Chp 4 Discussion

Torey

(Wang, Bosveld, and Bellaiche 2018; Daniel et al. 2018)

(Hartsock and Nelson 2008)

(Grashoff et al. 2010; Kim et al. 2015; Borghi et al. 2012)

(Tepass and Hartenstein 1994)

(Jameson et al. 2013)

(Gardel 2004; Zimmermann et al. 2017)

# Adherens and Tight Junctions: Structure, Function and Connections to the Actin Cytoskeleton

 In this dissertation, I have shown that epithelial cells remain connected to one another, maintain the barrier function of the tissue, and reinforce their adherens junctions in response to tension generated by the contractile ring. I have also demonstrated that Anillin regulates apical cell mechanics by organizing the medial-apical contractile network. Together these data demonstrate that epithelia are mechanical sensors and single proteins can have dramatic mechanical impacts at the cellular level that propagate to the tissue level.  While the work presented here answered several question about how epithelial cells regulate and respond to mechanical inputs in order to build and maintain a functional tissue, many spokes stemmed form this work that lead to interesting questions. In the following sections of “Epithelial cytokinesis” and  “Anillin’s role in epithelial mechanics” I explore these questions, put them in the context of the field, and describe how I would test them.

**Epithelial cytokinesis**

**Why don’t tight junctions feel the force?**

A relatively unanswered question in epithelial biology is how/do epithelia maintain their barrier when cells within the tissue are undergoing the dramatic shape changes and junction rearrangements that happen during cytokinesis. This is especially baffling in epithelial with rapid cell division, such as the small intestine which is turning over all of its epithelial cells in just a few days. This leads to the question, are forces from cell division linked to tight junctions and are those forces disrupting, maintaining, or not impacting barrier function? Even though tight junctions are stabilized in a tension sensitive manner (Higashi et al. 2016; Yu et al. 2010) I showed that tight junction components are not stabilized by forces from the contractile ring **(Fig. 2.3,4)**. This implicates adherens junctions as the major load bearing junction during cell division while the tight junction appears to be along for the ride. Tight junctions are, however, connected to the circumferential F-actin associated with junctions and tight junctions are mechanically sensitive and regulate epithelial mechanics (Fanning, Van, and Anderson 2012; Choi et al. 2016; Hatte, Prigent, and Tassan 2018; Spadaro et al. 2017). So why then are tight junctions uncoupled from the forces driving cytokinesis? Are they uncoupled because forces from cytokinesis would disrupt barrier function? It would be interesting to know if uncoupling tight junctions from tension is common among other events that drastically increase apical tension, such as apical constriction during gastrulation and neurulation or cellular rearrangements during convergent extension. While it is known that Myosin II driven contractility in the circumferential actin network attached to tight junctions is an important regulator of barrier function (Arnold, Stephenson, and Miller 2017) inhibiting Myosin II activity increases the permeability of tight junctions (Ivanov et al. 2007; Ivanov et al. 2004) while in other cases loss of Myosin II activity decreases permeability (Yu et al. 2010). Similar contradicting evidence can be found for increased apical tension where activation of Myosin II has no effect on barrier function in human epithelial colorectal adenocarcinoma cells (Acharya et al. 2018) while in endothelial cells it strengthens barrier function (Dudek et al. 2004). It is clear that Myosin activity is a regulator of barrier function, however, it is unclear, how elevated levels of acute tension affect barrier function.

Since much of work on barrier function in relation to forces has been done in cultured cells it would be informative to first test how barrier function is affect by forces in an intact animal such as *Xenopus*. To test how actomyosin forces affect barrier functions manipulations such as Calyculin A, or mosaic Anillin overexpression to increase tension, and drugs such as Y-27632, and blebbistatin to decrease tension could be used in conjunction a barrier assay more sensitive that the tracer dye experiment in **(Fig. 2.1).**A former postdoc in the lab developed a Zinc-based Ultrasensitve Microscopic Barrier Assay (ZnUMBA) that would perfectly serve this role (Tomohito Higashi, unpublished). After determining how actomyosin forces affect barrier breaches on the cellular scale using ZnUMBA it would be informative to look more closely at the epithelial barrier during cell division**.**It is possible that there are many small breaches around the dividing cells that are undetected via the tracer dye method used in **(Fig. 2.1)**, flares of RhoA activity, which are sites of barrier leaks, are increased around dividing cells (Rachel Stephenson, Torey Arnold, & Farah Huq, unpublished). Specifically there may be an increased number of breaches at the cleavage furrow where the force regimes are drastically changing along with the shapes of the junction and the membrane.  Additionally, using chimeric proteins would be informative about how linking forces from cytokinesis to the contractile ring would affect barrier function. For example, by making a chimerical proteins of ZO-1 and α-catenin or ZO-1 and Anillin and to see how directly connecting tight junctions to the forces from the contractile ring affects barrier function.  To summarize there is very little consensus on how tensile forces affect tight junction barrier function and even less known about how large acuter tensile forces such as those genereated during cytokinesis affect barrier functions making this an interesting question to pursue.

**Adherens junction reinforcement during cytokines**

Adherens junctions maintain epithelial integrity by mechanically linking cells together which is required for tight junction formation and barrier establishment. Through my research I showed that adherens junctions are reinforced viz Vinculin recruitment during cytokinesis **(Fig. 2.5)**. Vinculin reinforcement at the cleavage furrow was simultaneously expected and surprising. It was expected because adherens junction are well known to be mechanically sensitive and recruit Vinculin to their junctions under mechanical stress (Yonemura et al. 2010; Yao et al. 2014; Kim et al. 2015). Surprising because recent studies investigating cytokinesis in *Drosophila* showed a weakening or break in adherens junctions at the cleavage furrow (Herszterg et al. 2013; Guillot and Lecuit 2013; Founounou, Loyer, and Le 2013). Why then are vertebrates reinforcing their junctions at the cleavage furrow while invertebrates are disrupting theirs?  One possibility is the requirement and maintenance of barrier function in the embryo.  The studies in *Drosophila*were carrier out in stage 8-10 embryos which don’t form an epithelial barrier, in contrast, the experiments we conducted were in stage 10.5 *Xenopus*which form a complete tight junction seal.  In Drosophila stage 8-10 embryos, their is no requirement to maintain adhesion to preserve barrier function so adherens junctions can decouple in *Drosophila* without much consequence. *Xenopus*may require a strong barrier at stage 10.5 in order to develope properly, however, this had not been directly tested.  It would be interesting to know if *Drosophila* still disengage their adherens junctions later in development when they from epithelial barriers.

The work I presented in Chapter 2 showed forces from cytokinesis recruit Vinculin to adherens junctions and this in turn stabilizes junction components and reinforces the connection between the actin cytoskeleton and the junction, however, we have less evidence about why this is happening and if it is required for successful cytokinesis or maintaining the barrier functions during cell division. In Xenopus we had difficulty depleting Vinculin, I think Vinculin depletion will be a critical step towards understanding Vinculins role in cytokinesis. While our dominate negative use of Vinculin-D1 increased the speed of contractile ring closure and decreased E-cadherin and ZO-1 intensity at the cleavage furrow, which could increased barrier permeability, we saw no dramatic defects in cytokinesis using this perturbation **(Fig. 2.6)**. Moving to an epithelial system where we could more readily deplete Vinculin, such as culture epithelial cells could be informative about the role Vinculin reinforcement is actually playing. Staying is Xenopus we could use a combination approach, for example, using Vinculin-D1 in conjunction with a different Vinculin morpholinos than the one we previously experimented with. Another possibility would be to try knocking down α-catenin and replacing with a mutant α-catenin that cannot bind to Vinculin.  Together these experiments will be informative about the function of adherens junction reinforcement during cell division.

**Planar symmetry of junction reinforcement during epithelial cell division**

With the asymmetric nature of cell division in a tissue, where one cell is dividing and the surrounding cells are not, it brings many questions to mind about how are the neighboring cells are responding, adapting, and maybe even contributing to cell division. For example, are neighboring cells making themselves more compliant to allow cell division to occur successfully?  In order to understand cell division in a tissue it is equally important to understand the mechanics of the dividing cell and the neighboring cells. In Chapter 2 work performed by Tomohito Higashi revealed that Vinculin accumulates in the neighboring cells when the dividing cell expresses Vinculin-D1 and in the dividing cell when neighbors express Vinculin-D1. These experiments revealed that Vinculin is potentially accumulating in both dividing and neighboring cells, however, it is possible that perturbing with D1 expression is stimulating accumulation. It would be useful to use an unperturbed method such as mosica expression of green Vinculin and red Vinculin to see if both dividing and non dividing cells reinforce their junctions. If this is true, it would mean that pulling strain is being applied to both the diving cell and the neighboring cell. This makes sense for the dividing cell it’s adherens junctions are directly coupled to the contractile ring, however,  in the neighboring cell things are less clear. Is the circumferential actomyosin network in the neighboring cell providing a passive resistance and is this enough to trigger the strain across α-catenin or is their an active contractile response in the network? In either case, why does the neighboring cell need to build a strengthen adherens junctions at all? It is possible that i does not serve a function and is just a consequence of the properties of the proteins involved in the response. The resistive force of junctional interfaces in *Drosphila* has been measured to be on the 100 pN scale (Bambardekar et al. 2015) while forces generated from the contractile ring in the first embryonic division have been measured to be in the 10 nN range (Rappaport 1967; Miyoshi et al. 2006). The amount of force required to induce the conformational change in α-catenin is only 5 pN (Yao et al. 2014) so it seems likely that reinforcement not only occur on the dividing side but also the neighboring cell as a results of the restive force of the junction interface. One consequence of Vinculin recruitment is the the recruitment of the actin polymerizer Ena/VASP which binds directly to Vinculin (Leerberg et al. 2014). This raises the interesting possibility that increased actin polymerization is required at the cleavage furrow, possibly in both the dividing and neighboring cells. It would be interesting to perturb this function of Vinculin and observe the effects it has on actin and the barrier at the cleavage furrow. Our findings that Vinculin is recruit to cleavage furrow has generated many question that still need to be answered and further research exploring Vinculin’s role could lead to exciting discoveries about the mechanism of epithelial cytokinesis.

**Understanding the diversity of cytokinesis**

Studies in *Xenopus* and *Drosophila* have revealed that cells use dramatically different solutions to cell division in epithelial tissues **(Fig. 2.7 )**(Higashi et al. 2016; Herszterg et al. 2013; Guillot and Lecuit 2013; Founounou, Loyer, and Le 2013). Personally this fascinates me, and I want to know all of the different types of cell division that can occur! Looking at cell division at other stages and in other tissues of Xenopus development, and other organisms is likely to find exciting results. This may not seem apparently useful, however, I would argue that it is worth pursuing. The researchers exploring cytokinesis in Drosophila discovered something unexpected that shifted the paradigm of cell division, that cell division in a tissue can be a multicellular process. In the *Drosophila* tissues explored it is not just the dividing cell that contributes to cell division but the neighboring cells actively contribute as well. As the cleavage furrow ingresses the neighbors being pulled in accumulate Myosin II perpendicular to the axis of division in order to bring the furrowing interfaces close to one another, then the dividing cells polymerizes branched F-actin to push the neighboring cells membrane out of the furrow and adherens junctions are formed between the newly formed daughter cells (Herszterg, Pinheiro, and Bellaïche 2014). Investigations of cell division in other types of tissues could reveal equally or even more fascinating qualities, for examples cell division which isn’t driven at all by the dividing cell or cell division in a tissue that doesn’t require actomyosin. In less drastic examples, I think it is important to understand how cells undergo cytokinesis in different environments, for example in a highly proliferative intestinal vs. the less proliferative skin epithelium. There are also drastic differences in cell shapes of epithelial cells, columnar, cuboidal, squamous, how are these affecting the mechanics of cytokinesis in epithelial cells? I think a simple survey of cell division in several tissues in Xenopus and others, just observing actin and Myosin II would reveal interesting types of cytokinesis to further investigate.

**How does epithelial stiffness affect cytokinesis?**

It is well known that stiffness of the extracellular matrix increases proliferation and dedifferentiation of cells and this has major impacts on the disease progression of cancers (Handorf et al. 2015).  Much less is known about how the stiffness of surrounding cells rather than the extracellular matrix affects cell division. Even though tumors as a whole  tend to be more stiff than surrounding tissues the tumorgenic cells themselves tend to be less stiff than surrounding cells. This opens interesting questions about where in tumors cells are proliferating, is it at sites of cell-matrix adhesion instead of cell-cell adhesion? It would be interesting to test how surrounding cell stiffness affects cytokinesis. One possible method for perturbing cell stiffness comes from Chapter 3 where the scaffolding protein Anillin can modulate tissue stiffness. Mosaic depletion or over expression of Anillin could be used to perturb the stiffness of a population of cells in a tissue and cytokinesis phenotypes could be observed in cells next to the perturbed areas. *Xenopus* is also an ideal system to study the effects of cancer cell stiffness on cell division. Xenopus tadpoles are clear, making them amenable to imaging, and can be induced to generate tumors (Dahmane et al. 1997; Yang et al. 1998; Wallingford et al. 1997) or mammalian tumors can be seeded and grown in tadpoles (Haynes-Gimore et al. 2015). Understanding how cell division is regulated by the mechanical environment imposed by the surround cells will be useful for our understanding of disease progression and embryonic development.

**Anillin’s role in epithelial mechanics**

**What is Anillin’s role in cancer cells and tumor progression?**

Tumors are mechanical sensors and as the extracellular matrix of a tumor becomes more stiff the tumor cells sense this and become more invasive and metastatic (Reid and Zanivan 2017). In Chapter 3, I showed that Anillin organizes medial-apical actomyosin into a contractile unit and Anillin overexpression results in structural and mechanical consequence for cells that are similar to treatment with the actin stabilizing toxin jasplakinolide **(Fig. 3.1-6)**.Finally, I showed that Anillin depletion reduces the stiffness of tissues **(Fig. 3.7)**. Our result may shed light on how Anillin is affecting cancer progression making this topic of high interest to pursue further. Anillin is well known to be expressed in many human tumors (Hall et al. 2005) and correlating with poor survival rate (Hall et al. 2005; Suzuki et al. 2005; Wang et al. 2016; Idichi et al. 2017; Zhang et al. 2018). However, work examining the sub cellular localization of Anillin found that *nuclear*localization of Anillin correlates with poor survival rates, whereas *cytoplasmic* localization of Anillin is a marker of favorable prognosis, suggesting that Anillin’s localization rather than its expression level is key (Ronkainen et al. 2011; Liang et al. 2015). This brings forth several possibilities, one is that stiffening of cells we observed when Anillin was overexpressed is acting as a protective mechanism, preventing cells from becoming metastatic. This is plausible cancer cells become less stiff as they become metastatic (Swaminathan et al. 2011; Guo et al. 2014). Anillin also promotes normal adhesion between cells (Reyes et al. 2014; Wang et al. 2015)  and apical actomyosin contractility which has been shown to protect junctions from disassembly (Weng and Wieschaus 2016) again hinting that Anillin may function to mechanical maintain cells in a non metastatic or invasive state. In opposition to this idea, in cells potentiated for migration such as neurons and podocytes, Anillin actually promotes migration by organizing, protecting, and scaffolding F-actin at the leading edge (Tian et al. 2015; Gbadegesin et al. 2014). One possible reconciliation is that Anillin helps maintain an epithelial cell state by maintaining stiffness and adhesion, but in migratory cells Anillin is relocalized to promote migration. It would be interesting to test this, possibly by overexpressing Anillin and HIF-1a, which induces metastasis (Yang et al. 2008), and observing if Anillin can prevent epithelial cells from transitioning to motile mesenchymal cells. In contrast would cells expressing HIF-1a and with Anillin knocked down undergo the epithelial to mesenchymal transition but become less mobile compared to cells with normal levels of Anillin?

Another possible role for Anillin in cancer is the role that Anillin may perform in the nucleus.  There role of cytoskeletal proteins in the nucleus is an under studied area that still requires much scientific attack. Even actin, one of the most studied skeletal components which was first confirmed to be in the nucleus in the 70s (Clark and Merriam 1977), still has many unanswered questions about what functions it plays in the nucleus. That said it is clear that actin both in globular form and filamentous form has many functions in the nucleus and is more than just a “molecular wanderer” as was first posited (Pederson and Aebi 2002). Since then it has been found that actin helps shape the nucleus and reshape chromatin after mitotic exit (Baarlink et al. 2017; Moore and Vartiainen 2017). Actin even functions to regulate transcription, mRNA processing, and organization of the genome  (Visa and Percipalle 2010).  This likely means that actin associated proteins, such as Anililn, are also performing functions in there roles and high levels of Anillin in the nucleus may be potentiating metastatic through nuclear functions. Since Anillin is overexpressed in so many human tumors (Hall et al. 2005) and can have both negative or positive out comes for the patient it makes Anillin a enticing area to study in cancer biology as it may be a possible target to prevent tumors from entering a metastatic state.

**Is Anillin important for developmental events that require apical constriction?**

Developmental process such as, gastrulation, neurulation, and eye and gland formation require apical constriction (Sawyer et al. 2010). Apical constriction is a well conserved cellular shape change used to induce tissue folding in in urchin, worms, flies, frogs, chicks, and mice (Kimberly and Hardin 1998; Nance and Priess 2002; Young, Pesacreta, and Kiehart 1991; Keller 1981; Schoenwolf and Smith 1990; Sadler et al. 1982). In *Xenopus* gastrulation, a small population of cells on the bottom or vegetal side of the embryo undergo apical constriction to invaginate the tissue which initiates the formation of the primary tissue layers (Hardin and Keller 1988). This process involves actomyosin accumulation at the apical surface to drive constriction, however, in *Xenopus*it is unknown if the accumulation of actomyosin is medial-apical or junctional (Lee and Harland 2007). Later in *Xenopus* development the actin binding protein Shroom 3 induces apical constriction to form the neural tube (Haigo et al. 2003; Nishimura and Takeichi 2008). Interestingly even though there is clear medial-apical accumulation in Shroom 3 expressing cells the researchers at the time did not denote this as a the mechanism driving of apical constriction and instead attributed it to junctional actomyosin (Haigo et al. 2003). In *Drosohpila*, gastrulation occurs as a ventral furrow invaginates and this was the first time medial-apical actomyosin accumulation was shown to drive apical constriction (Martin, Kaschube, and Wieschaus 2009). Since then medial-apical actomyosin has also been found to drive apical constriction during lens pit formation (Plageman et al. 2011) and is likely to drive apical constriction in mouse neural tube formation as Shroom 3 is required there as well (Hildebrand and Soriano 1999).

With the diversity of morphological events associated with medial-apical actomyosin driven apical constriction and our findings showing Anillin affect media-apical contractility **(Fig. 3.3 & S3.2)** makes exploring Anillin’s role in development enticing.  Unpublished data from our lab suggest that Anillin functions during development. When Anillin is knocked down gastrulation is delayed and some embryos fail to gastrulate and and die soon after (Torey Arnold, unpublished). Xenopus is an ideal system for exploring  Anillin’s role in apical constriction during development as experiments can be performed in intact embryos or explants of embryonic tissue which still undergo morphogenetic events (Zhou, Kim, and Davidson 2009; Keller, Shih, and Sater 1992; Lee and Harland 2007). One hurdle with studying Anillin in morphogenic event’s is that Anillin participates in cytokinesis, cell-cell junction structure, and medial-apical contractility. Therefore’ regulating the amount of knockdown is critical but even then it is difficult to determine if the gastrulation defects we have observed are from failed cytokinesis, junctional, or medial-apical contractility defects. Explanting tissues would make direct imaging of bottle cell apical constriction and neural plate apical constriction easier and more consistent than whole embryo experiments, and to my knowledge this would allow the first en face images of bottles cells to be captured. This would allow the apical constriction to be tracked over time with Anillin perturbations looking at a variety of probes.

In *Xenopus* after apical constriction of the bottle cells the tissue migrates into the embryo and forms several cavities, including the archenteron and the blastocoel.  The tissue migration and elongation is drivine by actomyosin processes in mesnchymal cells that rearrange and elongate cells (Keller et al. 1985; Shindo 2018). When Anillin is knocked down these cavities are still present, however, when Anillin is overexpressed these cavities appear to be absent and are instead full of cells (Torey Arnold, unpublished). The exact mechanism that leads to the loss of the cavities is unknown, but could be a results of over elongations of the tissue, or increases migrations of meshncymal cells that break away from the tissue and proliferate in the cavities. Determining the origin of filling the embryo and a robust characterization of the mechanisms behind the defect could tie back into and provide insights into Anillin role in cancer and metastasis. As a whole Anillin’s role in development is uncharacterized and is likely a fruitful area of study given Anillin’s direct role in cellular events that are critical for development.

**How does Anillin organize medial-apical F-actin into a contractile unit?**

The first study that identified Anillin determined that a small region near the N-terminus of the protein can bind and bundle F-actin *in vitro (Field 1995)*. Recently using electron microscopy researchers found that Anillin’s F-actin binding domain has three separate actin binding regions (Jananji et al. 2017). When mutations were made and only one F-actin binding site was present the fragment bound F-actin, when two binding sites were present the fragment bundled F-acitn into two dimension sheets, and when all three binding sites were present the fragment bundled F-actin into three dimensional structures (Jananji et al. 2017).  Knowing that in vitro the F-actin binding domain alone can bundel F-actin (Field 1995; Kinoshita et al. 2002; Jananji et al. 2017), we hypothesized that the F-actin binding domain of Anillin was required to structure medial-apical F-actin. This prediction was correct **(Fig.3.5)**, however, it was unexpected that *in vivo* all threeC-terminal domains were also *required* for Anilliln to organize F-actin in bundles **(Fig 3.5)**. *In vivo* all of the C-terminal mutants localize fairly well to medial apical surface and they all contain the actin binding domain, so why aren’t these mutants bundling F-actin? The Rho binding domain, Calcium2 (C2) domain, and the, Pleckstrin Homology (PH) domain all help anchor Anillin to the membrane and they also impact Anillin’s ability to regulate active RhoA. The Rho binding domain directly binds active RhoA, which helps link Anillin to the membrane, and when Anillin binds to RhoA it is proposed that Anillin resets the timer for RhoA inactivation allowing it to remain active longer (Budnar et al. 2018; Piekny and Glotzer 2008; Sun et al. 2015). Anillin’s C2 domain binds to the membrane and to the negative regulator of RhoA, MgcRacGAP and the positive regulator, Ect2 (Gregory et al. 2008; Frenette et al. 2012). The PH domain binds to the membrane, septins, and the negative regulator of RhoA, P190RhoGAP-A (Manukyan et al. 2015). It is possible that membrane binding is important for F-actin bundling, however, since the C-term mutants localized properly it is not readily apparent how this would be the case. It seemsmore likely that RhoA activity is critical for Anillin to bundle and organize F-actin *in vivo*.

The *in vitro* studies that showed Anillin’s bundling capabilities used stabilized F-actin filaments (phalloidin/high salt concentration).(Field 1995; Sun et al. 2015; Kinoshita et al. 2002). *In vivo*, medial-apical F-actin is very dynamic with a t1/2 on the order of 10s **(Fig. 3.6)**. While we showed Anillin overexpression stabilizes F-actin **(Fig. 3.6)**, likely through protecting the actin filaments from Cofililn severing (Tian et al. 2015) this level of F-actin stabilization does not seem to be enough, as overexpression of C-terminal mutants could not bundle F-actin. I hypothesize that increased polymerization of F-actin through RhoA activation is required to bundle and organize medial-apical F-actin *in vivo*.  To test this idea, the technique of preparing actin-intact *Xenopus* egg extracts on a substrate supported lipid bilayer being optimized in our lab by Jennifer Landino could be used (Field, Pelletier, and Mitchison 2017).  With this method, a lipid bilayer is seeded on a cover slip and *Xenopus*egg extracts are added and a cortex of actin forms on the lipid bilayer (Field, Pelletier, and Mitchison 2017). To perturb the system purified proteins can be added for overexpression phenotype, antibodies can be used to deplete proteins, and various inhibitors can be directly added to the extract.  Purifying Anillin for creating overexpression phenotype will be hurdle for these experiments but would be useful here as well as other *in vtiro*experiments. For example, there is evidence that suggest Anililn is a tension sensitive protein like α-catenin (Manukyan et al. 2015) having purified Anillin would allow direct *in vitro* measurements that Anillin undergoes a mechanically induced conformational change (Buckley et al. 2014). This would pair nicely with a fret based in vivo experiments showing a loss of FRET when Anillin is under mechanical stress. Once set up the actin network can be visualized with Total Internal Reflection Fluoresence (TRIF), or confocal microscopy of F-actin probes, such as purified mCherry-Utrophin, which the lab already has, and perturbed through addition of Anillin, inhibition of RhoA, formins, and or depletion of RhoA regulators such as Ect2.  This would provide a more direct and easily perturbable system to determine the mechanism through which Anillin promotes a organized and contractile actin network.

While it is known that Anillin is involved in many different aspects of a contractile network such as, membrane anchoring, RhoA regulation, and actin and myosin biding, it is not know how all of these function culminate to establish a functional contractile network. A pure *in vitro* system where purified components are added such as F-actin, Anillin, and Myosin II could shed light onto the mechanism(s) through which Anillin builds a contractile network. For example, the interactions between Anillin and F-actin is fairly low with a kd in the 4 uM range (Jananji et al. 2017).  This may be for several reason, first Anillin can bind to Myosin II and formins, both of which are “processive” where Myosin II walks along actin filaments and formins polymerize F-actin at the plus end of actin filaments, so if the formin is anchored in place the actin filaments will grow out from the filament,  if the formin is free to move the formin will have a velocity associated with the growing end of the actin filament (Romero et al. 2004).  Anillin binds to both of these processive proteins, so one possiblity is that Anillin has multiple weak interactions with F-actin to allow the filaments to “slide” through Anillin. This would allow Anillin to ride along with a formin at the growing end of polymerizing actin filament or allow the filaments to slide trhough Anillin as Myosin II motors act on filaments. To test this hypothesis purfied tagged version of Anillin, Myosin II, and mDia2 (formin) could be viewed in the presence of stabilized actin filaments and TIRF microscopy could be performed to determine if Anillin is stationary when bound to F-actin or is processive via untethered formins/Myosin II.  Formins and and Myosin II could also be tethered to beads or the coverslip, and if for example Anillin does not allow sliding of actin filaments then high levels of Anillin should inhbit formin polymerization of F-actin or Myosin II induced sliding of actin filaments.

Finally, many of Anillin’s interaction and functions have been mapped therefore modeling of Anillin’s role in organizing a contractile actomyosin network could be done in conjunction with *in vitro* experiments. This would allow confirmation of *in vitro* results and may provide unexpecte insights for futuer experiments as variables and parameters can be adjusted more readily.  This could be particularly useful for guiging experimetns to teas apart Anillin role in regulting junctional vs. medial-apical F-actin.  While there is a great deal is known about *what* Anillin does in a contractile network, there is very little known about *how* Anillin actually does any of it. This multi pronged approach of *in vitro* , *in silco* and additional *in vivo*will helptotease apart the molecular mechanisms of *how*Anillin organizes and orchestrates F-actin, Myosin II, formins, and RhoA activity flux into a contractile network.

**What is the interplay between Anillin’s role in regulating junctional and medial-apical actomyosin?**

The apical surface of cells is organized into two contractile units junctional and medial-apical. These two distinct, and yet similar contractile networks can function to perform the exact same task such as embryonic wound healing, or distinct functions like how junctional actomyosin mechanically coupling cells together during cytokinesis, or how medial-apical actomyosin induces apical constriction during ventral furrow formation in *Drosophilia*. While it is clear that forces from medial-apical contractility are directed upon junctional actomyosin and transmitted to neighboring cells, it is less well understood how junctional actomyosin impacts medial-apical actomyosin. Specially it difficult to attribute Anillin’s role in regulating cellular mechanics at the apical surface of cells to one of the populations of actomyosin because Anillin is localized to both. For example, when Anillin is knockdown we see reduced levels of junction proteins, indicating possible junction dissaebmly (Reyes et al. 2014).  It is known that Myosin II dependent contractility prevents junction disassembly (Weng and Wieschaus 2016) but in the case of Anillin knock down is this disassembly caused by lack of forces generating by junctional actomyosin, the loss of medial-apical forces applied to junctions, or something else? This is a difficult question to tease apart and would require knockdown of Anillin and rescue with Anillin constructs that could localize separately to junctions or the medial-apical surface which could be accomplished by fusing Anillin to a junctional protein or a protein that only localizes to the medial-apical surface such as Endolyn (Ihrke et al. 2001; Hildebrand 2005). Possible issues might arise from this as Anillin might still target the chimeric proteins to both locations. A method independent of Anillin to tease apart how medial-apical forces affect junction maintenance would be to laser ablate medial-apical actomyosin and observe the affects on cell-cell junctions. Low levels of the actin deploymerizing drug latrunculin B could also be used as I have observed actin polymerization seems to occur first across the medial-apical surface, then junctionally at fairly high concentrations (25 µM) of Latrunculin B (Torey Arnold, unpublished results). A lower concentration of latrunculin B in the nano molar range might disrupts medal-apical F-actin while preserving the junctional network.

In addition to the force interplay, another area of interest to explore is the interplay of signaling and protein components between junctional and medial-apical actomyosin . For example, my data exploring apical contractility after the addition of ATP demonstrated that medial-apical accumulation of F-actin occurs first near cell-cell junctions and then sweeps across the apical surface of the cell **(Fig. 3.3)**. Additional, when Shroom 3 was overexpressed to induce apical constriction Anillin accumulated first near cell-cell junctions and then propagated across the apical surface of the cell **(Fig. S3.2)**. These data position junctions as potential signaling centers or protein stores for medial-apical contractility. In the case of ATP addition it is possible that the P2Y receptors that bind ATP and induce the signaling cascade are accumulated near junctions. Immunostaining or live imaging of taggged P2Y receptors could determine if this is true, the mislocalization of the receptor across the apical surface instead of at junctions could determine if junctional localization is the mechanism for propagation from junctional to medial-apical . Another possibility is that the large accumulation of active RhoA at cell-cell junctions is what initiates this propagation so even if the P2Y receptor is mislocalized the accumulation of F-actin will still sweep out from junctions to the medial-apical surface.  To test if protein populations are shared between junction and medial-apical, photoactivatable or photoswitchable tagged proteins could be used.  For example, Anillin tagged with a photo-switchable fluorophore could be activated at junctions, or in the cytoplasm and the medial-apical intensity of red to green florescence could be measured.  This would provide evidence as to the origin of the newly accumulating proteins across the apical surface. If for example the cytoplasmic pool was switched to red while the junctional population remained green and the accumulation population was of high red intensity, this would support the idea that the newly accumulated medial-apical Anillin is mostly from the cytoplasm. This would demonstrat that the observed propagation of Anillin from junctions to the medial-apical is not a result of protein spreading but likely through the propagation of a recruitment signal that spreads from junctions across the medial-apical surface. These experiments would be exciting because to my knowledge there is little evidence about how junctional and medial-apical networks communicate with one another to build a functional apical contractile network even though the two structures are so closely related and mechanically integrated with one another.

**Closing thoughts**

Being a part of the Miller lab for the past 5 years has given me the opportunity to expand our knowledge about how epithelial cells respond to and organize mechanical forces.  With my work and the work of others in the lab we have shown that epithelial cells reinforce their junctions in response to the forces generated by the contractile ring that pinches the cell in two during cell division. I think the most important questions to pursue next are why are these force only transmitted to adherens junction and not the barrier producing tight junctions and ultimately what is consequence to developmental and barrier functions if adherens junctions are not reinforced during cell division. My main project focusing on Anillin regulating forces across the apical surface of cells only started to come together in the past 2 years of my graduate career and the driving force behind it was the surprising and peculiar results of the laser ablation data. I feel extremely lucky to have such a “well that’s funny” moment fall into path during graduate school which motivated me to keep an open mind and explore what Anillin was really doing across the apical surface. This open mindset and curiosity allowed me to characterize a new role for Anillin in regulating medial-apical contractility. So thanks for leading me to a cool and interesting result Anillin. \*Fist bump\*. Anillin is a Swiss Army Knife of a protein, with so many protein interactions and cellular functions it makes it difficult to tease apart it’s functions and mechanism with acute accuracy and there are many future paths I would take in exploring Anillin’s function in regulating epithelial mechanics. However, the one I am most curious about is how my findings can tie into and explain the mechanisms for Anillin’s role in cancer progression.  This is saying a lot coming from me as I prefer basic science and don’t really have a particular interest in studying disease mechanism, but I guess I just like conflicting results, as it is really what drove this project, and I want to know why Anillin can both promote and inhibit cancer progression. Uncovering this conundrum would also be a noble path of research as Anillin is overexpressed in many tumors and could be a potential target for cancer treatment. There is still so much to be learned about how cells respond to and generate mechanical cues to establish a function epithelial sheet. I hope the data presented here have added useful pieces to the puzzle for future researchers to build off of so eventually we can unlock the mysteries of the mechanical information stored within these cells.

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