crb leads to a co-depletion of aPKC from a specific cell cortex (Roper, 2012). Baz also interacts with Crb via Sdt (Krahn et al., 2010), thus providing another possible mechanism for PAR complex localization to the apical surface. My work has shown that Crb is localized to apical surface puncta in amnioserosa cells, closely associated with PAR complex (David et al., 2010), suggesting close interactions between the Crb and PAR complexes in the amnioserosa. To test this further, it will be interesting to express Crb constructs incapable of binding to the PAR complex, or PAR protein constructs incapable of binding the Crb complex, and assess PAR protein localization.

It appears that actin, myosin, aPKC, and Baz are interdependent for efficient localization to the apical surface. Recent evidence has outlined the necessity of positive feedback and interdependent interactions for efficient polarization of Baz during cellularization, relying on Canoe and Rap1 (Choi et al., 2013). Here, the AJ-associated proteins Canoe and Rap1 are critical for apical positioning of Baz during cellularization, but Baz and aPKC are also required for proper positioning of Canoe (Choi et al., 2013). Thus, a circuit of multiple protein network interactions could function to establish polarity during *Drosophila* cellularization. This could be an example of positive feedback required for establishment of large polarized domains (Fletcher et al., 2012), and is reminiscent of later stages in *Drosophila* embryogenesis wherein maintenance of polarity requires a network of interactions between apical and basolateral regulators (Laprise et al., 2009) and multiple, redundant mechanisms of positioning Baz (McKinley et al., 2012; McKinley and Harris, 2012). In my results, actomyosin networks appear to have the “upstream” role in recruiting aPKC and eventually Baz to the apical surface. Expression of Baz incapable of binding to aPKC localizes instead to the apical circumference with the junctions, suggesting a reliance of aPKC – Baz interactions for apical surface localization. However, Baz is also required for efficient targeting of aPKC to the cell cortex of both amnioserosa (my data) and epidermal cells (McKinley et al., 2012). Together, these results suggest that a circuit of interactions between actomyosin networks and the PAR
complex may be required for their mutual robust apical surface enrichment. To further investigate the direct link between the PAR complex and actomyosin, I will attempt to identity putative aPKC phosphorylation targets that affect actomyosin networks, as to be outlined below. Alternatively, possible interaction partners with Baz and Par-6 that also affect actomyosin networks can also be pursued.

4.3 Delayed negative feedback circuits between networks in vivo

Investigations in other systems, mainly synthetic gene circuits, have described oscillatory networks arising from delayed negative feedback. Classic examples such as the “repressilator” have repressive genes in a circuit and the reporter gene GFP rises and falls with time (Elowitz and Leibler, 2000; Tigges et al., 2009). Interestingly, these networks can be “tuned” when there is addition of positive feedback such that their amplitude or period can be change depending on the magnitude of input into the system (Stricker et al., 2008; Tigges et al., 2009). Such work has thus informed the understanding of other oscillatory networks.

Actomyosin networks have some limited negative feedback, and have been seen to be oscillatory. For example, actin polymerization can induce recruitment of actin depolymerizing factor in the contractile ring of mitotic cells (Chen and Pollard, 2011; Nakano and Mabuchi, 2006). Furthermore, the tension produced by actomyosin contractility can act as both positive and negative feedback. Tension enhances myosin attachment to actin (Fernandez-Gonzalez et al., 2009; Kovacs et al., 2007; Ren et al., 2009). However, excessive tension can cause actin depolymerization and close mechanosensitive ion channels that would otherwise stimulate myosin activators (Lee et al., 2013; Soares e Silva et al., 2011). There are many recent examples of oscillatory actomyosin networks in vivo (e.g., David et al., 2010; Martin et al., 2009; Roh-Johnson et al., 2012) but the degree to which delayed negative feedback plays a role remains unclear.

My research suggests the PAR complex can form an aspect of this delayed negative feedback. Furthermore, I have found that actomyosin networks are somehow important to recruit the PAR complex to the apical surface of amnioserosa cells. However, I also
previously demonstrated that aPKC is an inhibitor of actomyosin (David et al., 2010). Possible antagonism of aPKC by Baz could be part of the delay in this circuit. To examine the importance of aPKC inhibition further, other aPKC inhibitors could be used, such as the pseudosubstrate region within the amino terminus of aPKC that functions as a competitive inhibitor to keep aPKC in an autoinhibited state (Graybill et al., 2012). However, since Baz contains multiple binding sites for aPKC both directly (Nagai-Tamai et al., 2002; Wodarz et al., 2000) and through Par-6 (Joberty et al., 2000; Lin et al., 2000; Morais-de-Sa et al., 2010), this may increase local Baz concentration around aPKC and thus make it a more effective competitive inhibitor.

Careful examination of the buildup of apical surface PAR complex components in relation to actomyosin can also be performed. Since recent mathematical modeling suggests that the levels of actomyosin activators in amnioserosa cells during dorsal closure can rise and fall with each actomyosin pulse (Wang, Q. et al., 2012), the activity of the actomyosin inhibitor may in turn rise and fall as well, albeit with a temporal delay. It will therefore be interesting if abundance and localization of the PAR complex at the apical surface of amnioserosa cells rises and falls with each actomyosin pulse. Our current data suggests that the abundance and brightness of PAR complex puncta appear relatively stable at the apical surface of amnioserosa cells in relation to each actomyosin pulse (David et al., 2010). More careful comparisons of PAR complex puncta levels in real time with high temporal resolution in relation to actomyosin pulses may reveal whether apical PAR complex levels increase with the appearance of each actomyosin network. In addition, although I note a stage-dependent difference in PAR complex localization from apical circumferential to apical surface puncta during early to late dorsal closure, I do not currently adequately capture the mode and mechanisms of this transition. Thus, more detailed live imaging may uncover whether apical surface PAR complex puncta increase step-wise in response to each actomyosin pulse, or whether they linearly increase through another unknown mechanism.
4.4 Possible targets for the aPKC phosphorylation

My research supports the idea that Baz acts as a potential competitive inhibitor for aPKC. Baz interaction with aPKC may tune the substrate preference of aPKC from Lgl towards Numb in *Drosophila* neuroblasts (Wirtz-Peitz et al., 2008). *In vitro*, an aPKC-binding fragment of Baz acts as a competitive inhibitor of aPKC (Graybill et al., 2012) as seen earlier in *in vitro* experiments on their mammalian homologs (Lin et al., 2000). In MDCK cells, overexpression of Par-3 that cannot be phosphorylated by aPKC also leads to disruption of TJ formation (Nagai-Tamai et al., 2002), presumably either by disrupting Par-3 functions or by disrupting aPKC functions elsewhere (Iden et al., 2012).

If apical surface Baz in amnioserosa cells functions as a competitive inhibitor of aPKC kinase activity, then the aPKC substrates must be considered. Work in mammalian cells has implicated possible aPKC phosphorylation targets affecting actomyosin networks. For example, aPKC and Par-6 can recruit an E3 ubiquitin ligase, leading to Rho1 degradation (Wang et al., 2003). Furthermore, mammalian aPKC phosphorylates p190RhoGAP to change its preference such that p190RhoGAP inactivates Rho1 instead of Rac1 (Levay et al., 2009; Zhang and Macara, 2008). Mammalian aPKC can also phosphorylate ROCK, leading to its displacement from the cortex (Ishiuchi and Takeichi, 2011). Thus, aPKC in mammalian cell culture can inhibit actomyosin networks. Whether these phosphorylation events are conserved in *Drosophila* remains to be seen.

To determine whether aPKC phosphorylation sites can affect actomyosin networks in *Drosophila*, I first attempted a bioinformatics approach to scan for putative aPKC phosphorylation sites in the *Drosophila* proteome. I obtained a list detailing entries from the predicted *Drosophila* proteome that have to been assigned with the gene ontology (GO) term for “actin”. Next, I scanned these proteins for high-confidence PKCζ phosphorylation sites using the computational tool Scansite 2.0 (Obenauer et al., 2003). This tool scans primary amino acid sequences and allows for prediction of specified motifs based on similarity to sites found from other databases.
Of the list of 63 genes with putative aPKC phosphorylation targets, I then hand-picked 5 target proteins on which to focus. These proteins were chosen based on either general interest, conservation of the site or protein, or possible links to Rho GTPases: Bitesize, Ena, and Hu-li tai shao are actin-interacting or regulating proteins (Gates et al., 2007; Petrella et al., 2007; Pielage et al., 2011; Pilot et al., 2006; Wang et al., 2011); TRIO (GEF of Rac1 or Rho1) and Still Life (SIF, the Rac1 GEF, homolog of Tiam1) were also chosen as GEFs of the Rho GTPases (Newsome et al., 2000; Sone et al., 2000).

To assess whether these are subjected to aPKC phosphorylation, these candidates can be pursued with localization studies and perturbations. First, I performed preliminary immunofluorescence staining with available antibodies against Ena, Hu-li tai shao, and TRIO. My preliminary results suggest that these three proteins possibly localize to the apical circumference and/or the apical surface of amnioserosa cells during dorsal closure, but further work needs to be done to better resolve their localization.

Next, testing for the importance of these putative phosphorylation sites can be pursued by mutating the phosphorylation sites to either mimic or abolish aPKC phosphorylation. These genes have already been subcloned into the Gateway System for cloning. The proteins are tagged at either the carboxy or amino terminus with mCherry for visualization live with or without dual imaging with actomyosin networks. The constructs are also under UAS control to allow for tissue-specific expression. Stable integration into the attP2 site in *Drosophila* allowed different constructs to be inserted into the same site in the genome (Groth et al., 2004), minimizing possible differences in expression of the constructs from position effects. First, to test whether the constructs are functional, their ability to rescue mutant phenotypes of these genes will be assessed most easily at first by measuring differences in overall lethality when expressing them in a mutant background. Differences in ability to rescue mutants when comparing WT, phosphomimetic, and nonphosphorylatable forms of the candidate genes would suggest a functional importance of that putative phosphorylation site. However, there are several caveats to this approach: available mutants may not be sufficiently null to abolish gene function, and mutants may not be lethal. To address these concerns, careful quantification of lethality of mutants must first be confirmed, with alleles that either delete large fragments of the gene or
alleles that are known to be null. If genes are not essential, then phenotypes other than lethality could still be assessed (such as behavioural or morphological), though this is more arduous than a screen of lethality. Furthermore, these UAS constructs will initially be driven with a ubiquitous GAL4 driver (such as da-GAL4), though ectopic expression of the constructs may in turn cause lethality. To evaluate possible effects of ectopic gene expression, lethality will also first be evaluated when these constructs are expressed. Ideally, GAL4 driven under the endogenous promoter of the relevant gene would be used, but this is pending availability.

Next, after testing the ability of candidate constructs to rescue endogenous gene function, candidates with possibly functional phosphorylation sites will then be pursued further. First, the subcellular localization of phosphorylation mutants will be compared to WT or endogenous protein. In addition, effects of these constructs on actomyosin networks can be studied either with overall morphology of the networks through immunofluorescence, or differences in dynamics as seen with dual live imaging. However, the endogenous form of the candidate protein may mask possible effects of the phosphorylation mutants. To address this possibility, the constructs can be expressed in a mutant background (as discussed above to assess rescue). Several caveats are present with this approach: zygotic mutants may have insufficient depletion of the protein from maternal gene contribution in the embryo, or alternatively, there may be earlier global defects. Maternal/zygotic mutants would eliminate maternal contribution in the embryo but may have significant defects during earlier embryogenesis or oogenesis. To address these concerns, the constructs can instead be co-expressed with RNAi against the endogenous genes that would leave the exogenous constructs intact. This can be achieved by using RNAi that targets the 5’ or 3’ untranslated regions (UTRs) that are present in the endogenous mRNA but are absent in my engineered constructs. The WT, phosphomimetic, and nonphosphorylatable forms of the genes are now in transgenic animals and are currently being investigated by Yixie Zhang, an MSc student in our lab.

Recent mathematical modelling of amnioserosa pulsatile behaviour (Wang, Q. et al., 2012) will allow us to make predictions about the putative aPKC phosphorylation target. The model from our collaborators (Wang, Q. et al., 2012) predicts that the activity of the
actomyosin activator rises and falls in concert with the actomyosin networks themselves. This allows us to screen for putative aPKC phosphorylation targets whose activities rise and fall. However, it is worth noting that activity and localization of a protein need not necessarily be the same; relatively stable protein localization can be activated or inactivated through other means. For example, Rho GTPase proteins are activated when GTP-bound, though this may also involve membrane localization (Bokoch et al., 1994; reviewed by Cherfils and Zeghouf, 2013); and mechanosensitive ion channels remain on the plasma membrane, but open and close in response to mechanical stress (Kapustina et al., 2008).

Recent advances in understanding aPKC phosphorylation targets may lead to further refinement in the search for relevant aPKC substrates. When I started the bioinformatics scan of the Drosophila proteome for aPKC phosphorylation targets (Obenauer et al., 2003), there was a relatively well-defined consensus phosphorylation site. However, recent updates to the databases from which Scansite derives its information have vastly reduced the predictive power of the consensus site; the consensus site appears now to be the phosphorylatable serine or threonine. A recent crystal structure of aPKC with its Par-3 substrate may shed light on this looseness of aPKC substrates. Here, the researchers found that atypical PKCs have a unique insertion in a region surrounding the catalytic site that would allow some substrate peptides to fill in and form interactions (Wang, C. et al., 2012). This may provide some allowance of some substrates with or without positively-charged residues to interact with the catalytic core of aPKC. Another issue that may play a role in determining the targets of aPKC are the interactions with aPKC binding partners. Par-6 can modulate the kinase activity of aPKC (Graybill et al., 2012; Wirtz-Peitz et al., 2008) and Par-6 has at least two protein binding domains other than its interaction with aPKC. Furthermore, other partners in Drosophila can interact with Par-6 and aPKC to alter aPKC substrate affinity: either Aurora-A or Bazooka (Wirtz-Peitz et al., 2008). Therefore, in an attempt to better characterize putative aPKC phosphorylation targets, it may be worthwhile to take into account the effects of 3D structure and binding partners of the PAR complex.
4.5 Overall conclusions

In summary, my thesis research has investigated dynamic actomyosin networks that mediate cell shape change during *Drosophila* development, and how these networks interact with regulators of apicobasal polarity. These actomyosin networks are at first highly pulsatile, then by later stages become more persistent and stable, leading to sustained constriction. These networks are therefore an interesting model with which to study cell shape change and regulation of actomyosin dynamics *in vivo*. Furthermore, investigations in other model systems suggest that dynamic actomyosin meshwork-like networks may be an evolutionarily-conserved configuration to mediate constriction. It will be interesting to see whether these networks are also conserved during mammalian morphogenesis. In addition, my research has shown that dynamic actomyosin networks of the amnioserosa are regulated by the PAR complex regulators of polarity. Baz enhances, whereas Par-6/aPKC suppresses actomyosin networks. My results suggest that Baz may enhance actomyosin networks by acting as a competitive inhibitor of aPKC. Delayed negative feedback may be at play in actomyosin network oscillations and the dynamics of these oscillations can be tuned by refinement of the regulatory circuits involved. Finally, dynamic actomyosin networks, apical surface Baz, and apical surface Par-6/aPKC appear to be mutually interdependent. Together, my results suggest circuits of regulatory networks that shift and change over time to drive a robust and tunable system.
References


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For Chapter 2: The PAR complex regulates pulsed actomyosin contractions during amnioserosa apical constriction in *Drosophila*


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For the Methods Chapter (Appendix 2)


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Appendix 1: List of Movies

These movies correspond to Chapter 2.

**Movie 2.1.** Assembly and disassembly of a Zip::GFP network in the amnioserosa at mid-DC. Each frame is a 4.5-µm-deep projection that is 49.7 µm wide. The imaging was performed at 11.1 seconds/frame and is shown at seven frames/second (77.7× real time). The amnioserosa is to the right and the epidermis is at the bottom left.

**Movie 2.2.** An actin network mediating apical constriction of a single amnioserosa cell. Each panel is a single plane that is 27.5 µm wide. The imaging was performed at 27.8 seconds/frame and is shown at seven frames/second (194.3× real time). Stills from this movie are shown in Fig. 2.5A.

**Movie 2.3.** Comparison of PAR complex and myosin dynamics at the apical surface of a single amnioserosa cell at DC. Each panel is a 3-µm-deep projection that is 22.4 µm wide. The imaging was performed at 44.8 seconds/frame and is shown at seven frames/second (313.6× real time). Stills from this movie are shown in Fig. 2.6D.
Appendix 2: Live Imaging of Drosophila Embryos: Quantifying Protein Numbers and Dynamics at Subcellular Locations

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I have used the Materials, Sample Preparation and Basic Imaging Considerations, and Statistical Considerations outlined below for my live imaging experiments during the course of my thesis work.

Contributions:

I wrote the following sections: Section 2 (Materials), Section 3.1 (Sample Preparation and Basic Imaging Considerations), and Section 3.4 (Statistical Considerations). I prepared Figure 1.

DJVD, MAM, and RFAM contributed equally.