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Publication Date

2017

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Mechanisms of 3D Organ Elongation

By

Dong-Yuan Chen

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Molecular and Cell Biology

in the

Graduate Division

of the

University of California, Berkeley

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Fall 2017

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by

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Abstract

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Elongation of a tissue along one body axis is a conserved morphogenetic process that happens repeatedly during embryo and organ morphogenesis. While the mechanisms of tissue elongation in 2D sheet-like tissues are increasingly well understood, the molecular and cellular mechanisms that elongate topologically distinct tubular and acinar organs may be distinct. In this dissertation, I utilize *Drosophila* egg chamber (follicle) morphogenesis in the adult female ovary as a model system to understand tissue elongation in the unbounded tube-like organs. The follicle grows spherically during early oogenesis and then elongates during mid-oogenesis along the anterior-posterior axis to become an ellipsoidal organ. Whole-tissue rotation of the follicle during the elongation phase is thought to provide Planar Cell Polarity (PCP) information that orients basal actin bundles and basement membrane Collagen IV fibrils around the circumference, through a process requiring the atypical cadherin Fat2. Despite its essential role in the process, how Fat2 regulates PCP symmetry-breaking to specify rotation in one chirality was unresolved.

With long-term live imaging, I showed that the follicle initiates rotation immediately after it buds off from the germarium. To trace the earliest sign of chiral symmetry-breaking, I developed an *in toto* 3D image analysis platform to comprehensively chart the cytoskeletal PCP in curved structures. In collaboration with my labmate Katherine Lipari, we found that microtubule growth polarity exhibits a bias within the germarium, prior to follicle budding and rotation. This germarial microtubule bias is dependent on Fat2, and provides chiral information that breaks the PCP symmetry prior to rotation. We propose a PCP relay mechanism that initiates from germarial microtubule chirality, to rotational polarity and actin bundle alignment, to finally a polarized basement membrane organization that elongates the organ.

The limitations of current *ex vivo* cultures for long-term morphogenetic analyses compelled me to develop a tool that can track cell motility over extended periods. I developed M-TRAIL, which utilizes cell-ECM interactions to retrospectively trace cell movements in fixed tissues. By making clones overexpressing GFP-tagged ECM proteins, cell motility trajectories are revealed based on the long-lasting traces of fluorescently-tagged ECM proteins deposited onto basement membrane. I applied M-TRAIL to compare *ex vivo* versus *in vivo* rotation and between WT and *fat2* to validate M-TRAIL's validity. Strikingly, a previously reported protein-truncating *fat2* mutant allele, which does not rotate *ex vivo* yet produces elongated follicles, actually rotates *in*

vivo at half the speed than WT. These results revalidate the model that tissue rotation is required to build a proper basement membrane that elongate the follicle. The M-TRAIL approach also demonstrates how reliance solely on *ex vivo* imaging can mislead.

By combining our previous work that chart the BM stiffness profile and by measuring BM stiffness in the slow rotating Fat2-truncated follicle, we show that rather than the absolute BM stiffness, an anterior-posteriorly patterned BM stiffness gradient elongate the follicle. By further refining the 3D image analysis platform I developed and use it to chart tissue and cellular scale morphometrics, I found three major cell behaviors that can contribute to follicle elongation, which are oriented cell division, polarized cell shape changes, and cell rearrangement. With analyses of either mutants that fail to elongate or by genetic manipulations that disrupt one particular cell behavior, I found cell shape changes and cell rearrangement play a major role in elongating the follicle. The tools I developed and my findings provide a platform and new insights in understanding the molecular, cellular, and mechanical mechanisms in organ morphogenesis.

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Chapter 1

Introduction

1.1.0 Morphogenesis: The Formation of Shapes

All living organisms have various sizes and shapes. Literature documenting characterization of animals based on these two properties can be found from the beginning of human history. Later, the diverse external morphologies of organisms served as criteria for classification by natural historians. Even within our body, our internal organs are shaped different ways that dictates their specialized functions. How these diverse shapes of organs arise is one of the long-standing questions in natural sciences, dating back to the ancient Greece long before the discoveries of cell and gene. Several centuries ago, developmental biologists began to study the formation of various structures during their embryonic stages by direct observation. While 18th century embryologists focused mainly on the physical properties of tissue morphogenesis, the more recent work of Christiane Nüsslein-Volhard and Eric Wieschaus defined the genetics of pattern formation giving spatiotemporal information to morphogenesis (Nüsslein-Volhard and Wieschaus, 1980). Now, we appreciate that there is a joint effort between genes, cells, and mechanics that unites geneticists, cell biologists and physicists in an intricate and integral view of morphogenesis.

1.1.1 Tissue Elongation as a Morphogenetic Process to Shape Organs

Starting from embryogenesis, the first significant morphogenetic event in animal is gastrulation. Gastrulation set the stage by defining the primary three germ layers for later tissue and organ morphogenesis. Various morphogenetic processes at the tissue scale occur to sculpt the embryos, including invagination, ingression, involution, epiboly, and convergent extension (Solnica-Krezel and Sepich, 2012) (**Fig. 1.1**). Similar morphogenetic processes can also be seen in developing organs. Examples include tissue bending, an invagination event that transforms a flat sheet into three-dimensional structures at the onset of tube formations for vertebrate neural tubes and the lens (Plageman et al., 2010). Tissue can also fold locally during vertebral gut looping, airway branching in the lung, generating tree of ducts, mammary glands, and salivary glands (Horne-Badovinac:2003iv, Chung:2010fe, Ochoa-Espinosa:2012ic, Andrew:2010bn, Varner:2014fl) (Andrew and Ewald, 2010; Chung et al., 2010; Horne-Badovinac et al., 2003; Ochoa-Espinosa and Affolter, 2012; Varner and Nelson, 2014). Finally, tissues can elongate to form long tubules such as those seen in the kidney. Among four basic types of animal tissues, the epithelium plays a major role in the morphogenetic events. In my dissertation, I focus in particular on tissue elongation and will review known molecular, cellular, and mechanical mechanisms that drive tissue elongation in several model systems.

1.1.2 Choreographing the Molecular and Cell Dynamics for Elongation

At the macroscopic level, tissue elongation can be defined by the gross morphology of the entire tissue or organ of interest, as it extends unproportionately in one dimension as compared to another. Typical elongation events happen along a body axis, such as the anterior-posterior or proximo-distal. At the cellular level, elongating a tissue may involve a combination of different cell behaviors. Basic behaviors documented in the field of cell

biology --including cell migration, division, shape changes, and growth-- can all serve as effectors of elongation at the tissue scale. However, individual cell behaviors alone cannot achieve tissue scale elongation. Like choreography, cell dynamics have to be coordinated collectively both locally and globally at the right time. Besides fine spatial and temporal execution, a 'GPS' system must exist to guide all cell behaviors toward the right direction with respect to either other and the organism. In the following sections, I briefly summarize the polarized cell behaviors that elongate tissues and organs, followed by the molecular players involved in setting up the positional cues and the cellular machineries that generate forces to shape the cells.

1.1.2 Cell Rearrangements by Convergent Extension

Convergent extension (CE) is the most studied collective cell behavior that elongates a body axis (**Fig. 1.2A**). During this process, cells intercalate between each other to converge along one axis for axis extension, and shrinking the length of the other axis. At the cellular level, there are two known modes to achieve CE. The crawling mode emerged as a hallmark of CE in the mesenchymal tissues from the pioneering study of embryonic notochord formation in African clawed frog (*Xenopus laevis*) (Keller et al., 1985; Shih and Keller, 1992). Live imaging of tissue explants from the presumptive notochord reveals that these mesenchymal cells exhibit bipolar actin-rich membrane protrusions that enable them to crawl along the narrowing axis. This concerted cell collective cell migration behavior are also conserved in mouse neural plate (Williams et al., 2014) and notochord formation in sea squirts (*Ciona intestinalis*) (Munro and Odell, 2002). The contraction mode of CE was originally discovered during fruit fly (*Drosophila melanogaster*) embryonic germband extension (Irvine and Wieschaus, 1994). Cells in the germband rearrange by remodeling their adherens junctions (AJ) to either shrink or extend the cell-cell contacts, based on their orientation with respect to the A-P and D-V axes. Termed T1 transition, AJs perpendicular to long axis shrink followed by the extension of the junctions parallel to the long axis. In addition to junctions between two cells, junctional rearrangements can also happen between multiple cells to form rosette-like structures that resolve several cell-cell contacts at once (Blankenship et al., 2006). Similar rosette-based CE can be seen during mouse kidney tubule elongation (Lienkamp et al., 2012).

Although it was originally thought that the crawling mode is utilized particularly in mesenchymal tissues while contraction is used in epithelial tissues, recent findings suggest that this boundary may not be so clear-cut. During *Drosophila* germband extension, Rac-mediated actin-based protrusive activity at the basal surface can contribute to rosette formation (Sun et al., 2017). In parallel, during *Xenopus* notochord intercalation, shortening cell-cell contact can be seen and is powered by mechanisms similar to the germband extension (Shindo and Wallingford, 2014). Studies in the mouse neural plate convergent extension also suggest 2 behaviors might cooperatively drive elongation (Williams et al.,

2014). Further studies on the interplay between these behaviors in coordinating tissue elongation will be interesting.

1.1.3 Polarized Cell Shape Changes

Deformation of cells that increase the cellular aspect ratio along the axis of elongation can be another behavior to achieve elongation to the overall tissue (**Fig. 1.2B**). Cell shape changes can contribute in part to elongating the germband, as demonstrated in embryos defective for cell intercalation (Butler et al., 2009) (Sawyer et al., 2011). Polarized shape changes also contribute to elongation of the *Drosophila* embryonic respiratory system. The epithelial cells forming the trachea tubular airway undergo a Src protein tyrosine kinase-mediated shape change that expands their apical surfaces in a polarized manner (Nelson et al., 2012) (Förster and Luschnig, 2012).

Unlike classical CE examples which usually occur at a timescale of minutes, cell shape changes can span days, concomitant with cell growth. This anisotropic cell growth that leads to polarized cell shape changes can also contribute to tissue elongation. The directional growth is exemplified in the mammalian palatal epithelium, where cell elongate during embryonic stages E12.5 to E14.5 to directly contribute to axis elongation (Economou et al., 2013).

1.1.4 Oriented Cell Division

In tissues and organs undergoing cell proliferation, the orientation of cell division can potentially contribute to tissue elongation (**Fig. 1.2C**). There are several systems where cells dividing in a polarized manner elongate the tissue. While the anterior *Drosophila* germband is elongated by CE, cell at the posterior germband are undergoing cell division. Cell division is primarily oriented along the elongating axis and is required to extend the germband (da Silva and Vincent, 2007). In the developing wing epithelium of *Drosophila*, oriented cell division have been found to contribute to the elongated shape of the wing blade (Baena-López et al., 2005). In the mouse kidney, disrupting oriented cell division by loss of the PCP components described in the next section also affect tubule elongation (Saburi et al., 2008). While the molecule mechanisms controlling spindle orientation that align with the plane of the epithelium are better understood (di Pietro et al., 2016), there is a prominent role of tissue mechanics in controlling spindle orientation along the plane of elongation. The role of forces will be discussed in section 1.1.6.

1.1.5 Patterning and Planar Cell Polarity orient tissue polarity for elongation

All the cell behaviors described above need be polarized and aligned within the tissue plane to execute tissue elongation. At the global level, patterning genes and morphogens set the stage by specifying cell fate and by providing spatial information that align with the tissue axis. Locally at the intercellular level, planar cell polarity (PCP) pathways polarize individual cells along the plane. The molecules that communicate PCP information have been

intensively studied, and PCP pathways can be categorized by the molecular components as Frizzled/Van Gogh PCP (also known as the core PCP or the non-canonical Wnt PCP) and Fat/Dachsous PCP pathways, though recent studies suggest two modules can intersect (Matis and Axelrod, 2013). In general, PCP is thought to be established within one cell by either asymmetrical localization of PCP protein complexes, or by the differential affinity of protein-protein interaction at distinct domains across the plane of the cell. This planar polarized cue can be further propagated from cell to cell based on the intercellular interactions between membrane receptors, ligands, and their intracellular effectors that ultimately leads to planar polarized cytoskeletal structures or dynamics (Aw and Devenport, 2017; Carvajal-Gonzalez and Mlodzik, 2014; Goodrich and Strutt, 2011).

Besides the core and Fat/Dachsous PCP pathways, patterning genes can also provide planar polarization cues to elongate the tissue. During *Drosophila* germband extension, global A-P patterning transcription factors Eve and Runt direct their downstream targets, the Toll receptors, to be expressed in stripes along the AP axis (Paré et al., 2014). These local stripes serve as positional information to polarize cell behaviors, as cell intercalation becomes misoriented when the stripes are disrupted (Paré et al., 2014).

Components of the PCP pathways were mainly revealed through mutant analyses, where inhibition of PCP genes leads to defects in polarized cellular behaviors (Gubb and Garcia-Bellido, 1982; Vinson et al., 1989). Here I focus on the role of PCP genes that polarize cell behaviors to contribute to tissue elongation. Dishevelled, a component of the core PCP pathway, orients CE in *Xenopus* notochord elongation by regulating localized Rho activity to polarize lamellipodia protrusions and elongate cell shapes (Habas et al., 2001; Tanegashima et al., 2008; Wallingford et al., 2000). The Fat/Dachsous pathway is necessary for spindle orientation and thereby axis elongation in the *Drosophila* wing (Mao et al., 2013). During mouse embryonic neural plate morphogenesis, CE contributes to elongation of this single layered pseudostratified epithelium. Interestingly, it utilized different molecular mechanisms for planar polarity rather than two well characterized PCP signaling pathways described above, suggesting various planar polarity mechanisms may still guide conserved polarized cellular behaviors among species (Williams et al., 2014).

In addition to tissue elongation in a 2D sheet, PCP proteins also contribute to elongation of a tubular structure. In vertebral kidney tubule morphogenesis, both core PCP and Fat PCP components are involved in regulating cell orientation, convergent extension, and oriented cell division during tubule elongation and the diameter of the tube (Karner et al., 2009; Kunimoto et al., 2017; Lienkamp et al., 2012; Yu et al., 2009). While asymmetrical distribution of PCP proteins can be seen locally in the renal tubules (Kunimoto et al., 2017), it will be interesting to test whether they share conserved mechanisms at the signaling level with the well characterized PCP pathways seen in a flat sheet.

1.1.6 Mechanical Forces driving elongation

The importance of mechanical forces in shaping cells in developing tissues has been appreciated and studied a century ago by D'Arcy Thompson in his influential book On Growth and Form (Thompson, 1917; 1942). In this book, he argued that all living matter including cells within tissues are bounded by mathematical and physical laws. By using foams as an analogy, he described in a quantitative manner how differences in pressure, tension, and viscosity can explain and predict the shape and the packing of the cells. Even though some assumptions were oversimplified and plainly wrong, his reductionist's concept and a quantitative approach has influenced biologists for decades, and also influenced and contributed to the current renaissance of mechanobiology in morphogenesis (Graner and Riveline, 2017). As Thompson insightfully foresaw, the polarized cell behaviors described above have to be executed by molecules generating and exerting forces either within, between or outside of the cell in a coordinated manner. Intracellularly, forces can be generated by actomyosin machinery. Between cell-cell contact, tensile forces can be transmitted through Cadherin-based adherens junctions. Finally, Extracellular Matrix (ECM) components and their receptors in the cell can exert traction forces. Below, I will summarize known mechanical mechanisms that drive tissues elongation.

During CE in the *Xenopus* notochord, while cells with actin protrusions crawling along other cells may produce traction forces (Keller et al., 2008), contractile forces generated by non-muscle myosin II (MyoII)-dependent actomyosin dynamics at the cortex provides resistance to cell deformation. This cell stiffening is proposed to facilitate cell intercalation rather than cell elongation, while the traction force from actin cytoskeleton remodeling occurs at the protrusion (Kim and Davidson, 2011). At the local cell-cell level, MyoII-dependent actomyosin dynamics can generate contractile forces within the cell. During T1 transition in the *Drosophila* germband convergent extension, MyoII is enriched at the junctions, and actomyosin filaments generate forces to shorten the junction. This planar polarized contraction of actomyosin induces coordinated junction shortening along one axis that produces concerted elongation at the tissue level (Rauzi et al., 2008). Further live imaging approaches demonstrate that 2 pools of MyoII exist in the same cell (Rauzi et al., 2010). MyoII from the medial pool flows to junctional pool to shorten the junctions and stabilize those shortened junctions. This actomyosin flow also collaborates with E-cadherin trafficking to regulate junctional protein binding affinities that facilitate AJ asymmetrical stability to change cell-cell contact dynamics in a polarized manner (Levayer and Lecuit, 2013; Levayer et al., 2011; Rauzi et al., 2010; Simoes et al., 2010). Analyses at the junctions that are about to extend hinted that medial pool of actomyosin contraction may provide tensile forces to the nascent junction for stabilizing a junctional lengthening event (Collinet et al., 2015; Yu and Fernandez-Gonzalez, 2016).

Cell behaviors executed in a polarized fashion may be a response to force anisotropy. Mechanical forces can orient cell division in developing animals. When tissues are stressed along one particular axis, cell divisions are oriented along the axis to relieve tension in the wing imaginal disc of *Drosophila* larvae (Mao et al., 2011). Tension anisotropy can come from the differential proliferation rates between different domains in the same disc, and are further strengthened by tension induced MyoII recruitment (Mao et al., 2013). To relay tissue

force imbalance to cell division, tricellular junctions in the *Drosophila* pupal notum and larval wing act as cues to store force anisotropy information that orient the mitotic spindles to the tension oriented long axis (Bosveld et al., 2016).

Apart from forces generated internally, external forces coming from adjacent tissues may facilitate the elongation of a sheet of tissues as global cues to orient cell behaviors. Concomitant with germband extension, other large-scale movements including mesoderm invagination, cephalic furrow formation, and endoderm invagination occur in the *Drosophila* embryo. While mesoderm invagination does not contribute to cell intercalation in the germband (Irvine and Wieschaus, 1994), shape changes during germband extension can be a response to the pulling force coming from the invaginating mesoderm (Butler et al., 2009). Furthermore, endoderm invagination was found to promote cells to extend during the first 15 min of germband extension (Lye et al., 2015). Pulling forces at the boundary with the invaginating posterior midgut also actively participate in tissue extension by orienting junction growth (Collinet et al., 2015). Similar inter-tissue force influence can be seen in the developing *Drosophila* wing. Pupal wing hinge can exert tension on the developing wing blade and this tension anisotropy realigns planar polarity along one axis in the blade. This tension induced planar polarity guides several polarized cell behaviors, including oriented cell division, cell deformation, and intercalation, that collectively result in tissue flow to generate an elongated wing blade (Aigouy et al., 2010).

Besides internal forces exerted by actomyosin or external forces from other tissues, by work from our lab which I will describe in section 1.2.11, extracellular mechanical force coming from the ECM may also serve as patterning cues to globally polarize the cell behaviors to elongate the tissues and organs.

1.1.7 A Tractable Model with an *in toto* Approach for Studying Organ Morphogenesis

The role of external forces on polarizing cell behaviors emphasizes the importance of treating the structure as a whole. Most studies focus a subset of cells or tissues in a 2D flat tissues that are easier to analyze, while few studies provide comprehensive analyses of topologically distinct three dimensional organs at its entirety. One factor for a lack of *in toto* analyses is that the 3D nature of most tissues and organs complicates the analysis for live imaging and cell tracking. Another factor to consider is the timescale of tissue and organ elongation among various species. For example, germband extension in *Drosophila* occurs rapidly within one hour, while the timescale of much mammalian organ morphogenesis can span days. Conserved cell behaviors found in gastrulation and organogenesis may utilize different molecular or mechanical mechanisms to achieve temporal accuracy. Finally, in many examples above, tissue and organs can use a combination of polarized behaviors to achieve overall elongation. To define the causal cell behaviors and the relative contribution of each behaviors, solely descriptive analyses without the manipulation are not sufficient. A tractable model system would be ideal to understand the intricate relationships of molecular, cellular, and mechanical mechanisms in organ morphogenesis.

1.2.0 *Drosophila* Egg Chamber as a Model for Studying Tissue Elongation during Organ Morphogenesis

Morphogenesis of the egg chamber (follicle) in the *Drosophila* ovary over the past decade has become a model system to study tissue morphogenesis. Due to its structural similarity to many mammalian tube-like acinar organs such as the breast duct, it has recently become an emerging model for studying 3D organ morphogenesis. With powerful *Drosophila* genetic tools and major advances in microscopy and *ex vivo* cultures for live imaging, these provide unique opportunity to dissect the molecular mechanisms of organ morphogenesis at the interface of cell biology and mechanobiology. The oogenesis of *Drosophila* was initially characterized in the pioneering works by Robert C. King in the 1950s and later on by others to study almost every aspect of biology. In section 1.2, I summarize the tissue architecture of ovary, several key events in follicle morphogenesis, and finally focus on the works on the mechanisms of follicle elongation.

1.2.1 Morphology of the Ovary

Oogenesis takes place in the female adult abdomen, where two lobes of ovaries reside (**Fig. 1.3A**). Each lobe has 10-20 ovarioles consisting of several individually developing egg chambers connected through stalk cells. About 6-8 developing egg chambers are aligned in each ovariole like an assembly line, from younger at the anterior to more mature ones toward the posterior (**Fig. 1.3B**). These developing egg chambers go through 14 morphologically subdivided developmental stages of oogenesis to produce a mature egg (Cummings and King, 1969) (**Fig. 1.4**). There are ovarian muscles that surround each ovariole. The contraction of muscle sheath pushes each egg chamber gradually into the oviduct during maturation, before the female lay eggs outside of the body.

An intact egg chamber consists of two main cell types, from the germline and somatic lineages. Within each egg chamber, there a cyst of 16 germ cells. Only one out of the 16 germ cells will become the oocyte while the other 15 become the nurse cells supporting the oocyte. These germ cells are surrounded by follicle cells of somatic lineage that form the follicle epithelium.

Both types of cells originate from a structure at the anterior tip of the ovariole called the germarium (**Fig. 1.3C**). The germarium is where follicle stem cells and germline stem cells reside. These two populations undergo several rounds of cell division to produce an intact follicle. Germline stem cells first undergo asymmetric divisions that give rise to one self-renewing stem cell and a cystoblast. The cystoblast next undergoes four rounds of incomplete mitotic divisions that eventually form a cyst made of interconnected 16 germ cells. These actin-rich cytoplasmic bridges called the ring canals provide nutrients exchange and later in oogenesis serve as channels to deposit cytoplasmic components to the oocyte. While most literatures estimated there are 2-3 follicle stem cells (FSCs) in the germarium (Nystul and Spradling, 2010a), a recent paper suggests there might be more FSCs than previously anticipated. In this paper (Reilein et al., 2017), the authors use multicolor

lineage analyses to infer that there may be 14-16 FSCs. The FSCs that lie more anterior in the germarium produce escort cells in the germarium that form the FSC niche and support the germline stem cell (Reilein et al., 2017; Song and Xie, 2002). The other population of FSCs at the posterior position in the germarium produce follicle epithelial cells that encapsulate 16 germ cells. These FSCs on average produce 4 follicle precursor epithelial cells in each of the egg chamber (Reilein et al., 2017).

1.2.2 The Formation of an Individual Egg Chamber

The first morphogenetic event in follicle development occurs at the posterior region of the germarium. The epithelial founder cells divide and migrate to encapsulate one 16-cell germline cyst. Concurrently, the epithelial progenies begin to adopt one of three different epithelial cell fates, either the polar, interfollicular stalk, or main body cell fate. Each egg chamber has a pair of 2 polar cells at the anterior pole and another pair at the posterior pole. Both anterior and posterior polar cell fates are specified roughly around the time during encapsulation, with Notch signaling from the germline required to specify polar cell fates (Nystul and Spradling, 2010a; Torres et al., 2003). Stalk cells on the other hand localize at the anterior of the encapsulating cyst with a cluster of 6-8 cells separating the younger cyst in the germarium.

Encapsulation marks the developmental stage 1 of egg chamber. From here on, it takes about 3-4 days, going through subsequent 13 stages of oogenesis to produce a mature egg that moves into the oviduct and ready to be fertilized (Cummings and King, 1969) (Spradling, 1993). Following encapsulation, the egg chamber buds off from the posterior end of the germarium and becomes a stage 2 egg chamber. Each intact egg chamber now contains a cyst of 16 germ cells surrounded by a single layer of follicle epithelial cells. Each egg chamber develops independently but are connected by stalk cells.

1.2.3 Anterior-Posterior Symmetry Breaking in the Egg Chamber

During the encapsulation process in the germarium, several events happen to break the Anterior-Posterior (A-P) symmetry in the germ cells and the somatic cells. The location of the oocyte in the budding egg chamber play a major role in defining A-P axis of the entire structure. The somatic cells facilitate oocyte placing at the posterior end. Both the posterior follicle cells and the oocyte upregulate E-cadherin (Godt and Tepass, 1998). This facilitates cell sorting event in the cyst so that the oocyte differentially adheres to the posterior follicle cells. In mutants where oocyte fails to reach either ends, the follicle fails to break A-P symmetry (González-Reyes et al., 1997) (González-Reyes and St Johnston, 1998). The anterior follicle cells on the other end induce the pre-stalk cells between the younger cyst to assume stalk cell fates. Through a combination of Notch and JAK/STAT signaling, the determination of stalk cell defines the long axis of the egg chamber and the A-P axis of the cyst, including the future A-P axis within the oocyte. (Torres et al., 2003) (Assa-Kunik et al., 2007). In summary, A-P asymmetry is established by the interactions between two follicles, the

anterior follicle cells of the older egg chamber define the posterior of the younger, budding egg chamber in the germarium.

A-P patterning in the follicle cell, however, initiates later on during stage 8 and is regulated by receptor tyrosine kinase Egfr signaling (Berg, 2005). The oocyte signals through the Gurken/Egfr pathway to induce the adjacent follicle cells to adopt a posterior fate (Poulton and Deng, 2006).

1.2.4 Major Morphogenetic Events During Egg Chamber Development

During the entirety of oogenesis, each egg chamber grows in size, increasing about 5000-fold in total volume (King, 1970). While much of the growth is contributed by the growth of germ cells, follicle cells continue to divide until the end of stage 6, reaching approximately between 650 cells (Margolis and Spradling, 1995)-1000s cells (King, 1970). At this point, follicle cells undergo a process controlled by Notch signaling called mitotic to endocycle switch, where cell cycle switch from a complete G1-S-G2-M cycle to only G2-S cycle (Calvi et al., 1998), that is, cell becomes polyploid through repeated DNA duplication without mitosis.

During this period, the follicle epithelial cells begin to change shape. The epithelium during the mitotic stages 1 to 6 remains cuboidal, but starting around stage 7, the anterior ~50 cells start to flatten and become squamous at stage 9. The flattening event of these cells, named stretch cells, is regulated by TGF β signaling that modulates the strength of E-cadherin based adherens junctions (Brigaud et al., 2015). The stretch cells eventually cover the nurse cell domain at stage 10, while the other follicle cells undergo a cuboidal to columnar transition and cover the oocyte domain. The process of redistribution of follicle cells relative to their inner germ cell position is either termed posterior migration (Grammont, 2007; Horne-Badovinac and Bilder, 2005) or anterior accommodation (Kolahi et al., 2009) depending on mode of mechanism suggested.

The anterior polar cells recruit 4-8 cells anterior follicle cells named the border cells to form a cluster of 6-10 cells. This cluster becomes motile during stage 9, invades the nurse cells and migrates posteriorly toward the oocyte (Montell et al., 2012; Rørth, 2009). Border cells migrate dorsally when they reach the oocyte surface. At a later stage, border cells build a canal inside the micropyle, a specialized structure of egg shell, for sperm entry (Montell et al., 1992).

From stage 9, the columnar follicle cells begin to synthesize and secrete proteins apically between the oocyte and the epithelium to form the egg shell (Cavaliere et al., 2008) (Margaritis et al., 1980). The egg shell forms several distinctive structures as a result of follicle epithelial morphogenesis, which serves as molds for shell architecture (Osterfield et al., 2017).

At the onset of stage 10B, a subset of columnar follicle cells at the columnar-squamous boundary migrate inward between the nurse cell and the oocyte. This morphogenetic event called centripetal migration ultimately enclose the anterior of the oocyte. This event ensures eggshell cover the anterior of the oocyte, as mutants that have impaired centripetal migration led to opened, cup-like shaped egg at the anterior (Schüpbach and Wieschaus, 1991). With live imaging the anterior most columnar cell first elongates apically toward the germ cells followed by the cell posterior to it. Though mechanisms not well understood, E-cadherin dependent germ cell-follicle cell adhesion may be required (Niewiadomska et al., 1999).

The final significant morphogenetic event is the formation of 2 dorsal appendages extending beyond the anterior pole that serve as a respiratory apparatus for the egg. Tube formation initiate at stage 10B from a 2D epithelial sheet into a 3D tube, which closely resembles wrapping and budding tube morphogenesis in other tube-like organs (Berg, 2008). Each dorsal appendage develops independently from 2 placodes separated apart by the dorsal midline. The placodes contain a subset of about 60 cells in the epithelium. 2 types of cells behave differently during the 3 phases for dorsal appendage morphogenesis, tube formation, tube elongation, and tube maturation. During tube formation at stage 11, the roof cells intercalate and narrow the apical surface to bend the tissue. Concomitantly, the floor cells become elongated and undergo cell reorganization to form new cell-cell junctions in order to join 2 groups of cells together. During the elongation phase, these cells utilized a combination of cell shape changes, convergent extension, and anterior cell migration to form an A-P elongated tube (Osterfield et al., 2013).

At the onset of stage 11, nurse cells transfer massive cytoplasmic contents, including organelles, RNAs and proteins to the oocyte (Cooley et al., 1992; Mahajan-Miklos and Cooley, 1994) in a process called nurse cell dumping. Oocyte at these period increases in volume massively and concurrently nurse cells shrink drastically. Failure of nurse cell dumping seen in mutants affecting ring canals leads to oocyte decrease in volume and ultimately produce a shorter egg, where in the mature egg the length of the A-P axis are shortened. The anterior stretch cells have recently been found to be crucial for nurse cell dumping, as nurse cells in squamous cells genetically ablated egg chamber fails to perform cytoplasmic transfer (Timmons et al., 2016). After the completion of nurse cell dumping, the stretch cells further promote non-cell autonomous, non-apoptotic programmed cell death of nurse cells by activating phagocytotic genes in the follicle cells (Timmons et al., 2016; 2017).

After the completion of the egg shell and approaching the end of oogenesis, posterior follicle cells degenerate mediated by matrix metalloproteinase 2 during the process of ovulation (Deady and Sun, 2015; Deady et al., 2015). This leads to follicle rupture at the posterior, causing the egg becomes separated from the anterior follicle cells and move in to the oviduct. The egg at this stage becomes activated and are ready for fertilization in the uterus.

1.2.5 Follicle epithelium plays a major role in tissue elongation

Concurrent to growth of the entire structure and the morphogenetic events described above, egg chambers also undergo tissue elongation. During stage 2-4 follicles grow spherically, but from stage 4 to 14 gradually assume an ellipsoid structure. In terms of the aspect ratio, defined by dividing the length of Anterior-Posterior axis by the length of Dorsal-Ventral axis, follicles elongate by two fold in 26 hours during stage 4 to 8 (Bilder and Haigo, 2012; Haigo and Bilder, 2011) (**Fig. 1.5A**). Besides nurse cell dumping event that impact elongation by affecting the egg volume (Cooley et al., 1992; Mahajan-Miklos and Cooley, 1994), the muscle sheath that surrounds the each ovariole also play a role in elongating the egg (Andersen and Horne-Badovinac, 2016).

One major player that defines the shape of the egg is the follicle epithelium (**Fig. 1.5B**). Besides its role as a factory for yolk protein production and later on generating materials for the egg shell, the epithelium is also known for its importance for oocyte patterning. The role of follicle epithelium on the shape of mature eggs emerged when a study generated genetic mosaics with female sterile mutations known to affect egg shape. In this study (Wieschaus et al., 1981), when generating germline specific clones rather than the whole mutants, they found the egg ends up being normally shaped. This result suggests that the somatic (follicle epithelial) cells, rather than the germ cells, play an active role in shaping the egg. Additional studies using various techniques, either generating clones solely in the follicle epithelium or using epithelial specific GAL4/UAS system for gene manipulations also support this notion (Bateman et al., 2001; Berns et al., 2014; Frydman and Spradling, 2001; Horne-Badovinac et al., 2012).

1.2.6 The Molecular Corset Model for Follicle Elongation

How does the epithelium facilitate elongation? A key hint came from visualization at the molecular level. By staining and visualizing the cytoskeleton at the microscopic level, actin bundles appear as linear fibrils present at the basal surfaces of the cell (Gutzeit, 1990) (**Fig. 1.5C-D**). These fibrils are aligned perpendicularly along the A-P axis in the follicle cells and the alignments between cells are polarized at similar direction along the A-P arc globally throughout the epithelium. Besides actin bundles at the basal cell surface, basement membrane components outside the epithelium also showed similar planar polarization (Gutzeit et al., 1991; Schneider et al., 2006) (**Fig. 1.5E**). Interestingly, in a female sterile mutant called *kugelei*, eggs are spherical compared to ellipsoidal WT eggs (**Fig. 1.5F**), both actin and basement membrane global bundle alignments are lost (Gutzeit et al., 1991) (**Fig. 1.5G-H**). *kugelei* later on was shown to disrupt function of the atypical cadherin Fat2 (Viktorinová et al., 2009), which has been found to be one of the crucial components for follicle planar cell polarity discussed in section 1.2.9.

Since there is a correlation between tissue elongation and the capabilities of these proteins in generating and exerting forces in and/or between the cells, a mechanical-centric model was proposed: A 'molecular corset' generated by the global and circumferential

alignments of actin and BM bundles provide forces that constrain the growing follicle toward anisotropic growth along the A-P axis.

1.2.7 Basal Actomyosin Contraction Contributes to Elongation

The first evidence of the molecular corset at play at the cellular level came from the analysis of actomyosin dynamics in late-stage follicles. With the advancement of the techniques to culture follicles outside of the female abdomen(*ex vivo*) for a period of time (Bianco et al., 2007; Prasad and Montell, 2007), live imaging to study follicle morphogenesis at the cellular and molecular level became possible (He et al., 2011; Peters and Berg, 2016; Prasad et al., 2011). During stage 9 to 10, actin bundles at the basal surface undergo oscillatory accumulation(He et al., 2010). This basal actomyosin contraction can exert tensile forces that contribute to follicle elongation, as manipulating the contraction rates acutely with drugs alter follicle shapes in a predictive manner(He et al., 2010).

1.2.8 Follicle Rotation Builds a Polarized Basement Membrane for Elongation

A mystery with the discovery of planar polarized actin and BM was how they arise. Saori Haigo, a previous graduate student in our lab, also took a live imaging approach to understand the cellular basis of follicle elongation. When she performed *ex vivo* time lapse imaging on early developing follicles, she found a previously unappreciated novel behavior, which she termed global tissue rotation, can be seen during stage 5 and ceases at stage 9(Haigo and Bilder, 2011) (**Fig. 1.6A**). The follicle cells collectively migrate along the circumference, making cells rotate around the A-P axis. The chirality of rotation in each intact follicle can be either dextral or sinistral within one ovariole. She demonstrated that rotation builds a polarized basement membrane that consists of planar polarized Collagen IV fibrils that wrap around the circumference (**Fig. 1.6B**). In conjunction with the actomyosin contraction at the later stages, her study suggested that it is the polarized ECM during stage 5-8 that serves as a molecular corset to constrain the shape of rapidly growing follicles(Bilder and Haigo, 2012) (**Fig. 1.6D-F**). Besides building a polarized BM through rotation(Isabella and Horne-Badovinac, 2016), it also aligns basal actin bundles (Cetera et al., 2014) .

Rotational movement in epithelial cell isn't just unique to *Drosophila* follicles. Dated back 40 years ago, D.F Went already reported the follicles of gall midge (*Heteropeza pygmaea*) rotate when he performed live imaging (Went, 1977). Beyond insects, in mammalian organoid culture system, similar behavior in other model systems has been documented. In the 3D mammary acini culture, when cells are seeded in the 3D matrix, the entire cyst shows rotational movements of multiple rounds spanning about 4 days. Strikingly, this rotation correlates with the establishment of planar polarized actin and assembly of their endogenous ECM around the spherical structure(Tanner et al., 2012; Wang et al., 2013) (Squarr et al., 2016) (**Fig. 1.6C**).

1.2.9 Fat2 as a Core Component of Follicle PCP

As opposed to most other PCP phenomena described previously, in the follicle PCP components such as Fat/Dachsous and Frizzled/Van Gogh are not required for planar polarization of either basal actin bundles or BM components (Viktorinová et al., 2009). Instead, Fat2 serves as a core regulator for ‘follicle PCP’. In contrast to apical localization of core PCP components, Fat2 localizes at the basal surface in a planar polarized manner at the trailing edge of the migrating follicle cells (Viktorinová and Dahmann, 2013; Viktorinová et al., 2009). Fat2 later was found to be required for follicle cell migration (Chapter 2 in this dissertation) and (Cetera et al., 2014; Viktorinová and Dahmann, 2013). Recently, Lar has been demonstrated to serve as Fat2’s counterpart in defining follicle PCP at the basal cell surface (Barlan et al., 2017). Previously found to be a round egg mutant (Frydman and Spradling, 2001), the receptor tyrosine phosphatase Lar is localized at the leading edge of migrating cell and is required for follicle cell migration. At the leading edge, Lar is induced by Fat2 from the cell behind to promote actin-rich protrusions non-cell autonomously (Barlan et al., 2017; Squarr et al., 2016). At the trailing edge, Fat2 facilitates retraction of the trailing edge in a cell autonomous manner via the signaling through Lar at the cells behind (Barlan et al., 2017). Further studies on the binding partners of Fat2 may provide more mechanistic insights for this basal PCP. Even though this local Fat2/Lar mediated PCP can propagate one cell away, how Fat2 provide global PCP cue across the entire tissue to orient planar polarity for follicle elongation remains to be determined.

1.2.11 An A-P patterned BM Stiffness Gradient Elongates the Follicle

Even though the prevailing corset model predicts that planar polarized BM fibrils might serve as mechanical cues to elongate the follicle, a direct force measurements was lacking. Justin Crest, a post doc in our lab, focus on the mechanical properties of developing follicle. He took the atomic force microscopy approach to probe the BM stiffness profile in the follicle (Crest et al., 2017) (**Fig. 1.7A**). While the BM in developing follicles become stiffer, strikingly, the stiffness does not increase uniformly throughout the entire BM. By probing the BM along the A-P arc, he found that follicle stiffness profile during stage 3-6 remains similar along the A-P, but becomes graded during stage 7-8, with a stiffer BM at the equator and softer at the ends (**Fig. 1.7B**). By genetic manipulations of the stiffness gradient and analyses of known mutants that produce round follicle, he demonstrated the relative BM stiffness along the A-P arc is required to elongate the follicle (Crest et al., 2017). Our work has provided a revised molecular corset model for follicle elongation: Rather than the absolute increase in stiffness, it is the A-P patterned BM stiffness gradient that actively instruct follicle elongation. The question on how BM stiffness gradient instructs follicle elongation at the cellular level remains to be elucidated.

1.3 Overview of Dissertation

As I began my dissertation project in 2012, our lab had just published the discovery of follicle rotation. Many questions emerged about the mechanisms of rotation itself and on its role in follicle elongation. We did not know how PCP symmetry is broken to initiate follicle rotation. We did not know how Fat2 played a role in rotation. We did not know how rotation

build a polarized BM. We did not know how the BM influences follicle elongation (mechanically or otherwise). Most importantly, we had no cellular basis on how follicle elongate. In my dissertation, I will describe the work that I have done to tackle these questions.

In Chapter 2, I describe collaborative work with Katherine Lipari, Yalda Dehghan, and Sebastian Streichan. We tackled the question of how PCP rotation initiates, and what molecular components define the earliest symmetry breaking event. Here, I developed an *in toto* 3D imaging analysis platform that enabled us to comprehensively chart PCP in the follicle, including its earliest stages. Combined with RNAi screen and live imaging, we found that microtubules play a key role in biasing PCP in the pre-follicle, prior to follicle budding and rotation. We proposed a PCP relay mechanism for tissue elongation: from microtubule chirality in the germarium, to follicle rotation chirality and actin bundle alignment, to finally a polarized BM that elongate the follicle.

Even though we utilized live imaging to chart follicle rotation, limitations of current *ex vivo* culture for long term live imaging in an environment that recapitulates *in vivo* follicle morphogenesis remains a rate-limiting factor. In Chapter 3, I describe the development of a cell tracking tool that allow us to track cell motility *in vivo*. This tool is called M-TRAIL, which tracks past cell migration trajectories based on the durable traces left by fluorescently tagged ECM proteins during migration. With M-TRAIL, I resolved a conflict in literature and reestablished that follicle rotation is required for elongation. M-TRAIL has potential applications beyond follicle morphogenesis, and beyond the fruit fly.

In Chapter 4, I further refined ImSAnE to extract morphometrics of the developing follicle. I comprehensively define both tissue level and cellular level morphometrics during the initial phase of tissue elongation. I found three major cell behaviors that can contribute to follicle elongation, which are oriented cell division, polarized cell shape changes, and cell rearrangement, with cell shape changes playing the major role. Through an RNAi screen for genes involved in follicle morphogenesis, I found that Rack1 may facilitate these polarized cell shape changes in post-mitotic follicles. Rack1 might regulate these polarized cell behaviors through regulating Src activity.

In summary, the follicle *in toto* image processing platform I developed has allowed our lab to execute a true quantitative approach on follicle morphogenesis. There is an increasing awareness in developmental and cell biology that such comprehensive and quantitative approaches are essential, and my tool provides a means to meet this demand not only for studying morphogenesis, but also other fields that requires 3D quantitative image analyses. With my development of a *in vivo* cell migration tracking tool, I have provided a new alternative (other than live imaging) to visualize cell motility. The tool allows quantitative analyses on long-term cell movements in tissues and organs that cannot be subjected to live imaging.

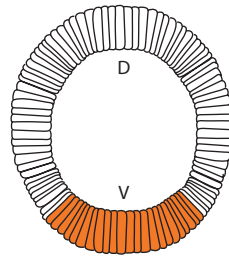
Besides tool building, the biological findings described in my dissertation first reveal the PCP symmetry breaking event and PCP relaying mechanisms for elongating the follicle edgeless epithelium. This PCP relay requires follicle rotation to build a A-P patterned BM stiffness gradient to sculpt the follicle. The A-P patterned BM stiffness gradient may provide cues globally to instruct polarized cellular behaviors I have discovered to elongate the follicle. Overall, my dissertation has expanded our understanding of cell and tissue symmetry breaking, collective cell migration, and follicle morphogenesis. These findings may provide potential insights for organ morphogenesis and tissue engineering for regenerative medicine.

Fig. 1.1 Morphogenetic behaviors that shape tissue and organs

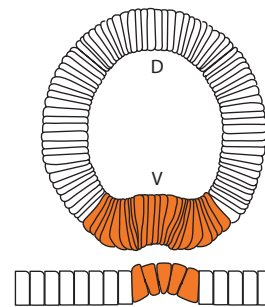
Various morphogenetic processes during animal gastrulation. Tissues in orange internalized in a process called emboly, while tissue in blue undergoes spreading and thinning, in a process called epiboly. (A) In fruit fly, cross section of embryo highlighting tissue invagination in the orange cells at the ventral region. (B) During zebrafish epiboly, mesoendodermal precursor cells in orange move beneath the prospective ectoderm, a process termed cell ingression. (C) During frog gastrulation, cell involution occurs during mesoderm internalization. (D) Cells in orange separate from the epithelium during chick and mouse gastrulation. Abbreviations: A, anterior; An, animal; D, dorsal; P, posterior; SMO, Spemann-Mangold organizer; V, ventral; Vg, vegetal. Drawings adapted from (Solnica-Krezel and Sepich, 2012).

Fruit fly
Drosophila melanogaster

A

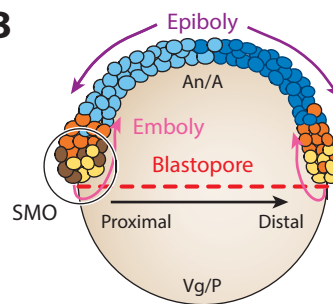


A' Invagination

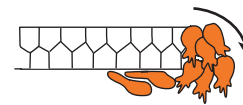


Zebrafish
Danio rerio

B

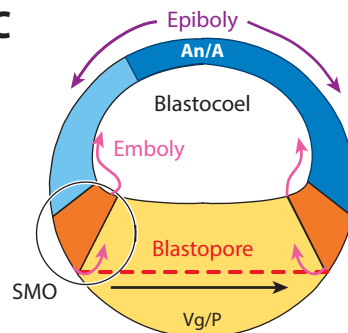


B' Synchronized
ingression



Frog
Xenopus laevis

C

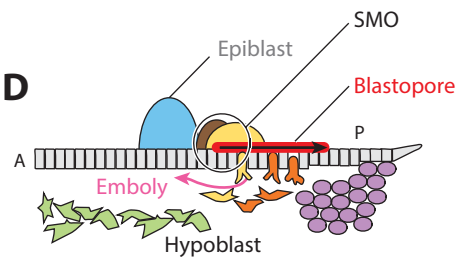


C' Involution



Chicken
Gallus gallus

D



D' Ingression



Mouse
Mus musculus

A

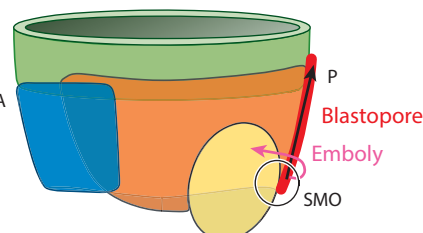


Fig. 1.2 Cellular behaviors for tissue elongation

(A) Cell rearrangement by convergent extension, where cells intercalate by changing neighbor relationship in the cell to extend one axis at the expense of the other. (B) Polarized cell shape changes can elongate tissues by orienting and extending the long axis in a coordinated manner. (C) Cell can orient their mitotic spindles along one axis and divide in order extend the axis.

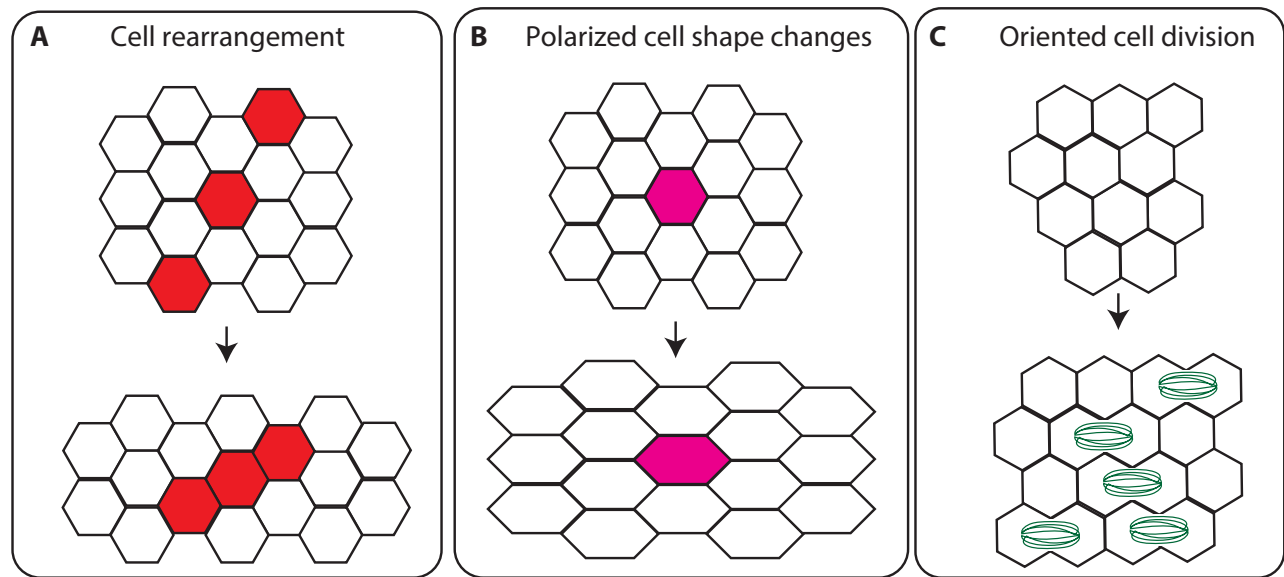


Fig. 1.3 *Drosophila* adult ovary and germarium structure

(A-B) Illustration adapted from (Rubin and Huynh, 2015) and (C) from (Reilein et al., 2017). (A) The structure of the ovary, which include several ovarioles with developing egg chambers and the reproductive tract. (B) The structure of an ovariole in cross section from anterior (a) to posterior (p). Germline cells are outlined in red with the oocyte as yellow and somatic cells outlined black. (C) The architecture of the germarium at the anterior of an ovariole. Terminal filament (TF) cells located at anterior tip (to the left), followed by Cap cells and the purple germline stem cells (GSCs), which divide and produce 16-germ cell cysts in grey. Escort cells (ECs) in yellow and follicle stem cells(FSCs) in white or green with their progenies in blue or green.

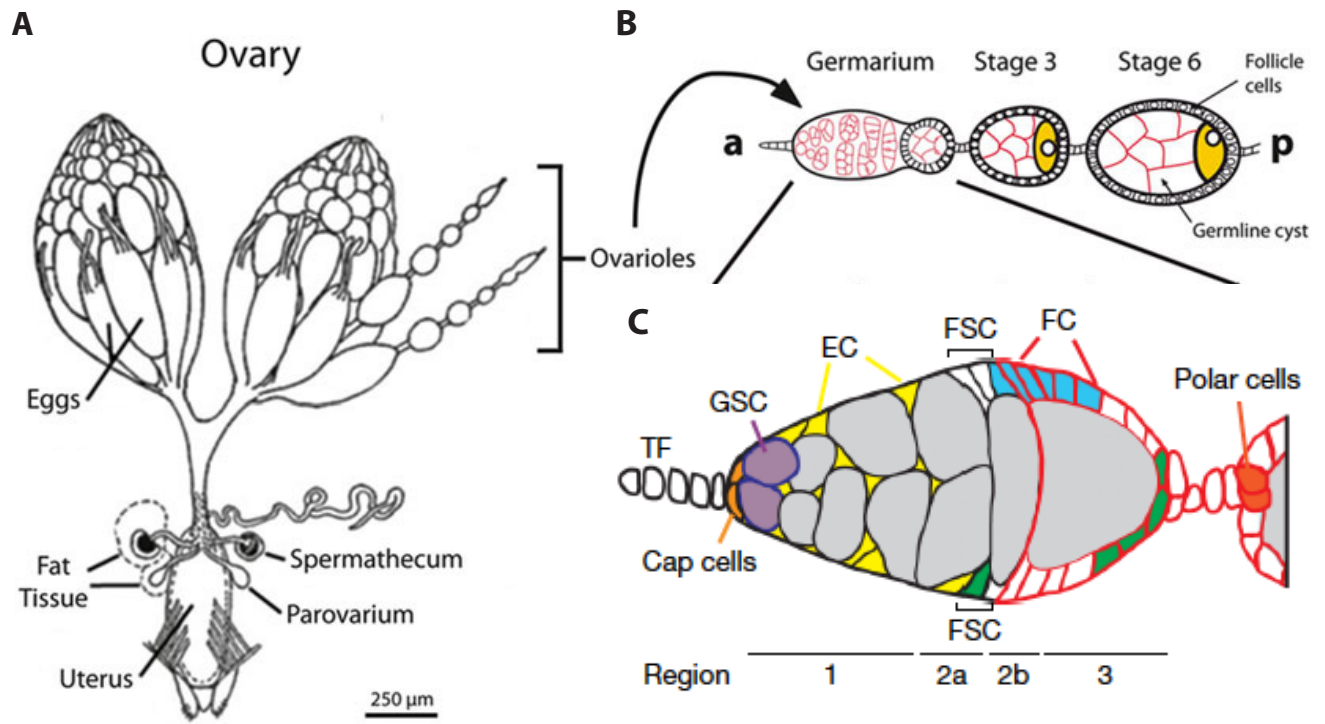


Fig. 1.4 Fourteen developmental stages of oogenesis

Illustrations adapted from (Peters and Berg, 2016) of 14 stages of oogenesis. Starting from the germarium, G, at the eleven O'clock position. Egg chamber undergoes 14 morphologically defined stages to become a mature egg, E with only the anterior portion drawn.

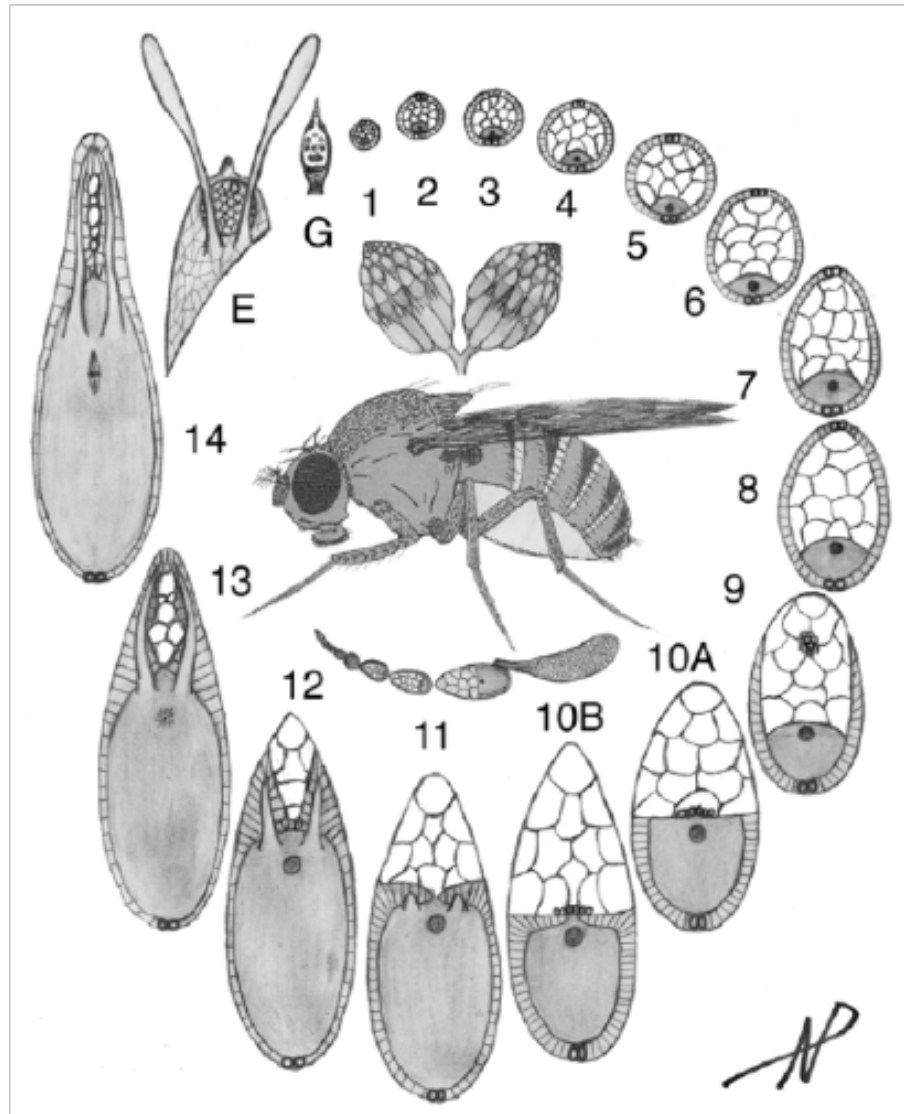


Fig. 1.5 Tissue Elongation in Follicle Morphogenesis

(A) Follicle elongation adapted from (Haigo and Bilder, 2011). Aspect ratio of the follicle, A-P axis length divided by D-V axis length, during stages of oogenesis. Follicle aspect ratio begins to increase around st.4 and around st.8 has increased almost 1-fold. (B) The architecture of the follicle epithelium. The epithelium in green with nucleus in red. Beyond the apical surface are the germline cells and beneath the basal surface lies the basement membrane in purple. (C) Actin bundles at the basal surface is planar polarized. Follicle cell outline in green and basal actin bundles in red. Illustration by Katherine Lipari. (D) Follicle is fixed and stained with TRITC-phalloidin to visualize F-actin(white). Volocity opacity projection was used to display 3D reconstruction of the confocal stacks. Scale bar: 10 μ m. (E) Collagen IV in the basement membrane(green) shows planar polarized circumferential fibrils that are perpendicular to the A-P axis. (F) Mature WT and *fat2* eggs. (G) *fat2* follicles exhibit mis-oriented basal actin bundles. (H) *fat2* follicles also exhibit mis-oriented Collagen IV fibrils.

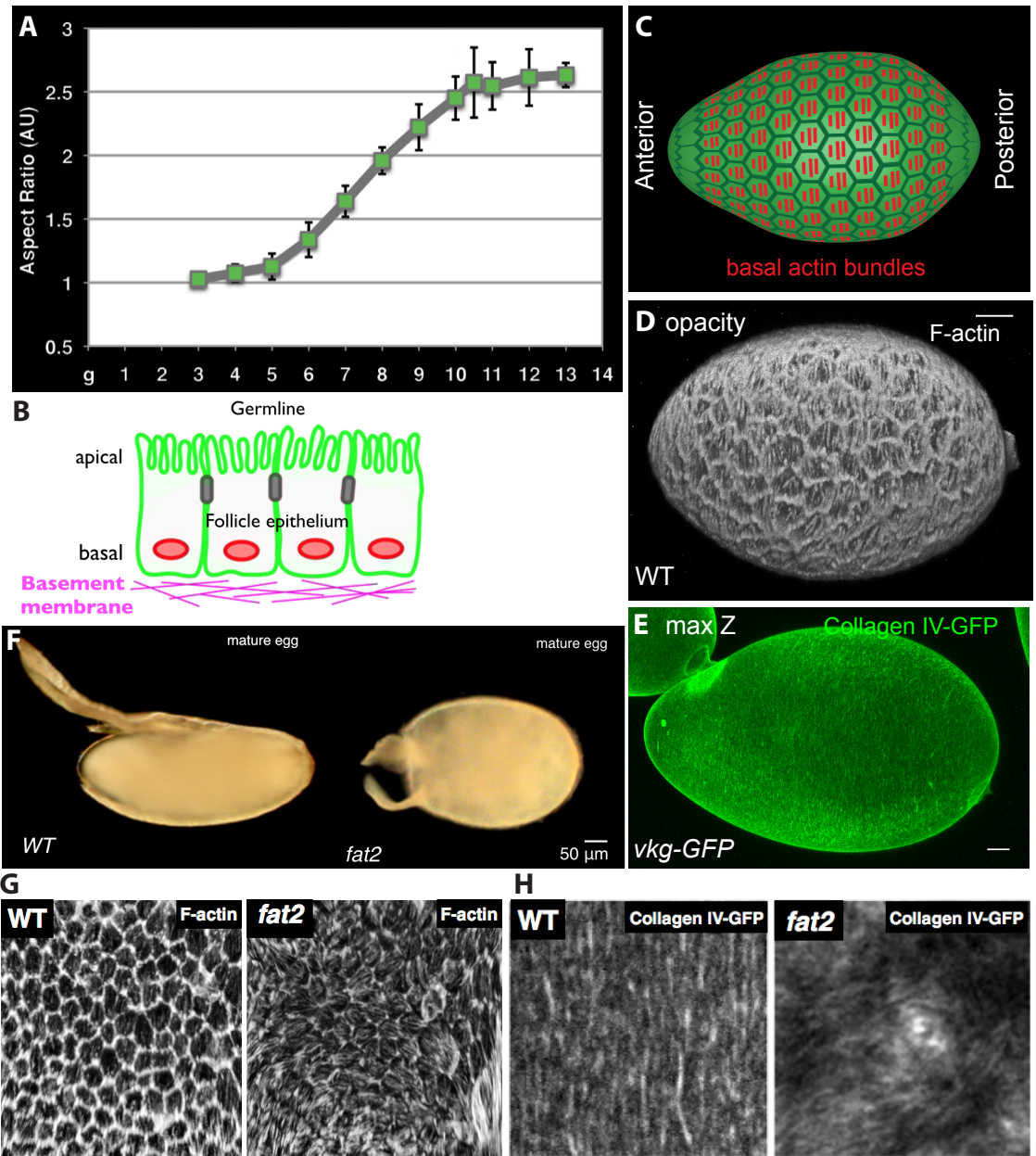
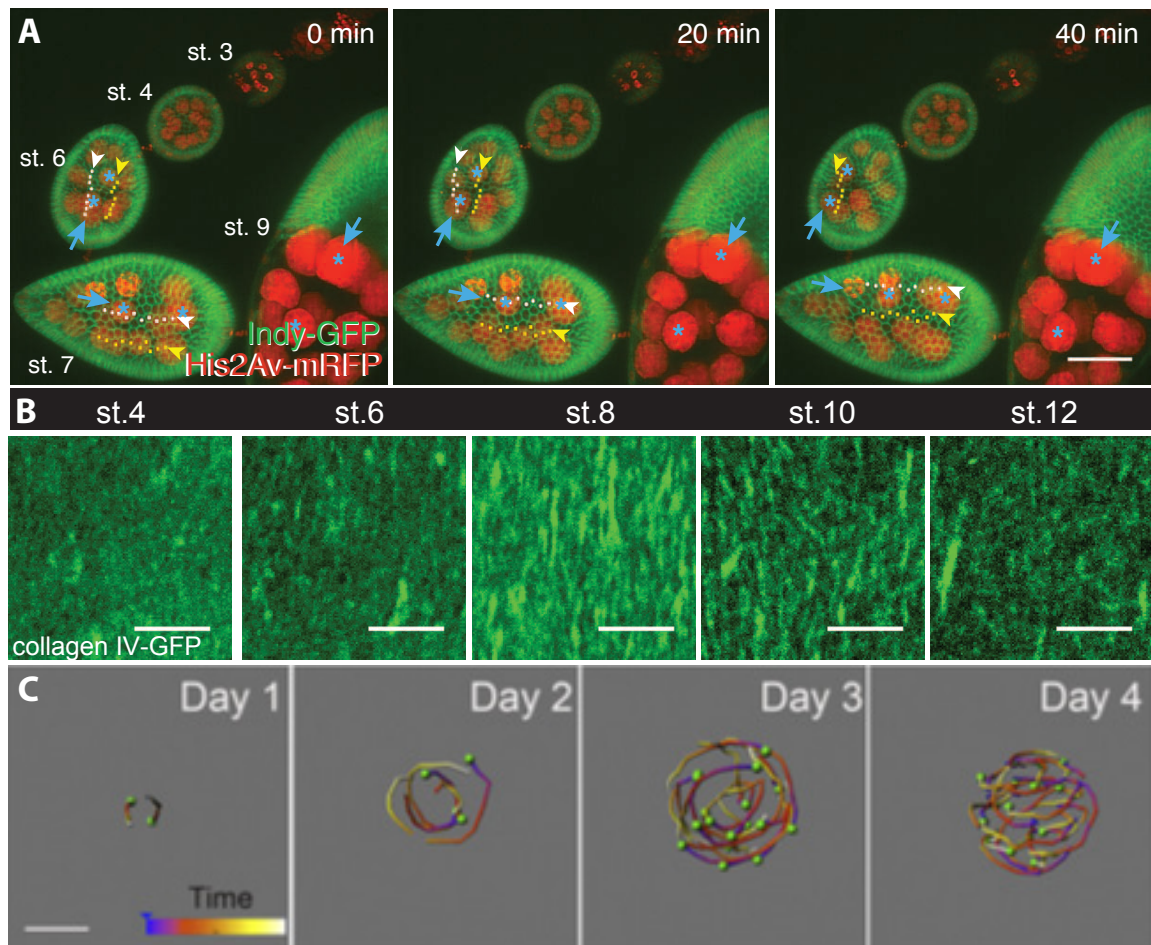
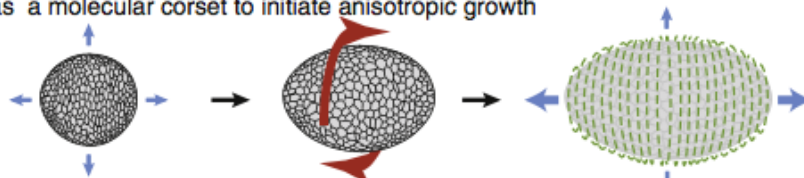


Fig. 1.6 Rotation builds a polarized BM

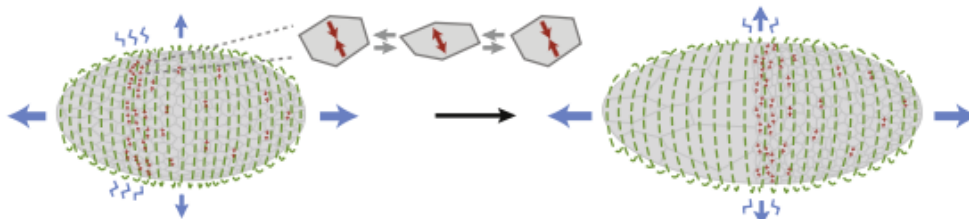
(A) Follicle rotation. Follicles with epithelial membrane marked in green and all nuclei, including the germ cells in red. Follicle cells collectively migrate circumferentially around the A-P axis. Subset of cells were manually tracked with blue dots and yellow dots across multiple time points. Images adapted from (Haigo and Bilder, 2011). (B) Polarized collagen IV fibrils are built during follicle rotation. Collagen IV fibrils at different stages of oogenesis. Scale bars: 5 μm . Images adapted from (Haigo and Bilder, 2011). (C) Rotational motion seen in the MCF-10A mammary epithelial cell in 3D culture. Image adapted from (Wang et al., 2013). (D-F) The molecular corset model revised. Follicle rotation builds a planar polarized collagen corset that serve as various function during different stages to elongate the follicle. Image adapted from (Bilder and Haigo, 2012).



D EARLY ELONGATION PHASE: Polarized tissue rotation builds planar polarized ECM fibrils that act as a molecular corset to initiate anisotropic growth



E MID ELONGATION PHASE: Actomyosin-driven polarized basal contractions, through interactions with polarized ECM fibrils, coordinate follicle cell morphogenesis with tissue elongation



F LATE ELONGATION PHASE: Polarized ECM fibrils maintain molecular corset function during continued anisotropic growth of egg chambers until the maturation of the eggshell at stage 12

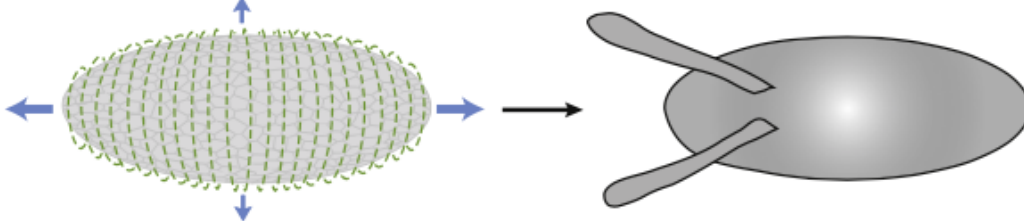
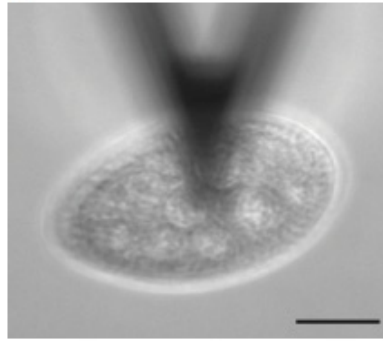
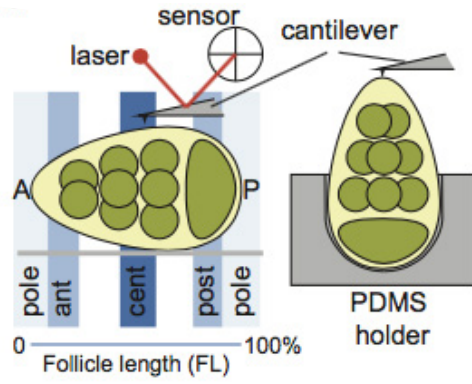
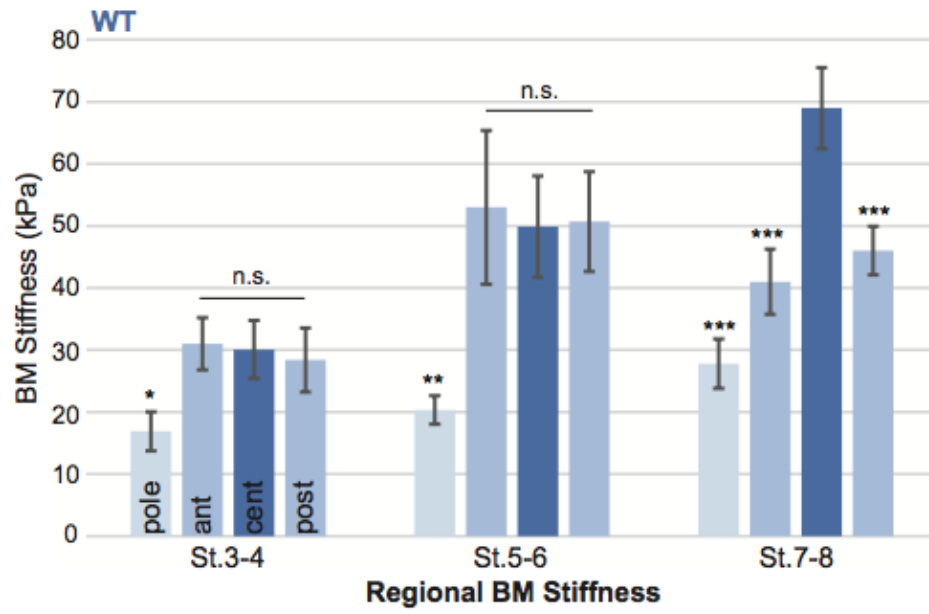


Fig. 1.7 A-P Patterned BM Stiffness Drive Follicle Elongation

Images showing BM stiffness measurement are adapted from (Crest et al., 2017). (A) Probing follicle BM stiffness with Atomic Force Microscopy (AFM). (B) Follicles are probed at different regions along the A–P axis. The poles are probed by using Polydimethylsiloxane (PDMS) holder to align follicles for AFM probes. (C) Regional BM stiffness along the follicle A–P axis; color intensity matches position as in(B).

A**B****C**

Chapter 2

Symmetry-breaking in an edgeless
epithelium by Fat2-regulated MT polarity

Symmetry-breaking in an edgeless epithelium by Fat2-regulated MT polarity

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Running Title: PCP in edgeless epithelia

2.1 Abstract

Planar cell polarity (PCP) information is a critical determinant of organ morphogenesis. While PCP in bounded epithelial sheets is increasingly well-understood, how PCP is organized in tubular and acinar tissues is not. *Drosophila* egg chambers (follicles) are an acinus-like 'edgeless epithelium' and exhibit a continuous, circumferential PCP that does not depend on pathways active in bounded epithelia; this follicle PCP directs formation of an ellipsoid rather than a spherical egg. Here we apply a novel imaging algorithm to 'unroll' the entire 3D tissue surface and comprehensively analyze PCP onset. This approach traces chiral symmetry-breaking to plus-end polarity of microtubules in the germarium, well before follicles form and rotate. PCP germarial microtubules provide chiral information that predicts the direction of whole-tissue rotation as soon as independent follicles form. Concordant microtubule polarity, but not microtubule alignment, requires the atypical cadherin Fat2, which acts at an early stage to translate plus-end bias into coordinated actin-mediated collective cell migration. Because microtubules are not required for PCP or migration after follicle rotation initiates, while dynamic actin and extracellular matrix are, polarized microtubules lie at the beginning of a handoff mechanism that passes early chiral PCP of the cytoskeleton to a supracellular planar polarized extracellular matrix and elongates the organ.

2.2 Introduction

Animal organs undergo development not in isolation, but in the context of global body axes that form in the early embryo. The process of planar cell polarity (PCP) allows organogenesis to occur with reference to these axes. In PCP, cells acquire a common subcellular organization within the plane of the often epithelial tissue. PCP organization involves two features: alignment with respect to a given axis, and a polarized orientation along this axis. This organization allows coordinated morphogenesis with respect to anterior-posterior, dorsal-ventral, or proximal-distal elements of the body plan (Wallingford, 2012). Our understanding of PCP in morphogenesis comes almost exclusively from tissues that approximate flat sheets, and are generally treated as bounded. In paradigmatic tissues such as *Drosophila* wing, notum, eye and abdomen, PCP is organized by gradients of factors that specify global directional cues within the epithelium (Goodrich and Strutt, 2011; Vladar et al., 2009). However, many organs contain tubular and acinar structures whose topology can be considered, in part, unbounded, and in which similarly localized sources of cartographic information may not be present. PCP around the circumference of these ‘edgeless epithelia’ is likely to involve distinct mechanisms of organization.

The *Drosophila* egg chamber (also called the follicle) is an acinar tissue that exhibits a non-canonical form of PCP (reviewed in (Bilder and Haigo, 2012; Cetera and Horne-Badovinac, 2015)). This PCP is seen within the ‘follicle epithelium’ that encases the germline. Like paradigmatic PCP tissues, the follicle epithelium exhibits polarized cytoskeletal organization that is coordinated between cells. Basal F-actin filaments align within each follicle cell around the circumference of the tissue, perpendicular to the A-P axis. Alignment of microtubules can also be seen, as well as of fibril-like structures of extracellular matrix in the basement membrane. This organization drives a coordinated collective cell migration that leads the entire tissue to rotate circumferentially in a PCP fashion around the A-P axis. In a further parallel to many PCP tissues, follicle PCP drives A-P tissue elongation, changing shape of the growing egg chamber from an initial sphere to the distinctively ellipsoid egg. However, unlike paradigmatic PCP tissues that are polarized toward specific tissue boundaries, follicle PCP is continuous around the circumference of the tissue. Moreover, follicle PCP does not rely on Fat/Ds or Fz/Vang, the two major signaling pathways that direct PCP in bounded tissues (Viktorinová et al., 2009).

While mechanisms controlling tissue rotation continue to be elucidated, how PCP symmetry in the follicle is broken, and how this directs coordinated cellular motility of the tissue remains unclear. In this work we identify the earliest sign of PCP in the follicle and show that chiral polarity of MTs in the follicle precursors, rather than the follicle itself, is regulated by the atypical cadherin Fat2 and sets the template for subsequent PCP organization, tissue rotation, and organ elongation.

2.3 Results

2.3.1 PCP rotation begins immediately following follicle budding

In order to investigate the origins of follicle PCP, we sought to examine its initial stages. Follicles form in region 3 of the germarium, where ~30 progeny of somatic stem cells residing in region 2b surround a germline cyst and establish a contiguous epithelium (**Supplemental Fig. 2.1**) (Nystul and Spradling, 2010b). This structure, also called a stage 1 follicle, then progresses through 13 additional developmental stages to create a mature egg (Spradling, 1993). Our previous work described robust rotation at stage 5 but did not identify the time when it initiates (Haigo and Bilder, 2011). Live imaging demonstrates that follicles rotate, perpendicular to the A-P axis and with a stable chirality, shortly after they are formed (**Fig. 2.1A**; **Supplemental Movie 2.1**). (Cetera et al., 2014) were first to report early follicle rotation, placing it at st. 1; we do not detect consistent axial movement at this stage. However, rotation was seen reliably at stage 2, after the stalk completes budding of the follicle from the germarium (**Fig. 2.1A-F**). We live-imaged the stage 1 to stage 2 transition and compared the timing of rotation initiation to the production of the follicle basement membrane (BM) over which the epithelial cells move. BM surrounds the stage 1 follicle at all regions except the anterior pole; BM is deposited in this region during stage 2 to isolate the follicle from the anterior stalk cells (**Fig. 2.1D**). Follicles in which Collagen IV (assessed by an α 5 chain (Flybase:Vkg)-GFP fusion protein) was not detected between the epithelium and the anterior stalk did not progressively rotate, but 92% (n=12) of follicles with continuous anterior Collagen IV-GFP rotated (**Fig. 2.1D-F**). Once initiated, the absolute speed of rotation increased over time, from 0.2 $\mu\text{m}/\text{min}$ at stage 2 to 0.5 $\mu\text{m}/\text{min}$ at stage 6 (measured at the equator, **Fig. 2.1B**). Interestingly, while rotation speed increased, the angular velocity of rotation was much less changed, approximating 40 degrees/hour from stage 2 to stage 6 (**Fig. 2.1C**). As follicles rotate with a determined alignment and orientation from the time that they first become isolated from external cells, the PCP symmetry-breaking that directs rotation must originate prior to follicle formation.

2.3.2 Alignment of microtubules and actin precede PCP rotation

To identify the earliest molecular sign of follicle PCP, we examined the germarium, including stage 1 follicles, for proteins known to show PCP organization at later stages. An obstacle to comprehensive quantitative analysis of the germarium is its architecture. Like many tubular and acinar tissues, the germarium and early follicle have highly curved surfaces. Confocal sections of such tissues capture only the tangential fraction of the epithelial plane, while volumetric projections provide 3D visualization but not quantification along the curved surface; moreover, these approaches overrepresent the equator and miss the poles (**Fig. 2.2A**). To comprehensively and accurately assess PCP in the follicle epithelium, we employed a newly-developed imaging algorithm that can accurately analyze the surfaces of contoured objects. This algorithm, ImSAnE (Imaging Surface Analysis Environment), extracts the plane of the epithelium from three-dimensional stacks and projects it two-dimensionally; projections can be chosen to maintain area or orientation, while geometric

observables, generally distorted in projections, can be correctly quantified using built-in correction methods (**Fig. 2.2B**) (Heemskerk and Streichan, 2015). Nematic order parameters (S , see Experimental Procedures), as well as angle of orientation to axes with standard deviations can then be automatically assessed. Values for PCP elements along the entire basal surfaces of the large stage 6 follicles correspond to those previously published (**Fig. 2.2C**) (Cetera et al., 2014; Viktorinová and Dahmann, 2013). Using ImSAnE, we confirmed the high degree of F-actin alignment in region 2b first reported by Frydman (**Fig. 2.2D-F**) (Frydman and Spradling, 2001). Notably, MTs are also strongly aligned in this region, with order parameters that parallel those of F-actin (**Fig. 2.2E-F**). Alignment of both actin and MTs is reduced but not lost as the follicle forms. Nematic order parameters, which are > 0.9 at stage 1, drop below 0.8 during stage 4 before recovering to 0.9 at stage 5; the standard deviation of nematic angles increases when order parameters drop. By contrast to cytoskeletal elements, we were unable to detect polarized organization of Rab10, Msn, or Ena in the germarium (Cetera et al., 2014; Lerner et al., 2013; Lewellyn et al., 2013). These data emphasize that coordinate alignment of the cytoskeleton precedes PCP rotation as well as PCP organization of several other molecular markers.

2.3.3 MTs are required in the germarium prior to PCP rotation

To assess the functional role of early cytoskeletal organization in follicle PCP, we employed genetic and chemical approaches. A forward genetic screen using transgenic RNAi expression specifically within the somatic gonad (unpublished results) identified several cytoskeletal regulators whose knockdown induced defects in follicle elongation. As expected from actin's well-established role in cell motility, these included regulators of actin organization. Disrupting dynamic actin by knocking down the SCAR complex regulators Hem (also known as Kette/NAP1) or Abi throughout follicle development prevents rotation and leads to round eggs (**Fig. 2.3E, Supplemental Fig. 2.2**) (Cetera et al., 2014; Chen et al., 2014). Sra-1 depletion had a similar effect, although it displayed a more general disruption of cortical F-actin that hindered analyses of molecular PCP. Acute chemical inhibition using the Arp2/3 inhibitor CK-666 demonstrated that Arp2/3-initiated actin polymerization is required during stages 1-5 for PCP follicle rotation (**Fig. 2.3E**) (Cetera et al., 2014).

Knockdown of the MT stabilizing protein CLASP (Flybase: Chromosome Bows) caused a potent loss of MT bundles, with minor effects on the formation of F-actin filaments, in both germaria and stage 1 follicles (**Fig. 2.3A, B**). Strikingly, CLASP-depleted follicles failed to rotate and gave rise to round follicles with mispolarized F-actin, as well as later to round eggs (**Fig. 2.3B-D**). Disrupting MTs by overexpressing the MT-severing protein Spastin also induced round eggs (not shown). Moreover, conditional RNAi expression revealed that CLASP knockdown after stage 3 slows but does not prevent rotation (**Fig. 2.3E**) and leads to only mildly round eggs (**Fig. 2.3F**). We confirmed these data by using chemical inhibitors. When monitoring follicles from colchicine-fed flies, we found that no stage 2 follicles initiated rotation (**Fig. 2.3E**). However, follicles that were at stage 3 and later when colchicine feeding commenced continued to rotate on-axis, albeit at a slower rate (**Fig. 2.3E**).

(Viktorinová and Dahmann, 2013). These data suggest that, while MTs are not required for cell motility driving follicle rotation per se, they are required at stage 1-2 to initiate it.

2.3.4 Germarial Fat2 regulates both rotation initiation and maintenance but not cytoskeletal alignment

Since MTs are required to initiate PCP follicle rotation, we considered potential regulators of germarial PCP organization. The atypical cadherin Fat2 is required to initiate rotation (Cetera et al., 2014) and for egg elongation and cytoskeletal PCP, but its role in the latter has been largely inferred from phenotypes seen ~16 hours after rotation has initiated (Viktorinová et al., 2011; Viktorinová et al., 2009). We identified RNAi lines that knockdown *fat2* either strongly or mildly; the strong knockdown line phenocopies the *fat2* null phenotype with 100% penetrance (**Supplemental Fig. 2.3**). We examined strongly *fat2*-depleted germaria and found that they failed to rotate at stage 2, despite stalk and basement membrane morphogenesis comparable to WT, and shortly after this stage, PCP organization fails to align (**Fig. 2.4A-B, Supplemental Movie S2.2**). To define the requirements for Fat2 in initiation and maintenance of follicle rotation, we employed conditional knockdown, with the limitation that we were unable to directly assess the completeness of Fat2 depletion. When follicles raised at permissive conditions and initiating rotation were shifted to knockdown *fat2* at st. 3 and following, they slowed rotation within 8 hours and arrested after 12 hours; the resultant eggs showed substantial elongation defects. If *fat2* was instead knocked down prior to stage 3, follicles always failed to rotate and gave rise to round eggs, even when shifted to permissive conditions that terminated *fat2* knockdown for the remaining 40 hours of oogenesis (**Fig. 2.4C,D**). These results indicate an absolute requirement for Fat2 in both rotation initiation as well as maintenance, as well as elongation, following st. 2. Despite this, we found no significant changes in the circumferential alignment of either actin or MT in *fat2*-depleted or null as compared to WT germaria (**Fig. 2.4A, B**). Thus, Fat2 regulates rotation by a mechanism that is independent of germarial cytoskeletal alignment.

2.3.5 Fat2-regulated microtubule polarity in the germarium predicts follicle PCP chirality

To explore this mechanism, we took advantage of the mild *fat2* RNAi line to test genetic interactions. Knockdown of *fat2* using the mild line gives a moderately penetrant round egg phenotype: ~20% of laid eggs are round (**Fig. 2.4E**). This is a sensitized background, as heterozygosity for *fat2* enhances this phenotype to over 80%. Remarkably, heterozygosity for *CLASP* causes a similar degree of enhancement: over 80% of eggs laid by such females are round. This result suggests that Fat2 regulates follicle elongation through close functional ties to the MT cytoskeleton.

The intimate and essential roles of both Fat2 and MTs in initiating follicle rotation prompted us to examine early MT organization more closely. Since Fat2 did not regulate germarial MT alignment (**Fig. 2.4A, B**), we instead evaluated the polarized organization of

the MTs. MTs have an intrinsic polarity, with a fast-growing + end and a slow-growing – end. We used the + end tracking protein EB1 to assess the polarity of the aligned MTs within live stage 1 follicles (**Fig. 2.4F, Supplemental Movie S2.3**), and calculated a bias parameter in which 0 represents unbiased MT+ end growth, while 1 represents all MT+ ends growing in the same direction. Interestingly, this analysis revealed a significant ($p < 0.003$) bias in the chirality of MT + end growth, and nearly 2/3 of germaria displayed a bias of 0.2 or greater (**Fig. 2.4G**). Moreover, the chirality of this MT bias was not identical in all stage 1 follicles. With respect to the ovariole A-P axis, the ratio of dextral (clockwise) to sinistral (counter-clockwise) MT +end biases seen in follicles prior to rotation was ~50% (**Fig. 2.4H**). This corresponds to the ratio of chiralities of rotation seen in later stage follicles (**Fig. 2.4H**). To determine the relationship between MT bias at stage 1 and chirality of rotation once it initiates at stage 2, we carried out live imaging on EB1-expressing budding follicles. Of follicles that remained healthy throughout imaging, 7 of 8 showed a, germarial MT bias exceeding that of *fat2*, and 100% of these later rotated with a chirality opposite to their earlier +-end bias ($n=7$; $p < 0.01$) (**Fig. 2.4I, Supplemental Movie 2.S4**). Strikingly, despite the fact that MT bundles in *fat2* mutant follicles retain PCP alignment at stage 1, their biased orientation was lost, with MT + end organization randomized (**Fig. 2.4G**). These results indicate that Fat2 acts in the germarium to organize MT chirality, and suggest that polarity of MT in the developing proto-follicle is the symmetry-breaking event that sets subsequent PCP including the direction of rotation after organ formation.

2.4 Discussion

In this work we identify a central symmetry-breaking role for microtubule polarity in PCP of an ‘edgeless’ epithelial organ. MTs are the earliest PCP molecule during follicle development, germarial MT polarity predicts the chirality of subsequent follicle PCP events, and disruption of either MT alignment or polarity in the germarium prevents all subsequent aspects of follicle PCP, including the coordinated cell motility that initiates follicle rotation. These requirements for MTs are not due to secondary effects on actin, which retains its organization in germaria with disrupted MTs. Importantly, unlike actin, which is required acutely and constantly for collective cell migration, MTs are not strictly required for follicle cell motility once rotation has initiated. We therefore propose that MTs provide the initial source of PCP information in the early follicle, and that actin, through its role in promoting tissue rotation, serves to amplify and propagate PCP.

Our data demonstrate that the MT polarity bias in the forming follicle predicts the chirality of PCP tissue rotation that initiates ~10 hours later. It was recently reported that, in st. 4 follicles, MT + end orientation anticorrelates with rotation direction at st. 7 (Viktorinová and Dahmann, 2013). However, in that work, stage 4 and earlier follicles were thought to represent pre-rotation stages, contrary to our identification here of rotation initiation at st. 2 and that of Cetera et al. (2014) who place it at st. 1. Since both MT alignment and polarity are present in the germarium, the st. 5 correlation is not predictive and reflects pre-existing PCP information rather than revealing its source. In the absence of an

independent and direct manipulation of MT + end orientation, we cannot conclusively state that the MT polarity that we document in the germarium is instructive for rotational direction. Nevertheless, the strong correlation between this chirality and the subsequent direction of follicle rotation, along with disruption of both in the absence of *fat2*, point to a model in which coordination of MT polarity in cells across the follicle is required for a unidirectional consensus amongst individual motile cells to initiate productive rotation.

Our data thus suggest that the atypical cadherin Fat2, a key regulator of follicle PCP, rotation, and elongation, acts through effects on early MT polarity. Through analyses of middle stages of follicle development, it has been argued that Fat2 regulates global PCP alignment of the cytoskeleton, as does the canonical PCP regulator Fat in the developing *Drosophila* wing (Harumoto et al., 2010; Viktorinova et al., 2011; Viktorinová et al., 2009). However, in *fat2* germaria, actin and MT alignment are maintained; it is coordinated MT polarity that is lost. These phenotypes, along with its strong genetic interaction with CLASP, argue that Fat2 promotes rotation initiation and follicle PCP via its effects on MT polarity. We have not excluded an additional role in actin regulation other than polarized alignment; the requirement for Fat2 and actin but not MTs to maintain rotation, as well as the direct binding of actin regulators by vertebrate Fat1, a possible ortholog of *Drosophila* Fat2 (Moeller et al., 2004; Tanoue and Takeichi, 2004), suggests such a role. Moreover, while this work was in revision, Squarr et al. (2016) showed that Fat2 can directly influence the actin cytoskeleton via binding to the WAVE complex.

As with PCP in the developing *Drosophila* wing disc (Matis et al., 2014; Olofsson et al., 2014; Shimada et al., 2006), the initial PCP bias provided by MT polarity within the early follicle precursors is mild, but becomes more robust as organogenesis progresses. A mechanism for amplification in the follicle involves whole-tissue rotation. Preventing rotation by disrupting actin or integrins causes a rapid loss of all PCP organization primed in the germarium (Bilder and Haigo, 2012; Cetera and Horne-Badovinac, 2015). Interestingly, just as MTs are largely dispensable for PCP after actin PCP becomes established (**Figs. 2.3 and 2.4**), actin PCP is dispensable after circumferentially aligned ECM becomes established (Cetera et al., 2014). Hence, PCP transitions from highly dynamic and intracellular MTs, to longer-lasting and sometimes juxtacellular actin filaments, and then finally to the durable ECM fibrils that span multiple cells. PCP information in the follicle is therefore passed along by a ‘handoff’ mechanism, to increasingly stable as well as larger scale components that can ultimately exert force to shape the organ.

Non-centrosomal microtubule arrays, and in particular their regulated polarized organization, have previously been implicated as central governors of global PCP in tissues such as *Drosophila* wings, zebrafish gastrulae, and mammalian airway epithelia (reviewed in Galic and Matis, 2015; Sepich et al., 2011). In ‘edged’ PCP tissues in *Drosophila*, a ‘global PCP’ module molecularly controlled by Fat is thought to use gradients of positional information from specific sources to bring individual cell PCP in alignment with overall body axes (Goodrich and Strutt, 2011; Vladar et al., 2009). In the circumferential ‘edgeless’ PCP axis of the follicle epithelium, where no such graded information is known, Fat2 seems to similarly

coordinate the PCP of individual cells. That both contexts involve important roles for polarized MTs and are controlled by related protocadherins raises the possibility of ancient links between the modes of epithelial PCP organization.

2.5 Acknowledgements

We thank Christian Dahmann, Nasser Rusan, Nina Tang Sherwood and Jeff Axelrod for sending reagents, and are grateful to Boris Shraiman and the Bilder lab, especially Justin Crest, for helpful discussions. We also acknowledge Lin Yuan, who first documented stage 2 follicle rotation. We thank the TRiP at Harvard Medical School (NIH/NIGMS R01-GM084947) and the Bloomington Stock Center for providing transgenic RNAi fly stocks used in this study. KRL is an American Heart Association graduate fellow. This work was supported by NIH RO1 GM068675 to DB.

2.6 Author Contributions

DYC, KRL, and YD conducted the experiments; SJS developed analytical tools; NYC, KRL, and DB designed the experiments and wrote the paper.

2.7 Experimental procedures

RNAi-mediated depletion in follicle cells used a Gal4 insertion in *tj* (Li et al., 2003) with UAS-RNAi transgenes. Conditional depletion was carried out in combination with *tubGAL80^{ts}*, using 18° as restrictive temperature and 29° as permissive temperature. Live follicles were cultured ex vivo under previously described conditions (Haigo and Bilder, 2011), and imaged on a Zeiss 700 confocal microscope. EB1-GFP comets were analyzed using Fiji (Schindelin et al., 2012). ImSAnE analysis was executed on full stacks of confocal sections, and nematic order was analyzed as described in **7. Supplemental Experimental Procedures**, where additional genetic and imaging details are provided.

2.8 Supplemental Experimental Procedures

2.8.1 Fly Strains, culture, and drug treatments

All flies were maintained at 25°C unless otherwise indicated. *fat2^{58D}* and *fat2^{103C}* are gifts from Dr. Christian Dahmann. *UAS-EB1::GFP* is a gift from Nasser Rusan, and *UAS-SpastinEP* is a gift from Nina Tang Sherwood. RNAi strains against the following genes were generated by the Harvard TRiP: *Fat2* (BDSC #40888), *CLASP* (BDSC #34669), *Hem* (BDSC #29406), *Abi* (BDSC #51455); *mCherry* (BDSC #35785) was used as a control. Mild *Fat2* knockdown was achieved using VDRC ID #27113. To temporally control knockdown, *tj-GAL4 tubP-GAL80^{ts}* was crossed to UAS-RNAi strains. Adult females were collected at 22° and shifted to 29°. Stage-specific temperature shifts were timed based on duration of stages of

ovarian development (Spradling, 1993), informed by the temperature dependence of *Drosophila* development. To depolymerize microtubules, adult flies were fed with yeast paste mixed with colchicine at 62.5 µg/ml (Sigma); to inhibit Arp2/3 activity, cultured ovarioles were treated with CK-666 at 500 µg/ml (Millipore). All flies were flipped onto fresh yeast daily to ensure no mid-stage follicle degeneration or egg retention.

2.8.2 Microscopy and Immunostaining

Ex vivo follicle culture was performed as previously described (Haigo and Bilder, 2011), with minor modifications. Images were acquired using an inverted Zeiss LSM700 with LD C-Apochromat 40x/NA 1.1 water-immersion lens. Other fluorescent images were acquired with a Zeiss LSM700 using Plan-Apochromat 40x/ NA 1.3 oil-immersion lens. Follicle and egg aspect ratios were calculated from images taken with an AxioImager.M1 (Zeiss) with 10x/NA 0.3 air objective lens; data was analyzed and displayed using GraphPad Prism 6 or Rstudio(Team, 2015) with ggplot2. Figures were assembled in Adobe Illustrator CS6.

Adult ovaries were dissected in PBS or Schneider's *Drosophila* medium (Gibco) and fixed with 4% formaldehyde in PBS for 15 min and stained as previously described. Stains with TRITC-phalloidin (Sigma) and DAPI (Molecular Probes) were mounted in SlowFade Antifade solution (Invitrogen). The following antibodies were also used: mouse monoclonal anti-acetylated- α -tubulin (1:300, Santa Cruz), and AlexaFluor-488, -563, and -633 conjugated secondary antibodies (1:400, Molecular Probes).

2.8.3 Image analyses and statistics

Follicle rotation velocities were either measured by the displacement of cell nuclei perpendicular to the AP axis, taken at the follicle equator, or plasma membranes stained with 5µg/mL FM4-64FX dye (Molecular Probes). Angular velocity was calculated using measured follicle maximal circumference. 3D Opacity projections were generated with Volocity (Perkin Elmer). Other images were processed in Fiji (Schindelin et al., 2012).

Orientation and alignment of the cytoskeleton was analyzed in fixed ovarioles stained with TRITC-phalloidin and anti-acetylated-tubulin, mounted on concave slides (Ted Pella) to prevent deformation. *in toto* images were collected with confocal microscopy with pixel width of 0.07-0.15 µm and voxel depth of 0.35-0.50 µm.

We used ImSAnE (Heemskerk and Streichan, 2015) to 'unroll' follicles and germaria. In brief, coarse identification of the Surface Of Interest (SOI) is followed by representing the SOI as a smooth mesh, equipped with a coordinate system for mapping to the plane. Images were first rescaled to uniform aspect ratio at the most fine resolution. The ovariole SOI was identified using the basal F-actin signal and the 'ilastik' (Sommer et al., 2011) segmentor interface implemented in ImSAnE, which produced a coarse point cloud. Meshlab (Cignoni et al., 2008) generated a smooth triangulation of the basal actin layer from the point cloud, following publicly available ImSAnE protocols. The ImSAnE CylinderMeshWrapper class automatically generates a coordinate system embedded in the triangulation for mapping the SOI to the plane: the long axis and azimuth around it are used as a discretized coordinate

pair on the surface and the image data interpolated at the corresponding embedding point of each discretized coordinate pixel, generating a planar projection of the data. Image contrast was enhanced with the contrast limited adaptive histogram enhancement (CLAHE) plugin (Fiji). To automatically determine local orientation of actin filaments, region 2 germaria and stage 1 follicles were first screened with a uniform raster of 20×20 px/ $4 \mu\text{m}^2$, overlapping by $2.23 \mu\text{m}$; for stage 2 and older, cells were segmented based on cortical actin signal. To determine the polarization axis of basal actin filaments *A* in boxes or segmented cells, we measured spatial frequency patterns using Fourier's method, from which a nematic order parameter defined as $S = 1/N \sum_{k=1}^N e^{2iA}$ was constructed. Orientation to the A-P axis (defined by polar cell position in early follicles, and long axis in germaria and elongated follicles) and the standard deviation (σ) of angles within a follicle were measured using the curvature-corrected dot product of unit vectors with corresponding orientation. Error bars in cytoskeletal alignment analyses represents SEM and statistical significance was assessed by Welch's unequal variances *t*-test. Error bars in charts for rotation velocity (**Figure 2.3E, 4C**), mature egg aspect ratio (**Figure 2.3F, 4D**), percentage round eggs (**Figure 2.4E**) represent standard deviation. Unless stated otherwise, statistical significance was assessed by Welch's unequal variances *t*-test.

2.8.4 EB1-GFP comet tracking

tj>UAS-EB1:GFP flies were raised on yeast for 5 days at 29°C , flipped onto fresh yeast for 6-12 hours. Ovarioles were dissected in modified Schneider's medium and mounted using low-melting point agar. Movies of basal EB1 comet growth were made on the Zeiss LSM700 with LD C-Apochromat 40x/NA 1.1 water-immersion lens. Individual EB1 comets were tracked manually in Fiji by generating kymographs perpendicular to the AP axis of the germarium and measuring the slope of EB1:GFP comet growth. Microtubule growth bias was calculated by the formula $|(D-S)/T|$ where D equals the number of dextral comets, S equals the number of sinistral comets and T is the total number of comets. Statistical significance was assessed by Unpaired T-test.

2.9 Supplemental Movie Legend

Supplemental Movies can be download online via this link:
[http://cell.com/cell-reports/fulltext/S2211-1247\(16\)30427-2](http://cell.com/cell-reports/fulltext/S2211-1247(16)30427-2)

Supplemental Movie S2.1, related to Fig. 2.1: Live imaging of ovariole (genotype: *vkgGFP, His2aVmRFP*) shows rotation commencing as follicle buds at st. 2.

Supplemental Movie S2.2, related to Fig. 2.4: Live imaging of *fat2*-depleted ovariole (genotype: *tj>fat2 RNAi; vkgGFP, His2aVmRFP*) shows failure to initiate rotation.

Supplemental Movie S2.3, related to Fig. 2.4: EB1-GFP comet imaging in the germarium.

Supplemental Movie S2.4, related to Fig. 2.4: Imaging of EB1-GFP stage 1-2 follicle during the initiation of rotation. MT growth bias for this follicle is 0.48 sinistral at st 1.

Figure 2.1. PCP rotation initiates during follicle budding.

(A) Stills (opacity projections) from live imaging of germarium through stage 4 follicles, with angular velocity (ω) of each given above. His2AV-mRFP (red) and Vkg-GFP (green) show nuclei and basement membrane respectively; colored dots mark tracked nuclei. The st. 1 follicle initially does not rotate, but between 72 and 132 minutes transitions to st. 2 and begins rotation **(B, C)** Maximum linear and angular velocity of rotating follicles ($n > 5$ for each stage, bars represent SEM). Color-coded bar indicates relative time length of stages of oogenesis. **(D)** BM closure during st. 1-2 transition, shown in single confocal section from live imaging. In the non-rotating st. 1 follicle, BM at the anterior is non-continuous (arrowheads). Rotation initiates after BM becomes continuous around the entire follicle. **(E, F)** Quantitation of relationship between BM completion and follicle rotation ($n = 11$, bars represent SEM).

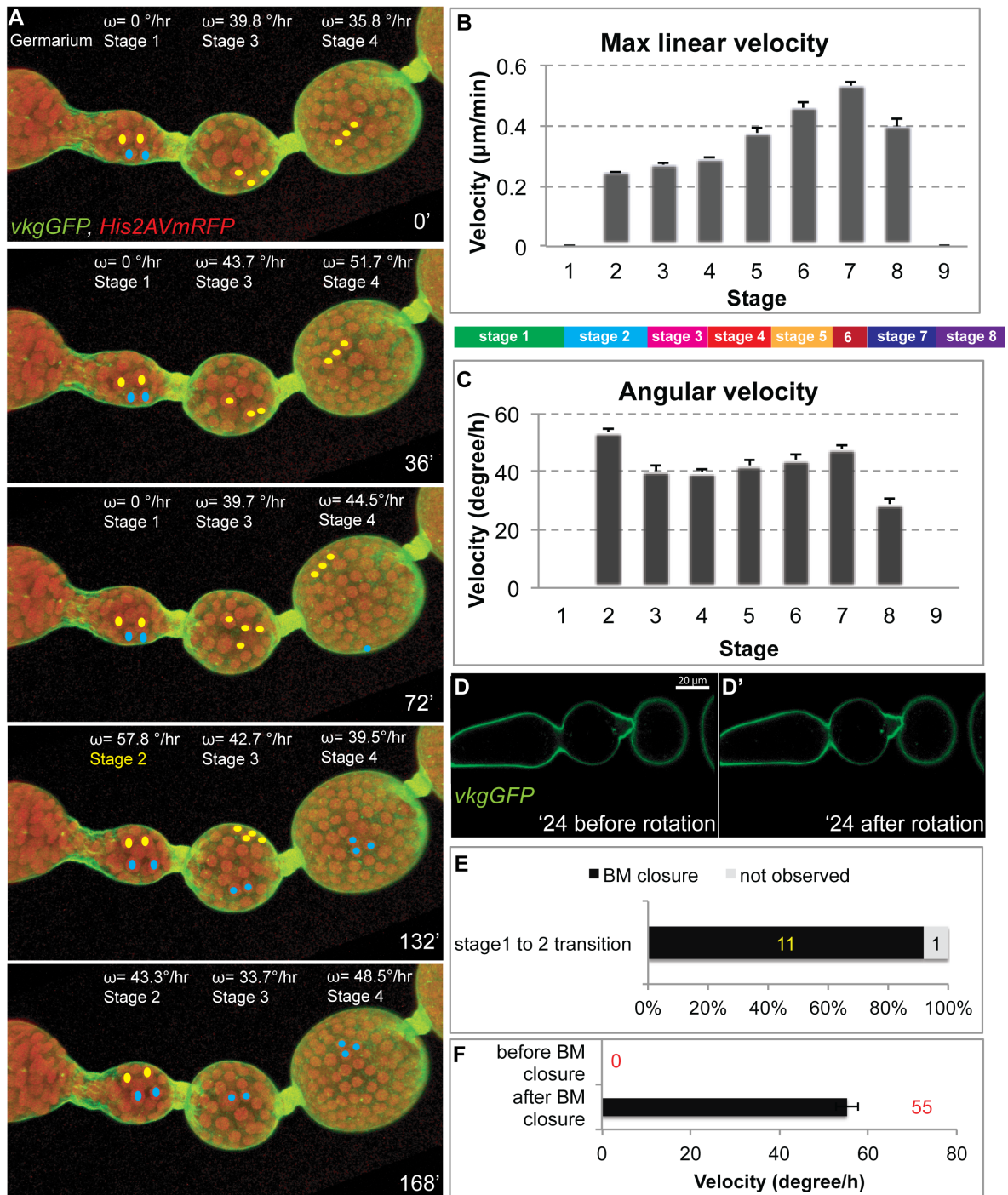


Figure 2.2. Germarial PCP revealed by ‘unrolling’ algorithm.

(A) Phalloidin-stained st. 6 follicle, shown in (A) single confocal section at basal surface, (A') maximum intensity projection of confocal stack, and (A'') 3D opacity projections. **(B)** Diagram of ImSAnE unrolling of the basal surface of the follicle. **(C)** ImSAnE unrolling of stage 6 follicle stained with phalloidin and (C') anti-acTub. Nematic order parameters (quantitated in upper left) demonstrate PCP organization. **(D)** Confocal cross-section of germarium stained with phalloidin (red) and anti-acTub (green). **(E)** ImSAnE unrolling of region 2b through stage 3 basal surface of ovariole in D. **(F)** Nematic order quantitation of MTs demonstrate dynamics of PCP organization in the germarium and early follicle. Data for Actin and acetylated Tubulin is shown to the immediate left and right (respectively) of each stage. Scale bars: 10 μ m.

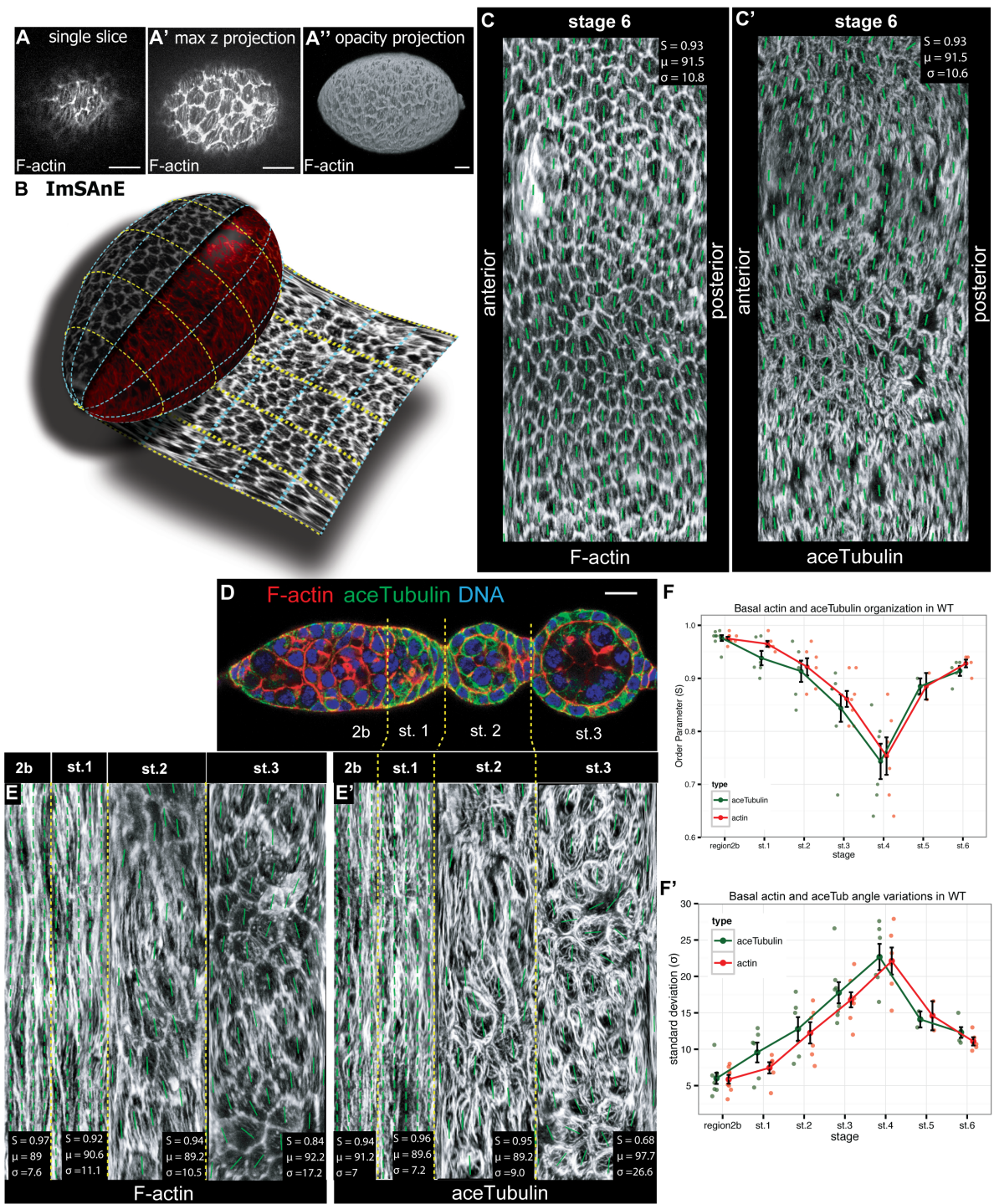


Figure 2.3. PCP MTs in the germarium direct rotation.

(A) ImSAnE unrolling of basal surface of *CLASP*-depleted ovariole, showing regions 2b through st. 3 stained with phalloidin and anti-acTub. **(B)** Nematic order shows intact actin alignment when MTs are disrupted. ($n \geq 5$ for each stages, $**p < 0.01$) **(C, D)** Control stage 9 follicle and mature eggs are elongated, while *CLASP-RNAi* stage 9 follicles and mature eggs are round. **(E)** Rotation speeds of st. 6-7 follicles, depleted at specified times using *tj > CLASP RNAi tubGAL80ts*, or treated with the MT polymerization inhibitor colchicine or the Arp2/3 inhibitor CK-666. **(F)** Quantitation of *tj > CLASP RNAi tubGAL80ts* egg shape.

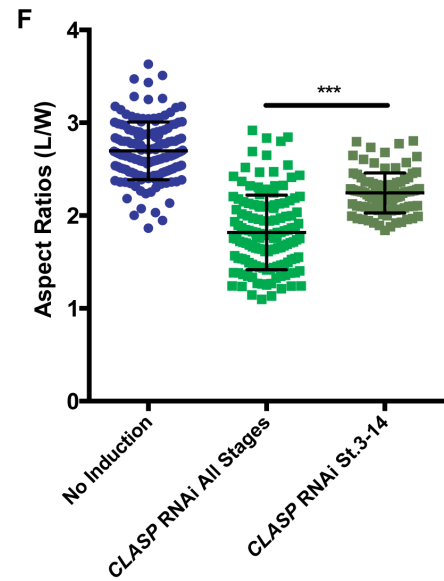
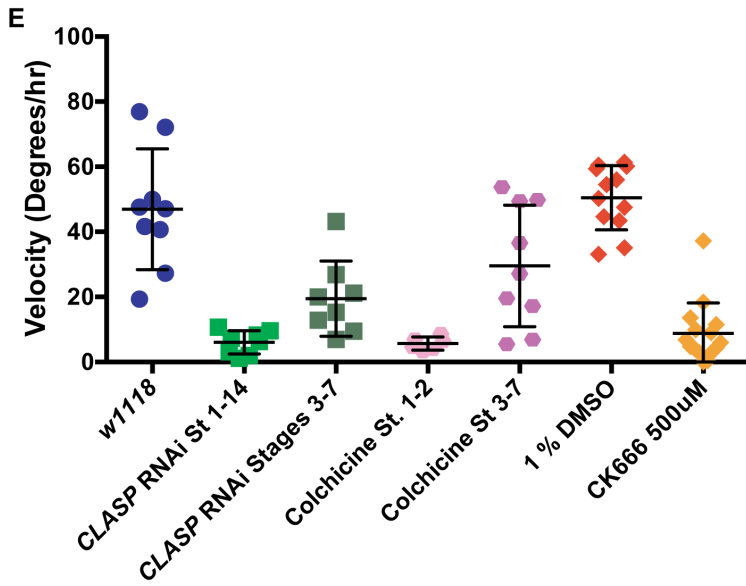
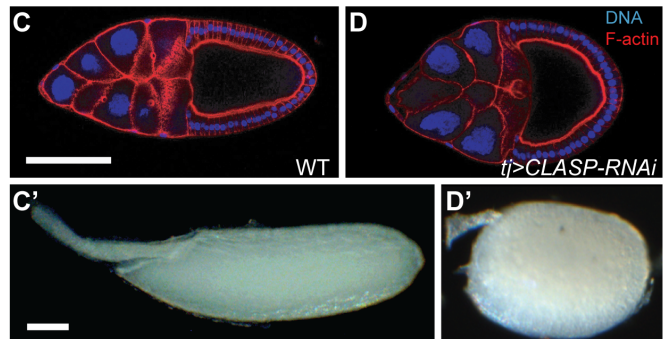
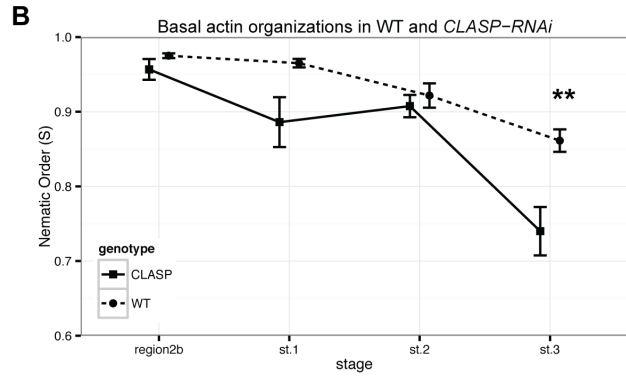
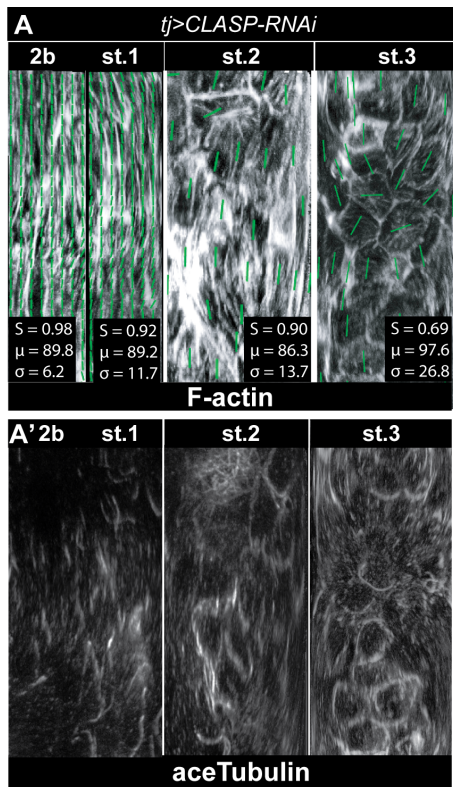
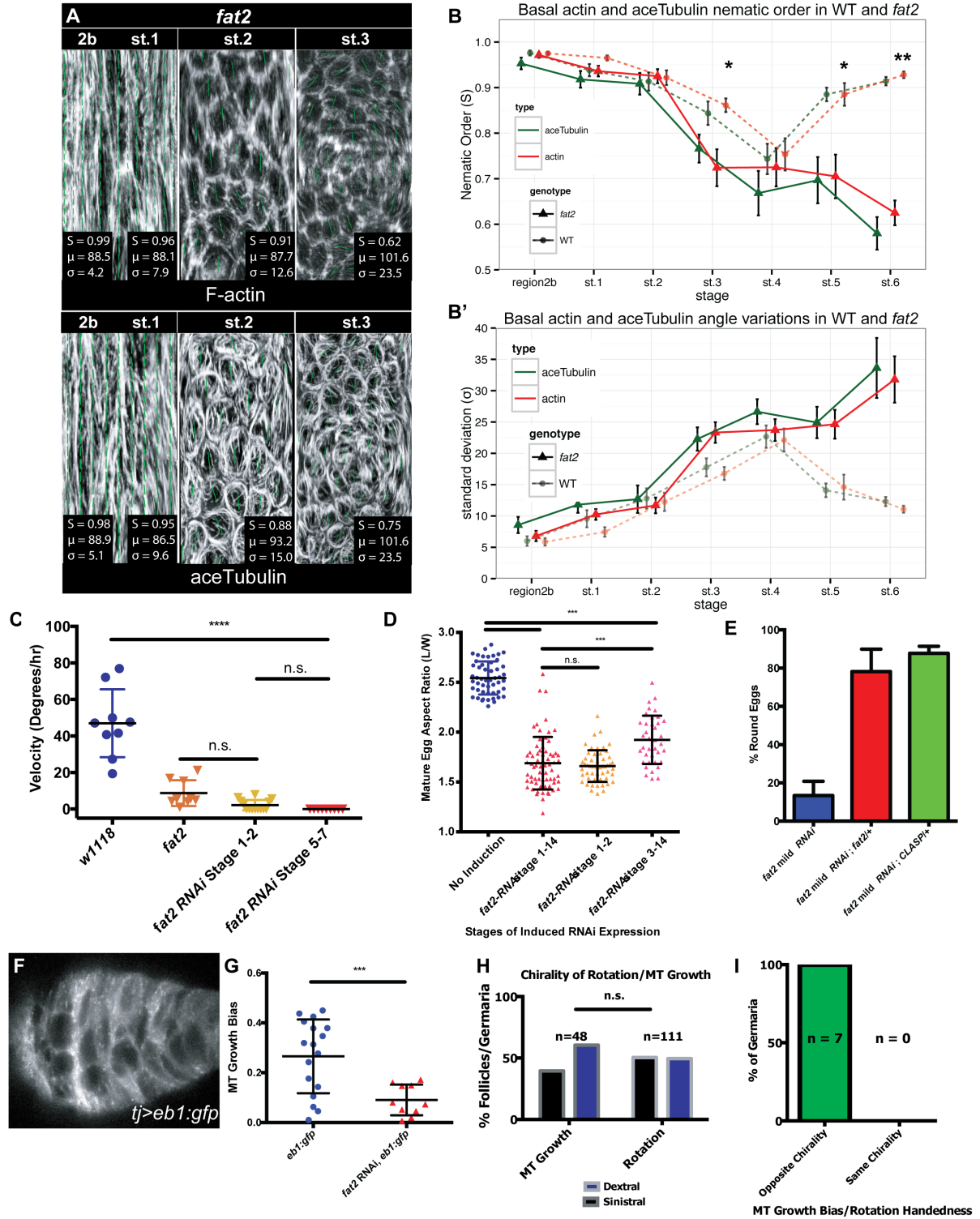


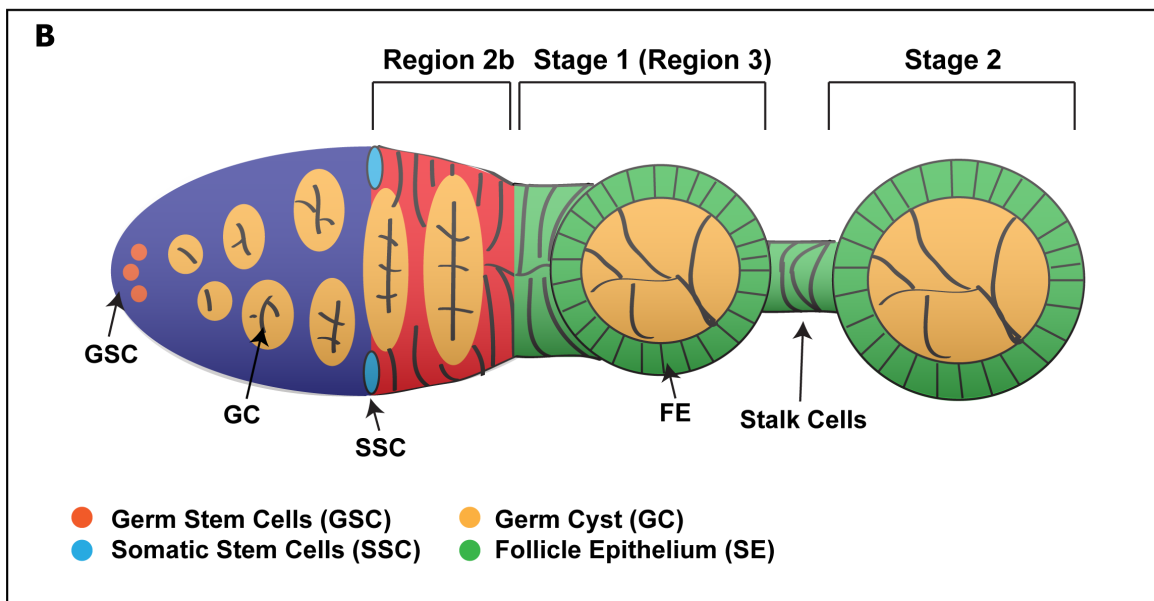
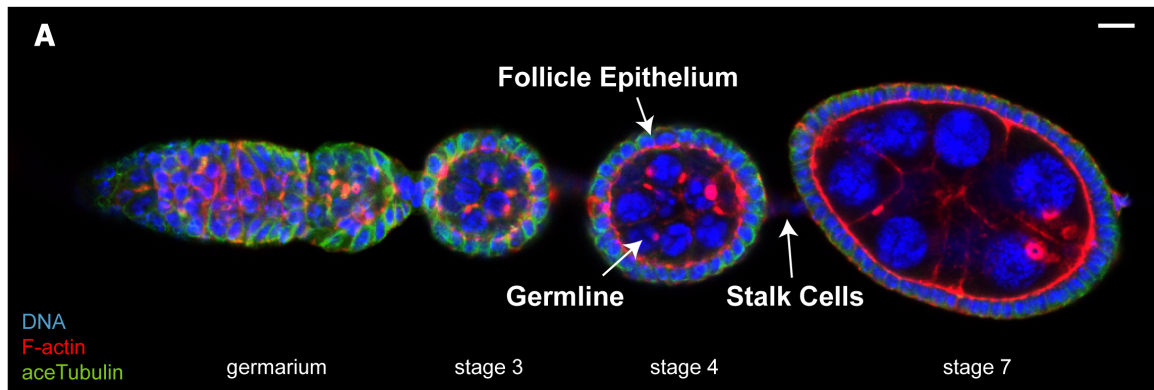
Figure 2.4. Fat2 regulates rotation initiation and MT chirality.

(A) ImSAnE unrolling of basal surface of *fat2*-depleted ovariole, showing regions 2b through st. 3 stained with phalloidin and anti-acTub. **(B)** Nematic order shows MT and actin alignment resemble WT until st. 2, but become disrupted following st. 3. **(C)** Rotation speeds at st. 7-8 of follicles depleted of *fat2* by RNAi at specified stages. Follicles depleted of *fat2* either prior to or following st. 3 fail to rotate. **(D)** Aspect ratios of eggs from conditional depletion of *fat2*, showing strong early and late requirements for egg shape. **(E)** Egg shapes from *fat2*-RNAi-mild genetic interaction tests. Heterozygosity for either *fat2* or *CLASP* enhances the round egg phenotype. **(F)** Still frame from live imaging of MT +end growth in WT st. 1 follicle. **(G)** Quantification of EB1 growth bias in WT and *fat2*-depleted st. 1 follicle (0=unbiased direction of growth, 1=fully concordant direction of growth), shows that significant MT growth bias in WT is lost when *fat2* is depleted. **(H)** Population-level MT growth biases at st. 1 and follicle rotation directions at st. 6 show similar proportions of chiralities. **(I)** Live imaging of EB1-GFP expressing follicles during st.1 to st. 2 transition reveals rotation with a chirality opposite to that of MT growth bias.



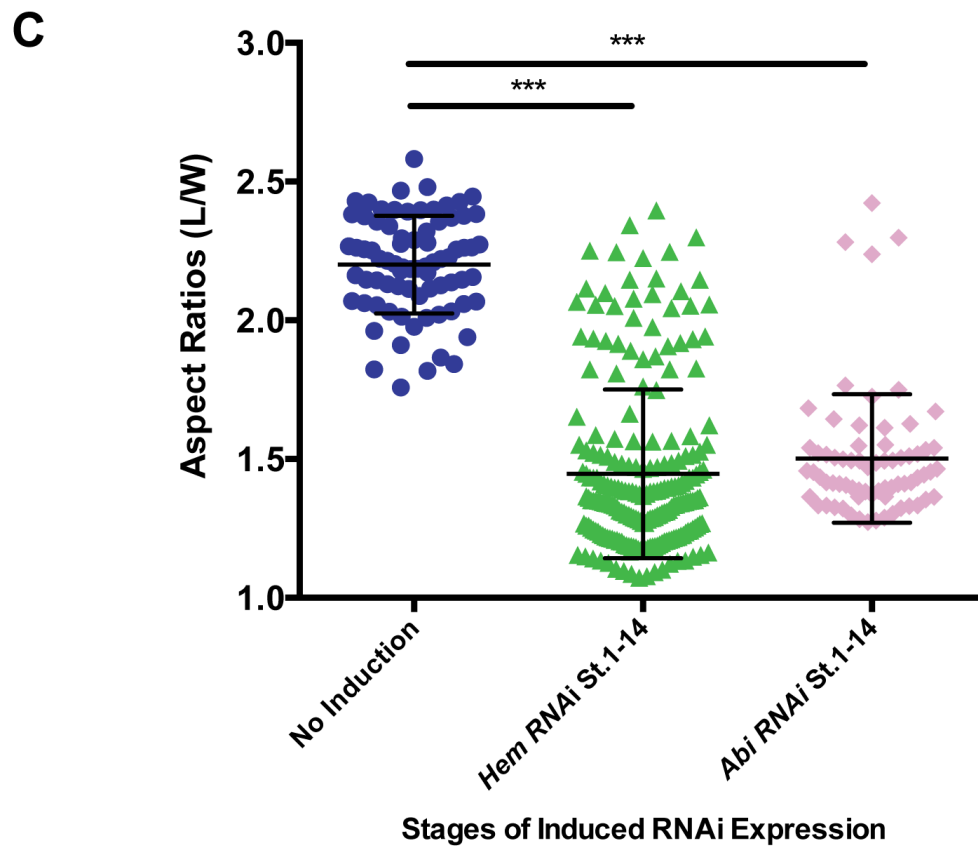
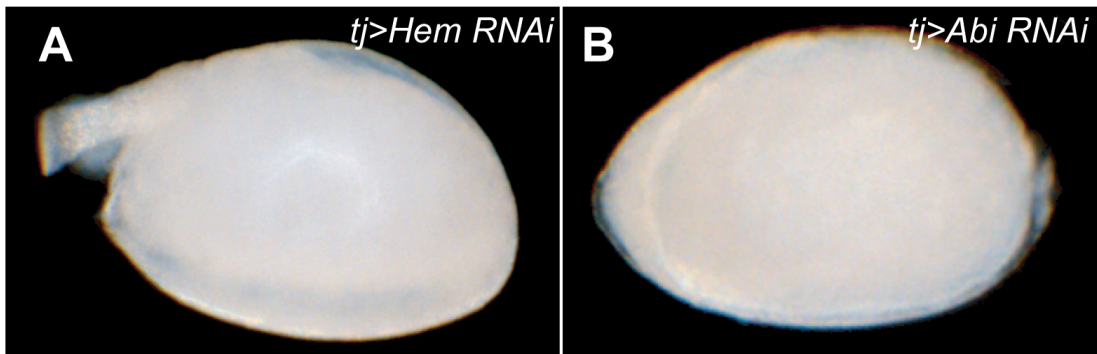
Supplemental Fig. 2.1: Early stages of follicle development (related to Fig. 2.1)

(A) Confocal section of anterior ovariole, stained for DNA (blue), F-actin (red) and acetylated tubulin (green). **(B)** Diagram of germarium and st. 2 follicle, showing position of stem cells, somatic and germline cells, and stalk cells.



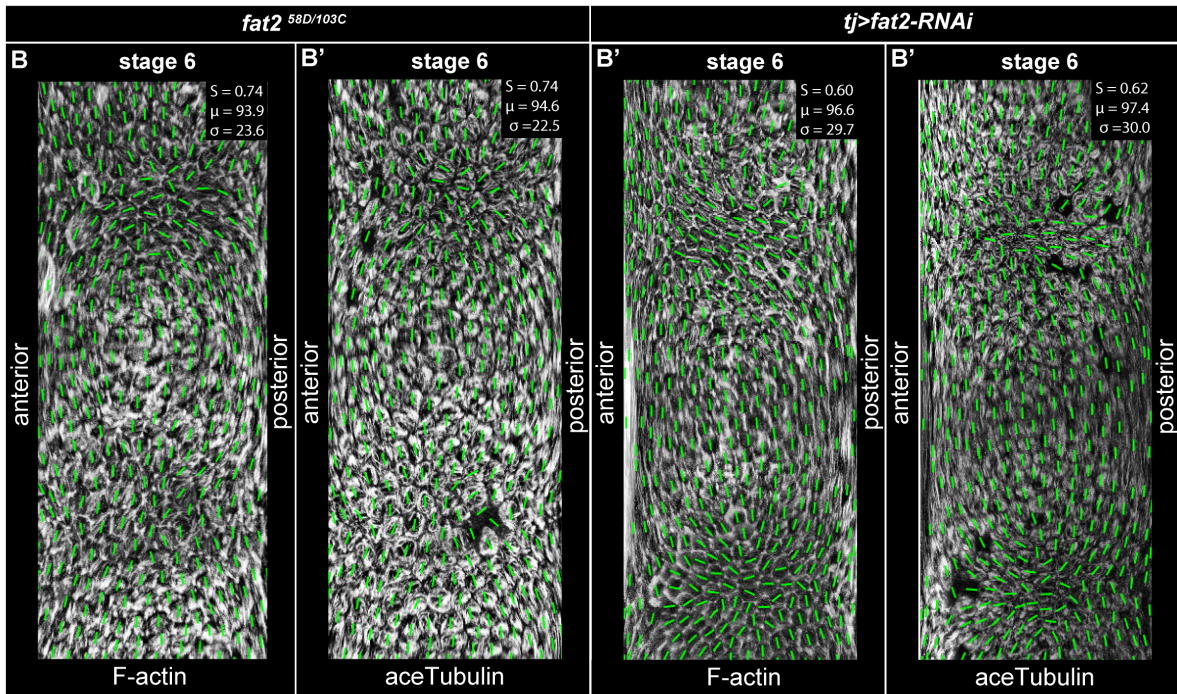
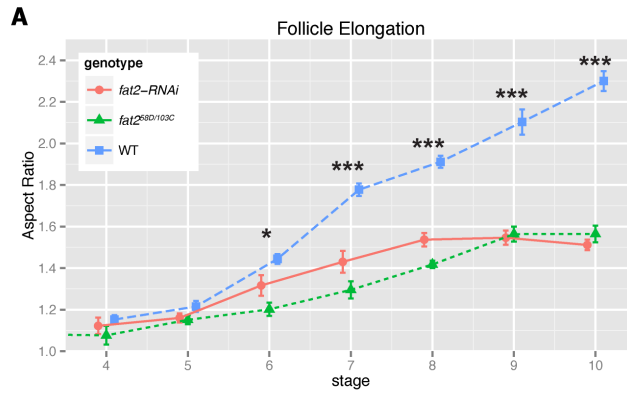
Supplemental Fig. 2.2: Arp2/3 regulators are required for egg elongation (related to Fig. 2.3)

Knockdown of Arp2/3 regulators *Hem* **(A)** and *Abi* **(B)** by *tjGAL4 UAS-RNAi* in genetic screen produces round eggs; aspect ratios are quantitated in **(C)**.



Supplemental Fig. 2.3: Characterization of *fat2 RNAi* phenotype (related to Fig. 2.4)

(A) Aspect ratio and **(B)** st. 7 actin alignment of *fat2* null mutant vs *tj>fat2 RNAi* follicles demonstrates effective depletion of *fat2* function by RNAi.



Chapter 3

A novel cell migration tracking tool
supports coupling of tissue rotation to
elongation

A novel cell migration tracking tool supports coupling of tissue rotation to elongation

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3.1 Abstract

Cell migration is indispensable to morphogenesis and homeostasis. Live imaging allows mechanistic insights, but long-term observation can alter normal biology, and tools to track movements *in vivo* without perturbation are lacking. We develop here a tool called M-TRAIL, which reveals migration histories in fixed tissues. Using clones that overexpress GFP-tagged ECM components, motility trajectories are mapped based on durable traces deposited onto basement membrane. We applied M-TRAIL to *Drosophila* follicle rotation, comparing *in vivo* and *ex vivo* migratory dynamics. The rate, trajectory, and cessation of rotation in WT follicles measured *in vivo* and *ex vivo* were identical, as was rotation failure in *fat2* mutants. However, follicles carrying intracellularly truncated Fat2, previously reported to lack rotation *ex vivo*, in fact rotate *in vivo* at a reduced speed, thus revalidating the hypothesis that rotation is required for tissue elongation. The M-TRAIL approach could be applied to track and quantitate *in vivo* cell motility in other tissues and organisms.

3.2 Introduction

Cell migration is critical to metazoan biology. Developing organisms require massive morphogenetic movements to shape their organs, while tissues that encounter physical or chemical challenges induce cell motility during immune responses, wound healing and regenerative processes to maintain homeostasis. In cancer, cells' acquisition of the ability to migrate and metastasize is a hallmark of progression. To understand these fundamental biological processes, tools to accurately track and quantitate cell motility in tissues are crucial.

Tracing movements of individual or collectively migrating cells in non-transparent systems *in vivo* remains a challenge. Advances in *ex vivo* preparations, allowing an explanted tissue to be imaged for a period of hours, allow motility to be studied at cellular resolution (Shamir and Ewald, 2014). Nevertheless, few current techniques fully recapitulate development or homeostasis over long periods of time. Furthermore, imaging *per se* can lead to artifacts that may alter biological processes, via heat production, phototoxicity, and ROS generation (Liu et al., 2015). Overall, tools allowing long-term tracing of cell migration routes and velocities in intact but opaque organisms are highly limited.

One biological system that exemplifies the above is the *Drosophila* follicle (egg chamber). This epithelium-encased organ develops in the adult female abdomen, and undergoes a whole-tissue collective cell migration that spans several days (Bilder and Haigo, 2012; Cetera and Horne-Badovinac, 2015). This migration leads to rotation of the entire organ through multiple rounds of revolution, and has been proposed to drive its elongation from spherical to ellipsoid (Haigo and Bilder, 2011). Because of its long-term nature and internal site, follicle rotation and its dynamics have only been imaged and quantified through explants in *ex vivo* culture. Whether the dynamics of cell migration differ *in vivo* remains unknown.

In this report, we develop a tool called M-TRAIL that allows unperturbed tracking of cell migration *in vivo*, and show its applicability in the follicle and other *Drosophila* tissues. We further use M-TRAIL to critically evaluate results that have challenged the model coupling follicle rotation to elongation. We show that limitations of *ex vivo* culture account for the conflict, while the M-TRAIL-derived *in vivo* results are fully consistent with the model. These data demonstrate the value of M-TRAIL, which should be applicable to a range of other tissues and systems.

3.3 Results

3.3.1 M-TRAIL as a tool to trace cell migrations *in vivo*

A valuable tool to track long-term cell migration *in vivo* would meet several criteria. First, it should minimally perturb normal physiology. Second, it should allow spatial mapping of past location at cellular resolution. Third, it should allow extraction of temporal information for quantifying migratory dynamics. We moved to develop such a tool, based on the interactions between cells and extracellular matrix (ECM). Cell-ECM interactions are central to migration, as cells in physiological conditions often crawl over an ECM substrate such as the basement membrane (BM); migrating cells can also remodel the local BM environment (Wolf and Friedl, 2011). On the timescale of most cell motility, BMs are relatively static and long lasting.

We exploited these properties in an approach called ‘Matrix-labelling Technique for Real-time And Inferred Location (M-TRAIL)’ (**Fig. 3.1A**). In M-TRAIL, migrating cells are engineered to inducibly and constitutively express tagged BM components. Following induction, these components will be secreted and deposited by the cell into the local BM. If an induced cell moves, a trail of relatively stable tagged ECM molecules will be deposited in the BM along the route of migration, and this durable trail can be imaged following fixation. Knowing the onset of BM component deposition allows one to infer average velocity as well as the trajectory of the migrating cell.

We tested whether the M-TRAIL concept could be successfully implemented, by exploring cell migrations in *Drosophila*. In this organism, stochastic single-cell expression clones can be generated using heat-induced FLP recombinase (hsFLP) to drive expression of a GAL4 gene separated from a constitutive promoter by cis-paired FRT sites (‘FLPout GAL4’) (Blair, 2003). We paired *hsFLP* and *FLPout GAL4* transgenes with a UAS-driven, GFP-tagged version of one of the two *Drosophila* Collagen IV chains (GFP-Cg25c; Flybase: Col4a1), along with *UAS-Histone RFP* (**Fig. 1C**). Histone-RFP expression identifies the GAL4-expressing clone, although we noted that high levels of intracellular GFP-Cg25c puncta, presumably within the secretory system, were also sufficient.

We first applied M-TRAIL to motile cells in the follicle of *Drosophila* ovaries. In this organ, epithelial cells migrate collectively, circumferentially around the anterior-posterior axis, in a morphogenetic process known as follicle rotation (Haigo and Bilder, 2011). We heat-shocked females carrying M-TRAIL transgenes and then dissected, fixed, and examined ovaries 13 hours later. At 13 hour post heat shock (phs), a clear trail of GFP in the BM contiguous with the GAL4-expressing clone could be detected, extending in a single direction away from it in a swath extending around most of the circumferential axis (**Fig. 3.1B**). This is the axis in which follicle cells migrate in *ex vivo* preparations, and in which a previous BM-based *in vivo* assay inferred that migration occurs (Haigo and Bilder, 2011). Trails on the BM were not seen when clones expressed a soluble GFP-tagged secreted protein (preproANF-EMD) rather than GFP-Cg25c (**Fig. 3.1D**).

To determine when M-TRAILS could first be detected and how long they persisted, we examined follicles at various time points phs. In st. 8 follicles, which have been

undergoing rotation for at least 36 hours, Cg25c-GFP was detected inside the clone at 6 hours phs, but no trails were visible (**Fig. 3.1E**). Trails were first detected at 7 hours phs, and increased in length as well as strength with time (**Fig. 3.1F, 3.1G**). Weak intracellular puncta and trails can be seen as early as st.5 follicles within an ovariole 17 hours phs (**Fig. 3.1H**). Trails were difficult to detect in st. 4 or earlier follicles, possibly because of the weak efficacy of the *act5c* promoter during these stages. We explored the stability of follicle M-TRAILS by examining ovarioles at 25 hours phs. Robust trails were detected in st. 10 follicles, which were likely at st. 6 when trail deposition began (**Fig. 3.1H**). Overall, these results demonstrate the applicability of M-TRAIL to mapping cell migration.

3.3.2 Exploring criteria for M-TRAIL applicability

We evaluated the parameters within which M-TRAIL is useful for migration analysis by testing cells other than the follicle epithelium. The best-characterized motile cells in *Drosophila* are the border cells, a specialized subset of follicle cells that delaminate from the epithelium and migrate through the central germline towards the oocyte at st. 9 (Pocha and Montell, 2014). Interestingly, migrating border cells expressing M-TRAIL left only scant and transient trails along their route (**Fig. 3.2A and A'**). Since germ cells do not express BM components (**Fig. 3.2B, C**), this result suggests that a local BM may be necessary to provide a depot for the ECM components induced by M-TRAIL.

We then asked whether only cells that endogenously produce large amounts of BM components are suitable for M-TRAIL analysis. While follicle cells secrete Collagen IV into surrounding BM, imaginal disc epithelia instead construct their local BM predominantly from soluble Collagen IV synthesized by the fat body (Isabella and Horne-Badovinac, 2015a; Pastor-Pareja and Xu, 2011). We tested the ability of discs to secrete M-TRAIL-induced Cg25c-GFP. Staining for extracellular proteins revealed incorporation of disc cell-derived Cg25c-GFP into the local BM (**Fig. S3.1A**). Although cells in imaginal discs do not obviously migrate, the M-TRAIL approach appears applicable to cells that do not professionally secrete BM.

We turned to a third tissue to ask whether M-TRAIL might be used for tracking motility in cells outside of the ovary. *Drosophila* hemocytes are macrophage-like cells that undergo cell migration in response to tissue damage (Pastor-Pareja et al., 2008). We expressed GFP-Vkg specifically in hemocytes and then compared control imaginal discs to those that had been physically wounded *in situ*. Consistent with the documented production of basement membrane by hemocytes (Lunstrum et al., 1988), unwounded discs displayed a low level of GFP accumulation at their basal surface (**Fig. 3.2D**). In wounded discs, linear trails of basal GFP were often associated with hemocytes at the site of damage (**Fig. 3.2E**). These results suggest that M-TRAIL can be implemented in additional motile cell types.

Biogenesis, secretion, and incorporation of mature Collagen IV trimers into BM involves a specialized set of proteins, raising the question of whether alternative BM resident

proteins might work more efficiently for M-TRAIL (Saito et al., 2009). We compared Cg25c-GFP to SPARC-HA, a tagged version of another secreted protein that is incorporated into the BM, by inducing M-TRAIL clones expressing both (Martinek et al., 2002). In follicles, SPARC-HA appeared to be more efficiently secreted than Cg25c-GFP, as less protein was detected intracellularly, but it appeared to diffuse more from the cell trajectory (**Fig. 3.2F**). However, SPARC overexpression can modify local BM properties and alter follicle shape (Isabella and Horne-Badovinac, 2015b). By contrast, Cg25c represents only one component of the obligate heterotrimeric Collagen IV, suggesting that its overexpression would not structurally alter the BM (Van De Bor et al., 2015). Consistent with this hypothesis, overexpression of Cg25c in the entire epithelia does not lead to any evident perturbation of morphogenesis (**Fig. S3.1B-D**).

3.3.3 M-TRAIL analysis of WT follicle morphogenesis *in vivo*

Having established the general utility of M-TRAIL, we applied it to analyze follicle rotation in more detail, comparing the *in vivo* data to that inferred from other approaches. Rotation has previously been analyzed using *ex vivo* live imaging, from which linear and angular velocities (ω) have been measured (Chen et al., 2016) (Cetera et al., 2014) (Haigo and Bilder, 2011) (Viktorinová and Dahmann, 2013). Current *ex vivo* culture protocols support follicle viability for a maximum of ~10 hours, representing only a fraction of the 42 hours through which rotation is thought to occur *in vivo*. We used M-TRAIL to compare *in vivo* rotation velocities and those *ex vivo*, analyzing the length of trails in fixed M-TRAIL-induced st. 8 follicles 9 hours phs by using the ‘unrolling’ algorithm ImSAnE (Chen et al., 2016) (**Fig. 3.3A-C**). Accounting for the 7 hour onset time of M-TRAIL induction, at st.8 *in vivo* ω equals 50°/hr, very similar to that measured *ex vivo* (**Fig. 3.3D**). These results demonstrate that in WT follicles, short-term *ex vivo* conditions recapitulate the speed of cell motility *in vivo*.

In addition to rotation from st. 2 to 8, follicles undergo a morphogenetic event variously termed ‘posterior migration’ or ‘anterior accommodation’ from st. 9 to 10, after rotation ceases (Kolahi et al., 2009). During this process, the anterior follicle epithelium undergoes rapid reorganization: cells alter their morphology from cuboidal to squamous and concurrently elongate anisotropically towards the anterior pole (Brigaud et al., 2015). Interestingly, M-TRAIL captured this movement: in trails initiating at late st. 7, both cessation of rotation and a posteriorly-directed shift were seen in the squamous ‘stretch’ cells at the anterior, while only the former process was seen in the columnar cells at the posterior (**Fig. 3.3E**). This result confirms that the anterior ‘stretch’ cells change their position relative to the BM, while posterior cells are relatively static. Moreover, the lengthy (>24 hour) time courses that capture complete rotations as well as anterior morphogenesis (**Fig. 3.3F**) far exceed the limits of follicle viability under current *ex vivo* culture conditions; M-TRAIL uniquely allows their visualization.

3.3.4 M-TRAIL analysis of *fat2* mutant follicles

As M-TRAIL can be used to study WT follicle development, we then turned to mutant analysis. The most dramatic perturbation of follicle cell migration occurs in flies mutant for *fat2*, which encodes an atypical cadherin implicated in planar polarity and symmetry-breaking (Chen et al., 2016; Viktorinová et al., 2009). In *fat2* null follicles, rotation *ex vivo* completely fails, never initiating at st. 2 and not measurably moving throughout st. 8 (Chen et al., 2016) (Cetera et al., 2014); (Viktorinová and Dahmann, 2013). We tested whether the *ex vivo* results accurately reflect the *in vivo* situation by inducing M-TRAIL in *fat2* null follicles. *Fat2* follicles produced ectopic Cg25c-GFP and deposited it into the BM comparable to WT. However, M-TRAIL clones induced either at st. 5-6 or st. 7-8 did not produce a measurable circumferential trail, even 17 hours phs (**Fig. 3.4A, B**). Importantly, posteriorly-directed trails in stretch cells at st. 9 were visible (**Fig. S3.2A**), consistent with the observation that acquisition of squamous morphology and anterior follicle morphogenesis are undisturbed by *fat2* mutation.

Thus, the lack of circumferential M-TRAILS in *fat2* mutants rigorously establishes the absence of rotation *in vivo*, as previously seen *ex vivo*.

3.3.5 Follicles lacking the *Fat2* intracellular region rotate *in vivo*

A recent paper interrogated the role of *fat2* in rotation and elongation by designing and testing novel alleles, carried on fosmids in a *fat2* null mutant background (Aurich and Dahmann, 2016). These authors found that deletion of either the intracellular region (Δ ICR) alone or the transmembrane domain in conjunction with the ICR (Δ TMICR) prevented rotation *ex vivo*. Interestingly, follicles lacking ICR alone (*fat2* Δ ICR) were able to elongate appropriately and achieve late-stage PCP cytoskeletal organization, despite their observed lack of PCP rotation. The authors proposed that this genotype, which apparently uncouples the two processes, demonstrates that PCP rotation is not required for follicle elongation and PCP morphogenesis.

We investigated this proposition using M-TRAIL to analyze rotation of the new *fat2* alleles *in vivo*. We first confirmed that *fat2* Δ ICR follicles elongate normally but fail to rotate *ex vivo* (**Fig. S3.2B-E**). As previously reported, no circumferential rotation could be detected under *ex vivo* live imaging during a 2 hour course; in this *fat2* Δ ICR follicles resembled *fat2* nulls (**Fig. S3.2B**). Surprisingly, when we utilized M-TRAIL to visualize *in vivo* migration, clear circumferentially oriented trails were visible in *fat2* Δ ICR follicles but not *fat2* null nor *fat2* Δ TMICR 13 hours phs (**Fig. 3.4B, C, and D**). Interestingly, trails appeared shorter than those seen in WT follicles (**Fig. 3.4A**). We therefore tested if *fat2* Δ ICR-carrying follicles have altered rotation dynamics by directly comparing them with heterozygous sibling follicles under the same M-TRAIL induction conditions (**Fig. 3.4E**). While st.8 follicles carrying either a WT *fat2* allele or a functional *fat2*-GFP fosmid have $\omega=44^\circ/\text{hr}$, follicles carrying only *fat2* Δ ICR were $\sim 50\%$ slower, with $\omega=25^\circ/\text{hr}$. We confirmed these results using a second *fat2*

mutant background (**Fig. S3.2F**). This result demonstrates that *fat2ΔICR* follicles indeed rotate *in vivo*, although an undefined limitation of current culture conditions does not support *ex vivo* rotation. Importantly, it further establishes that this genotype does not in fact uncouple rotation from elongation.

3.3.6 Slow-rotating *fat2ΔICR* follicles have functionally softer but anisotropic BMs

Does the normal elongation of *fat2ΔICR* follicles establish that even a low rate of PCP follicle rotation is sufficient to generate the tissue organization and mechanical properties required for appropriate morphogenesis? We have recently shown that follicle shape is determined by mechanical properties provided by its fibril-like BM, with key parameters including BM PCP organization and A-P patterned levels (Crest et al., 2017). We analyzed both parameters in *fat2ΔICR* follicles with ImSAnE. In agreement with previous data, *fat2ΔICR* follicles generate PCP Collagen IV fibrils but these fibrils are shorter as well as less dense than those seen in WT (**Fig. 3.5A-D**) (Aurich and Dahmann, 2016). However, the anisotropic A-P expression gradient of Collagen IV resembled WT (**Fig. 3.5E-F**). The mechanical stiffness of the *fat2ΔICR* follicle BM was then tested, using both Atomic Force Microscopy (AFM) along the A-P and circumferential axes (**Fig. 3.5G**) and an osmotic swelling assay in which follicles are placed in distilled water (**Fig. 3.5H**). We have previously used these techniques to show that WT follicles display an anisotropic A-P stiffness gradient that directs elongation (Crest et al., 2017). AFM analysis on *fat2ΔICR* follicle BM showed the existence of an anisotropic A-P stiffness gradient, but revealed that it appears softer than WT (**Fig. 3.5I**). Indeed, when challenged with osmotic stress, *fat2ΔICR* follicles burst more frequently than WT (**Fig. 3.5J**). Nevertheless, bursting is restricted to poles (**Fig. 3.5K**), consistent with the anisotropic stiffness measurements. Thus, reduced fibril formation and a softer BM correlate with the slower albeit normally-oriented tissue rotation revealed by M-TRAIL. Overall, the results are consistent with the model that a degree of PCP migration of the follicle epithelium, generating PCP fibrils and consistent circumferential stiffness, is indeed essential for appropriate organ elongation.

3.4 Discussion

3.4.1 M-TRAIL tracking of cell migration *in vivo*

Ex vivo live imaging has led to important discoveries about cell motility during development and homeostasis. Whether explant culture systems fully recapitulate *in vivo* biology, however, remains an issue, especially for biological processes that span longer time scales. Here we provide a tool to track long-term cell motility *in vivo*, by quantitating ECM traces stably deposited by migrating cells. The genesis of this strategy lies in a previous experiment in the *Drosophila* follicle, where large clones endogenously expressing tagged

Collagen IV were marked by mitotic recombination *in trans* and used to demonstrate that follicle rotation organizes BM *in vivo* (Haigo and Bilder, 2011). M-TRAIL uses *cis*-recombination to allow temporal control over ECM component secretion, as well as to free induction from cell division, and can be utilized even in natively non-ECM-producing cells. Importantly, the ability to generate single cell clones allows cellular resolution of migratory tracks. Overall, M-TRAIL enables long-term, quantitative measurements of cell migration in fixed tissues and can potentially track other cellular dynamics, such as cell division and rearrangements, under physiological conditions.

Several points are relevant to applying and interpreting M-TRAIL. First, cells must be able to secrete the M-TRAIL protein. Export of Collagen chains, including Collagen IV, can involve specialized machinery that may not be active in all cells (Saito et al., 2009). In our experiments, an alternative ECM protein, SPARC, was more efficiently secreted, but appeared to diffuse farther and also has the ability to alter ECM stiffness. Inactive mutant forms of SPARC or other ECM proteins may be appealing directions for future refinements (Morrissey et al., 2016). Second, M-TRAIL depends on the pre-existence and stability of the BM. In early animals, there may be insufficient BM for incorporation, and throughout development there are migrations that do not involve integrin-ECM interactions (Paluch et al., 2016). Moreover, trail stability is subject to BM remodeling, such as degradation by matrix metalloproteases. BMs can also be more dynamic than commonly appreciated (Loganathan et al., 2016); this should be considered when plotting speed and trajectory of the source cell relative to the BM and the rest of the organism. Third, it is necessary to determine the lag time to trail detection in the cells under analysis. This includes not just genetic induction but also microscopic visualization. Since the amount of ECM protein deposited will correlate with the cellular ‘dwell time’ over a region of BM, rapid migrations as well as those that occur over short periods of time will require more sensitive detection. Improved secretion, brighter fluorophores, and multiple epitope tags can benefit this. While we have demonstrated M-TRAIL’s value in *Drosophila*, the relationship of migrating cells to the ECM is conserved throughout metazoans (Brown, 2011), so M-TRAIL should be useful for a wide variety of biological systems.

3.4.2 Coupling of follicle elongation to tissue rotation

The original discovery of tissue rotation in WT follicles, and its failure in non-elongating mutants, led to the proposal that rotation is required for elongation, by creating PCP organization of cytoskeletal and BM elements to form the previously hypothesized ‘molecular corset’ (Gutzeit et al., 1991; Haigo and Bilder, 2011). This initial model has guided numerous subsequent studies, whose data are largely consistent with its formulation. The recent report that *fat2ΔICR* follicles uncouple rotation and elongation thus presents an important challenge to the model (Aurich and Dahmann, 2016). Here we resolve this discrepancy by showing that *fat2ΔICR* follicles indeed rotate *in vivo*, although they do not in *ex vivo* culture for reasons currently unknown. At this time, we are unaware of any non-rotating follicle genotype that is capable of elongation, consistent with the proposition that

rotation is a morphogenetic mechanism that generates the mechanical environment necessary to shape the *Drosophila* egg.

Importantly, although *fat2ΔICR* follicles show appropriate aspect ratios, they are not fully normal in several parameters. Migration is slowed, the BM fibrils that are produced are attenuated, and the result is a functionally softer BM than in WT follicles. Although these phenotypes could point to a direct role of the *fat2ΔICR* in BM secretion, an attractive alternative hypothesis is that they instead reflect a role for tissue rotation rates in constructing BM networks and their mechanical properties. Integrin-mediated traction forces are known to drive a positive feedback loop linking ECM stiffness to cell migration speeds in many systems (Parsons et al., 2010); this conserved molecular mechanism, amplified by the continuous topology of the follicles' axis of rotation, could drive the construction of a robust BM that instructs elongation.

The demonstration that *fat2ΔICR* follicles undergo PCP cell migration sheds further light on the activity of Fat2 itself. Fat2 shows PCP localization in rotating follicles and binds to actin polymerization-stimulating WAVE complex proteins; both of these properties are mediated by the ICR (Barlan et al., 2017; Squarr et al., 2016; Viktorinová and Dahmann, 2013). The ability of follicles lacking the Fat2 ICR to rotate demonstrates that a direct Fat2-WRC interaction is not essential for collective cell motility, placing more emphasis on the Fat2 extracellular domain, perhaps in a ligand-like role (see also (Barlan et al., 2017)). This ability also emphasizes that it is PCP rotation, rather than Fat2 localization, that is correlated with proper organization of cytoskeleton and ECM components. Recent work identifies an initial and essential requirement for Fat2 in regulating symmetry-breaking microtubule polarity in the germarium, in a manner independent of actin organization (Chen et al., 2016). The localization of Fat2 during these stages is not known but the ability of *fat2ΔICR* follicles to break chiral symmetry is consistent with an ECD-mediated role. In general, the use of M-TRAIL to both reveal unexpected migration and quantitate its rate in this genotype, as well as in future manipulations, will guide testable hypotheses for mechanisms that couple rotation of a tissue to the mechanical properties that drive its elongation.

While M-TRAIL uncovered one limitation of *ex vivo* follicle imaging, reconciling contradictory results, it is important to emphasize that in other aspects it was concordant with prior analyses. In particular, the migration rate, trajectory, and cessation of rotation in WT follicles measured *in vivo* by M-TRAIL were identical to those observed *ex vivo*, as was the failure of rotation in *fat2* mutants. This validates the general *ex vivo* approach to study follicle rotation, but points out that live imaging of some mutant genotypes may not accurately reflect what occurs in the animal. The creation of more genetically convenient as well as more sensitive M-TRAIL variants will expand the range of situations in which this new tool can be used.

3.5 Experimental Procedures

All fluorescent images were acquired on a Zeiss LSM700 confocal microscope. M-TRAIL follicle clones were induced by 15 or 20 minute heat shocks of 3-day old, fed adult females in a 37°C water bath. Ovaries were dissected and stained after hours post heat shock (phs) indicated on the figures. Over 50% follicles show multiple single clones along the A-P arc and ~10% have non-overlapping single clones. Non-permeablized immunostains were performed without detergent. ImSAnE unrolling and analyses were executed as previously described on full stacks of confocal sections of follicles stained with Alexa Fluor647-conjugated phalloidin (Chen et al., 2016). The phalloidin channel was used to generate point clouds for unrolling. Conditions for *ex vivo* follicle culture were as previously described (Chen et al., 2016). AFM measurements, bursting assays, and Collagen IV-GFP fibril intensity measurements were done as described (Crest et al., 2017). Fibril density and length were measured as in (Isabella and Horne-Badovinac, 2015b) on the central region of ImSAnE-flattened images. Error bars in charts represents SEM and statistical significance was assessed by Welch's unequal variances *t*-test. Additional details are described in **Supplemental Experimental Procedures**.

3.6 Supplemental Experimental Procedures

3.6.1 Immunostaining and Imaging

Immunostaining and imaging were previously described (Chen et al., 2016). Antibodies used were listed in **Table S3.1 KEY RESOURCE TABLE**.

Except for **Fig. S3.2C-E** with 100 μm scale bars, all scale bars are 10 μm .

3.6.2 In Situ Larval Disc Wounding

Larvae carrying *5XQE-dsRed* (Zecca and Struhl, 2007) were used to visualize wing pouch in situ and *UAS-GFP-Vkg* is driven by *Hml Δ -Gal4* and *He-Gal4* (gifts from Katja Brückner). Third instar larvae were immobilized in ice-chilled PBS filled petri dish on ice. In situ wing pouch wounding was performed by holding the larva with one forcep while pinching the wing pouch with a dull tungsten needle. Larvae were transferred to a vial containing yeast paste to allow hemocyte migration and Vkg-GFP deposition to the basement membrane before fixation.

3.6.3 Image Analyses

ImSAnE was used to 'unroll' follicles as previously described (Chen et al., 2016) (Heemskerk and Streichan, 2015). Cylinder projections from slices containing the basement membranes showing the full length of M-TRAIL were used for M-TRAIL quantification. Lengths of M-TRAIL were measured from ImSAnE-unrolled cylinder projection by Fiji. M-TRAIL migration percentages were calculated by dividing the M-TRAIL pixel length over the total pixel length of the D/V circumference. Angular velocity of rotation (ω) were calculated

based on the percentage of follicle circumference covered by M-TRAIL, divided by the time post-heat shock minus M-TRAIL induction time. Data were analyzed and charts displayed using Prism 6 (GraphPad) and Excel (Microsoft). Figures were assembled by Illustrator CC 2017 (Adobe).

3.6.4 Biomechanical Assays

Basement membrane stiffness was measured by Atomic Force Microscopy (AFM), indenting live-cultured follicles 50 nm using a pyramidal tip cantilever as described previously (Crest et al., 2017). Young's Modulus of elasticity was calculated using a modified Hertz model; 4 measurements at each position were averaged for each data point.

For bursting assays, live follicles were first adhered to a poly-D lysine glass bottom dish (MatTek) in Schneider's media. Media was then replaced twice with distilled water and images were collected at 15 second intervals to identify time and site of follicle rupture, as described previously (Crest et al., 2017).

3.6.5 Fly Strains and Husbandry

Fly Strains used are listed in **Table S3.1 KEY RESOURCE TABLE**. Fly strains were obtained from Bloomington Drosophila Stock Center except the following: *UAS-GFP-Vkg* and *UAS-GFP-Cg25c* are gifts from Stéphane Noselli (Van De Bor et al., 2015). *fat2^{58D}-FRT80*, *FRT42D-fat2-GFP*, *fat2^{ΔICR-GFP}*, *fat2^{ΔTMICR-GFP}* are gifts from Dr. Christian Dahmann (Viktorinová et al., 2009) (Aurich and Dahmann, 2016). *UAS-SPARC-HA* is a gift from Eduardo Moreno (Portela et al., 2010). *vkg-GFP* and *trol-GFP* are from FlyTrap (Buszczak et al., 2007). *5xQE-DsRed* is a gift from Gary Struhl (Zecca and Struhl, 2007). *Act5C>CD2>Gal4*, *UAS-his2Av-mRFP* is a gift from Melanie Worley (Worley et al., 2013). Adult flies and larvae were maintained at 25°C. For ovary samples, adult females were flipped onto fresh food daily for 1-2 days and were fed with yeast paste overnight before heat shock or dissection. Detailed genotypes in each figure are listed in **Table S3.2**.

3.7 Author Contributions

D.Y.C. and J.C. conducted the experiments; D.Y.C and D.B. designed the experiments and wrote the paper.

3.8 Acknowledgements

We thank Christian Dahmann, Stéphane Noselli, Eduardo Moreno, Katja Brückner, Iswar Hariharan, and the Bloomington Stock Center for sending reagents, Geert de Vreede

for assistance in discs wounding experiments, and are grateful to the Bilder lab for helpful discussions. This work was supported by NIH RO1s GM068675 and GM111111.

Figure 3.1. M-TRAIL, a novel tool to track cell migration *in vivo*.

- (A)** Schematic of M-TRAIL strategy. A one-cell clone (red) overexpressing a tagged ECM protein (green) is generated through genetic mosaic techniques. Secreted ECM protein is deposited in local BM, leaving a trail if the cell migrates before fixation.
- (B)** Implementation of M-TRAIL. Early st. 9 follicle with M-TRAIL induced, 13 hrs phs. Single epithelial clones reveal trajectory of cell migration during follicle rotation. 'Anti-GFP' here and below indicates that anti-GFP immunohistochemistry was performed without cell permeabilization. Left panel shows maximum Z projection of confocal stacks. Right panels show rotated views of opacity projection. Scale bar:10 μ m.
- (C)** Genetic components used to generate M-TRAIL in **Figs. 3.1-5**. A heat shock-inducible recombinase causes excision of a stop cassette to initiate GAL4 production. GAL4 in turn drives expression of a nuclear RFP and GFP-tagged chain of Collagen IV.
- (D)** Early st. 9 follicle with non-ECM secreted protein tagged with GFP, 12 hrs phs. No trails are seen. Scale bar:10 μ m.
- (E-F)** Intracellular GFP-Cg25c can be detected 6 hrs phs, but trails are not evident until 7-8 hrs phs. Scale bar:10 μ m.
- (G)** Quantitation of M-TRAIL length, measured by percent of follicle circumference. Data are represented as mean +/- SEM.
- (H-I)** Trails can be seen in follicles from st. 5 to st. 10. Scale bar:10 μ m.

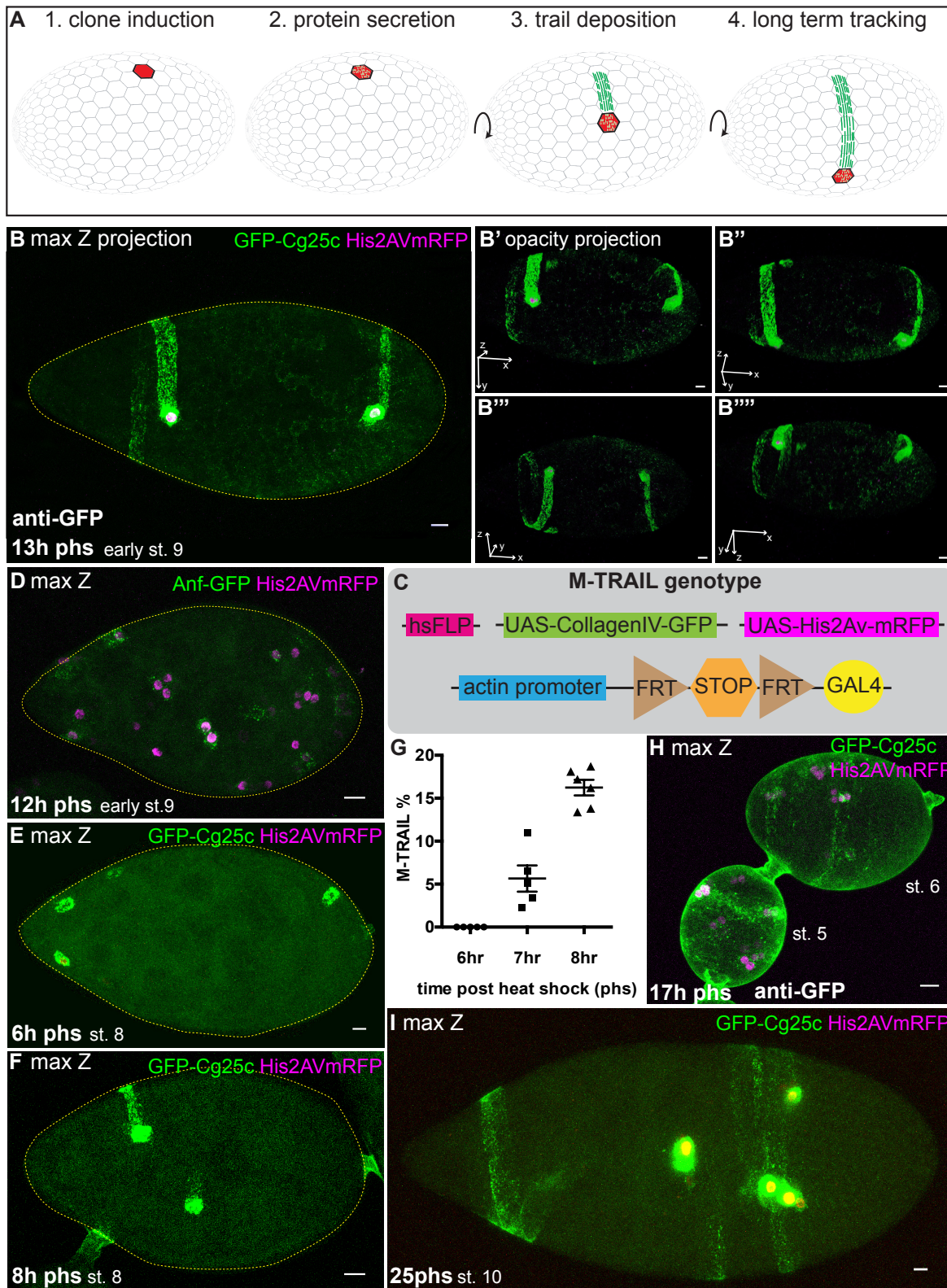


Figure 3.2. Criteria for M-TRAIL application

- (A)** In contrast to follicle epithelial cells, migrating border cells expressing GFP-Cg25c do not leave trails. Scale bar:10 μm .
- (B-C)** Germline cells lack expression of BM components Vkg and Perlecan. Scale bar:10 μm .
- (D)** In larvae expressing GFP-Vkg in hemocytes, unwounded wing imaginal discs (expressing a red fluorophore in the pouch) show only low-level GFP signal in the BM. Scale bar:10 μm .
- (E)** 7 hours following wounding, hemocytes (arrowhead) adhere close to wound sites (white dotted line) and are often found next to BM trails of GFP (grey arrow). Scale bar:10 μm .
- (F)** The ECM protein SPARC-HA can be used in M-TRAIL, although it diffuses farther from cells than GFP-Cg25c. Scale bar:10 μm .

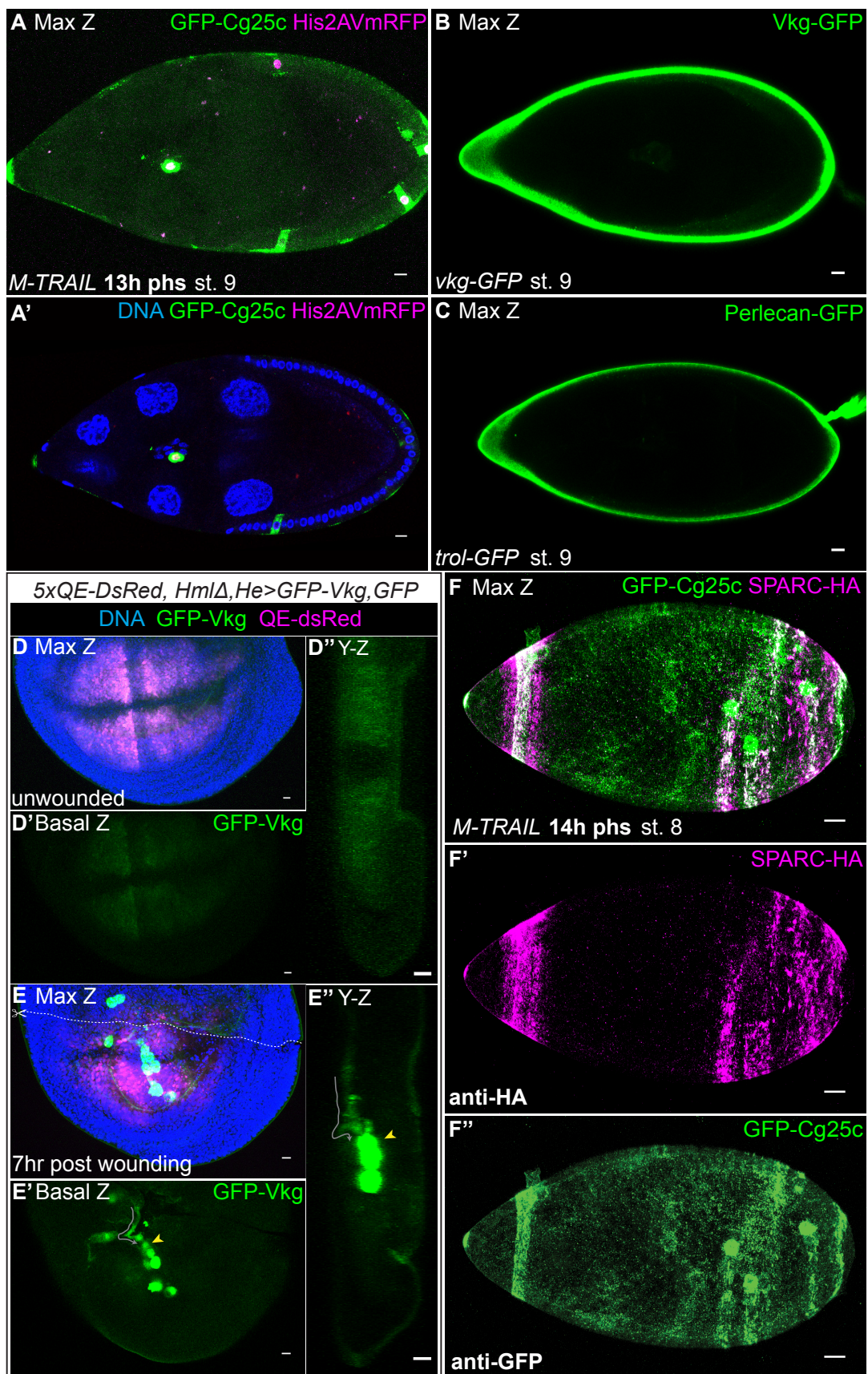


Figure 3.3. Characterization of follicle morphogenesis *in vivo* using M-TRAIL

(A) Use of ImSAnE 'flattening' algorithm to project 3D follicle epithelium onto 2D plane.

(B-C) Flattened trails present in M-TRAIL follicles cultured for 2 hrs *in vivo* **(C)** vs *ex vivo* **(B)**.

(D) Rotational angular velocity (ω , calculated by trail length as a fraction of follicle circumference) reveals no difference *ex vivo* vs *in vivo*. Data are represented as mean +/- SEM.

(E) Anterior clones (arrow) leave trails that are circumferential during st. 7-8, but then shift toward the posterior after rotation ceases at st. 9. Posterior clones (arrowhead) show rotation but no A-P shift. Scale bar:10 μm .

(F) St. 9 follicle with two clones showing long term tracing. Note the complete rotations evident; also note the greater posterior shift in the more anterior clone. Scale bar:10 μm .

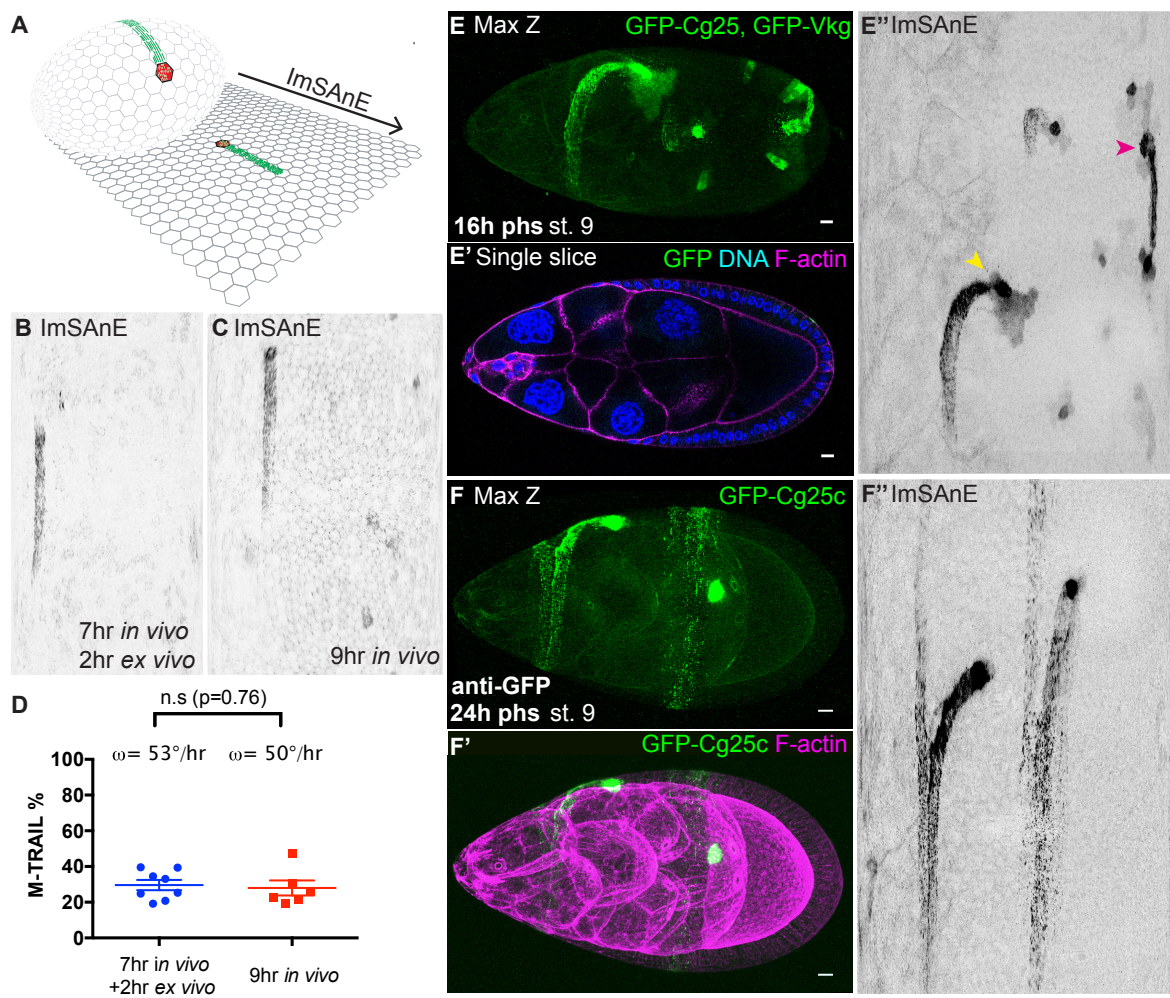


Figure 3.4. *fat2 Δ ICR* follicles rotate *in vivo*, albeit at a slower rate

(A-D) Maximum intensity Z and ImSAnE-flattened projections of M-TRAIL induced follicles carrying different *fat2* alleles. Scale bars:10 μ m. **(A)** Follicles carrying a WT allele generate long trails; **(B)** homozygous *fat2* follicles show no trails; **(C)** follicles carrying *fat2 Δ ICR* show short trails; **(D)** follicles carrying *fat2 Δ TMICR* show no trails.
(E) Angular velocity calculated by trail length; *fat2 Δ ICR* follicles rotate at 57% the rate of those carrying a WT allele, while *fat2 Δ TMICR* do not rotate at all. Data are represented as mean +/- SEM. ****p $\leq$ 0.001.

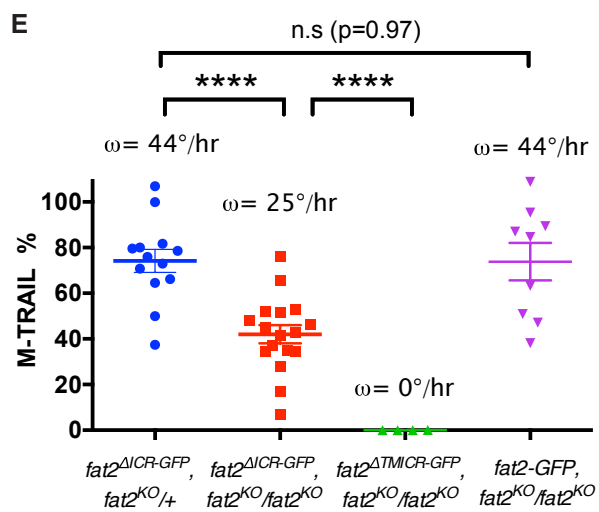
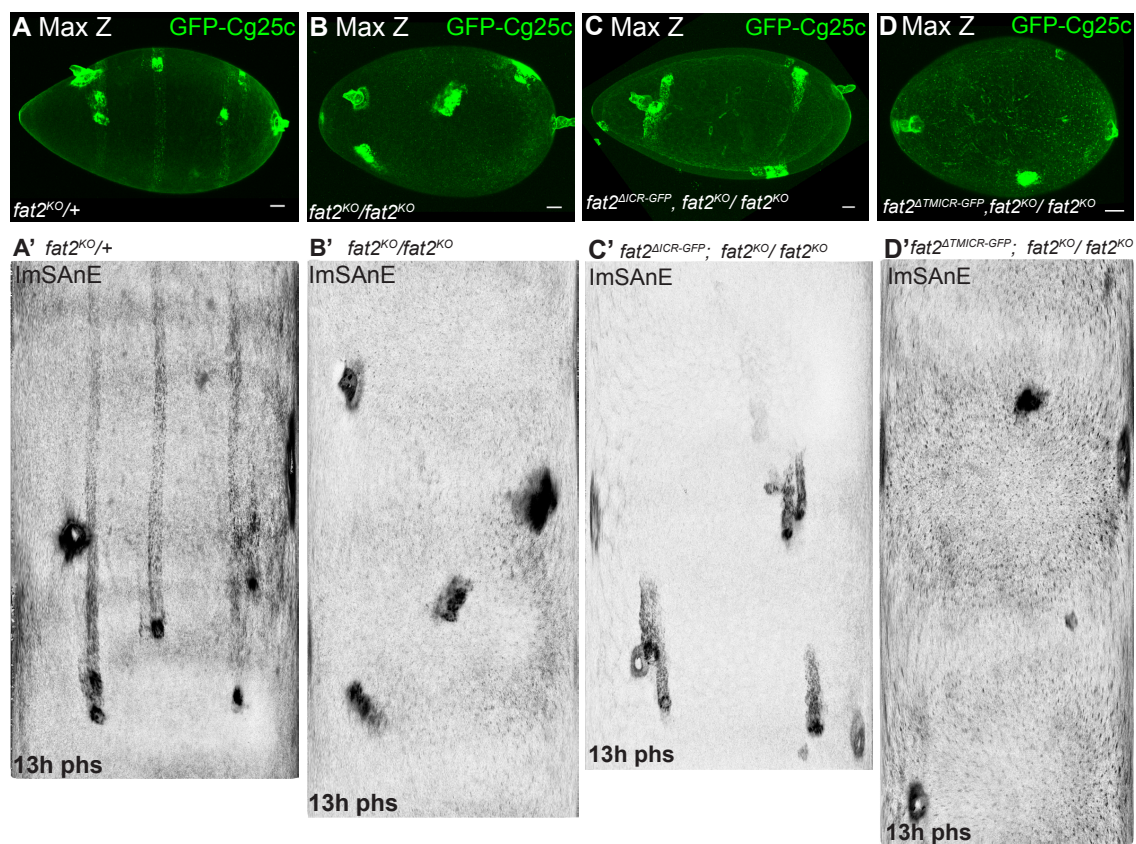


Figure 3.5. *fat2ΔICR* follicles show altered mechanical properties

(A-B) Confocal projections of Collagen IV-GFP in WT **(A)** and *fat2ΔICR* **(B)** follicles. Scale bar:10 μm.

(C-D) Collagen IV fibril analysis quantitates shorter **(C)** and less dense **(D)** fibrils in *fat2ΔICR* vs control (*fat2* heterozygous) follicles. Data are represented as mean +/- SEM.

(E-F) Anisotropic A-P distributions of Collagen IV-GFP are similar between *fat2ΔICR* **(F)** vs control (*fat2* heterozygous) follicles **(E)**.

(G) Schematic of Atomic Force Microscopy experiments. Follicles are probed at different regions along the A-P axis to measure BM (green) stiffness.

(H) Schematic of bursting assay. Follicles are placed in distilled water to swell tissue; timing and position of BM (green) rupture reflects BM mechanics.

(I) AFM measurements reveal that *fat2ΔICR* follicles maintain an AP stiffness gradient similar to WT, but display modest softening. Data are represented as mean +/- SEM.

(J) Following osmotic challenge, *fat2ΔICR* follicles burst more frequently than WT follicles, but less frequently than *fat2ΔTMICR* follicles. Data are represented as mean +/- SEM.

(K) Like WT, bursting in *fat2ΔICR* follicles does not occur in the central region, in contrast to *fat2ΔTMICR* follicles.

p≤0.05, *p≤0.01, ****p≤0.001

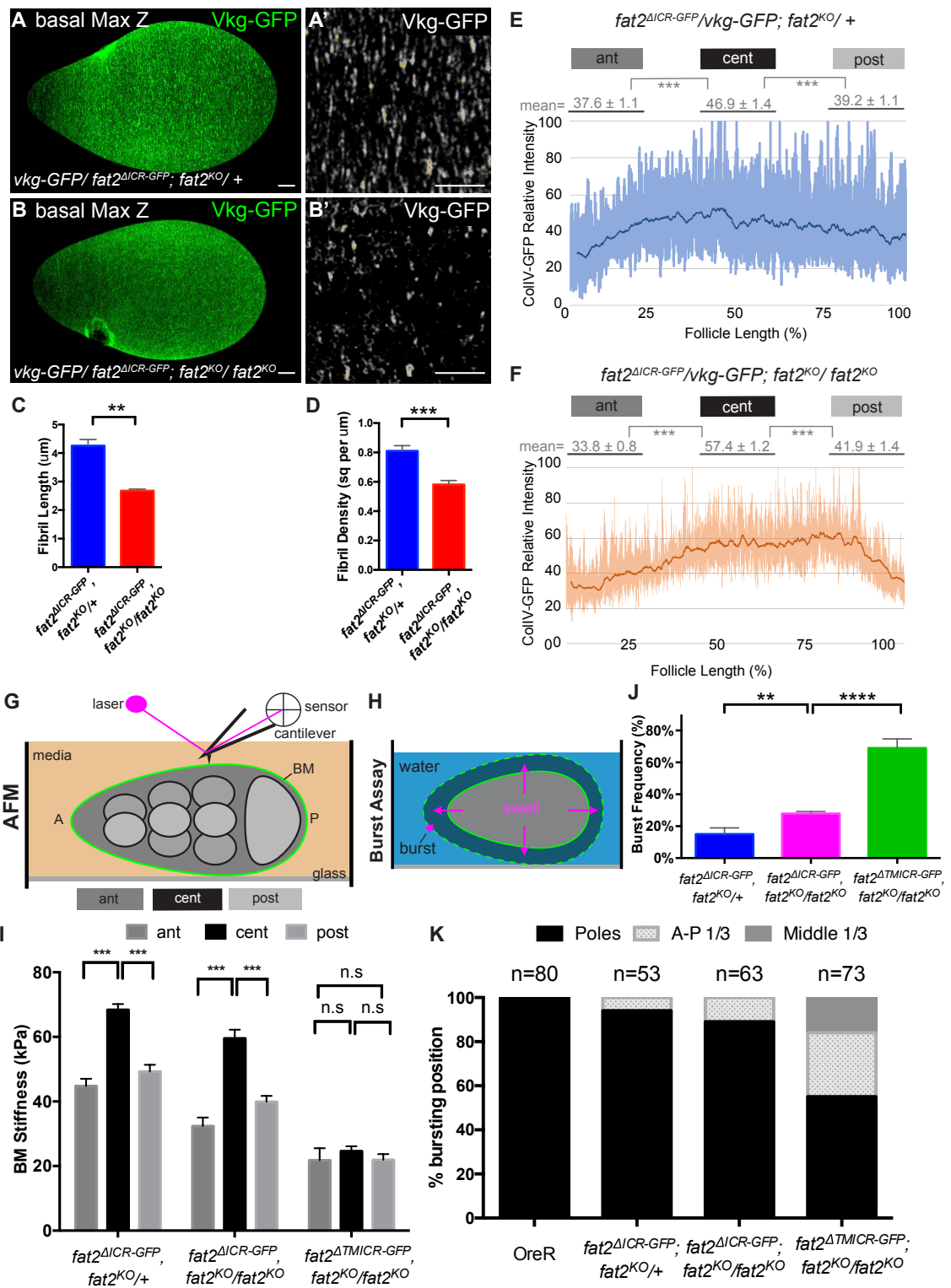


Fig.5

Figure 3.6. Cover Art for *Cell Reports*

On the cover: Chen et al. report development of an in vivo cell tracking tool called M-TRAIL that reveals past migratory trajectories in fixed tissues. Histone-RFP-labeled cells (Hansel and Gretel in black) express and deposit collagen-GFP (green bread crumbs) onto the basement membrane, leaving a stable record of the trail traveled. M-TRAIL can allow quantitative analysis of cell movements over extended periods of time without perturbation in opaque, as well as transparent, samples. Artwork by Dong-Yuan Chen.

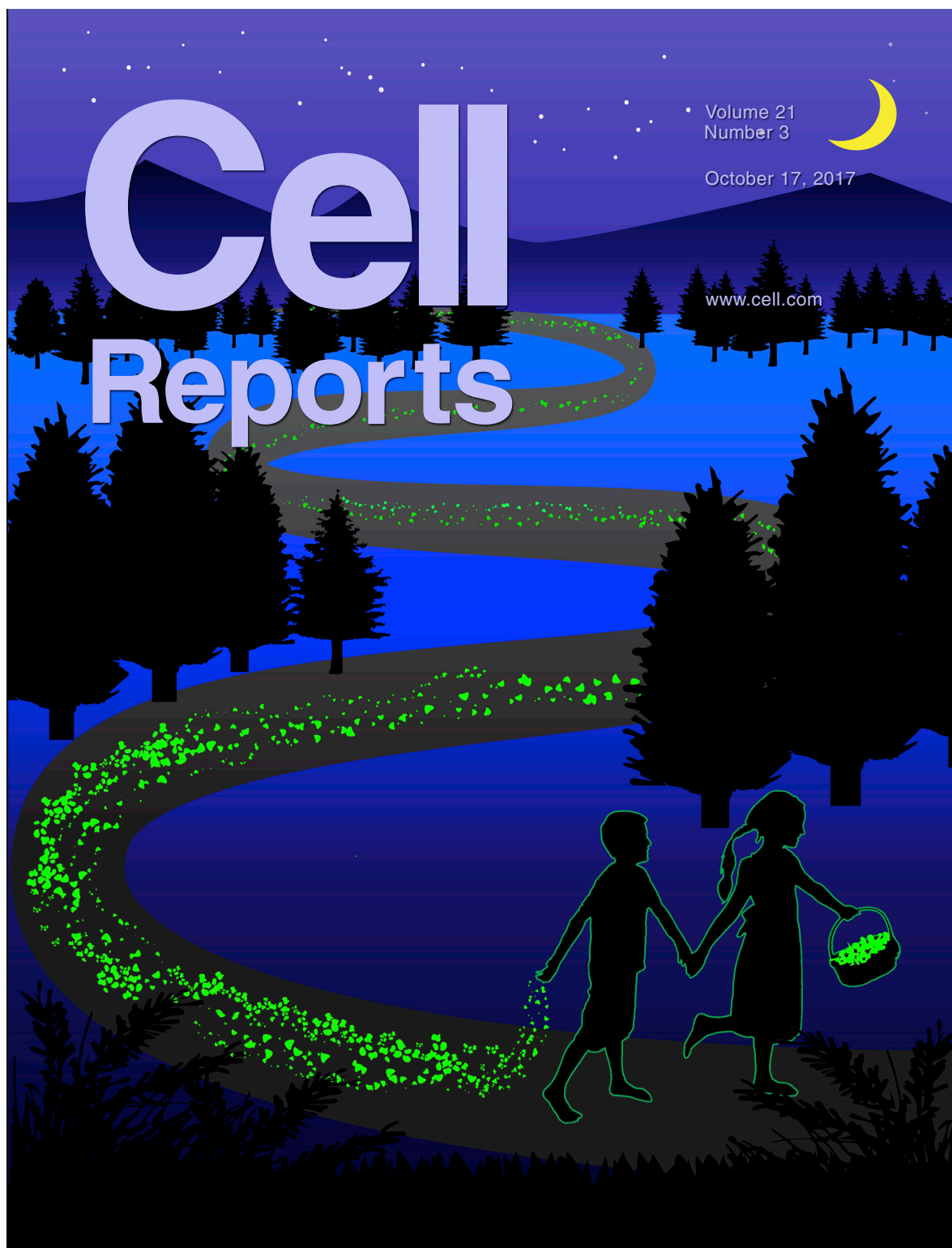
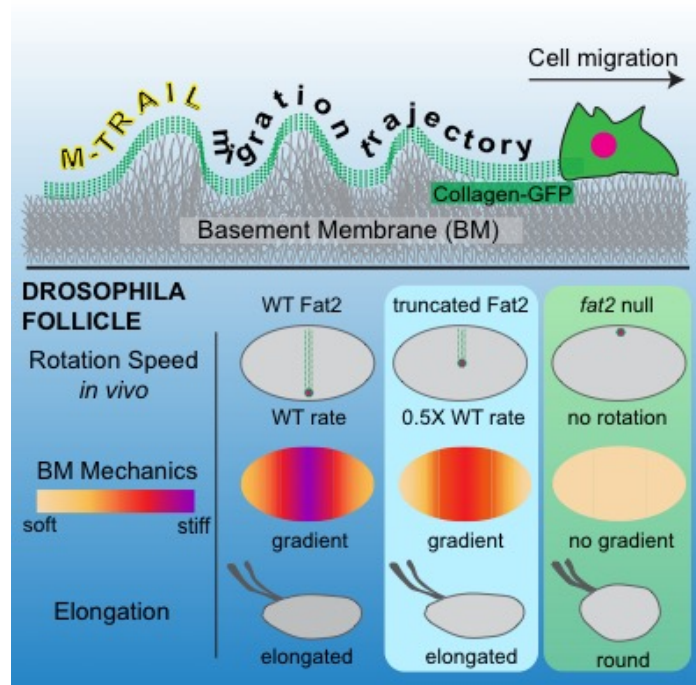


Figure 3.7. Graphical Abstract

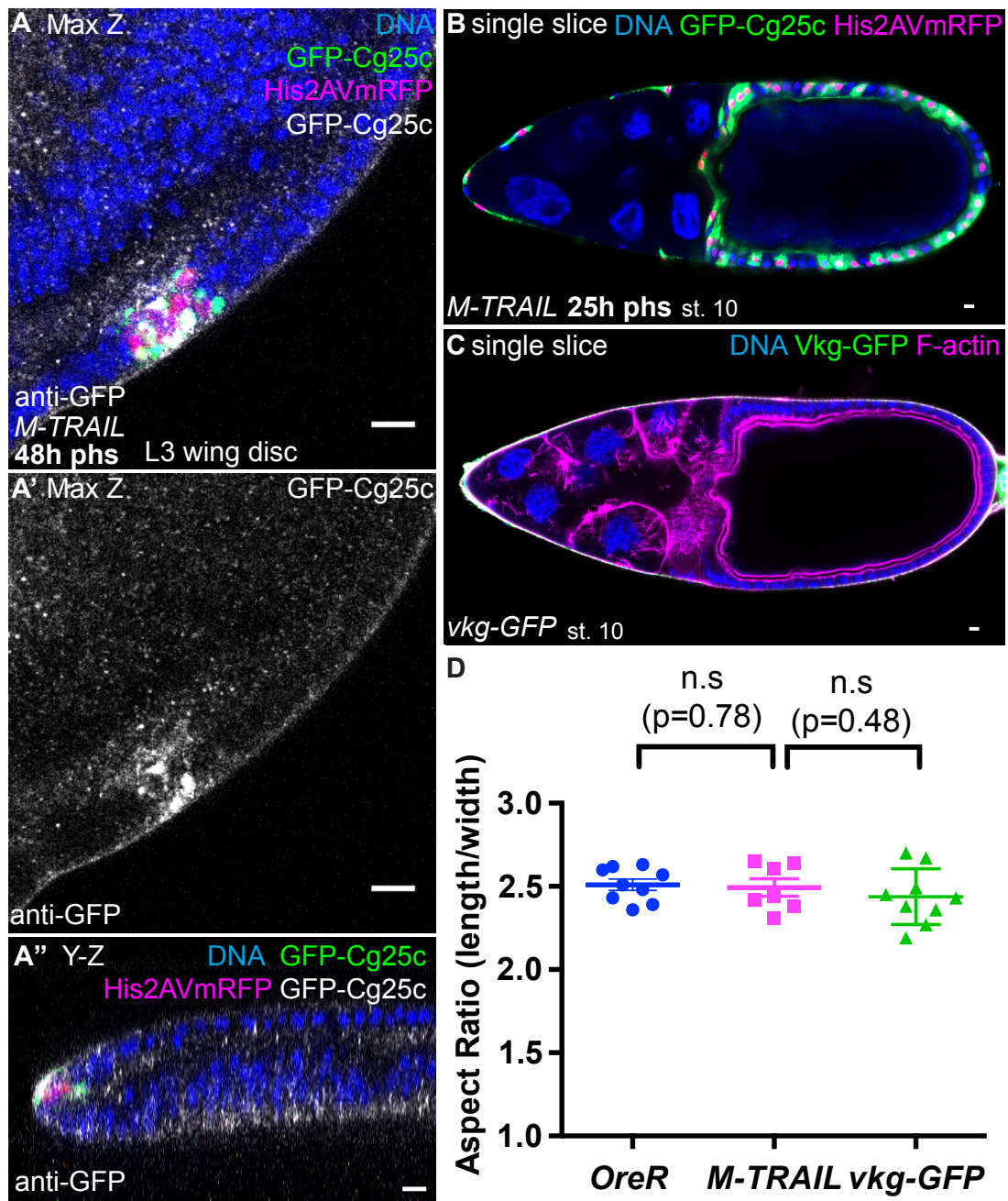
- M-TRAIL allows retrospective tracking of unperturbed cell migration in vivo
- Fluorescent proteins incorporate into BM, allowing visualization and quantitation
- In vivo rotation of fly follicle can differ from ex vivo
- Elongating follicles, even those with aberrant mechanics, rotate in vivo



Supplemental Figure 3.1

(A) Wing imaginal disc cells secrete M-TRAIL-derived GFP-Cg25c into the BM, as shown by GFP fluorescence and anti-GFP antibody staining under non-permeabilized conditions (white channel).

(B-D) Ubiquitous M-TRAIL-driven GFP-Cg25c does not alter follicle elongation at st. 10, compared to WT (OreR) or Vkg-GFP protein trap controls.



Supplemental Figure 3.2

fat2 anterior M-TRAIL clones show anterior accommodation (yellow and blue arrowheads).

(A') ImSAnE image of the same follicle.

(B) *fat2* ^{Δ ICR} follicles, imaged via the membrane dye FM4-64FX, show no rotation throughout 90 minutes *ex vivo*. Same 5 cells are labelled with yellow dots.

(C-E) *fat2* ^{Δ ICR} fosmid rescues egg elongation in *fat2*^{KO} flies. Scale bars: 100 μ m.

(F) M-TRAIL reveals slowed but rotating follicles in *fat2* ^{Δ ICR}; *fat2*^{58D} / *fat2*^{KO} flies.

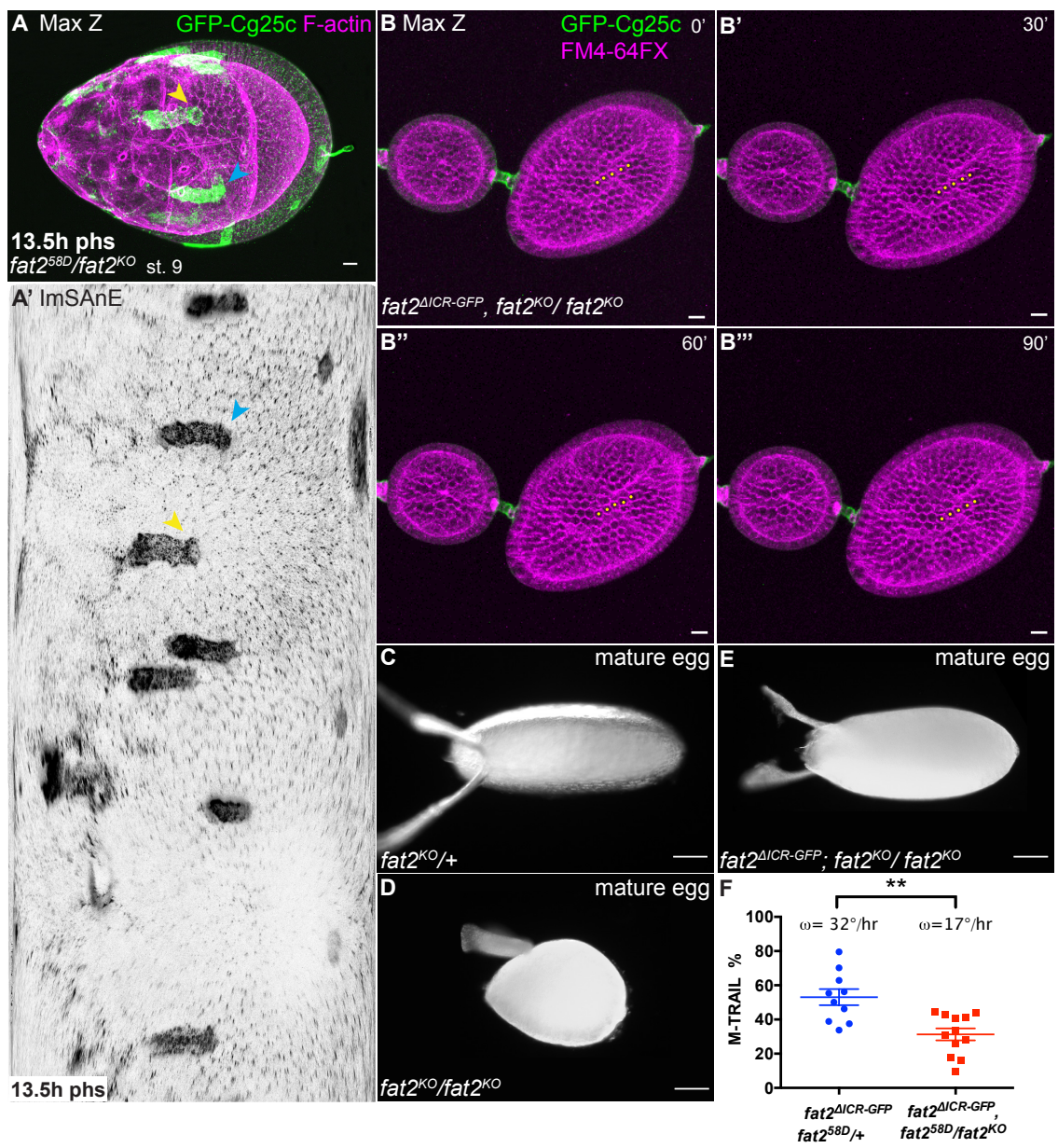


Table S3.1 KEY RESOURCES TABLE (Related to Figs. 3.1-5 and Figs. S3.1-2)

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal Anti-GFP(3E6)	ThermoFisher	A11120
Rabbit HA-Tag mAb (C29F4)	Cell signaling	3724
Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	ThermoFisher	A11001
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 647	ThermoFisher	A21244
Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 647	ThermoFisher	A31571
Chemicals		
FM4-64FX	ThermoFisher	F34653
Phalloidin-TRITC	Sigma	P1951
Alexa Fluor 647 Phalloidin	ThermoFisher	A22287
DAPI	Sigma	D9542
Experimental Model: <i>D. melanogaster</i>		
<i>UAS-GFP-Vkg</i>	Laboratory of Stéphane Noselli; (Van De Bor et al., 2015)	N/A
<i>UAS-GFP-Cg25c</i>	Laboratory of Stéphane Noselli; (Van De Bor et al., 2015)	N/A
<i>fat2^{KO}</i>	Laboratory of David Bilder; (Crest et al., 2017)	N/A
<i>fat2^{58D}-FRT80</i>	Laboratory of Christian Dahmann;(Viktorinová et al., 2009)	Flybase ID: FBal0241217
<i>FRT42D-fat2-GFP</i>	Laboratory of Christian Dahmann;(Viktorinová et al., 2009)	Flybase ID: FBal0241218
<i>fat2^{ΔICR-GFP}</i>	Laboratory of Christian Dahmann;(Aurich and Dahmann, 2016)	Flybase ID: FBal0319177
<i>fat2^{ΔTMICR-GFP}</i>	Laboratory of Christian Dahmann;(Aurich and Dahmann, 2016)	Flybase ID: FBal0319178
<i>P{UAS-SPARC.HA}</i>	Laboratory of Eduardo Moreno; (Portela et al., 2010)	Flybase ID: FBtp0085610
<i>HmlΔ-GAL4, UAS-GFP; He-GAL4 5xQE-DsRed</i>	Laboratory of Katja Brückner	N/A
<i>P{GAL4-Act5C(FRT.CD2).P}S, P{UAS-His-RFP}3/TM3, Sb1</i>	Laboratory of Gary Struhl; (Zecca and Struhl, 2007)	Flybase ID: FBtp0040071
<i>vkg-GFP</i>	Laboratory of Iswar Hariharan; (Worley et al., 2013)	Flybase ID: FBst0051308
<i>trol-GFP</i>	Flytrap; (Buszczak et al., 2007)	Flytrap ID: CC00791
	Flytrap; (Buszczak et al., 2007)	CA06698

<i>w[1118]</i>	Bloomington Drosophila Stock Center	Flybase ID: FBst0003605
<i>P{ry[+t7.2]=hsFLP}22, w[*]</i>	Bloomington Drosophila Stock Center	Flybase ID: FBst0008862
<i>P{ry[+t7.2]=hsFLP}1, y[1] w[1118]; Dr[1]/TM3, Sb[1]</i>	Bloomington Drosophila Stock Center	Flybase ID: FBst0026902
<i>y1 w* P{GAL4-Act5C(FRT.CD2).P}D</i>	Bloomington Drosophila Stock Center	Flybase ID: FBst0004779
<i>P{w[+mC]=UAS-preproANF-EMD}136.3, y[1] w[*]</i>	Bloomington Drosophila Stock Center	Flybase ID: FBst0007001
Software and Algorithms		
ImSAnE	(Heemskerk and Streichan, 2015)	N/A
Volocity	Perkin Elmer	N/A
Fiji	(Schindelin et al., 2012) http://fiji.sc/	N/A
Matlab 2015a	Mathworks	N/A
Prism V6	Graphpad	N/A
Illustrator CC 2017	Adobe	N/A
Excel	Microsoft	N/A

Table S3.2. Detailed Genotypes (Related to Figs. 3.1-5 and Figs. S3.1-2)

Figure	Panel	Genotype
3.1	B	<i>hsFLP/+; UAS-GFP-Cg25c; Act5C >CD2>GAL4, UAS-His2Av-mRFP</i>
	D	<i>hsFLP/preproANF-EMD; ;Act5C >CD2>GAL4, UAS-His2Av-mRFP</i>
	E	<i>hsFLP/+; UAS-GFP-Cg25c; Act5C >CD2>GAL4, UAS-His2Av-mRFP</i>
	F	<i>hsFLP/+; UAS-GFP-Cg25c; Act5C >CD2>GAL4, UAS-His2Av-mRFP</i>
	G	<i>hsFLP/+; UAS-GFP-Cg25c; Act5C >CD2>GAL4, UAS-His2Av-mRFP</i>
	H	<i>hsFLP/+; UAS-GFP-Cg25c; Act5C >CD2>GAL4, UAS-His2Av-mRFP</i>
	I	<i>hsFLP/+; UAS-GFP-Cg25c; Act5C >CD2>GAL4, UAS-His2Av-mRFP</i>
3.2	A	<i>hsFLP/+; UAS-GFP-Cg25c; Act5C >CD2>GAL4, UAS-His2Av-mRFP</i>
	B	<i>vkg-GFP</i>
	C	<i>yw,trol-GFP</i>
	D	<i>5xQE-DsRed/HmlΔ-GAL4, UAS-GFP; He-GAL4/ UAS-GFP-Vkg</i>
	E	<i>5xQE-DsRed/HmlΔ-GAL4, UAS-GFP; He-GAL4/ UAS-GFP-Vkg</i>
	F	<i>hsFLP/+; UAS-GFP-Cg25c; Act5C >CD2>GAL4, UAS-His2Av-mRFP, UAS-SPARC-HA</i>
3.3	B	<i>hsFLP/+; UAS-GFP-Cg25c; Act5C >CD2>GAL4, UAS-His2Av-mRFP</i>
	C	<i>hsFLP/+; UAS-GFP-Cg25c; Act5C >CD2>GAL4, UAS-His2Av-mRFP</i>
	D	<i>hsFLP/+; UAS-GFP-Cg25c; Act5C >CD2>GAL4, UAS-His2Av-mRFP</i>
	E	<i>hsFLP/+; UAS-GFP-Cg25c; UAS-GFP-vkg, Act5C >CD2>GAL4, UAS-GFP</i>
	F	<i>hsFLP/+; UAS-GFP-Cg25c; Act5C >CD2>GAL4, UAS-His2Av-mRFP</i>
3.4	A	<i>hsFLP/ Act5C >CD2>GAL4; UAS-GFP-Cg25c/+; fat2^{KO} /+</i>
	B	<i>hsFLP/ Act5C >CD2>GAL4; UAS-GFP-Cg25c/+; fat2^{KO} /fat2^{KO}</i>
	C	<i>hsFLP/ Act5C >CD2>GAL4; UAS-GFP-Cg25c/ fat2^{ΔICR}-GFP; fat2^{KO} /fat2^{KO}</i>
	D	<i>hsFLP/ Act5C >CD2>GAL4; UAS-GFP-Cg25c/ fat2^{ΔMICR}-GFP; fat2^{KO} /fat2^{KO}</i>
	E	<i>hsFLP/ Act5C >CD2>GAL4; UAS-GFP-Cg25c/ fat2-GFP; fat2^{KO} /fat2^{KO}</i>
3.5	A	<i>vkg-GFP/ fat2^{ΔICR}-GFP; fat2^{KO} / +</i>
	B	<i>vkg-GFP/ fat2^{ΔICR}-GFP; fat2^{KO} / fat2^{KO}</i>
	E	<i>vkg-GFP/ fat2^{ΔICR}-GFP; fat2^{KO} / +</i>
	F	<i>vkg-GFP/ fat2^{ΔICR}-GFP; fat2^{KO} / fat2^{KO}</i>
S3.1	A	<i>hsFLP/+; UAS-GFP-Cg25c; Act5C >CD2>GAL4, UAS-His2Av-mRFP</i>
	B	<i>hsFLP/+; UAS-GFP-Cg25c; Act5C >CD2>GAL4, UAS-His2Av-mRFP</i>
	C	<i>vkg-GFP</i>
	D	<i>hsFLP/+; UAS-GFP-Cg25c; Act5C >CD2>GAL4, UAS-His2Av-mRFP</i>
S3.2	A	<i>hsFLP/ Act5C >CD2>GAL4; UAS-GFP-Cg25c/+; fat2^{58D}-FRT80/fat2^{KO}</i>
	B	<i>hsFLP/ Act5C >CD2>GAL4; UAS-GFP-Cg25c/ fat2^{ΔICR}-GFP; fat2^{KO} /fat2^{KO}</i>
	C	<i>fat2^{KO}/+</i>
	D	<i>fat2^{KO}/ fat2^{KO}</i>
	E	<i>fat2^{ΔICR}-GFP/+; fat2^{KO} / fat2^{KO}</i>
	F	<i>hsFLP/ Act5C >CD2>GAL4; UAS-GFP-Cg25c/ fat2^{ΔICR}-GFP; fat2^{58D}-FRT80/+</i>
		<i>hsFLP/ Act5C>CD2>GAL4; UAS-GFP-Cg25c/ fat2^{ΔICR}-GFP; fat2^{58D}-FRT80/fat2^{KO}</i>

Chapter 4

Morphogenetic behaviors that elongate
the *Drosophila* follicle

Morphogenetic behaviors that elongate the *Drosophila* follicle

Dong-Yuan Chen, Justin Crest, Sebastian Streichan, and David Bilder

4.1 INTRODUCTION

A full understanding of the mechanisms that shape a functional organ is a long-time goal of biology. Early morphogenesis studies focused on only a part of the organ, either by culturing dissected portions *ex vivo*, or by restricting *in vivo* imaging to an optically convenient region. Fundamental morphogenetic principles have emerged from classical examples of these systems like Keller explants of *Xenopus* embryos (Keller, 2002), as well as contemporary examples like the *Drosophila* germband (Zallen, 2007). Nevertheless, these systems remain limited because creation of artificial boundaries prevents evaluation of outside influences such as tissue-wide mechanics. Only now are comprehensive analyses of systems like the *Drosophila* notum and wing disc, zebrafish gastrula and avian embryo in play (Heisenberg and Bellaïche, 2013). However, these often flat tissues are treated primarily as 2D sheets, in contrast to the many *in vivo* organs that are fully 3D. Thus, there is a need to study true 3D organs with *in toto* approaches.

The *Drosophila* egg chamber, or follicle, provides an excellent model for such studies (Horne-Badovinac and Bilder, 2005). Follicles have an architecture that is typical of many animal organs, with multiple components that associate to form a 3D acinar structure with an epithelial sheet surrounding a lumen. At the same time, the simplicity and highly regular development of the follicle lend themselves to comprehensive analyses. The follicle exhibits straightforward and symmetric geometry for much of its development, while its cells originate from only two stem cell populations and show limited differential fates. Follicles can be genetically manipulated using the powerful *Drosophila* toolkit, and are well-suited for imaging either in fixed preparations or when cultured live *ex vivo*.

Development of the follicle involves several conserved morphogenetic behaviors including initial primordial assembly, epithelial diversification, and collective cell migration. A major focus for mechanistic studies has been follicle elongation, during which the initially spherical organ transforms into a more tubular ellipsoid shape (Bilder and Haigo, 2012; Cetera and Horne-Badovinac, 2015). ~2-fold elongation is seen in ~40 hours between follicle budding at st. 3 to the end of st. 8; eventually there is ~2.5 fold overall elongation when the egg is laid ~25 hours later. This degree of elongation is similar to that in paradigmatic morphogenetic systems such as the amphibian neural plate and mesoderm, or the *Drosophila* germband. In the latter tissues, the main cellular behavior that drives elongation is convergent extension, as cells intercalate mediolaterally toward a specific landmark that is defined anatomically and/or molecularly. These tissues also have defined borders, which create boundary conditions to instruct and orient cell behaviors. No such boundary is evident along the ‘edgeless epithelium’ of the *Drosophila* follicle (Chen et al., 2016), and the cellular changes that drive follicle morphogenesis are not known.

We recently showed that mechanical heterogeneity patterned within the BM, rather than within the cells, is instructive for follicle elongation (Crest et al., 2017). Specifically, a gradient of matrix stiffness that is low at the poles and peaks in the follicle center provides differential resistance to germline growth, leading to tissue elongation. But how the cells of

the follicle epithelium respond to this stiffness gradient, and engage in the behaviors that actually elongate the organ remains unexplored. Here we comprehensively catalog morphogenetic and morphometric dynamics during follicle elongation, and evaluate their involvement in organ shaping.

4.2 RESULTS

4.2.1 *in toto* morphometrics of *Drosophila* follicles

We established an imaging and computational platform to acquire morphometric data from follicles during their major elongation phase, from st. 4 to mid-st. 8 prior to major asymmetries between the anterior and posterior hemispheres (see **Methods**). Because morphometric measurements can be altered by mounting preparation (e.g. flattened on a slide and/or coverslip) and by artifacts of imaging plane (e.g. XY slices on cells that also have Z orientation in the epithelial plane), complete XYZ images of follicles within a depression slide were captured on a confocal microscope, and analyzed using analytical software including the recently described package ImSAnE (Chen et al., 2016; Heemskerk and Streichan, 2015). ImSAnE detects the surfaces of 3D objects and projects them onto 2D planes to facilitate segmentation and quantitative analysis of the follicle epithelium. Projections can be selected that preserve the size or orientation of the epithelial cells with respect to follicle axes (defined in **Supp. Fig. 4.1**), which were segmented via basal profiles. The veracity of ImSAnE projections was confirmed by the ability to reconstruct 3D follicles ‘*in silico*’ (**Supp. Fig. 4.1**). Importantly, this approach allowed complete analysis of the epithelium, including the polar regions, which are seldom analyzed due to their high curvature as well as their position perpendicular to the standard imaging plane.

Accurate morphometric comparisons require clear identification of developmental stages. We compared the classic staging summarized in (Spradling, 1993) to our morphometric data on follicle volume and epithelial cell number (**Suppl Fig. 4.2**). For stages where definitive morphological criteria exist, we generally found good accordance, allowing boundaries based on follicle volume to mark the end of st. 4, 7, and 8 (**Suppl Fig. 4.2**). St. 5 and 6 are not clearly distinguished on morphological criteria, and there is debate about whether cell proliferation ends prior to st. 6 or continues during this stage (Jia et al., 2016; Spradling, 1993; Sun and Deng, 2005). We found that cell counts of follicles that would be deemed st. 6 by the criteria of Jia. et al contained well fewer than 900 cells (Jia et al., 2016). Moreover, the frequency of such follicles *in vivo* (our results and (David and Merle, 1968)) is higher than would be expected for the length of st. 6 given by (Spradling, 1993). Timings of developmental stages in Spradling (Spradling, 1993) are based on transplant of a single germarium into the abdomen of an *ovoD1* female host, and are likely faster than follicles in a native ovariole *in vivo* (Lin and Spradling, 1993). Using 9.6 hours as the average follicle cell cycle time (Margolis and Spradling, 1995), the durations of st. 4, 5 and 6 based on our epithelial cell counts are 9.6, 4.8, and 8.4 hours respectively, in reasonable agreement with the durations suggested by David and Merle based on relative follicle representation in WT hosts (David and Merle, 1968). Due to the wide ranges observed in classically defined st. 6 follicles, we divided these into substages 6A and 6B using cell number and volume

boundaries. Finally, we limited our analysis of st. 8 follicles to the presumed youngest half, excluding the larger volume follicles in which the squamous transition is clearly evident.

Our analysis of WT follicles confirmed, quantified, and refined general parameters of oogenesis previously derived using conventional analyses. Follicle volume grows exponentially with stage (**Fig. 4.1A**); discrepancies with (Jia et al., 2016) are likely due to mounting and imaging conditions. Cell numbers in the follicle epithelium increase linearly, reaching a final number of 873 $\pm$ 27 SD, similar to early estimates from King (1000) (King, 1970), and more recent analyses (905) (Kolahi et al., 2009) (**Fig. 4.1B**). As expected, basal surface area of epithelial cells decreased during division stages and increased after the onset of endoreplication at st. 7 (**Fig. 4.1C**). Both basal surface areas and apicobasal profiles were similar along the AP axis through st. 6, emphasizing that cells have largely equal volumes (**Suppl. Fig. 4.2**). However, at st. 7 basal surface area significantly increased in cells at the anterior one third, as apicobasal profiles decreased, suggesting the first onset of the transition of this population to eventually become fully squamous. Overall, we measure a linear increase in elongation, from aspect ratio 1.1 to 1.9 between st. 4 and st. 8, for a net elongation of 54% in the mature egg (**Fig. 4.1D**).

4.2.2 Cellular behaviors during tissue elongation

We then considered the morphogenetic behaviors that could drive follicle elongation. In general, tissue elongation could be driven by oriented cell division, oriented cell shape changes, or oriented cell rearrangements (Solnica-Krezel and Sepich, 2012). We analyzed follicles using ImSAnE to capture parameters for every cell, eliminating artifacts associated with the imaging plane and preserving the orientation along the arc of the curved epithelium, which at the poles is discrepant from the A-P follicle axis. To analyze cell division, we expressed the microtubule-binding protein Jupiter-GFP to mark the mitotic spindle and midbody (**Fig. 4.2A and B**); we then live-imaged follicles from st. 3 through st. 6, when mitotic divisions terminate. Midbody position, reflecting the ultimate plane of cytokinesis, was variably aligned in the epithelial plane during st. 3-4, but during st. 5-6 became preferentially aligned with respect to the AP arc (**Fig. 4.2C**). Thus, oriented cell divisions could potentially contribute to follicle elongation prior to st. 7.

To analyze cell shape changes, we measured the eccentricity of each follicle cell; that is, the degree of elongation of its best-fit ellipse. During st. 5-8, follicle cells maintain a very consistent average eccentricity, ranging 0.65-0.7 (**Fig. 4.2D**). There was no significant difference between cell eccentricities from st. 4 and st. 8 follicles, despite close to 1 fold change of aspect ratio increase during this period. Distribution of cell eccentricity also show no significant difference from st. 5-8 (**Fig. 4.2E**). Importantly, we also determined the orientation of cell eccentricity within the epithelial plane and found that at st. 6, most cells' long axes are oriented circumferentially, perpendicular to the A-P arc (**Fig. 4.3A-C**). Thus, more cells are more elongated in the direction of rotation but not in the axis of tissue elongation. However, at st. 8, many cells in the anterior third reorient their long axis parallel to the A-P axis, potentially contributing to tissue elongation (**Fig. 4.3B and 4.3D**).

To analyze cell rearrangements, we first analyzed cellular topology in fixed follicles (**Fig. 4.3E-F**). Changes in topological order can be caused by either cell proliferation or by neighbor exchange (Guirao and Bellaïche, 2017). St. 4 follicles were the most disordered, likely reflecting their high rate of cell division rather than tissue elongation, which is limited at this time (**Fig. 4.3F**). Topological order then increased; this was true even following st. 6, indicating that cell junctions rearrange during these stages independent of mitoses. We next examined follicles live-imaged *ex vivo*, searching for intercalation events. We did not see rosette-like junctional arrangements, and while we saw occasional 4-cell (T2-like) junctions, we were unable to consistently identify T1>T3 transitions in movies from st. 6-8. However, follicles in these movies showed signs of deterioration after 6 hours, limiting the window of detection during the ~15 hours in which elongation takes place. Moreover, follicle growth (i.e. change in volume) *ex vivo* was inconsistent to that expected *in vivo*, raising the possibility that this might alter elongation-driving cell rearrangements. We then attempted to use a recent *in vivo* cell migration tracking technique (M-TRAIL) to follow single cells undergoing follicle rotation (Chen et al., 2017), reasoning that deviations from a true circumferential trajectory could reveal rearrangements in the follicle axis. These experiments revealed consistent slight posterior-directed shifts in all samples, regardless of whether the tracked cell was located at the anterior or posterior of the follicle (**Fig. 4.3G**). Finally, we directly counted cells along the follicle arcs. These counts revealed an 90% increase in the ratio of meridian to equatorial cells between st. 4-8 (**Fig. 4.3H**). These data indicate that cell rearrangements occur during follicle elongation, leading to intercalation of cells from the circumferential to the AP axis.

4.2.3 Cell behaviors in elongation-defective mutants

To test the functional involvement of the above behaviors, we analyzed them in mutant backgrounds that altered the behaviors specifically or follicle elongation in general. The most frequently-used elongation mutant disrupts *fat2*, which encodes an atypical cadherin required for follicle planar cell polarity and tissue rotation (Viktorinová and Dahmann, 2013; Viktorinová et al., 2009). *Fat2* follicles fail to create a BM stiffness gradient, and their elongation diverges from WT follicles at st. 5; by st. 8 they have aspect ratios of 1.6 instead of 1.9 (**Fig. 4.4A**). When we analyzed cell divisions in *fat2* follicles at st. 6, we found that, in contrast to WT, the plane of cytokinesis did not orient along the AP arc, but remained randomly aligned as in earlier stages (**Fig. 4.4B**). Because *fat2* follicles show attenuated BM stiffness at st. 5, before defects in elongation are evident (Crest et al., 2017), we considered the possibility that oriented divisions in WT follicles are a consequence of the stiffness gradient, rather than a cause of tissue elongation. We therefore examined the initial orientation of the mitotic spindle in WT follicle cells and found that it was not preferentially AP oriented, even at st. 5-6. Instead, AP alignment of the spindle, often perpendicular to the initial cell long axis, generally occurred after metaphase, consistent with the outcome of division being organized by tissue-wide tension. Additionally, RNAi to the *mushroom body defective* (*mud*) gene (Bergstralh et al., 2015), which regulates the mitotic plane in follicle cells, did not induce any elongation defects. Finally, we inhibited mitoses by overexpressing *fizzy-related* (*fzr*), a Cdh1 homolog that is sufficient to prevent mitoses switch cells to an

endocycle program (Hassel et al., 2014). Fzr overexpression from st. 4 enabled epithelial cell numbers to reach only 415 +/- 30 cells instead of the 872 of WT. Nevertheless, aspect ratio in these follicles, which do not undergo cell division during st. 5 and 6, was unchanged at st. 8 (**Fig. 4.4C-D**). We conclude that oriented cell divisions are not required for follicle elongation.

Growth of the follicle in *fat2*-depleted follicles overall, as well as proliferation of epithelial cells, were unchanged (**Suppl. Fig. 4.3A-B**). We then investigated cell shape and cell rearrangements in *fat2* disrupted follicles. Eccentricity of *fat2* follicle cells was similar to WT at st. 6, indicating that cell migration does not dictate elongated cell shape (**Fig. 4.4E**). Strikingly, and in contrast to WT, fewer cells in the anterior of st. 8 *fat2* follicles were oriented circumferentially (**Fig. 4.4F-G**); cell orientation resembled a normal distribution than the skewed distribution of WT. This phenotype was seen before significant differences in follicle elongation were evident and represents the earliest morphometric defect seen in *fat2* cells. Moreover, although *fat2* cells became less eccentric at st. 7 and 8, overall cell orientation did not change. Cell topology in st. 6-8 *fat2* follicles did not differ from WT, but M-TRAIL analyses failed to display a posterior shift in migration trajectories, suggesting a failure of rearrangement (**Fig. 4.4 H-I**). Consistent with such a failure, cell counts along A and P axes in *fat2* reveal that cell numbers do not alter in either axis (**Fig.4J**). Thus, failures of cell orientation and shape change correlate with the failure to rearrange cells to elongate *fat2* follicles.

4.2.4 *Rack1* is required for cells to reorient in response to BM mechanical cues

Most of the manipulations above eliminated the gradient of BM stiffness that is proposed to sculpt the follicle. We therefore searched for a genotype that might retain the gradient, exploiting an ongoing genetic screen in the lab. In this screen, transgenic RNAi is expressed specifically within the follicle epithelium, and defects in oogenesis are scored as 'hits'. We noted that RNAi targeting *Receptor for Activated C Kinase-1* (*Rack1*) gave rise to round follicles and round eggs, with a high degree of penetrance (**Fig. 4.5A-B and Suppl. Fig. 4.3C**). This phenotype was seen with two independent RNAi lines targeting different regions of the transcript, and was rescued by overexpression of a *Rack1*-encoding transgene. It was further confirmed by making mitotic clones of *Rack1* null cells in the follicle epithelium, wherein moderately-sized clones clearly altered follicle aspect ratio (**Suppl. Fig. 4.3D**). Thus, *Rack1* is a new regulator of follicle elongation.

Analysis of *Rack1*-depleted follicles revealed that they did not differ from WT in several assays. Proliferation of epithelial cells was unchanged (**Suppl. Fig. 4.3E-F**), and in *ex vivo* culture, *Rack1*-depleted follicles rotated with normal speed and trajectory. At st. 6, cell divisions were oriented along the elongation axis, comparable to WT follicles (**Fig. 4.5C**). Moreover, cell eccentricity at st. 7-8 did not differ from WT (**Fig. 4.5D**). However, defects were seen in the orientation of this eccentricity (**Fig. 4.5E**). This also resulted in changes in tissue topology, where *Rack1*-depleted follicles have lower percentages of hexagonally packed cells at st. 8 (**Fig. 4.5F**). Unfortunately, the GAL4- and FRT-dependent nature of our

current M-TRAIL technique prevented its use to analyze cell rearrangements, but counts along arcs confirmed that *Rack1*-depleted follicles fail to rearrange cells. We conclude that *Rack1* elongation failure may associate with an inability to execute proper polarized cell shape changes and rearrangements.

We next assayed BM stiffness in *Rack1*-depleted follicles, using AFM. The data showed a moderate reduction in overall stiffness (**Fig. 4.5G**); however, A-P anisotropy remained, with anterior and posterior poles significantly less stiff than the central region. When challenged with osmotic pressure induced by placement in distilled water, *Rack1*-depleted follicles burst slightly more rapidly than WT follicles, but not as rapidly as *fat2* or *Perl o/e* follicles (Crest et al., 2017); bursting position also more closely resembled WT. A-P differences in Collagen IV incorporation within the BM were still evident, although overall levels were lower than in WT. Together, these data indicate that *Rack1*-depleted follicles display a moderately softer BM that nonetheless retains its A-P gradient of stiffness.

BM mechanical properties in *Rack1*-depleted follicles resemble those seen in follicles overexpressing SPARC or carrying *fat2* alleles that delete the intracellular domain of the protein (Chen et al., 2017; Crest et al., 2017). Strikingly, these latter genotypes elongate normally, while *Rack1*-depleted follicles do not. This contrast suggests that Rack1 might be required for cellular behaviors triggered in response to the BM stiffness gradient. Rack1 is a scaffolding protein whose seven WD-40 domains have been shown to interact with the Src tyrosine kinase, maintaining it in an inactive state (Duff and Long, 2017; Li and Xie, 2015). Because Src has been implicated in cell junction remodeling in other systems (Förster and Luschnig, 2012; Nelson et al., 2012), we asked whether Rack1 might act as a negative regulator of Src activity during follicle elongation. Indeed, immunostaining to active phosphorylated Src showed an elevation in *Rack1*-depleted follicles (**Fig. 4.5H-I**), and overexpression of Src42A, one of two *Drosophila* Src orthologs, was sufficient to induce elongation defects in otherwise WT follicles (**Suppl. Fig. 4.3G**). Interestingly, depletion of *Src42A* also induced elongation defects (**Suppl. Fig. 4.3H**). These results suggest that RACK1 may restrict Src activation levels to allow elongation-driving junctional remodeling in response to extracellular mechanical constraints.

4.3 DISCUSSION

The *Drosophila* follicle is an emerging model for 3D morphogenesis that exhibits a number of distinctive features, including global tissue rotation and shaping in response to BM stiffness. Here we use *in toto* image analysis to analyze the cell dynamics that elongate this organ. We find that polarized growth in a subset of cells, allowing reorientation of their axes from circumferential to AP, is a major driver of elongation. This process fails when the BM is isotropically soft or stiff, and is regulated by Src activity. This unusual mechanism highlights differences between the elongation of flat/bounded and tubular/edgeless tissues.

The three cellular behaviors that drive tissue elongation in other systems –cell deformation, cell division, and cell rearrangement- are all seen during follicle elongation.

Our manipulations show that polarized cell division is not required for elongation, and its absence can be compensated by other cell dynamics, as also seen in e.g. the pupal thorax (Guirao et al., 2015). Additionally, an initial cell shape change --stretching around the organ circumference-- is not perturbed in several 'round egg' mutants. Instead, our data suggest that the major tissue-elongating behavior involves changes in cell orientation, in which cells in the follicle anterior shift the direction of their long axis from circumferentially-oriented to AP-oriented. Without changing average cell shape, altering the orientation of cell shape anisotropy changes neighbor relationships, and results in net cellular intercalation along the AP axis. It is important to note that, despite the nearly twofold relative elongation of the tissue overall along Cartesian axes, only a small number of cells actually switch places between the AP and circumferential arcs. Simple modeling of a 3D ellipsoid surface indicates that this switch is sufficient to account for elongation. This behavior resembles that previously seen during *Drosophila* tracheal morphogenesis (see below), highlighting a repeatedly utilized mechanism for elongation of tissues with tubular topology.

This relative paucity of frank intercalation events contrasts with the frequent intercalation seen in elongating tissues such as the *Drosophila* germband, pupal wing and thorax, and vertebrate neural tube and mesoderm (Walck-Shannon and Hardin, 2014). The elongation rate of the follicle more closely resembles these other systems than the rapid *Drosophila* germband, but a number of differences stand out. First, significant growth of both epithelial cells and the follicle overall occur during the period under study, while the other systems largely rearrange a constant tissue volume. Second, the other systems have defined boundaries that exert identified mechanical or signaling influences across the tissue field, allowing vectorial orientation of cell behaviors. While the follicle differentiates its polar regions to define an anterior-posterior axis and its concordant information, the epithelium itself is 'edgeless', that is, topologically continuous and without a boundary in the circumferential axis. Third, follicle elongation does not require conventional PCP signaling components that guide morphogenetic behaviors in many other systems, nor is there evidence to date of active behaviors that autonomously drive junctional dynamics by polarized Myosin localization. Instead, the instructive force seems to be anisotropic resistance to growth, induced by stiffness differences patterned into the BM. These differences with bounded epithelia undergoing elongation via convergent extension emphasize the new perspectives required for analysis of elongating tubular organs.

Relative differences in BM stiffness seem to be the defining feature driving the morphogenetic behaviors of follicle cells. We do not find that tissue rotation *per se* directs elongation-driving cell dynamics, as a genotype that preserves tissue rotation but nevertheless induces a soft and isotropic BM shows similar defects to a non-rotating mutant. Rather, an anisotropic BM that is softer at poles may be sensed by cells and trigger changes in cell shape.

How do cells sense BM stiffness to change their growth characteristics? The elongation-defective phenotype of *Rack1*-depleted follicles points to a possible mechanism, involving the Src tyrosine kinase. *Rack1* is a Src-interacting protein that inhibits its activity,

and both increases or decreases in Src expression also perturb follicle elongation. In *Rack1* mutants, mean cell shape is not altered, but the orientation of cell eccentricity is compromised. This suggests that proper regulation of Src activity is required for dynamic reapportionment of cell shape that is reflected in this switch. Src's most familiar regulatory targets are integrin-ECM adhesions, but Src has also been implicated in directly regulating adherens junction(AJ) remodeling, through effects on the dynamics of E-cadherin trafficking (Fujita et al., 2002; Swaminathan and Cartwright, 2012). We propose that Src-mediated junctional remodeling underlies the elongation defect.

The requirement for Src in elongation, as well as the specific cellular phenotypes seen when altering Src activity, raises striking parallels with the *Drosophila* trachea, a model for tubular morphogenesis. In trachea, loss of *Src42A* results in shortened tubules with increased luminal diameter, because mutant cells are deficient in growth along the A-P axis but not the circumferential axis (Förster and Luschnig, 2012; Nelson et al., 2012). This imbalance in polarized growth leads to inappropriate circumferentially-biased cell shape orientation, which impairs cell intercalation that normally extends the organ. Since follicle cells also undergoes significant growth between st.6-8, *Src42A* could play a similar role in the ovary, mediating AJ dynamics to allow a preferential expansion in the A-P axis that changes cell eccentricity orientation. Like the follicle, the trachea is an 'edgeless epithelium'; moreover, chitin-like apical extracellular proteins in the tracheal lumen are proposed to provide signaling cues that regulate *Src42A* phosphorylation. These results suggest that ECM-mediated control of anisotropic cellular growth, mediated by Src, may be a general mechanism for the elongation of acinar and tubular tissues.

Overall, this work emphasizes the necessity of a comprehensive approach to studying the development of highly curved 3D organs. Our specific approach is enabled by the use of ImSAnE, which accurately provides morphogenetic parameters of all cells as well as their orientation with the epithelial plane. ImSAnE identified critical cell dynamics near the follicle poles, which are the area most subject to distortion from conventional analyses. These dynamics required quantitation not only of the gradual shape change phenotype, but also its alignment within the epithelial axes, which conventional methods cannot do accurately. The ImSAnE-based platform was also capable of distinguishing the cellular basis underlying overtly similar elongation phenotypes, for instance the distinct defects in anterior cell dynamics between *Rack1* and *fat2* follicles. As more 'round egg' mutants emerge in the literature and from forward screens, such a workflow will be important for phenotypic interpretation (Horne-Badovinac et al., 2012). Finally, we have focused here on follicle elongation between st. 5-8, which is dependent on BM mechanics and molecules such as *fat2*. This work does not address the cell dynamics that elongate the follicle prior to and following these stages, nor other aspects of follicle morphogenesis that recapitulate those seen in other developing tissues. It does provide a framework for true *in toto* analysis of this simple organ, at all stages and in multiple genotypes.

4.5 Acknowledgments

Experiments using Zeiss Lightsheet Z.1 were conducted at the CRL Molecular Imaging Center. We would like to thank Holly Aaron and Jen-Yi Lee for their microscopy training and assistance. We thank the TRiP at Harvard Medical School (NIH/NIGMS R01-GM084947), Kyoto Stock Center, and the Bloomington Stock Center for providing stocks. We Thank Tian Xu and Trudi Schüpbach for sending fly strains to us.

4.6 Materials and Methods

4.6.1 Fly Strains and Husbandry

Fly Strains used are listed in **Table 4.1**. Fly strains were obtained from Bloomington Drosophila Stock except the following: *UAS-Src42A* from Tian Xu, *GR1-GAL4*, *UAS-FLP* from Trudi Schüpbach, *Indy-GFP* from Flytrap (Kelso et al., 2004), and *TrafficJam-Gal4* from Kyoto Stock Center. Adult flies were maintained at 25°C unless noted. Adult females were flipped onto fresh food daily for 1-2 days and were fed with yeast paste overnight before dissection.

4.6.2 Immunostaining and Imaging

Immunostaining and imaging were previously described (Chen et al., 2016; 2017). Antibodies used are listed in **Table 4.1**. Fluorescent images were acquired either on a Zeiss LSM700 confocal microscope or Zeiss lightsheetZ1. We used ImSAnE to unroll the follicle epithelium as previously described (Chen et al., 2016; Heemskerk and Streichan, 2015). M-TRAIL induction and analysis were previously described (Chen et al., 2017). Conditions for *ex vivo* follicle culture were as previously described (Chen et al., 2016). Follicles were staged according to Spradling, with the modifications described in Results.

4.6.3 Morphometrics extraction

We used ImSAnE to unroll the follicle epithelium as previously described (Chen et al., 2016; Heemskerk and Streichan, 2015). All measurements were computed using the metric tensor as previously described (Heemskerk and Streichan, 2015). Follicle Anterior-Posterior (A-P) orientation was defined either automatically by the longest axis or manually based on the position of the polar cells at the stages when follicles are close to spherical. A-P length was computed by determining the anterior and posterior poles as extreme points along the AP axis, and then measuring the length of the geodesic connecting these two points. circumferential length was computed by choosing an arbitrary point on the equator of the follicle, and measuring the length around the circumference passing through this point. Aspect ratio is defined as the ratio of A-P and circumferential length. Follicle volume was computed by adding up all pixels contained within the 3D surface. The number of cells along the A-P arc and D-V circumference was determined by first segmenting all cells in a pullback. Cells whose centroids are a given distance away from the line used to measure the A-P and circumferential length were then counted accordingly. For fusing 3D representations of the

surface, pullbacks showing anterior, posterior, and two cylinder projections were first segmented, and then stitched together in 3D. The pullbacks were based on a maximum intensity projection of 3x0.5 micron thick pullbacks from 3-4 micron inward from the basal-most surface was extracted. Cell outline segmentation used simple thresholding of prediction maps obtained by using Ilastik (Sommer et al., 2011). The thresholded images were treated with standard morphological operations to obtain skeletonized cell outlines. Automated methods for detecting branching points then enabled construction of a lattice. Lattice consists of a lookuptable, with vertices, bonds connecting vertices, and cells arranging bonds (cell-cell interfaces) to a closed outline. The number of neighbors in each cell was determined by the number of bonds. Surface area was obtained by integrating the square root of the metric tensor over the cell. Adding up all cell areas gives the surface area of the follicle. Cell eccentricity is determined based on the long and short axis of the cell, while cell orientation is defined as the angle between the long axis of the cell and the circumferential axis of the follicle.

4.6.4 BM Stiffness Assays

Basement membrane stiffness was measured by Atomic Force Microscopy (AFM) as described previously (Crest et al., 2017).

4.6.5 Data presentation and Statistical analyses

Data were analyzed and charts displayed using Matlab (Mathworks), Excel (Microsoft), and ggplot2 Rstudio. Figures were assembled by Illustrator CC 2018 (Adobe). Error bars in charts represents SEM and statistical significance was assessed by Welch's unequal variances *t*-test, while two-sample Kolmogorov-Smirnov test was used to analyze probability distribution functions in cell Eccentricity and cell orientation.

Figure 4.1. Morphometrics of WT follicles

in toto imaging of fixed, unflattened follicles between st. 4 and mid-st. 8. See text for details and stage definitions, and note that the x-axis shows stage, not time of development. **(A)** Follicle volume increases ~6-fold over this period. **(B)** Epithelial follicle cell(FC) number increases from ~180 to a final count of ~870. Note that proliferation continues until the end of st. 6 (see text). **(C)** Areas of basal profiles of FCs decrease during divisions at st. 4, and then increase during FC endocycles following st. 6. **(D)** Aspect ratio increases 60% over the study period, linearly with stage.

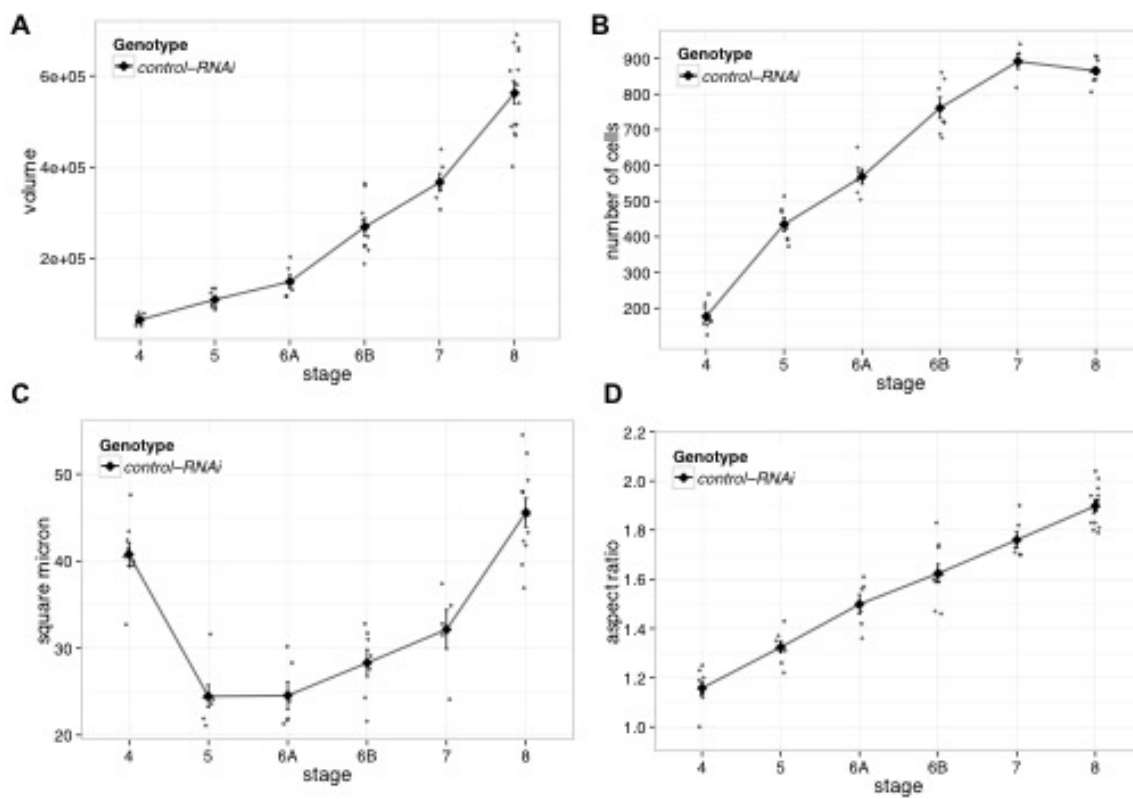


Figure 4.2. Oriented cell divisions and cell eccentricity

(A) Still from confocal movie of live-imaged follicle expressing Jupiter-GFP to visualize mitotic spindles and midbodies in the FCs. **(B)** Stills from ImSAnE projection of A, at 9 and 15 minutes, demonstrating assessment of orientation with respect to axes in plane of follicle epithelium. **(C)** Quantitation of final mitotic division orientation based on midbodies shows no bias in st. 3-4 follicles, but significant bias along the A-P axis at st. 5-6. **(D)** Average eccentricity of FCs shows mild elongation and no change throughout the period under study. n.s: not statistically significant. **(E)** Distribution (density function) of cell eccentricities was unchanged from st. 5-7. **** $p \leq 0.0001$.

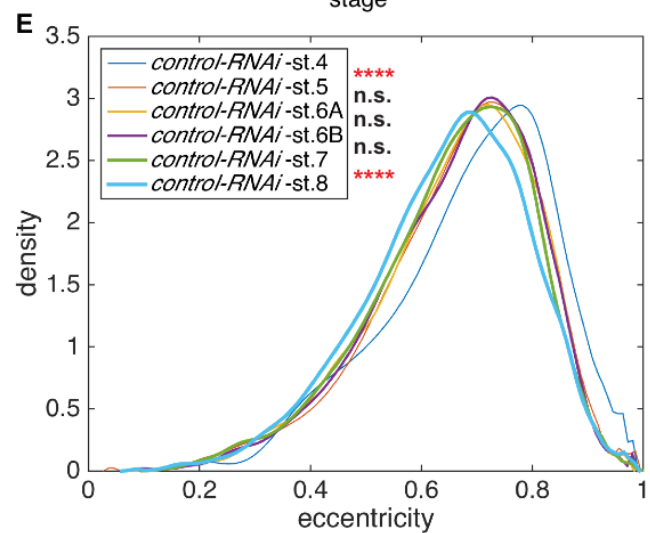
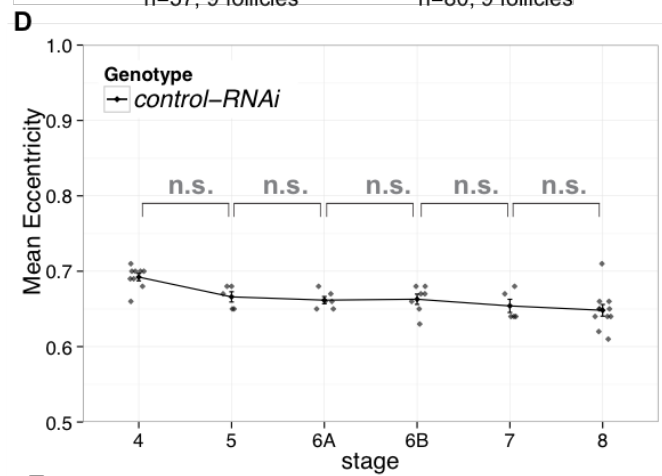
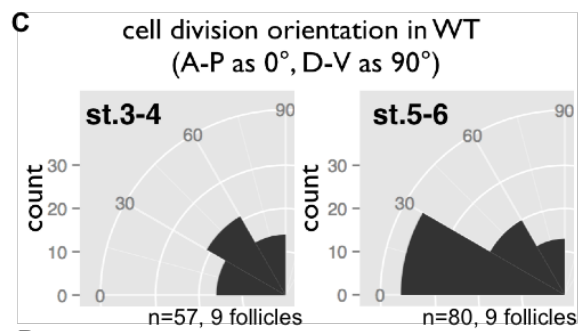
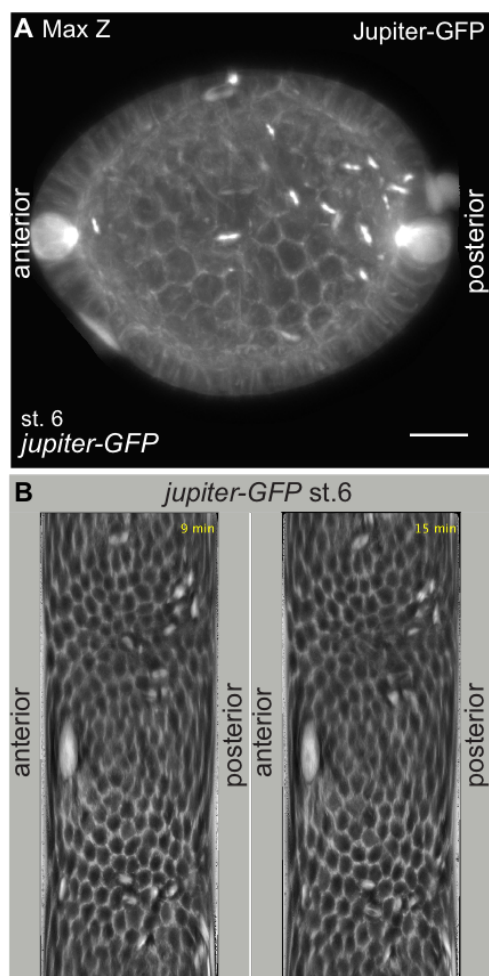


Figure 4.3. Cell orientation, topology, and evidence for relative movement

(A-B) Heat maps displaying orientation of cell long axis with respect to circumferential axis of follicle at st. 6 and st. 8 plotted on cylinder projection; cold colors align with this axis whereas warm colors orient perpendicular to it (degree from the circumferential axis). **(C)** Distribution of orientation data; note the shift in distribution of orientations towards the elongation axis during st. 7-8. **(D)** Positional analysis (see 3G) reveals that cells shift orientation primarily in the anterior 30% of the follicle. **(E)** Reconstruction of ImSAnE-flattened follicle, color coded for number of cell-cell contacts. **(F)** Quantitation of topological distributions; note a slight increase of the percentage of 6-sided cells during post-mitotic epithelium from st. 7 to 8, as an evidence of cell rearrangement by junctional remodeling. **(G)** M-TRAIL tracking of cell migratory paths along A-P axis over a ~6 hour period at st. 8 shows consistent shifts towards the follicle posterior. **(H)** Direct counting of cells along follicle axes provides evidence for intercalation of cells between st. 6B and 7. * $p \leq 0.05$.

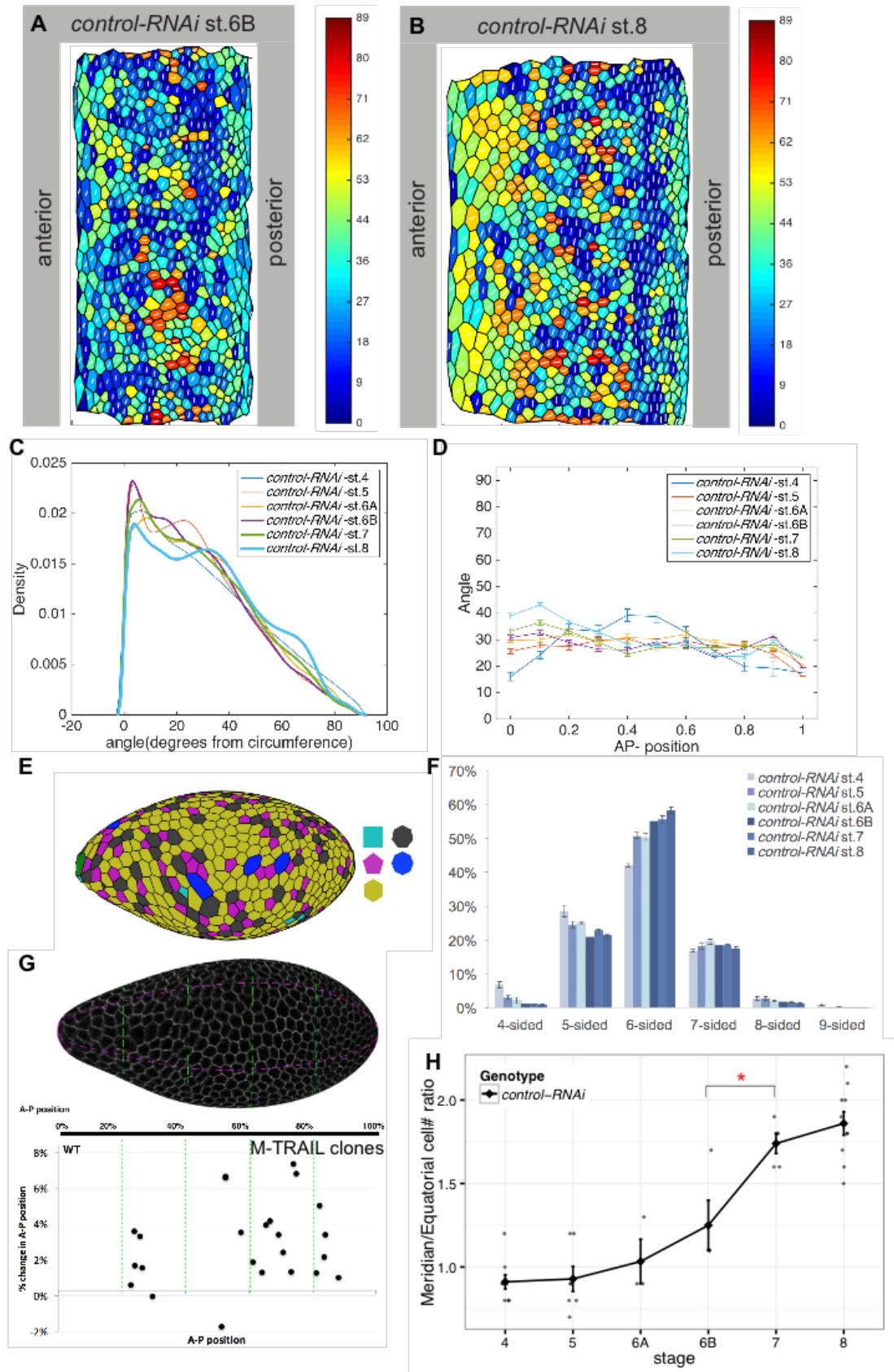


Figure 4.4. Morphometric analysis of *fat2*

(A) *fat2*-depleted follicle elongation deviates from WT controls significantly at st. 7. **(B)** *fat2*-depleted follicles do not display oriented cell divisions at st. 5-6. **(C-D)** Blocking cell division with Fzr overexpression, which halves FC numbers (449 cells), does not prevent elongation. **(E)** Cells become less eccentric in *fat2* follicles, concomitant with elongation defects. **(F-G)** Orientation of the long axis of *fat2*-depleted cells displays a more normal distribution at st. 8 than the circumferentially-skewed distribution of WT follicle cells. **(H)** Topology analysis of *fat2*-depleted follicles still exhibit evidence of cell rearrangements by junctional remodeling. **(I)** M-TRAIL demonstrates that *fat2*-depleted FCs do not change A-P position over 6 hours at st. 8. **(J)** Axial cell counting shows no evidence for A-P oriented intercalation in *fat2*-depleted follicles. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

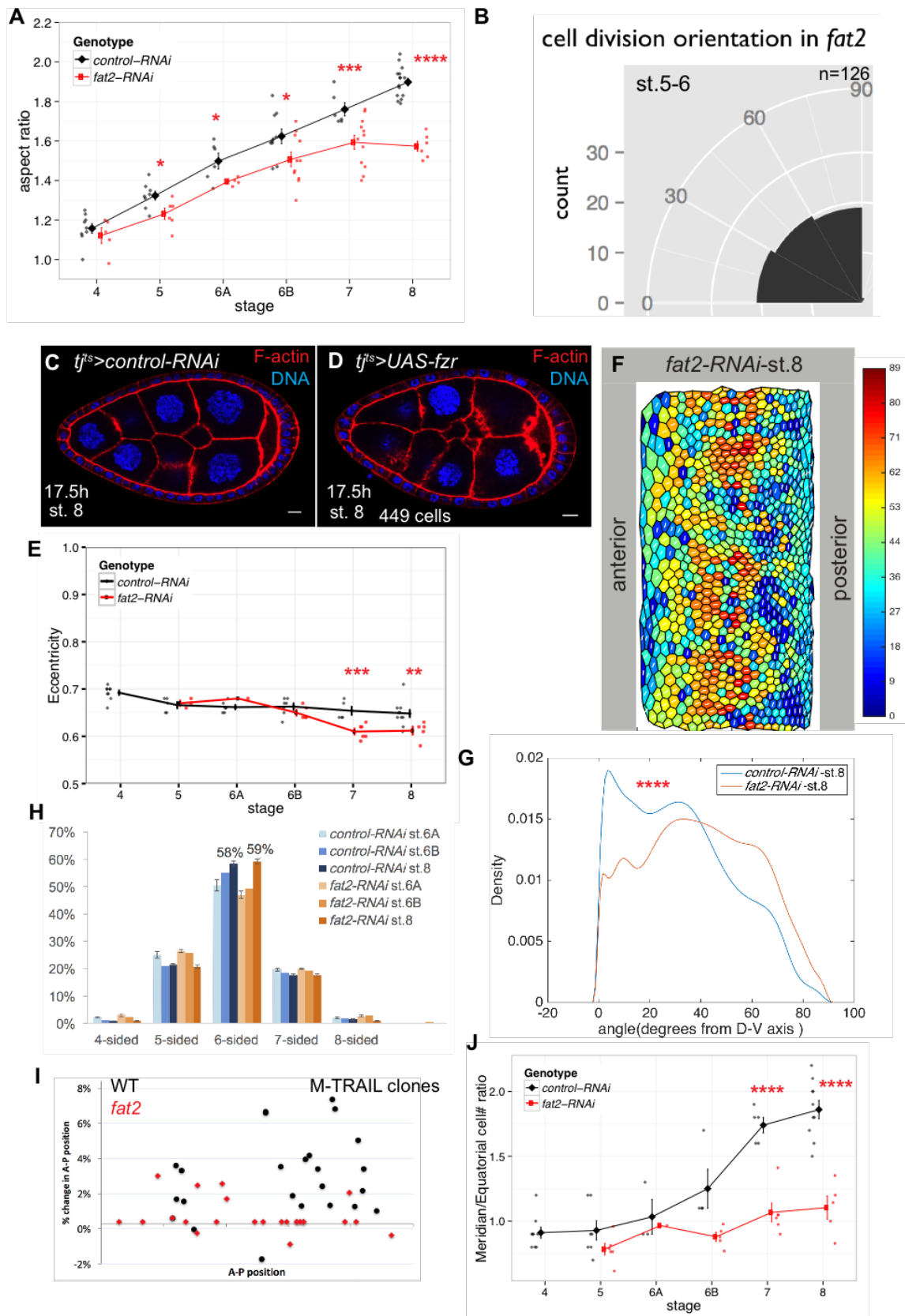
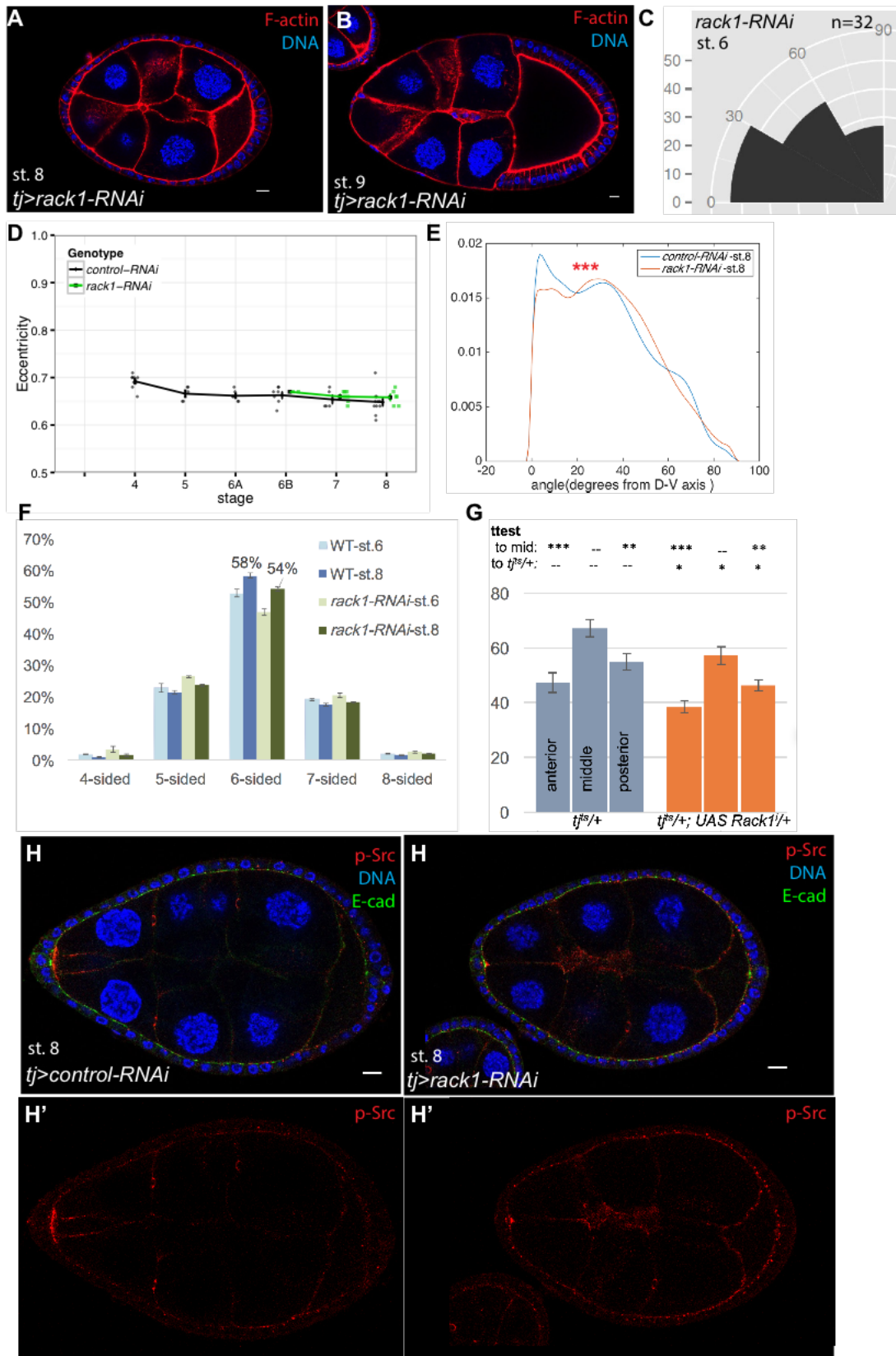


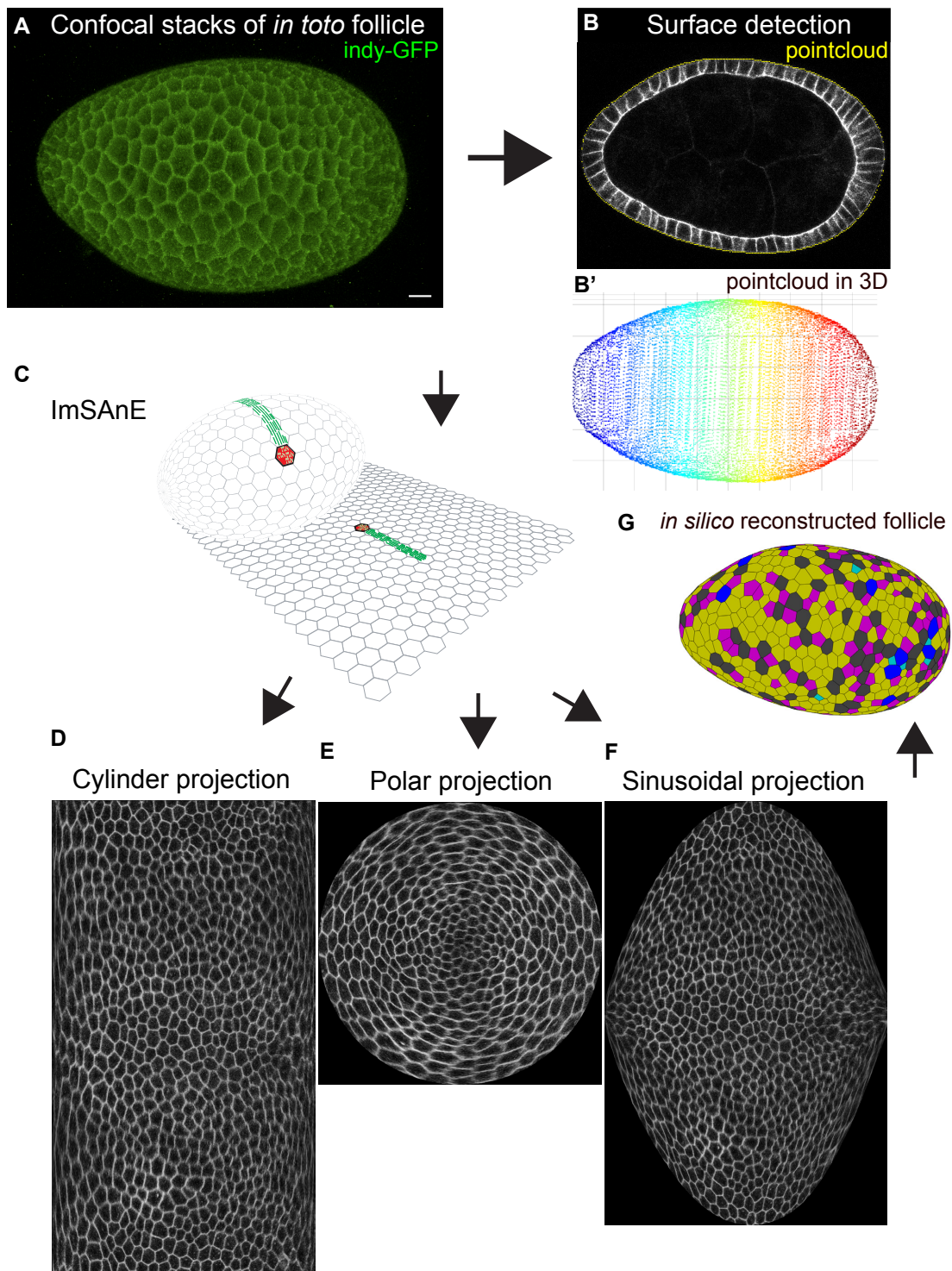
Figure 4.5. RACK1 is required for follicle elongation and proper rearrangement.

(A-B) Rounded follicle phenotype displayed by follicles with epithelial cells depleted of *Rack1*. **(C)** Oriented cell divisions persist in *Rack1*-depleted follicles. **(D)** Cell eccentricity in *Rack1*-depleted follicles is indistinguishable from WT. **(E)** *Rack1*-depleted follicles show fewer cells oriented along follicle circumference at st. 8. **(F)** Topology analysis of *Rack1*-depleted follicles shows lack of full cell rearrangements as percentage of hexagonal packing decreased. **(G)** AFM-measured analysis of BM stiffness in *Rack1*-depleted follicles shows that they are mildly softer than WT but maintain anisotropy, with a stiffer center and softer termini. **(H-I)** *Rack1*-depleted follicles display increased phosphorylated Src, localized to adherens junctions at the apical-lateral domains.



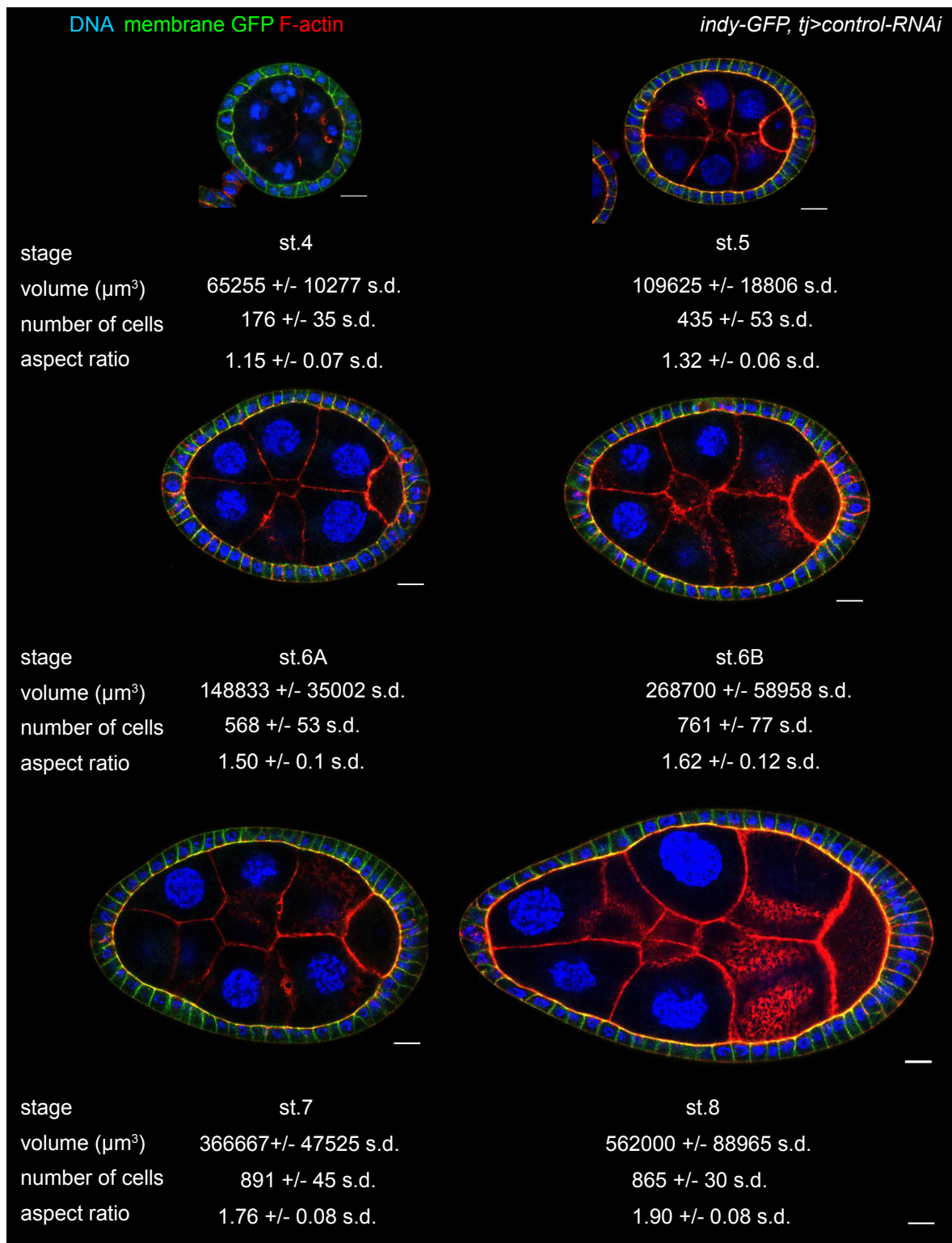
Supplemental Figure 4.1 Workflow for follicle morphometrics.

(A) Confocal stacks of membrane-tagged GFP(indy-GFP) were acquired with a lateral resolution between 0.1-0.2 μm and axial resolution $\sim 0.5 \mu\text{m}$. **(B)** 3D pointcloud was detected with iLastik based on the basal-most pixels of the follicle. **(C)** ImSAnE was used to 'unroll' the surface that **(D)** Cylinder projection **(E)** Polar projection and **(F)** Sinusoidal projection can be generated. **(G)** Cells in the projections were segmented based on the membrane marker by iLastik, skeletonized, and 4 projections (2 cylinders and 2 poles) fused to generate an *in silico* reconstructed follicle.



Supplemental Figure 4.2 Staging criteria and the summary of morphometrics for follicles.

Graph showing the cross sections of the follicles at each stage with morphometrics including mean volume, number of cells, and follicle aspect ratio (s.d: standard deviation). Anterior to the left.



Supplemental Figure 4.3 Additional morphometric and genetic data.

(A) Volume of *fat2*-depleted follicles is indistinguishable from WT. **(B)** Cell proliferation of *fat2*-depleted follicles tracks that of WT, except for apparently excess divisions during st. 7. **(C)** Elongation of *Rack1*-depleted follicles diverges from WT at st. 6B. **(D)** *Rack1* null clones also cause follicle elongation defects. **(E-F)** Proliferation and volume of *Rack1*-depleted follicles are similar to WT. **(G-H)** Both overexpression and depletion of *Src42A* result in defective follicle elongation. **(I-J)** Overexpression of WT *Rack1* rescues the elongation defect of *Rack1*-depleted follicles

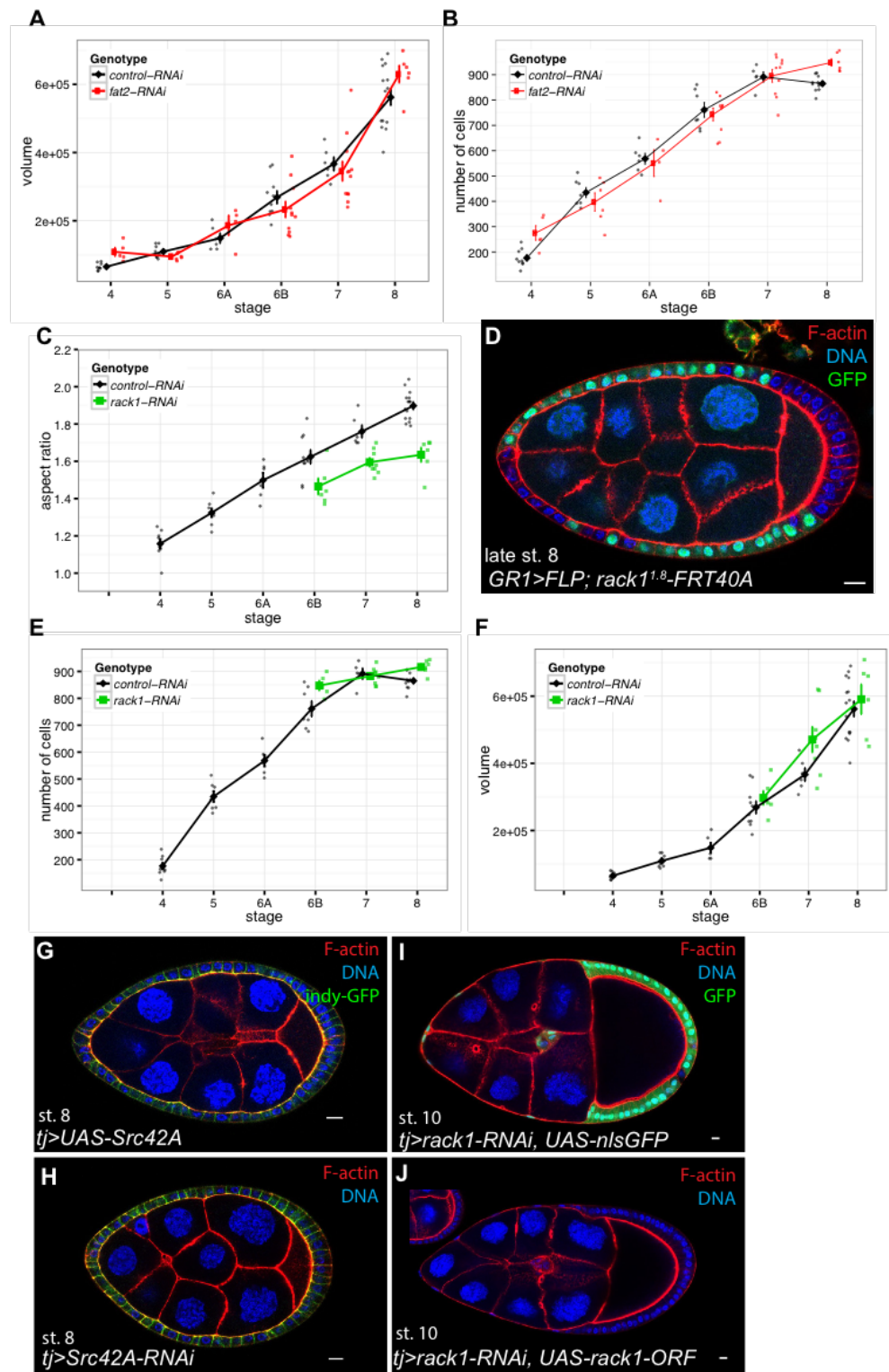


Table 4.1 Reagents and Strains

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal Anti-GFP(3E6)	ThermoFisher	A11120
Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	ThermoFisher	A11001
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 647	ThermoFisher	A21244
Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 647	ThermoFisher	A31571
Phospho-Src Family (Tyr416)	Cell Signaling	#2101
Chemicals		
Phalloidin-TRITC	Sigma	P1951
Alexa Fluor 647 Phalloidin	ThermoFisher	A22287
DAPI	Sigma	D9542
Experimental Model: <i>D. melanogaster</i>		
<i>GR1-GAL4, UAS-FLP</i>	Trudi Schüpbach lab	Flybase ID: FBst0036287
<i>TrafficJam-Gal4</i>	Kyoto Stock Center #104055	Flybase ID: FBst0302922
<i>Indy-GFP</i>	Flytrap #YC0017 (Kelso et al., 2004)	#YC0017
<i>mCherry-RNAi</i>	Bloomington Drosophila Stock Center	Flybase ID: FBst0035785
<i>Fat2-RNAi</i>	Bloomington Drosophila Stock Center	Flybase ID: FBst0040888
<i>Rack1-RNAi</i>	Bloomington Drosophila Stock Center	Flybase ID: FBst0034694
<i>Rack1^{1,8}-FRT40A/Cyo</i>	Bloomington Drosophila Stock Center (Kadrmas et al., 2007)	Flybase ID: FBst0024152
<i>UAS-Src42A-WT</i>	Tian Xu's lab (Read et al., 2004)	Flybase ID: FBtp0019448
<i>UAS-Rack1.ORF</i>	FLY-ORF # F001448	Flybase ID: FBst0500163
Software and Algorithms		
ImSAnE	(Heemskerk and Streichan, 2015)	N/A
Fiji	(Schindelin et al., 2012) http://fiji.sc/	N/A
Matlab 2015a	Mathworks	N/A
RStudio	(RStudio Team, 2015)	N/A
ggplot2	(Wickham, 2009)	N/A
Illustrator CC 2018	Adobe	N/A
Excel	Microsoft	N/A

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