Mechanisms of Morphogenetic Regulation During Gastrulation

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Abstract

The shape of an embryo is achieved through tissue-scale morphogenetic movements. These force-dependent events are guided by adhesive, mechanical and chemical cues. During morphogenesis, the cells within a tissue adhere to their neighbors primarily through cadherin-catenin complexes and to their extracellular matrices through integrin-based focal adhesions. Mechanical forces are generated at sites of adhesion through actomyosin contractility. Not only are these contractile forces important for cell migration, but they also function in force-dependent mechanosensitive signaling pathways. One such mechanosensitive process is the fibrillar assembly of the extracellular matrix protein, fibronectin.

Integrin receptors are important for attachment to the extracellular matrix as well as its fibrillar assembly. This force-dependent process requires cadherin-catenin adhesive complexes and actomyosin contractility. One catenin found at these adhesions, plakoglobin (also called γ-catenin), mediates the attachment of actin stress fibers to the cytoplasmic tails of classical cadherins through interactions with actin-binding proteins. In *Xenopus* gastrulae, plakoglobin has been identified as an essential member in the force-induced collective migration of the mesendoderm tissue. In Chapter 2 of this thesis I present data indicating that plakoglobin is required for the morphogenetic processes of convergent extension and epiboly. When plakoglobin expression is reduced, fibronectin fibrillogenesis fails as a result of cell adhesion defects. Plakoglobin morphant cells are unable to form strong attachments to cadherin or fibronectin substrates, processes that are required for assembly of the soluble dimers into fibrils.

Contractile forces applied at cell adhesions function as mechanical cues that can also activate intracellular signaling pathways in neighboring cells. Mitochondrial enzymes have been found to undergo conformational changes, likely associated with changes in activity states, in response to mechanical stimuli. During the collective migration of the mesendoderm, cells within the leading edge extend broad lamellipodial protrusions that apply high traction stresses to a fibronectin substrate. In Chapter 3 of this thesis I provide evidence indicating that these protrusions contain densely packed, punctate mitochondria. The mitochondria in these protrusions are dynamic, in a high activity state, and likely coordinate the collective migration of the tissue through the localized production of ATP and purinergic signaling.

The data presented in these studies focus on two different force-dependent processes in the context of morphogenesis. In addition, my work identifies plakoglobin as a regulator of fibronectin fibril assembly, which emphasizes the role of cadherin adhesion in fibrillogenesis. My work on the mesendoderm suggests that a mechanical activation of mitochondria functions in collective cell migration. Together, these findings provide a more comprehensive understanding of the morphogenetic processes involved in embryonic development as well as in the progression of diseases such as fibrosis and cancer metastasis.

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"An investment in knowledge pays the best interest." -Benjamin Franklin

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List of Abbreviations

ADP	Adenosine diphosphate
AFM	Atomic force microscopy
AJ	Adherens junction
AMPK	AMP-regulated protein kinase
APC	Adenomatous polyposis coli
ATP	Adenosine triphosphate
BCR	Blastocoel roof
CAM	Cell adhesion molecule
CS-MS	Cysteine shotgun- mass spectrometry
DIC	Differential interference contrast
DMZ	Dorsal marginal zone
DRP	Dynamin-related protein
DSHB	Developmental studies hybridoma bank
ECM	Extracellular matrix
EMT	Epithelial to mesenchymal transition
ER	Endoplasmic reticulum
FA	Focal adhesion

FAK	Focal adhesion kinase
FN	Fibronectin
FRET	Förster resonance energy transfer
GFP	Green fluorescent protein
GFP-MA	Mitochondria-anchored GFP
Hep II	Heparin-binding domain
IF	Intermediate filament
LEF	Lymphocyte enhancer factor
MBS	Modified Barth's saline
MBT	Midblastula transition
MFN	Mitofusin
MHC	Major histocompatibility complex
MT	MitoTracker
OXPHOS	Oxidative phosphorylation
OPA1	Optic atrophy 1
p120	p120 catenin; $p120^{ctn}$
PDGF	Platelet-derived growth factor
PG	Plakoglobin

PLL	Poly-l-lysine
qPCR	quantitative reverse transcription polymerase chain reaction
RGD	Arginine–glycine–aspartic acid
ROS	Reactive oxygen species
TCA	Tricarboxylic acid
TCF	T cell factor
TEM	Transmission electron microscopy
TIRF	Total internal reflection fluorescence
TJ	Tight junction
TMRE	Tetramethylrhodamine, ethyl ester
UDP	Uridine diphosphate
UTP	Uridine triphosphate

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Chapter 1

Introduction

Morphogenesis

Large, tissue-scale movements are essential processes in the development and repair of tissues. These coordinated cell movements aid in wound healing (Friedl and Gilmour, 2009), in forming branched structures within the mammary gland, kidney, and lung (Ochoa-Espinosa and Affolter, 2012) and are responsible for shaping embryos during development (Keller et al., 2008; Rozario and DeSimone, 2010). Morphogenetic events are force-driven processes that involve cells pulling on one another as well as on their substrate, which is composed of a glycoprotein-rich extracellular matrix (ECM) (Collins and Nelson, 2015). Investigating the proteins and signaling pathways involved in normal morphogenesis is important for understanding how these processes are perturbed in cancers and diseases.

Xenopus laevis embryos are a powerful model system for studying development and complex tissue movements. The availability of embryos makes biochemical and *in vivo* experiments possible. The large size of the externally developing embryos and intracellular nutrient stores in the form of yolk platelets allows for extended *in vivo* studies, transplantation, *ex vivo* explants, and single cell experiments. We have taken advantage of these benefits to establish the functions of various proteins in morphogenesis.

Shortly after fertilization, embryos begin a period of rapid cell division. After 12 synchronous divisions, the time between cleavage events lengthens and divisions become asynchronous. This stage of development is termed the midblastula transition (MBT) and

is also the point at which zygotic transcription begins (Newport and Kirschner, 1982). In the stages following the MBT the embryo begins taking shape, changing from a sphere of dividing cells to an elongated body with numerous cell types and tissues. Gastrulation is the developmental time frame during which the three primary germ layers (mesoderm, endoderm, and ectoderm) are first created through a combination of growth factor signaling and morphogenesis.

The morphogenetic events that occur during gastrulation rely on coordinated cellular shape changes and movements. Five of the main morphogenetic movements that occur within this developmental time frame include epiboly, vegetal rotation, invagination, collective migration, and convergent extension (Figure 1.1). The oriented division and radial intercalation of the ectodermal cells that line the roof of the fluid-filled blastocoel drive epiboly. During epiboly, the blastocoel roof (BCR) tissue thins radially and spreads towards the marginal zone (Szabó et al., 2016b). The ectoderm is induced to become mesoderm along the marginal region of the embryo. During the same time frame, the vegetal cell mass within the embryo rotates, contributing to the involution of the newly induced mesodermal tissue (Ibrahim and Winklbauer, 2001; Winklbauer and Damm, 2011; Winklbauer and Schürfeld, 1999). The first few rows of post-involuted mesoderm are fated to become both mesodermal and endodermal structures in the embryo and the tissue is termed the mesendoderm. The cells within the mesendoderm adopt a shingled arrangement, overlapping one another. The mesendoderm tissue migrates as a sheet of cells from the vegetal pole towards the animal pole. This collective cell migration of the mesendoderm along with epiboly, contribute to the convergent

extension of the dorsal mesoderm (Hara et al., 2013; Tada and Heisenberg, 2012). The two main cell movements that occur during convergent extension are mediolateral and radial intercalation. The dorsal mesoderm extends along the anteroposterior axis as the cells within the tissue elongate and intercalate between one another in the mediolateral and radial directions (Keller et al., 2008). Although there are differences in the proteins and signaling pathways involved in these morphogenetic movements, they all rely on cell migration machinery including cell adhesion and actomyosin contractility.

Cell adhesion molecules

Nearly all living organisms exhibit some form of adhesion. Many bacteria adhere to one another and to secreted polymers through both receptor-mediated and nonspecific adhesions in biofilms (Garrett et al., 2008). Single cell migration events and coordinated large-scale movements of tissues in more complex organisms are orchestrated by cell-cell adhesions as well as adhesion to an ECM. Cells attach to one another and to their substrates through transmembrane adhesion proteins termed cell adhesion molecules (CAMs). CAMs are often cell-type specific and are regulated both spatially and temporally (Collins and Nelson, 2015).

The immunoglobulins are a superfamily of CAMs that can form homotypic and heterotypic adhesive interactions. Cadherins and integrins, the CAMs on which this dissertation focuses, are among the receptors to which immunoglobulin-family members form heterotypic interactions. One subgroup of immunoglobulin superfamily, the nectins,

is especially important for cadherin adhesions. Nectin accumulation and adhesion precedes and is required for cadherin recruitment at cell junctions (Takai and Nakanishi, 2003). Other immunoglobin CAM family members include the major histocompatibility complex (MHC) and T-cell receptors that form heterotypic interactions during antigen recognition (Aricescu and Jones, 2007).

Immune cell homing relies on selectin-family CAMs. There are 3 known selectin family members, including L, E, and P-selectin. Selectins require calcium for adhesion, and recognize various carbohydrates. L-selectin is found on lymphocytes, E-selectin on endothelial cells, and P-selectin on endothelial cells and activated platelets. The ligands for E and P-selectin are expressed by lymphocytes, while L-selectin ligands are present on endothelial cells. The coordination of the expression patterns of these selectins and their respective ligands is essential for lymphocyte homing to sites of inflammation (Cummings and Smith, 1992; Varki, 1994).

The two classes of CAMs that will be discussed in depth in this thesis are the cadherin and integrin families. Cadherins are calcium-dependent transmembrane adhesion molecules that form homotypic interactions across cells to facilitate cell-cell adhesion. These adhesion proteins contain cadherin-type repeats in their extracellular domains, which are responsible for the homotypic interactions between cells. The number of sequential cadherin-type repeats varies depending on the protein, ranging from as few as 4 to more than 30 (Oda and Takeichi, 2011). Cadherin proteins form cis interactions with other cadherins on the same cell as well as trans interactions across cells. Calcium is

required for trans but not cis interactions (Kim et al., 2011). Removal of calcium from cell culture medium results in the dissociation of cadherin adhesions and subsequent endocytosis of the associated protein complex (Troyanovsky et al., 2006).

Adhesion to and assembly of ECM fibrils is dependent on various receptors that are specific for certain ECM molecules. The integrins are the largest and most ubiquitously expressed family of ECM receptors. Integrins are cation-dependent heterodimeric transmembrane receptors. Substrate specificity is determined by the integrin α and β-subunits expressed by the cell. In humans, there are 18 α- and 8 β-subunits, with 24 different integrin heterodimers that have been identified thus far. The central and most promiscuous β-subunit is β_1, which interacts with 12 known α-subunits to confer adhesion to a variety of substrates (Hynes, 2002). The folding and dimerization of integrins requires intracellular calcium, which also acts to keep the integrin in an inactive state until it is secreted to the cell surface (Tiwari et al., 2011). Surface-bound integrins also require divalent cations for ligand binding. Increasing concentrations of calcium or magnesium results in increased affinity for the ECM substrate. Furthermore, manganese is capable of outcompeting these cations for integrin binding and confers a significant increase in affinity, presumably by maintaining a high affinity conformation (Gailit and Ruoslahti, 1988; Humphries, 2002). During *Xenopus* gastrulation the primary integrin heterodimer is $\alpha_5\beta_1$ (Whittaker and DeSimone, 1993). Cadherin and integrin-family CAMs will be the focus of this thesis.

Cell-cell adhesion

In a typical epithelium, there are four major kinds of cell-cell contacts on the lateral junctions that are distinguishable by electron microscopy (Figure 1.1). In order of most apical to basal, the junctions are [1]tight junctions (TJs), [2]adherens junctions (AJs), [3]gap junctions and [4]desmosomes. The most apical cell contact is the TJ (also called occludin junctions or zona occludens). The lateral membranes of two cells are closest in proximity at TJs, which form many of the physiological barriers throughout the body. The main CAMs within a TJ are members of the claudin family of proteins (Zihni et al., 2016). Both AJs and desmosomes utilize cadherin-family CAMs. AJ and desmosomes are discussed in more detail below. The most basal of the cell junctions are gap junctions. Instead of utilizing cell adhesion molecules, gap junctions are mediated by channel-forming proteins in the connexin and innexin-families. Cytosol and small molecules such as ions and metabolites are be transferred between cells through these channels, allowing for cytoplasmic coupling and rapid signal transduction across cells and tissues (Skerrett and Williams, 2017).

Adhesion at AJs and desmosomes occurs through cadherins. Although cadherins do not form strong adhesions upon initial attachment, adhesive strength increases drastically upon lateral clustering (Yap et al., 1997) and with the reinforcement of actin and intermediate filaments (IFs) at AJs and desmosomes, respectively. Cadherins are divided into two major groups: the classical and the nonclassical. Classical cadherins include those found in AJs, whereas desmosomal cadherins fall within the category of

nonclassical cadherins. Differences in amino acid sequence of the cytoplasmic tail domains determine the proteins recruited to cadherins. The proteins found in AJs and desmosomes are for the most part mutually exclusive, with very few proteins shared between the two complexes (Gumbiner, 2005).

The classical cadherins found in AJs are often cell-type specific. E-cadherin is predominantly found in epithelial cells, N-cadherin in neural tissue, and VE-cadherin in vascular endothelial cells. I have focused my studies on C-cadherin as it is the most abundant cadherin found in *Xenopus* during early embryonic development. C-cadherin was originally named EP-cadherin because of its sequence homology to both E- and P-cadherin (found in the placenta) (Ginsberg et al., 1991; Levi et al., 1991). The tendency of cadherins to form homotypic interactions facilitates *in vitro* cell sorting. For example, cells overexpressing N-cadherin group together in a pellet of wildtype cells. There is conflicting evidence suggesting that cadherins are more promiscuous *in vivo*, forming heterotypic interactions with other cadherins (Gumbiner and Niessen, 2002; Ninomiya et al., 2012). Nonetheless, cell-type specificity of classical cadherins is important for tissue structure and function. During epithelial to mesenchymal transition (EMT) events, cells differentiate from an epithelial cell-type that favors cell-cell adhesions to a more migratory mesenchymal cell-type relying on attachment to the ECM. One of the best examples of a *bona fide* EMT is the migration of the cells within the neural crest during neurulation. Neural crest cells downregulate E-cadherin and begin to express high levels of N-cadherin as they become highly migratory and exhibit contact inhibition of locomotion. This contact inhibition limits cell-cell contacts forcing more transient N-

cadherin AJs as opposed to the stable E-cadherin junctions found in epithelia (Scarpa et al., 2015).

Although most cells exhibit AJs, only certain tissues including epithelia and cardiomyocytes have desmosomes. Desmosomes are significantly stronger and longer-lived adhesions compared to AJs. The desmosomal cadherins include cell-type specific desmogleins and desmocolins. The cortical IF network, made up of vimentin or keratin, is anchored and organized at the cytoplasmic tails of these desmosomal cadherins (Garrod and Chidgey, 2008). Cells within an epithelium are structurally reinforced by desmosomes, whereas more migratory cell types typically favor stronger ECM attachments and do not form desmosomes. During EMT events, transcription factors such as *Slug* are expressed, leading to the dissociated of desmosomes and subsequent cell spreading (Savagner et al., 1997).

Cadherin adhesions in *Xenopus* development

The primary structural role of the cadherin/catenin complexes is in maintaining tissue integrity. Cadherin complexes achieve this structural integrity through the organization of cortical IF and actin cytoskeletal networks at cell-cell adhesions. Inhibition of maternal cadherin proteins in *Xenopus* embryos results in severe adhesion defects and the dissociation of blastomeres (Angres et al., 1991; Heasman et al., 1994; Kurth et al., 1999). Overexpression of dominant negative constructs yields disruptions in cell-cell adhesion and morphogenesis (Kuhl et al., 1996; Lee and Gumbiner, 1995;

Levine et al., 1994). Furthermore, perturbations in the actomyosin contractility (Lee and Harland, 2007; Pfister et al., 2016; Zhou et al., 2009) or keratin IF (Klymkowsky et al., 1992; Torpey et al., 1992; Weber et al., 2012) cytoskeletal networks also result in defects in adhesion and morphogenesis. These studies stress the importance of cell-cell adhesions in development and morphogenesis.

Catenin proteins at cell junctions

Although cadherins are partially responsible for maintaining cortical actin and IF networks, they do not directly interact with these cytoskeletal components. Attachment of the cytoskeleton to cadherin adhesions is mediated through catenin-family proteins (Figure 1.2). The majority of the catenins are related to the armadillo protein, initially discovered in *Drosophila.* These proteins contain a series of armadillo repeats that facilitate most interactions with other proteins. The catenins have divergent, yet occasionally overlapping functions. In AJs there are four major catenins: α-catenin, β-catenin, plakoglobin (PG; also known as γ-catenin), and p120-catenin (p120; $p120^{ctn}$) (Nelson, 2008).

Unlike most other catenins at cell contacts, p120 does not facilitate the recruitment of cytoskeletal elements; instead, it binds to classical cadherins at the juxtamembrane domain within the cytoplasmic tail, enhancing lateral clustering (Yap et al., 1998) and regulating cadherin recycling (Ireton et al., 2002; Perez-Moreno and Fuchs, 2012). α-catenin is the only one of the four main AJ catenins that does not directly bind

to cadherin cytoplasmic tails, but is anchored at the AJs by PG and β-catenin. α-catenin can interact with actin directly (Figure 1.3) as well as through additional binding partners such as vinculin and α-actinin (Knudsen et al., 1995; Nieset et al., 1997).

β-catenin and PG bind directly to the cadherin cytoplasmic tail domain (Figure 1.3). The two proteins share high amino acid sequence similarity, and serve redundant functions in AJs where they recruit and bind to α-catenin. Unbound cytoplasmic β-catenin and PG are targeted for degradation by the same axin and adenomatous polypsis coli (APC) destruction complex. β-catenin and PG contain 13 sequential armadillo repeats that form a single structural unit comprised of α-helices that together form a superhelix (Huber et al., 1997). This structure is responsible for most β-catenin and PG protein interactions, including the ability to bind to cadherin, α-catenin, and the APC-destruction complex (Hulsken et al., 1994; Kodama et al., 1999).

In addition to its role in AJs, PG performs an essential function at desmosomes where it interacts with the cytoplasmic tails of desmogleins and desmocolins. PG is the only AJ protein that is also required for desmosome assembly. β-catenin does not normally localize to desmosomes. However, β-catenin can compensate for a loss of PG at desmosomes in knockout mice (Bierkamp et al., 1999). As stated above, PG does not bind directly to the cytoskeleton at desmosomes. Instead, it recruits adapter proteins that bind and anchor IFs including vimentin and keratin. The two most important proteins that PG recruits at desmosomes are plakophilin and desmoplakin (Garrod and Chidgey, 2008). Plakophilin is another member of the catenin family of proteins. In addition to

binding to PG and IFs, plakophilin also is important in recruiting desmoplakin, another IF-binding protein (Bonné et al., 2003; Bornslaeger et al., 2001; Kowalczyk et al., 1999). Recruitment of desmoplakin is required in the formation of desmosomes, leading to further accumulation of IFs (Figure 1.3) (Choi et al., 2002; Gallicano et al., 1998).

IFs are relatively long-lived with slow turnover and disassembly rates. Interestingly, some cell types express what are thought to be more dynamic desmosome-like structures. Although these adhesive structures have many of the essential components of a typical desmosome, they utilize the classical cadherins found in AJs. These adhesive structures are smaller than desmosomes, and may be involved in different cellular processes. During lens development, there is a differentiation event during which the epithelial cells of the lens proliferate and a subset of daughter cells become fiber cells. Fiber cells migrate and elongate along the lens epithelia (Rao, 2008; Zelenka, 2004). This migratory cell type exhibits a desmosome-like structure, complete with an enrichment of PG and vimentin IFs at an N-cadherin adhesion. These cells express β-catenin, but only the N-cadherin/PG complex associates with vimentin IFs (Leonard et al., 2008). Furthermore, C-cadherin-based desmosome-like structures perform an essential role in the collective migration of the *Xenopus* mesendoderm. Removing PG from mesendoderm cells results in a loss of keratin IFs, depolarization of protrusion orientation and subsequent loss of directional migration (Weber et al., 2012). Desmosome-like structures have not been thoroughly investigated, but it is intriguing to think that they may serve as a more transient mechanism of attaching the IF cytoskeleton to cell junctions of motile cells that break and reform adhesions as they migrate.

Catenin protein functions outside of cell-cell adhesions

Most of the catenin family proteins localize to sites of cell-cell adhesion where they interact with cadherins. However, many catenins have been found to serve additional functions outside of cell adhesion. The most widely studied non-adhesive function of a catenin is the role of β-catenin in Wnt signaling. Wnt ligands are secreted molecules that are involved in large-scale planar cell polarity events among other things. Canonical Wnt signaling is characterized by the nuclear translocation of β-catenin and subsequent increases in the transcription of Wnt target genes. On its own, β-catenin does not have a high affinity for DNA. Instead, it functions as a transcriptional coregulator, relying on T cell factor (TCF) and lymphocyte enhancer factor (LEF)-family proteins for nuclear import and transcriptional regulation (Clevers, 2006). This process is essential for mesoderm induction (Schohl and Fagotto, 2003) as well as dorsalization of the embryo. Overexpressing β-catenin in a UV-ventralized embryo results in the formation of a dorsal axis (Guger and Gumbiner, 1995) and overexpression on the ventral side of an otherwise wildtype embryo results in the formation of a second axis (McCrea et al., 1993). When β-catenin is knocked down the result is a ventralized embryo that does not develop anterior structures. These dorsalization phenotypes are distinct hallmarks of canonical Wnt signaling (Heasman et al., 1994).

Interestingly, overexpressing PG results in secondary axis formation similar to that which is seen in β-catenin overexpressing embryos (Karnovsky and Klymkowsky,

1995; Klymkowsky et al., 1999; Merriam et al., 1997). These data were the first indications that PG is involved in Wnt signaling. Further experiments revealed that PG localizes to the nucleus in some cell types, albeit at a lower concentration than β-catenin. This lower level of nuclear localization of the protein is in part due to a lower binding affinity of PG to TCF/LEF transcription factors (Simcha et al., 1998; Zhurinsky and Shtutman, 2000). The two catenins are thought to regulate many of the same (Martin et al., 2009), but also some mutually exclusive target genes. For instance, PG, but not β-catenin, can regulate the expression of desmocolins 2 and 3 (Tokonzaba et al., 2013). However, evidence suggests that β-catenin is the primary Wnt signaling transcriptional coregulator (Shimizu et al., 2008).

Most of the research surrounding the catenins has focused on their role in adhesion and Wnt signaling, but there are other catenin functions that have not yet been well characterized. Both PG and plakophilin interact with mRNAs in some systems. PG can regulate mRNA transcript stability (Todorović et al., 2010). Within stress granules, plakophilin recruits elongation initiation factor 4A1, promoting 5' cap-dependent RNA translation (Wolf and Krause-Gruszczynska, 2010). Plakophilin can also be found within the nucleus where it is enriched at nucleoli after DNA damage. It is thought to play a role in DNA damage repair (Sobolik-Delmaire et al., 2010).

One understudied function of the AJ-associated catenins surrounds their ability to interact with pre-processed cadherins. Cadherins are translated as long pro-cadherin precursor proteins that are proteolytically cleaved prior to being trafficked to the cell

membrane. The pro-cadherins are only found in the endoplasmic reticulum and golgi apparatus, and are absent from cell contacts. Interestingly, catenins are able to bind to pro-cadherins. The functional consequence of the catenin-pro-cadherins interaction remains to be determined, however an intriguing thought is that the interaction may provide a potential link to the regulation of the basolateral polarization of cadherin secretion (Chen et al., 1999; Curtis et al., 2008; Wahl et al., 2003).

ECM proteins, their receptors, and fibrillogenesis

Cells not only adhere to one-another, but they also adhere to their ECM substrates. The composition of an ECM varies depending on the tissue and stage of development, and it is ever changing. The ECM in the skin and connective tissues is mainly composed of collagens (Ricard-Blum, 2010). Laminins are major components of the basement membranes that line the basal surfaces of epithelia (Shaohua Li et al., 2003). During *Xenopus* embryonic development, the notochord is lined with laminin during neurulation (Fey and Hausen, 1990), but fibrillin and fibronectin (FN) are predominate in earlier developmental stages (Davidson et al., 2004; Skoglund et al., 2006). Prior to gastrulation there is no significant accumulation of ECM proteins in the *Xenopus* embryo. At the onset of gastrulation, assembly is initiated with FN fibrillogenesis along the BCR (Lee et al., 1984; Winklbauer and Stoltz, 1995). This FN deposition along the BCR is required for epiboly (Rozario et al., 2009). Similarly, convergent extension of the dorsal mesoderm (Davidson et al., 2004; 2006; Marsden and

DeSimone, 2003; 2001) and mesendoderm migration (Davidson et al., 2002) are disrupted in FN morphants. FN will be the ECM protein of focus throughout this thesis (Rozario and DeSimone, 2010).

The FN protein consists of three kinds of repeated amino acid sequences, termed the types I-III repeats. There are a total of 12 type I, 2 type II, and 15-17 type III repeats depending on the FN isoform, in addition to a V-region that contains multiple alternative splice sites. This diversity in the type III repeat and V-regions yields multiple variations of FNs. Most integrins interact directly with the Arg, Gly, Asp (RGD) sequence within the tenth type III repeat region (III_{10}). This RGD sequence is a common feature found in many ECM components. Although most integrins require RGD for attachment, some instead interact with the CS1 domain within the V-region. Integrins also often utilize a synergy site found in the III_9 for stronger engagements of the integrin receptors. *Xenopus* cells attach and spread on a FN fusion protein containing only type III repeats 9-11. However, when a mutation is introduced at the synergy site, cells attach and form filopodial protrusions but do not spread and extend large lamelli (Ramos et al., 1996). Towards the c-terminus of the FN protein is a heparin-binding domain (Hep II) that facilitates interactions with heparin, heparan sulfate, and glycosaminoglycan chains (Ramos and DeSimone, 1996). Cells are able to attach to the substrate through various glycosaminoglycan chain-containing glycoproteins including the syndecan receptors (Woods et al., 2000).

When bound to a ligand, integrins undergo a conformational change (Su et al., 2016) that results in the recruitment of cytoplasmic adapter proteins. These adapter proteins attach to cytoskeletal components. With the exception of $\alpha_6\beta_4$, which binds to IFs at hemidesmosomes, integrins form focal adhesion (FA) structures rich in actin. These FAs begin as nascent adhesions and mature to form larger complexes as proteins are recruited and integrin receptors begin to cluster. Actin is anchored to the cytoplasmic tails of integrins through actin-binding proteins including talin, α-actinin, filamin, vinculin, and kindlin. Integrins have no enzymatic activity. Instead, they recruit different proteins that can affect cell migration, proliferation, polarity, and differentiation among other things (Hynes, 2002). One of the proteins that bind directly to the ligated integrin, paxillin, recruits many of these actin-binding proteins and kinases. Among signaling proteins recruited are focal adhesion kinase (FAK) (Sieg et al., 1999) and Src (Arias-Salgado et al., 2003; S. J. Parsons and J. Parsons, 2004). When inactive, many of these kinases adopt an autoinhibitory conformation in the cytoplasm. Upon binding to integrin receptors, the kinases undergo a conformational change that together with the high concentrations of the protein found at FAs leads to autophosphorylation and activation. FAK and Src in particular regulate FA turnover, an essential process in cell migration (Fincham and Frame, 1998; llić et al., 1995). FAK and Src can also initiate a kinase cascade resulting in the activation of signaling pathways that further regulate cell migration, contractility, and proliferation. Activation of these cascades in response to integrin interaction with external ligands is referred to as “outside-in” signaling.

Kinase promiscuity is essential to integrin function, allowing for not only outside-in mechanisms of signaling, but also inside-out signaling and transactivation of other transmembrane receptors. Inside-out signaling relies on activation of kinases and actin-binding proteins that then bind to the cytoplasmic tails of integrins and initiate a conformational change leading to a higher ligand-binding affinity state. In a similar mechanism, kinases also support the transactivation of other receptors. Integrins accumulate in lipid rafts on the cell surface and cluster upon activation (Leitinger and Hogg, 2001). Lipid rafts also have high concentrations of growth factor receptors. The close proximity of integrin and growth factor receptors and overlapping signaling pathways between the different receptors results in transactivation (Pike, 2005). Interestingly, there is also overlap between integrin and cadherin signaling pathways.

Integrins and cadherin adhesions crosstalk

Much like integrin and growth factor receptor transactivation, integrins and cadherins also exhibit overlapping signaling pathways. FAK and Src are two proteins that can affect both integrin and cadherin adhesions. Knocking down FAK results in a reduction of PG at cadherin adhesions (Bjerke et al., 2014). Src has been shows to phosphorylate and activate p120 both *in vitro* and *in vivo*, affecting cadherin clustering and recycling (Mariner et al., 2001). FAs and AJs also share connections to the same cortical actin networks, resulting in what we hypothesize is a direct mechanical linkage.

Activating or suppressing one class of adhesion molecule can affect the other through cytoskeletal rearrangements or changes in contractility.

Integrin-based adhesions have been shown to enhance or suppress cadherin adhesion strength depending on the experimental setup. In endothelial cells, engaging integrins with FN-coated microspheres results in the dissociation of the catenins from VE-cadherin adhesions (Wang et al., 2006). However, experiments performed in other systems suggest that the two families of adhesions predominantly work together, either redistributing forces or increasing adhesiveness. Traction force microscopy using mouse keratinocytes indicate that traction stresses exerted by cells in a cluster are balanced by cell-cell adhesions (Mertz et al., 2012). This cell-cell adhesion-mediated balancing of traction forces also occurs in collectively migrating sheets of mammary epithelial cells (Bazellières et al., 2015).

In addition to the redistribution of forces, integrin engagement affects cell-cell adhesive strength. E-cadherin AJs are stronger in single cells that are stimulated with FN-coated microspheres. This mechanism of crosstalk is Src-mediated and requires actin remodeling (Martinez-Rico et al., 2010). Similarly, in *Xenopus* tissues, the engagement of integrin adhesions strengthens cadherin-based adhesions. Adding soluble FN fragments to cells results in stronger attachment to cadherin (Marsden and DeSimone, 2003), whereas an overexpression of cadherin leads to an increase in traction stresses on a FN substrate and precocious integrin-mediated fibrillogenesis. Much of the integrin-cadherin crosstalk can be attributed to a mechanical coupling of the two adhesive complexes

through the actin cytoskeleton (Dzamba et al., 2009). This mechanical coupling allows for rapid crosstalk between the two spatially distinct complexes.

Mechanotransduction at adhesions

Cascades of post-translational modifications have been the focus of most research into mechanisms of cell signaling. More recently there has been an effort to understand mechanically-induced changes in protein activity and protein-protein interactions in response to external stimuli (mechanotransduction). An example of *bona fide* mechanically-sensitive proteins are the force-gated ion channels of the hair cells in the inner ear. As sound enters the inner ear it bends bundles of actin-rich stereocilia extending from the apical surface of the sensory hair cells. This physical bending of the sterocilia opens force-gated ion channels depolarizing the hair cell, releasing neurotransmitters from the basal side of the cell (Corey, 2009). The list of force-gated channels continues to grow and includes pannexin hemichannels that pass purines for purinergic signaling. Pannexins are discussed in more detail in Chapter 3.

Cell-cell and cell-matrix adhesions play crucial roles in mechanotransduction. Early observations of endothelial cells indicated that the application of fluid flow resulted in cell alignment in the direction of flow. This shear stress was designed to mimic the forces generated by blood flow (Davies et al., 1986). Since these initial discoveries CAMs including VE-cadherin have been identified as the proteins affected by this shear

stress (Conway et al., 2013). Not only do cadherin adhesions function as mechanotransducers, but also force is required for their formation.

For years, biologists were unable to generate complete AJs, rich with actin filaments, *in vitro*. Cadherins were capable of binding to β-catenin and α-catenin and forming nascent adhesions *in vitro*, but they did not bind to actin filaments. Studies showed that α-catenin bound with a high affinity to actin and with a lower affinity to cadherin/β-catenin complexes, but it could not associate with both actin and AJs at the same time. However, in 2014, Buckley *et al* found that the missing component in the complex was an applied force. By pulling on *in vitro* adherens junctions they could induce actin recruitment and reinforce the adhesion (Buckley et al., 2014). X-ray crystallography data indicate that α-catenin adopts a tertiary structure that inhibits its ability to bind to vinculin. Applying mechanical strain results in a force unfolding of α-catenin and recruitment of vinculin (Yao et al., 2014). This evidence suggests that the entire AJ protein complex requires force in order to perform its primary function of attaching cells to one another and anchoring the cortical actin network.

Cadherin/catenin complexes serve as actin-mediated mechanosensors, but the extent of influence from IFs in mechanical signaling remains unclear. One way that IFs are important in mechanotransduction is through the organization of the actin cytoskeletal network (Gregor et al., 2014). The IF and actin networks are interconnected by various proteins, primarily through plectins. Plectin-family proteins are capable of binding multiple cytoskeletal components at one time, functioning as molecular bridges between

actin, IF, and microtubule networks (Gad et al., 2008; Sutoh Yoneyama et al., 2014; Wiche et al., 2015). When the IF network is disrupted or disconnected from actin the result is morphological changes in the actin cytoskeleton (Fujiwara et al., 2016; Jiu et al., 2017; Sonavane et al., 2017). Therefore, IFs are important for organizing the actin network, which has a defined role in mechanical signaling.

Keratin and vimentin IFs are attributed to maintaining the mechanical properties of the cell (Lowery et al., 2015). The viscoelastic properties of IFs are unique compared to actin stress fibers and microtubules, which are significantly more rigid. The flexibility of IFs allows for the dissipation of forces, a favorable property in tissues that are exposed to large mechanical stresses such as vascular smooth muscle cells in the heart or epithelial cells of the skin (Ackbarow and Buehler, 2007; Charrier and Janmey, 2016). The fact that desmosomes are exposed to such large forces lends them to be potentially as important in mechanical signaling as actin-bound adhesions. IFs have only been associated with a few mechanosensation pathways, including a C-cadherin, PG, and keratin complex that is essential to the forward persistent migration of the mesendoderm tissue (Weber et al., 2012).

At the substrate level, FAs participate in mechanical signaling. Pulling on integrin receptors with a FN-coated microsphere leads to cytoskeletal reinforcement of the adhesion and contractility (Choquet et al., 1997). In a similar set of experiments, pulling on integrin-engaged ECM-coated microspheres induces vinculin recruitment and FA maturation. Cells also apply a contractile force onto the microspheres in an inside-out

mechanism of mechanical signaling (Galbraith et al., 2002). This application of force onto the microsphere is comparable to the traction forces that are applied to a FN substrate during cell migration (Legant et al., 2010; Tambe et al., 2011; Trepat et al., 2009a) and is important for the process of fibrillogenesis (Lemmon et al., 2009).

FN assembly and morphogenesis

The assembly of ECM proteins into fibrils is a complex process that is unique to the matrix component. ECM components are secreted from the cell as subunits that are then assembled into fibrils. Although some, such as the fibrillar collagens, self-assemble (Fang et al., 2013), many ECM proteins require a receptor for their assembly. Not all receptors that bind to an ECM proteoglycan are capable of assembling a matrix. However, many ECM components can be assembled by multiple receptors. One such proteoglycan in which assembly has been characterized is FN. FN is secreted as a dimer and assembled into a homopolymeric fibril. The receptor responsible for most FN assembly is integrin $\alpha_5\beta_1$ (Fogerty et al., 1990). FN assembly is receptor-mediated, during which the FN dimer must be physically stretched, resulting in a force-induced unfolding. Dimers are folded in such a way that FN-FN interaction sites are unexposed, buried within the tertiary protein structure. As the integrin receptors bind to the FN dimers, a force is applied via actomyosin contractility and the FN undergoes a conformational change. This unfolding event exposes the cryptic FN-FN interaction sites and fibrillogenesis proceeds (Baneyx et al., 2001; Lemmon et al., 2009; Smith et al., 2007).

Many of the processes involved in assembly have been characterized in monolayer cultures where cells assemble a matrix on the surface to which they adhere. This allows cells to attach to the dish as they apply contractile forces to the FN dimer through integrin receptors. One example of this has been documented using human foreskin fibroblasts, which express integrin $\alpha_5\beta_1$, a FN receptor, as well as $\alpha_V\beta_3$, a vitronectin receptor. Vitronectin fibrils and $\alpha_V\beta_3$ localize to the cell periphery where the cell attaches to the substrate without the requirement for FN or $\alpha_5\beta_1$. FN assembly begins near the periphery and extends centripetally as the FN-bound $\alpha_5\beta_1$ integrins are pulled towards the center of the cell. During this process, cells are held to the vitronectin substrate by $\alpha_V\beta_3$ (Pankov et al., 2000). During *de novo* FN assembly *in vivo*, there is no existing ECM substrate to which cells anchor themselves. Furthermore, at the BCR FN is assembled along a free surface. We propose that *in vivo* cells rely on cell-cell adhesions for stability as contractile forces are applied to growing FN fibrils. This hypothesis is explored further in Chapter 2.

The assembly of the FN matrix is an essential process in development, and is required for many morphogenetic events. During epithelial branching in the mouse salivary gland cadherin is displaced from nascent clefts as FN is deposited. Knocking down FN results in a reduction in the number of branches whereas overexpressing FN results in increased branching (Larsen et al., 2006; Sakai et al., 2003). During zebrafish segmentation stages of development the mediolateral convergence of the myocardial precursors requires FN. Disorganized tight junctions and adhesion defects are also noted

in these tissues in FN mutants (Trinh and Stainier, 2004). In *Xenopus* gastrulae, treating explants with function blocking antibodies specific for $\alpha_5\beta_1$ or FN results in defects in epiboly (Marsden and DeSimone, 2001) and convergent extension (Davidson et al., 2006). Epiboly is also perturbed when fibrillogenesis is inhibited by the expression of a 70kD c-terminal FN fragment, which incorporates into forming fibrils and prevents further assembly (Rozario et al., 2009). Interestingly, although fibrillar FN is important for the polarized protrusive activity of the mesendoderm cells, collective cell migration of the tissue is not entirely dependent on a FN matrix (Davidson et al., 2002; Winklbauer and Keller, 1996).

Summary and dissertation outline

Embryos acquire their shape through morphogenetic processes that rely on actomyosin contractility and cell adhesion. Both cadherin-based cell-cell and integrin-mediated cell-matrix adhesions are essential mediators of these processes. The forces exerted by cells onto their neighbors and surrounding microenvironments guide these tissue-wide movements. In this dissertation, I further characterize these movements that shape the embryo. Work described in Chapter 2 identifies PG as a novel regulator of morphogenesis, and is the primary focus of this thesis. This cadherin-binding protein is important for FN attachment and assembly. Chapter 3 focuses on understanding the influence of mechanical force on collective cell migration. Evidence suggests that mechanical strain is involved in activating mitochondrial ATP synthesis and inducing the

extracellular release of ATP through mechanosensitive pannexin channels. This purinergic signaling pathway has proven to be an important regulator of mesendoderm migration. Finally, Chapter 4 is a summary of the two projects and discusses both future experimental approaches and the significance of this work. Together, this dissertation is an overview of two separate mechanical processes that regulate morphogenesis and influence embryo shape.

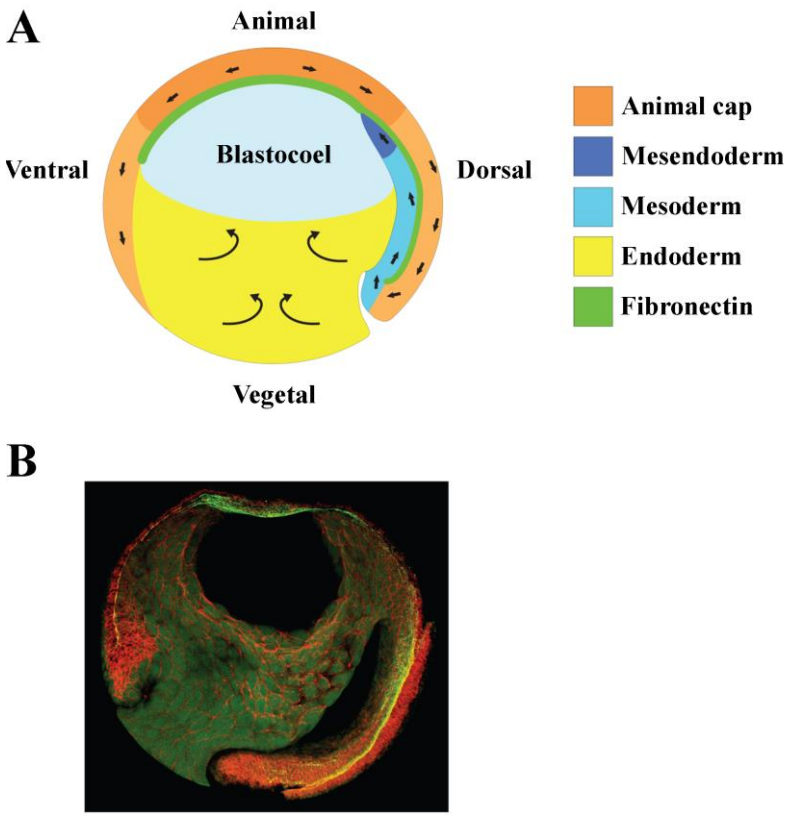

A
Animal
Ventral
Blastocoel
Dorsal
Vegetal
Animal cap
Mesendoderm
Mesoderm
Endoderm
Fibronectin
B

Figure 1.1: Morphogenetic movements during gastrulation.

(A) Depiction of a gastrula-stage embryo and the morphogenetic processes that occur during this stage of development. Direction of cell movements are denoted by arrows. Vegetal rotation of the endoderm occurs within the vegetal cell mass (yellow). At the animal pole, cells within the animal cap intercalate radially (dark orange). On the dorsal side of the embryo, involuted mesoderm cells intercalate mediolaterally (light blue). The mesendoderm (dark blue) collectively migrates along a FN substrate (green). (B) Cross-section of a gastrulating embryo with FN in green and cadherin in red. Figure provided by Bette Dzamba.

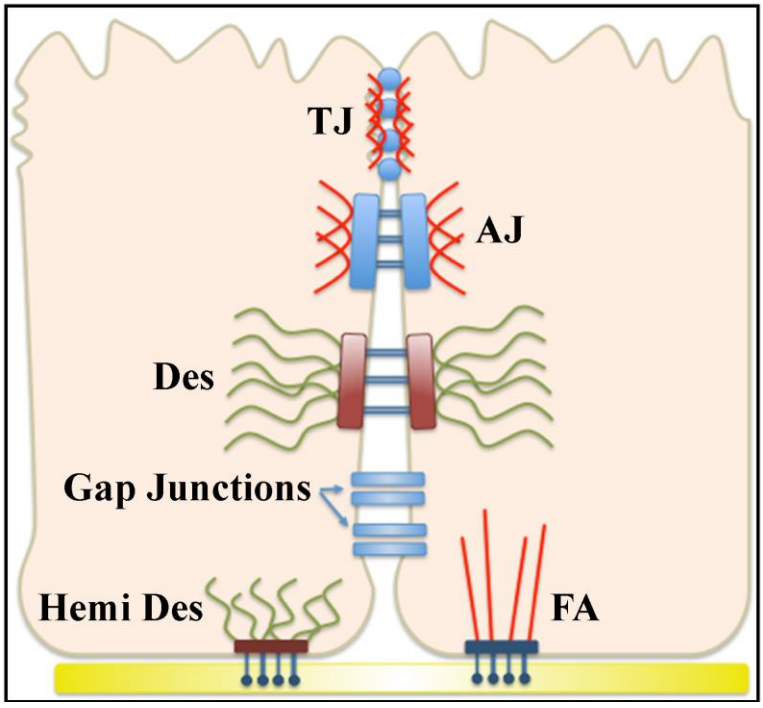

TJ
AJ
Des
Gap Junctions
Hemi Des
FA

Figure 1.2: Cell-cell junctions and matrix adhesions

Diagram depicting the four kinds of cell-cell junctions and the two integrin-mediated cell-matrix adhesions. TJ= tight junction, AJ= adherens junction, Des= desmosome, FA= focal adhesion, Hemi Des= hemi desmosome. Actin (red) is anchored at TJs, AJs, and FAs. Intermediate filaments (green) are attached at desmosomes and hemi desmosomes.

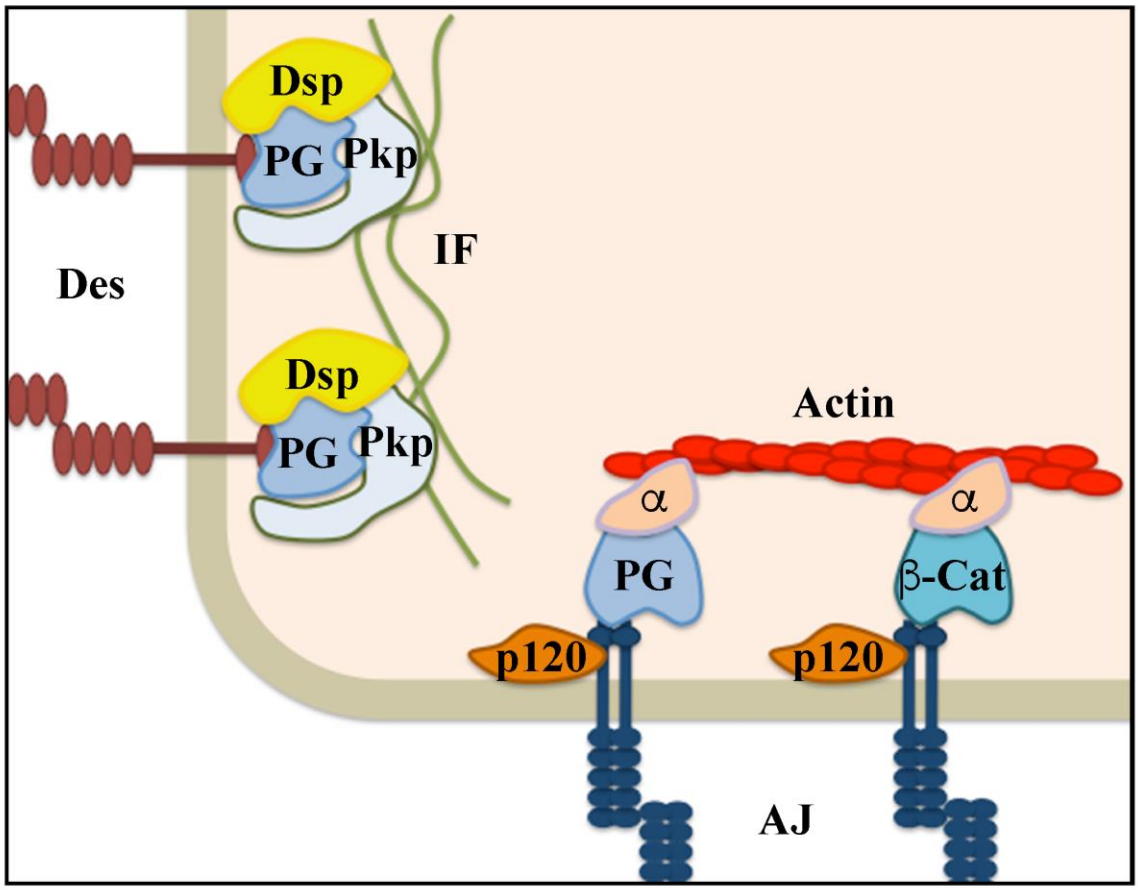

Dsp
PG
Pkp
IF
Des
Dsp
PG
Pkp
Actin
α
α
PG
β-Cat
p120
p120
AJ

Figure 1.3: Catenins in cell-cell adhesions

Intermediate filaments (green) are anchored to desmosomal cadherins through interactions with desmoplakin (Dsp) and plakophilin (Pkp). Desmoplakin and plakophilin are recruited to the cytoplasmic tails of desmosomal cadherins by PG. Actin (red) is anchored to the cytoplasmic tails of classical cadherins through PG and β-catenin. PG and β-catenin recruit actin-binding proteins including α-catenin. p120 catenin binds to the juxtamembrane region of cadherins in AJs, regulating cadherin clustering and turnover. Des= desmosome. AJ= adherens junction. Dsp= desmoplakin. PG= plakoglobin. Pkp= plakophilin. α= α-catenin. β-cat- β-catenin. p120= p120 catenin.

Chapter 2

A novel role for plakoglobin in *de novo* fibronectin assembly and morphogenesis

This chapter is composed of data from

a manuscript in preparation

Introduction

The extracellular matrix is a common feature of all multicellular organisms and is involved in cell adhesion and signaling, cell migration, cell survival, homeostasis, and maintenance of tissue structural integrity. Spatiotemporal regulation of ECM production and deposition occurs throughout embryonic development resulting in multiple tissues containing different ECM molecules with distinct tensile and viscoelastic properties. These distinct properties ensure proper functioning of the cells, tissues, and organs that interact with assembled ECM. FN is a multifunctional prototypical matrix glycoprotein for which many of the steps involved in fibrillogenesis have been identified. FN is secreted from cells as a dimer, and assembled into fibrils at the cell surface (Rozario and DeSimone, 2010; Schwarzbauer and DeSimone, 2011). Binding of FN to integrin receptors, primarily $\alpha_5\beta_1$, followed by actin-based contractility applies strain to FN dimers resulting in conformational changes thus exposing cryptic FN-FN binding sites. This contractility and unfolding event is required for fibrillogenesis (Baneyx et al., 2001; Geiger et al., 2001; Smith et al., 2007; Wu et al., 1995; Zhong et al., 1998).

In *Xenopus,* FN is the earliest known matrix glycoprotein expressed in the embryo, with protein translation and fibrillogenesis beginning at the MBT and early gastrulation, respectively. Throughout gastrulation fibrils are assembled onto the BCR, serving as a substrate for mesendoderm cell migration (Lee et al., 1984; Winklbauer, 1998) and as a repository of PDGF secreted by BCR cells (Ataliotis et al., 1995; Smith et al., 2009). Aside from collective migration of the mesendoderm, FN is also required for

radial cell intercalation and epiboly of the BCR tissue (Rozario et al., 2009) and for mediolateral cell intercalation in the dorsal mesoderm during convergent extension (Davidson et al., 2006; Marsden and DeSimone, 2003; Skoglund and Keller, 2010). FN functions have been a focus of much research, however, most of what is known about steps involved in the expression, secretion, and assembly of FN and other ECM proteins are derived from studies using cell culture monolayers. It is unclear how this translates to assembly along a free surface of a tissue *in vivo*, such as at the BCR.

Cultured cells in a monolayer are relatively flat, maximizing the surface in contact with the substrate. This flattening minimizes the area in contact with neighboring cells. During *in vitro* fibrillogenesis, cells generate traction forces onto their substrates at integrin-rich FAs (Fournier et al., 2010). Cell culture dishes provide a substrate to which cells and forming FN fibrils are anchored, and cells rely on this substrate attachment for the generation of forces required for fibrillogenesis (Pankov et al., 2000). During *de novo* FN assembly in a tissue such as along the BCR, cells have no substrate on which to apply forces other than the surfaces of neighboring cells. Calcium-dependent transmembrane adhesion proteins called cadherins mediate most cell-cell adhesions. Cadherin adhesions have been implicated in ECM fibrillogenesis, through kinase cascades as well as force-dependent mechanisms of crosstalk (Dzamba et al., 2009; Weber et al., 2011).

Cadherin-based cell-cell adhesions are divided into two main categories, AJs and desmosomes. In AJs, the actin cytoskeleton is anchored to the cytoplasmic tails of classical cadherins such as E-, N-, and C-cadherin. At desmosomes, keratin or vimentin

intermediate filaments (IFs) are anchored to the cytoplasmic tails of desmosomal cadherins, including desmoglein and desmocolin (Saito et al., 2012). C-cadherin (also called EP-cadherin), is the earliest cadherin expressed in *Xenopus* embryos (Ginsberg et al., 1991; Levi et al., 1991). While it is a *bona fide* classical cadherin based on sequence homology and localization at AJs, C-cadherin is also capable of assuming the function of a desmosomal cadherin, interacting with the keratin IF network in mesendodermal cells (Weber et al., 2012). Localization of classical cadherins at desmosome-like structures has been documented in other cell-types including the lens fiber cells of the eye, however, these structures have not been thoroughly explored (Leonard et al., 2008; Rao, 2008; Zelenka, 2004).

The cytoplasmic tails of desmosomal cadherins distinguish their functions from that of classical cadherins, through interactions with different cadherin-binding proteins. The catenin family of proteins are responsible for anchoring cytoskeletal networks at adherens junctions (AJs) and desmosomes (Shapiro and Weis, 2009). β-catenin and plakoglobin (PG; γ-catenin) exhibit high amino acid sequence homology, and have overlapping functions. In AJs, β-catenin and PG bind directly to the cytoplasmic tail domains of classical cadherins as well as to α-catenin. α-catenin anchors actin stress fibers at AJs through direct interactions with filamentous actin as well as by recruiting actin binding proteins such as vinculin and α-actinin (Fagotto and Gumbiner, 1994; Heasman et al., 2000; Knudsen et al., 1995; Nieset et al., 1997; Peifer et al., 1992). In an analogous manner, PG binds to the tails of desmosomal cadherins and recruits the IF-

binding proteins desmoplakin and plakophilin (Garrod and Chidgey, 2008). This function is specific to PG. β-catenin only partially compensates for a loss of PG at desmosomes in PG knockout mice, which eventually die of desmosome-associated heart defects (Ruiz et al., 1998).

In the cytoplasm, β-catenin and PG are targeted for proteasomal degradation by an axin, adenomatous polyposis coli (APC), and glycogen synthase kinase 3 (GSK3)-containing destruction complex. The destruction complex is sequestered at the cell membrane during canonical Wnt signaling as cytoplasmic β-catenin and PG protein levels increase. Cytoplasmic β-catenin or PG interact with T-cell factor (TCF) and lymphoid enhancer factor (LEF) and are translocated into the nucleus where these complexes regulate the expression of canonical Wnt target genes (Baker et al., 2010; Guger and Gumbiner, 1995; Vonica and Gumbiner, 2007). The list of transcripts regulated by β-catenin and PG overlap, with some regulated by both catenins (Martin et al., 2009) and others controlled by either β-catenin or PG alone (Tokonzaba et al., 2013). During *Xenopus* gastrulation canonical Wnt signaling is required for mesoderm induction and axis specification (Fagotto et al., 1997; Guger and Gumbiner, 1995). Overexpression of β-catenin on the ventral side of an embryo results in the formation of a second axis (McCrea et al., 1993; Wylie et al., 1996). The same axis duplication phenotype is achieved when PG is expressed on the ventral side, likely through competitive binding to the destruction complex and the subsequent accumulation and nuclear translocation of β-catenin (Karnovsky and Klymkowsky, 1995; Klymkowsky et al., 1999; Merriam et al.,

1997). Furthermore, the LEF-1/PG complex exhibits less nuclear translocation and a lower affinity for DNA compared to the LEF-1/β-catenin complex (Simcha et al., 1998; Zhurinsky et al., 2000). For these reasons, PG likely plays a more dispensable role in transcriptional regulation.

Although it is involved in Wnt signaling, the major role of PG is at cell-cell adhesions. PG morphant *Xenopus* embryos are flatter in appearance compared to wildtype embryos likely due to a reduction in the formation of actin stress fibers (Kofron et al., 2002). Within the collectively migrating mesendoderm tissue, PG localizes to dynamic desmosome-like structures. In place of desmosomal cadherins desmosome-like structures in the mesendoderm contain C-cadherin, which are attached to keratin IFs through PG. These mechanosensitive protein complexes are essential for mesendoderm cell protrusion polarity and for maintaining the persistent forward migration of the tissue (Weber et al., 2012). This study further characterizes the role of PG in morphogenesis. Interestingly, PG morphant embryos do not extend along their anteroposterior axis as well as control embryos. This phenotype cannot be explained simply by a defect in collective cell migration and suggests perturbations in other morphogenetic movements. Here we report a role for PG in convergent extension and radial intercalation. The data demonstrate that PG regulates these movements through the assembly of the fibrillar FN matrix. In the absence of PG cell-cell adhesion is perturbed, resulting in defects in matrix assembly.

Results

PG regulates convergent extension

Relative to the abundance of β-catenin literature, PG is a vastly understudied protein in the context of embryonic development. Most PG studies have relied on overexpression of the protein, which can result in changes in β-catenin-mediated signaling through competition for the destruction complex and the cytoplasmic tails of cadherins at AJs (Karnovsky and Klymkowsky, 1995; Klymkowsky et al., 1999; Merriam et al., 1997). While these studies are crucial for understanding the potential role of PG in Wnt signaling, overexpression of a protein that involved in such an essential signaling pathway can result in activities that do not reflect the normal physiology of the affected tissues. Loss-of-function studies in which PG was knocked down in whole embryos have identified the importance of the protein in maintaining cortical actin structures, but further characterization of whole embryo phenotypes were not determined (Kofron et al., 2002; 1997). When PG is knocked down specifically in the collectively migrating mesendoderm tissue, protrusion orientation and persistent migration are perturbed. The requirement of PG in other morphogenetic movements has not yet been reported (Weber et al., 2012). We have found that knocking down PG in whole *Xenopus* embryos results in a significant decrease in axial extension (Figure 2.1 A). PG morphant tadpoles are 40% shorter than controls. Overexpressing a morpholino-insensitive PG RNA construct partially rescues embryo length (Figure 2.1 A, B). Equivalent concentrations of PG rescue or wildtype RNA were injected into embryos along with PG morpholino and total PG levels were compared to embryos injected with control morpholino. Western blots

show that the rescue construct accumulates to a higher concentration than wildtype PG indicating a resistance to morpholino knockdown (Figure 2.1 C, D).

The shortened embryo phenotype seen in Figure 2.1 A cannot be explained by mesendoderm migration defects alone and suggests perturbations in additional morphogenetic events, namely radial and mediolateral intercalation, that contribute to anteroposterior elongation. During epiboly and convergent extension, cells intercalate between one another, resulting in a thinning of the animal cap and narrowing of the dorsal mesoderm respectively. These processes require the rearrangement of cell-cell and cell-ECM contacts and actomyosin contractility (Keller et al., 2008; Pfister et al., 2016; Skoglund et al., 2008). Keller sandwich explants were used to assess the effects of PG knockdown on convergent extension movements. Keller sandwiches were constructed by dissecting the dorsal sides of two early gastrula stage embryos and sandwiching the inner surfaces together (Keller et al., 1985). Explants elongate as the dorsal tissues converge mediolaterally and extend along the anteroposterior axis. PG morphant explants extend 25% less than their control counterparts (Figure 2.1 E). This reduction in axial extension in whole embryos and explants is significant (Figure 2.1 F), and the data are consistent with defects in convergent extension.

During convergent extension, the dorsal mesoderm cells extend protrusions and elongate in the mediolateral direction. Cells use these protrusions to attach to neighbors through cadherin-based adhesions as they intercalate between one another (Keller et al., 2000; Pfister et al., 2016; Skoglund et al., 2008). The notochord, a product of convergent

extension, is a dorsal mesoderm-derived structure that serves as a signaling center during neurulation (Choi and Harfe, 2011; Fan and Tessier-Lavigne, 1994). The axial mesoderm cells within the notochord and the paraxial mesoderm cells surrounding the notochord elongate in the mediolateral direction and shorten along the anteroposterior axis as they intercalate. These elongated cells in control embryos exhibit high length-to-width ratios of nearly 3:1. In PG morphants, the length-to-width ratios of both axial and paraxial mesoderm cells are reduced to 1:1 (Figure 2.2 A, B). Morphant notochords are also significantly wider than control notochords, another result of defects in axial extension (Figure 2.2 A, C). Surrounding the notochord, along the notochord-somite-boundary, is a sheath of ECM containing FN, fibrillin, and laminin. This ECM is necessary for proper mediolateral elongation and intercalation of the dorsal mesoderm cells (Aszódi et al., 1998; Davidson et al., 2004; Pagnon-Minot et al., 2008; Parsons et al., 2002; Pollard et al., 2006; Rongish et al., 1998; Skoglund et al., 2006; Skoglund and Keller, 2007). This notochord sheath is disrupted in PG morphant embryos, exhibiting a reduction in FN and fibrillin staining (Figure 2.2 D).

PG is a regulator of radial intercalation

When maternal PG is knocked down, morphant embryos exhibit a delay in blastopore closure, suggestive of defects in epiboly (Kofron et al., 1997). This intercalation-driven event thins and spreads the tissue and is required for blastopore closure. During gastrulation, the BCR thins as the deep ectoderm cells intercalate radially

between one another and orient cell divisions in the horizontal plane (Marsden and DeSimone, 2001; Rozario et al., 2009). Much like the converging and extending mesoderm, radial intercalation during epiboly requires directed cell migration (Damm and Winklbauer, 2011; Szabó et al., 2016) and cadherin-mediated cell-cell adhesion (Babb and Marrs, 2004; Shimizu et al., 2005). To determine if PG is required for radial intercalation we bisected control and PG morphant embryos and measured the thickness of the BCR tissue. By mid-gastrula stage the BCRs of control embryos have thinned to just two cell layers, measuring about 35 μm thick. However, BCRs of PG morphants remain more than twice as thick and multilayered, with rounded cells protruding into the blastocoel cavity (Figure 2.3 A, B). This rough appearance along with a reduction in basolateral cadherin accumulation in morphant embryos is suggestive of cell-cell adhesion defects, and may contribute to epiboly defects.

In addition to cell-cell adhesion defects, PG-deficient embryos exhibit a reduction in FN assembly along the BCR (Figure 2.3 A). This is consistent with the reduced FN and fibrillin staining at the notochord-somite-boundary (Figure 2.2 B). For high magnification imaging with single-fibril resolution, immunostained animal caps were dissected and visualized *en face*. The BCRs of control cells are coated with a dense network of FN fibrils. This network of fibrils is drastically reduced in PG morphants. This fibril loss is rescued by the overexpression of a morpholino-insensitive PG construct (Figure 2.3 C, D).

Localization and expression patterns of PG during development

Knocking down PG results in a disruption in epiboly and convergent extension. Although β-catenin and PG share a high amino acid sequence homology, and β-catenin is also serves an essential function during gastrulation, its main role is in Wnt signaling and tissue patterning. β-catenin morphants exhibit defects in mesoderm induction, resulting in ventralized embryos that develop no dorsal structures (Heasman et al., 1994). These phenotypes are not seen in PG morphants. To gain further insight into the divergent roles of β-catenin and PG we performed western blots and whole mount immunofluorescence microscopy for the two proteins. Western blots of whole embryo lysates indicate that PG is expressed at relatively low levels pre-MBT (stage 6) and begins to accumulate gradually at MBT (stage 8) and through gastrulation (stages 10-13; Figure 2.4 A). In contrast, β-catenin expression does not increase until later stages of gastrulation, towards neurulation when it is involved in neural crest induction (stages 12-13; Figure 2.4 B). These data are similar to a previous report that focused on time points that span embryonic development (DeMarais and Moon, 1992) instead of discrete stages within gastrulation.

Spatially, PG and β-catenin colocalize at cell contacts in some cells while remaining distinct in others, such as at the blood-brain barrier (Lampugnani et al., 1995; Liebner et al., 2000). Using confocal microscopy, we found that in early gastrula stage animal cap cells, β-catenin and PG primarily colocalize at punctae along cell boundaries. Separate from these areas of colocalization, PG displays a “beads-on-a-string”

appearance adopted by desmosomal proteins as they decorate IFs at nascent desmosomes (Jones and Grelling, 1989; Todorovic et al., 2014) (Figure 2.4 C). β-catenin and PG primarily localize to cell membranes along the BCR, suggesting that the major role for the proteins in this tissue is in adhesion. Although PG and β-catenin serve redundant functions at AJs, their spatiotemporal expression patterns differ in *Xenopus* tissues.

Mesoderm patterning is normal in PG-deficient embryos

PG and β-catenin are paralogues with high sequence homology, serving some common functions in AJs, but also independent functions, such as the requirement for PG, but not β-catenin, in desmosomes (Peifer et al., 1992). β-catenin is a transcriptional coregulator in Wnt signaling, regulating mesoderm induction and dorsalization. Similarly, PG overexpression is sufficient to induce dorsalization and secondary axis formation, phenotypes associated with canonical Wnt signaling (Karnovsky and Klymkowsky, 1995; Klymkowsky et al., 1999). However, unlike β-catenin knockdown, a loss of PG does not result in ventralization (Heasman, 2002; Kofron et al., 1997). These results suggest that PG is likely more dispensable for canonical Wnt signaling and tissue patterning (Maeda et al., 2003; Simcha et al., 1998; Williams et al., 2000; Zhurinsky and Shtutman, 2000). To establish whether PG knockdown affects patterning and axis formation, *in situ* hybridizations and immunofluorescence staining of embryos were performed. *Xenopus Brachyury (XBra)* (Smith et al., 1991a) and *Chordin (chrd)* (Plouhinec et al., 2013; Sasai et al., 1994) were expressed in the early mesoderm and

chordomesoderm, respectively, in both control and PG-deficient embryos (Figure 2.5 A, B). Additionally, immunostaining of dorsal isolates using the 12101 antibody to label the presomitic mesoderm (Kintner and Brockes, 1985), revealed that the tissue remained properly patterned in PG morphant embryos (Figure 2.5 C).

Cytoplasmic pools of β-catenin and PG are targeted by the same APC/GSK-3 destruction complex (Rubinfeld et al., 1995; Stamos and Weis, 2013). Therefore, altering the levels of one protein can result in subsequent changes in the other (Li et al., 2007). In order to determine if β-catenin protein levels are affected by the knockdown of PG we performed western blots. Densitometric analyses of western blots indicate that β-catenin protein levels are maintained when PG is knocked down (Figure 2.5 D, E). Overall, data suggest that embryo patterning is maintained in PG morphant embryos and the observed phenotypes are more likely the result of defects in cell adhesion rather than Wnt signaling.

FN protein levels are reduced in PG morphant embryos

Although PG knockdown does not affect β-catenin protein levels or cell fate, it does result in reduced FN deposition at the notochord-somite-boundary as well as along the BCR. A loss of fibrils can be caused by a decrease in the FN protein level in the embryo, defects in fibrillogenesis, or a combination of the two. Western blot analysis confirmed a reduction in FN protein levels in PG deficient embryos at gastrulation

(Figure 2.6 A). At stage 13, there is a 40% reduction in total FN protein in PG morphants compared to control embryos (Figure 2.6 B).

One mechanism by which PG may regulate the FN protein is through transcription or transcript stability. β-catenin has been shown to regulate FN transcription in *Xenopus laevis* through canonical Wnt signaling (Gradl et al., 1999). PG is also reported to function in transcriptional regulation in some cell types (Aktary et al., 2013; Maeda et al., 2003; Tokonzaba et al., 2013). One study indicates that PG stabilizes the FN transcript in keratinocytes. Knocking down PG results in rapid degradation of FN mRNA (Todorović et al., 2010). To determine if PG regulates FN on the transcript level in *Xenopus* gastrulae, we performed quantitative reverse transcription PCR (qPCR). FN transcript levels are maintained in PG morphant embryos, suggesting that PG does not regulate FN transcription or mRNA stability at this stage in development (Figure 2.6 C). This agrees with the data indicating that the major function of PG during gastrulation and morphogenesis is in cell adhesion.

Integrin and cadherin surface protein levels are intact in PG morphants

PG morphants exhibit round cells in the dorsal mesoderm and the BCR, suggesting cell adhesion defects. One possible reason for these defects in adhesion as well as the reduced FN assembly along the notochord and BCR is a decrease in cell surface receptors involved in these processes. Cell-cell adhesions in *Xenopus* gastrulae are mediated by C-cadherin, while integrins are responsible for assembling FN dimers

into fibrils. Integrin and cadherin receptors are trafficked to the cell surface where they perform their functions in cell adhesion. The catenin family of proteins can physically interact with the uncleaved pro-cadherin in the golgi apparatus and endoplasmic reticulum and are thought to regulate trafficking of the cadherin receptors to the cell membrane (Chen et al., 2009; Curtis et al., 2009; Wahl et al., 2003). Cell-surface biotinylation was used to address the possibility that PG is involved in cadherin or integrin trafficking. Whole animal caps were treated with non-cell-permeable biotin, lysed, and precipitated using NeutrAvidin agarose. Densitometric analyses of western blots indicate equivalent integrin β_1 and C-cadherin surface levels in control and PG morphants (Figure 2.6 D-F). This confirms that changes in cell-cell adhesion or FN assembly state are not due to reductions in surface C-cadherin or integrin levels.

PG is required for FN assembly

Cadherin overexpression is sufficient to induce precocious FN assembly through an actomyosin-based mechanism. This suggests that cell-cell adhesions perform an important role in fibrillogenesis (Dzamba et al., 2009). PG is one of the proteins involved in anchoring the actin cytoskeleton to cadherin adhesions and may regulate fibrillogenesis through the same mechanism. In order to study fibrillogenesis in PG morphants without affecting overall FN protein levels, we knocked down PG in a subset of cells while maintaining expression in the rest of the embryo. PG morpholino was injected into one animal cap cell at 32-cell stage (stage 6) and *de novo* FN fibrillogenesis

along the BCR was visualized at stage 11 (mid-gastrulation). Adjacent to the BCR, the blastocoel cavity is filled with soluble FN dimers (Lee et al., 1984). Therefore, knockdown cells remain exposed to an equivalent FN concentration as their adjacent uninjected counterparts (Figure 2.7 A). Fixed and immunostained animal caps revealed that FN fibrillogenesis is perturbed in PG morphant cells, while the surrounding uninjected cells are able to assemble FN into fibrils (Figure 2.7 B). In a similar experimental approach, mosaic overexpression of PG was injected into one animal cap cell at 32 cell-stage and embryos were allowed to develop until stage 10 (early gastrulation). At this stage of development, uninjected cells do not assemble FN into fibrils. Cells overexpressing PG were able to assemble FN fibrils precociously, a phenotype similar to that of cadherin overexpression (Figure 2.7 C).

Knocking down PG in a small subset of animal cap cells leads to a loss of fibrillogenesis in a cell autonomous manner (Figure 2.7 B). The BCR cells are thought to acquire soluble FN dimers from the blastocoel fluid for the assembly of FN fibrils. One possible explanation for the decrease in assembly is that the local concentration of soluble FN is decreased at the surface of the morphant cells. To avoid this possibility while testing for the requirement of PG in fibril assembly we knocked down PG in whole embryos and reintroducing exogenous FN protein. Embryos were injected with PG morpholino immediately following fertilization. Prior to gastrulation, a fluorescently-tagged *Bovine* FN protein was injected directly into the blastocoel cavity of morphant embryos. At stage 11, animal caps were dissected, fixed, and endogenous FN identified using an antibody that recognizes *Xenopus* but not *Bovine* FN. Control embryos are able

to assemble fibrils containing both exogenous *Bovine* FN and endogenous *Xenopus* FN and show extensive overlap between the two species of protein. PG morphant embryos were unable to assemble *Xenopus* or *Bovine* FN into fibrils, confirming a defect in fibrillogenesis (Figure 2.7 D, E).

PG morphant cells do not form strong adhesions to cadherin or FN

Crosstalk between cadherin and integrin-based adhesions has been identified in both cell culture and *in vivo* systems (Dzamba et al., 2009; Langhe et al., 2016; Weber et al., 2011). It is possible that through direct mechanical linkage or indirect signaling pathways, the observed defects in fibronectin fibrillogenesis in PG morphants are a result of reductions in adhesion strength. To address the possibility that PG is involved in attachment to FN we performed atomic force microscopy (AFM). A single mesendoderm cell was attached to a Cell-Tak-coated cantilever and brought into contact with cadherin or FN substrates. After a short five-second incubation, cells were detached from the substrates as adhesive strength was measured. Force-distance curves indicate a reduction in the maximum adhesive strength to both cadherin and FN substrates in PG morphant cells when compared to controls (Figure 2.8 A-C). To test for specificity of decreased attachment strength, forces associated with attachment the second heparin-binding domain of FN (Hep II) (Ramos and DeSimone, 1996) and non-specific attachment to poly-L-lysine (PLL) and were measured. Mesendoderm cells attach to a Hep II substrate via syndecan receptors rather than integrins, therefore, Hep II serves as a receptor-

mediated control for attachment. PLL serves as a control for non-receptor-mediated attachment. There is no significant difference in the force of attachment between control and PG morphants on a Hep II substrate (Figure 2.8 D) or a non-specific PLL substrate (Figure 2.8 E) indicating that the reduction in adhesion to cadherin and FN is integrin receptor-mediated (Figure 2.8 B, C).

Discussion

Gastrulation is a complex process that involves numerous coordinated tissue movements. These morphogenetic events rely on ECM assembly and cellular adhesion. The importance of cadherins in cell-cell adhesion and integrins in attachment to and assembly of an ECM has been well documented. However, there is still much that we do not understand about the coordination of these two processes. In this study, we have further characterized the role of PG, a cadherin-associated protein in the context of gastrulation and morphogenesis. Our data indicate that PG plays an essential role in FN attachment and assembly.

The main role that PG plays in the cell is to anchor actin stress fibers and keratin IFs at AJs and desmosomes, respectively. Previously reported *in vivo* loss-of-function studies in mice and *Xenopus* have been performed in PG-null or maternal-RNA KD embryos. Using these methods of perturbation leads to severe phenotypes. PG knockout mouse embryos die as a result of heart defects associated with a loss of desmosomal integrity (Ruiz et al., 1998). When maternal PG is knocked down in *Xenopus* oocytes using antisense oligonucleotides, the resulting embryos exhibit a loss of cortical actin

stress fibers, mild adhesion defects, and significant developmental delays (Kofron et al., 2002; 1997). In contrast, here we have knocking down PG after fertilization allowed embryos to develop through initial cleavage stages before the protein levels were reduced, and we were able to study the function of PG in embryos with a milder phenotype. Our previous study identified PG as a regulator of mesendoderm cell polarity and migration, characterizing the function of PG in mechanosensitive desmosome-like adhesions. These PG and keratin-rich structures are essential for the elongation of mesendoderm cells along the anteroposterior axis (Weber et al., 2012). The current study demonstrates that PG serves additional functions in convergent extension and epiboly through cell-cell and cell-matrix adhesion and FN fibrillogenesis.

Immunofluorescence microscopy of the BCR shows that PG localizes to cell-cell junctions overlapping with β-catenin in areas while occupying distinct regions in others (Figure 2.4). Our data complement previous studies confirming that regardless of the high sequence homology between β-catenin and PG, the two proteins have both analogous and divergent functions in embryonic development. Overexpression of PG on the ventral side of the embryo can result in anteroposterior axis duplication as seen in β-catenin overexpressing embryos, a phenotype typical of ectopic canonical Wnt signaling (Karnovsky and Klymkowsky, 1995; Klymkowsky et al., 1999; Merriam et al., 1997; St Amand and Klymkowsky, 2001). However, PG and β-catenin knockdown phenotypes are different from each other. β-catenin knockdown embryos become ventralized, exhibiting defects in mesoderm patterning consistent with a loss of canonical Wnt signaling

(Heasman et al., 1994). In contrast, knocking down PG results in short (Figure 2.1), albeit properly patterned embryos (Figure 2.1). Additionally, knocking down PG does not affect β-catenin protein levels, indicating that the defects seen in PG morphants are not due to a loss of β-catenin or changes in overall tissue patterning (Figure 2.5).

In the current study, we have found that PG plays an essential function in morphogenesis through its role in cell adhesion and FN fibrillogenesis. Short morphant embryos exhibit defects in convergent extension and epiboly (Figure 2.1- 2.3), two processes that require cell-cell and cell-matrix adhesion (Babb and Marrs, 2004; Keller et al., 2008; Marsden and DeSimone, 2001; Pfister et al., 2016; Rozario et al., 2009; Shimizu et al., 2005; Skoglund et al., 2008). PG morphants have normal surface integrin β_1 and C-cadherin levels (Figure 2.6), but do not form strong attachments to cadherin or FN substrates (Figure 2.8) and do not efficiently assemble FN into fibrils (Figure 2.7). Reports indicate mechanisms of crosstalk between cadherin and integrin-based adhesions (Bazellières et al., 2015; Marsden and DeSimone, 2003; Mertz et al., 2012; Wang et al., 2006), at least in part through Rac and Pak-mediated actomyosin contractility (Dzamba et al., 2009). This contractile force generated at cadherin-based adhesions is transduced through the actin cytoskeleton to integrin adhesions. Force applied to FN dimers induces a conformational change, exposing cryptic FN-FN interacting domains leading to fibril extension (Lemmon et al., 2009). In the current study we have provided further evidence supporting the essential role for the cadherin and PG-containing adhesive complex in *in vivo* matrix assembly, a contributor largely overlooked in fibrillogenesis studies that rely on cell culture monolayers.

While there are benefits to studying matrix assembly in a cell monolayer where much of what is known about fibrillogenesis has been characterized, it does not mimic the tissue geometry present *in vivo* or the process of *de novo* ECM assembly. When cultured in a monolayer, cells attach to their substrate and assemble an ECM on the surface to which they are attached. Cells are capable of applying traction forces to the substrate as they assemble fibrils. Within a tissue such as the BCR where FN is assembled along a free surface, the only substrate on which cells can anchor themselves is their neighbors. This makes cell-cell adhesions important players in the process of fibrillogenesis.

Prior to this study, the function of the PG protein had not been well characterized in the context of embryonic development. We have found that one function of PG during gastrulation is in the assembly of a FN ECM. The function of PG in fibrillogenesis is likely through anchoring of the actin cytoskeleton at AJs and maintaining actomyosin contractility (Figure 2.9). The cortical actin network is connected to actin filaments at cadherin/catenin AJs as well as at integrins in focal adhesions. The generation of actomyosin contractility and formin-dependent actin polymerization (Acharya et al., 2017; Leerberg et al., 2014) at AJs is likely important for the assembly of the FN matrix. As the two halves of a FN dimer are bound to integrins on adjacent cells, the forces generated by formin-dependent actin polymerization and actomyosin contractility as well as cortical actin flow moves the integrins centripetally along the basal surface towards the center of the cell. Separation of the adjacent integrin receptors force unfolds the FN, exposing cryptic FN-FN attachment sites that allow for polymerization and fibril

assembly. In the absence of PG, cadherin-mediated adhesion is perturbed (Figure 2.8 A, B) the actin cytoskeletal network is disrupted (Kofron et al., 2002) and subsequent attachment strength to FN is reduced (Figure 2.8 C). Together, our data suggest that PG is indispensable for matrix assembly, a process required for morphogenesis and embryonic development.

Materials and Methods

Embryos and explants

Xenopus laevis embryos were obtained and fertilized *in vitro* using standard methods and staged according to Nieuwkoop & Faber, 1967. Keller sandwiches were prepared as described in Keller & Danilchik, 1988. Single cell experiments were performed using mesendoderm and animal cap cells that were dissociated in calcium- and magnesium-free Modified Barth's Saline (MBS) (Sive et al., 2000).

Morpholino and RNA constructs

Fertilized embryos were injected with morpholinos or RNA constructs into both hemispheres of stage 2 embryos or into 1 blastomere located on the animal cap at 32 cell stage. Morpholinos were injected into whole embryos at a concentration of 25 pg/embryo or into 1 blastomere at 32 cell stage at a concentration of 0.78 pg/embryo. RNA was injected into whole embryos at a final concentration of 500 pg/embryo and into 1

blastomere at 32 cell stage at 15.6 pg/embryo. Injections were performed in a solution of 3% Ficoll in 0.1x MBS and incubated in 0.1x MBS until the specified developmental stages (Sive et al., 2000). Standard control (CCTCTTACCTCAGTTACAATTTATA) and PG morpholinos (TTTCCACTACGTCTCCCAAATCCAT) (Weber et al., 2012) were purchased from Gene Tools.

Whole mount *in situ* hybridizations

In situ hybridizations were performed according to Sive, Grainger, & Harland, 1997 using probes for *Xenopus brachyury (Xbra)* and *Chordin.* Plasmids for probe synthesis were gifts from J. Smith (Smith et al., 1991) and E.M. DeRobertis (Sasai et al., 1994).

Antibody staining

Cadherin, fibrillin, FN, 12101 or actin stained samples were fixed for 15 min in MBS containing 3.7% formaldehyde and 0.25% glutaraldehyde. For PG and β-catenin staining, samples were fixed in Dent's solution (80% methanol and 20% DMSO) overnight at 4˚C. Dorsal isolates were bleached, stained, and cleared as previously described (Wallingford, 2010). Primary antibodies were used at the following dilutions: PG at 1:100 (γ-catenin; BD Biosciences), β-catenin at 1:1000 (C2206; Sigma Aldrich), cadherin at 1:2,000 (XC; polyclonal from Barry Gumbiner), fibrillin at 1:400 (JB3;

Developmental Studies Hybridoma Bank [DSHB]), FN at 1:1000 (monoclonal 4H2 and polyclonal 32FJ were developed in the DeSimone lab; 4H2 is available from DSHB), 12101 at 1:10 (DSHB).

Optical Microscopy

Confocal microscopy was performed on a Nikon C1 microscope, with Nikon Plan Fluor 20x, Plan Apo 60x, and Plan Apo TIRF 100x objectives. Whole embryo images were taken on a Zeiss Stemi SV 6 with an Excelis HDS camera or on a Zeiss SteREO Lumar.V12 with a Zeiss AxioCam MRm.

Atomic force microscopy

A JPK Instruments NanoWizard 4a AFM head with the CellHesion 200 module mounted on a Zeiss AxioObserver were used for all adhesion measurements. NanoWorld Arrow TL-2 cantilevers were coated with a 20 μg/ml solution of Corning Cell-Tak adhesive according to product information sheet. Cantilevers were calibrated using the thermal noise-based contact-free method built into the JPK software. Dissociated mesendoderm cells were attached to cantilevers maintaining a constant height for 20 seconds using 5 nN of force. After cells were lifted from the dish, they were incubated on the cantilever for 5 minutes to adhere more before force measurements were taken. Once attached to cantilevers, nascent adhesions were formed to substrates by applying 1 nN of

force for 5 seconds. Substrates tested were 20 μg/ml of *Bovine* plasma FN, *Xenopus* C-Cadherin-FC, and PLL. Three independent measurements were taken for each cell on a given substrate and statistics were performed on averages of the maximum adhesive strengths for all three measurements.

Western blots

Embryos were lysed in a modified RIPA buffer and proteins were separated on 6% acrylamide SDS–PAGE gels as described in Bjerke *et al.*, 2014. Western blot membranes were probed using antibodies at the following dilutions: Plakoglobin at 1:1000 (γ-catenin; BD Biosciences), β-catenin at 1:1000 (C2206; Sigma Aldrich), β-tubulin at 1:10000 (DM1A; Sigma Aldrich), FN at 1:10000 (4H2; DSHB), C-cadherin at 0.5 μg/ml (6B6; DSHB), integrin β_1 at 1:1000 (8C8; DSHB).

Length-to-width ratio and notochord width measurements

Dorsal isolates cut from control and PG morphant embryos were stained for β-catenin to visualize cell outlines. Length-to-width ratios of 5 axial and 5 paraxial mesoderm cells were measured and averaged for each analyzed image. Statistical analyses were performed using these calculated averages. For each image, notochord widths were measured at the anterior and posterior ends as well as in the center. The three

width measurements were averaged and used as a single data point for statistical analysis. All measurements were taken from single confocal sections.

Fibronectin fibrillogenesis measurements

Fibrillogenesis was measured by determining the total area of an immunofluorescence image occupied by fibronectin fibrils. Stage 11 animal caps were stained for FN and imaged using confocal microscopy. Analyses were performed in ImageJ Version 1.50C. Z-stacks were collapsed into z-projections and lower thresholds were set to remove background fluorescence and kept constant across all analyzed images for each experiment. Fluorescent areas corresponding to FN fibrils above threshold levels were measured.

Injecting embryos with fluorescent FN

Bovine plasma fibronectin (Alfa Aesar) was fluorescently labeled with DyLight 550 according to the protocol provided by Thermo Scientific. Embryos were injected with control or PG morpholinos at stage 2. At stage 8, embryos were injected with 20 ng of fluorescent FN directly into the BC. Animal caps were fixed and stained for *Xenopus* FN at stage 11.

qPCR

RNA was isolated from 10 embryos per condition using the Absolutely RNA Microprep Kit from Agilent. cDNA was synthesized from RNA using the Bioline SensiFAST cDNA Synthesis Kit. cDNA was quantified in the Thermo Scientific PikoReal Real-Time PCR System using the Bioline SensiFAST SYBR No-ROX kit. A plasmid containing the *Xenopus* FN mRNA sequence was used to generate a standard curve for absolute mRNA quantification. Primers used for qPCR reactions were:

FN Forward: ACCTCAACTACACAGACTCGAC

FN Reverse: AGCTGCCATGGGACAGAATC

PG rescue construct

A GFP-tagged PG construct (a gift from M.W. Klymkowsky; University of Colorado, Boulder) (Merriam et al., 1997) was mutagenized using site-directed mutagenesis according to the Agilent QuikChange Site-Directed Mutagenesis Kit protocol. A total of 6 silent mutations within the morpholino target sequence were made across 6 amino acids. Mutations were inserted in pairs. PG morpholino target sequence and mutated PG sequence are shown below, with bases targeted for mutagenesis underlined:

PG morpholino target sequence: ATG GAT TTG GGA GAC GTA GTG GAA A

Mutated PG sequence: ATG GAT TTA GGT GAT GTC GTC GAG A

Surface biotinylation

Surface biotinylation experiment procedures were modified from Gaultier *et al,* 2002. In brief, 25 animal caps were cut per condition and treated with EZ-Link Sulfo-NHS-LC-Biotin (Thermo) diluted in 0.1x MBS to a concentration of 0.33 mg/ml for 30 minutes at room temperature. Animal caps were transferred to eppendorf tubes and washed five times in 100 mM glycine. Animal caps were then lysed in TBS with 1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride 5 mM EDTA, and Protease Inhibitor Cocktail (Sigma). Yolk and cellular debris was removed by centrifuging lysate for 20 minutes at 14,000 G. Biotinylated proteins were precipitated by NeutrAvidin agarose (Pierce) for one hour at room temperature. NeutravAdin agarose was spun down at 3,000 G for 5 minutes, washed three times with lysis buffer, and biotinylated proteins were removed by boiling for 5 minutes in sample buffer containing SDS.

Statistics

Graphs were made and statistics were performed using the Prism V5 software package. Wilcoxon match-paired signed rank tests were performed to compare conditions within a given clutch of embryos for western blot analyses. Mann-Whitney tests were used to test for significant differences between two samples across multiple clutches of embryos and experiments, such as embryo and explant lengths or fibronectin fibril formation. Significance was reported according to the following annotation: ns = not significant, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

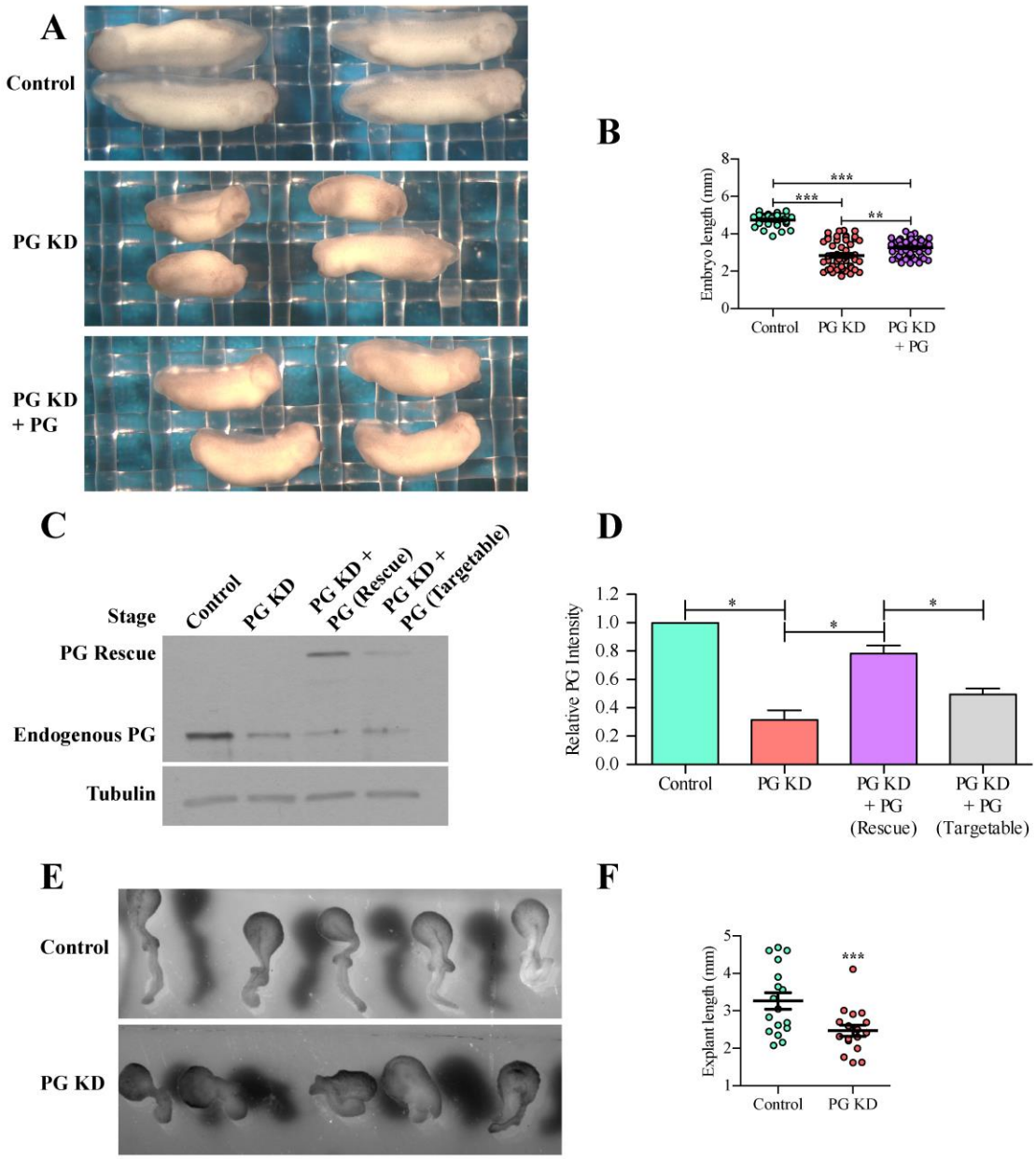
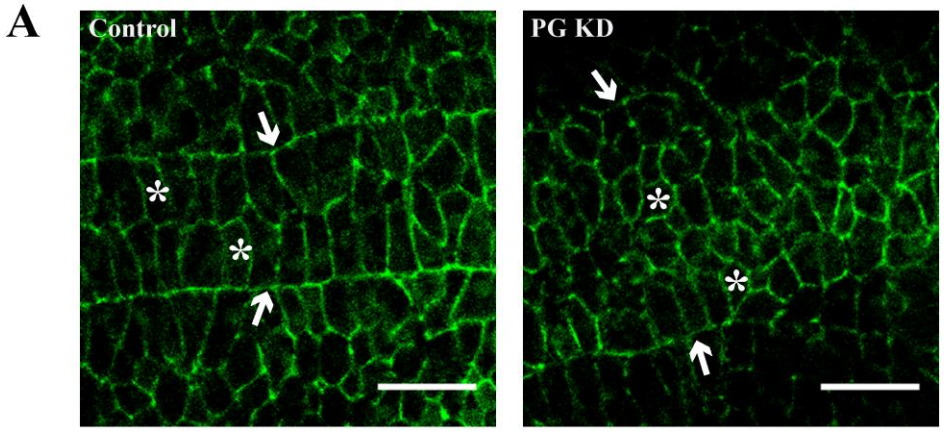
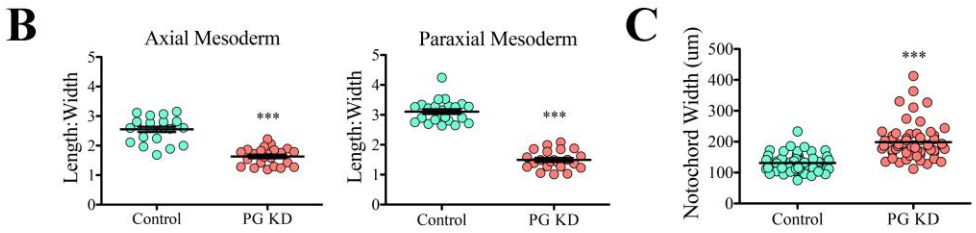

A
Control
PG KD
PG KD
+ PG
B
Embryo length (mm)
***
***
**
Control
PG KD
PG KD
+ PG
C
Stage
Control
PG KD
PG KD +
PG (Rescue)
PG KD +
PG (Targetable)
PG Rescue
Endogenous PG
Tubulin
D
Relative PG Intensity
Control
PG KD
PG KD
+ PG
(Rescue)
PG KD
+ PG
(Targetable)
E
Control
PG KD
F
Explant length (mm)
***
Control
PG KD

Figure 2.1: Plakoglobin morphants exhibit deficiencies in convergent extension movements.

(A) Embryo length comparisons between control (n=35), PG knockdown embryos (n=53) and PG morphant embryos coinjected with a morpholino non-targetable PG rescue construct (n=56). (B) Quantification of embryo length measurements from (A). (C) Representative western blot of lysates from whole embryos injected with control morpholino, PG morpholino, or PG morpholino with the nontargetable PG RNA construct (rescue) or the wildtype PG construct from which the rescue was generated (targetable). (D) Quantification of both endogenous PG and overexpressed PG constructs n=4). (E) Keller sandwich explants from control embryos (n=17) and PG knockdown embryos (n=17). (F) Quantification of explant lengths from (E). Statistics were calculated based on Wilcoxon matched pairs signed rank test. *$p \leq 0.05$, ***$p \leq 0.001$. Differences are not significantly different where not specified.


A
Control
PG KD


B
Axial Mesoderm
Length:Width
***
Control
PG KD
Paraxial Mesoderm
Length:Width
***
Control
PG KD
C
Notochord Width (um)
***
Control
PG KD

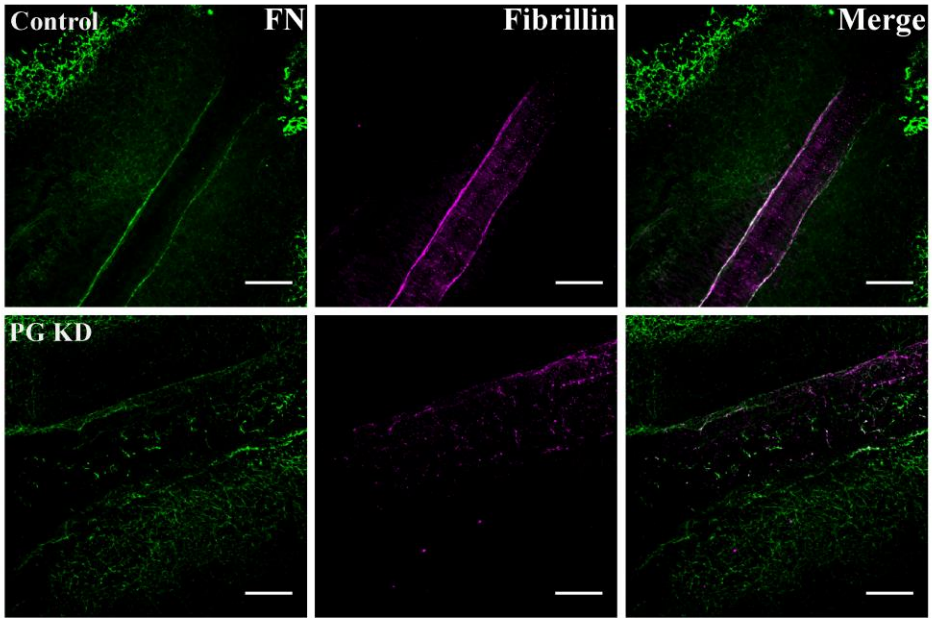

D
Control
FN
Fibrillin
Merge
PG KD

Figure 2.2: Notochord defects in plakoglobin morphants.

Representative single confocal slice of stage 13 dorsal isolates from control and PG morphant embryos. (A) β-catenin staining (green) outlines cell boundaries. Axial mesoderm cells (asterisks) are shown within the notochord-somite boundaries (arrows) while paraxial mesoderm cells are outside the boundaries. (B) Axial (n=22 control; n=23 PG KD) and paraxial mesoderm (n=23 control; n=22 PG KD) length:width ratio measurements from (A). (C) Notochord width measurements (n=53 control; n=58 PG KD) from (A). (D) Dorsal isolates stained for FN (green) and fibrillin (magenta). Statistics were calculated based on Mann-Whitney tests. ***$p \leq 0.001$. Scale bars are 50 μm in (A) and 100 μm in (B).

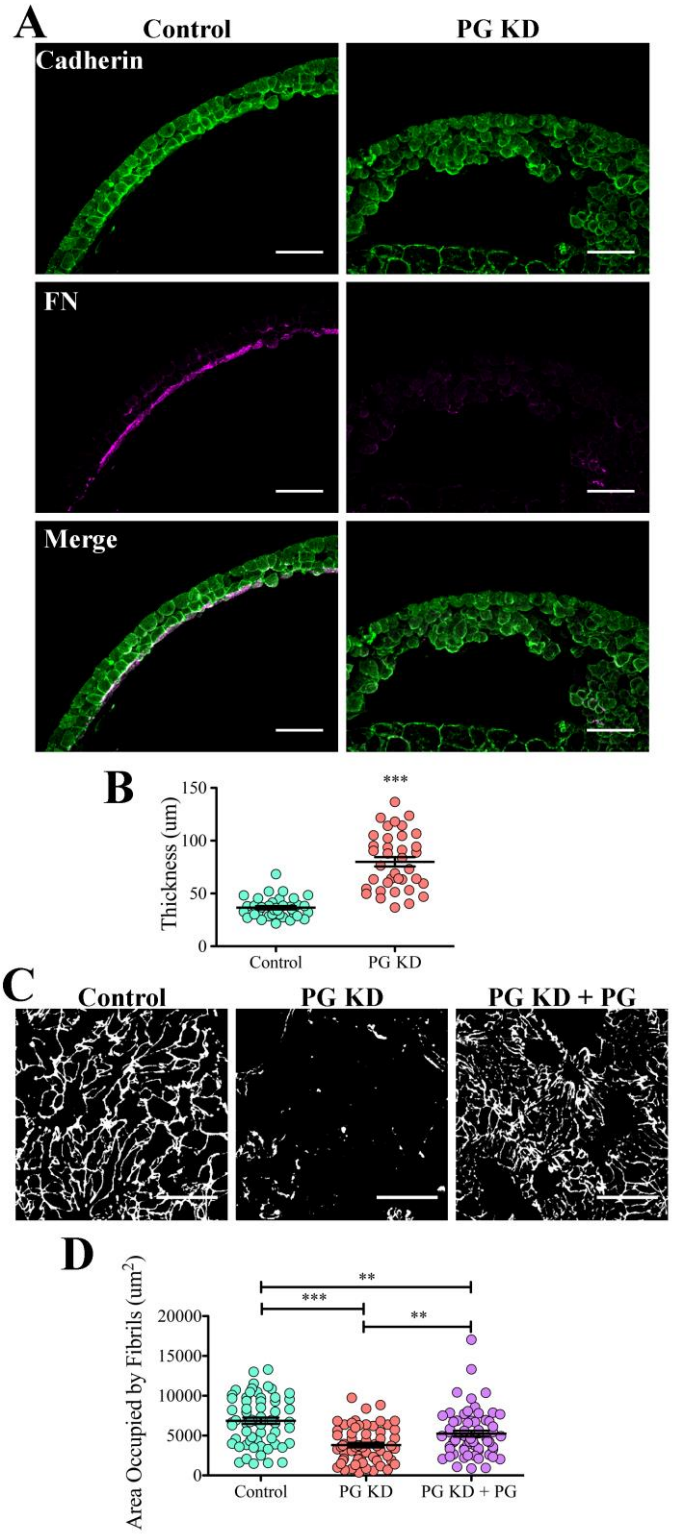

A
Control
PG KD
Cadherin
FN
Merge
B
Thickness (um)
150
100
50
0
***
Control
PG KD
C
Control
PG KD
PG KD + PG
D
Area Occupied by Fibrils (um2)
20000
15000
10000
5000
0
**
***
**
Control
PG KD
PG KD + PG

Figure 2.3: Radial intercalation is disrupted in plakoglobin-deficient embryos.

(A) Transverse sections of stage 11 control (left; n=37) and PG KD animal caps (right; n=37). Embryos were stained for C-cadherin (green) and FN (magenta). (B) Quantification of animal cap thickness from (A). (C) Stage 11 animal caps from embryos injected with control or PG morpholino, or coinjected with PG morpholino and a morpholino-insensitive PG rescue construct stained for FN (white). (D) Area occupied by fibrils in (C) was quantified (n= 61 control; n=64 PG KD; n=63 PG KD + PG embryos). Statistics were calculated based on Wilcoxon matched pairs signed rank tests. ***$p \leq 0.001$. Scale bars in (A) are 100 µm and (C) are 15 µm wide.

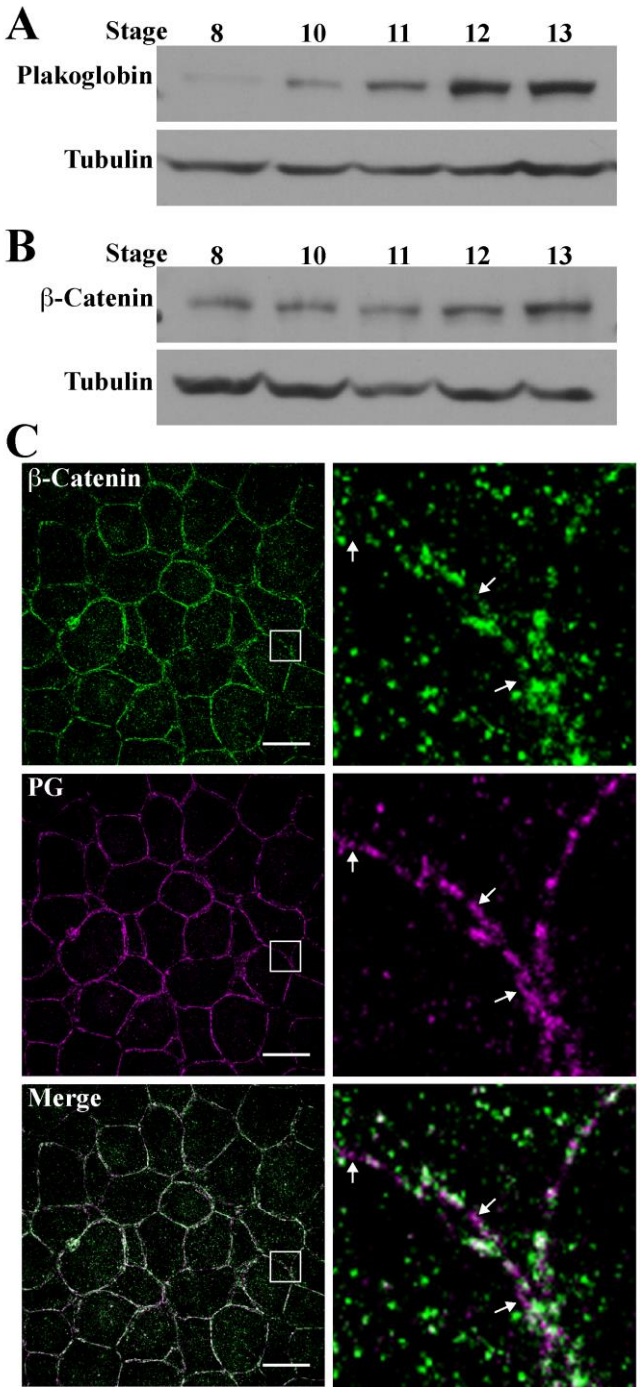

A
Stage 8 10 11 12 13
Plakoglobin
Tubulin
B
Stage 8 10 11 12 13
β-Catenin
Tubulin
C
β-Catenin
PG
Merge

Figure 2.4: Expression and localization of the plakoglobin protein during gastrulation.

A representative western blot showing a time course of PG (A) and β-catenin (B) expression prior to the MBT (stage 8) and throughout gastrulation (stages 10-13). Tubulin was used as a loading control. (C) Confocal Z-stack image of immunofluorescence in a stage 10 animal cap showing β-catenin (green) and PG (magenta) localization. Box in left column outlines magnified area shown in right column. Arrows indicate areas where PG is present without β-catenin. Scale bar is 20 μm wide.

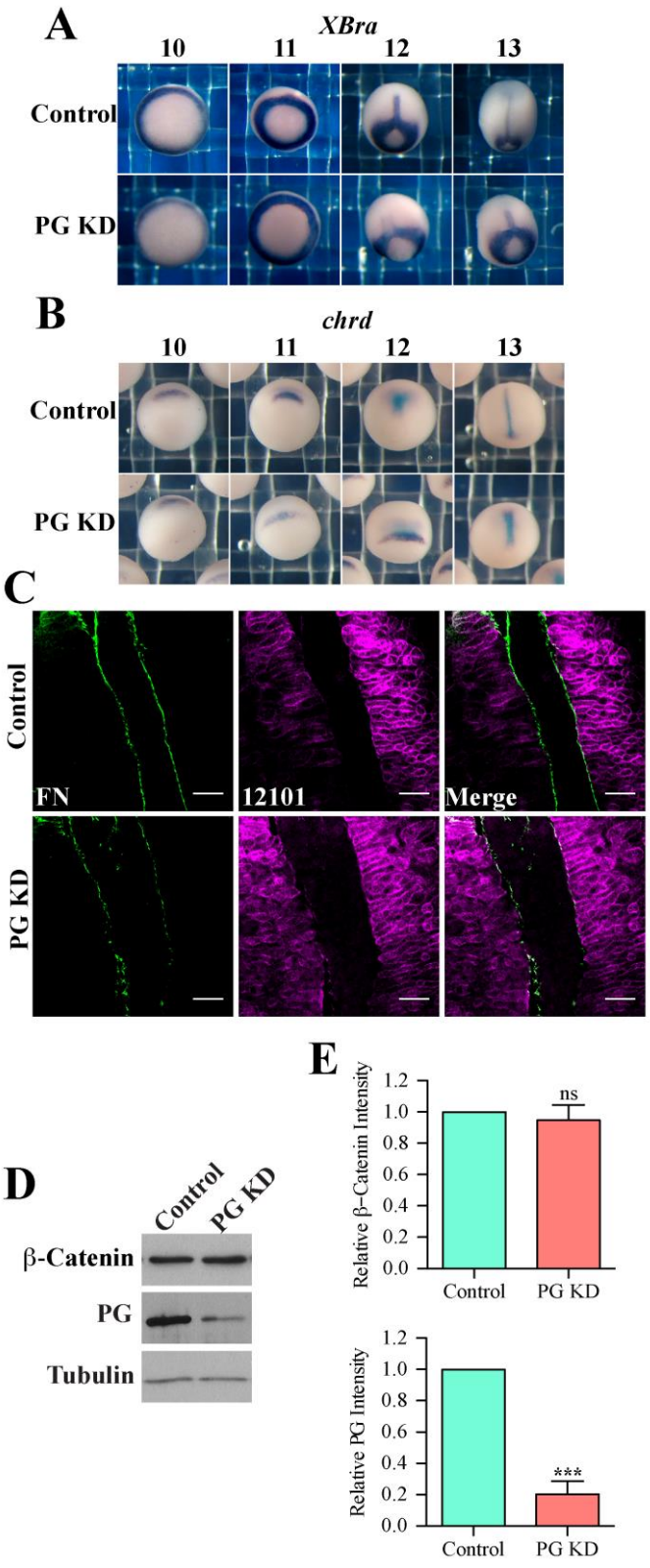

A
XBra
10
11
12
13
Control
PG KD
B
chrd
10
11
12
13
Control
PG KD
C
Control
PG KD
FN
12101
Merge
D
Control
PG KD
β-Catenin
PG
Tubulin
E
Relative β-Catenin Intensity
1.2
1.0
0.8
0.6
0.4
0.2
0.0
ns
Control
PG KD
Relative PG Intensity
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
Control
PG KD

Figure 2.5: Mesoderm patterning is intact in PG-deficient embryos.

(A, B) Control and PG morphant *in situ* hybridization time courses of *Xenopus Brachyury* and *Chordin* expression patterns at stages throughout gastrulation. (C) Dorsal isolates stained for FN (green) and 12101 (magenta). (D) Western blots from whole embryo lysates blotted for β-catenin, PG, and tubulin. (E) Comparison of β-catenin and PG protein levels in control embryos (n=13) and embryos treated with PG morpholino (n=13). Statistics were calculated based on Wilcoxon matched pairs signed rank test. ns = not significant, ***$p \leq 0.001$. Scale bars in (C) are 50 μm wide.

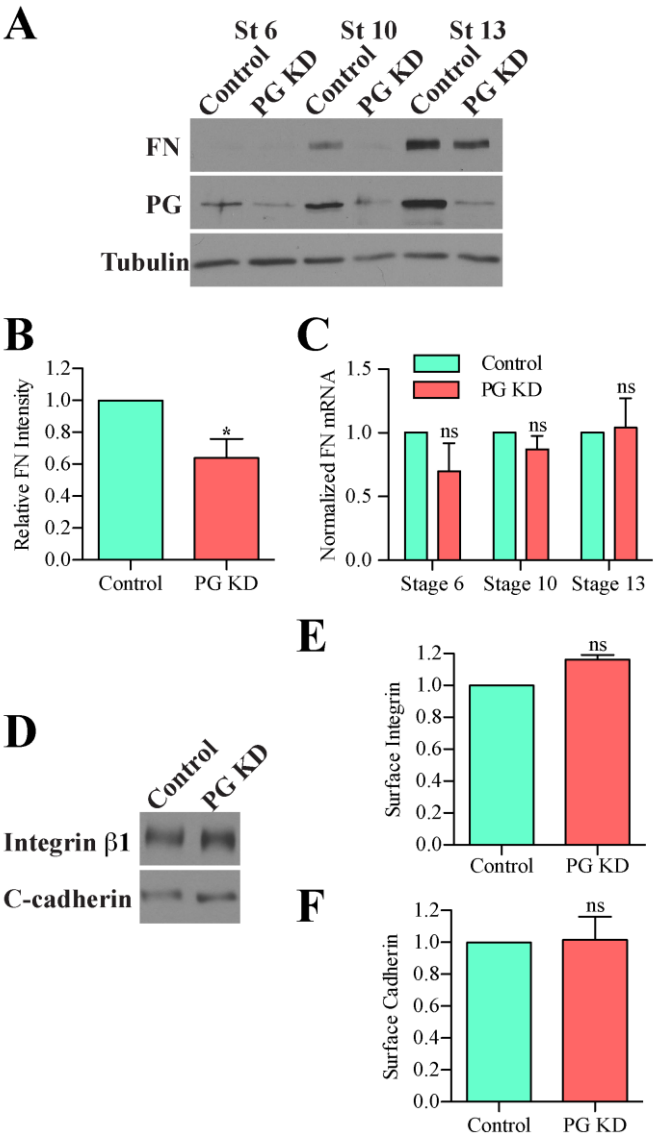

A
St 6
St 10
St 13
Control
PG KD
Control
PG KD
Control
PG KD
FN
PG
Tubulin
B
Relative FN Intensity
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Control
PG KD
C
Normalized FN mRNA
1.5
1.0
0.5
0.0
Control
PG KD
ns
ns
ns
Stage 6
Stage 10
Stage 13
D
Control
PG KD
Integrin β1
C-cadherin
E
Surface Integrin
1.2
1.0
0.8
0.6
0.4
0.2
0.0
ns
Control
PG KD
F
Surface Cadherin
1.2
1.0
0.8
0.6
0.4
0.2
0.0
ns
Control
PG KD

Figure 2.6: FN protein levels are reduced in PG morphants.

(A) Western blots of control and PG morphant whole embryo lysates from stages 6, 10, and 13. (B) FN protein levels relative to tubulin (n=13 control; n=PG KD). (C) Comparison of FN mRNA levels as determined by absolute quantification methods of qPCR (n=3 control; n=3 PG KD). Values within a given experiment were normalized to the level of FN mRNA in control embryos. (D) Surface biotinylated proteins were pulled down with NeutrAvidin agarose and run on an SDS-PAGE gel. Western blots for integrin β_1 and cadherin were performed on the samples. (E) Densitometry analyses of integrin β_1 (E) and cadherin (F) blots in (D) to determine surface integrin and cadherin levels. Statistics were calculated based on Wilcoxon matched pairs signed rank tests on samples from three individual clutches of embryos. ns= not significant. Statistics were calculated based on Wilcoxon matched pairs signed rank test. ns = not significant, $^{**}p \leq 0.01$.

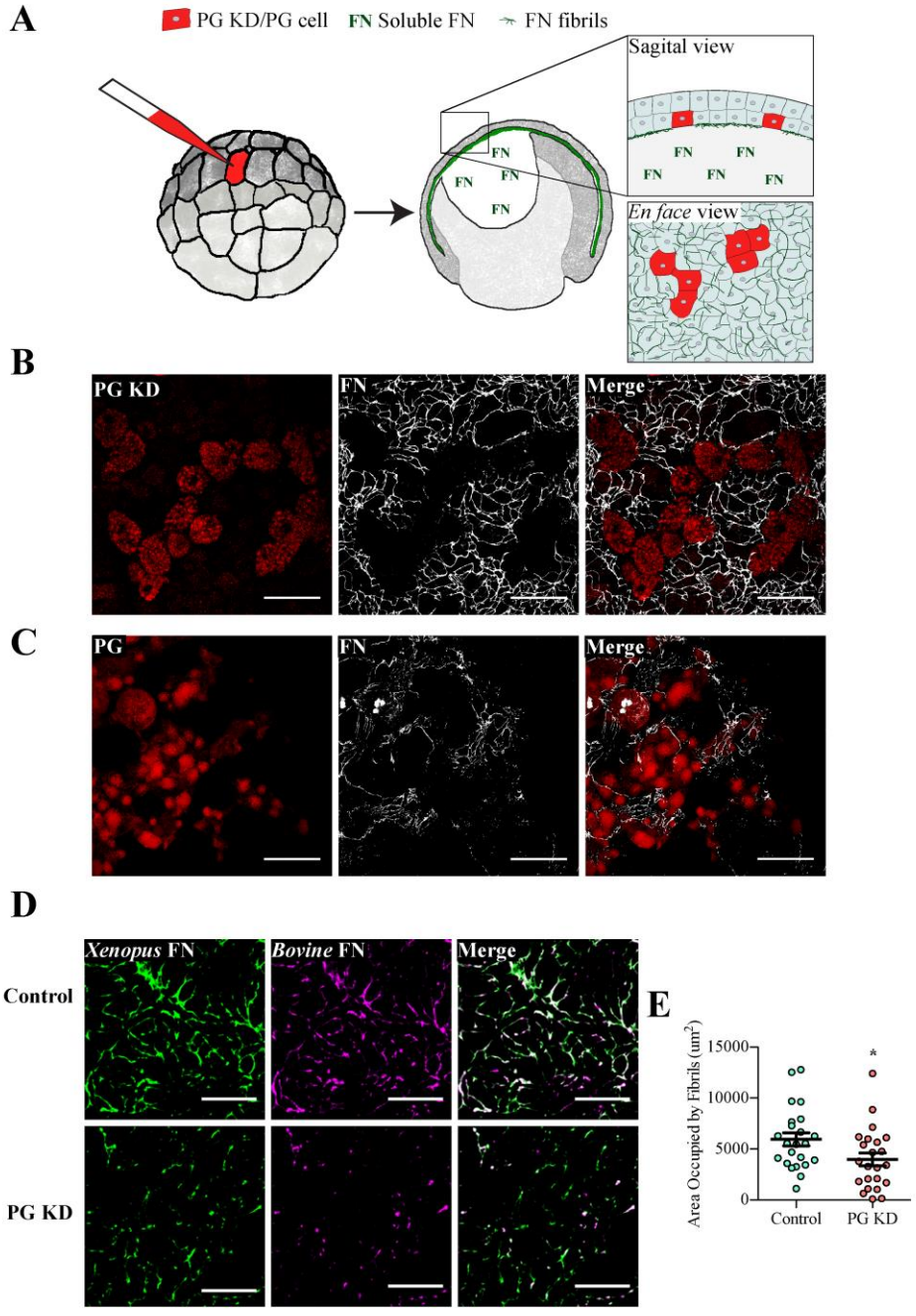

A
PG KD/PG cell
FN Soluble FN
FN fibrils
Sagital view
En face view
FN
B
PG KD
FN
Merge
C
PG
FN
Merge
D
Xenopus FN
Bovine FN
Merge
Control
PG KD
E
Area Occupied by Fibrils (um²)
15000
10000
5000
0
Control
PG KD
*

Figure 2.7: PG plays an essential role in FN assembly.

(A) Depiction of experimental design used in (B) and (C). Embryos at 32 cell stage were injected with PG morpholino (A) or a PG overexpression construct (B) into a single blastomere (red). At gastrulation, all cells within the BCR are exposed to the same pool of soluble FN in the BC which is assembled into fibrils along the roof. Animal caps were isolated and immunostained for FN fibrils (green and white). Uninjected cells surround the KD cells and were unlabeled. (B) Stage 11 animal caps from embryos coinjected with dextran and PG morpholino into 1 of 32 cells. (C) Stage 10 animal caps from embryos overexpressing PG in 1 of 32 cells. (D) Animal caps from control or PG morphant embryos injected with Bovine FN (magenta) and stained for Xenopus FN (green). (E) Area occupied by Bovine FN fibrils in (D) was quantified (n=23 control; n=23 PG KD). Statistics were calculated based on Mann-Whitney tests. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. Scale bars are 50 µm wide in (B) and (C) and 15 µm wide in (D).

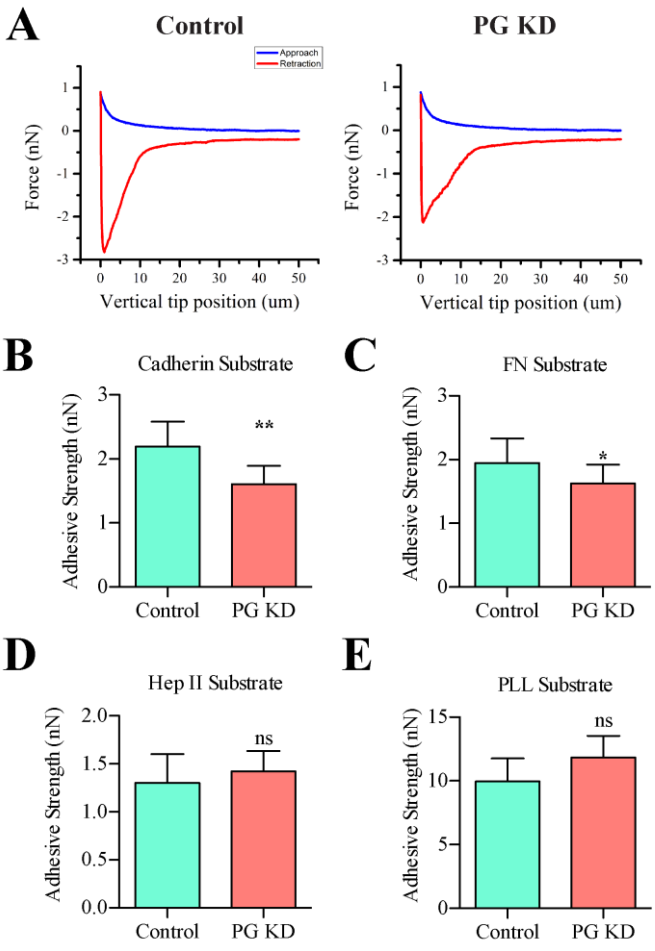

A
Control
PG KD
Approach
Retraction
Force (nN)
Vertical tip position (um)
B
Cadherin Substrate
Adhesive Strength (nN)
**
Control
PG KD
C
FN Substrate
*
D
Hep II Substrate
ns
E
PLL Substrate

Figure 2.8: Adhesive strength to cadherin and FN are reduced in PG-deficient cells.

(A) Representative force-distance curves of single cell adhesion experiment on a C-cadherin substrate. Single control or PG KD cells were attached to cantilevers and allowed to attach to C-cadherin (B), FN (C), HepII (D), or poly-l-lysine (E) substrates. Approach and retraction curves are shown in blue and red, respectively. Adhesive strength was determined for 10-12 cells per condition for each substrate. Cells from three independent clutches were analyzed for each substrate. Statistics were calculated based on Wilcoxon matched pairs signed rank test. ns = not significant, *$p \leq 0.05$, **$p \leq 0.01$.

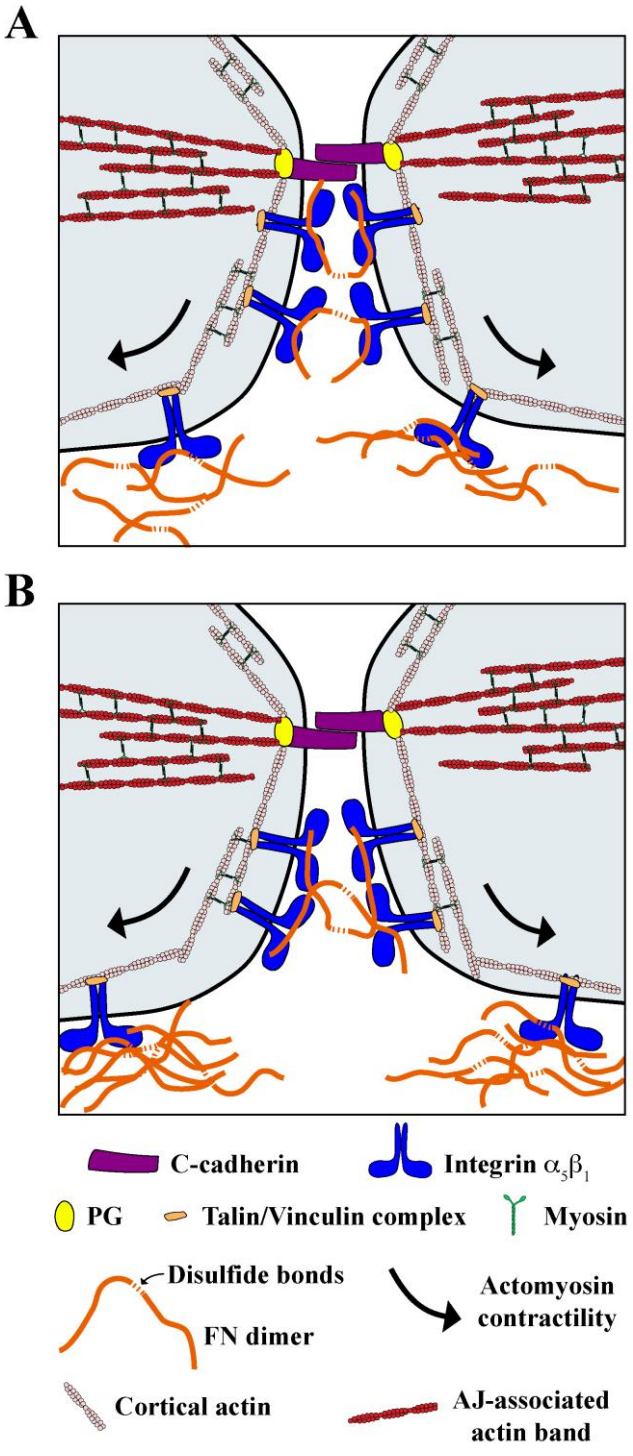

A
B
C-cadherin
Integrin $\alpha_5\beta_1$
PG
Talin/Vinculin complex
Myosin
Disulfide bonds
FN dimer
Actomyosin contractility
Cortical actin
AJ-associated actin band

Figure 2.9: A role for PG in FN assembly.

A model depicting FN assembly over time (A-B). Adherens junctions between animal cap cells mediated by C-cadherin (purple) adhesions. On the cadherin cytoplasmic tails, PG (yellow) recruits actin-binding proteins. Actin filaments (red) are anchored to these adhesions. Actin stress fibers at adherens junctions are bundled and contractility is mediated by myosin (green). $\alpha_5\beta_1$ integrins (blue) bind to extracellular fibronectin dimers (orange) and cortical actin stress fibers. Myosin-based contractility drives the centripetal movement of integrin receptors (arrows), applying strain to the bound FN dimers. Force-unfolded FN dimers attach to one another and form a network of fibrils.

Chapter 3

Mitochondrial localization and activation in collective cell migration

This chapter is composed of unpublished data.

Introduction

The majority of the ATP within a cell is synthesized in mitochondria through oxidative phosphorylation (OXPHOS). As part of OXPHOS, transmembrane ATP synthase proteins that span the inner mitochondrial membrane are responsible for transferring protons from the intermembrane space to the matrix. The energy generated by this process is used to synthesize ATP from ADP and an inorganic phosphate. These ATP synthases are the producers of the most of the ATP synthesized during OXPHOS. The process requires a membrane potential established by the separation of protons from electrons that pass down the electron transport chain and drive the protons in to the intermembrane space. This results in higher membrane potentials in mitochondria that are more metabolically active (Hüttemann et al., 2007). Some of the byproducts of OXPHOS are negatively charged reactive oxygen species, which can damage mitochondrial proteins and lipids. Reactive oxygen species-induced mitochondrial damage is minimized through the processes of mitophagy and the formation of mitochondrial-derived vesicles (Cadete et al., 2016).

The mitochondrial network is a dynamic organelle that is constantly changing. Mitochondria fuse, divide, and can be trafficked rapidly throughout the cell along cytoskeletal networks. Morphology is often linked to the cell cycle, metabolic state, and even migration rates. Highly fused mitochondria are more efficient at OXPHOS and are associated with reduced rates of apoptosis. The ROS that accumulate within the matrix during OXPHOS cause damage over time. Damaged mitochondria are recycled through

mitophagy, a mitochondria-specific autophagic process. During mitophagy, damaged portions of the mitochondria are sequestered, separated from the main mitochondrial network, engulfed by a phagophore, and trafficked to the lysosome for destruction. This constant selective turnover is essential to maintain a functional mitochondrial network (Kubli and Gustafsson, 2012).

Mitochondrial morphology and mitophagy are achieved through a balance of fusion and fission. Both processes rely on dynamin-related proteins (DRPs), which facilitate the fusion of both the inner and outer mitochondrial membranes. DRP1 is the main protein involved in fission, forming a ring around the mitochondria at sites of fission. Interestingly, endoplasmic reticulum (ER)-associated proteins facilitate mitochondrial fission at sites of contact (Friedman et al., 2011). Fusion, on the other hand, is regulated by two different DRPs, mitofusin (MFN) and optic atrophy 1 (OPA1). Mitofusin regulates fusion of the outer mitochondrial membrane, and OPA1 is required for fusion of the inner membrane (Friedman and Nunnari, 2014).

One of the major functions of the mitochondrion is ATP production. ATP is consumed at a relatively rapid rate at the leading edge of a migratory cell. Processes important for pushing the membrane forward at the leading edge, such as actin polymerization (Pollard and Borisy, 2003) and actomyosin contractility (Murrell et al., 2015), are highly energetic. Mitochondria accumulate at the leading edge of some migratory cell types, at least in part for the localized production of ATP at sites of high consumption (Rangaraju et al., 2014). AMP-activated protein kinase (AMPK) is an

important sensor of ATP and AMP levels and is active at the leading edge of migratory cells. This active AMPK leads to the recruitment of mitochondria, ATP production, and subsequent membrane ruffling and forward migration (Cunniff et al., 2016). In T cells, mitochondrial accumulation and activation at the leading edge is essential for persistent migration (Ledderose et al., 2015). Similarly, mitochondria accumulate on the anterior side of the nucleus in migratory cancer cells. Cells in which the mitochondrial networks are positioned behind the nucleus exhibit significantly lower migration rates (Desai et al., 2013). These data demonstrate the importance of mitochondrial activation at the leading edge of single cells during directional migration. How this translates to morphogenesis and large-scale collective cell migration events has not yet been investigated.

The subcellular localization of mitochondria is dynamic, with constant rapid movement throughout the cell. Actin filaments (Sunan Li et al., 2015), IFs (Matveeva et al., 2015; Nekrasova et al., 2011), and microtubules (Campello et al., 2006; Yaffe et al., 1996) associate with mitochondria, and perturbations in any of these cytoskeletal components can lead to changes in mitochondrial localization and morphology. Knockdown or inhibition of the fission and fusion machinery can result in changes in subcellular localization and subsequently, migration rates (Zhao et al., 2012). These cytoskeletal components, although important for mitochondrial localization, also serve as a means of mechanically coupling the mitochondrion to cellular adhesive complexes and organelles throughout the cytoplasm (Fletcher and Mullins, 2010).

Not only is ATP used as an energy source, but it is also an important signaling molecule. In addition to intracellular kinase cascades, ATP is involved in autocrine and paracrine mechanisms of signaling. For example, ATP is released from apoptotic cells, acting as a “find-me” signal. Phagocytes are recruited by the ATP for clearance of the dying cells (Chekeni et al., 2010). Extracellular purines are sensed by membrane-bound purinergic receptors. These receptors fall into three categories: the adenosine receptors (P1s), the ligand-gated ion channels (P2Xs) and the G-protein coupled receptors (P2Ys). ATP signaling occurs through P2 receptors. In the presence of ATP, P2X channels open for the passage of cations. The P2Y receptors are responsible for initiating most of the intracellular signaling cascades that result from extracellular di- and triphosphorylated purines. The most ubiquitously expressed P2Y receptor, P2Y2, is activated by ATP and UTP, whereas many other P2Y receptors are specific to either ATP or UTP (Faas et al., 2017). Purines are hydrolyzed as they bind to P2Y receptors, resulting in the activation of various intracellular signaling pathways that lead to actin polymerization, cell contractility, opening of ion channels (Erb and Weisman, 2012), and even activating mitochondria (Bao et al., 2015).

The extracellular release of ATP is achieved through exocytic mechanisms, such as at synapsis in the central nervous system where it functions as a rapid excitatory neurotransmitter, or through various channels and hemichannels in the cell membrane (Pankratov et al., 2006). The connexin and pannexin hemichannels have been a recent focus in the field of purinergic signaling. Connexins are the channel-forming gap junction proteins that cytoplasmically couple adjacent cells. Although they are most often

associated with gap junctions, they have also been shown to form hemichannels, releasing ATP and other small molecules into the extracellular space (Kang et al., 2008). Pannexins are a functionally similar family of proteins that form hemichannels with the main purpose of purine release. Pannexins do not form gap junctions and are the primary family of hemichannels that are investigated in the context of purinergic signaling (Lohman and Isakson, 2014).

There are three pannexin proteins in humans. Pannexin-1 is the most ubiquitously expressed family member and is a homohexameric channel. In migratory immune cells, pannexin-1 accumulates at the leading edge where it releases ATP to guide the cells directionally in an autocrine mechanism of signaling (Bao et al., 2013; 2014). The pannexin channels open in response to extracellular ATP (Locovei et al., 2006), an applied voltage, and through mechanical strain applied to the cell membrane (Bao et al., 2004; Maroto and Hamill, 2001; Nakamura and Strittmatter, 1996).

In vascular endothelial cells, shear stress (Rongsong Li et al., 2009) and cyclic stretch (Ali, 2004) have been shown to increase mitochondrial activity. Cyclic stretch has also been shown to activate mitochondria in smooth muscle cells (Bartolák-Suki et al., 2015). Most studies that have shown a mechanical activation of the mitochondrion utilize constant or cyclic mechanical stimulation over the course of several hours. After long-term mechanical stimulation, it becomes difficult to distinguish between proximal mechanosensitive events and more distal downstream effects. In systems such as the vasculature or lungs where constant stimulation is physiologically relevant, this poses no

problem. However, understanding the cellular mechanisms involved in rapid mechanosensation requires shorter time frames.

The collective migration of the *Xenopus* mesendoderm relies on mechanosensation. The cells within the mesendoderm adopt a shingled arrangement as they migrate, with cells extending protrusions beneath the cells in front of them. As they migrate, cells pull on one another through their cadherin-based adhesions. These transient mechanical pulls direct protrusions in the forward orientation, allowing for the cohesive migration of the tissue (Weber et al., 2012). The first line of evidence suggesting that this process relies on mechanical activation of the mitochondrion came from an unbiased approach to identify mechanosensitive proteins within the mesendoderm. Mechanical strain was applied to mesendoderm cells plated on C-cadherin or FN substrates, and proteins that had undergone conformational change in response to the mechanical strain were identified by a cysteine-labeled shotgun mass spectroscopy technique (CS-MS) (Johnson et al., 2007). Of the proteins identified as undergoing conformational change within 1 minute of stretch were two TCA enzymes: aconitase and malate dehydrogenase (Bjerke, 2014). In the present study, we investigate the role of mechanical stimulation on mitochondrial activation and purinergic signaling in the mesendoderm. We have found that mitochondrial localization and activity likely serve important roles in the collectively migrating tissue. Additionally, we have found that the mesendoderm cells are sensitive to purines. Current efforts aim to further characterize the influence of mechanosensitive purinergic signaling on collective cell migration.

Results

Active mitochondria accumulate in protrusions

Morphology and distribution of mitochondria vary greatly from one cell type to another. Transmission electron microscopy (TEM) focusing on the lateral contacts between mesendoderm cells reveals small oval-shaped mitochondria (Figure 3.1). Most of these punctate mitochondria are less than 1 μm long and can be found throughout the cell. To visualize mitochondria in live mesendoderm cells, a mitochondria-anchored GFP RNA construct (GFP-MA) was expressed. GFP-MA contains a mitochondria-targeting sequence from the *Listeria monocytogenes* ActA protein (Pistor et al., 1994) attached to a GFP construct. This protein labels all mitochondria and does not reflect changes in membrane potential. At gastrula stage, the mesendoderm was dissected, dissociated and treated with tetramethylrhodamine, ethyl ester (TMRE). TMRE is a rhodamine-based dye that accumulates in mitochondria based on membrane potential (Emaus et al., 1986). Accumulation of the dye is proportional to membrane potential and is reversible, allowing for live visualization of changes in the activity states of individual mitochondria.

ME cells labeled with TMRE were visualized at high magnification to gain a better understanding of subcellular localization and activity states of mitochondria. Live imaging confirmed TEM observations (Figure 3.1) indicating that mitochondria adopt a small punctate morphology throughout the cell. These punctate mitochondria accumulate

in cell protrusions where they are highly active (Figure 3.2 A). When TMRE is compared to that of GFP-MA labeling in the same cell, it is apparent that the highest mitochondrial activity is seen in protrusions (Figure 3.2 B). The ratio of activity level in protrusions compared to activity in the cell body is significantly greater in the TMRE channel compared to that of GFP-MA (Figure 3.2 C). This high activation in protrusions is not specific to single mesendoderm cells. The intact, collectively migrating mesendoderm tissue can be isolated in a dorsal marginal zone (DMZ) explant (Davidson et al., 2002). In a TMRE-treated DMZ explant, leading edge cell protrusions contain densely packed and active mitochondria (Figure 3.2 D). These data suggest that the mitochondria in the protrusions exhibit a high activity state compared to mitochondria in the cell body in single cells as well as in the intact tissue.

Mitochondria in the protrusion are dynamic

Mitochondria within protrusions are highly dynamic. TMRE-labeled mitochondria move in and out of protrusions rapidly. As they enter a protrusion, mitochondria become highly active, and they maintain this activity for a short period of time after exiting the protrusion (Figure 3.3 A). High speed imaging on a spinning disk microscope shows the speed at which mitochondria move within the protrusion. An overlay of two pseudocolored images acquired one second apart reveals several regions where the images do not overlap (Figure 3.3 B). When two images acquired five seconds apart are overlaid, there is minimal colocalization remaining (Figure 3.3 C). This rapid

movement and activation of the mitochondria within the protrusion may prove to be essential to cell migration.

Mesendoderm cells are sensitive to purinergic signals

In addition to generating ATP at sites of high consumption, mitochondria in protrusions may participate in purinergic signaling. *Xenopus* ectodermal tissues have been shown to react to ATP through rapid actomyosin contractility (Kim et al., 2014), but the role of purinergic signaling in mesendoderm migration has not yet been investigated. To determine if mesendoderm cells respond to purines, either adenosine or ATP was added to single cells. Adenosine signaling occurs through P1 receptors, whereas ATP signaling requires P2 receptors. Cells respond within minutes after the addition of adenosine (Figure 3.4 A) or ATP (Figure 3.4 B). In the presence of adenosine, cells begin protruding and migrating (Figure 3.4 A). ATP addition to the medium results in cell spreading (Figure 3.4 B). These results indicate that mesendoderm cells likely express the P1 and P2 receptors necessary for sensing a purinergic signal. Current efforts aim to identify the function and specific mechanism of purinergic signaling in the collectively migrating mesendoderm tissue.

Discussion

The mitochondrial network is essential for generating the majority of ATP within a cell. This ATP serves as both an energy source and an extracellular signaling molecule. Data indicate that mitochondrial enzymes within mesendoderm cells are sensitive to mechanical stimulation of cadherin and integrin adhesions (Bjerke, 2014). The protrusions, where the traction stresses applied to a substrate are highest, contain densely packed and hyperactivated mitochondria in both dissociated mesendoderm cells as well as in isolated mesendoderm explants. The role of the mitochondria in these protrusions is currently under investigation.

Most cell types display a tubular network of mitochondria. Interestingly, this is not the case in the mesendoderm where the mitochondria are small punctate structures with nearly no tubules. Punctate mitochondria are often observed during mitosis and in protrusions of migratory cells. During mitosis, cellular contents are divided into two daughter cells. Instead of being actively segregated, mitochondria are stochastically distributed between daughter cells. Fission is upregulated during mitosis, resulting in a more even distribution of mitochondria (Kashatus et al., 2011; Taguchi et al., 2007). Cell division occurs rapidly during embryonic development, likely requiring smaller mitochondria for proper segregation. Mesoderm cells (including the ME), however, rarely divide during gastrulation (Saka and Smith, 2001). Small mitochondria in the mesendoderm may be a remnant of high rates of cell division from pre-gastrula stages of development.

Small mitochondria are also beneficial during cell migration. Migratory cells that accumulate mitochondria in the protrusions tend to have smaller mitochondria. Platelet-derived growth factor (PDGF)-treatment of vascular smooth muscle cells results in increases in mitochondrial fission and cell migration rates. PDGF-induced migration rates are reduced when fission is inhibited (Wang et al., 2015). Presumably, smaller mitochondria are more efficiently trafficked to the protrusions where larger organelles are size-excluded. Inhibiting fission or increasing fusion in invasive breast cancer cells leads to larger mitochondrial networks, fewer lamelliform protrusions and reduced migration rates. These perturbations also result in less mitochondrial accumulation in protrusions (Zhao et al., 2012).

Our CS-MS data indicate that mitochondrial enzymes undergo conformational changes in response to force (Bjerke, 2014). The protrusions are the sites of greatest traction stresses and correlate with high mitochondrial activity levels. The mitochondria that accumulate in the protrusions of mesendoderm cells have a high activity level. In single cells, although most had at least one protrusion filled with active mitochondria, not all cells or protrusions exhibited this accumulation. Similarly, not all protrusions extended from a single cell apply high traction stresses (Bjerke et al., 2014). Patterns of force distribution are more predictable in DMZ explants where the protrusions of leading edge cells apply nearly all of the measurable traction stresses (Sonavane et al., 2017).

The majority of the protrusions at the leading edge of a DMZ explant are full of active mitochondria, while protrusions in following cells are not. Cells within a DMZ

explant protrude underneath one another, with most cells extending forward-directed protrusions under the cells in front of them. However, TMRE-treated explants indicate that the mitochondria are active in the protrusions of the leading-edge cells, but not in protrusions of following cells. This further supports the hypothesis that mechanical strain in the form of traction stresses can lead to the accumulation and activation of mitochondria. The result of this mitochondrial activation remains to be determined.

There are two likely functions of the mitochondria within the protrusions of leading edge cells. The first is for localized ATP production. Extending protrusions in a migratory cell requires actin polymerization and ATP hydrolysis. Producing ATP at sites of high consumption is one way to ensure that the ATP is in the location where it is required, without relying on passive diffusion. The second likely function of the mitochondria in the protrusion is for purinergic signaling. mesendoderm cells spread on a FN substrate in response to treatment with ATP. This suggests that the cells are capable of responding to purinergic signaling. The role of the mitochondria in protrusions may be in producing ATP for subsequent release and purinergic signaling.

Taken together, our data suggest that mitochondrial activity is sensitive to mechanical stretch. We hypothesize that the accumulation and activation of mitochondria within leading edge protrusions is essential for purinergic signaling in the collectively migrating tissue. Mechanically-induced release of ATP through pannexin hemichannels at the leading edge may result in autocrine and paracrine mechanisms of ATP signaling through P2Y receptor activation. P2Y receptors can then induce localized mitochondrial

activation and more ATP production (Figure 3.5). This polarized ATP release may serve an important role in the persistent migration of the mesendoderm tissue. However, the specific mechanism of mitochondrial localization and activation remains to be determined. Future directions for this project are discussed further in Chapter 4.

Materials and Methods

Embryos and explants

Xenopus laevis embryos were obtained, fertilized *in vitro*, and staged using standard practices (Nieuwkoop and Faber, 1967). Single mesendoderm cells were dissociated in calcium- and magnesium-free MBS (Sive et al., 2000). DMZ explants were isolated from embryos as previously described (Davidson et al., 2002). Cells and explants were attached to glass-bottom dishes coated with 10 µg/ml FN (Alfa Aesar).

Labeling mitochondria

The majority of the images depicted in this chapter utilized TMRE for labeling mitochondria. Cells or explants were treated with TMRE (Invitrogen, #T-669) at a final concentration of 50 nM if HEPES-free MBS. Cells or explants were plated in a TMRE solution and allowed to attach for 30 min prior to imaging. Imaging was performed with TMRE in solution. For labeling with GFP-MA construct, cells were injected with 500 pg of RNA per embryo.

Microscopy

Structured illumination microscopy was performed on a Zeiss AxioObserver Z1 with an Apotome 2 and Axiocam 506 camera using Plan Apochromat 63x and EC Plan-neofluar 40x objectives. Spinning disk microscopy was performed on a custom system based on a Nikon TE2000-E microscope with a Plan apo TIRF 60x objective.

Mitochondrial activity measurements and protrusion: cell body calculations

Relative mitochondrial activity in protrusions was calculated based on fluorescence in the TMRE or GFP-MA channels. The rate of mitochondrial movement is rapid enough that acquiring two channels in series results in a shift in some mitochondria. This makes it impossible to determine TMRE relative to GFP-MA fluorescence on a per pixel basis. Instead, we have statistically compared the ratios of fluorescence in the protrusions versus the cell body for both the TMRE and GFP-MA channels.

In ImageJ, a threshold was applied to an image of a single spread cell and converted to a binary mask. Protrusions and cell body were selected and mean fluorescence and area per mitochondrion was determined. Mean fluorescence was multiplied by area for each particle, and values were averaged for all particles in the protrusion or cell body of each cell. This average brightness per area measurement was then divided by total fluorescence area to get final average fluorescence values. Average

protrusion fluorescence was divided by cell body fluorescence for each individual cell and ratios were statistically compared.

Statistics

The Prism V5 software package was used to conduct statistical analyses and generate graphs. A Wilcoxon match-paired signed rank test was performed to compare the fluorescence in protrusions to fluorescence in the cell body.

Purine addition experiments

Single cells were plated on FN substrates for at least 30 min before experiments began. Cells were imaged for 5 min by differential interference contrast prior to addition of adenosine or ATP. Purines were added at a final concentration of 100 μM and imaged for another 15 min to track cell behavior. Higher concentrations of ATP, such as 1 mM, resulted in apoptosis, a known effect of high concentrations of extracellular ATP (Wen and Knowles, 2003; Zheng et al., 1991).

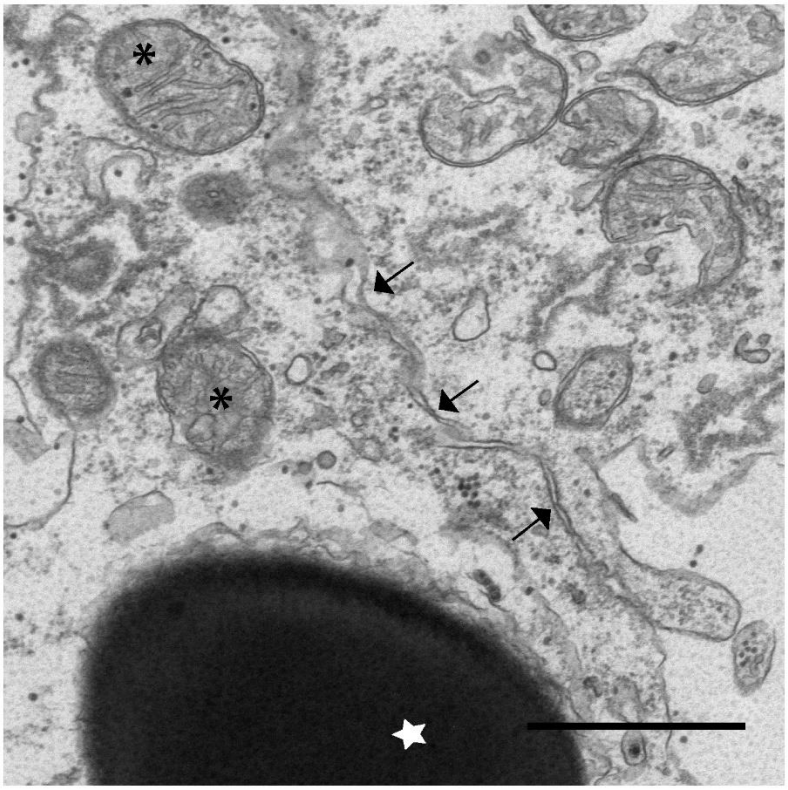


Figure 3.1: Transmission electron microscope image of mitochondria in mesendoderm cells.

TEM of the mitochondria located adjacent to lateral cell-cell contacts between two mesendoderm cells in a DMZ explant. Asterisks denote examples of mitochondria. Arrows indicate cell-cell contacts. Star marks a yolk platelet. Scale bar is 1 μm wide. Image acquired by Bette Dzamba and David Castle.

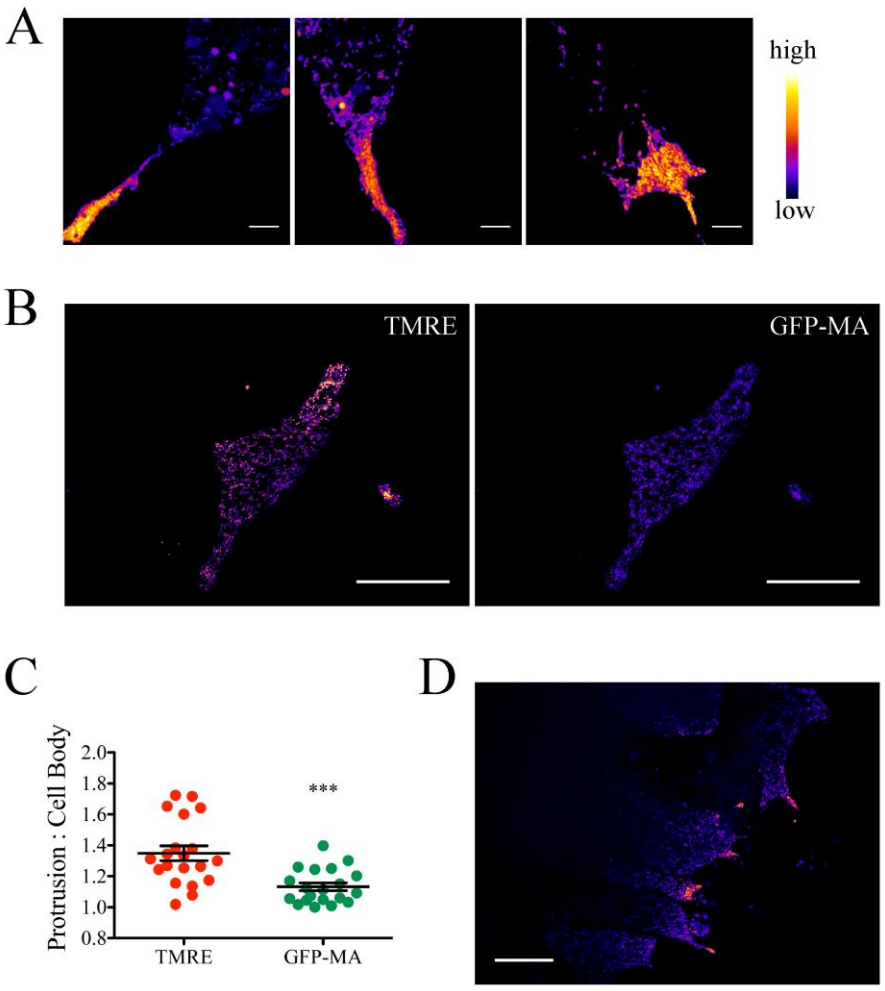

A
high
low
B
TMRE
GFP-MA
C
Protrusion : Cell Body
2.0
1.8
1.6
1.4
1.2
1.0
0.8
***
TMRE
GFP-MA
D

Figure 3.2: Accumulation and activation of mitochondria in protrusions.

Images of TMRE-labeled mitochondria at mesendoderm cell protrusions are pseudocolored (brighter/warmer colors represent higher levels of activation). (A) High magnification representative images of protrusions from three different cells acquired on a spinning disc microscope. Cells in (B) were labeled with TMRE (left) and express the GFP-MA construct (right). Cells in (B) were imaged on a widefield microscope. (C) Quantification of relative mitochondrial fluorescence calculated from TMRE and GFP-MA images. Statistics were calculated based on Wilcoxon matched pairs signed rank test. ***$p \leq 0.001$. (D) TMRE-labeled DMZ explants migrate towards the right. Scale bars are 5 µm wide in (A) and 50 µm wide in (B) and (D).

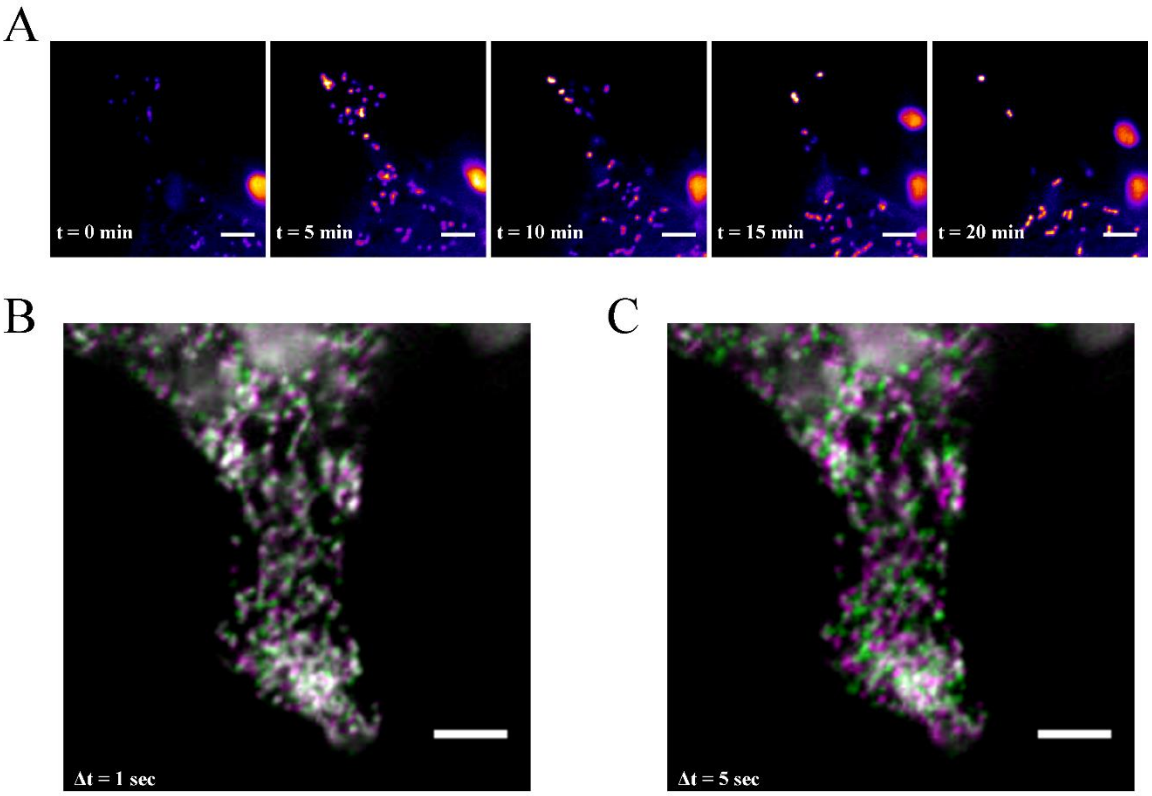
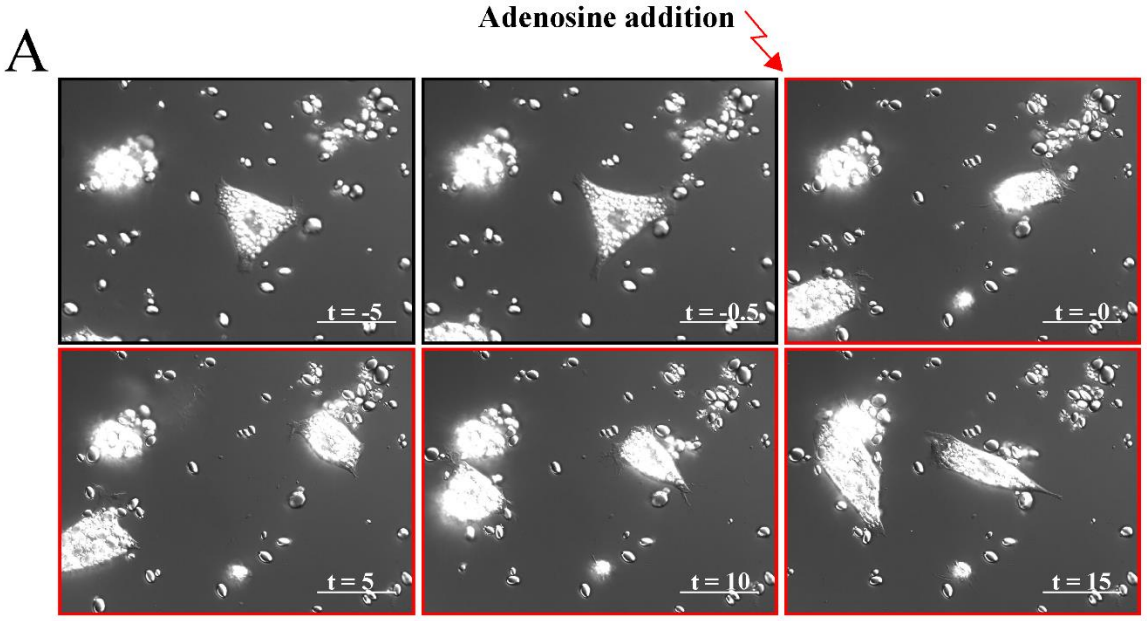

A
t = 0 min
t = 5 min
t = 10 min
t = 15 min
t = 20 min
B
Δt = 1 sec
C
Δt = 5 sec

Figure 3.3: Mitochondria in protrusions are dynamic.

Isolated mesendoderm cells were labeled with TMRE and imaged on a spinning disk microscope. (A) Psuedocolored TMRE in a protrusion displaying dynamics in mitochondrial distribution and membrane potential from t=0 to 20 min. (B) Overlay of images taken at t=0 and one (B) or five (C) seconds apart. Image at t=0 sec in magenta and images at t=1 sec or t=5 sec in green.


A
Adenosine addition
t = -5
t = -0.5
t = -0
t = 5
t = 10
t = 15

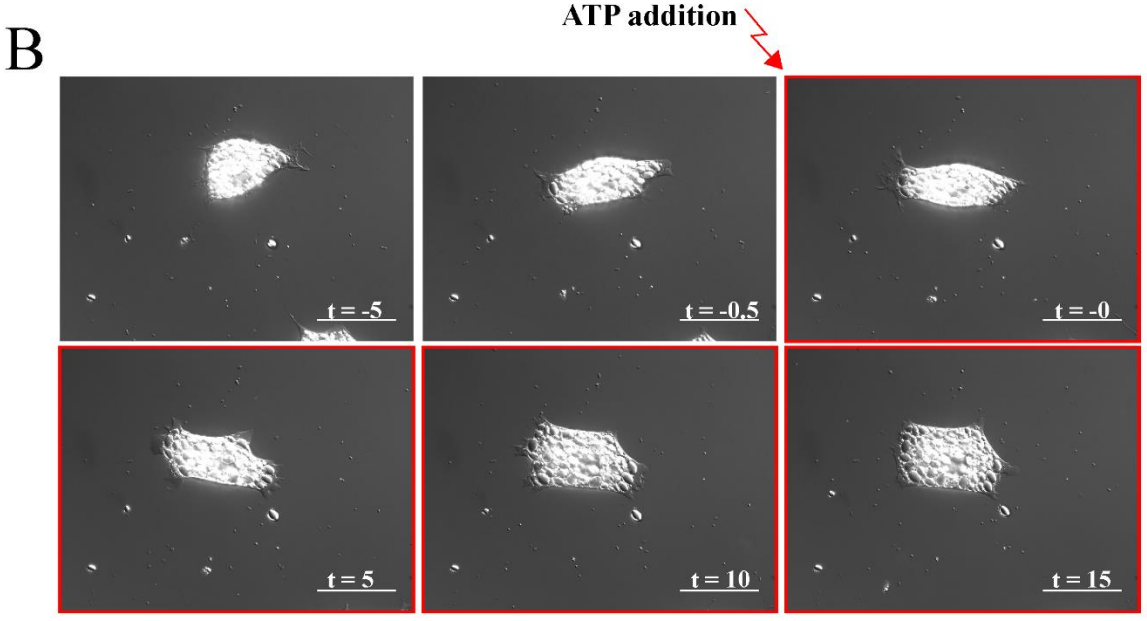

B
ATP addition
t = -5
t = -0.5
t = -0
t = 5
t = 10
t = 15

Figure 3.4: Mesendoderm cells are sensitive to purinergic signals.

Isolated mesendoderm cells were treated with 100 µM (A) adenosine or (B) ATP and imaged. Cells were imaged with DIC microscopy at t=-5s and t=-0.5s prior to treatment, treated at t=0 (arrow), and imaged at t=5s, t=10s, and t=15s after treatment. Scale bars are 50 µm long.

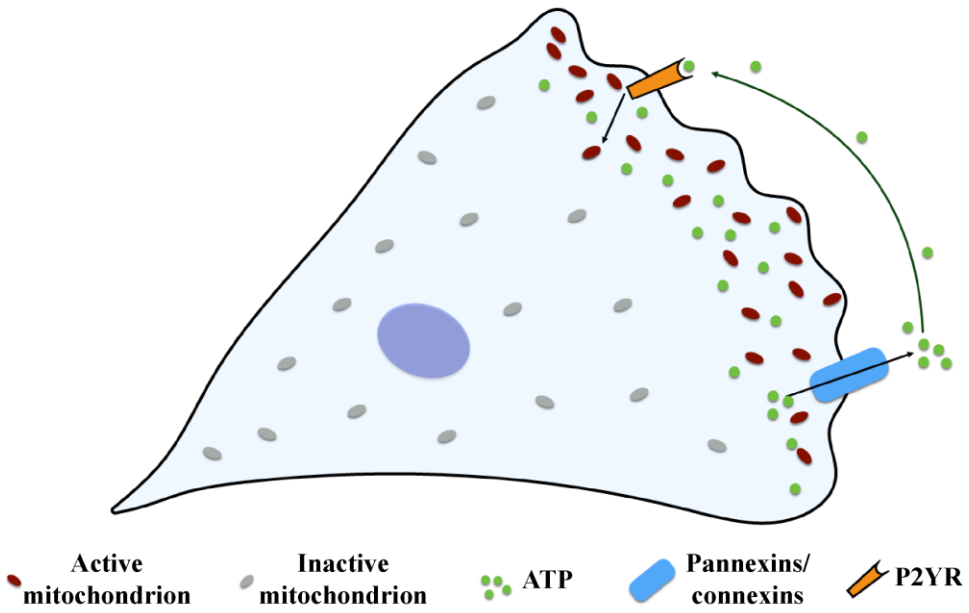

Active mitochondrion
Inactive mitochondrion
ATP
Pannexins/ connexins
P2YR

Figure 3.5: Mechanism of autocrine purinergic signaling in the mesendoderm

Active mitochondria accumulate in protrusions of leading edge cells during collective migration. High traction stresses at the leading edge are conducive to the opening of pannexin and connexin hemichannels for extracellular ATP release. This ATP can activate G-protein coupled P2Y receptors, further activating mitochondria and promoting cell migration through various kinase cascades.

Chapter 4

Conclusions, Future Directions, and Significance

Summary and major findings

The shape of an organism is achieved through coordinated morphogenetic movements. These movements rely on actomyosin contractility as well as cell adhesion (A. C. Martin, 2010). Cells assemble and adhere to their ECM through integrin receptors at FAs and they adhere to one another through cadherin-mediated AJs and keratin-rich desmosomes. The structural integrity and cohesion of the tissue, essential to coordinated morphogenesis, relies on these adhesive complexes. The work described in this thesis further characterizes the role of cell adhesion and mechanical signaling in morphogenesis.

Chapter 2 of this thesis focuses on understanding the role of cell-cell adhesion complexes in morphogenesis. Knocking down the cadherin-associated catenin, PG, leads to defects in radial intercalation and convergent extension. These defects result in short tadpoles in which tissue patterning is maintained, and FN deposition is reduced. The loss of FN assembly is cell autonomous and does not rely on a general reduction in FN protein levels. Experiments confirm reduced attachment forces applied to both cadherin and FN substrates when PG is knocked down. This reduction in attachment strength is likely the cause of the loss of matrix assembly and subsequent defects in morphogenesis. These data promote a better understanding of cadherin-integrin crosstalk mechanisms.

Although preliminary, the data in Chapter 3 suggest that mitochondria serve an important role in collective cell migration. Previous CS-MS experiments from our laboratory (Bjerke, 2014) have identified mitochondrial enzymes as undergoing

conformational changes in response to physical stretch. I have since found that mitochondria accumulate and are hyperactivated in the protrusions of single cells where traction stresses are highest. These mitochondria in the protrusions are dynamic, with rapid changes in localization and activity state. This accumulation of active mitochondria at leading edge protrusions is more pronounced in intact DMZ explants where traction forces in the tissue are highest (Sonavane et al., 2017). My data suggest that the mitochondria in these protrusions are likely important for the localized production of ATP to fuel energetic processes and for autocrine and paracrine signaling that directs collective cell migration.

Taken together, data described in this thesis reveal novel mechanisms of ECM deposition and morphogenetic regulation. I have investigated these processes in the context of the developing *Xenopus* embryo; however, the data are applicable to various diseases and processes where morphogenesis and ECM fibrillogenesis are essential. For example, during wound healing, large sheets of cells migrate collectively to close an open wound. Not only does this process require coordinated directional migration of sheets of cells within a tissue, it also relies on the assembly of a new ECM as a substrate on which cells migrate. Similarly, during metastasis, cancerous cells from a tumor migrate collectively while remodeling the dense ECM in the surrounding microenvironment (Friedl and Gilmour, 2009). The findings in this thesis give us more insight into some of the processes involved in morphogenesis in development and disease progression. However, new knowledge often yields new questions. Future directions and implications of the work described in Chapters 2 and 3 are discussed below.

Cadherin and integrin crosstalk in FN assembly

Cadherin-integrin crosstalk mechanisms have been documented in *Xenopus* development (Dzamba et al., 2009; Marsden and DeSimone, 2003) as well as in various cell lines (Mariner et al., 2001; Martinez-Rico et al., 2010; Wang et al., 2006). Many of these crosstalk mechanisms rely on signaling proteins that are shared between AJs and FAs such as Src and FAK, whereas others are more indirect, relying on β-catenin-mediated Wnt signaling and transcriptional regulation. Wnt-mediated methods of crosstalk have been documented in fibrotic disorders where cadherin protein levels are altered. In idiopathic pulmonary fibrosis, crosstalk between E-cadherin, TGF-βR1 and $\alpha_3\beta_1$ mediate an EMT (Kim et al., 2008; Kim et al., 2009). Increased cadherin-11 expression is also associated with systemic sclerosis (Wu et al., 2014) and idiopathic pulmonary fibrosis where it has been shown to regulate EMT through TGF-β signaling (Schneider et al., 2012). The influence of cadherin adhesion on fibrillogenesis in the context of fibrosis tends to be indirect, through transcriptional regulation and changes in cell fate. By shifting cells from an epithelial to a mesenchymal-like state, they increase transcription of ECM molecules resulting in fibrotic disease (Agarwal, 2014).

Some mechanisms of cadherin-integrin crosstalk rely on changes in transcriptional regulation. This is not the case in *Xenopus* gastrulae (Figure 2.6). Overexpressing cadherin in animal cap cells leads to precocious assembly of FN. This fibrillogenesis requires Rac and Pak for actin remodeling and contractility (Dzamba et al.,

2009). FAs and AJs are connected to the actin cytoskeletal network, providing a direct mechanical linkage between the two complexes. Cadherin engagement at AJs on the lateral contacts results in actomyosin contractility that can be transduced to the integrin-based FAs on the basal surface of the cells. The application of this contractile force to the integrin receptors may result in increased assembly of FN fibrils (Figure 4.1). This direct mechanical linkage and transfer of force from AJs to FAs may contribute, in part, to the observed PG-mediated FN assembly. However, there is likely a second mechanism involved in this process.

PG does not directly anchor actin to the cytoplasmic tails of cadherins at AJs. Instead, it recruits α-catenin, an actin binding protein. If the only role for PG in actin organization were through the recruitment of α-catenin, then depleting α-catenin or PG should yield the same actin phenotypes. Work from the Wylie lab indicates that this is not the case. Depleting maternal PG results in a severe reduction in the amount of filamentous actin stress-fibers, whereas depleting α-catenin does not affect cortical actin organization to nearly the same extent (Kofron et al., 2002). This suggests a secondary mechanism by which PG is involved in organizing actin, possibly through the recruitment of additional as-yet unidentified actin-binding proteins at AJs, or through an AJ-independent mechanism.

An interconnected cytoskeletal network

There are three major components of the cytoskeletal network: actin, IFs, and microtubules. Cytoskeletal components within the cell are interconnected through the scaffolding protein plectin. Plectin is a member of the plakin family of proteins, and it is capable of binding to multiple cytoskeletal networks simultaneously. Through plectin, IFs and actin stress fibers are interconnected, and removal of one network can result in restructuring of the other (Wiche et al., 2015). One explanation for the extensive loss of cortical actin architecture in PG morphants is that the PG-associated IF network is responsible, in part, for organizing actin stress fibers. A loss of PG resulting in disrupted IFs can lead to a reduction in actin stress fibers (Fujiwara et al., 2016).

PG morphant cells display a partial loss of the keratin basket structures that form at the rear of the cells at the leading edge of a migrating DMZ explant. Most of the keratin network in these cells is maintained, including the keratin cable that forms along the lamellipodia of the leading-edge cells (Weber et al., 2012). Similarly, knocking down XCK1(8) expression shortly after fertilization causes a reduction in the posterior basket, but there is no change in the cable or other keratin structures (Figure 4.2). Keratin IFs are obligate heteropolymers made up of acidic (type I) and basic (type II) keratins. Early *Xenopus* embryos have multiple type I keratins, but XCK1(8) is the only type II keratin expressed (Franz and Franke, 1986; Franz et al., 1983). Knocking down XCK1(8) should consequently result in a reduction in the formation of new IFs. The fact that most of the IF cytoskeleton remains unperturbed in XCK1(8) knockdown embryos suggests that two distinct keratin networks exist: one that is susceptible to knockdown and one that is resistant. Resistance or susceptibility to knockdown is likely related to filament stability.

One interesting possibility is that the knockdown-insensitive IFs are stabilized by other cytoskeletal components such as actin stress fibers.

Interestingly, integrins do not bind to IFs directly, but plectin can anchor IFs to the actin stress fibers anchored at FAs. These IFs can be essential to proper FA function. In plectin knockout fibroblasts, FA turnover is slowed and cells exhibit larger, more stable lamellipodial protrusions. This reduction in FA turnover is likely due to a loss of mechanical tension associated with anchoring IFs to the actin at FAs (Gregor et al., 2014). Another location where the two networks interact is at invadopodia, where paxillin-rich FAs form a large ring-like structure on the basal side of the cell. Actin attaches to the integrins within the FAs, and the structure is also linked to a keratin network through plectin (Gad et al., 2008). We have confirmed the existence of keratin and paxillin-rich invadopodia-like structures in animal cap cells through total internal reflection fluorescence (TIRF) microscopy (Figure 4.2).

Cytoskeletal crosslinking between a dynamic PG-organized keratin network and actin stress fibers may be an important part of FN assembly. It would be interesting to investigate this possibility by using live cell TIRF microscopy to visualize keratin at FAs in the presence and absence of PG and plectin. If PG is important for stabilizing the FA-associated keratin network, one would expect to see a reduction in this network in PG morphants. If the keratin network is anchored at FAs through plectin, knocking down plectin should yield similar results.

Identifying the mechanism of PG-mediated FN attachment

I have identified PG, a cadherin-binding protein, as a regulator of matrix attachment and assembly. The specific mechanism through which PG regulates these processes remains unclear. Attachment of integrin receptors to ECM components relies on both outside-in and inside-out mechanisms of signaling. My findings indicate that PG morphant cells do not form strong attachments to FN substrates after 5 seconds of contact. Within 5 seconds there is little time for FA maturation or actin assembly, and defects in attachment are likely due to changes in inside-out integrin signaling.

There are two main mechanisms by which cytoplasmic signals and proteins can increase the strength of integrin attachment to a matrix: through increases in receptor affinity or through integrin clustering (avidity). To increase affinity, intracellular proteins such as talin and kindlin can bind to the cytoplasmic tails of integrin receptors and lock the integrin in a high affinity conformation (Ginsberg, 2014; Ye et al., 2014). This high affinity state of the integrin results in a stronger substrate attachment by individual integrin receptors. This increases attachment strength without affecting the number of engaged integrin receptors. Changes in integrin conformation and affinity are the primary means of inside-out signaling. A second mechanism through which integrin-ECM attachment strength can be modulated is by integrin clustering. Attachment strength to a substrate increases as the local concentration of integrin receptors increases (Campbell and Humphries, 2011). PG morphant embryos do not have lower levels of integrin β_1 on the cell surface. However, I have not assayed for localized integrin clustering. Live

imaging of a GFP-tagged integrin construct via TIRF microscopy can give insight into the extent of integrin clustering. More clustering will result in larger FA structures on the substrate level. Unfortunately, defects in FA turnover can also lead to larger FAs. Others have developed Förster resonance energy transfer (FRET) (Smith et al., 2007) and β-galactosidase-based clustering assays (Buensuceso et al., 2003) that result in increased signal where integrins cluster, but these assays suffer from the same pitfalls. Although distinguishing between FA maturation and clustering is difficult, similar signal intensity in control and PG morphants would suggest that both clustering and FA maturation are maintained.

In order to assay for changes in integrin affinity state, the most useful tools available are conformation-specific integrin antibodies (Su et al., 2016). These antibodies recognize epitopes that are only exposed after ligand binding (ligand-induced binding sites) (Byron et al., 2009). One way to determine if there are conformation-dependent changes in integrin affinity state is to perform immunofluorescence microscopy using conformation-specific antibodies, comparing PG-deficient to control cells. These antibodies work well in some systems, including human and mouse cells, but no conformation-specific integrin antibodies exist for use in *Xenopus*. Human or mouse cell lines could be used to perform these experiments, however, it is possible that a 3D *in vivo* or *ex vivo* system is necessary for studying this mechanism of fibrillogenesis regulation. In 2D culture systems, cells are attached to a substrate onto which they assemble a matrix. In contrast, *in vivo* cells adhere to one another through cadherin adhesions on lateral edges and assemble a matrix on the basal surface of the cell. This geometry, which

may be important for assembly, is drastically different in a 2D cell culture system. Performing experiments in another system in order to utilize these conformation-specific antibodies would first require the validation of the existence of a PG-mediated FN assembly process. If the PG-regulated assembly only exists in a 3D system, it may be necessary to design new conformation-specific antibodies for use in *Xenopus*.

Intracellular integrin priming: a prerequisite to fibrillogenesis?

FN fibrillogenesis is thought to be a purely extracellular process. FN is secreted into the extracellular space as a dimer and is assembled into fibrils by the integrin receptors along the cell surface (Schwarzbauer and DeSimone, 2011). Because this process occurs extracellularly, when FN from another species is added, cells can assemble the protein into fibrils (Darribere and Schwarzbauer, 2000). I have found that PG-deficient cells are incapable of assembling a FN matrix. This is true when PG is knocked down in a small subset of cells, but also when *Bovine* FN is added back to PG-deficient whole embryos (Figure 2.7). Interestingly, I see similar results when the same experiments are performed in cells where FN is knocked down (Figure 4.3), suggesting that cells must produce their own FN in order to assemble extracellular FN into fibrils. This phenomenon has not been described in any *in vitro* or *in vivo* system and indicates that there is still much that we do not understand about the process of fibrillogenesis.

PG morphants display a reduction in FN protein expression, however, the embryos still express the protein at a level of about 60-65% of that seen in control

embryos. If FN production is a prerequisite for extracellular assembly, this should not be the cause of assembly defects in PG morphants, which express more than half of the normal level of FN. Thus, our finding suggests that assembly defects in PG morphants are not due to the loss of FN expression. The machinery responsible for assembly of FN into fibrils should be unaffected in FN morphants. The fact that there are defects in assembly suggests that cells must express their own FN in order to assemble extracellular FN. This evidence supports the hypothesis that integrin receptors may need to interact with the FN protein intracellularly. This intracellular interaction may serve as a priming mechanism where the integrin receptor must be secreted to the cell surface while bound to FN before it can assemble extracellular FN fibrils.

Intracellular interactions of receptors and ligands have been investigated extensively in the field of immunology. MHC receptors bind to intracellular peptide fragments and are secreted to the cell surface. These peptide fragments are then presented to passing T cells. For example, when a cell is infected with a virus, the MHC class I receptors present viral peptides to passing cytotoxic T cells, which then kill the infected cell (Hewitt, 2003). A similar mechanism of intracellular interaction between receptor and ligand may be necessary for FN assembly.

It would be difficult to directly visualize FN-primed integrin receptors within the cell, however, I propose a few key experiments that may support or refute this hypothesis. First, scaling down the extent of FN knockdown should result in a recovery in assembly when exogenous FN is added. If integrin receptors require a priming

mechanism, it should be possible to rescue fibrillogenesis by adding exogenous FN to embryos expressing a low level of FN. Additionally, TEM should reveal where the two proteins are in close proximity to one another. If they interact intracellularly, immunogold-labelled FN and integrin receptor should be juxtaposed in TEM images, likely within the ER and golgi apparatus.

Regulated ECM assembly in disease and wound repair

In addition to its essential role in embryonic development, ECM assembly is crucial for the progression of various diseases and for wound repair. Open wounds are closed through the processes of regeneration and scar formation. This involves directed cell migration of the healing tissue as well as the deposition of new ECM. A moderate level of fibrillogenesis is required for wound healing, but excess leads to the formation of a scar. Scar formation is an undesirable result of tissue injury in which the tissue becomes more mechanically rigid and dysfunctional (Atala et al., 2011; Xue and Jackson, 2015). Small scars in the skin may be mainly of cosmetic concern, but those in the heart can be fatal. Following myocardial infarction, damaged cardiac tissue is often repaired through the formation of a scar. The elastic nature of the tissue is compromised within the fibrous scar, resulting in reduced blood flow (Ma et al., 2012). In order to prevent scar formation or treat fibrotic disease we must first understand the process of fibrillogenesis and the players involved. Identifying PG in the process of matrix assembly exposes new potential targets for therapeutics. A complete loss of PG or desmosomal cadherins leads to serious

desmosomal defects within the heart and skin, and over time, reductions may affect Wnt signaling and β-catenin protein levels (Karnovsky and Klymkowsky, 1995; Kodama et al., 1999; Martin et al., 2009). Therapeutic targeting of PG is likely not ideal, however there may be other members of the PG-mediated ECM assembly machinery that are more appropriate candidates (Agarwal, 2014). Further investigations into the specific mechanism of PG-mediated matrix assembly may prove crucial to our understanding of fibrillogenesis not only in development, but also in disease and injury repair.

Mitochondrial localization and morphology in collective cell migration

Coordinating the collective migration of a tissue relies on the distinction between leading and following cells (Friedl and Gilmour, 2009; Poujade et al., 2007). Leader cells are phenotypically different from following cells, displaying larger lamellipodial protrusions and exerting greater traction stresses (Sonavane et al., 2017; Collins and Nelson, 2015; Trepat et al., 2009b). Chapter 3 of this thesis presents evidence suggesting that the mitochondria within the leader cells of the migrating mesendoderm adopt a distinct subcellular localization. I have found that punctate mitochondria within the leading-edge cells are heavily concentrated in the cell protrusions. Data in single cells suggest that the localization of mitochondria in protrusions is important for directing cell migration (Desai et al., 2013; Ledderose et al., 2015; 2014), at least in part through purinergic signaling (Bao et al., 2014; 2013). This mechanism of directed migration may also occur in the collectively migrating ME; however, this has not yet been confirmed.

Localization and morphology of mitochondria are closely linked. Perturbations in cytoskeletal components or motor proteins responsible for localization also result in changes in morphology and activity (Sunan Li et al., 2015; Matveeva et al., 2015). Interestingly, inhibiting fission or stimulating fusion can also result in mislocalized mitochondria (Zhao et al., 2012). To investigate the role of mitochondrial localization and morphology in the migrating mesendoderm, one could inhibit fission and fusion through inhibition by small molecule (Cassidy-Stone et al., 2008) in addition to RNA knockdowns. Considering the naturally punctate morphology of the mitochondria in the mesendoderm, it would be most advantageous to inhibit fission machinery while measuring migration rates and protrusion directionality in DMZ explants. Reduced rates of migration in DMZ explants would indicate a requirement for mitochondrial fission. Although this would implicate mitochondrial localization and activation in collective cell migration, it does not address the function of the small mitochondria in the protrusions.

Purinergic signaling in collective cell migration

I have shown that single mesendoderm cells are responsive to exogenous purine addition, exhibiting changes in cell spreading and migration upon addition of ATP or adenosine. Therefore, I hypothesize that one function of mitochondria at the leading-edge protrusions is in producing ATP for purinergic signaling. This hypothesis can be tested by determining the effects of purinergic signaling inhibitors on DMZ explant migration. Pannexin-specific small molecule inhibitors can be used to inhibit the secretion of ATP

and prevent most ATP-related purinergic signaling (Poon et al., 2014). A second method to inhibit ATP signaling is through the extracellular hydrolysis of ATP after treatment of explants with the non-cell membrane permeable enzyme apyrase. If purinergic signaling is found to be involved in mesendoderm migration it would be important to then begin characterizing the receptors and pannexin proteins in the context of *Xenopus* development.

Not much work has been done to characterize the role of purinergic signaling during *Xenopus* development. Because of this, if purinergic signaling is important for mesendoderm migration it will become necessary to characterize the expression of Pannexin channels and P2Y ATP receptors. The best way to characterize the expression of these genes spatially and temporally is through *in situ* hybridization. Pannexin-1 and P2Y2 are the most ubiquitously expressed channel and receptor in purinergic signaling and are most likely to be the proteins involved in a purinergic response. After a pannexin and receptor have been characterized, specific knockdowns of these proteins in DMZ explants would be the next step to identifying them as participants in the purine-guided collective migration of the mesendoderm.

Force-induced mitochondrial activation and purinergic signaling

Our preliminary CS-MS data indicate that two different mitochondrial enzymes undergo conformational changes in response to uniaxial stretch (Bjerke, 2014). Previous studies have shown that shear stress (Rongsong Li et al., 2009) and cyclic stretch (Ali,

2004; Bartolák-Suki et al., 2015) can activate mitochondria. We hypothesize that the conformational changes in enzymes identified in the CS-MS experiments are indicative of mitochondrial activation. The most direct way to test this hypothesis is by measuring ATP in stretched mesendoderm cells compared to unstretched cells through luciferase-based assays. These assays are sensitive enough to measure intracellular ATP in cell lysates as well as ATP released into the medium. Results of this experiment will indicate if the mesendoderm is sensitive to mechanically-induced changes in both ATP production and release.

The importance of the mitochondrion and mechanotransduction in collective cell migration

The influence of mechanical forces on cell behavior has been investigated in detail over the past few decades (Wozniak and Chen, 2009). These external forces are translated to intracellular signals that influence the activities of mechanosensitive proteins. We have found that mitochondrial enzymes are mechanosensitive, undergoing conformational changes rapidly in response to mechanical stretch on a cadherin or FN substrate. Mitochondrial function and subcellular localization are essential for immune cell homing (Bao et al., 2014; 2013) and apoptotic cell clearance (Chekeni et al., 2010). My data are the first implicating purinergic signaling and subcellular localization of mitochondria in the collective migration of the mesendoderm. Although there is still much work that needs to be done to characterize the function of the mitochondrion in the

mesendoderm, my data suggest that localized ATP production and signaling is an unappreciated contributor to this morphogenetic process.

Conclusions

Normal developmental processes are adapted and misregulated in different diseases and during wound healing. By investigating morphogenesis in the context of development we can gain a better understanding of diseases that rely on these same processes, such as cancer and fibrosis. The work in this thesis focuses on two different mechanisms of morphogenetic regulation. My work has identified the cadherin-binding protein, PG, as a novel regulator of FN assembly. Without PG, cells exhibit decreased attachment strengths to FN substrates, and fibrillogenesis is perturbed. The resulting embryos are defective in radial intercalation and convergent extension. Both of these processes require the application of force from a cell onto its neighbors as well as its ECM substrate. We have found that in the mesendoderm, these mechanical forces applied to cadherin or integrin adhesions lead to conformational changes in mitochondrial enzymes. The punctate mitochondria in these cells, which are active and enriched in protrusions, are likely participating in purinergic signaling. These findings suggest that the mitochondrion and purinergic signaling, which are involved in single cell chemotaxis, also play an important role in collectively migrating tissues. Together, the data presented in this thesis help uncover new regulators of morphogenesis. My data provide insight into

how these movements are coordinated and how a physical force applied to adhesive complexes can affect cell behavior.

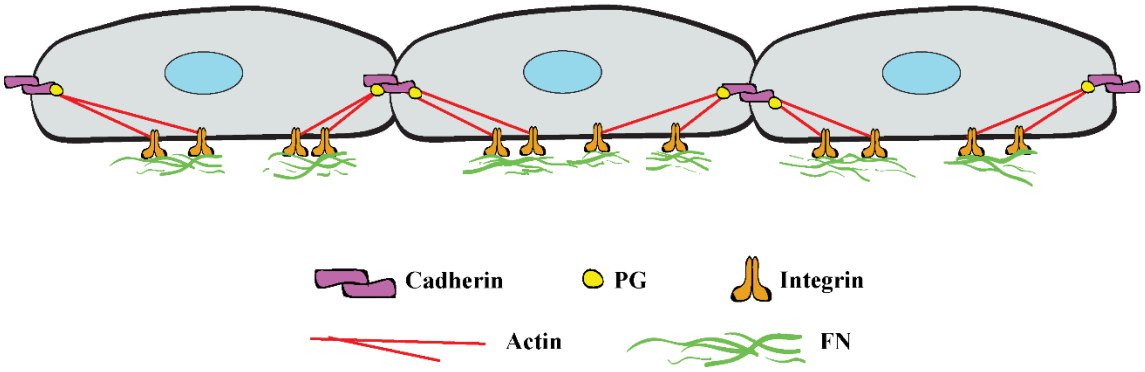

Cadherin
PG
Integrin
Actin
FN

Figure 4.1: Mechanical coupling of AJs and FAs.

Cells in a tissue form AJs where actin stress fibers (red) are anchored to the cytoplasmic tails of cadherin molecules (purple) through PG (yellow). Stress fibers are also attached to integrin receptors (orange), which assemble FN fibrils (green).

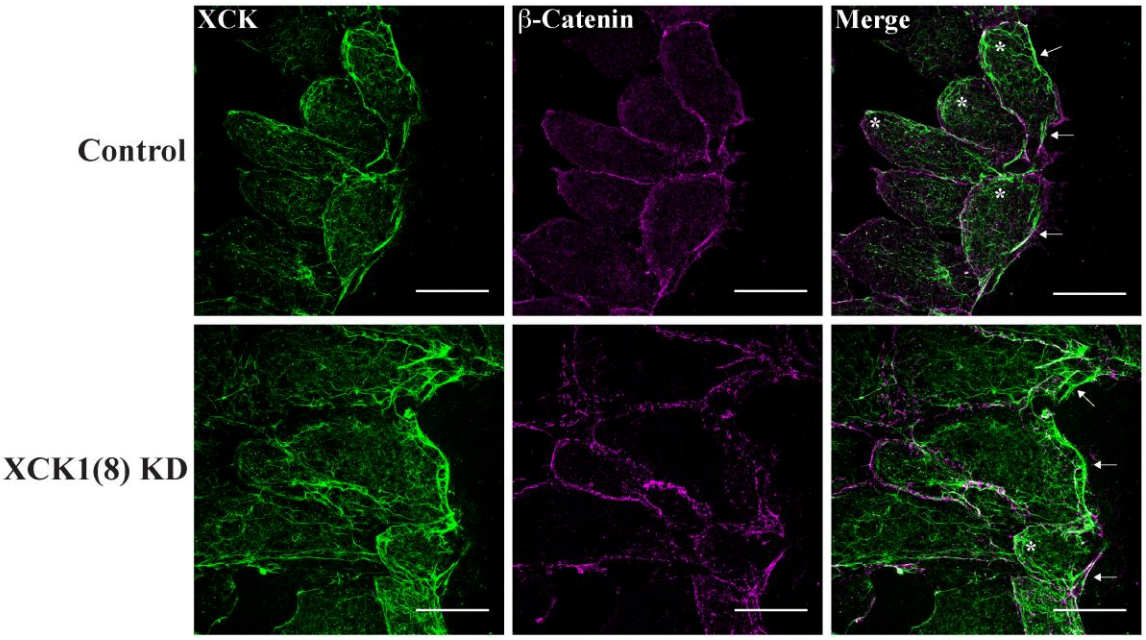

XCK
β-Catenin
Merge
Control
XCK1(8) KD

Figure 4.2: A reduction in XCK1(8) only affects a subset of keratin IFs.

Control (top) and XCK1(8) morphant (bottom) DMZ explants immunostained for β-catenin (purple) and keratin (green) using a pan-cytokeratin antibody (C11). Keratin cables at the leading edge are marked by arrows. Asterisks denote keratin baskets are the rear of leading edge cells. Scale bars are 50 μm. Images courtesy of Pooja Sonavane.

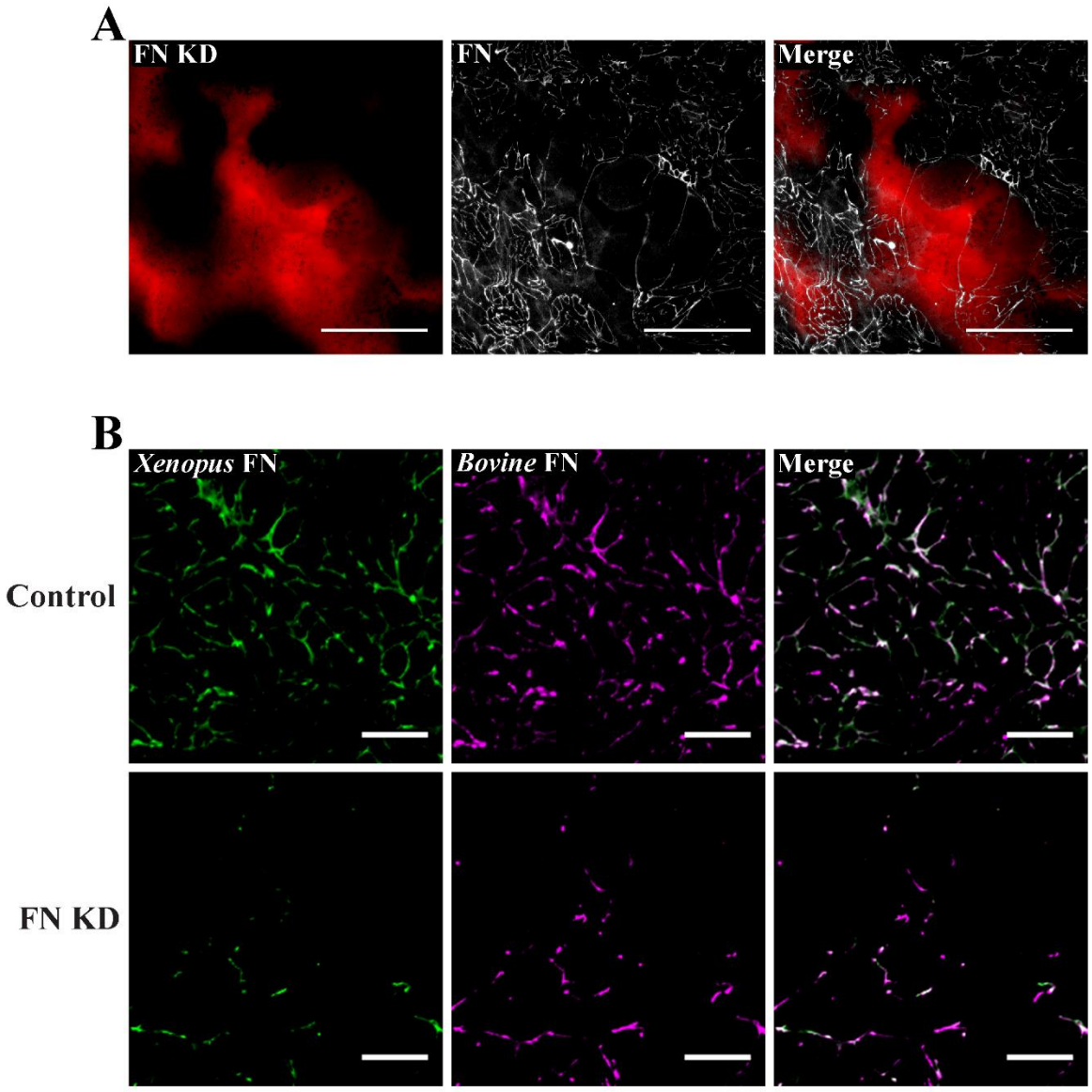

A
FN KD
FN
Merge
B
Xenopus FN
Bovine FN
Merge
Control
FN KD

Figure 4.3: FN morphant cells do not assemble extracellular FN.

(A) Embryos were coinjected with a fluorescent dextran (red) and FN morpholino into 1 of 32 blastomeres at the animal cap. Embryos were fixed and immunostained for FN (white) at stage 11 (midway through gastrulation). (B) Morphant embryos in which FN was knocked down in the whole embryo following fertilization. At the MBT, *Bovine* FN (magenta) protein was injected into the blastocoel cavity. At stage 11, embryos were fixed and stained using a *Xenopus*-specific FN antibody (green). Scale bars are 50 μm in (A) and 15 μm in (B).

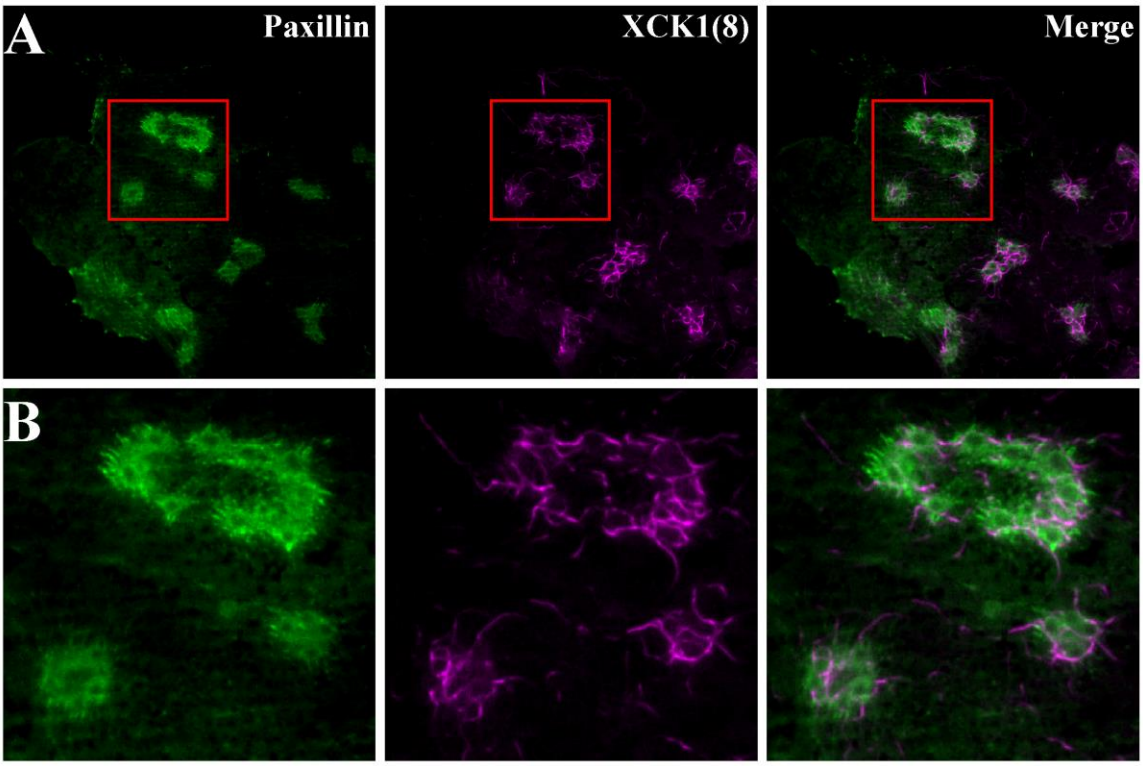

A
Paxillin
XCK1(8)
Merge
B

Figure 4.4: Invadopodia-like structures in animal cap cells.

Live TIRF microscopy images of animal cap cells expressing GFP-paxillin (green) and mCherry-XCK1(8) (magenta). Red outlines in (A) mark regions that were cropped and magnified in (B). Microscopy performed by Pooja Sonavane.

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