The role of cell division during tissue morphogenesis

Lindsay Rathbun
Syracuse University

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Abstract

During embryonic tissue morphogenesis, cell division increases both the number of cells and cellular diversity. This is often regulated by the positioning of daughter cells post-mitosis. The goal of this dissertation research is to determine the mechanisms that place daughter cells, and how this contributes to tissue development. First, the zebrafish left-right organizer, Kupffer’s vesicle (KV) is used as a model to investigate the role of cytokinesis and abscission during de novo lumen formation. The cytokinetic bridge places at the center of the developing KV rosette, where it acts as a landmark for Rab11-mediated vesicle trafficking to bring polarity components such as CFTR to the site of future lumen formation. Next, the early zebrafish embryo is used as a model to determine how the spindle is oriented to create a monolayer grid formation prior to three-dimensional embryo expansion. Here, the mitotic spindles are oriented parallel to each other and perpendicular to the previous cell division plane. A stark asymmetry in spindle pole size creates a directionality in this spindle positioning, where the larger spindle pole points towards the center of the embryo in a PLK1- and PLK4-dependent manner. Lastly, a local drug delivery system was developed in zebrafish embryos to target a PLK1 inhibitor to the centrosome. Through this system, it was revealed that centrosomal PLK1 is responsible for spindle organization and mitotic progression in zebrafish embryos. Taken together, this work describes how the contribution of cell division to tissue morphogenesis is tissue-specific, raising the argument that further cell division studies should be conducted in vertebrate systems to understand mitosis in a three-dimensional tissue context.
The role of cell division during tissue morphogenesis

by

Lindsay Rathbun

B.S., State University of New York at Geneseo, 2015

Dissertation
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Introduction: The regulation of post-mitotic daughter cell size, placement, and fate drives tissue morphogenesis
1.1. Abstract. During early development, rapid cell divisions give rise to a large mass of embryonic cells that will eventually differentiate and build complex tissue structures. Beyond increasing the number of cells in a tissue, cell division also regulates the positioning and contents of daughter cells post-division. The work highlighted in this dissertation focuses on the mechanism of tissue morphogenesis through cell division in the vertebrate zebrafish model. In this introductory chapter, we define mechanisms that place the mitotic spindle to give rise to daughter cells of differing position, size, and content. Additionally, we highlight what is currently known about these mechanisms in vertebrate and invertebrate settings, as well as discuss the advantages and limitations of cell division studies in various model systems. Overall, the work detailed in subsequent chapters aims to contribute to this understanding, detailing novel mechanisms by which cell division contributes to tissue morphogenesis and introducing new tool sets to continue this research in the future.

1.2. Introduction. During development, rapid cell divisions give rise to a cell mass that must organize into functional tissues in order for the organism to survive. Multicellular structures can have a variety of shapes and functions, spanning from early monolayer grids in vertebrate development to networks of tubes in an organ system. For example, a monolayer grid is crucial for the early development of a vertebrate zebrafish embryo, as it provides a foundation upon which the cell mass will expand on top of a yolk. The specific placement of cells within this monolayer will impact the integrity of the cell mass as it grows (Figure 1.1a). Additionally, rosettes are a common multicellular intermediate
Figure 1.1. Examples of multicellular structures in vivo. (a) Model of early monolayer grid formed in zebrafish embryo development at 1.5 hours post-fertilization (hpf). Top view of embryo shown on left with yolk behind gray cells. Side view of same embryo shown in center panel, rotated such that gray cells are on top and white yolk on bottom. Later embryonic stage shown in right panel with multiple gray cell layers on top of white yolk. (b) Model depicting transition from multicellular rosette intermediate (left) to lumen (center) and subsequent tubule formation (right). Lumen space shown in black in center and right panels.
structure that precedes the formation of lumens and tubules in tissues within the gut, renal system, and cardiovasculature\(^1\) (Figure 1.1b). These shapes can be formed through the positioning of cells during and after cell division. The final placement of a daughter cell post-mitosis is often coordinated through a multi-step process. First, by the orientation of the mitotic spindle, a macromolecular machine that orchestrates cell division (Chapter 3)\(^2\). Second, by determining the cytokinetic cleavage plane placement and the directionality of cleavage plane constriction\(^3\). Third, through the decision to sever the cytokinetic bridge connecting two daughter cells within a tissue (Chapter 2). It is therefore important to understand how the mitotic spindle is positioned, how the cleavage plane is placed, and when the final severing of the cytokinetic bridge occurs within a developing tissue to understand the crucial role of cell division in tissue morphogenesis.

1.3. The role of mitotic machinery in spindle placement during tissue morphogenesis. In order to create two genetically identical cells from one, the mitotic spindle is required to physically separate duplicated genetic material into two daughter cells. This macro-molecular machine is organized by two bipolar microtubule organizing centers, termed centrosomes (centrosomes are cyan in Figure 1.2). To make two centrosomes, the single interphase centrosome is duplicated at the same time as the genetic material during S phase, prior to the construction of the mitotic spindle (Figure 1.3). Centrosomes, microtubules, and microtubule motor proteins (e.g. dynein and kinesin) are used to build the bipolar mitotic spindle drawn in Figure 1.2. There are
Figure 1.2. Components of a mitotic spindle.
Model of metaphase mitotic spindle including
kinetochore fibers and astral microtubules (purple),
kinetochores (gold), chromosomes (blue), centrosomes (cyan), and cell cortex (black).
Example proteins found in centrosomes and at the cell cortex are listed in model.
multiple microtubule populations within the spindle, including kinetochore fibers and astral microtubules (Figure 1.2), required to successfully segregate and pull chromosomes into two daughter cells and to position the mitotic spindle within the cell.

Kinetochore fibers connect the centrosomes to the chromosomes at the kinetochores. Kinetochores are multi-protein structures that function to connect microtubules to the chromosomes at the centromere region\(^4\). The microtubules that make up kinetochore fibers can both push and pull on the kinetochores during chromosomes segregation\(^5\) (Figure 1.2). Checkpoints ensure that kinetochore fibers are attached completely and equally to each duplicated chromosome to ensure segregation fidelity\(^6\). Defects at the interface between kinetochore fibers and chromosomes can result in defects such as abnormal chromosome number termed aneuploidy. One consequence of this are genetic conditions such as Down’s syndrome where trisomy occurs resulting in an extra copy of chromosome 21\(^7\). A second population of microtubules called astral microtubules connect the centrosomes to the cell cortex to anchor the spindle within the cell. These microtubules attach in regions where proteins such as NuMA and LGN reside at the cell cortex, guiding the placement of the mitotic spindle within the cell volume (Figure 1.2). This placement can determine how the spindle orients in context with its surrounding tissue and subsequently determine where each daughter cell is positioned as a tissue is developing (refer to models Figure 1.4-1.5).

While chromosome segregation defects are typically the focus when discussing mitotic errors, spindle orientation defects can also arise when the connection between
centrosomes and the cell cortex is lost. These occur with loss-of-function mutations in cell cortex (LGN\textsuperscript{8,9}) and/or centrosome genes (e.g. Cep215/CDK5RAP2\textsuperscript{10} or pericentrin\textsuperscript{11}), which manifest in disease phenotypes such as microcephaly, heart malformation, small stature, and/or hearing loss (Figure 1.2).

Mitotic spindle orientation is orchestrated by centrosomes, astral microtubules, and anchoring proteins at the cortex. A major driver of spindle orientation is signaling at the oldest centrosome\textsuperscript{12}. During centrosome duplication, one centriole within the centrosome (the oldest) acts as a template for the other (youngest)\textsuperscript{13}. Once the centrioles duplicate and centrosomes separate from one another, one of the centrosomes within the bipolar spindle is innately older than the other as a result (Figure 1.3). This age-dependent asymmetry between the two centrosomes is thought to modulate spindle placement through astral microtubule regulation by the oldest centrosome\textsuperscript{14}. For example, the protein cenexin is asymmetrically distributed between the two centrosomes, localizing to the appendage structures unique to the oldest centriole in the oldest centrosome\textsuperscript{14}. Cenexin is a scaffold protein for the essential mitotic kinase PLK1, which is required for the recruitment of pericentriolar matrix proteins such as pericentrin and CEP215\textsuperscript{15}. The asymmetry in cenexin and other related asymmetries between centrosomes can therefore result in downstream biochemical asymmetries that contribute to mitotic spindle orientation.

1.4. Mitotic spindle drives daughter cell position, size, and composition. Based on mitotic spindle placement, daughter cells can vary in their post-mitotic positions, size
Figure 1.3. Model of centrosome duplication and cell division. Model depicting the cell cycle with corresponding centrosome duplication stages. Kinetochore fiber microtubules (purple), chromosomes (blue), kinetochores (gold), centrosomes (cyan), centrioles (magenta), and midbody (green) shown. Older and younger centrioles denoted.
and composition. In cases where cells divide in relation to an extracellular matrix, mitotic spindles orient such that either both daughter cells interface with the substrate (planar division), or one daughter cell remains at the substrate and one is located away from the substrate (oriented division, Figure 1.4)². By changing the orientation of the spindle, daughter cells can be placed specifically to contribute to the directional growth of a tissue. Additionally, cell division can be regulated to result in unequal daughter cells in regards to their size or composition¹⁶. First, the mitotic spindle can be positioned either at the center of the cell or off-center, resulting in daughter cells of equal size or unequal size, respectively (Figure 1.5). This occurs in contexts like mammalian germline cell divisions where cell divisions result in one large daughter cell and one smaller daughter cell¹⁷. The larger of the two daughter cells becomes the ovum, which will go on to be fertilized and produce a zygote. The smaller daughter cell is extruded as a polar body, which degenerates through apoptosis in most species and is not capable of fertilization and organism development¹⁸. Secondly, proteins can be asymmetrically segregated during division to produce daughter cells with different protein composition. This frequently results in a difference in cell fate for the daughter cells. For example, polarity proteins such as Par proteins are asymmetrically segregated during early C. elegans cell divisions to give rise to different cell types in later development¹⁹ (Figure 1.6). After this initial polarity establishment, cells derived from the original anterior cell are primarily fated to become hypodermis, neural, or pharynx cells, compared to cells derived from the original posterior cell that are also fated to function as muscle, germline, or intestine cells²⁰.
Planar cell division:

Oriented cell division:

Figure 1.4. Planar and oriented cell divisions are driven by spindle orientation in relation to extracellular substrate. Model depicting examples of planar (left) and oriented (right) cell divisions in relation to an extracellular substrate (gray, bottom). Microtubules (purple), chromosomes (blue), kinetochores (gold), centrosomes (cyan), and cell cortex (black) shown.
Daughter cells of same size:

Daughter cells of different size:

Figure 1.5. Cell divisions can result in daughter cells of symmetric or asymmetric size. Model depicting examples of cell divisions producing daughter cells of equal (a) or unequal size (b). Microtubules (purple), chromosomes (blue), kinetochores (gold), centrosomes (cyan), and cell cortex (black) shown.
Daughter cells with the same protein composition:

Daughter cells with different protein composition:

Figure 1.6. Daughter cells can differ in protein composition post-division. Model depicting examples of cell divisions producing daughter cells of symmetric (a) or asymmetric composition (b). Microtubules (purple), chromosomes (blue), kinetochores (gold), centrosomes (cyan), and cell cortex (black) shown. Example of proteins segregated symmetrically or asymmetrically shown in grayscale.
Mitotic spindles can be placed to create divisions that fit into more than one of these categories as well. For example, in the context of brain development, planar cell divisions are symmetric in nature and produce two progenitor daughter cells residing at the extracellular matrix, expanding the progenitor pool (Figure 1.4). Alternatively, oriented and asymmetric divisions result in one progenitor daughter cell at the extracellular matrix and one daughter cell placed away from the niche that will go on to differentiate and create cellular diversity in the developing tissue\(^{21}\) (Figure 1.4). Here, both daughter cell positioning and fate are regulated by spindle orientation.

Alternatively, \textit{Drosophila} neuroblasts undergo cell divisions that result in daughter cells of different size, composition, and subsequent fate\(^{22}\). Careful coordination of daughter cell placement and composition is crucial for the proper development of a functional tissue. The wide range of daughter cell phenotypes created by the positioning and orientation of the mitotic spindle ensures that cell division during tissue morphogenesis not only increases cell number, but cellular diversity as well.

Once the spindle has formed and chromosomes segregated, the process of cytokinesis constricts the cell cortex into a cleavage furrow to begin daughter cell separation. This cleavage furrow is typically positioned over the center of the spindle, further defining the plane by which the daughter cells will be positioned post-mitosis\(^3\) (Figure 1.3). This process resolves the cleavage furrow into a cytokinetic bridge connecting the two daughter cells that is eventually severed through the process of abscission. During abscission, endocytic and secretory vesicles are trafficked to the cytokinetic bridge to coordinate the final severing of the bridge by the ESCRT
(endosomal sorting complex required for transport) complex, completing the final separation of the two daughter cells\textsuperscript{23} (Figure 1.3).

Here we will focus on four mechanisms that drive cell placement following division during tissue morphogenesis: mitotic spindle placement, asymmetric cell division, cleavage plane determination, and abscission.

1.5. Mitotic spindle placement. During mitosis, a cell needs to be able to sense its shape in order to properly place its mitotic spindle. The majority of spindles are placed along the longest axis of the dividing cell, which has become known as Hertwig’s rule\textsuperscript{24}. This allows cell division to align along the plane of tissue tension in some cases, where generation of daughter cells during division will alleviate the existing tension\textsuperscript{25}, or regulate the geometry. However, the mechanism by which cells sense their shape is unknown in contexts such as early embryo cell divisions, where the size of the cell is disproportionately large compared to that of the spindle.

During early embryonic cell divisions, cells become smaller and smaller over time as the number of cells increases and the overall cell mass remains constant. These early divisions are termed cleavage divisions, and they build an embryonic cell mass of sufficient size to begin forming functional tissues during development\textsuperscript{26}. However, it has remained unclear how the mitotic spindle adjusts to cell size to determine the longest cell axis throughout these early divisions where drastic changes in cell size occur. One particular study surveyed the embryos of multiple metazoan organisms to understand how the size of mitotic spindles related to their surrounding cell size during early...
divisions. This study determined that across various organisms including nematodes, annelids, echinoderms, chordates, and nemertea, spindle size scaled linearly with cell size during the first few cell divisions of development. However, this study also concluded that this finding was limited to cells below 140 microns in diameter\textsuperscript{27}. Since vertebrate organisms such as zebrafish and \textit{Xenopus} begin development with cell diameters in this range or higher, it is crucial to determine how mitotic spindles can span these large cells in order to properly segregate genetic material in early development if not by this type of spindle scaling mechanism.

It has been suggested that there is a limit to the size of the mitotic spindle even in extremely large cells. For example, while early \textit{Xenopus} cells can reach diameters of over 1000 microns, spindles do not reach lengths of more than about 60 microns\textsuperscript{28}. This leaves a large cellular distance between the mitotic centrosomes and cell cortex that needs to be spanned for the mitotic spindle to be able to separate chromosomes. One theory is that microtubule nucleation can occur in locations other than the mitotic centrosomes. This would allow for shorter astral microtubules to span this distance by positioning their minus-ends between the centrosome and the cell cortex\textsuperscript{29}. However, this theory has been tested in a cell-free system, where extract from \textit{Xenopus} embryo was utilized instead of dividing cells in a live embryo. It is therefore difficult to ascertain whether this is representative of cell divisions in the \textit{Xenopus} embryo as well, or whether these findings are specific to the cell-free system.

Several studies have sought to determine how mitotic spindles are able to sense their shape to successfully facilitate cell division in cells that seem disproportionately
large. For example, in early zebrafish cleavage divisions, a monolayer grid needs to be constructed to serve as the foundation upon which three-dimensional embryo expansion will subsequently occur. In order for complex multi-cellular structures to be built in early embryos, a shape-sensing mechanism likely exists to facilitate spindle placement in these disproportionately large cells.

Studies in the early zebrafish embryo have proposed that large, uniquely structured centrosomes may account for how astral microtubules are able to span the large distance from mitotic centrosome to cell cortex in large embryonic cells (see Chapter 3). Here, the mitotic centrosomes decreased in size as cells decreased in size throughout early cell divisions, providing a possible shape-sensing mechanism to aid in mitotic spindle placement in large zebrafish embryonic cells. These dramatically enlarged centrosomes eventually focus into smaller centrosomes in later developmental stages, closely resembling those seen in smaller somatic cells in tissue culture settings. This suggests that the dramatically large centrosomes (~125 \( \mu \text{m}^2 \) in diameter at 8-cell stage) found in the early zebrafish embryo is specific to cell divisions in large embryonic cells. In order to gain a more complete understanding of the mechanism for longest axis sensing, it is necessary to utilize both \textit{in vivo} model organisms and \textit{in vitro} settings. For example, the centrosome size and morphology found in large embryonic cells can be more thoroughly characterized in multiple embryonic model systems to determine which characteristics are conserved. In parallel, an \textit{in vitro} setting can be utilized to study how centrosome morphology changes under different conditions of cell size constraint, and whether the shape-sensing mechanism of the cell is altered. \textit{In vivo} studies have also
been conducted in this manner to determine the mechanism of spindle and nucleus centering under cell geometry constraint. Here, sea urchin embryos were placed in chambers of various shapes and sizes to determine how effective the shape-sensing mechanism is in various cellular aspect ratios\textsuperscript{30}. Here, the nucleus positioned at the center of the cell to divide along the longest axis of symmetry across a variety of cell shapes and aspect ratios, demonstrating a cellular shape-sensing mechanism for division plane positioning. Moving forward, both \textit{in vitro} and \textit{in vivo} studies should be used in tandem to determine the mechanism by which a cell is able to sense its shape and appropriately place a mitotic spindle.

1.6. Asymmetric cell division. Spindle positioning can also be regulated to produce daughter cells of two different sizes during asymmetric cell division. This is an important mechanism that ensures cellular diversity. Asymmetric spindle placement occurs through the breaking of cellular symmetry, such as during the asymmetric placement of signals in the early \textit{C. elegans} embryo that causes the spindle to shift to one side\textsuperscript{31}. During the first cell division in a \textit{C. elegans} embryo, proteins are asymmetrically segregated into the two daughter cells (refer to Figure 1.6). An example of this asymmetric protein segregation is the distribution of Par proteins, named after the partitioning defects seen upon mutation, knockout, or mislocalization of these proteins\textsuperscript{32}. At the start of the first \textit{C. elegans} cell division, PAR-3 and PAR-6 localize to the anterior region of the one-cell stage embryo, whereas PAR-1 and PAR-2 localize posteriorly during the one-cell stage. After the first embryo cell divides, the larger, anterior cell of
the embryo becomes enriched for PAR-3 and PAR-6 protein and the smaller, posterior of the embryo contains more PAR-1 and PAR-2\textsuperscript{19}. This asymmetry in Par protein localization across the anterior-posterior axis of the embryo results in a downstream asymmetry of components necessary for spindle pulling-force generation. GRP-1/2 are proteins required to generate a pulling force through the astral microtubules during cell division. They are also enriched at the posterior end of the embryo in a PAR-2- and PAR-3-dependent manner. This asymmetry in protein localization translates to an asymmetry in spindle pulling forces, which shifts the spindle towards the posterior end of the embryo during asymmetric division. Here, mislocalization or loss of GPR-1/2, PAR-2, or PAR-3 result in a symmetric positioning of the spindle in the one-cell embryo \textsuperscript{33}. While early \textit{C. elegans} establish polarity at the one-cell stage, this is not a common mechanism for polarity establishment in other \textit{in vivo} contexts such as vertebrate embryos.

Asymmetric cell division is not an event specific to the early \textit{C. elegans} embryo, as vertebrates also employ it to establish cellular diversity. However, the establishment of an anterior-posterior axis in vertebrate embryos such as mice or zebrafish does not occur until much later in embryonic development. Axis establishment typically occurs either hours (in zebrafish) or days (in mice) after fertilization when the embryo has established a sizeable cell mass. In zebrafish for example, it has been suggested that the anterior-posterior axis is determined during oogenesis through the asymmetric distribution of maternal mRNAs\textsuperscript{34,35}. After fertilization, a cell mass is constructed on top of a large yolk through multiple synchronous rounds of cell division followed by
asynchronous rounds of division. These early divisions occur within the first few hours post-fertilization to help further refine the anterior-posterior axis prior to gastrulation\textsuperscript{36}. Alternatively in mouse embryos, early asymmetric divisions beginning at the 8-cell stage result in the distinction between inner and outer cell mass cells, which lead to important cell fate decisions in the later embryo\textsuperscript{37}. The mouse anterior-posterior axis is not formed until approximately five days after fertilization, after transitioning from the previously established proximal-distal axis earlier in development\textsuperscript{38}. When comparing these two vertebrates to \textit{C. elegans} embryos that establish their anterior-posterior axis on their first cell divisions, it is clear that divergent mechanisms have been established to set up an anterior-posterior axis.

In addition to the asymmetric distribution of proteins or mRNAs post-mitosis, membrane organelles can also be segregated unequally into daughter cells. In \textit{Drosophila} sensory organ precursor (SOP) cells, fate can be determined based on the presence of Rab11-associated recycling endosomes in daughter cells post-mitosis. Rab11 is a small GTPase that regulates a specific compartment within the endocytic pathway, recycling endosomes\textsuperscript{39,40}. Strikingly, one daughter cell inherits a large pool of Rab11-associated vesicles between anaphase exit and abscission which transport a ligand in the Notch pathway to specify a certain cell fate. The other daughter cell lacks the crucial binding partner of Rab11, nuclear fallout (Nuf), so recycling endosomes are unable to form and transport the ligand\textsuperscript{41}. This creates two different cell fates through the asymmetric distribution of Rab11-associated endosomes in post-mitotic daughter cells. While SOP cells are specific to the invertebrate \textit{Drosophila} model, a study has
found that a Rab11 asymmetry exists in mammalian cells grown \textit{in vitro} where recycling endosomes interact specifically with the oldest mitotic centrosome\textsuperscript{39}. An additional study has implicated Rab11 in spindle organization and orientation as well, where mitosis is delayed, astral microtubules are disrupted, and centrosome proteins are misorganized when Rab11 is depleted or mutated\textsuperscript{40}. It is therefore possible that Rab11 plays a more pivotal role in driving spindle orientation in asymmetric division contexts through directly regulating the spindle itself.

The mitotic spindle is asymmetric in nature, containing an older and a younger mitotic centrosome that assemble the microtubule based spindle (Figure 1.3). Interestingly, this innate asymmetry has been linked to asymmetric cell division in a stem cell niche, such as the \textit{Drosophila} germline. Here, the division plane is placed perpendicular to the stem cell niche resulting in one daughter cell that remains in the niche and one that is positioned away from the niche (Figure 1.4). The oldest centrosome is specifically segregated into the self-renewed stem cell, whereas the youngest centrosome is inherited by the daughter cell fated to differentiate\textsuperscript{42}. While this work was conducted in an invertebrate system, it is similar to findings in the mouse brain where radial glia progenitors are more likely to inherit the oldest centrosome after division, and daughter cells destined to differentiate inherit the youngest centrosome\textsuperscript{43}. Alternatively, a separate study concluded that the opposite scenario occurs in \textit{Drosophila} neuroblasts, where the older of the two mitotic centrosomes is inherited by the daughter cell fated to differentiate\textsuperscript{44}. Another conflicting study suggested that an asymmetry in centrosome inheritance does not exist in the developing mouse
cerebellum, where the localization of the oldest centrosome was not correlated with cell fate despite a stark asymmetry in centrosome composition\textsuperscript{45}. Although one centrosome contained the protein ODF2, an appendage protein that denotes the oldest centrosome, there was no trend noted in the fate of post-mitotic cells and the age of the centrosomes they inherited. These differences in centrosome inheritance across various tissues in both vertebrates and invertebrates reiterate the need to conduct studies into the role of cell division during tissue morphogenesis in various organisms and tissue types. The caveat here is that live tracking of centrosome inheritance during asymmetric cell divisions is difficult or impossible in many vertebrate systems like mice. However, the optical transparency of the zebrafish embryo make it a great candidate for continued study of asymmetric centrosome inheritance in a variety of vertebrate tissues.

1.7. **Lumen establishment through cell division.** Lumenogenesis in the zebrafish Kupffer’s vesicle (KV) is one example of how the role of cell division during tissue formation has been elucidated using the vertebrate zebrafish model (see Chapter 2). KV is a cyst of polarized cells surrounding a fluid filled lumen and functions as the left-right organizer in zebrafish. Within the KV lumen, motile cilia beat and produce a leftward fluid flow within the lumen space that is thought to establish the left-right axis. This organ is conserved across most vertebrates and is analogous to the node in mammals\textsuperscript{46}. Interestingly, the formation and function of the KV lumen is analogous to lumens in other tissue and organ contexts. The main function of a lumen is to move air or fluid through an organ. This movement allows for processes such as digestion,
filtering, and transport of components to different regions of an organism. Some lumens like that within KV remain as small sphere-shaped hollows, while many other are elongated into tubules such as blood vessels or the intestine. Defects in lumen formation can cause a variety of diseases, such as polycystic kidney disease, hypertension from vascular lumen defects, and reversed organ placement due to left-right organizer lumen defects. One contributor to defective lumen formation is defective cell division, where either inappropriate placement of the mitotic spindle or inappropriate timing of cytokinetic bridge cleavage result in disrupted lumenogenesis.

Lumens can form through a variety of different mechanisms. One such mechanism is called cavitation, where apoptosis at the center of a polarizing cell mass creates a hollow lumen or tubule. This occurs in contexts such as mammary glands and salivary glands. Conversely, hollowing is a separate process of lumen formation, where directed membrane trafficking during cell division brings membrane to the future apical membrane, which gives rise to a lumen in contexts such as blood vessels and the zebrafish left-right organizer, KV. A third mechanism of lumen formation occurs in the neural tube, where an epithelial sheet invaginates, seals, and separates itself from the original cell sheet to form an elongated lumen tube. All three of these mechanisms result in a hollow lumen surrounded by an epithelial sheet. However, the role of cell division in each of these contexts has not been carefully explored.

To understand the mechanisms that contribute to lumen formation, in vitro three-dimensional mammalian tissue culture systems have been frequently employed. These
*ex vivo* systems are advantageous because they allow cellular and intracellular events to be easily monitored using live cell imaging unlike many *in vivo* mammalian models. In these systems, the placement of the cytokinetic bridge is correlated with the initiation of an apical membrane. One cell in a matrix suspension divides into two cells, and a lumen begins to form where the cytokinetic bridge is positioned and likely cleaved between these cells (modeled in Figure 1.7)\(^{65,66}\). While cytokinetic bridge cleavage has yet to be clearly resolved in this context, the directed membrane trafficking of apical polarity components such as tight junctions and aPKC has been clearly defined\(^{46,50,67–69}\). These proteins are transported to the cytokinetic bridge between two dividing cells in a three-dimensional culture, and this location will eventually become the apical membrane of the nascent lumen\(^{65,70,71}\). This directed membrane transport requires the small GTPase Rab11 and its associated effector protein, FIP3 (Family of Rab11-Interacting Proteins 3), for appropriate delivery of apical polarity proteins to the bridge and membrane remodeling at this site\(^{72}\). Once remodeling occurs on either side of the cytokinetic midbody, bridge cleavage (abscission) occurs through the use of ESCRT\(^{73}\). After the lumen is formed during this first cell division, subsequent cell divisions occur with mitotic spindles oriented parallel to the plane of the lumen\(^{74}\). Ingression then occurs asymmetrically with the furrow constricting towards the apical membrane\(^{75}\) (modelled in Figure 1.7). This *ex vivo* system created a testable model in which the first cell division initiates lumen formation and subsequent divisions are oriented appropriately to expand the lumen.
Figure 1.7. Cell division drives lumen formation \textit{ex vivo} and \textit{in vivo}. (a) Model depicting lumen formation \textit{ex vivo} from single cell (left) to lumen formation within two cells (center) and subsequent lumen expansion (right). (b) Model depicting lumen formation \textit{in vivo} from group of cells (left) to lumen formation from a rosette intermediate (center) and subsequent lumen expansion (right). Note position of cytokinetic bridge marked by midbody (orange). Cytokinetic midbody (orange), apical membrane (magenta), and nuclei (blue) shown within cell boundaries (black).
The idea that apical membranes could be expanded through alignment of the mitotic spindle along the face of a lumen originated with studies using MDCK (Madin-Darby canine kidney) monolayer cultures. In this system, molecular cues were identified that are required to orient the spindle in polarized cells\textsuperscript{76–78}. It was determined that astral microtubules are anchored to the cell cortex via cadherins, where LGN binds and recruits NuMA. The location of this interaction is dictated by the Cdc42-dependent positioning of aPKC, as the NuMA-LGN complex is excluded by aPKC. Astral microtubules are able to anchor to the NuMA-LGN complex through the interaction of dynein with NuMA\textsuperscript{79}. Astral microtubules therefore connect the centrosome to the cell cortex through these proteins to position the spindle within the confines of the cell. When centrosome appendage proteins\textsuperscript{14}, cadherins\textsuperscript{76–78}, aPKC\textsuperscript{80}, Cdc42\textsuperscript{74}, NuMA\textsuperscript{81}, or LGN\textsuperscript{9} are mutated or mislocalized, the mitotic spindle misorients in relation to the cells substrate. In the case of an expanding monolayer, the misregulation of these proteins would therefore result in randomized spindle placement in relation to the monolayer. Daughter cells would instead protrude away from the monolayer instead of incorporating into and expanding the monolayer\textsuperscript{76–78}. When using MDCK cells in a three-dimensional culture, a single MDCK cell resuspended in an extracellular matrix will expand into a multi-cellular cyst with a hollow lumen. Studies in this system found that in order for the cells to organize into a cyst with a single lumen, spindles needed to orient parallel to the apical membrane. Here, disruptions in components such as Cdc42\textsuperscript{82} or the centriole appendage protein cenexin\textsuperscript{14} result in spindle misorientation and subsequent multi-
lumen phenotypes. Studies in this system introduced the idea that spindle orientation was essential in the development, specifically expansion, of a lumen.

The connection between proper spindle orientation and lumenogenesis originally studied in *in vitro* settings was corroborated by several *in vivo* mouse studies as well. For example, defects in spindle orientation caused by disruption of E-cadherins resulted in randomized spindle orientation in relation to the luminal plane in mouse prostate development\(^\text{83}\), and similar phenotypes were observed in mouse seminiferous tubules upon depletion of a centrosome scaffold protein, Gravin\(^\text{84}\). Additional studies were performed in a developing zebrafish, where depletion of the spindle pole protein IFT88 resulted in misoriented spindles in the developing zebrafish pronephric duct\(^\text{85}\). One caveat here is that these studies are correlative, since live imaging of a misoriented spindle could not be accomplished to determine if a multi-lumen phenotype resulted. In order to definitively determine whether spindle orientation defects results in disrupted lumenogenesis, live imaging would have to be feasible. This suggests that zebrafish are an ideal model to investigate the role of spindle orientation during lumenogenesis. Additionally, further studies would be necessary to determine whether proper spindle orientation is required for lumen formation, lumen expansion, or both.

1.8. Studies in zebrafish have demonstrated a separate mechanism for lumen formation and expansion. The zebrafish KV utilizes a transient rosette as a prerequisite structure before transitioning to polarized cells surrounding a fluid filled lumen. Rosettes are common intermediate structures that consist of five or more cells
interfacing at a single locus (Figure 1.1 and 1.7). The cells that make up the rosette adopt wedge shape through actomyosin contractility at the apical membrane\(^1\). Rosette formations are seen in a variety of organs and tissue types \textit{in vivo} such as the eye\(^86\), kidney\(^87\), pancreas\(^88\), KV, and lateral line\(^89\). In cases such as pancreas branching, the rosette structure will resolve and contribute to tissue elongation. Conversely, in cases like KV or kidney tubule formation, a lumen will open and expand at the central locus where the rosette cells interface. Interestingly, while rosettes are seen prior to lumen formation \textit{in vivo}, the rosette intermediate is not seen in \textit{in vitro} contexts. The transition from rosette to lumen likely uses conserved intracellular mechanisms similar to the \textit{ex vivo} models described (and modeled in Figure 1.7), however it is also likely that unique mechanisms exist that need to be identified through \textit{in vivo} exploration.

In our recent studies, we identified for the first time that the placement and severing of the cytokinetic bridge at the center of the rosette drives KV lumen formation (Chapter 2). One of the most striking advances in this study was the live imaging of an abscission event \textit{in vivo}, which has not been accomplished before. Similarly, we were able to follow live mitotic cells during KV development, and we determined that spindle positioning from metaphase to anaphase was random with respect to the lumen. If spindle orientation was the driving factor in lumen expansion, we would expect that mitotic spindles to be placed parallel to the apical membrane or plane of the lumen\(^14,74\). However, since spindle orientation was determined to be random, we concluded that spindle orientation was not a driving factor in lumen establishment or maintenance as was seen in \textit{in vitro} contexts. Instead, we observed that daughter cells are repositioned
as they enter cytokinesis in order to project the cytokinetic bridge towards the center of the rosette (modeled in Figure 1.7). It is therefore likely that various mechanisms of lumen expansion are utilized across different tissue types, requiring the study of lumen expansion in many in vivo and in vitro settings to fully understand the process.

In addition to these mechanistic differences, our studies also identified similarities between ex vivo and in vivo lumen formation. For instance, we determined that Rab11-mediated vesicle motility is required in the KV to bring apical polarity proteins such as CFTR (cystic fibrosis transmembrane conductance receptor)\textsuperscript{50} to cytokinetic bridges at the center of the rosette (refer to model Figure 1.7) to aid in the establishment of the apical membrane and a subsequent lumen. This is similar to findings in ex vivo three-dimensional acini\textsuperscript{71}. We expanded upon both the ex vivo and our in vivo models by acutely inhibiting abscission or by prematurely severing the cytokinetic bridge. In both instances, a lumen was unable to form or was much decreased in size\textsuperscript{61}. Our studies are unique in that they demonstrate the importance of comparing ex vivo and in vivo studies to identify cellular and intracellular mechanisms of cell division during construction of a three-dimensional tissue.

1.9. Implications of tissue morphogenesis studies in an in vivo model. Overall, it is becoming clear that a move towards the use of various in vivo models that can be coupled with live-cell imaging approaches is necessary to elucidate both the conserved and unique mechanisms by which cell division contributes to tissue morphogenesis. The research detailed in the subsequent chapters of this dissertation highlight the various
imaging, optogenetic, pharmacological, and molecular toolsets that can be utilized to unravel how cell division is regulated during vertebrate development. Future research in model systems such as zebrafish will ensure a greater understanding of the tissue-specific mechanisms that build complex three-dimensional structures through mitosis.
Chapter Two:

Cytokinetic bridge triggers \textit{de novo} lumen formation \textit{in vivo}
This chapter features work published in Nature Communications in 2020:

**Cytokinetic bridge triggers *de novo* lumen formation *in vivo***

**Rathbun LI**, Colicino EG<sup>1,2,6</sup>, Manikas J<sup>1</sup>, O’Connell J<sup>1</sup>, Krishnan N<sup>1</sup>, Reilly NS<sup>3</sup>, Coyne S<sup>2,4</sup>, Erdemci-Tandogan G<sup>5</sup>, Garrastegui A<sup>1</sup>, Freshour J<sup>1</sup>, Santra P<sup>2</sup>, Manning ML<sup>5</sup>, Amack J<sup>2</sup>, Hehnly H<sup>1</sup>

<sup>1</sup>Biology Department, Syracuse University, Syracuse, NY

<sup>2</sup>Department of Cell and Developmental Biology, SUNY Upstate Medical School, Syracuse, NY

<sup>3</sup>Department of Physics and Astronomy, University of Rochester, Rochester, NY

<sup>4</sup>Department of Biology, SUNY Geneseo, Geneseo, NY

<sup>5</sup>Department of Physics, Syracuse University, Syracuse, NY

<sup>6</sup>Current location: Department of Cell and Developmental Biology, University of Michigan Medical School Ann Arbor, MI

2.1. Abstract. Multicellular rosettes are transient epithelial structures that serve as intermediates during diverse organ formation. We have identified a unique contributor to rosette formation in zebrafish Kupffer’s Vesicle (KV) that requires cell division, specifically the final stage of mitosis termed abscission. KV utilizes a rosette as a prerequisite before forming a lumen surrounded by ciliated epithelial cells. Our studies identify that KV-destined cells remain interconnected by cytokinetic bridges that position at the rosette’s center. These bridges act as a landmark for directed Rab11 vesicle motility to deliver an essential cargo for lumen formation, CFTR (cystic fibrosis transmembrane conductance regulator). Here we report that premature bridge cleavage through laser ablation or inhibiting abscission using optogenetic clustering of Rab11 result in disrupted lumen formation. We present a model in which KV mitotic cells strategically place their cytokinetic bridges at the rosette center, where Rab11-associated vesicles transport CFTR to aid in lumen establishment.

2.2. Introduction. Tissue morphogenesis is a fundamental process that contributes to building and maintaining organs, as well as orchestrating overall embryogenesis. How these morphogenic changes are coordinated at a molecular and cellular level remains a central question to developmental biology. One common cellular rearrangement that occurs during tissue morphogenesis is the generation of a transient epithelial rosette that remodels to form a finalized organ with apical-basal polarity and a central lumen. Rosettes are multicellular structures that interface at a central point. Rosette formation has been observed in many contexts including Drosophila eye morphogenesis,
zebrafish lateral line development, mouse and *Xenopus* kidney tubule formation, and pancreatic branching in mice. Our studies here utilize the left-right organizer, Kupffer’s vesicle (KV), in the vertebrate model *Danio rerio* to characterize a mechanism of rosette and subsequent lumen formation.

KV is a conserved organ of asymmetry that is required in all vertebrates to place visceral and abdominal organs with respect to the two main body axes and requires the formation of a rosette structure before it fully develops. The mechanism of asymmetry establishment in some mammals (humans, mouse, rabbit), fish, and amphibians is that the organ of asymmetry creates a leftward flow through motile cilia in the extracellular lumen to initiate the asymmetrical expression of 3 genes, Nodal, Lefty, and Pitx2, across the embryo. Due to the conservation of this organ, the ease of transgenics, and live cell imaging of a transparent embryo, KV was used as an *in vivo* model for lumen formation. The current framework for KV development is that non-polarized mesenchymal cells organize into a two-dimensional (2D) rosette-like structure that will assemble into a three-dimensional (3D) sphere with a fluid-filled lumen. During rosette assembly the individual cells start to establish apicobasal polarity. While events downstream of KV’s leftward fluid flow have received much attention, little is known about the mechanism required for KV assembly.

KV rosette formation may require the actin-myosin network at the apical membrane. This same actin-myosin network drives contractile ring formation during cytokinesis, a process of separating the two daughter cells following mitosis. Following cytokinesis, a cytokinetic bridge remains between the two daughter cells for a duration...
of 1-3 hours depending on cell type\cite{96,97}. The remaining bridge is cleaved in a process called abscission. To accomplish this, the bridge is first resolved to a diameter of approximately 1-2 micrometers permitting the Endosomal Sorting Complexes Required for Transport (ESCRT) to sever the bridge\cite{98,99}. In 3D kidney epithelial cell cultures that form a sphere with a central lumen, the cytokinetic bridge furrow ingress occurs towards the center of the sphere where the apical membrane is established\cite{75,100}.

Here, our studies demonstrate that the cytokinetic bridge acts as a symmetry breaking event to signal where the apical membrane of the dividing cell is positioned. We find in the developing zebrafish embryo that the process of cell division is required for KV morphogenesis. Specifically, the placement of the cytokinetic bridge and its appropriate cleavage is involved in KV transition from a rosette structure to a sphere with a fluid filled lumen.

2.3. Results and Discussion

2.3.1. Mitosis is required for lumen formation. KV uses a rosette intermediate before forming a lumen (Figure 2.1a-b)\cite{46}. KV precursor cells are visualized in live embryos by decorating the plasma membrane with GFP (Sox17:GFP-CAAX, Figure 2.1b). The current framework for KV development is that mesenchymal precursor cells transition to epithelial KV cells (MET), which requires establishment of apicobasal polarity, apical clustering, and the expansion of apical cell surfaces to facilitate the formation of a central lumen\cite{101} (Figure 2.1a-b). To investigate the contribution of cell division to KV development, we first calculated the mitotic index of cells destined to form KV compared
Figure 2.1. Mitosis is required for lumen formation. (a) Model depicting zebrafish embryo (top) and KV morphology (bottom) during development. Approximate location of KV denoted by magenta spot. KV membrane (magenta) and regions of apical polarity (black) depicted in model below. (b) Top: maximum confocal projections of KV at developmental stages denoted in panel a. pH3 (mitotic nuclei, cyan) and KV membrane marker (Sox17:GFP-CAAX, magenta) shown. Bottom: KV membrane marker (Sox17:GFP-CAAX, gray) and lumen trace (orange) shown. Bars, 50μm. (c) Mitotic indices (%) represented as violin plot with endpoints depicting minimum and maximum values, quartiles depicted by thin black lines, median depicted by thick black line. n > 247 cells/stage, n = 43 embryos, two-tailed, unpaired Students t-test. Statistical results detailed in Methods Table 5. (d) Representative 3D renderings of KV under conditions of DMSO vehicle control, microtubule inhibition (1μM nocodazole) or PLK1 inhibition (1μM BI2536). Sox17:GFP-CAAX (magenta), pH3-positive nuclei (cyan), and DAPI (blue) shown on left. Sox17:GFP-CAAX (gray) and lumen trace (orange) shown on right. Percentages indicate mitotic index of image, lumen area denoted. Bar, 20μm. (e) Violin plot depicting normalized 2D lumen area under conditions represented in (d) with endpoints depicting minimum and maximum values, quartiles depicted by thin black lines, median depicted by thick black line. One-way ANOVA with Dunnett’s multiple comparison, statistical results detailed in Methods Table 5 (**, p<0.0001 for n>41 embryos).
to other stages of development. The mitotic index was measured during the first 24 hours post-fertilization (hpf). In this time frame, embryos transition through four basic developmental stages: the cleavage period (0-2.25 hpf), the blastula period (2.25-5.25 hpf), the gastrula period (5.25-10 hpf), and the segmentation period\textsuperscript{102} (10-24 hpf, Supplementary Figure 2.1a). KV formation occurs between the gastrula and segmentation period. During the cleavage period the mitotic index is 100% and steadily decreases to ~3% during the subsequent periods (Supplementary Figure 2.1a-c).

Between the gastrula and segmentation period, KV cells had a mitotic index between 5-10% as seen by pH3-positive cells in fixed embryos (Figure 2.1b-c) or with PLK1-mCherry in live embryos (Polo-like Kinase 1). This index was significantly greater than the mitotic index in cells outside the KV (Figure 2.1c), suggesting division entry is upregulated in KV-destined cells, providing a program where division is incorporated to contribute to KV morphogenesis.

To determine if cell division is required for KV lumen formation, we treated cells with two different mitotic synchronizing agents: a small molecule inhibitor of an essential mitotic kinase, Polo-Like Kinase 1 (PLK1, BI2536, used in \textsuperscript{103,104}), or a low dose of a microtubule destabilizing drug to disrupt spindle dynamics (nocodazole, used in \textsuperscript{105}). PLK1 inhibition can result in cells arresting in G2, prometaphase, metaphase, or cytokinesis\textsuperscript{15}. Nocodazole treatment can cause overall microtubule destabilization at high doses leading to disruption of intracellular trafficking (usually at 10\textmu M)\textsuperscript{106,107}, but at lower doses (100nM) the majority of microtubules are intact and defects in prometaphase exit occur \textsuperscript{15,103,108}. During 75-90% epiboly, dechorionated embryos were
Supplementary Figure 2.1. Mitosis is required for lumen formation. (a) Timeline depicting zebrafish embryo development from 0-12 hours post-fertilization (hpf, top) and models with embryo morphology during these stages (bottom). Adapted from Kimmel et al 1995. (b) Representative confocal projection of nuclei during the blastula, gastrula, and segmentation periods. Mitotic nuclei (cyan) and non-dividing nuclei (blue) displayed. Percentages indicate mitotic index of image. Bar, 20μm. (c) Histogram displaying mitotic index from 0-12hpf. Cleavage (C, orange), blastula (B, green), gastrula (G, purple), and segmentation (S, blue) periods highlighted. n > 80 embryos ± SEM. n=6 (0-2hpf), 10 (2-4hpf), 11 (4-6hpf), 9 (6-8hpf), 32 embryos (10-12hpf) from 11 clutches. (d) Representative 3D renderings of KV under conditions of microtubule inhibition (100nM nocodazole) or PLK1 inhibition (100nM BI2536). Sox17:GFP-CAAX (magenta), pH3-positive nuclei (cyan), and DAPI (blue) shown on left. Sox17:GFP-CAAX (gray) and lumen trace (orange) shown on right. Bar, 20μm. Percentages indicate mitotic index of image. (e-f) Violin plot depicting KV mitotic index (e) or cell number (f) under conditions of DMSO (black), BI2536 (orange), or nocodazole (blue) treatment. Endpoints depict minimum and maximum values, quartiles depicted by thin black lines, median depicted by thick black line. (e-f) One-way ANOVA, p<0.00001 (****). (e) DMSO: n=27 embryos, 100nM BI2536: n=35 embryos, 1μM BI2536: n=30 embryos, 100nM nocodazole: n=39 embryos, 1 μM nocodazole: n=40 embryos per treatment over four independent experiments. F(4,166)=8.733, df=166. (f) DMSO: n=32 embryos, 100nM BI2536: n=40 embryos, 1μM BI2536: n=37 embryos, 100nM nocodazole: n=48 embryos, 1 μM nocodazole: n=45 embryos per treatment over four independent experiments. F(4,197)=6.137, df=197. (g) Scatter plot depicting relationship between number cells in KV (normalized to control mean, x-axis) and 2D lumen area (normalized to control mean, y-axis). DMSO (black), BI2536 (orange), and nocodazole (blue) treatment conditions included. n>31 embryos per treatment across five independent experiments. Pearson’s correlation: r=0.3653, p<0.0001 (****), n=206 pairs of x,y values. Detailed statistical analysis in Methods Table 5.
treated with a vehicle control (DMSO), nocodazole (100nM or 1μM), or BI2536 (100nM or 1μM, Figure 2.1d and Supplementary Figure 2.1d). The embryos were allowed to develop to 6-somite stage, where control embryos had a fully developed lumen (Figure 2.1d-e). However, lumen area was significantly lower after BI2536 or nocodazole treatments (Figure 2.1d-e). BI2536 and nocodazole treatments resulted in significantly increased mitotic indices compared to control DMSO-treated embryos (Supplementary Figure 2.1d-e), as well as significant decreases in KV cell number (Supplementary Figure 2.1f) suggesting that defects in cell proliferation resulted in abnormal lumens. When comparing the lumen area and number of KV cells for each embryo analyzed, a positive relationship between these two variables occurred such that an increase in the KV cell number correlates with an increase in the KV lumen area (Supplementary Figure 2.1g). These studies suggest that defects in lumen formation occur when cell division is disrupted.

2.3.2. Cytokinetic midbodies localize to apical membranes. We hypothesized that KV cell placement post-division may be driving KV development, and therefore needed to establish both live and fixed markers of mitotic machinery during zebrafish embryo development. In addition to the nuclear marker H2B-Dendra that marks all nuclei (Supplementary Figure 2.2a), two markers were developed for live-cell microscopy in zebrafish: Polo-Like Kinase 1 (PLK1-mCherry, Supplementary Figure 2.2b) and/or mitotic kinesin-like protein (GFP/mKate-MKLP1, Supplementary Figure 2.2c-d). PLK1 is an essential mitotic kinase that localizes to mitotic spindle poles, kinetochores, and
Figure 2.2. Cytokinetic midbodies localize to apical membranes of lumens in vivo. (a-c) Maximum confocal projections of KV in zebrafish embryos during apical clustering (a), lumen formation (b), and lumen expansion (c). Immunolabeled for midbodies (MKLP1 (a) or RacGAP (b-c), cyan), a polarity marker (aPKC (b), white), and a membrane marker (Sox17:GFP-CAAX, magenta). Bars, 50μm (a, c), 20μm (b), and 10μm (c, inset). Midbodies localizing to apical membrane during KV lumen formation and lumen expansion denoted by yellow arrowheads (b, c). (d) Representative images of midbody localization (RacGAP, white) within KV (Sox17:GFP-CAAX, magenta and DAPI, blue). Pre-rosette (top), rosette (middle), and lumen (bottom) stages of KV development depicted. Orange arrowheads denote apical midbodies, cyan arrowheads denote peripheral midbodies. Bar, 50μm. (e) Violin plot depicting percentage of apical midbodies in KVs at pre-rosette (n=21 embryos), rosette (n=16 embryos), and lumen (n=35 embryos) stages. Endpoints depict minimum and maximum values, quartiles depicted by thin black lines, median depicted by thick black line. n>4 independent experiments. One-way ANOVA, p<0.0001 (****), F(2,69)=104.7, df=69.
**Supplementary Figure 2.2. Cytokinetic midbodies localize to apical membranes of lumens in vitro and in vivo.**

(a-b) Maximum confocal projections of live cell divisions shown by nuclear marker (H2B-Dendra, a) and mitotic marker (mCherry-PLK1, b). Bars, 10μm. (c) 3D rendering of a live Sox17:GFP-CAAX-positive cell (blue) expressing PLK1-mCherry (white) as it progresses through mitosis. Bar, 10 μm. (d) Maximum confocal projections of a live mitotic cell as it progresses from metaphase (0 sec) to cytokinesis (720 sec). PLK1-mCherry (magenta) and GFP-MKLP1 (cyan) depicted. Bar, 5μm. (e) Maximum confocal projections of fixed mitotic cells during metaphase, anaphase, cytokinesis, and pre-abscission. Nuclei (blue), MKLP1 (cyan), PLK1 (magenta), and acetylated tubulin (gold) shown. Green arrowheads depict locations of centrosomes during cytokinesis. Bar, 5μm. (f-g) Maximum confocal projections of fixed embryos during lumen formation and lumen expansion stages of KV development. Midbodies (RacGAP, cyan) and KV membrane (CAAX, magenta) shown. Bar, 20μm. (h) Maximum STED microscopy projections of fixed embryos depicting the cytokinetic midbody (RacGAP, cyan), apical polarity (aPKC, magenta), and acetylated tubulin (blue). Bar, 5μm. (i) Maximum projection depicting a midbody (RacGAP, cyan) and associated cytokinetic bridge (tubulin, magenta). Bar, 10μm. (j) MDCK 3D-acini immunolabeled for actin (magenta), midbodies (MKLP1, cyan), and nuclei (DAPI, white). Bar, 20μm. (k) Diagram depicting the boundary between apical midbodies (inner KV) and peripheral midbodies (outer KV) shown by dashed black line. KV cell membranes (magenta) shown at pre-rosette (left), rosette (center), and lumen (right) shown.
cytokinetic midbodies in dividing cells\textsuperscript{15}. Following division, the separation of two daughter cells occurs through the ingression of a cleavage furrow. A complex containing MKLP1 and RacGAP, called centralspindlin, contributes to cleavage furrow ingression\textsuperscript{109}. After furrow ingression, dividing animal cells stay interconnected by a narrow intercellular bridge that contains a proteinaceous structure known as the midbody, containing RacGAP and MKLP1 (Supplementary Figure 2.2e-i). While daughter cells remain interconnected, the PLK1-positive centrosomes stay on the far side of the nucleus in relation to the cytokinetic bridge and associated midbody (Supplementary Figure 2.2b-e). These studies demonstrate that PLK1-mCherry and GFP/mKate-MKLP1 can be used for monitoring cell cycle progression \textit{in vivo} due to their similar localization patterns as in \textit{in vitro} contexts\textsuperscript{103,108,110}.

During zebrafish apical clustering, endogenous MKLP1 is enriched at sites where apical membranes are initiated (as shown by antibody staining in Figure 2.2a). In KVs with newly initiated lumens, RacGAP-positive midbodies organize at the apical membrane (decorated with aPKC, Figure 2.2b). With stimulated emission depletion (STED) microscopy, we noted aPKC localizing to the cytokinetic bridge adjacent to midbody (positive for RacGAP and the bridge positive for acetylated microtubules, Supplementary Figure 2.2h). Midbodies were also noted in the newly formed lumen still connected to the apical membrane (using KV membrane marker GFP-CAAX, Supplementary Figure 2.2f). During KV lumen expansion, cytokinetic bridges are located closest to the lumen edge and have an associated midbody (Figure 2.2c). Midbodies were noted within the lumen surrounded by membrane that were likely
released after the bridge was abscised (Supplementary Figure 2.2g). To quantify midbody localization throughout KV development, midbodies were scored based on their location, either as apical or peripheral (quantification modelled in Supplementary Figure 2.2k). We determined that the percentage of apical midbodies significantly increases as KV develops from pre-rosette to rosette to lumen stages (Figure 2.2d-e). These findings are consistent with an analysis of human fetal tissues where the accumulation of KIF14-positive midbodies were identified in the lumen of ureteric bud tips\textsuperscript{111}.

Using an \textit{in vitro} 3D epithelial cell model (MDCK), we noted MKLP1-positive midbodies organizing to the site of apical membrane assembly (Supplementary Figure 2.2j). This is consistent with previous \textit{in vitro} 3D tissue culture studies demonstrating that the cytokinetic bridge constricts towards the apical lumen\textsuperscript{71,75}. This finding corroborates our data in zebrafish embryos during KV apical clustering, lumen formation, and lumen expansion (Figure 2.2a-c) and together suggest a role for placement of the cytokinetic bridge in lumen formation.

### 2.3.3. Cytokinetic bridges are placed at lumen formation site

To examine whether cytokinetic bridge placement is associated with lumen formation, a developing KV was monitored where the cells expressed mKate-MKLP1 (Figure 2.3) or PLK1-mCherry (Supplementary Figure 2.3a). Upon examination of a cell exiting mitosis, we note that pinching of the cytokinetic bridge places the two daughter cells such that the cytokinetic bridge is positioned where the lumen will form (Figure 2.3, Supplementary Figure 2.3a).
Figure 2.3. Cytokinetic bridges are placed at the site of future KV lumen formation. (a) 3D rendering of a cell (mKate-MKLP1, cyan) dividing within KV (Sox17:GFP-CAAX, magenta) over time. Bar, 20μm. (b) Cell within KV highlighted during cytokinesis onset (left), pre-abscission (center), and cytokinetic bridge cleavage (right). Region denoted with dashed line in (a) are shown in (b). mKate-MKLP1 (top) and Sox17:GFP-CAAX (bottom) shown in grayscale and in merge below (mKate-MKLP1 in cyan, Sox17:GFP-CAAX in magenta). Green arrowhead denotes the locations of cytokinetic bridge cleavage, orange regions indicate lumen location. Bars, 10μm.
Supplementary Figure 2.3. Cytokinetic bridges are placed at the site of future KV lumen formation. (a) 3D renderings of a mitotic cell (PLK1-mCherry, cyan) within KV (Sox17:GFP-CAAX, magenta) during metaphase (top) and cytokinesis (bottom). Orange asterisk denotes position of forming lumen during cytokinesis. Bar, 10 μm. (b) Distance between cell pairs quantified from cytokinesis (-10 to 0 min) through pre-abscission (0-70 min, n = 17 pairs from n = 7 embryos, range with mean shown). (c) Representative images of midbody localization (mKate-MKLP1, cyan) within KV (CFTR-GFP, grayscale). Pre-rosette (top), rosette (middle), and lumen (bottom) stages of KV development depicted. Orange arrowheads denote apical midbodies, cyan arrowheads denote peripheral midbodies. Bar, 20μm. (d) Violin plot depicting percentage of apical midbodies in live KVs at pre-rosette (n=12 embryos), rosette (n=15 embryos), and lumen (n=15 embryos) stages. Endpoints depict minimum and maximum values, quartiles depicted by thin black lines, median depicted by thick black line. n>4 independent experiments. One-way ANOVA, p<0.0001 (**), F(2,39)=12.74, df=39. (e) Diagram for spindle orientation calculations (left). Angle calculation example within KV (right). Bar, 20 μm. (f-g) Individual spindle angles (f) and angle frequency (g, n = 28 cells, n = 25 embryos) for mitotic cells during KV development. Inset, scatter-plot of individual angles ± SD.
At this time, the bridge is cleaved at one side of the midbody (Figure 2.3b, 38min.), then on the other side (Figure 2.3b, 80min.), depositing the midbody into the lumen (Figure 2.3b). During pre-abscission (22 min, Figure 2.3b), daughter cells are noted to start at >10 μm apart and then move to <5 μm apart (Supplementary Figure 2.3b), suggesting that daughter cells remain interconnected to pack next to each other into the forming KV.

To quantify changes in cytokinetic bridge/midbody positioning during KV development, we expressed live midbody markers (mKate/mCherry-MKLP1 or PLK1-mCherry) in zebrafish embryos with a KV marker (Sox17:GFP-CAAX or CFTR-GFP). In the same manner as previously used in fixed embryos (Figure 2.2d-e, Supplementary Figure 2.2h), midbodies were scored based on their location (Supplementary Figure 2.2h). We calculated a similar trend in live embryos, where the percentage of apical midbodies increased as embryos progressed through pre-rosette, rosette, and lumen formation stages of KV development (Supplementary Figure 2.3c-d).

While spindle orientation is generally thought to be a deciding factor in the placement of daughter cells post-division\textsuperscript{14,51,95}, we found that daughter cell positioning continues to change throughout cytokinesis and abscission suggesting that spindle orientation is not always the deciding factor (Figure 2.3a-b, Supplementary Movie 5). To directly test whether spindle orientation is utilized in KV development, spindle orientation was measured during the apical clustering and lumen formation stages (Supplementary Figure 2.3e-g). If metaphase spindle positioning was a deciding factor in daughter cell placement as in other \textit{in vitro} and \textit{in vivo} contexts\textsuperscript{11,14,65}, we would
predict a Gaussian distribution of spindle orientation values where the majority of spindles orient at a 90° angle to a line passing through the center of a developing KV lumen (predicted Gaussian drawn in Supplementary Figure 2.3g, grey line). However, a random distribution of spindle angles in relation to the center of KV was calculated (Supplementary Figure 2.3f-g), suggesting that the placement of the cytokinetic bridge is a driving factor in daughter cell positioning during KV development as opposed to spindle positioning.

2.3.4. Cytokinetic bridge ablation disrupts lumen formation. Since the cytokinetic bridge is placed at the site of future lumen formation, and that from cytokinesis to abscission it takes one to three hours in vitro\(^{12,13}\), we hypothesized that proper spatiotemporal control of abscission is required for lumen formation in KV. To test this idea, we utilized laser ablation to prematurely sever cytokinetic bridges during rosette formation/apical clustering (see Supplementary Figure 2.4a for diagram of ablation experiment conditions). In a control embryo, lumen formation begins approximately 20 minutes after the apical clustering stage, where cytokinetic midbodies can be seen decorating the site of future lumen formation (Figure 2.4a, top). However, when the cytokinetic bridge is prematurely severed through the targeting of a single midbody at the site of lumen formation during apical clustering, lumen formation is either severely diminished or fails altogether (Figure 2.4a bottom, 4d-f). Successful ablation was marked by the lack of mKate-MKLP1 fluorescence recovery, with failed midbody ablations resulting in a recovery of midbody fluorescence (Figure 2.4b, Supplementary
Figure 2.4. Premature cleavage of the cytokinetic bridge via laser ablation results in disrupted lumen formation. (a) Top: Unablated control embryo during apical clustering (left) and lumen formation (center, right). Bottom: central KV midbody ablated during apical clustering from experimental ablation group (left). Subsequent failed lumen formation shown (center, right). KV membrane (Sox17:GFP-CAAX, magenta) and midbody marker (mKate-MKLP1, cyan) shown in left and center panels, KV membrane (Sox17:GFP-CAAX, gray) and lumen trace (orange) on right. Bar, 20 μm. (b) Midbody ablated in (a). Pre-ablation, immediately post-ablation, and at 5- and 10-minutes post-ablation shown. KV cell membrane (Sox17:GFP-CAAX, magenta) and midbodies (mKate-MKLP1, cyan) shown. Additional unablated midbodies depicted with asterisks. Bar, 10μm. (c) Representative 3D renderings of KV pre-ablation (left), immediately post-ablation (center), and after lumen formation (right) in control groups. Ablation control conditions shown: midbody ablation outside KV (top), KV cell cytosol ablation (middle), KV cell-cell interface ablation (bottom). KV membrane (Sox17:GFP-CAAX, magenta or grayscale), midbodies (mKate-MKLP1, cyan), and lumen trace (orange) shown. Ablation location shown by dotted white circle. Grayscale inset in bottom panel depicts ablation at cell-cell interface within KV (Sox17:GFP-CAAX). (d-e) Graphs depicting average lumen area over time for unablated (gray) embryos and embryos with midbody ablated outside KV (blue, d), or for embryos with ablation at midbody outside KV (blue, e), KV cell cytosol (green, e), KV cell membrane (purple, e), and midbody within KV (red, e). Lumen areas averaged and binned every 30 minutes. (f) Bar graph depicting rate of lumen area expansion over time. Dots represent individual values. (d-f): n>6 embryos/condition across n>3 experiments. ANOVA with Dunnett’s multiple comparison test completed for panels d-f, compared to embryos with midbody ablated outside KV (blue). Mean displayed ± SEM (f). Statistical results detailed in Methods Table 5.
Supplementary Figure 2.4. Premature cleavage of the cytokinetic bridge via laser ablation results in disrupted lumen formation. (a) Diagram depicting locations of laser ablation for unablated embryos and embryos with ablation of midbody outside KV (blue), KV cell-cell interface (purple), KV cell cytosol (green), or midbody inside KV (red). KV cell membrane (magenta), midbody marker (cyan), and ablation location (orange arrowhead) shown. (b) Maximum projections depicting a successful (top) and failed (bottom) midbody ablation. KV marker (CFTR-GFP, magenta) and midbodies (mKate-MKLP1, cyan) shown. Pre-ablation and post-ablation images shown on right of panels (mKate-MKLP1, grayscale). Bar, 10μm. (c) Time lapse images depicting a single cytokinetic bridge (mKate-MKLP1 magenta) within KV (CFTR-GFP, cyan). Inset shows cytokinetic bridge magnified at 3x. Cyan arrowhead depicts midbody with ablation location shown with magenta dashed line. Bar, 20μm.
Figure 2.4b). An additional example of a successful cytokinetic bridge ablation is under conditions where the cytokinetic bridge is resolved and the section next to the midbody is ablated (Supplementary Figure 2.4c). Control ablation experiments were conducted to ensure that lumen formation failure was due to premature bridge severing and not solely to embryo ablation trauma. Control ablation conditions included ablating cytokinetic bridges/midbodies outside KV, ablating KV cell-cell interfaces, and ablating KV cytosol (Figure 2.4c-f, modeled in Supplementary Figure 2.4a). While control ablations result in slightly delayed lumen formation compared to unablated controls (Figure 2.4d-e), there is no significant difference in lumen formation between control ablation conditions (Figure 2.4d-f). However, lumen growth rate was significantly decreased in embryos where a KV cytokinetic bridge was severed during apical clustering compared to control groups (Figure 2.4c-f). These experiments suggested that cytokinetic bridges during rosette formation/apical clustering are required for lumen formation.

2.3.5. Rab11-vesicles are required for abscission in vivo. Since premature severing of the cytokinetic bridge perturbed lumen formation, we sought to establish whether blocking abscission altogether would perturb lumen formation as well. Previous work in an in vitro model has identified that apical-targeted endosomes containing the Par3/aPKC polarity complex assemble adjacent to the cytokinetic midbody

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These endosomes contain a small monomeric GTPase, Rab11, required to initiate abscission, making inhibition of the Rab11 vesicle trafficking pathway an ideal method to block abscission in KV development. In zebrafish, Rab11-depletion is associated with
KV morphology defects such as lumen size depletion\textsuperscript{69,112}. To determine the role of Rab11-associated vesicles in lumen formation and abscission, we acutely inhibited Rab11-associated membrane vesicles through an optogenetic oligomerization approach (modeled from \textsuperscript{113}, Supplementary Figure 2.5a).

To test the efficacy of this system we expressed cryptochrome 2-mCherry (CRY2-mCherry) and CIB1-mCerulean-Rab11 in HeLa cells. A blue-light-inducible (488 nm) hetero-interaction between CRY2 and CIB1 is induced within a specific Region of Interest (ROI) to initiate cellular aggregation of Rab11-associated membranes (Supplementary Figure 2.5a-b). To examine whether the cellular aggregation of Rab11-associated membranes disrupts function, HeLa cells expressing the optogenetic constructs in pre-abscission were treated with normal light conditions or 488nm blue light (Figure 2.5a-b). Under control conditions, where cells are imaged in the absence of blue light, cells can progress through cytokinesis to abscission within approximately 90 min. (Figure 2.5a). Note CIB1-Cerulean-Rab11 transporting into the cytokinetic bridge where a cleavage event occurs at one side of the midbody (blue arrow, Figure 2.5a), and another event occurs on the other side of the midbody 10 min. later (blue arrow, Figure 2.5a). This is consistent with the events we find in KV with cytokinetic bridge cleavage during lumen formation (Figure 2.3b). When cells are exposed to 488nm light throughout the 90-minute time course, Rab11-associated vesicles are unable to move into the cytokinetic bridge and remain clustered within the cell body, inhibiting the ability of this cell to abscise (Figure 2.5b). Under conditions of CRY2-mCherry and CIB1-mCerulean-Rab11 expression with 488nm blue light exposure, a significant increase in
Figure 2.5. Optogenetic clustering of Rab11-associated vesicles results in failed abscission in vitro and in vivo. (a-b) Time-lapse of cytokinetic HeLa cells transfected with CRY2-mCherry and CIB1-mCerulean-Rab11 (black) in the absence (a) or presence of 488 nm light (b). Bar, 10 μm. Note cleavage events of cytokinetic bridge (blue arrows, a), but not in (b). (c) Bar graph depicting the percentage of total HeLa cells displaying a binucleate phenotype after being released from a metaphase synchronization for two hours in the presence or absence of 488nm light. Cells were transfected with CRY2-mCherry and CIB1-mCerulean-Rab11 as in (a). Unpaired, two-tailed Mann-Whitney test, p=0.0043 (**). Mean displayed ± SEM. n=100 cells per treatment for n>5 experiments. Dots represent individual values. Statistical results detailed in Methods Table 5. (d) 3D rendering of embryos expressing CRY2 and CIB1-mCherry-Rab11 in the absence (left) and presence (right) of 488nm light. Sox17:GFP-CAAX (magenta), CIB1-mCherry-Rab11 (cyan) and nuclei (DAPI, white) shown. Bar, 5 μm. (e) Representative images of single nuclei, binucleate, or multinucleate cells. Nuclei shown in grayscale (DAPI). Bar, 5 μm. (f) Bar graph depicting percentage of binucleate and/or multinucleate cells per KV in uninjected embryos and embryos expressing CIB1-mCherry-Rab11 or CRY2 and CIB1-mCherry-Rab11 plus or minus 488nm light. One-way ANOVA with Dunnett’s multiple comparison test used, compared to uninjected embryos in the absence of 488nm light exposure. Statistical results detailed in Methods Table 5. Analyses performed in n>5 embryos over three experiments. Mean displayed ± SEM. Dots represent individual values.
Supplementary Figure 2.5. Optogenetic clustering of Rab11-associated vesicles results in failed abscission in vitro and in vivo. (a) Model depicting CRY2/CIB1 optogenetic system. Since multiple fluorescent molecules are used, FP (fluorescent protein) is used in model for simplicity. CRY2-FP and CIB1-FP- Rab11 shown under conditions of no light (no clustering) or 488nm light (clustering). (b) Example micrograph of HeLa cells before (left) and after (right) exposure to 488nm light within a circular ROI. CRY2-mCherry (magenta) and CIB1-mCerulean-Rab11 (cyan) shown. Bar, 5μm. (c) Western blot depicting Rab11 protein expression in wild-type (left) and Rab11-null CRISPR HeLa cells (right). Rab11 (25kDa) and GAPDH (37kDa) loading control shown. (d) Representative maximum projections of nuclei in wild-type (left) and Rab11-null CRISPR cells (right). Brightfield (grayscale) and nuclei (DAPI, cyan) shown. Bar, 10μm. (e) Bar graph depicting percentage of binucleate cells in wild-type (Rab11-positive, gray) and Rab11-null CRISPR cells (Rab11-negative, cyan). Two-tailed Mann-Whitney p=0.0079 (**), u=0. n=100 cells across n=5 experiments. (f) Time lapse images of cell divisions in wild-type (control, top) and Rab11-null CRISPR cells (bottom). Metaphase, anaphase, pre-abscission stages shown with either subsequent completed (white arrowhead) or unsuccessful abscission with binucleate cell resulting (yellow arrowhead). Bar, 50μm. (g) Model depicting optogenetic experiment protocol. (h) Line graph depicting embryo survival during the first 33hpf. Wild-type (TAB) embryos (green, n=655 embryos from 3 clutches), uninjected transgenic embryos +/- 488nm light exposure (grayscale, n=831 embryos from 4 clutches), CRY2-FP-injected transgenic embryos +/- 488nm light exposure (red), CIB1-FP-Rab11-injected transgenic embryos +/- 488nm light exposure (blue), and CRY2-FP + CIB1-FP-Rab11-injected transgenic embryos +/- 488nm light exposure (purple, n=555 embryos from 4 clutches) shown. Mean ± SEM shown.
the percentage of binucleated cells occurred when compared to cells not exposed to blue light (Figure 2.5c). Previous *in vitro* studies reported that increases in binucleate formation can be indicative of cytokinesis or abscission failure\textsuperscript{114}. We generated Rab11-null cells (Supplementary Figure 2.5c-d), and found that while cytokinesis occurred as expected, binucleate formation occurred after the formation of the cytokinetic bridge due to abscission failure (Supplementary Figure 2.5d-f, Supplementary Movie 8). Rab11-null cells had a significantly higher percentage of binucleate cells when compared to control (Supplementary Figure 2.5d-e), similar to clustering Rab11 *in vitro* using optogenetics (Figure 2.5a-c).

We next sought to determine whether this binucleate phenotype could be recapitulated in zebrafish. We injected mRNA into zebrafish embryos to express CRY2-Fluorescent Protein (FP, mCherry or no FP) and CIB1-FP-Rab11 (FP, either mCerulean or mCherry, Supplementary Figure 2.5g). Uninjected embryos (control), embryos injected with CRY2-FP mRNA only (control), CIB1-FP-Rab11 mRNA only (control), or injected with both CRY2-FP and CIB1-FP-Rab11 mRNA (experimental) were exposed to normal light or 488nm blue light conditions starting at 50-60% epiboly until a late lumen expansion stage (14 hpf, experimental protocol diagram in Supplementary Figure 2.5g). Embryonic lethality during optogenetic experiments was similar in all injection groups (Supplementary Figure 2.5h), suggesting that acute clustering of Rab11-membranes did not result in embryo mortality. Embryos were fixed and the number of binucleate cells were evaluated (Figure 2.5d-f). Strikingly we found a significant
increase in the number of binucleated cells in KV under experimental conditions (Figure 2.5f), suggesting that clustering Rab11 vesicles in vivo blocks abscission.

2.3.6. Rab11 vesicles are required for lumen formation. We next examined whether the clustering of Rab11-membranes resulted in KV lumen formation defects. Due to the mosaic nature of mRNA expression in zebrafish, embryos were categorized into five groups: uninjected (control), CRY2-FP mRNA only (control), CIB1-FP-Rab11 mRNA only (control), CRY2-FP plus CIB1-FP-Rab11 mRNA without KV expression (control), and CRY2-FP plus CIB1-FP-Rab11 mRNA with KV expression (experimental). Injected embryos were exposed to 488nm light at either 50-60% epiboly or 75-90% epiboly (Figure 2.6b, Supplementary Figure 2.5g). Zebrafish developmental speed can vary due to variations in ambient room temperature\textsuperscript{115}. To control for this, lumen area was normalized to the mean of uninjected control embryos within each clutch. This minimized the variation in lumen area due to differences in clutch developmental speed, since control groups demonstrated a range in basal lumen area dependent on the clutch (Supplementary Figure 2.6b).

In double injected embryos where KV cells have clustered Rab11-associated membranes (488 nm exposure beginning at 50-60% or 75-90% epiboly), significant defects in KV lumen formation occurred such as decreased lumen area or an inability to form a lumen at all (Figure 2.6a-b, Supplementary Figure 2.6b) compared to control conditions (Figure 2.6b, Supplementary Figure 2.6a-b). When clustered Rab11-membranes only occurred in a proportion of KV cells, lumen formation in the non-
Figure 2.6. Optogenetic clustering of Rab11 during KV development results in abnormal lumen formation and perturbed polarity establishment. (a) Representative 3D renderings of KV under conditions of CRY2-mCherry/CIB1-mCerulean-Rab11 plus 488nm light with partial (top) or majority KV mRNA expression (bottom). 3D rendering with lumen trace (orange), cell membrane (GFP-CAAX, white), CRY2-mCherry (magenta), and CIB1-mCerulean-Rab11 (cyan) shown. Bar, 50 μm. (b) Box and whisker plot depicting two-dimensional lumen area normalized to uninjected control values plus or minus 488 nm light beginning at 50-60% epiboly (left, n>15 embryos) or 75-90% epiboly (right, n>21 embryos). Dots represent individual KV values. Whiskers denote minimum and maximum values, 25th and 75th percentiles denoted by box boundaries. Median denoted by line within box, mean denoted by plus sign. One-way ANOVA with Dunnett’s multiple comparison test, compared to uninjected embryos. Statistical results detailed in Methods Table 5. (c) Representative 3D renderings of KV in CFTR-GFP (magenta) embryos under conditions of CIB1-mCherry-Rab11 (cyan, top) or CRY2 + CIB1-mCherry-Rab11 (cyan) + 488nm light exposure (bottom). Dashed box represents insets shown at right. Bars, 20μm. (d) Bar graph depicting the Pearson’s coefficient for CFTR-GFP and CIB1-mCherry-Rab11 in embryos treated plus or minus 488nm light exposure. ANOVA with Dunnett’s multiple comparison test, compared to embryos expressing CIB1-mCherry-Rab11 under normal light conditions. Statistical results detailed in Methods Table 5. Mean displayed ± SEM. Dots represent individual values. (e) Bar graph depicting the percentage of puncta per KV expressing CFTR-GFP (magenta), CIB1-mCherry-Rab11 (cyan), or both (white). One-way ANOVA with Dunnett’s multiple comparison test completed for each cluster type, compared to percentages from embryos expressing CIB1-mCherry-Rab11 under normal light conditions. Statistical results detailed in Methods Table 5. (d-e) n>10 embryos analyzed from five experiments. Mean displayed ± SEM. Dots represent individual values.
Supplementary Figure 2.6. Optogenetic clustering of Rab11 during KV development results in abnormal lumen formation and perturbed polarity establishment. (a) Representative 3D renderings of KV under conditions of CRY2-mCherry mRNA only, or CRY2-mCherry + CIB1-mCerulean-Rab11 with KV-positive mRNA expression. KV cells (CFTR-GFP, white), CRY2-mCherry (magenta), and CIB1-mCerulean-Rab11 (cyan) shown. Bars, 50 μm. (b) Box and whisker plots comparing raw (left) and normalized (right) lumen area resulting from optogenetic experiments (Figure 2.6b). Normalized data shown as a ratio compared to uninjected control mean. Whiskers denote minimum and maximum values, 25th and 75th percentiles denoted by box boundaries. Median denoted by line within box, mean denoted by plus sign. Data from 10 individual clutches shown by colors described in legend. n>15 embryos per treatment. Results from unpaired, two-tailed Student's t-test of raw data set (left) and one-way ANOVA with Dunnett's multiple comparison test in normalized data described in detail in Methods Table 5. (c) Midbodies (mCherry-MKLP1, cyan) localize at sites of apical polarity (CFTR-GFP, magenta) during MET stage of KV development. Bar, 20 μm (top), 10 μm (bottom). (d) Apical polarity (monitored by CFTR, Fire LUT) increases within a 4 minute time frame adjacent to the cytokinetic midbody (mCherry-MKLP1). Bar, 5 μm. (e) Quantification of CFTR-GFP intensity over time. n = 12 midbodies ± SEM, n = 4 embryos, One-way ANOVA, p = 0.0023 (**), F(2,33) = 7.323, df=33.
clustered areas occurred (Figure 2.6a, left). In embryos with clustered Rab11 in cells surrounding KV, but not KV cells, KV lumen size was comparable to unclustered-Rab11 control conditions (Figure 2.6b). Overall, these findings suggest that acute inhibition of Rab11-associated vesicles within KV-destined cells disrupts lumen formation.

Rab11 is involved in the targeted apical exocytosis of cystic fibrosis transmembrane conductance regulator (CFTR) to the apical membrane in mammalian tissue culture. In zebrafish, CFTR apical localization is required for KV lumen expansion. When monitoring the positioning of cytokinetic bridge/midbody in relation to CFTR in KV, we found that CFTR organizes on either side of the cytokinetic bridge midbody during MET (Supplementary Figure 2.6c). CFTR-GFP was highly dynamic within regions proximal to the midbody, where a significant increase in CFTR-GFP integrated intensity was measured over time adjacent to the midbody (Supplementary Figure 2.6d-e). These findings suggest a model that cytokinetic bridges provide a locale for directed membrane transport of apical polarity proteins (e.g. CFTR) for lumen establishment.

To test whether CFTR utilizes Rab11 for its apical distribution in KV, we examined whether Rab11-associated vesicles trapped CFTR when optogenetically clustered during KV formation. CIB1-FP-Rab11 mRNA-injected embryos were compared to embryos injected with CRY2 plus CIB1-FP-Rab11 mRNA. Both groups of embryos were exposed to 488nm light during late epiboly until a fully developed KV should be formed (described in Supplementary Figure 2.5c). Under conditions where only CIB1-FP-Rab11 mRNA was injected, CFTR-GFP clearly organizes to the apical
membrane surrounding the lumen and a population of it colocalizes with CIB1-FP-Rab11 (Figure 2.6c, top). However, under conditions of optogenetic clustering of Rab11, CFTR-GFP is trapped in Rab11-membrane associated clusters and is unable to organize at the apical membrane (Figure 2.6c, bottom). Under these conditions, there is a significant increase in CFTR-GFP colocalization with CIB1-FP-Rab11 compared to non-clustered controls (Figure 2.6d). Under control conditions (CIB1-FP-Rab11 plus or minus 488nm light, CRY2 + CIB1-FP-Rab11 minus 488 nm light), we found that the percentage of puncta per KV that contained both CIB1-FP-Rab11 and CFTR was <40%. However, under experimental conditions of CRY2 plus CIB1-FP-Rab11 plus 488nm light exposure, we found a significant increase in CIB1-FP-Rab11 puncta that contained CFTR (79.4 ± 9.87%, Figure 2.6e). These findings suggest that CFTR-GFP utilizes Rab11-associated vesicles for its delivery to the apical membrane during KV formation. This is likely occurring both during abscission and in cells post-abscission. It also presents an interesting model, where premature severing of the cytokinetic bridge (Figure 2.4) limits the time for CFTR trafficking to the cytokinetic bridge to create an apical membrane. CFTR is a master regulator of fluid secretion through control of chloride transport to generate osmotic gradients that drive the movement of water through a tissue\textsuperscript{117}. Here, we propose that when abscission occurs prematurely or Rab11-associated vesicles carrying CFTR are clustered, CFTR cannot assemble at the apical membrane resulting in a loss of fluid flow and defects in lumen formation and/or expansion.
2.3.7. CFTR transport to cytokinetic bridge aids in lumenogenesis. In conclusion, these studies have highlighted the importance of cell division during the development of KV and the de novo formation of its lumen. We provide evidence that cell division is upregulated in cells destined for KV, and these cells retain their cytokinetic bridges post-division. The cytokinetic bridges are then projected to the site of future lumen formation during rosette formation/apical clustering, where Rab11-associated vesicles can traffic important apical polarity components to the bridge during epithelialization to allow for lumen formation (Figure 2.7).

2.4. Materials and Methods

2.4.1. Fish Lines. Zebrafish lines were maintained using standard procedures approved by the Syracuse University IACUC committee (protocol #18-006). Embryos were staged as described in ¹. See Supplementary Table 4 for list of transgenic zebrafish lines used.

2.4.2. Plasmid and mRNA Constructs. Plasmids were all made using Gibson Cloning methods (NEBuilder HiFi DNA Assembly Cloning Kit (NEB no E5520S)) and maxi-prepped before injection (BioBasics Cat: 9K-006-0023). mRNA was made using mMESSAGE mMACHINE™ SP6 transcription kit (Invitrogen AM1340). See Supplementary Table 3 for list of plasmid constructs used and concentrations injected.

2.4.3. Imaging. A SP5 or SP8 (Leica, Bannockburn, IL) laser scanning confocal microscope was used throughout this manuscript. An HC PL APO 20x/0.75 IMM CORR
Figure 2.7. Targeted membrane transport of CFTR towards the cytokinetic bridge is used to establish a lumen. Model depicting lumen formation through Rab11-mediated vesicle transport to the cytokinetic bridge. KV membrane (GFP-CAAX, magenta), midbodies (RacGAP/MKLP1/PLK1, cyan), vesicles (CFTR/Rab11, green), and nuclei (blue) shown.
CS2 objective, HC PL APO 40x/1.10 W CORR CS2 0.65 water immersion objective, and an HCX Plan Apochromat 63x/1.40-0.06 NA OIL objective were used. Images were acquired using LAS-X software. A Leica DMi8 (Leica, Bannockburn, IL) with a X-light v2 confocal unit spinning disk was also used, equipped with an 89 North – LDI laser and a Photometrics Prime-95B camera. Optics used were either 10x/0.32 NA air objective, HC PL APO 63X/1.40 NA oil CS2, HC PL APO 40X/1.10 NA WCS2 CORR, a 40X/1.15 N.A. Lamda S LWD, or 100x/1.4 N.A. HC Pl Apo oil emersion objective. Additionally, a Nikon Eclipse Ti-E microscope using a Hammamatsu C9100-50 EMCCD camera coupled to a PerkinElmer spinning disk confocal system was used with a CFI Apo LWD Lambda S 20x water immersion objective or a CFI Apo Lambda S LWD 40x water immersion objective. Images were acquired using Volocity software. STED imaging was performed using a Leica TCS SP8 (Leica, Bannockburn, IL) equipped with STED 3X, a supercontinuum laser (white light laser 470–670 nm) for excitation, 592/546/600-nm STED depletion lasers, and an HCS PL APO 100x/1.40 oil STED white objective. Images were acquired using the Leica LAS software and post image processing of STED images was performed using SVI Huygens deconvolution software.

2.4.4. Laser Ablation. Tg(sox17:GFP-CAAX) zebrafish embryos were injected with 300 picograms of MKLP1-mKate mRNA at the 1-cell stage. Embryos were embedded in agarose at the 1-somite stage and imaged on either an Andor Dragonfly spinning disk confocal microscope with a pulsed nitrogen pumped tunable dye laser at 100%, or X-light v2 Confocal Unit spinning disk with VisiView kinetics unit coupled to a 355 nm
pulsed laser used at 50% both equipped with a 40x 1.15NA water objective. An image was obtained prior to laser ablation to record midbody positioning within the embryo. Ablation conditions included midbodies ablated within KV or outside KV, KV cytosol, or KV cell-cell interfaces. Images of KV post-ablation were captured using the 488nm and 561nm lasers, obtained a z-stack with a 0.8 μm step size every 2 min.

2.4.5. Zebrafish Optogenetics Experiments. Optogenetic experiments were performed by injecting CRY2-mCherry and/or CIB1-mCerulean-Rab11 (or CRY2 and/or CIB1-mCherry-Rab11) mRNA into zebrafish embryos at the 1-cell stage. Embryos were exposed to 488nm light using the NIGHTSEA fluorescence system from 60% or 75-90% epiboly (late exposure experiments) until 6-8 somite stage. Embryos were either fixed with 4% paraformaldehyde + 0.5% Triton-X 100 in PBS or incubated overnight in the absence of 488nm light to evaluate death rates. Fixed embryos were then imaged on a confocal microscope as described above.

2.4.6. Pharmacological treatments. For Nocodazole and BI2536 treatments, zebrafish embryos were dechorionated and soaked in the desired concentration of drug diluted in zebrafish embryo water (refer to Figure 2.2.1 and Supplementary Figure 1.1). Embryos were manually dechorionated and treated from 60% epiboly until 6-8 somite stage on petri plates coated with 3% agarose, when they were washed with embryo water and fixed in 4% paraformaldehyde containing 0.5% Triton-X 100 overnight at 4°C. Staining, imaging, and lumen size quantification were then completed as described.
2.4.7. Immunofluorescence of zebrafish embryos. Zebrafish embryos were fixed using 4% paraformaldehyde containing 0.5% Triton-X 100 overnight at 4°C. Zebrafish were then dechorionated and incubated in PBST (phosphate buffered saline + 0.1% Tween) for 30 minutes. Embryos were blocked using a Fish Wash Buffer (PBS + 1% BSA + 1% DMSO + 0.1% Triton-X 100) for 30 minutes followed by primary antibodies incubation (antibodies diluted in Fish Wash Buffer in concentrations stated in Supplementary Table 2) either overnight at 4°C or 3 hours at room temperature. Embryos are then washed five times in Fish Wash Buffer before incubating with secondary antibodies for 3 hours at room temperature. After five more washes, embryos were incubated with 4',6-diamidino-2-phenylindole (NucBlue® Fixed Cell ReadyProbes® Reagent) for 30 minutes. For imaging, embryos were either halved and mounted on slides using Prolong Diamond (Thermo Fisher Scientific cat. # P36971) or whole-mounted in 2% agarose (Thermo-Fisher cat. # 16520100).

2.4.8. Cell Culture. 3D Madin-Darby canine kidney (MDCK) cultures were grown in Dulbecco’s Modified Eagle Medium (DMEM, Gibco™) supplemented with 10% Seradigm FBS (VWR) and 1% penicillin-streptomycin (10,000U/ml) (Gibco™) with 40% Matrigel (Fisher cat no CB40234C; Corning no 356237). Rab11 Optogenetic clustering in HeLa cells: HeLa cells were transfected with CIB1-mCerulean-Rab11 and CRY2-mCherry using Mirus TransIT-LT1 and then synched at prometaphase in nocodazole (100nM) and released after 6 hours in the presence or absence of 488nm
light. Cells were imaged on a spinning disk confocal microscope. Images of dividing

cells were acquired for a time lapse series, or cells were imaged two hours post-release
to quantify binucleate cells.

**2.4.9. Rab11 CRISPR.** HeLa cells expressing FIP3-GFP stably were used throughout

the study, maintained at 37°C with 5% CO₂. Rab11A CRISPR vector (Santacruz SC-

400617) and Rab11A HDR vector (Santa Cruz SC-400617-HD) were transfected into
cells using the Mirus TransIT-LT1 transfection reagents (Cat# MIR2305) using

manufacturers specifications. Cells were grown in puromycin selection medium

(5ug/ml). Three single clones were isolated and tested for Rab11 levels using Western

blot. HeLa cells are maintained at 37°C with 5% CO₂.

**2.4.10. Immunofluorescence of 3D acini.** Using a pipette, media was carefully

removed from cultures. Cultures were rinsed with PBS and fixed with 4%

paraformaldehyde (PFA) at room temperature for 30 minutes with light shaking. The

PFA was removed and replaced with fresh PFA for an additional 30 minutes with light

shaking. After PFA was removed, 50mM NH₄Cl was added for 10 minutes. Cells were

washed with PBS for 30 minutes, with light shaking, and then treated for 5 minutes with

0.1% Triton-X, blocked with PBSΔT (PBS, 1% BSA, 0.5% Triton X-100), and incubated

with primary antibodies for 4 hrs at room temperature. Cultures were washed three
times with PBSΔT and incubated with secondary antibodies for 4 hours at room

temperature. For actin and DAPI staining, acini were incubated with ActinRed
555vReady Probes reagent (Thermo Fisher Scientific R37112) and NucBlue Fixed Cell Stain from Ready Probes (Thermo Fisher Scientific R37606) for 30 minutes. Cultures were kept in PBS containing DABCO (1,4-Diazabicyclo [2.2.2] octane) antifade reagent (200μM) for imaging. See Supplementary Table 2 for list of antibodies and concentrations used.

2.4.11. Image and Data Analysis. Images were processed using both FIJI/ImageJ software, IMARIS (Bitplane), and/or Adobe Photoshop. Angles were calculated using FIJI/ImageJ software and Microsoft Excel. All graphs were generated, and statistical analysis performed using GraphPad Prism software. 3D images, movies, and surface rendering were performed using Bitplane IMARIS (Surface, Smoothing, Masking, and Thresholding functions). Surface renderings: Imaris surface renderings were created through the manual surface protocol by outlining fluorescence regions of interest using the Isoline function for each z-plane and timepoint. Once the surface rendering was created for each cell, individual cell renderings were pseudocolored and each frame was captured. To isolate and pseudocolor specific cells, the same surface rendering protocol was completed, and masks were created from the surface renderings to isolate the new channel. CFTR intensity measurements: To measure the integrated density of CFTR at the cytokinetic midbody, rectangular regions of interest (ROIs) were drawn around the midbody (MKLP1). The larger ROI (ROI\textsuperscript{l}) is used to measure background whereas the center, smaller ROI (ROI\textsuperscript{s}) measures the CFTR signal. The following equation was used: integrated intensity of ROI\textsuperscript{s} -((integrated intensity of ROI\textsuperscript{l} –
integrated intensity of ROI^S)\times(\text{area ROI}^S / (\text{area ROI}^L - \text{area ROI}^S))}{118}. Intensities were normalized to the final intensity at 4 minutes (percentage). Calculation of spindle orientation in relation to lumen: Spindle orientation was measured during the apical clustering and lumen formation stages of KV development. As shown in Supplementary Figure 2.2.3e, a line was drawn through the DNA plate of a metaphase cell in KV (solid black line). A second line was then drawn perpendicular to the first line to denote the position of the mitotic spindle poles (dashed black line). A third line was drawn passing through the center of KV and the center of the metaphase DNA plate (dashed gray line). Lastly, the angle between the dashed gray line and the dashed black line were calculated to determine the spindle position in relation to the KV center. Lumen area quantifications: Prior to lumen area measurements, images were turned using Imaris software such that the equatorial plane of the lumen could be measured, resulting in a representative lumen area measurement regardless of initial embryo positioning during imaging. This dataset was then transferred to FIJI/ImageJ for lumen area calculations. A region was drawn around the lumen perimeter and area calculated using the measure function. Where applicable, values were normalized to the control mean by dividing each lumen area by the mean value of the control lumens for that particular experiment. This controlled for KV size fluctuations based on slight differences in ambient room temperature and difference growth rates of clutches in different experimental setups. Mitotic index and cell number calculations: Mitotic index and cell number counts were completed with embryos after a DAPI stain and/or antibody staining with a phospho-H3 antibody. For mitotic index, the number of mitotic cells was divided by the
total number of cells to result in a percentage of mitotic cells out of the entire population.

**Colocalization quantification:** For optogenetic experiments, colocalization quantification was performed using Imaris software. In the “Colocalization” menu, a Region of Interest (ROI) around Kupffer’s vesicle was defined by masking the channel depicting CFTR fluorescence. CFTR was defined as Channel 1, and CIB1-FP-Rab11 was defined as Channel 2. Threshold values were calculated with the “Automatic Threshold” option to define colocalization parameters, and the “Pearson’s coefficient in colocalized volume” was recorded for each embryo. Additionally, optogenetic clusters were scored for presence of CFTR, CIB1-FP-Rab11, or both and this was presented as percentages per embryo.

**2.4.12. Statistics and Reproducibility.** Unpaired, two-tailed Student’s t-tests, Mann-Whitney, and one-way ANOVA analyses were performed using GraphPad Prism software. **** depicts a p-value <0.0001, *** p-value <0.001, **p-value<0.01, *p-value <0.05. See Supplementary Table 5 for detailed information regarding statistics.

All graphs, micrographs, images, and blots in this paper are representative of at least three independent experiments.
### 2.4.13. Methods Table 1: Key Resources Table

<table>
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<th>Reagent Type (species or resource)</th>
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### 2.4.14. Methods Table 2: Antibodies

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### 2.4.15. Methods Table 3: Plasmid Constructs

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### 2.4.16. Methods Table 4: Zebrafish Transgenic Lines

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### 2.4.17. Methods Table 5. Detailed statistical analysis results

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**Note:** Mann-Whitney test (two-tailed)
Chapter Three:

A novel embryonic centrosome structure coordinates zebrafish spindle directionality in a PLK1- and PLK4-dependent manner
This chapter features work from a manuscript prepared for publication:

A novel embryonic centrosome structure coordinates zebrafish spindle directionality in a PLK1- and PLK4-dependent manner

Rathbun LI¹, Alijiboury A¹, Bai X²,⁵, Bembenek JA²,³, Amack JD⁴, Hehnly H¹

¹Biology Department, Syracuse University, Syracuse, NY
²Department of Biochemistry, Cellular and Molecular Biology, University of Tennessee, Knoxville, TN
³Department of Molecular, Cellular, Developmental Biology, University of Michigan Medical School Ann Arbor, MI
⁴Department of Cell and Developmental Biology, SUNY Upstate Medical School, Syracuse, NY
⁵Current location: National Institute of Health, Bethesda, MD
3.1. Abstract. While factors that regulate mitotic spindle positioning have been elucidated in vitro, it still remains unclear how a spindle is placed within the confines of extremely large cells. A previous study postulated that the presence of acentrosomal microtubule nucleation sites would allow astral microtubules to more easily span the large distance between spindle pole and cell cortex when the cell is disproportionately large. Here, we expand upon this idea with the discovery of a large, novel centrosome structure in the early zebrafish embryo. During early embryonic cell divisions when cell size changes rapidly, we find that mitotic spindle pole area scales more closely with the changing cell size than spindle length during consecutive rounds of cell division in C. elegans and zebrafish. Strikingly, we also discovered that spindle poles in the zebrafish embryo are not only much larger than expected, they are also asymmetric in size. This asymmetry in size creates a vectoral directionality in the mitotic spindle placement that points the larger of the two spindle poles towards the embryo center. We find that this placement is dependent on both PLK1 and PLK4 activity during embryogenesis. With this work, we propose a model in which large, uniquely-structured centrosomes direct spindle placement within the disproportionately large zebrafish embryo cells to orchestrate cell divisions during early embryogenesis.

3.2. Introduction. During early embryogenesis, rapid cell divisions increase the number of cells in an embryo to ensure proper tissue and organ formation can proceed during later development. However, it remains unclear how the mitotic spindle is able to position itself within the confines of a cell when that cell is disproportionately large.
Previous studies have investigated whether a limit to mitotic spindle size exists in large embryonic cells, and the overarching trend was that larger embryonic cells have larger spindles to compensate\textsuperscript{27}. Across a wide variety of organisms spanning multiple phyla, large spindle size correlated with large cell size in cells of less than 140 microns in diameter. However, this study has two caveats. First, many vertebrate embryos have cells of greater than 140 microns in diameter, including \textit{Xenopus} and \textit{Danio rerio} (zebrafish) embryos. Knowing this, the question still remains whether spindle scaling applies to these large cells in order to coordinate division. Additionally, this study focused on comparing spindle and cell sizes across a variety of organisms. This poses the question of how the mitotic spindle adapts to the rapidly changing cell size during early cell divisions during the development of a single organism. This study aims to understand the previously unknown mechanism by which cell division is regulated during early development in extremely large cells.

A previous study proposed that large embryonic cells take advantage of microtubule nucleation sites outside the mitotic centrosomes/spindle poles in order to span large cells to coordinate division. These sites outside the spindle poles could allow microtubules to more easily span the large distance from spindle pole to cell cortex in order to anchor and provide pulling forces during division\textsuperscript{29}. The mitotic centrosome/spindle pole is the traditional site that assembles the microtubule-based spindle. In most eukaryotic systems, one spindle pole consists of two centrioles surrounded by pericentriolar material that contains microtubule nucleation sites\textsuperscript{13}. Our studies have expanded upon the idea that acentrosomal nucleation sites contribute to
cell division in extremely large cells. Building on this, we propose that these remote nucleation sites are derived from uniquely-organized spindle poles. Using the developing zebrafish embryo as a model organism for division in giant cells, we present a testable model where spindle pole size scales with cell size instead of the previously proposed spindle scaling mechanism, and these large spindle poles regulate mitotic spindle positioning during zebrafish embryo monolayer formation in a PLK1- and PLK4-dependent manner.

3.3. Results

3.3.1. Spindle pole area scales with cell length in *C. elegans* and zebrafish embryonic cell division. In order to elucidate a conserved mechanism of embryonic spindle placement, we utilized the invertebrate *C. elegans* and vertebrate zebrafish embryo as model systems. These organisms were chosen based on their stark differences in size, embryo morphology, and organism complexity. Additionally, both organisms have been extensively utilized in previous spindle positioning studies, where *C. elegans* is a classic model for spindle positioning during embryonic polarity establishment and zebrafish are an ideal model to study spindle positioning in disproportionately large embryo cells.

*C. elegans* embryos develop within the confines of an eggshell during their earliest cell divisions (modeled in Figure 3.1a). These divisions occur asynchronously, and anterior-posterior polarity is established from the first asymmetric cell division. In contrast, early zebrafish embryos undergo rapid cleavage stage cell divisions on top of
Figure 3.1. Spindle and cell size decrease rapidly during early *C. elegans* and zebrafish cell divisions. (a) Model of early *C. elegans* developmental stages (1-cell through 5-cell stage). Embryonic cells shown in gray. (b) Model of early zebrafish developmental stages (8-, 16-, 32-, 64-, and 128-cell stage). Embryonic cells shown in gray, yolk shown in white. (c) Representative three-dimensional rendering of a zebrafish embryo at interphase and metaphase at the 8- and 16-cell stage. Microtubule marker (EMTB-3xGFP) shown in grayscale. Bar, 250μm. (d) Bar graph depicting two-dimensional cell area of single cells during *C. elegans* (left) and zebrafish embryo development (right). Mean ± SEM shown. One-way ANOVA, p < 0.0001 (****). n=10 *C. elegans* embryos and n=3 zebrafish embryos measured. (e) Bar graphs depicting spindle length (orange) and cell length along spindle axes (gray) during *C. elegans* (left) and zebrafish development (right). Mean ± SEM shown. One-way ANOVA, p < 0.0001 (****) for *C. elegans* and zebrafish cell length, p = 0.0005 (****) for *C. elegans* spindle length, p = 0.0002 (****) for zebrafish spindle length. n=10 *C. elegans* embryos and n=3 zebrafish embryos measured. (f-g) Representative images of metaphase cell at the 1-cell (top) and 3-cell stage (bottom) in a *C. elegans* embryo (f), and at the 8-cell (top) and 16-cell stage (bottom) in a zebrafish embryo (g). Chromosomes and γ-tubulin shown in white, chromosomes denoted by blue arrowhead. Mitotic spindle poles highlighted in insets on right. Bar, 15μm. (h) Violin plot with box and whiskers depicting two-dimensional spindle pole area (μm²) at the 1-cell and 3-cell stage in *C. elegans* (left), and at the 8-cell and 16-cell stage in zebrafish (right). n>24 *C. elegans* embryos and n>12 zebrafish embryos quantified. Student’s t-test, p < 0.0001 (****). (i) Violin plot depicting cell length, spindle length, and spindle pole area for *C. elegans* at the 1-cell and 3-cell stage (left), and zebrafish at the 8-cell and 16-cell stage (left). Values normalized to mean of earliest developmental stage (1-cell for *C. elegans*, 8-cell for zebrafish), dashed line at value of 1. (j) Scaled model depicting cell (gray), spindle (orange), and spindle pole (purple) sizes during the 1-cell and 3-cell stage in *C. elegans* embryos, and the 8-cell and 16-cell stage in zebrafish embryos. Bar, 20μm. For violin plots: Plot boundaries depict minimum and maximum, 25th and 75th quartiles represented by thin black line, median represented by thick black line.
a yolk (Figure 3.1b). Cell divisions occur synchronously for the first ten cell divisions, at which point the divisions begin to occur in an asynchronous wave\textsuperscript{102}. During the first five cycles of cell division, blastomeres divide along a single plane to create a cellular monolayer on top of the yolk. Each cell division during this stage occurs perpendicular to the plane of the previous division within this monolayer, leading to the construction of a cellular grid (2x1 cells at the 2-cell stage, 2x2 at the 4-cell stage, 4x2 at the 8-cell stage, 4x4 at the 16-cell stage, 8x4 at the 32-cell stage)\textsuperscript{102}. This is clearly visualized through the use of a transgenic zebrafish line, where the fluorescently labelled microtubule binding protein ensconsin (EMTB-3xGFP) is used as a microtubule marker. Here, the 16-cell stage embryo can be seen with mitotic spindles oriented perpendicular to the previous division at the 8-cell stage (Figure 3.1c).

In the early development of many organisms, rapid rounds of division result in a stark decrease in cell size during the cleavage stage\textsuperscript{123}. We first measured cell area during the 1- to 5-cell stage in \textit{C. elegans} and the 8- to 128-cell stage in zebrafish embryos. We found that while both organisms had a significant decrease in cell area during these five rounds of division, the change was of differing magnitudes between \textit{C. elegans} and zebrafish. In \textit{C. elegans} a 43.54±0.84\% decrease in cell area was noted between the 1- and 2-cell stages, and a cell area decrease of 8.08±3.89\% occurred between the 4- and 5-cell stages. This indicates that changes in cell area becomes less drastic over time in \textit{C. elegans}. In contrast, a cell area decrease of 46.62±2.25\% occurred between the 8- and 16-cell stage in zebrafish embryos, and this decrease remained consistent from the 64- to 128-cell stage at 35.91±1.66\% (Figure 3.1d). This suggests that while a marked decrease in cell size occurs during the first several rounds
of cell division in many organisms, the magnitude of this change is not always similar. However, the consistent decrease in size across both organisms suggests that cells do not expand in size between cell divisions during early proliferation (Figure 3.1d).

We next sought to determine whether the spindle adjusted to changes in cell length and if this was conserved between \textit{C. elegans} and zebrafish embryos. To accomplish this, we measured spindle length in \textit{C. elegans} embryos that stably expressed a centrosome marker (\(\alpha\)-tubulin-GFP), cell membrane marker (PH:mCherry) and a nuclear marker (H2B-GFP/his-58) in order to measure cell and spindle length specifically at metaphase (Figure 3.1f, Supplementary Figure 3.1a-b). Similarly, we used the EMTB-3xGFP transgenic zebrafish line to visualize microtubules (Figure 3.1c, Supplementary Figure 3.1a-b), or stained spindle poles (\(\gamma\)-tubulin) and nuclei (DAPI) in fixed zebrafish embryos for measurements (Figure 3.1f) as an alternative method to clearly label the mitotic spindle. Metaphase mitotic spindle length was measured from spindle pole to spindle pole, and cell length was measured from cell membrane to cell membrane along the same plane of the metaphase spindle (Supplementary Figure 3.1f). The trend calculated in cell length through the cell cycles of interest in \textit{C. elegans} and zebrafish embryos was similar to that identified for cell area (Figure 3.1d-e), where cell length decreased with every division over time (Figure 3.1d, gray). Interestingly, we found that while spindle length also decreased through multiple rounds of cell division in \textit{C. elegans} and zebrafish embryos, this decrease was not as drastic as the decrease in cell length (Figure 3.1d, orange). When considered as a ratio between spindle length and respective cell length, this results in mitotic spindles occupying a higher percentage
Supplementary Figure 3.1. Spindle and cell size decrease rapidly during early C. elegans and zebrafish cell divisions. (a) Representative images of a single cell at the 8-cell stage in a C. elegans embryo (left) and zebrafish embryo (right). Microtubules (cyan), cell membrane and histone marker (magenta) shown with cell boundary highlighted in white. Bar, 10 μm. (b) Representative images of C. elegans (left) and zebrafish embryos (right) during the first few hours after fertilization. Microtubules (cyan), cell membrane and histone marker (magenta) shown. Bar, 20 μm and 200 μm, respectively. (c) Bar graph depicting the ratio of spindle length to cell length in C. elegans and zebrafish embryos. Values were calculated by dividing the spindle length (as displayed in the orange bars in Fig. 1e) by the cell length (shown in gray bars of Fig. 1e) to determine the percentage of the cell length occupied by the spindle. Mean ± SEM shown. One-way ANOVA, p < 0.0001 (***) for both C. elegans and zebrafish. n=10 C. elegans embryos and n=3 zebrafish embryos measured. Detailed statistical analysis in Methods Table 4. (d) Bar graph depicting the distance from spindle pole to cell cortex in C. elegans and zebrafish embryos during early development. Mean ± SEM shown. One-way ANOVA, p < 0.0001 (***) for both C. elegans and zebrafish. n=10 C. elegans embryos and n=3 zebrafish embryos measured. Detailed statistical analysis in Methods Table 4. (e) Representative image of spindle pole morphology at the 512-cell stage of zebrafish development. γ-tubulin (green) and chromosomes (blue) shown. Bar, 10μm. (f) Scaled model of C. elegans and zebrafish embryo at the 8-cell stage during early development. Microtubules (orange), chromosomes (blue), and spindle poles (purple) shown within cell area (green). Representative spindle length and cell length depicted. Bar, 20μm.
of the cell length in later cell divisions compared to earlier divisions in both organisms (Supplementary Figure 3.1c). Additionally, this leads to a significant decrease in the distance from mitotic spindle poles to the cell membrane with each cell division (Supplementary Figure 3.1d). Despite the stark size difference between *C. elegans* embryo cells (22μm cell with 8μm spindle at 8-cell stage) and zebrafish embryo cells (127μm cell with 18μm spindle at 8-cell stage, Figure 3.1i, Supplementary Figure 3.1f), this data suggests a conserved trend of disproportional changes in cell and spindle dimensions during early cell divisions.

Previous studies theorized that acentrosomal nucleation sites made it possible for astral microtubules to more easily span the distance from spindle pole to cell cortex in disproportionately large cells29. Based on this idea, we next measured the size of mitotic spindle poles in *C. elegans* and zebrafish embryos to determine whether they scaled to the changes in cell size. Spindle pole area was measured using γ-tubulin signal (live transgenic fluorescence, Figure 3.1f, or antibody staining, Figure 3.1g) in 1- and 3-cell stage *C. elegans* (Figure 3.1f) and 8- and 16-cell stage zebrafish embryo cells (Figure 3.1g). In both organisms, we found a significant decrease in spindle pole area over time (Figure 3.1h). In *C. elegans*, spindle pole area decreased from 6.75±0.28μm² at the 1-cell stage to 4.06±0.14μm² at the 3-cell stage, and zebrafish embryos decreased from 391.5±27.79μm² at the 8-cell stage to 173.6±6.64μm² at the 16-cell stage (Figure 3.1h). We were surprised that the mitotic spindle poles in zebrafish embryos were so large, as we expected to see condensed punctate spindle poles similar to those seen at the zebrafish 512-cell stage (Supplementary Figure 3.1e) or
with *C. elegans* embryos (Figure 3.1f). Instead, we noted giant, uniquely-structured spindle poles at the 8- and 16-cell stages of zebrafish development that often resembled a wheel-like structure (Figure 3.1g).

The values obtained from cell length, spindle length, and spindle pole area measurements were normalized to determine the relative change in these parameters. Size values in *C. elegans* were normalized to the mean value at the 1-cell stage, and values from zebrafish embryos were normalized to the mean value at the 8-cell stage. In both *C. elegans* and zebrafish embryos, we determined that the change in cell length scaled more closely with the change in mitotic spindle pole area than that of spindle length (Figure 3.1i). This is interesting since spindle size has been suggested to scale with cell size during early development27. We found that both cell length and spindle pole area decreased by approximately 30-40% over time. Cell length decreased 39.96±1.23% in *C. elegans* and 27.24±1.37% in zebrafish embryos during the cell cycles measured, and spindle pole area decreased 33.12±1.99% in *C. elegans* and 35.34±6.03% in zebrafish during the same time frame. Spindle length, however, decreased less than 20% during this time, 18.22±3.05% in *C. elegans* and 19.21±2.37% in zebrafish (Figure 3.1i). Taken together, these data suggest that decreases in cell size scale more closely with spindle pole size than spindle length. This regulation of spindle pole area during changes in cell size seem to be conserved between both organisms measured. This leads to a model where spindle poles adjust to cell size changes during early cleavage stage divisions (Figure 3.1j).
3.3.2. Centrosomes in early zebrafish development are uniquely-structured. In order to further characterize the spindle positioning and spindle pole morphology during early zebrafish development, we employed two transgenic lines to follow mitotic cells in a live zebrafish embryo. One line specifically marks microtubules (βactin::EMTB-3xGFP) and the second line marks spindle poles (βactin::centrin-GFP). We employed both lines to monitor spindle and spindle pole shape changes as cells transition from a 4x2 grid at the 8-cell stage to a 4x4 grid at the 16-cell stage (modeled in Figure 3.2a). Using a spinning disk confocal microscope, a four-dimensional time series of EMTB-3xGFP embryos was obtained and presented as a volumetric projected micrograph of depth-coded EMTB-3xGFP embryos (Figure 3.2b). The positioning of the labelled mitotic spindles within the depth-coded image indicated that spindles were positioned parallel to the yolk boundary during the 8- and 16-cell stage. We also visualized labelled mitotic spindles positioning perpendicular to the previous plane of division within the plane of the monolayer when using both EMTB-3xGFP marking the microtubule based spindle (Figure 3.2b) and centrin-GFP embryos that denote the mitotic spindle pole positioning (Figure 3.2c).

Through the use of these transgenic lines, we are able to determine the mitotic stage of dividing cells through changes in microtubule spindle morphology (Figure 3.2d) and identify novel changes in early embryo centrosome morphology (Figure 3.2e). Microtubule spindle morphology is similar to that documented in tissue culture settings, with a clear central spindle and astral microtubules (Figure 3.2d). However, the centrosome morphology visualized through centrin-GFP fluorescence is
Figure 3.2. Spindles are placed parallel to each other and perpendicular to previous division plane in early zebrafish divisions. (a) Model depicting the placement of mitotic spindles within embryonic zebrafish cells from the 1-cell stage to the 16-cell stage. Cells are viewed from top of cell mass with yolk placed below (XY view). Spindle poles (purple) and metaphase plate (blue) shown. Embryo midline placed perpendicular to spindle positioning drawn in 8- and 16-cell models. (b) Representative three-dimensional rendering images of mitotic spindle positioning during early embryonic divisions in zebrafish embryos. Microtubules (EMTB-3xGFP) shown in depth-coded z-stack such that z-slices closest to the embryo yolk are colored red and z-slices furthest from the yolk are colored blue. (c) Representative three-dimensional rendering of transgenic embryos expressing centrin-GFP (inverted grayscale) at the 8- and 16-cell stage. Cell highlighted by dashed box magnified in (e). Bar, 100μm. (d) Stills from timelapse of a cell division in EMTB-3xGFP transgenic embryo. Microtubules (inverted grayscale) shown from a single spindle over time. Mitotic stages denoted. (e) Single cell from centrin-GFP embryo highlighted in (c) in a timelapse through one cell division. Centrosomes (centrin-GFP, fire LUT) depicted over mitotic stages denoted. (f) Single mitotic cells from fixed embryos in prometaphase, metaphase, anaphase, and telophase. Centrin-GFP (magenta/inverted grayscale), γ-tubulin (cyan/inverted grayscale), and nuclei (DAPI, blue) shown. Bar, 20μm.
distinct compared to tissue culture settings. Here, mitotic centrosomes are uniquely-structured and much larger within the cell compared to cell culture settings\textsuperscript{103,108} (Figure 3.2e). During prophase, centrin-GFP wraps around the nucleus as a condensed structure, accumulating on the polar ends where the mature spindle pole will form from prometaphase to metaphase (Figure 3.2e). During prometaphase, the centrin-GFP remains moderately focused but as the dividing cell transitions into metaphase, the spindle pole reaches its maximum area and starts to present with a wheel-like structure (Figure 3.2e). Once the cell transitions to anaphase, the spindle poles begin to fragment and enlarge to the approximate size of the nucleus. They then disperse and start to reform during telophase to prepare for immediate re-entry of the daughter cells into the cell cycle (Figure 3.2e). This pattern of centrin morphology throughout mitosis was found to be similar to that of $\gamma$-tubulin (Figure 3.2f). Centrin is traditionally enriched at centriole barrels that are surrounded by pericentriolar matrix (PCM). $\gamma$-tubulin is a microtubule nucleating protein enriched at the PCM\textsuperscript{125}. When we fixed centrin-GFP embryos and additionally immunostained for $\gamma$-tubulin, we found that both proteins exhibited a similar structure at the centrosome throughout each mitotic stage (Figure 3.2f), suggesting that a significant population of centrin resided within the PCM in dividing cells in the zebrafish embryo. Later at the 512-cell stage of zebrafish development, both proteins localize to centrosomes that appear smaller and more punctate at various mitotic stages (Supplementary Figure 3.2a). We analyzed images with centrin-GFP signal and $\gamma$-tubulin immunostaining and performed a Pearson’s correlation coefficient to determine colocalization between the two proteins over time.
Supplementary Figure 3.2. Spindles are placed parallel to each other and perpendicular to previous division plane in early zebrafish divisions. (a) Maximum confocal projections of fixed mitotic zebrafish cells at the 512-cell stage of development. Mitotic stages denoted. Centrin-GFP (magenta, inverted grayscale), γ-tubulin (cyan, inverted grayscale), and nuclei (DAPI, blue) shown. Bar, 10μm. (b) Violin plot depicting Pearson’s correlation coefficient between centrin-GFP and γ-tubulin signal in fixed zebrafish embryo cells at the 8-, 16-, and 512-cell stage. One-way ANOVA, p=0.0488 (*). n>14 embryos. Plot boundaries depict minimum and maximum, 25th and 75th quartiles represented by thin black line, median represented by thick black line. Detailed statistical analysis in Methods Table 4.
We found that from the 8- to 16- to 512-cell stages of development, there was a significant increase in the colocalization of centrin-GFP and γ-tubulin (Supplementary Figure 3.2b). This suggests that the localization of centrin-GFP may change over time during early zebrafish development, adopting a dispersed localization in the PCM of dividing cells in early cell divisions (8-, 16-cell stage). However, during later divisions when cells become smaller in size (512-cell stage embryos and later), centrin may localize more predominantly with the centriole as seen in mammalian somatic cells\textsuperscript{103} with the PCM tightly organizing around the two centrioles\textsuperscript{13}.

3.3.3. **Spindle poles are asymmetric during early zebrafish cell divisions.** An asymmetry in spindle pole size was identified across a single spindle during early zebrafish embryonic divisions (8- and 16-cell stage embryos in Figure 3.1g, 16-cell stage embryos in Figure 3.3a) that wasn’t identified in \textit{C. elegans} embryos (Figure 3.1f). We quantified the area of zebrafish spindle poles using both centrin-GFP signal and γ-tubulin immunofluorescence labelling at the 8-cell and 16-cell stage (Figure 3.3b-e). We binned these values based on size, separating the larger and smaller spindle poles within each spindle. We calculated a significant different between the area of the larger and smaller spindle poles within a cell at both the 8- and 16-cell stage using both γ-tubulin (Figure 3.3b) and centrin-GFP (Figure 3.3d). When considered as a ratio between the larger and smaller pole area, the larger pole is approximately two-fold larger than that of the smaller pole when measured with γ-tubulin signal (2.21\(\pm\)0.12 at 8-cell, 2.16\(\pm\)0.08 at 16-cell stage, Supplementary Figure 3.3a) and approximately 1.5-fold
Figure 3.3. Mitotic spindle poles are asymmetric and oriented to place larger pole towards embryo center. 

(a) Model depicting a 16-cell embryo with maximum confocal projections of a representative cell (denoted with purple box). Fixed 16-cell metaphase embryo expressing centrin-GFP (magenta/inverted grayscale) and immunostained for γ-tubulin (cyan/inverted grayscale) and nuclei (DAPI, blue). Embryonic midline denoted with orange dashed line. Bar, 10μm. (b–c) Violin plot depicting the spindle pole area at the 8- and 16-cell stage binned by size (larger/smaller, b) or position relative to midline (inner/outer, c). Pole areas measured from γ-tubulin antibody signal in fixed embryos. Students t-test, p<0.0001 (****). n>13 embryos. (d–e) Violin plot depicting the spindle pole area at the 8- and 16-cell stage binned by size (larger/smaller, b) or position relative to midline (inner/outer, c). Pole areas measured from centrin-GFP signal in fixed embryos. Students t-test, p<0.0001 (****), p=0.1183 (ns), and p=0.0030 (**), respectively. n>6 embryos. (f) Model depicting the positioning of the asymmetric mitotic spindle poles in relation to the embryonic midline during the 8-cell and 16-cell stages. The larger of the two mitotic spindle poles (purple) is placed closest to the embryonic midline (orange), providing a directionality (turquoise arrow). For violin plots: Plot boundaries depict minimum and maximum, 25th and 75th quartiles represented by thin black line, median represented by thick black line. Detailed statistical analyses detailed in Methods Table 4.
Supplementary Figure 3.3. Mitotic spindle poles are asymmetric and oriented to place larger pole towards embryo center. (a-d) Violin plot depicting the ratio of spindle pole areas binned by size (larger-to-smaller pole ratio, a, c) or position in relation to the midline (inner-to-outer pole ratio, b, d). Spindle pole areas measured from γ-tubulin (a-b) or centrin-GFP (c-d) signal from fixed zebrafish embryos at the 16-cell stage. Student’s t-test, p=0.7101 (ns, a), p=0.7221 (ns, b), p=0.2015 (ns, c), p=0.2063 (ns, d). Plot boundaries depict minimum and maximum, 25th and 75th quartiles represented by thin black line, median represented by thick black line. Detailed statistical analysis in Methods Table 4.
when measured with centrin-GFP signal (1.48±0.08 at 8-cell, 1.64±0.09 at 16-cell stage, Supplementary Figure 3.3c). No significant difference was noted between ratios when comparing the two cell cycles (Supplementary Figure 3.3a, 3.3c), suggesting that spindle poles maintained a consistent asymmetry despite changing cell and spindle pole sizes from the 8- to 16-cell stages.

When considering the position of the asymmetric mitotic spindle poles within the embryo itself, we identified that the larger of the two poles more frequently pointed towards the center of the embryo (Figure 3.3a). We first identified the embryo midline, which is the line that passes through either the 4x2 cell grid (8-cell stage embryo) or 4x4 cell grid (16-cell embryo, refer to dashed orange line in Figure 3.3a, modeled in Figure 3.3f). We found that the largest spindle pole always pointed towards this embryonic midline. When binning spindle pole area values as either toward the midline (noted as inner) or away from the midline (noted as outer), we calculated that γ-tubulin-decorated spindle poles were significantly larger in size towards the midline (313.16±16.80μm²) compared to those positioned away from the midline (180.63±13.10μm²) at an 8-cell embryonic stage (Figure 3.3c). This asymmetry was also calculated at the 16-cell stage (222.96±10.71μm² at inner pole, 124.26±5.75μm² at outer pole, Figure 3.3c). A ratio was calculated to represent the inner pole area over the outer, identifying more than a two-fold difference in the two areas (2.14±0.13 at 8-cell, 2.09±0.09 at 16-cell stage, Supplementary Figure 3.3b), which is very similar to the values obtained when binning pole areas by size. This indicated that more often than not, the larger of two mitotic spindle poles within a spindle is positioned closest to the center of the embryo.
This trend was not consistent when considering pole area based on centrin-GFP signal. Here, spindle pole area was not significantly different at the 8-cell stage when binning values based on position (p-value of 0.1183, Figure 3.3e). Additionally, pole areas at the 16-cell stage were not nearly as significant (p-value of 0.003, Figure 3.3d) compared to those generated from values binned by size (p-value < 0.0001, Figure 3.3e). When a ratio between inner and outer poles was generated based on centrin-GFP, these ratio values were closer to a value of 1 (1.25±0.09 at 8-cell, 1.43±0.11 at 16-cell stage, Supplementary Figure 3.3d) suggesting that the pole with the larger area based on centrin-GFP localization is not as consistently placed at the center of the embryo compared to the data obtained with γ-tubulin signal. Taken together, these data suggest a model in which zebrafish spindle poles present with an asymmetry in PCM components such as γ-tubulin across a single spindle, and this asymmetry creates a vectoral directionality that positions the larger spindle pole towards the embryo center at the 8- and 16-cell stage (Figure 3.3f).

3.3.4. Spindle positioning, but not asymmetry, is PLK1- and PLK4-dependent. To determine the mechanism by which the spindle pole asymmetry is governed, we targeted the mitotic kinases PLK1 (Polo-like kinase 1) and PLK4 (Polo-like kinase 4). PLK1 is an essential mitotic kinase that regulates many aspects of cell division such as spindle assembly, checkpoints, and centrosome maturation. The related kinase PLK4 regulates centriole duplication prior to the assembly of the mitotic spindle15. Transcripts for PLK1 and PLK4 have been detected as early as the 1-cell stage in zebrafish.
embryos, indicating that they are maternally supplied prior to zygotic genome activation\textsuperscript{126}. Inhibitors for PLK1 (BI2536) and PLK4 (centrinone) have been previously published and have been effective in the inhibition of these kinases \textit{in vitro} \textsuperscript{103,108,127}, and BI2536 has recently been used in early zebrafish embryo studies\textsuperscript{103,124}.

PLK1 has been implicated in the phosphorylation of pericentriolar matrix proteins such as pericentrin and CEP215 at the centrosome which aids in their organization at this site. Pericentrin and CEP215 anchor the \(\gamma\)-TURC (\(\gamma\)-tubulin ring complex) to the PCM, a structure containing \(\gamma\)-tubulin that is responsible for microtubule nucleation at the centrosome\textsuperscript{15,128}. This occurs in a PLK1-dependent manner\textsuperscript{129}. Additionally, it has been determined that PLK1 localizes asymmetrically to centrosomes in later zebrafish embryos\textsuperscript{103}, leading us to ask whether the stark asymmetry in \(\gamma\)-tubulin distribution across the spindle could be dependent on PLK1 in the early zebrafish cell divisions. With PLK4 inhibition, in monopolar spindles, acentrosomal spindle poles, and mitotic delay have been reported to occur \textit{in vitro}\textsuperscript{15}. Given the unique architecture of the zebrafish spindle poles noted by \(\gamma\)-tubulin and centrin-GFP morphology, we sought to determine the response to either PLK1 or PLK4 inhibition.

When injecting a vehicle control (1\% DMSO), two concentrations of BI2536 (100nM or 1 \(\mu\)M), or two concentrations of centrinone (100nM or 1 \(\mu\)M) at the 1-cell stage, spindle pole asymmetry was not disrupted under any of the conditions. This was determined by measuring spindle pole areas in fixed embryos at the 16-cell stage after immunostaining for \(\gamma\)-tubulin. We binned spindle pole areas by size and separated the smaller and larger spindle pole areas. We found that the larger spindle poles were significantly larger than the smaller spindle poles (Figure 3.4b). When we converted
Figure 3.4. Placement of asymmetric zebrafish spindle poles is PLK1- and PLK4-dependent. (a) Representative model images of 16-cell stage embryos during metaphase under conditions of DMSO (left), 1μM BI2536 (center), or 1μM centrinone treatment (right). γ-tubulin (magenta/inverted grayscale), and nuclei (DAPI, blue) shown. Pole areas labelled in image. Model depicts spindle pole positioning in respective cells. Large and smaller poles denoted, poles not drawn to scale. Bar, 30 μm. (b-c) Violin plot depicting spindle pole area under conditions of DMSO (gray), BI2536 (100nM or 1μM, blue), or centrinone (100nM or 1μM, gold) exposure. Spindle pole area measured with γ-tubulin antibody signal and binned by size (larger/smaller in b) or position relative to midline (inner/outer, c). Student’s t-test performed within each treatment group, detailed statistical analysis in Methods Table 4. n>48 cells quantified per treatment. Plot boundaries depict minimum and maximum, 25th and 75th quartiles represented by thin black line, median represented by thick black line. (d) Bar graph depicting percentage of spindles with largest pole pointed towards midline under conditions of DMSO (gray), BI2536 (100nM or 1μM, blue), or centrinone (100nM or 1μM, gold) exposure. One-way ANOVA with Dunnett’s multiple comparison test performed with DMSO control. Detailed statistical analysis in Methods Table 4. n>10 embryos quantified per treatment. (e) Model depicting the positioning of the asymmetric mitotic spindle poles in relation to the embryonic midline during the 16-cell stages under conditions of DMSO (gray), BI2536 (blue), or centrinone (gold) exposure. Spindle poles (purple), metaphase plate (blue), and embryonic midline (orange dashed line) depicted.
these values into a ratio to compare the area of the largest pole to that of the smallest, we found a slight but significant decrease in the asymmetry under conditions of PLK1 or PLK4 inhibition compared to vehicle control (Supplementary Figure 3.4a). Interestingly, spindle pole area for both small and large poles increased under conditions of PLK1-inhibition in a dose-dependent manner (100nM BI2536: 127.25±7.14μm² large poles, 83.59±5.00μm² small poles; 1μM BI2536: 141.63±5.87μm² large poles, 91.17±3.44μm² small poles) compared to DMSO-treated control embryos (101.65±4.91μm² large poles, 52.28±272μm² small poles, Figure 3.4b). However with centrinone treatment, spindle pole area increased similarly with both drug concentrations (100nM centrinone: 199.33±9.96μm² large poles, 120.58±6.35μm² small poles; 1μM centrinone: 151.01±9.25μm² large poles, 97.80±6.62μm² small poles, Figure 3.4b). Contrary to findings in tissue culture settings\textsuperscript{127,130}, we did not observe instances of monopolar spindle formation or loss of centrin signal at centrosomes in zebrafish embryos upon PLK4 inhibition with centrinone. This could be due to the observed centrin localization in the PCM and not to an identifiable centriole. However, both PLK1 inhibition and PLK4 inhibition cause structural changes within the PCM architecture, causing a significant increase in PCM size while still able to retaining the asymmetry, albeit not as well as under control conditions (ratios calculated in Supplementary Figure 3.4a).

We next examined whether there was still a vectoral directionality in spindle placement under conditions of PLK1 and PLK4 inhibition, with large spindle poles pointed towards the midline and smaller spindle poles pointed away. When binning spindle pole area calculations based on position in relation to the midline, the difference in spindle pole area based on position was not significant under BI2536 conditions.
Supplementary Figure 3.4. Placement of asymmetric zebrafish spindle poles is PLK1- and PLK4-dependent. (a-b) Violin plot depicting the ratio of spindle pole areas binned by size (larger-to-smaller pole ratio, a) or position in relation to the midline (inner-to-outer pole ratio, b) under conditions of DMSO (gray), BI2536 (100nM or 1μM, blue), or centrinone (100nM or 1μM, gold) exposure. Spindle pole areas measured from γ-tubulin signal from fixed zebrafish embryos at the 16-cell stage. One-way ANOVA with Dunnett's multiple comparison test, n>10 embryos quantified. (c) Violin plot depicting mitotic spindle pole area measured from γ-tubulin signal from fixed zebrafish embryos at the 16-cell stage under conditions of DMSO (gray), BI2536 (100nM or 1μM, blue), or centrinone (100nM or 1μM, gold) exposure. One-way ANOVA, p<0.0001 (****). n>96 cells quantified. (d) Violin plot depicting spindle angle in relation to midline fixed zebrafish embryos at the 16-cell stage under conditions of DMSO (gray), BI2536 (100nM or 1μM, blue), or centrinone (100nM or 1μM, gold) exposure. One-way ANOVA, p=0.0514. n>48 cells quantified. For all plots: Plot boundaries depict minimum and maximum, 25th and 75th quartiles represented by thin black line, median represented by thick black line. Detailed statistical analysis in Methods Table 4.
compared to DMSO controls (Figure 3.4c). With centrinone treatment, there was a slight but significant difference noted in 100nM centrinone injections (p-value of 0.0341, Figure 3.4c). This was corroborated by the calculation of a ratio between the inner and outer pole areas, where ratios for BI2536 (100nM: 1.425±0.12, 1μM: 1.142±0.06) and BI2536 (100nM: 1.418±0.11, 1μM: 1.29±0.15) approached a value of 1 whereas DMSO control injections demonstrated a ratio of 2.00±0.14 (Supplementary Figure 3.4b). When calculating the percentage of spindles with the larger spindle pole positioned towards the embryonic midline, we determined that 79.65±4.56% of embryonic cells injected with the vehicle control positioned the largest pole towards the midline. This value represents the majority of cells evaluated. However, a dosage-dependent decrease in this value was noted under conditions of PLK1 (BI2536) or PLK4 (centrinone) inhibition. With BI2536 conditions, 60.87±4.07% (100nM) and 48.12±5.02% (1μM) of spindles positioned larger poles towards the midline, and 66.07±9.13% (100nM) and 54.52±7.76% (1μM) did so with centrinone treatment (Figure 3.4d). When considering that a 50% rate of positioning towards the midline would represent a random distribution, these percentages indicate that vectoral spindle placement becomes deregulated under PLK1 or PLK4 inhibition (Figure 3.4a, d). While the inherent asymmetry in spindle pole size functions under a PLK1-/PLK4-independent mechanism, these studies suggest that the placement of the asymmetric spindle poles with respect to the center of the embryo require PLK1 and PLK4 function (Figure 3.4e).
3.4. Discussion. In previous studies, PLK1 has been proposed to regulate PCM architecture by facilitating its phase separation in *C. elegans*. Additionally, PLK1 has a defined role in recruiting the pericentrin-CEP215 complex that anchors the γ-TURC at the centrosome\textsuperscript{15,128}. Based on this information, it is possible that PLK1 is working to orchestrate proper PCM architecture in the large cells of the early zebrafish embryo as well. This could explain why spindle pole area increased in a dosage-dependent manner with BI2536 treatment. Here, the loss of PLK1 activity would result in a loss of this PCM architecture regulator, causing the surrounding PCM to lose its tight matrix configuration and occupy a larger space.

Strikingly, centrinone treatment did not exhibit the same dosage-dependent change in spindle pole area, instead a similar increase was seen regardless of treatment dosage. A possible explanation is that PLK4 is present at much lower concentrations in the early zebrafish embryo compared to PLK1\textsuperscript{126}. It is therefore likely that lower drug concentrations are needed to target the small pool of embryonic PLK4, leading to a similar phenotype with various drug concentrations above this small threshold. The opposite would then be true for PLK1, where dosage-dependency was observed because the larger pool of PLK1 would take a higher drug concentration to completely render inactive. Further studies would be required to determine whether these two proteins behave similarly in zebrafish compared to previous tissue culture studies, and how the relative quantities of these proteins may change during development.
These studies aimed to understand how mitotic spindles are able to coordinate cell division in disproportionately large cells. One striking finding was that spindle orientation was not significantly impacted under inhibition of PLK1 or PLK4 (Supplementary Figure 3.4d). We theorized that the large mitotic spindle poles characterized in zebrafish embryos would allow for astral microtubules to be projected closer to the cell cortex to anchor and generate necessary pulling forces to orchestrate division in giant cells. We were therefore surprised to note that spindle orientation was not significantly changed in relation to the embryonic midline under drug treatment (Supplementary Figure 3.4d). A possible explanation is that although spindle directionality was changed through the randomization of larger pole placement in relation to the midline, Hertwig’s rule may still apply here and regulate spindle positioning independent of PLK1 or PLK4 activity. Hertwig’s rule describes how a spindle is typically placed along the longest axis of a dividing cell24, and it is possible that spindles are placed at specified angles that follow this rule and are PLK1- and PLK4-independent. Further studies will be necessary to understand the exact mechanism that placed the mitotic spindle at a defined angle in relation to the embryo midline.

Overall, these data contribute to a model where asymmetrically-structured spindle poles position themselves within the zebrafish embryo with vectoral directionality in respect to the embryo center (Figure 3.3f). We determined spindle pole asymmetry is governed by a PLK1- and PLK4-independent mechanism, but PLK1 and PLK4 are required to properly place the asymmetric poles with respect to the center of the
embryo. Through these studies, a novel centrosome structure has been characterized that may contribute to a better understanding of how mitotic spindles are able to coordinate cell division in disproportionately large cells.

3.5. Materials and Methods

3.5.1. Fish Lines. Zebrafish lines were maintained using standard procedures approved by the Syracuse University IACUC committee (protocol #18-006). Embryos were stages as described in Kimmel et al 1995. See Supplementary Table 3 for list of transgenic zebrafish lines used.

3.5.2. Zebrafish Imaging. A Leica SP5 or SP8 (Leica, Bannockburn, IL) laser scanning confocal microscope was used throughout this manuscript. An HC PL APO 20x/0.75 IMM CORR CS2 objective, HC PL APO 40x/1.10 W CORR CS2 0.65 water immersion objective, and an HCX Plan Apochromat 63x/1.40-0.06 NA OIL objective were used. Images were acquired using LAS-X software. A Leica DMi8 (Leica, Bannockburn, IL) with a X-light v2 confocal unit spinning disk was also used, equipped with an 89 North – LDI laser and a Photometrics Prime-95B camera. Optics used were either 10x/0.32 NA air objective, HC PL APO 63X/1.40 NA oil CS2, HC PL APO 40X/1.10 NA WCS2 CORR, a 40X/1.15 N.A. 19 Lamda S LWD, or 100Å~/1.4 N.A. HC Pl Apo oil emersion objective.
3.5.3. C. elegans imaging. Live cell imaging of C. elegans embryos was performed on a spinning disk confocal system that uses a Nikon Eclipse inverted microscope with a 60X 1.40NA objective, a CSU-22 spinning disc system and a Photometrics EM-CCD camera from Visitech International. Images were obtained every 2 minutes with a 1 micron z-stack step size. See Supplementary Table 3 for list of transgenic lines used.

3.5.4. Pharmacological treatments. Embryos were injected with either 1% DMSO, or BI2536 or centrinone (final concentration 100nM or 1µM) at the 1-2-cell stage. Embryos are incubated at 30°C until they reach the developmental stage of interest, at which time they are fixed with 4% paraformaldehyde in PBS. Immunohistochemistry then proceeds as detailed below.

3.5.5. Zebrafish immunohistochemistry. Zebrafish embryos were fixed using 4% PFA containing 0.5% Triton-X 100 overnight at 4°C. Zebrafish were then dechorionated and incubated in PBST (phosphate buffered saline + 0.1% Tween) for 30 minutes. Embryos were blocked using a Fish Wash Buffer (PBS + 1% BSA + 1% DMSO + 0.1% Triton-X 100) for 30 minutes followed by primary antibodies incubation (antibodies diluted in Fish Wash Buffer in concentrations stated in table above) either overnight at 4°C or 3 hours at room temperature. Embryos are then washed five times in Fish Wash Buffer before incubating with secondary antibodies for 3 hours at room temperature. After five more washes, embryos were incubated with 4',6-diamidino-2-phenylindole (NucBlue® Fixed Cell ReadyProbes® Reagent) for 30 minutes. For imaging, embryos were either halved
and mounted on slides using Prolong Diamond (Thermo Fisher Scientific cat. # P36971) or whole-mounted in 2% agar (Thermo-Fisher cat. # 16520100).

**3.5.6. Image and Data Analysis.** Images were processed using both FIJI/ImageJ software and Adobe Photoshop. Angles were calculated using FIJI/ImageJ software and Microsoft Excel. All graphs were generated and statistical analysis performed using Graphpad Prism software. 3-D images, movies, and surface rendering were performed using Bitplane IMARIS software (Surface, Smoothing, Masking, and Thresholding functions). For mitotic spindle angle calculations, a line was drawn in ImageJ/FIJI to measure the angle of the midline or yolk boundary in relation to the image boundary. Next, a second line was drawn along the mitotic spindle to measure its angle in relation to the image boundary. The difference between these two values was then calculated to represent the angle of the mitotic spindle in relation to the midline or yolk boundary. To calculate two-dimensional area, a boundary was drawn around the structure of interest (cell, spindle pole, etc.) in ImageJ/FIJI and the area within this shape was calculated. To calculate spindle length, cell length, aspect ratio, etc., a line was drawn in ImageJ/FIJI from one end of the structure of interest to the other. This length was then measured and recorded. To calculate aspect ratios, the length along the plane of the mitotic spindle was divided by the length perpendicular to the mitotic spindle and displayed as a ratio.
3.5.7. Statistical analysis. Unpaired, two-tailed Student's t-tests and one-way ANOVA analyses were performed using GraphPad Prism software. **** depicts a p-value <0.0001, *** p-value <0.001, **p-value<0.01, *p-value <0.05. See Supplementary Table 4 for detailed information regarding statistics.

3.5.8. Methods Table 1: Key Resources Table

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3.5.9. Methods Table 2: Antibodies

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3.5.10. Methods Table 3: Transgenic Lines

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### 3.5.11.Methods Table 4: Detailed statistical analysis results

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Chapter Four:

PLK-1 regulates spindle organization and mitotic progression during zebrafish development
This chapter features work from two publications:

**Part I: Evaluating the localization and activity of PLK1 in mammalian cells and vertebrate zebrafish embryos.**


Author contributions: E.C. and H.H. designed and E.C., M.B., L.R., K.S., J.M. and H.H. conducted experiments and analyzed the data. J.A. provided zebrafish embryos and husbandry knowledge. J.F. constructed all vectors utilized. E.C. and H.H. wrote the manuscript, contributing to multiple rounds of edits.

Lindsay Rathbun conducted and analyzed zebrafish studies in Figure 4.2d-f.

**Part II: Development of an inhibitor to selectively target centrosomal PLK1.**


Author contributions: Paula J Bucko, Conceptualization, Data curation, Formal analysis, Supervision, Validation, Investigation, Visualization, Methodology, Writing—original draft, Project administration, Writing—review and editing; Chloe K Lombard, Data curation, Formal analysis, Investigation, Methodology; Lindsay Rathbun, Data curation, Formal analysis, Investigation; Irvin Garcia, Data curation, Formal analysis, Validation, Investigation; Akansha Bhat, Validation, Investigation; Linda Wordeman, Conceptualization, Resources, Formal analysis; F Donelson Smith, Conceptualization, Supervision; Dustin J Maly, Conceptualization, Supervision, Funding acquisition, Methodology; Heidi Hehnly, Data curation, Supervision, Funding acquisition, Methodology; John D Scott, Conceptualization, Supervision, Funding acquisition, Visualization, Writing—original draft, Project administration, Writing—review and editing

Lindsay Rathbun conducted and analyzed zebrafish studies in Figure 4.4-6.
4.1. Abstract. PLK1 is an essential mitotic kinase that regulates spindle assembly, microtubule nucleation, and centrosome maturation. During mitosis, it localizes to the centrosomes, kinetochores, and cytokinetic midbody. However, it is not known if PLK1 populations at these distinct subcellular locales have different functions, and how these populations function in a vertebrate model system. In this chapter, studies are presented that demonstrate similar asymmetric localization in zebrafish PLK1 to previous studies in tissue culture models, and that this asymmetry is altered by the presence of a missegregated chromosome. Additionally, a new pharmacological tool termed LoKI (localized kinase inhibition) is introduced, where PLK1 inhibition is targeted directly to the centrosomal pool. With the LoKI system, a PLK1 inhibitor, BI2536, is attached to a CLP moiety, which binds to a SNAP-tag that is localized to the centrosome through the use of the PACT domain. With the use of this system, it was determined that the centrosomal pool of PLK1 functions in spindle maintenance and mitotic progression, as monopolar, multipolar, and misorienting spindles were observed along with mitotic delays under conditions of targeted PLK1 inhibition. This chapter highlights a new toolset to determine the mechanisms regulating cell division in a vertebrate model system.

Part I: Evaluating the localization and activity of PLK1 in mammalian cells and vertebrate zebrafish embryos.

4.2. Introduction. Mitotic cell division is a process whereby genetic material is duplicated, separated, and packaged to yield two daughter cells. This process relies heavily on the spatial and temporal synchronization of signaling activity at the mitotic spindle, a structure that segregates the chromosomes and guides them towards the daughter cells. The
mitotic kinase, polo-like kinase 1 (PLK1), is a major regulator of this process that works to ensure bipolar spindle formation and chromosome alignment at the metaphase plate. This is accomplished by PLK1-scaffold interactions at the mitotic centrosomes/spindle poles which modulate the recruitment of centrosome components SAS-4, γ-tubulin, γ-TuRC, pericentrin, and CEP215 (reviewed in 108). Their recruitment is initiated after PLK1-dependent SAS-4 phosphorylation131. This phosphorylation allows SAS-4 expansion to occur, followed by the recruitment of CEP215 and γ-tubulin and subsequent expansion of the pericentriolar material (PCM), playing a crucial role in mitotic centrosome/spindle pole formation during division131. However, it is unclear whether PLK1 is additionally regulated between the two spindle poles during cell division.

Due to the nature of centriole duplication, the two spindle poles are inherently asymmetric from one another. The oldest (mother) spindle pole is enriched with the centriole appendage protein cenexin, compared to the youngest spindle pole (daughter)14,51. During interphase, mother centriole appendages assist in centrosome positioning14 and primary cilia formation by anchoring the oldest centriole (known here as the basal body) to the cell membrane to form the primary cilia (reviewed in 132,133). Prior to mitotic onset, PLK1 is recruited to the basal body where it assists in ciliary disassembly134. Cenexin regulates appendage formation and has also been identified as a PLK1 binding partner135,136. Previous work utilizing Ground State Depletion (GSD) identified a modest, but significant, enrichment of PLK1 at the mother (cenexin-positive) spindle pole in fixed in vitro metaphase cells84. This study suggests an inherent asymmetry in PLK1 distribution that is dependent on centrosome age. During division,
cenexin has been implicated in multiple processes, including modulating preferential chromosome misalignment toward the oldest spindle pole in the event of mitotic error\textsuperscript{137}. Knowing this, we wanted to test the hypothesis that PLK1 localization and activity is asymmetrically regulated between the two spindle poles through the presence of cenexin at the mother spindle pole, which can modulate directional chromosome misalignment.

Using a multidisciplinary approach, we found a significant asymmetry in PLK1 localization and activity between spindle poles in \textit{in vivo} zebrafish studies and \textit{in vitro} tissue culture. From here, we tested whether the propensity for chromosomes to misalign towards one spindle pole altered PLK1 activity. Lastly, we developed a tool to specifically inhibit the PLK1 population at the centrosome and determined that this population regulates spindle organization and mitotic progression.

\textbf{4.3. Asymmetric distribution of PLK1 in mammalian cells and zebrafish.} In mammalian dividing cells, PLK1 is upregulated during mitosis. During this time, it is enriched at spindle poles and kinetochores, specifically from prometaphase to metaphase\textsuperscript{15,138}. Following metaphase exit, PLK1 transitions from kinetochores to the cytokinetic furrow, where it is subsequently concentrated at the forming midbody (\textsuperscript{108,138,139}, modeled in Figure 4.1a). The subcellular distribution of PLK1 in mammalian cells has predominately been studied in \textit{in vitro} cell culture models. However, \textit{in vitro} systems do not always represent what is happening \textit{in vivo}. Here, we examine the temporal and spatial regulation of PLK1 during division first in live mammalian cells to
Figure 4.1. PLK1 asymmetric distribution between spindle poles is conserved in vivo (zebrafish) and in vitro (mammalian cell culture). (a) Model depicting PLK1 (magenta) localization to spindle poles, kinetochores, and cytokinetic midbody. DNA = blue. (b-c) are data from human retinal pigment epithelial (RPE) cells stably expressing GFP-PLK1. (b) Representative images of fluorescence recovery after photobleaching (FRAP) of GFP-PLK1 expressing RPE cells at spindle poles during metaphase. (Fire-LUT, ImageJ). 3-D surface plot of a single metaphase cell displaying GFP-PLK1 integrated intensity between the two spindle poles. Spindle pole 1 and 2 are marked. (c) GFP-PLK1 integrated intensity at the highest spindle pole (pole 1) was normalized to 100% and compared to the lowest spindle pole within a single mitotic spindle, over n=44 cells in n=3 experiments ± S.E.M, Student’s paired t-test, p<0.001. (d) Model depicting zebrafish embryo 4.5 hours post fertilization (hpf). Drawn inset represents area imaged in (e) along the yolk boundary. (e) A maximum confocal projection is presented for PLK1-mCherry. Bar = 50μm. Inset of single metaphase cell. Bar = 5μm. Magenta dotted line represents yolk boundary. (f) PLK1-mCherry expression at 4.5hpf. Cells positive for PLK1-mCherry are indicative of dividing cells. Bar = 10μm. Inset (bottom panel): A maximum confocal projection of a single metaphase cell expressing PLK1-mCherry at spindle poles and kinetochores. Bar = 5μm. (g) Confocal maximum projections from a 4.5 hpf embryo expressing PLK1-mCherry. Single mitotic cell shown from prometaphase through cytokinesis. Images taken every 30 seconds, over 6 minutes. Bar = 10μm. (h) Maximum projection of single metaphase cell expressing PLK1-mCherry (left panel), Bar = 5μm. 3-D surface plot of metaphase cell (right panel) displaying PLK1-mCherry integrated intensity measurements ranging between 0 and 250. Spindle poles marked 1 and 2. Fire-LUT (ImageJ). (i) PLK1-mCherry integrated intensity at the highest spindle pole (pole 1) was normalized to 100% and compared to the lowest spindle pole within a single mitotic spindle, (n=49 cells measured across 10 embryos ± S.E.M, Student’s t-test p<0.0001). (j) Shown is a single prometaphase cell expressing PLK1-mCherry with pole 1 and 2 marked by a region of interest (ROI) at time point 0 sec. PLK1-mCherry integrated intensity is displayed through a Fire-LUT where high intensity white pixels are 35000 and lower intensity black pixels are 0. The ROIs where PLK1 intensity between pole 1 and 2 is symmetric is highlighted in gray (0 sec). Where PLK1 intensity is asymmetric is highlighted in blue 120 sec. Bar = 5μm. (k) Line graph of PLK1 intensity over 2.5 minutes at pole 1 (magenta) and 2 (cyan) featured in (j), illustrating periods of symmetric (gray) and asymmetric (blue) PLK1 intensity between the spindle poles.
establish its in vitro localization (Figure 4.1), then validate in a developing vertebrate embryo (Figure 4.2-3).

Previous work using GSD (ground-state depletion) in fixed in vitro cells suggests that an inherent asymmetry exists in the amount of PLK1 between the two spindle poles. To determine whether this inherent PLK1 asymmetry between metaphase spindle poles is conserved in live mammalian cells, we employed a retinal pigment epithelial (RPE) cell line that stably expresses GFP-PLK1 at endogenous levels. Fluorescence recovery after photobleaching (FRAP) was performed. To do this, a ROI was placed over both spindle poles (Figure 4.1b), where a 488 nm laser was applied. Upon application of the laser, GFP-PLK1 fluorescence within the regions was bleached. After 1.6 seconds, GFP-PLK1 signal returns to that region (Figure 4.1b). A 3-D surface plot was performed for the metaphase cell pre-FRAP (-1.2 seconds), during the FRAP (0 seconds), and post-FRAP (1.6 seconds) (Figure 4.1b). At -1.2 seconds (pre-FRAP), pole 1 contained significantly more GFP-PLK1 than the other (pole 2). At 0 seconds, GFP-PLK1 at both poles was successfully bleached. At 1.6 seconds, the pole 1 returned to have an elevated amount of GFP-PLK1 compared to spindle pole 2 (Figure 4.1b), suggesting an increased exchange of GFP-PLK1 at pole 1. Along these same lines, we determined over multiple metaphase cells that spindle pole 2 contained $14.70 \pm 4.12\%$ less GFP-PLK1 compared to pole 1 (Figure 4.1c). This suggests a mechanism for an asymmetric distribution of PLK1 between the two spindle poles.

We next tested whether this asymmetry was conserved in the dividing cells of the vertebrate zebrafish embryo. Fertilized embryos were injected with 100pg PLK1-mCherry
mRNA. Injected embryos were imaged using confocal microscopy 4.5 hours post fertilization (hpf, modelled in Figure 4.1d). At this time, embryonic cells are proliferating asynchronously\textsuperscript{102}, and proliferating cells can be distinguished via PLK1 expression (Figure 4.1e-g). By magnifying the PLK1-mCherry-positive sub-population, a distinct subcellular distribution of PLK1-mCherry at spindle poles and kinetochores was noted (Figure 4.1e-f). The spatial and temporal distribution of PLK1-mCherry in a single dividing cell was monitored over a 360 second time span. PLK1-mCherry transitions from spindle pole and kinetochore localization in metaphase to cytokinetic furrow localization during cytokinesis where it becomes concentrated at the cytokinetic midbody. (Figure 4.1g).

Upon investigation of the integrated intensity of PLK1-mCherry between spindle poles in metaphase cells within the zebrafish embryo, we noted that one spindle pole has a significantly larger proportion of PLK1-mCherry compared to the other (Figure 4.1h, Fire look up table (LUT)). This is clearly demonstrated when the maximum projection of a single metaphase cell (Figure 4.1h, left panel) is presented as a three-dimensional (3-D) surface plot (Figure 4.1h, right panel), where each peak represents a spindle pole (labeled with 1 and 2). The spindle pole peak on the left (1) presents with 10\% greater PLK1 fluorescence intensity than its partnering spindle pole peak on the right (2, Figure 4.1h). To validate this finding, we measured PLK1 fluorescence intensity between spindle pole pairs over 49 dividing metaphase cells from 10 embryos. The spindle pole with the highest intensity was binned as pole 1 and the pole with the lowest intensity was binned as pole 2. From this dataset, one spindle pole consistently contained 10.31\%± 1.14\% less PLK1-mCherry compared to the other (Figure 4.1i). We then examined whether this asymmetry
was present throughout a 150 second time course of a prometaphase cell transitioning through metaphase (Figure 4.1j-k). This was measured by placing a region of interest (ROI) over spindle pole 1 and 2. The integrated intensity of PLK1-mCherry within this region was plotted over 150 seconds with images taken every 30 seconds. The graph demonstrates that spindle poles present with asymmetric PLK1 distribution as cells exit prometaphase (Figure 4.1k, beginning at 60 second timepoint). These findings are strikingly similar to the differences in GFP-PLK1 between the two spindle poles observed in metaphase cells within mammalian cells, where one spindle pole contained 14.70±4.12% less PLK1 than the other (Figure 4.1c). Together, this suggests a conserved mechanism for an asymmetric distribution of PLK1 between the two spindle poles.

4.4. Chromosome misalignment drives asymmetry in PLK1 distribution. A possible mechanism to respond to misaligned chromosomes is to adjust PLK1 distribution between spindle poles. During prometaphase exit and metaphase, misaligned chromosomes can be found that realign with the metaphase plate (Figure 4.2a). During these situations, we imaged GFP-PLK1 RPE cells every 2 minutes across the full volume of the cell until it passed through anaphase (approximately 20 minutes in duration). GFP-PLK1 intensity was then measured at each spindle pole over time. The spindle pole with the misaligned chromosome in closest proximity was binned as spindle pole 1 and the other as spindle pole 2. When a misaligned chromosome occurred, spindle pole 1 contained an elevated GFP-PLK1 signal compared to spindle pole 2 (Figure 4.2a-b). To examine if this was a consistent phenomenon, a ratio was calculated for GFP-PLK1
Figure 4.2. PLK1 asymmetric distribution between spindle poles is driven by chromosome misalignment. (a) GFP-PLK1 (16-colors LUT, Image J) RPE cells treated with NucBlue to stain DNA (white) were imaged every 2 minutes. Shown is a time point with a misaligned chromosome (2 min, arrow) that then assembles within the metaphase plate by 6 min. Bar = 2 μm. The spindle pole on the side with the misaligned chromosome is marked as 1, and the opposite pole is 2. Ratio values for GFP-PLK1 between pole 1 and 2 shown in lower right corner. (b) The intensity of pole 1 and 2 from (a) was measured over a 20 min time course and plotted. Chromosome misalignment marked on plot. (c) The ratio GFP-PLK1 intensity at the spindle pole (labeled 1) with a misaligned chromosome divided by the GFP-PLK1 intensity of spindle pole without a misaligned chromosome (labeled 2) was measured during misalignment (magenta) and post misalignment (cyan) in the same cell over n=10 live cell data sets. Violin plot shown. Dashed line at median, dotted lines at interquartile range. Student’s paired t-test, *** p<0.001. (d) Maximum projection of a zebrafish embryo expressing PLK1-mCherry (cyan) and DAPI (white). Examples of metaphase cells with proper chromosome alignment (orange) and chromosome misalignment (magenta) denoted by boxes. Bar, 100 μm. (e) Example images of mitotic cells from (d) with proper chromosome alignment (top, orange box in (d)) and chromosome misalignment (bottom, magenta box in (d)). PLK1-mCherry (cyan) and DAPI (white) shown in left and center images. PLK1-mCherry (16-colors LUT) in right images to denote areas of high PLK1 intensities. Ratio values for PLK1-mCherry between mitotic spindle poles shown in upper right corner. Bar = 5 μm. (f) Violin plot depicting the ratio between highest PLK1-intensity spindle pole over lowest PLK1-intensity spindle pole in mitotic cells with an aligned metaphase plate (magenta) or misaligned (cyan). n>45 cells/treatment across n=11 embryos. Student’s paired t-test, **** p<0.0001.
intensity at the spindle pole with a misaligned chromosome (pole 1) over the spindle pole without the misaligned chromosome (pole 2) during time points of misalignment compared to time points post misalignment over 10 dividing cells (Figure 4.2c). During misalignment, a mean ratio of 1.33 occurs compared to post misalignment where a mean ratio is at 1.01 (Figure 4.2c), suggesting that asymmetry in PLK1 between mitotic spindle poles is due to adjustments in chromosome alignment.

Next, we tested whether this occurs in vivo by examining division in a zebrafish embryo expressing PLK1-mCherry and chromosomes stained with DAPI. In a fixed, 50% epiboly embryo (Figure 4.2d), we noted metaphase cells with misaligned chromosomes compared to cells with a clearly aligned metaphase plate (Figure 4.2e). Under these conditions, we calculated a ratio of the spindle pole with highest intensity over the pole with lowest intensity and determined that the mean ratio is significantly higher under conditions of misaligned chromosomes (mean at 1.27) compared to dividing cells with an aligned plate (mean at 1.12, Figure 4.2f). Taken together, these studies suggest that chromosome misalignment is causing an elevated asymmetric distribution of PLK1 at spindle poles both in tissue culture and in vivo.

Part II: Development of an inhibitor to selectively target centrosomal PLK1

4.5. Targeting a PLK1 inhibitor to the centrosomal population of PLK1. Protein kinase inhibitor drugs are an emerging class of therapeutics for a variety of clinical indications\textsuperscript{140}. These small molecules are also powerful research tools that can be used to discover new aspects of kinase signaling\textsuperscript{141}. While “drugging” individual kinases can
establish their role in cellular events, this global approach cannot discriminate where or when these signaling enzymes operate inside the cell. Thus, designing pharmacological strategies that influence the spatial and temporal action of kinases is at the frontier of precision medicine.

Polo-like kinase 1 (PLK1) is an important regulator of cell division. Accordingly, ATP-competitive drugs that block its activity, such as BI2536, ascribe functions to these kinases and are promising anticancer therapies. However, elucidating the spatial and temporal action of PLK1 remains challenging as enzymes like PLK1 continually change their location and activity throughout mitosis. As a result, global drug delivery strategies mask the unique contributions of each kinase at distinct mitotic structures. Moreover, standard drug regimens that saturate dividing cells with these compounds may increase off-target effects and toxicity.

PLK1 has been implicated in the control of mitotic progression. During cell division, PLK1 becomes enriched at the centrosomes, kinetochores, and midbody. Scaffold proteins such as Gravin and cenexin anchor PLK1 to the centrosome, creating a specific subcellular locale for PLK1 signalling. While the subcellular localization of PLK1 has been characterized, it is unclear whether each locale represents a specific subpopulation of PLK1 and whether these populations regulate different aspects of mitosis. To investigate this, a tool was developed to test the function of PLK1 at a specific subcellular locale, the centrosome.

In the present study develop a novel chemical-biology tool, LoKI (Localized Kinase Inhibition), to probe the actions of PLK1 at a defined subcellular location. We next
demonstrate that local inhibition of PLK1 kinase at centrosomes disrupts spindle formation and mitotic duration. Together, these studies decipher how activities of individual kinases at precisely defined microenvironments contribute to the global signaling events that underlie mitosis.

4.6. LoKI platforms direct kinase inhibitor drugs to specific subcellular locations. To decipher how centrosome-associated pools of PLK1 coordinate mitotic signaling events, we needed to selectively target drugs to this location. This led to the development of the LoKI tool which allows us to target kinase inhibitor drugs to specific subcellular locations. In this case, we utilized the pericentrin AKAP450 centrosomal-targeting (PACT) domain\textsuperscript{151} to target the LoKI system to the centrosome. This domain was fused to a SNAP-tag moiety which can be covalently labeled with chloropyrimidine (CLP)-linked substrates inside cells\textsuperscript{152} (Figure 4.3a). A CLP-conjugated analog of BI2536 (CLP-BI2536) was generated to selectively target PLK1 (Figure 4.3a-b). In vitro kinase activity measurements demonstrated that CLP-BI2536 potently inhibits PLK1 (IC50 = 49 ± 26 nM; Figure 4.3c).

To generate stable cell lines, U2OS osteosarcoma cells were infected with lentiviral constructs encoding the SNAP-PACT moieties fused to an mCherry reporter. Inducible protein expression was accomplished by a doxycycline-inducible promoter. Immunoblot detection of mCherry-SNAP-PACT persisted up to 4 hours upon removal of doxycycline. As anticipated, mCherry-SNAP-PACT associates with centrosomes during interphase and mitosis. Super-resolution structured illumination (SIM) imaging revealed
Figure 4.3. Validation of the LoKI platform. (a) Schematic of a centrosome-directed LoKI platform. SNAP-PACT fusion proteins conjugate CLP-linked PLK1 inhibitors at centrosomes. Inset depicts BI2536 in the ATP-binding pocket of PLK1. (b) Chemical structure of CLP-BI2536. (c) Dose-response curve of in vitro PLK1 inhibition with CLP-BI2536. (d) Structured illumination microscopy (SIM) of a LoKI-on U2OS cell labeled with CLP-fluorescein, α-tubulin (green), DNA (blue), mCherry-SNAP-PACT (magenta) and CLP-fluorescein (yellow) shown. Bar = 5µm. Magnification of SNAP and CLP-fluorescein co-distribution at a centrosome (inset, bar = 1µm). (e) SIM micrographs of LoKI-off (left) and LoKI-on (right) U2OS cells. SNAP expression (top, magenta), CLP-fluorescein conjugation (mid, yellow) and composite images (bottom) are depicted. Bar = 1µm. (f) Pulse-chase experiments measuring CLP-BI2536’s ability to block CLP-rhodamine conjugation to LoKI-on. In-gel rhodamine fluorescence (top), immunoblot of SNAP loading controls (mid), and fluorescence quantification of pulse-chase experiments (bottom). (g-h) Immunofluorescence of representative mitotic LoKI-off (g) and LoKI-on (h) U2OS cells treated with DMSO or 250 nM CLP-BI2536 for 4 hr. Composite images (left) show α-tubulin (green), DNA (blue), and SNAP (magenta). Immunofluorescent detection of pT210-PLK1 (mid, grey) as an index of kinase activity. Bar = 5µm. 5X magnification of centrosomal pT210-PLK1 signals and surface plots measuring integrated intensity of pT210-PLK1 signal (insets, bar = 1µm.). (i-j) Quantification of centrosomal pT210-PLK1 immunofluorescence for LoKI-expressing cells. Points represent individual cells (n). Data normalized to DMSO. Application of DMSO or CLP-BI2536 for 4 hr, (i, 100 nM, LoKI-off, n = 46, LoKI-on, n = 59, *P = 0.0059; 250 nM, LoKI-off, n = 46, LoKI-on, n = 46, ****P < 0.0001) and drug treatment followed by 1 hr washout (j, 250 nM, LoKI-off, n = 24, LoKI-on, n = 42, **P < 0.0001). Experiments were conducted at least 3 times (N=3) and P values were calculated by unpaired two-tailed Student’s t-test. Data are mean ± s.e.m. NS, not significant.
that the SNAP-PACT construct (magenta) was labeled by CLP-fluorescein (yellow) at centrosomes (Figure 4.3d-e). Counterstaining with α-tubulin (green) revealed the mitotic spindle and DAPI (blue) detected DNA (Figure 4.3d). Collectively these results demonstrate that centrosomal targeting of SNAP-PACT creates a platform for the delivery of CLP-conjugates. This new drug targeting method is herein referred to as LoKI-on (Localized Kinase Inhibition-on). In parallel a LoKI-off vector containing an inactivating mutation (C144A) in SNAP-tag was constructed. LoKI-off is unable to incorporate CLP-conjugates and serves as the control platform (Figure 4.3e).

Pulse-chase experiments were used to determine how efficiently CLP-BI2536 labeled SNAP-PACT. U2OS cells were treated with CLP-BI2536 (over a range of concentrations) to block CLP-rhodamine conjugation (Figure 4.3f). Incubation with 250 nM CLP-BI2536 for 4 hours at 37°C was defined as the optimal drug regimen (~50% labeling of SNAP-PACT; Figure 4.3f). Next, we measured the pT210-PLK1 immunofluorescence signal as an index of active kinase153 (Figure 4.3g-j). In mitotic cells expressing LoKI-off, incubation with 250 nM CLP-BI2536 reduced pT210-PLK1 immunofluorescence to 58.1% of DMSO-treated controls (Figure 4.3g, i). Strikingly, the pT210-PLK1 signal was reduced to 21.4% in cells expressing LoKI-on (Figure 4.3h, i). This trend persisted with lower CLP-BI2536 concentrations and even after a 1-hour washout of drug (Figure 4.3i-j). Further validation confirmed that the reduction of pT210-PLK1 does not result from a loss in total PLK1 protein at centrosomes. Additional controls established that inducible expression of LoKI-on was necessary to attenuate the pT210-PLK1 signal. Immunoblot analyses of nocodazole-synchronized cells collected via mitotic
shake-off further support these findings. Parallel analyses were conducted in HeLa and hTERT-immortalized RPE retinal pigment epithelial cells\textsuperscript{124}. Collectively, these findings establish LoKI as a new pharmacological tool to selectively block PLK1 activity at centrosomes.

4.7. Implementation of LoKI in live zebrafish embryos implicates PLK1 activity at centrosomes in coordinating mitoses during early development. Zebrafish provide an excellent model organism to test local drug action using the LoKI system because their transparency simplifies imaging analysis\textsuperscript{154}. Zebrafish embryos were microinjected with mCherry-LoKI-on mRNA and allowed to develop for 5 hours until they reached \~50% epiboly (Figure 4.4a-b). Detection of mCherry fluorescence confirmed expression of the local drug-targeting construct (Figure 4.4b). Higher resolution imaging of fixed embryos confirmed accumulation of LoKI-on at centrosomes during interphase and mitosis (Figure 4.4c). Co-distribution of the SNAP moiety (magenta) with CLP-647 dye (yellow) confirmed assembly of the drug-targeting platform at centrosomes (Figure 4.4c).

Microinjection of the PLK1 inhibitor adduct CLP-BI2536 (250nM) permitted local drug delivery. Live-cell imaging 5 hours post injection exposed a range of adverse mitotic phenotypes. Mitotic spindles were visualized using a microtubule binding protein, EMTB-3xGFP (Figure 4.5a-d). Multipolar spindles, monopolar spindles, spindle orientation defects, and prolonged mitoses were evident in drug-treated embryos expressing LoKI-on (Figure 4.5a-d). Fixed-cell imaging of whole embryos revealed intact microtubule organization and few mitotic cells in LoKI-off embryos treated with CLP-BI2536 (Figure
Figure 4.4. *In vivo* implementation of LoKI in zebrafish embryos.

(a) Schematic of experimental scheme. Microinjection of LoKI mRNAs into zebrafish embryos occurs at the 1-2 cell stage. Live-cell imaging is conducted at ~50% epiboly. (b) Zebrafish embryos (left, brightfield) depicting regional expression of SNAP (mid, magenta) at ~50% epiboly. Composite images (right) depict expression of SNAP only in the cells of the zebrafish embryos. Bar = 500μm. (c) In vivo validation of drug delivery. Immunofluorescent detection of microtubule marker (EMTB-3xGFP, blue), SNAP (cyan) and CLP-647 dye (magenta) in a cell embedded in a living zebrafish embryo. Bar = 10μm.
Figure 4.5. PLK1 activity at centrosomes coordinates spindle organization during early zebrafish development. (a) Time-lapse images of a cell dividing under LoKI-off control conditions. SNAP-PACT (top, magenta) and microtubule marker (EMTB-3xGFP, center, grayscale). Bar = 10 μm. (b-d) Centrosomal delivery of PLK1 inhibitors perturb cell division in zebrafish embryos. Time-lapse images of dividing cells embedded in LoKI-on zebrafish embryos 5 hr post-application of 250 nM CLP-Bi2536. Representative examples of normal bipolar spindles (a), multipolar spindles (b), monopolar spindles (c), and spindle orientation defects (d) are presented. Composite images show microtubule marker EMTB-3xGFP (white) and SNAP (magenta). Bar = 10 μm.
4.6a). In contrast, drug-treated LoKI-on embryos exhibited microtubule abnormalities and a higher incidence of mitotic cells (Figure 4.6b). Fluorescent detection of the SNAP moiety confirmed centrosomal targeting of LoKI platforms (Figure 4.5D, E). Additional analyses correlated centrosomal inhibition of PLK1 with increased mitotic indices in LoKI-on embryos (Figure 4.6c-d). Conversely, control experiments in LoKI-off embryos showed that CLP-BI2536 had minimal effect, as indicated by a significantly higher proportion of interphase cells (Figure 4.6c-d). Thus, targeted delivery of kinase inhibitor drugs to mitotic centrosomes induces a range of adverse mitotic phenotypes in developing embryos relative to global drug application.

4.8. Discussion. Cells have evolved a highly organized architecture that is segregated into functionally distinct microenvironments. However, traditional methods of drug delivery do not account for this exquisite degree of molecular organization. Conventional approaches flood cells with bioactive compounds, masking the unique contributions of individual kinases at distinct subcellular locations. Although it is well established that PLK1 coordinates various aspects of cell division, current drug-targeting strategies limit our ability to decode the spatiotemporal regulation of these events. Studying molecular scaffolds that form complexes with these key mitotic enzymes provides important mechanistic insight into how these processes are coordinated. Moreover, designing pharmacological tools that restrict the spatial and temporal action of kinase inhibitor drugs is paramount to deciphering local kinase action. In this study, we determined similarities between PLK1 localization and activity in mammalian cells and
Figure 4.6. Inhibition of PLK1 activity at centrosomes results in an increased mitotic index. (a-b) 3D-rendered images depict incidence of mitotic cells and general organization of whole LoKI-off (a) or LoKI-on (b) zebrafish embryos treated with 250 nM CLP-BI2536. Mitotic cells (cyan & top inset), SNAP (magenta & mid inset), and EMTB (white & bottom inset) are shown. Bar = 50µm. (c) Graph depicting mitotic index measurements for LoKI-expressing embryos (LoKI-off, n = 23, LoKI-on, n = 20, ****P < 0.0001). Each point represents % of cells per individual embryo (n). Experiments were conducted at least 3 times (N=3) and P values were calculated by unpaired two-tailed Student’s t-test. Data are mean ± SEM. (d) Representative 3D projections of dividing cells at prophase/prometaphase (left), metaphase/anaphase (center), and telophase/cytokinesis (right). SNAP-PACT (grayscale and cyan) and microtubule marker (EMTB-3xGFP, grayscale and magenta) shown. Bar=10µm.
zebrafish embryos. We then developed a novel chemical-biology tool, LoKI, to more precisely probe the actions of PLK1 at centrosomes. By combining biochemical approaches, quantitative imaging, and live-cell microscopy we uncover that local targeting of PLK1 kinase inhibitor drugs disrupts spindle formation and mitotic duration more profoundly than global drug distribution. Thus organellar targeting of drugs offers a new means to advance the investigation of broad-spectrum kinases at precise locations.

Previous studies suggest that PLK1 phosphorylates pericentriolar substrates that coordinate γ-tubulin accumulation at mitotic centrosomes to facilitate microtubule nucleation\textsuperscript{129,155,156}. We advance this concept and extend these findings by demonstrating that centrosomal inhibition of PLK1 prevents correct assembly of bipolar mitotic spindles in zebrafish. Thus, by using the LoKI system we are able to definitively establish that PLK1 activity at mitotic centrosomes is a driver in these processes. Furthermore, the utility of LoKI drug targeting was underscored by our \textit{in vivo} studies using zebrafish embryos. We provide evidence that embryos treated with centrosome-targeted PLK1 inhibitors have more microtubule abnormalities than those treated with a non-localized inhibitor (Figure 4.5-6). These data implicate centrosomal-localized pools of PLK1 in coordinating mitotic events such as spindle organization and mitotic progression during early zebrafish development. In a broader context we have been able to show that local targeting of PLK1 inhibitors in developing organisms offers an innovative precision technique to probe local drug action.

The versatility of this new chemical-biology platform is demonstrated in three ways. First, this approach works in a variety of cell types and microinjection of LoKI mRNA into
live zebrafish embryos permits local drug targeting in vivo (Figure 4.4-6). We foresee that LoKI platforms will be adapted to acutely probe local signaling in other genetically tractable organisms. Second, while derivatized PLK1 drugs delineate roles for the mitotic kinase, conjugation of chloropyrimidine (CLP) to other ATP analogs offers a general method to synthesize localizable inhibitors for additional members of the kinome\textsuperscript{157}. However, it is worth noting that the reduced cell permeability of certain CLP-drug conjugates, including CLP-BI2536, may necessitate their use at approximately 10-fold higher concentrations than the unmodified drugs. Additionally, derivatization of certain inhibitors may sterically hinder their access to the ATP-binding pockets of some kinases or, as is the case of the PKA antagonist H89, the lack of a functional groups prevents CLP derivatization. Third, plasma membrane and mitochondrial targeting domains from AKAP79 and dAKAP1 expand the repertoire of subcellular compartments reached by LoKI platforms. By exploiting our knowledge of how AKAPs compartmentalize signaling enzymes we have developed tools that define the local kinase terrain at the angstrom level. This will allow investigators to probe local signaling events at a level of precision that has not been possible before.

4.9. Materials and Methods: Part I

4.9.1. Zebrafish. Zebrafish embryos (provided by Dr. Jeffrey Amack’s laboratory, SUNY Upstate Medical University) were injected with 100-150pg PLK1-mCherry mRNA immediately following fertilization. Injected zebrafish were then grown at 30°C until 4.5hrs post-fertilization. For live-imaging, embryos were mounted in MatTek dishes using 2%
agar and imaged at 30°C. For fixed imaging, embryos were fixed at 4.5hpf in 4% PFA + 0.5% TritonX-100 overnight at 4°C. The following day, embryos were washed in PBS+0.5% Tween for 20 minutes, dechorionated, and placed in NucBlue Fixed Cell Stain from Ready Probes (Thermo Fisher Scientific R37606) for 30 minutes prior to imaging.

4.9.2. Cell culture. PLK1-GFP RPE cells were used in this study. All cultures were grown in 1X Dulbecco’s Modified Eagle Medium (DMEM, Gibco™) supplemented with 10% Seradigm FBS (VWR) and 1% penicillin-streptomycin (10,000U/ml) (Gibco™) and maintained at 37°C with 5% CO2.

4.9.3. Imaging. Zebrafish and tissue culture cells were imaged using a Leica SP8 scanning confocal microscope (Leica, Bannockburn, IL) or a Leica DMI8 equipped with a X-light V2 Confocal Unit spinning disk. The Leica SP8 was equipped with an HC PL APO 40×/1.10 W CORR CS2 objective equipped with Leica LAS-X software (Leica). The Leica DMI8 STP800 (Leica, Bannockburn, IL) equipped with a Lumencor SPECTRA X (Lumencor, Beaverton, Or) with a Hamamatsu ORCAflash 4.0 V2 CMOS C11440-22CU camera or 89 North – LDI laser with a Photometrics Prime-95B camera taken with a Crest Optics: X-light V2 Confocal Unit spinning disk. Optics used were either HC PL APO 63X/1.40 NA oil CS2, HC PL APO 40X/1.10 NA WCS2 CORR, a 40×1.15 N.A. Lamda S LWD, or 100×/1.4 N.A. HC PL Apo oil emersion objective. Metamorph or VisiView software was used to acquire images.
4.9.4. Image Analysis. 0.2 μm Z-steps of cell volumes are presented as maximum projections using ImageJ. Integrated intensities were measured on sum projections as described in 118. Spindle pole integrated intensities were measured from sum confocal projections. Graphs and statistical analysis (unpaired-student t-tests or ANOVA analysis as labeled) were completed using Graphpad Prism software. Error bars represent ± SEM, p < 0.05 were considered to be statistically significant.

To quantify PLK1 intensity ratios between poles in zebrafish embryos, a maximum projection was created using a z-stack that encompassed both spindle poles of a metaphase cell. A region of interest (ROI) was drawn around one pole of a single cell within an embryo using FIJI/ImageJ. The same ROI was used for all images. The mean intensity was calculated by subtracting the minimum intensity from that measured region. A ratio was calculated by dividing the value of the pole with the smaller intensity by the value of the pole with the larger intensity. This yielded a value greater or equal to 1. Graphs and statistical analysis (unpaired-student t-tests or ANOVA analysis as labeled) were completed using Graphpad Prism software.

4.10. Materials and Methods: Part II

4.10.1. Reagents:

Anti-α-Tubulin mouse mAb, clone DM1A (Sigma-Aldrich, T9026)
Anti-α-Tubulin–FITC mouse mAb, clone DM1A (Sigma Aldrich, F2168)
Anti-centromere human polyclonal antibody (Antibodies Inc,15-234-0001)
Anti-gamma Tubulin rabbit antibody (abcam, 11317)
Anti-phospho PLK1 (T210) antibody (Biolegend, 6186)
Anti-PLK1 mouse mAb 35-206 (Millipore, DR1037)
Anti-GAPDH–Peroxidase mouse mAb, clone GAPDH-71.1(Sigma Aldrich)
Anti-Gravin mouse mAb, clone JP74 (Sigma Aldrich, G3795)
Anti-phospho-Gravin (T766), rabbit antibody (Canton et al., 2012)
Anti-phospho-Hec1(S69), rabbit antibody (Deluca et al., 2018)
Anti-SNAP-tag® rabbit antibody (New England Biolabs, P9310S)
Anti-rabbit, anti-mouse, anti-goat Alexa Fluor 488, 647, 568 secondary antibodies (Invitrogen)
DyLight™ 405 AffiniPure Donkey Anti-Human IgG (H+L) antibody (Jackson Labs, 2340553)
Anti-rabbit, anti-mouse, anti-goat HRP-conjugated secondary antibodies (GE Healthcare)
DAPI Solution (Thermo Fisher 62248)
ProLong® Diamond Antifade Mountant (Life Technologies).
SNAP-Cell® 647-SiR (New England Biolabs, S9102S)
SNAP-Cell® Fluorescein (New England Biolabs, S9107S)
CLP-rhodamine (made in-house, see “synthesis of CLP-reagents”)
CLP-B12536 (made in house, see “synthesis of CLP-reagents”)
CLP-MLN8237 (made in house, see “synthesis of CLP-reagents”)
B12536 (AdooQ, A10134-50)
Dimethylsulfoxide (DMSO), Sequencing Grade (Pierce, TS-20688)
DMEM, high glucose (Life Technologies, 11965118)
DMEM/F-12, Hepes (Life Technologies, 11330057)
DMEM FluoroBrite™ (Life Technologies, A1896701)
Opti-MEM® I Reduced Serum Medium, no phenol red (Life Technologies, 11058021)
Puromycin dihydrochloride (Santa Cruz, 58-58-2)
Polybrene (Santa Cruz 134220)
Doxycycline hyclate (Sigma-Aldrich 24390-14-5)
Lipofectamine 2000 Transfection Reagent (Invitrogen, 11668027)
TransIT®-LT1 Transfection Reagent (Mirus)
Scienceware cloning discs (Sigma-Aldrich Z374431)
QuikChange II XL kit (Agilent 200522)
1.5 poly-D-lysine coated coverslips (neuVitro GG-12-1.5-pdl)
µ-Slide 4 Well Glass Bottom: # 1.5H (170 μm +/- 5 μm) D 263 M Schott glass (Ibidi)Pierce™
BCA Protein Assay Kit (Thermo Fisher 23227)
Bolt® 4-12% Bis-Tris Plus Gels (Invitrogen)
AnykD™ Criterion™ TGX™ Precast Midi Protein Gel (Biorad, 5671124)
NuPAGE™ LDS Sample Buffer 4X (Thermo Fisher, NP0008)
β-mercaptoethanol, BME (Sigma-Aldrich, M6250)
shRNA lentiviral particles (Santa Cruz Biotech)
SuperSignal™ West Dura Extended Duration Substrate (Thermo Fisher, 34075)

4.10.2. Plasmid constructs. SNAP, mCherry, eGFP, PACT, Mis12, AKAP79, and
dAKAP1 components and were individually PCR amplified with overlapping ends and/or
Gateway “att” sites and assembled using Gibson Cloning. Gateway cloning was carried out to subclone SNAP constructs into pLIX402 (a gift from David Root; Addgene plasmid #41394) for PACT and Mis12 studies or pcDNA3.1+ (Life Technologies) for AKAP79 and dAKAP1. To generate mutant SNAP, site-directed mutagenesis was performed with a QuikChange II XL kit (Agilent). GFP-H2B and EMTB-3xGFP constructs were used for live-cell imaging studies. Gravin-SNAP was cloned into pEYFP-N1 (Clontech) and used for Gravin experiments. Constructs were verified by Sanger sequencing.

4.10.3. Cell culture and virus generation. U2OS, HeLa, HEK293, and immortalized MEF, generated as described in 84, cells were maintained in DMEM, high glucose and hTERT-RPE cells were maintained in DMEM/F-12, Hepes (Life Technologies) at 37°C and 5% CO2. All media was supplemented with 10% FBS. Infections for generation of stable SNAP cells were performed using lentiviral particles created in-house. In brief, SNAP pLIX402 vectors were transfected alongside pMD2.G and psPAX2 plasmids (gifts from Didier Trono; Addgene plasmid #12259 [RRID:Addgene_12259] and plasmid #12260 [RRID:Addgene_12260]) into HEK293 cells using Lipofectamine 2000 reagent (Invitrogen) in Opti-MEM® (Life Technologies) media. Virus-containing supernatant was collected, passed through a .45 μm filter, and transduced into cells in the presence of 1μg/ul Polybrene (Santa Cruz). Cells were selected and maintained in supplemented media with 4 μg/mL Puromycin dihydrochloride (Santa Cruz). Single clones were isolated using Scienceware cloning discs (Sigma-Aldrich). Infections for generation of stable knockdown in HEK293 cells were performed with shRNA lentiviral particles (Santa Cruz...
Biotech). For expression of AKAP79, dAKAP1, and Gravin constructs in U2OS cells, transient transfections were performed using TransIT-LT1 reagent (Mirus) in Opti-MEM® (Life Technologies) media according to manufacturer’s instructions.

4.10.4. CLP-linker synthesis:

1a CLP-Amine, created as previously described (Hill et al. 2012), 1 Eq of 1a (0.2 M) and 1.1 Eq of 1b (Abachemscene) were dissolved in DMF at RT. The reaction was placed on ice. While stirring, 1.3 Eq HOAt (1-Hydroxy-7-azabenzotriazole) and 3 Eq DIEA (N,N-Diisopropylethylamine) were added. After 5 minutes on ice, 1.3 Eq of EDCI (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide) was added. The reaction was allowed to stir for 24 hours (letting the ice melt and the reaction slowly come to RT). Reaction was dissolved in ethyl acetate, washed with NaHCO3 and brine, and dried with Na2SO4. Remaining solvent and DMF were removed via rotovaping and lyophilization. 1c was deprotected with 30% TFA in DCM (0.2 M 1c final). Solid 1c was dissolved in CH2Cl2 and cooled on ice. TFA was added dropwise until it reached 30% v/v. Reaction was stirred for 1 hour at RT. Toluene was added (to help remove TFA) and the reaction was rotovapped to near dryness. Reaction was dissolved in ethyl acetate, washed with K2CO3, dried Na2SO4 and dried via rotovapping and lyophilization. Identity at each step was verified with MS.
### 4.10.5. BI2536 functionalization (+CLP-linker):

![Chemical Structure](image)

1 Eq 1d (0.2 M) and 1.1 Eq of 2a (Chem Scene) were dissolved in DMF at RT. The reaction was placed on ice. While stirring, 1.3 Eq HOAt and 3 Eq DIEA were added. After 5 minutes on ice, 1.3 Eq of EDCI was added. The reaction was allowed to stir for 24 hours (letting the ice melt and the reaction slowly come to RT). DMF was removed and 2b (BI2536-CLP) was purified with HPLC. Identity was verified with MS. [M+H]+ = 817.7 m/z.

### 4.10.6. CLP-rhodamine Preparation.

1 Eq 1a (0.2 M) and 1 Eq 5(6)-carboxytetramethylrhodamine N-succinimidyl ester (Thermo Fisher) were dissolved in DMF at RT. While stirring, 3 Eq DIEA were added. The reaction was allowed to stir for 24 hours. DMF was removed and product was purified with HPLC. Identity was verified with MS. [M+H]+ = 676.2 m/z.

### 4.10.7. Protein expression and purification.

His6-SNAP-tag in pMCSG7 (Addgene) was expressed in Escherichia coli BL21(DE3) cells in 250 mL LB Miller broth. The evening prior to expression, 5 mL LB Miller broth, containing 50 μg/mL Ampicillin, was inoculated with transformed cells, and they were grown at 37 °C overnight. The following day, the starter culture was used to seed 250 mL LB Miller broth in a 500 mL baffled flask. Cells were grown to OD600 ~0.3 and the temperature was then reduced to 20°C. Cells were
allowed to grow to OD600 ~0.8, and then induced with 500 μM isopropyl β-D-thiogalactopyranoside. Induced cells were grown at 20 °C overnight. Subsequent purification steps were carried out at 4 °C. Cells were spun down at 6500 g, suspended in 10 mL of wash/lysis buffer [50 mM HEPES (pH 7.5), 300 mM NaCl, 20 mM imidazole, and 1 mM phenylmethanesulfonyl fluoride], and lysed via sonication. The lysate was centrifuged at 10000 g for 20 minutes, and the supernatant was allowed to batch bind with 0.7 mL of Ni-NTA (Ni2+-nitrilotriacetate) beads for 60 minutes. The resin was collected by centrifugation at 500 g for 5 minutes and washed with 10 mL of wash/lysis buffer. The wash step was repeated three times. The Ni-NTA/His6-SNAP-tag was added to a BioRad purification column, and washing was continued until the wash showed no remaining protein by Bradford. The protein was eluted using ~ 5 mL of elution buffer [50 mM HEPES (pH 7.5), 300 mM NaCl, 200 mM imidazole]. The eluate was dialyzed against 50 mM HEPES (pH 7.5), 200 mM NaCl, 5% glycerol, and 1 mM fresh dithiothreitol (DTT). Protein was aliquoted, flash-frozen in liquid N2, and stored at – 80 °C.

4.10.8. SNAP Labeling experiments and pulse-chase labeling assays:

In vitro labeling: 50 μM SNAP-tag was incubated with 75 μM CLP-linker-inhibitors (or DMSO alone for control reactions) [2.5% (v/v) final DMSO concentration] in buffer [20 mM Tris-Cl (pH 8), 200 mM NaCl, 1 mM DTT (added fresh)] at 26 °C for 1.5 hours. The reactions were purified using Zeba columns (Thermo Fisher) and exchanged into a MS compatible buffer (50 mM NH4HCO3, 0.2% HCO2H). Ratios of unlabeled to labeled protein were determined using Native MS (Thermo Scientific LTQ Orbitrap XL/Bruker...
Esquire LC-Ion Trap). **Cellular labeling:** SNAP expressing (dox-induced) cells were treated with SNAP-Cell® Fluorescein or SNAP-Cell® 647-SiR (NEB) for 30 minutes in serum free DMEM at 37°C and 5% CO2. Cells were washed one time and incubated in fresh serum free DMEM for 30 minutes. Cells were fixed and stained as described under “immunofluorescence”. For pulse-chase labeling experiments, SNAP expressing (dox-induced) cells were treated with DMSO or increasing doses of CLP-BI2536 or CLP-MLN8237 for 1, 2, or 4 hours in serum free DMEM at 37°C and 5% CO2. Cells were washed one time and incubated in fresh serum free DMEM for 30 minutes. Cells were treated with 3 μM CLP-rhodamine (made in-house) in serum free DMEM for 30 minutes. Cells were washed one time and incubated in fresh serum free DMEM for 30 minutes. One wash with PBS was carried out and cells were lysed using immunoblotting protocol. Samples were resolved on an AnykD™ Criterion™ TGX™ Precast Midi Protein Gel (Biorad). Gels were scanned and fluorescence was measured with a GE Typhoon FLA 9000 scanner. Fluorescence measurements and densitometry was performed using NIH ImageJ (Fiji) software.

Total SNAP labeling was determined by normalizing fluorescence signal of rhodamine bands to total SNAP protein expression as determined by densitometry.

\[
\% \text{ SNAPs Labeled by CLP-rhodamine} = \frac{\text{CLP-rhodamine signal}}{\text{SNAP western blot signal}}
\]

\[
\% \text{ SNAPs Labeled by CLP-inhibitor} = 100 - \% \text{ SNAPs Labeled by CLP-rhodamine}
\]

**4.10.9. Drug treatments.** For induction of SNAP expression cells were treated for 48-72 hours in FBS-supplemented DMEM with 1 μg/mL (for SNAP-PACT) or 4 μg/mL (for
SNAP-Mis12) doxycycline hyclate (Sigma-Aldrich) prior to inhibitor treatments. For degradation assays, cells were dox-induced for 72 hours after which doxycycline was washed out (cells were incubated in normal media). At selected time point plates were collected, cells were washed once with PBS, plates were dried quickly, and frozen at -80°C until lysis. For nocodozole synchronization experiments, dox-induced cells were treated for 16 hr with nocodozole and 4 hr with nocodozole plus DMSO, 250 nM CLP-BI2536, or 100 nM CLP-MLN8237. Cells were washed once with PBS, collected via mitotic shake-off, and spun at 2000 rpm for 5 minutes at 4°C. Supernatants were discarded and pellets were kept for lysis. All lysates were prepared as described under “immunoblotting”. For fixed cell experiments, both dox-induced and non-induced cells were grown on 1.5 poly-D-lysine coated coverslips (neuVitro) for at least 16 hours in complete DMEM and then treated with DMSO or CLP-compounds in serum-free DMEM for 1-4 hours. For washout experiments (pT210-PLK1 1 hour washout and γ-tubulin data), cells were incubated in serum-free DMEM without inhibitors for an additional 1 hour. Cells were washed once with PBS prior to fixation. For live-imaging experiments, cells were treated with CLP-compounds for 18 hours (see “microscopy” for more details).

4.10.10. Zebrafish Studies. Zebrafish were bred and embryos were collected. Embryos were injected with EMTB-3xGFP (100pg mRNA or 20pg pCS2 plasmid construct), SNAP-Cell® Fluorescein (300 μM final embryo concentration), and/or CLP-BI2536 (250 nM final embryo concentration), and/or SNAP-PACT active or dead (200pg mRNA) at the 2-cell stage using a microinjector (Warner Instruments PLI-100A) with a Kanatec magnetic base
(MB-B), and a micromanipulator (Marzhauser Wetzlar MM33). Embryos were raised at 30℃ until ~50% epiboly, at which point they were imaged on stereoscope, mounted in 2% agarose for live confocal imaging, or fixed using 4% paraformaldehyde + 0.5% Triton-X overnight at 4℃. Embryos were dechorionated in PBST (phosphate buffered saline + 0.1% Tween-20) and incubated in DAPI solution (1 μg/mL in PBS) for 2 hours at room temperature. Fixed embryos were then mounted in 2% agarose and imaged on confocal microscope.

4.10.11. Immunoblotting. Cells were lysed in RIPA lysis buffer (50 mM Tris HCl pH 7.4, 1% Triton X-100, 0.5% Sodium Deoxycholate, 0.1% SDS, 50 mM NaF, 120 mM NaCl, 5 mM β-glycerophosphate) supplemented with protease and phosphatase inhibitors (1 mM benzamidine, 1 mM AEBSF, 2 μg/mL leupeptin, 100 nM microcystin-LR). Lysed samples were boiled for 5 minutes at 95℃ in NuPAGE™ LDS Sample Buffer 4X (Thermo Fisher) + 5% BME (Sigma-Aldrich). Protein concentration was determined using a Pierce™ BCA Protein Assay Kit (Thermo Fisher). Samples were resolved on Bolt® 4-12% Bis-Tris Plus Gels (Invitrogen) or AnykD™ Criterion™ TGX™ Pre cast Midi Protein Gel (Biorad). Proteins were transferred to nitrocellulose for immunoblotting and probed with Anti-SNAP-tag® rabbit antibody (NEB) and Anti-GAPDH–Peroxidase mouse mAb, clone GAPDH-71.1(Sigma Aldrich). Detection was achieved with a HRP-conjugated rabbit secondary antibody (GE Healthcare) followed by enhanced chemiluminescence with SuperSignal™ West Dura Extended Duration Substrate (Thermo Fisher). Densitometry was performed using NIH ImageJ (Fiji) software.
4.10.12. **Immunofluorescence.** Cells grown on 1.5 poly-D-lysine coated coverslips (neuVitro) for at least 16 hours were fixed for 10 minutes in 4% paraformaldehyde in PBS or in ice-cold methanol. Cells were permeabilized and blocked in PBS with 0.5% Triton X-100 and 1% BSA (PBSAT) for 30 minutes. Primary antibodies were diluted in PBSAT and cells were stained for 1 hour. Secondary antibodies conjugated to Alexa Fluor dyes (Invitrogen) were diluted in PBSAT and applied for 1 hour. Staining with FITC-tubulin antibodies and/or DAPI staining always followed secondary incubation step and was carried out for 10-45 minutes in PBSAT. Washes (quick on and off) with PBSAT were carried out 10X between antibody and/or dye incubation steps and prior to mounting. Coverslips were mounted on slides using ProLong® Diamond Antifade Mountant (Life Technologies).

4.10.13. **Spindle Classification Measurements.** To de-identify cell type and dox-treatment conditions and allow for blinded analysis of mitotic spindle differences, all identifying information on microscope slides was masked by a third-party individual. All mitotic cells within a coverslip were classified as either having normal bipolar, abnormal bipolar, or monopolar spindles based on morphology of the DNA and microtubules.

4.10.14. **Microscopy:**

**Fixed cell:** Super-resolution 3D-SIM images were acquired on a Deltavision OMX V4 (GE Healthcare) system equipped with a 60x/1.42 NA PlanApo oil immersion lens (Olympus), 405-, 488-, 568-, and 642-nm solid-state lasers and sCMOS cameras
(pcolal). For SIM, 15 images per optical slice (3 angles and 5 phases) were acquired. For both SIM and widefield (conventional) acquisitions, image stacks of 2.5-7 um with 0.125-um optical thick z-sections were acquired using immersion oil with a refractive index 1.516 or 1.518. Z-stacks were generated using the SNAP-PACT or SNAP-Mis12 channel to define the upper and lower regions of the plane with a 0.5 μM step size. SIM images were reconstructed using Wiener filter settings of 0.003 and optical transfer functions measured specifically for each channel with SoftWoRx software (GE Healthcare) to obtain super-resolution images with a twofold increase in resolution both axially and laterally. Images from different color channels were registered using parameters generated from a gold grid registration slide (GE Healthcare) and SoftWoRx. Widefield images were deconvolved using SoftWoRx. Live-cell: For LoKI experiments cells were first induced with doxycycline for 48-72 hours prior to transfection. For all time-lapse experiments cells were reverse transfected with GFP-H2B plasmid and plated onto μ-Slide 4 Well Glass Bottom: # 1.5H (170 μm +/- 5 μm) D 263 M Schott glass (Ibidi) in complete DMEM. Transient transfections were performed using TransIT-LT1 reagent (Mirus) with Opti-MEM® (Life Technologies) media. The next day all cells were treated with 2 mM thymidine for 24 hours. The following day thymidine was washed out and after 4 hours cells were incubated with DMSO or CLP-inhibitors (LoKI experiments) or with no reagents (WT/KO Gravin experiments) in serum-free FluoroBrite™ DMEM. Time-lapse images were acquired on a Keyence BZ-X710 microscope using a 10X objective with 25% transmitted light and 100% aperture stop, with 1/60s exposure for 488 channel. Images were captured every 5 minutes for 18 hours. Zebrafish imaging: Images were
acquired on a Leica DMi8 (Leica, Bannockburn, IL) equipped with a Crest Optics X-light v2 Confocal Unit spinning disk, an 89 North – LDI laser with a Photometrics Prime-95B camera using a Nikon 40×1.15 N.A. Lamda S LWD objective. Stereoscope images were acquired on a Leica M165 FC stereoscope with a DFC9000 GT camera and a PLANARPO 10X objective.

4.10.15. Image Analysis. Maximum intensity projections from z-stack images were generated using SoftWoRx (GE Healthcare) or NIH ImageJ (Fiji) software. All immunofluorescence signal measures were carried out using Fiji software. Sum slice 32-bit Tiff projections were generated from z-stack images for analysis of immunofluorescence at centrosomes. For kinetochore measurements the ImageJ "Subtract Measured Background" macro was first applied and sum slice 32-bit Tiff projections were generated. For centrosomes measurements, the oval selection tool in Fiji was used to draw a circle (ROI) around the centrosome in the 568 (SNAP-PACT) channel. The area of the circle remained consistent for all measurements and all replicates of an experiment. Measurements were taken in the 647 channel (which contained pT210-PLK1, Total PLK1, or γ-tubulin) using the predefined centrosome ROI. Using the measure function in Fiji, with “Area” and “Raw Integrated Density” predefined as measurements, values were determined for each centrosome and for an arbitrarily selected background region. The raw integrated density was recorded for each centrosome and the background. The average raw integrated density for the centrosomes was determined by adding together the raw integrated densities for each centrosome in
a cell and dividing that value by 2. The integrated density for the background was subtracted from the average centrosome integrated density to yield a background-subtracted average integrated density signal for a centrosome. For kinetochore measurements, the selection tool in Fiji was used to draw an arbitrary region (ROI) around the kinetochore in the 405 (ACA, centromeric DNA) channel or in the 568 (SNAP-Mis12) channel. Measurements were taken in the 647 channel (which contained pS69-Hec1) using the predefined kinetochore ROI. Using the measure function in Fiji, with “Area” and “Raw Integrated Density” predefined as measurements, values were determined for each kinetochore. The raw integrated density was recorded for each kinetochore. For both centrosome and kinetochore experiments and average was calculated for each control and experimental condition. To do this the normalized average integrated densities were added together and divided by the total number of cells for that condition. This yielded a value that represents the background-normalized average integrated density at a centrosome or at the kinetochore for a particular condition. Values for drug-treated cells were then normalized to their respective DMSO-treated control. Integrated intensity surface plots were generated from sum-slice 32-bit Tiff projections of representative images using the 3D Surface Plot function in Fiji software. Maximum intensity heat maps were generated from maximum projection representative images using the 3D Surface Plot function with Fire LUT in Fiji software. Zebrafish three-dimensional renderings: Three-dimensional renderings were created using Imaris software (Bitplane). Individual mitotic cells were isolated and assigned a new color channel using the “Surfaces” function to create a surface rendering. Surface renderings were created through the use of the
Isoline function, where regions of individual mitotic cells were isolated based on intensity. Completed surface renderings were then merged and masked to create a channel that encompassed the mitotic cells of each embryo.

4.10.16. **Statistical analysis.** Statistics were performed using an unpaired two-tailed Student's t-test in GraphPad Prism software. All values are reported as mean ± standard error of the mean (s.e.m) with p-values less than 0.05 considered statistically significant. Number of independent experiments (N) and number of individual points over several experiments (n) are presented. For γ-tubulin experiments a ROUT (Q=1%) outlier test was performed and two values were removed prior to performing an unpaired Student’s t-test.

4.10.17. **Sample size and replicates.** The sample size was not statistically determined. Where applicable, n > 15 independent measurements were conducted across N ≥ 3 independent experiments. For doxycycline removal experiments, at least 2-3 independent experiments were conducted per time point.
Chapter Five:

Discussion and Future Directions:

Examining the role of subcellular PLK1 populations during Kupffer’s vesicle lumenogenesis and early zebrafish monolayer establishment
5.1. Discussion: Overview of cell division regulation during tissue development

5.1.1. Summary of Key Findings. The overall goal of my thesis research was to understand how cell division orchestrates tissue morphogenesis. I defined the mechanism by which abscission (Chapter 2) and metaphase spindle positioning (Chapter 3) lead to the assembly of complex structures in an in vivo vertebrate setting. Additionally, my research established a protocol to utilize a system for localized kinase inhibition in a vertebrate setting to elucidate the role of an essential mitotic kinase at a specific subcellular locale (Chapter 4). With this chapter I will discuss the most novel points of these studies and consider possible future directions for each project.

5.1.2. Chapter 2 Summary. In Chapter 2, I presented a testable model in which cytokinetic bridges position at the site of future KV lumen formation where apical polarity proteins are transported in a Rab11-dependent manner. Here, I provided evidence that cell division was not only occurring during KV formation, but also that it was essential for lumen establishment. Additionally, I determined that while spindle orientation was not a driving factor in lumen growth, the later mitotic process of abscission was essential. This study was the first to visualize the cleavage of a cytokinetic bridge in an in vivo vertebrate model, as well as utilize an optogenetic approach to disrupt abscission in live animals. From a broader perspective, this work represents an important transition to studying lumen formation in an in vivo context to investigate tissue-specific mechanisms.
5.1.3. Chapter 3 Summary. The research featured in Chapter 3 utilized the early zebrafish embryo to determine how mitotic spindles are able to position themselves within the confines of disproportionately large cells. As cell size rapidly decreases during consecutive cell divisions, I determined that the mitotic spindle poles scale with the changing cell size rather than the spindle as previously suggested (Chapter 3). Mitotic spindle poles in the early zebrafish embryos are uniquely-ordered structures that contain a large, novel pericentriolar matrix to facilitate cell division in large cells. Furthermore, spindle poles are asymmetric across a mitotic spindle, with a two-fold difference in size between the large pole pointed towards the center of the embryo and the smaller pole facing the exterior. This vectoral directionality was also found to be dependent on PLK1 and PLK4 activity. This research introduces a novel centrosome structure present in embryonic zebrafish cells that may explain a mechanism for cell division in extremely large cells.

5.1.4. Chapter 4 Summary. Lastly, the research highlighted in Chapter 4 illustrates the use of an innovative drug delivery system employed in an in vivo vertebrate context. PLK1 kinase inhibition was targeted to the centrosomal population of PLK1 through the use of a centrosome-localized SNAP moiety and an engineered BI2536, a PLK1 inhibitor, conjugated to a CLP-tag (LoKI system). This resulted in an efficient manner of directing kinase inhibition to a specific subcellular locales, as verified by extensive in vitro validation assays. Advantages of this system include the ability to use a lower
concentration of PLK1 inhibitor due to its specificity to the centrosomal PLK1 population, resulting in fewer off target effects on a cellular scale. Through this system, I was able to identify that the centrosomal PLK1 population is responsible for mitotic spindle organization and mitotic progression. It has been previously unknown whether PLK1 populations at distinct subcellular locales serve unique purposes in vitro, and this research was the first to inhibit the population of endogenous PLK1 at the centrosome to determine its function. Beyond this, the function of PLK1 in a vertebrate system has been largely undocumented prior to these publications. This research is therefore novel in that we first characterized the localization of PLK1 during cell division in a developing embryo, then expanded upon this and determined the distinct role of a population of PLK1 at the centrosome locale in a zebrafish embryo. This study represents a significant advance in the use of chemical-genetic tools to investigate molecular mechanisms during vertebrate development for the first time.

5.2. Future Directions: Determining the function of subcellular PLK1 populations during tissue morphogenesis

5.2.1. Chapter 2- Expanding upon the role of PLK1 during lumenogenesis. In order to expand upon these studies, I propose to utilize the kinase inhibition system detailed in Chapter 4 to determine the molecular mechanism by which PLK1 regulates cell division during KV lumenogenesis. These studies would accomplish two important aims. First, they would provide an understanding of the role of PLK1 during vertebrate tissue
morphogenesis. KV development encompasses multiple steps such as polarity establishment, rosette formation, and lumenogenesis, and the role of PLK1 at each of these steps is not currently understood. Secondly, the discrete roles of PLK1 at various subcellular locales are not currently understood. Bucko and colleagues were the first to create the LoKI system to determine the function discrete pools of PLK1, and only the centrosomal population of PLK1 has been targeted \textit{in vivo} so far. Additional populations such as the midbody and kinetochore pools could therefore by targeted during KV development in zebrafish to gain a more complete understanding of PLK1 function during tissue development.

In Chapter 2, I determined that disrupting mitosis through PLK1 inhibition interfered with lumenogenesis in Kupffer’s vesicle. However, these studies were conducted using a greater concentration if BI2536 that had been previously published. While this was necessary to ensure a global inhibition of PLK1 in the zebrafish embryo, a caveat to this approach is that off-target effects are possible. By employing the LoKI system to target BI2536 directly to the centrosomal population of PLK1, I would use a lower concentration of PLK1 inhibitor to minimize off-target effects, as well as elucidate the role of centrosomal PLK1 during KV lumenogenesis. To accomplish this, I would inject early zebrafish embryos expressing the transgenic Sox17:GFP-CAAX KV marker with mRNA coding for SNAP-PACT, similar to studies performed in Chapter 4. Instead of injecting the CLP-BI2536 into the embryos, I would instead dechorionated and soak the embryos in drug during the early stages of KV development (8 hours post-fertilization). Because the drug covalently binds to the centrosome via the SNAP-CLP
bond, I would then wash the extra unbound drug out of the embryos to increase drug specificity to the centrosomal PLK1 population. I would then fix embryos at the 6-somite stage (approximately 12 hours post-fertilization), at which point control embryos would be expected to have a large KV lumen developed. Lumen area would be quantified to determine if inhibition of centrosomal PLK1 results in decreased lumen area.

Additionally, I would image live embryos under this condition to determine whether there is a significant change in lumen growth rate over time with localized PLK1 inhibition.

Since studies in Chapter 4 determined that centrosomal PLK1 is responsible for mitotic progression, I predict that lumen area will be decreased in CLP-BI2536 embryos due to the delay in mitosis. Alternatively, it is possible that I do not see a disruption in lumen area under conditions of localized PLK1 inhibition. Since inhibition of centrosomal PLK1 resulted in misoriented spindles, it is possible that the centrosomal pool of PLK1 does not play a crucial role during KV morphogenesis since spindle orientation is not a driving factor in KV lumen formation. If this is the case, I would hypothesize that centrosomal PLK1 is not as important for mitotic progression, and that pools of PLK1 at kinetochores or midbody could instead be more crucial for KV development. A separate population of PLK1 plays a more essential role in lumenogenesis in this particular tissue.

In order to investigate the role of PLK1 at additional subcellular locales such as the kinetochores and midbody, I would target the LoKI system to these locations. In Bucko et al 2020, a kinetochore-localized LoKI system was developed through the localization of the SNAP-tag to Hec1, a kinetochore protein. The zebrafish protein ndc80 is a homolog to Hec1 and is present during embryogenesis, so I would target this
protein to localize the LokI system to kinetochores\textsuperscript{158}. Additionally, a midbody-localized LoKI system could be developed through the localization of the SNAP-tag to a canonical midbody protein such as MKLP1. I propose to utilize these two systems in the experimental setups mentioned above to determine whether PLK1 populations at these additional subcellular locales play a role in KV lumenogenesis. During experiments that were featured in Chapter 2, I expressed fluorescently-tagged PLK1 in the Sox17:GFP-CAAX transgenic line to follow PLK1 in a developing KV. Strikingly, I identified a population of PLK1 that localized to the apical membrane of the developing lumen (Figure 5.1). An additional future direction would be to specifically inhibit this novel population of PLK1 at the apical membrane to determine if it is essential for lumenogenesis. This could be accomplished by adapting the LoKI system to inhibit PLK1 at the apical membrane by targeting the conjugated BI2536 to the membrane-localized motif CAAX. While CAAX would localize the CLP-BI2536 to the whole cell membrane, it would overlap with the PLK1 population present at the apical membrane and deliver targeted PLK1 inhibition to this site. These experiments would provide insight as to the specific roles of PLK1 at various subcellular locales during \textit{in vivo} vertebrate lumenogenesis.

5.2.2. Chapter 3- Determining the mechanism of PLK1-dependent pole asymmetry during monolayer construction. To continue with the project for Chapter 3, I again propose to utilize the LoKI system for targeting PLK1 inhibition in order to gain a more detailed understanding of how PLK1 regulates monolayer formation in early embryos.
Figure 5.1. PLK1 population noted around apical membrane of nascent lumen in zebrafish KV. Representative three-dimensional renderings of embryo expressing KV marker (Sox17:GFP-CAAX, blue/inverted grayscale) and PLK1-mCherry (white/inverted grayscale) during KV development. PLK1 populations localized to centrosomes (magenta), kinetochores (orange), and lumen apical membrane (cyan) denoted by arrowheads. Forming lumen denoted by purple asterisk. Bar, 10μm.
With these studies, I determined that the vectoral directionality created by spindle pole asymmetry pointed at the embryo center was dependent on active PLK1. When embryos were exposed to global BI2536 conditions, spindle pole positioning was random with respect to the embryo center. Again, a caveat to this study was that a targeted system of PLK1 inhibition had not been developed, leaving the possibility for off-target effects due to the higher concentration of inhibitor needed. Employing the LoKI system here would allow for a better understanding of PLK1 regulation from distinct subcellular locales during monolayer formation.

While mRNA injections can be used to introduce protein chimeras to embryos during KV developmental stages, this is not a feasible method for use in the early embryo. Therefore, a transgenic line would need to be created that expresses SNAP-PACT for drug targeting to the centrosome. The line would need to maternally express the construct so that it would be present early enough to accomplish experiments at the 16-cell stage of development. I would cross this line with the centrin-GFP transgenic zebrafish line so that once embryos are fixed at the 16-cell stage, I could immunostain for gamma-tubulin to evaluate spindle pole size with both markers as well as quantify the positioning of the larger spindle pole with respect to the center of the embryo. Since we measured a randomization of spindle pole positioning with global PLK1 inhibition in Chapter 3, I predict that we would see a similar or more severe randomization of pole positioning with the targeted LoKI system. This would be demonstrated by a randomization of spindle pole positioning with respect to the center of the embryo with a lower dose of CLP-BI2536 under the LoKI system. This result would suggest that the
centrosomal population of PLK1 regulates positioning of the asymmetric spindle poles in the early zebrafish embryo, which would explain why a small concentration of CLP-BI2536 targeted to the centrosome would have the same effect as a large concentration of global BI2536. If this is not the case, I would investigate whether a kinetochore or midbody population of PLK1 was present at this early zebrafish developmental stage, and whether this population was driving spindle pole directionality instead. Through this proposed use of the LoKI system during monolayer formation in zebrafish, the function of PLK1 during cell division in extremely large cells can be unraveled.

5.3. Broader implications in the field of tissue morphogenesis. Overall, the research presented in this dissertation begins to piece together the mechanism by which cell division contributes to tissue morphogenesis in a vertebrate system. Previous studies have focused on determining molecular mechanisms that drive formation of complex three-dimensional structures in *in vitro* contexts. While many genetic and pharmacological tools exist to elucidate molecular mechanisms in *in vitro* settings, it is becoming clear that many of these mechanisms may differ *in vivo* or are tissue- or organ-specific. Therefore, studies such as those described here are necessary to gain a full understanding of how cell division can be harnessed to build tissues, organs, and organisms. Continued work to develop genetic and pharmacological toolsets for vertebrate research will encourage future studies that will have translational benefits in both basic science and medical research.
Appendix: Rathbun et al 2020 Nature Communications Response to Reviewers

For reference, responses from the Nature Communications review of Rathbun et al 2020 is included here. Responses and revisions from the most recent reviewer comments are shown in orange (resubmitted 12/16/2019), those from the previous rounds of reviewer comments are shown in blue (resubmitted 11/19/2019) and green (resubmitted 07/22/2019). This manuscript was initially sent to reviewers by Nature Communications on 06/05/2018.

A.1. The following Response to Reviewers is from the 12/16/2019 resubmission:

Reviewer #2 (Remarks to the Author):

“In this revised version the authors have carried out additional experiments and expanded and clarified the text to address the concerns of the Reviewers.

I am personally still convinced that the only significant advance of this study is the evidence that the midbody is required for lumen formation in vivo. This is why, in my opinion, the paper would be more suitable for a specialised developmental/cell biology journal rather than Nature Communications. However, this is ultimately an editorial decision and therefore I will only address here the authors' replies to my specific comments.

I am satisfied by the authors' replies to my comments 1, 2, 3, 7 and 8.”

Thank you.
“As for the others:

Point 4: The authors are wrong in stating that the severing of the intercellular bridge is “often termed abscission”. Abscission unequivocally indicates the final cut between the two daughter cells, which often coincides with the severing of the microtubules in the intercellular bridge. The authors incorrectly use the term abscission to indicate the progressive thinning of the microtubule bundles at the intercellular bridge. This is extremely confusing for both specialist and non-specialist readers and therefore I must insist that the authors use a different term, such as ‘pre-abscission’ for example, to indicate this stage.”

We have edited the text accordingly to achieve the terminology you request. Please see highlighted regions of the text and Figure 3b, and Supplementary Figure 2e, 2i, Supplementary Figure 3b, and Supplementary Figure 5f.

“Point 5: Although I appreciate the efforts to show better images of RacGAP1 immunostainings, there are still way too many dots, some even outside the sample. I think that this antibody is not very specific and am not sure if it is actually worth including these images.”

Please see updated Figure 2d, where we have added a nuclei marker to denote that there are also cells that surround KV along with KV cells. In these images, the midbody marker RacGAP is seen outside KV because there are cells undergoing division in the surrounding tissue as well, highlighted by the presence of nuclei (see Fig. 1c for mitotic indices). These images are also maximum projections of an approximately 100 m z-
stack, thus capturing approximately 7-10 cell layers and subsequently many cytokinetic bridges. In regions without a KV or nuclear marker, there are no cells present and therefore no midbodies are seen. Additionally, please refer to Supplementary Figure 2e and h-i, where images are included of RacGAP localizing to microtubule-rich cytokinetic bridges to highlight antibody specificity.

“Point 6: I know from direct experience that overexpression of MKLP1 does affect cytokinesis. Therefore I am afraid that I cannot simply trust the “confidence” of the authors on this point and some experimental evidence in support of the authors’ “confidence” should be presented.”

To address Reviewer 2’s concern, we have provided a comprehensive overview of the mitotic duration of zebrafish cells when expressing a variety of fluorescently tagged markers (Rebuttal Figure 1) compared to transgenic lines (βactin::EMTB-3xGFP). We have done these studies in both KV cells (Rebuttal Figure 1a, d, e) and cells outside of KV (Rebuttal Figure 1b, c, f-h). The location of these panels in the manuscript are outlined in the figure legend for Rebuttal Figure 1. Based on the similarity of mitotic timing between these various markers including MKLP1 overexpression (~8-12 minutes from metaphase to midbody visualization), we argue that MKLP1 is not affecting mitotic progression and in zebrafish dividing cells can be used to monitor cytokinetic bridge placement and abscission. We also find no developmental abnormalities nor embryonic lethality when overexpressing MKLP1, arguing that ectopic expression of MKLP1 can be used to follow cytokinesis and abscission in a developing embryo.
Rebuttal Figure 1. Time lapse images of live zebrafish cell divisions as seen by fluorescent markers. GFP-MKLP1 (a, see Fig. 3, Movie 5), PLK1-mCherry + GFP-MKLP1 (b, see Supplementary Fig. 2d, Movie 4), H2B-Dendra (c, see Supplementary Fig. 2a), PLK1-mCherry (d-f, see Supplementary Fig. 2b-c, 3a, Movie 3), and EMTB-3xGFP (g-h).
“Point 9: Just a clarification here. My comment was intended to simply point out that multinucleation is a general readout for cytokinesis failure and not only for abscission failure as stated in the manuscript. I did not mean to imply that Rab11 knockout would not cause multinucleation. I was basically asking to simply change the wording, but I do appreciate the authors’ extra effort.”

We have altered the text for this section to reflect that multinucleation can also be a readout for abscission failure. Please refer to page 13, line 270-277.

Reviewer #3 (Remarks to the Author):

“The authors have addressed most of the concerns I had during my previous read. I have only two comments left.

1-Supplementary Table 5 is extremely useful to follow the statistical tests performed for most experiments. Some experiments seem to be missing from this list (for example Fig S5e). Also, I would suggest the authors to perform a non-parametric test instead of a Student t test when comparing percentages, such as for Fig S5e for example for which the sample size is small (n = 3) and it is therefore difficult to assume a normally distributed population.”

We have reanalyzed the data from Supplementary Fig. 5e and Fig. 5c with a non-parametric Mann-Whitney test and updated Supplementary Table 5 to include all statistical analyses.

“2-While I agree that the study supports the conclusions stated in the last paragraph of
the study:

“In conclusion, these studies have highlighted the importance of cell division during the development of KV and the de novo formation of its lumen. We provide evidence that cell division is upregulated in cells destined for KV, and these cells retain their cytokinetic bridges post-division. The cytokinetic bridges are then projected to the site of future lumen formation during rosette formation/apical clustering, where Rab11-associated vesicles can traffic important apical polarity components to the bridge during epithelialization to allow for lumen formation (Fig. 7).”

I think the statement about rosette packaging which the authors use to motivate the study in the introduction is no longer appropriate or relevant:

“One identified molecular mechanism for rosette formation is actin-myosin dependent apical constriction to form a rosette structure. Herein, our studies examining the formation of Kupffer’s Vesicle (KV) in Danio rerio (zebrafish) have identified another unclassified mechanism where cells packaged into a rosette utilize the last stages of cell division, cytokinesis and abscission.”

We agree that this statement may no longer provide the most appropriate rationale for this study given the removal of the theoretical model. Please see page 3, line 55-59 for our updated introductory text.

Best wishes

Jean-Léon Maître
A.2. The following Response to Reviewers is from the 11/19/2019 resubmission:

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

“The authors have revised their original manuscript in depth, adding several new experiments that addressed satisfactorily all my original concerns and, in my opinion, also concerns from the other reviewers. The text has also been greatly improved and now reads and flows very well.

Overall, this is a comprehensive and very interesting study that reports new in vivo findings that should be of interest to cell and developmental biologist.

Well done!”

Thank you.

Reviewer #2 (Remarks to the Author):

“This new version of the manuscript by Hehnly and co-workers presents some new data in support of their claim that the midbody/cytokinetic bridge is involved in the formation of the lumen during the development of the Kupffer’s Vesicle (KV) in zebrafish embryos. As I no longer have access to either the previous version of the manuscript or my full review, I have to consider this manuscript as a new submission.”

We apologize for using an abbreviated version of your previous comments in our first rebuttal letter, we’ve attached your full review here. This rebuttal includes your review in its entirety as requested:
Previous comments from Reviewer #2:

“The midbody is an organelle that forms at the center if the intercellular bridge during cytokinesis. Recent studies have indicated that the midbody is not only required for the final abscission of the two daughter cells, but it is also involved in establishing cell fate, apical-basal polarity, lumen and cilium formation. In this manuscript, Rathbun et al. investigates the correlation between midbody positioning and lumen formation in the Kupffer’s vesicle (KV) of zebrafish embryos. The authors show that KV cells assemble their intercellular bridges at the apical site and that spindle orientation is not required for KV organization. They then report that intercellular bridges are positioned at the site of apical clustering and that abscission seems associated with lumen formation. Finally, they show that prematurely severing the intercellular bridge by laser-mediated midbody ablation prevented lumen formation.

This a very descriptive paper that does not provide any novel insights into the role of the midbody in lumen formation, which was already described in a different system by the Prekeris lab (Magan et al, 2016, Nat Commun., PMID: 27484926). Moreover, the only evidence that the midbody is required for lumen formation is based on a single ablation experiment (Fig. 4). For these reasons, in my opinion this paper does not meet the standards for publication in Nature Communications.

I have no major technical comments, as I am not very familiar with the system used by authors, but I can say that the images are not very clear and the
number of markers used in this study is very limited. It is also unclear to me why
the authors did not use the zebrafish KIF23/MKLP1 gene instead of the human
homolog in their live-imaging experiments in Fig. 3.”

Original Response to Reviewer 2:
“The study mentioned above (Mangan et al, 2016)¹ did not examine this process
in vivo, but instead used a 3D in vitro tissue culture system. There are some key
findings that our study highlights that are unique from an in vitro 3D culture
system: (1) unlike MDCK 3-D acini, zebrafish KV do not utilize spindle orientation
in expanding a lumen. (2) Uniquely, zebrafish KV utilizes a prerequisite
temporary rosette structure before evolving into a sphere. Our study, for the first
time, has identified that rosette formation incorporates cell division. Unlike
zebrafish, in vitro MDCK 3D acini do not utilize a rosette scenario. Instead one
cell divides, forms two daughter cells, and then a lumen forms between the two
cells where the cytokinetic bridge was once placed, bypassing the formation of a
multicellular rosette. While this system can identify many molecular players that
are involved in lumen formation, it does not define the steps of forming a de novo
lumen in vivo, which we have done in our study in regard to KV. This suggests
that there are differences between these two systems, and the importance of
identifying what is occurring in vivo. These two points make our study broadly
applicable to both developmental and cellular biologists.
We have increased the number of mitotic markers used in this study to denote the cytokinetic bridge and/or mitotic stages (please refer to new Supplementary Fig. 2). We think that this comment has increased the significance of our manuscript in that none of the sub-cellular localization patterns of these markers have been previously described during zebrafish development. The new data and markers we have used for this resubmission include acetylated tubulin, RacGAP, PLK1, and MKLP1 (highlighted in our new Supplementary Fig. 2, Fig. 2 and 3). These studies were completed through immunostaining or fluorescently tagged constructs. We also have confirmed that the localization of the expressed human MKLP1 localizes in the same locale as two zebrafish midbody proteins, MKLP1 and RacGAP, by immunohistochemistry (Supplementary Fig. 2). All of this is included in new Fig. 2, and new Supplementary Figure 2.”

“The authors start by reporting that mitosis is required for lumen formation in KV by showing that cells involved in KV formation have a higher mitotic index than cells outside the KV and that lumen formation is impaired after treatment with mitotic drugs,(Fig 1). They then show that midbodies accumulate at the apical membranes prior to lumen formation and that this placement occurs during cytokinesis (Figs 2 and 3). They subsequently report that ablating the cytokinetic bridge impairs lumen formation (Fig. 4) and that optogenetic clustering of Rab11-vesicles results in abscission failure, abnormal lumen formation and reduced delivery of the cystic fibrosis transmembrane conductance regulator (CFTR) at the apical surface during KV formation (Fig. 5 and 6).
Most of the results presented in this paper are confirmatory. The Prekeris lab has published that midbodies are involved in lumen formation in an ex-vivo 3D culture system (Mangan et al, 2016, Nat Commun., PMID: 27484926) and it has been known for a while that Rab11 and its effector FIP3 are important for membrane trafficking during cytokinesis (reviewed in PMID: 19704869). As the authors mentioned in the paper, previous studies have shown a requirement for Rab11 in KV morphology and lumen formation (page 6) as well as in CFTR exocytosis (page 7). Therefore, the only novelty of this study seems to be that it is the first in vivo study showing a requirement for midbody/cytokinetic bridge in lumen formation in zebrafish embryos. Although I appreciate the importance of confirming ex-vivo studies in animal models, in my opinion this study does not represent a significant advance in the field to warrant publication in Nature Communications. Moreover, the ablation experiments, that probably are the most important part of the paper, do not seem well controlled (see comments below)."

Specific comments:

1. “The introduction is very succinct and does not provide sufficient information to the readers about the current knowledge of KV formation in zebrafish and of midbodies and their role in lumen formation.”

We thank the reviewer for this feedback. We have added more citations and a more comprehensive introduction of abscission and cytokinetic bridge cleavage. Please refer to page 4, lines 74-87, which are highlighted with blue font for your convenience.
2. "In Fig 1d, the mitotic index after treatment with BI or nocodazole should be properly quantified and plotted; how many replicates?"

As per Reviewer 2’s comments, we have carefully quantified both the cell number and mitotic index within KV for each of the drug treatments (new Supplementary Figure 1e-g). With this new data, we have correlated an increase in mitotic index and decrease in KV cell number with a decrease in KV lumen area (correlation plot shown in new Supplementary Figure 1g for cell number versus lumen size), suggesting that as cells are either inhibited from entering and/or progressing through mitosis (PLK1 inhibition with BI2536) or arrested in mitosis (nocodazole synchronization), lumen defects arise. All n values are included in Supplementary Table 5 and included in figure legends, n>27 embryos were used for each treatment in Supplementary Figures 1e-g across n>3 experiments.

3. "It is not specified what cell type(s) are shown in Supplementary figure 2a-c. Moreover, it should be made clear in the text that these figures only confirm a well established distribution of MKLP1 and Plk1 during mitosis."

Supplementary Figure 2a-c are images of early embryonic zebrafish cells expressing markers that have never before been resolved and/or shown in a live zebrafish embryo. However, we have confirmed that their distribution is similar to that reported in other contexts (refer to updated text on page 7-9, lines 134-179). Panel A shows an example of a live mitotic division within Kupffer’s Vesicle with PLK1-mCherry expression (denoted by Sox17::GFP-CAAX marker). Panel b is an example of another
live mitotic division in the zebrafish embryo, this time in cells expressing GFP-MKLP1 and PLK1-mCherry. Lastly, panel C is a representative image of mitotic stages within fixed zebrafish cells, highlighting specificity of GFP-MKLP1 and PLK1-mCherry expression at the cytokinetic midbody as shown by acetylated tubulin antibody staining.

4. “The term ‘abscission’ indicates a precise and unique time point, when the two daughter cells are physically separated. However, the authors use this term incorrectly. For example, there cannot be an ‘early’ and ‘late’ abscission as indicated in Supplementary figure 2c, and it is unclear what is the difference between ‘abscission’ and ‘cytokinetic bridge cleavage’ (Fig. 3b), as they may actually mean the same thing. In that figure, the two daughter cells appear still connected at 22 min, but the authors define this stage as ‘abscission’. Moreover, why the authors used the term ‘cytokinetic bridge’ instead of ‘intercellular bridge’, which is more appropriate?”

While the final severing of the cytokinetic/intercellular bridge is often termed “abscission”, in this manuscript we are referring to the resolution of the cytokinetic bridge in preparation for abscission, the final cleavage step. We have edited the manuscript to clarify on the confusion of abscission terminology (see page 4, lines 74-87). This is now outlined carefully in our expanded introduction based on your specific comment #1.

In short, we are using the term abscission to describe the end of cytokinesis when the cytokinetic/intercellular bridge is resolving to a diameter of approximately 1-2 microns and the bridge is subsequently cleaved2. Furthermore, studies have shown that
membrane trafficking pathways are required for abscission to bring new membrane to the sever site \(^3,^4\), which is required to allow for ESCRT complexes to complete the final severing step\(^5\). This process is now carefully laid out in the introduction and we have edited the language to achieve the consistency you requested.

5. “I can’t really see any specific accumulation of RacGAP1 in Fig. 2c-d, there are dots everywhere.”

In order to clarify the positioning of RacGAP-positive midbodies throughout this paper, we have carefully quantified their positioning in relation to the inner or outer 50% of KV both for endogenous midbodies (immunostained for RacGAP in new Figure 2d-e) and for midbodies that we follow live (new Supplementary Figure 3c-d). In both Figure 2d-e and Supplementary Figure 3c-d, the percentage of midbodies localized to the inner 50% of KV volume (“apical midbodies”) significantly increases as KV progresses from pre-rosette to lumen. We have also provided additional representative images depicting midbody localization (shown by RacGAP or MKLP1) at regions of apical polarity establishment, shown by CFTR-GFP expression (new Figure 2d-e and new Supplementary Figure 3c-d).

6. “The distribution of MKLP1 in Fig. 3 does not reflect the normal localization of this kinesin (compare with Supplementary figure 2b-c). It is clearly much more diffuse along microtubules instead of accumulating at the plus ends. This might indicate that the
construct is highly overexpressed, which would affect cytokinesis. Have the authors measured the level of expression of their constructs?”

A plasmid construct was utilized to visualize GFP- or mKate-MKLP1 in the case of Figure 3, as its localization on the microtubule cytoskeleton was useful in following the formation and severing of the cytokinetic bridge over time. We also utilize mRNA injections to visualize GFP- or mKate-MKLP1. While this plasmid construct is overexpressed compared to the mRNA injections, the timing of mitosis including cytokinesis and abscission was consistent with other experiments in this paper. Since the mitotic timing was not different between cells that ectopically expressed mKate-MKLP1 or PLK1-mCherry when compared to two transgenic lines where we can monitor division timing (actin:H2B-Dendra or actin:EMTB-3xGFP), we are confident that this overexpression is not detrimental to mitotic progression in this case.

7. “There are major issues with the ablation experiments (Fig. 4). First, the author claim that they ablated cytokinetic bridges while it is evident from the images (Fig. 4a-b) that they ablated midbody remnants (marked by MKLP1) instead. In order to correctly sever the cytokinetic bridge, the authors should visualise the central spindle microtubules. The most important control in these experiments is to show that ablation of cell-cell junctions adjacent to midbodies during apical clustering does not affect lumen formation. These are indicated by the authors as KV cell-cell interface controls, but only 3 of these experiments were carried out (Figure 4d-g) and it is impossible to identify the location of
these ablation sites from the kymograph (why a kymograph?) shown in Supplementary Figure 4.”

While we appreciate the concern for distinguishing midbodies within cytokinetic bridges versus midbodies post-abscission, we are confident that the midbodies ablated were still housed within a cytokinetic bridge at the time of laser ablation. When identifying a midbody/cytokinetic bridge we first had to image pre-ablation. This process takes time and a midbody remnant would not remain in frame. Specifically, we began by identifying a midbody for ablation and taking a two-color z-stack image to capture the midbody and its position within Kupffer’s vesicle. Then we proceeded to snap a pre-ablation image of the midbody plane, ablate the midbody with multiple pulses of laser, and snap a post-ablation image to ensure complete ablation (refer to new Supplemental Figure 4a-b). Therefore, midbodies that remain in the same position for this lengthy process are deemed to be housed within a cytokinetic bridge pre-severing.

To directly address your concern about midbody remnants, we’ve used improved imaging parameters to visualize the cytokinetic bridge on either side of the midbody during ablation (refer to new Supplemental Figure 4c). We identified the same defects under these conditions as in our previous experiments reported in the manuscript. Here, we noted that the midbody was ablated, leaving the severed cytokinetic bridge floating in the embryo, and KV lumen formation defects were identified when cytokinetic bridges within KV were ablated in this manner.
Rebuttal Figure 1. Live markers for microtubules during zebrafish development. Top panels: Examples of EMTB-3xmCherry mRNA expression (grayscale) during early (left) and later (right) zebrafish development. Bottom panels: Expression of EMTB-3xGFP (cyan) and Sox17:RFP (magenta) resulting from the crossing of two stable transgenic zebrafish lines. Early (left) and later (right) developmental stages shown. Note the loss of clear microtubule signal during late developmental stages (~8-10hpf) in both cases.
Unfortunately, it is not feasible for us to visualize microtubules in a live embryo during KV development. We have a stable zebrafish line that expresses a fluorescently-labelled microtubule binding protein (EMTB-3xGFP) that is commonly used for this purpose, however fluorescence is dim at best by the time the fish reaches KV developmental stages and we are unable to use it for careful microtubule identification (examples in rebuttal Figure 1). Additionally, we have attempted to use injectable mRNA expressing EMTB-3xmCherry to obtain live microtubule fluorescent labelling, however we experience the same difficulties as the transgenic line and are unable to visualize microtubules at this developmental stage in this locale. We have attached representative images of these tools during early (~2-4hpf) and later (~8-10hpf) zebrafish development for clarity (Rebuttal Figure 1). This has been a recurring problem in KV development and there are no publications that have demonstrated live microtubules in KV to date.

Additionally, we have included added controls and representative images as requested for cell-cell interface ablations (updated Figure 4c-f). We doubled the number of cell-cell interface ablation controls and added cell cytosol control ablations to make this section more rigorous (See updated Figure 4c-f, refer to updated Supplementary Table 5, n>6 embryos used for each ablation condition). We have also included added representative images of each type of ablation treatment (midbodies within and outside of KV, KV cell-cell interface control, KV cell cytosol control) for clarity and transparency (see updated Figure 4a-c). An inset is included for an ablation of cell-cell interface (updated Figure 4c) to replace kymograph.
8. “The authors did not indicate the number of cells and experiments for the graph in Fig. 5c and the number of cells for the graph in Fig. 5e.”

For the cell culture experiments in Figure 5c, n=100 cells were counted per experiment over three independent experiments for normal light conditions and 488nm blue light conditions. For the zebrafish experiments in Figure 5f, n>161 cells were counted for each experimental condition over n>5 embryos. This information is now in the revised figure legend and detailed in Supplementary Table 5.

9. “Multinucleation is a general readout for cytokinesis failure and not specifically for abscission failure as wrongly stated several times by the authors. Depletion or inactivation of factors specifically required for abscission, like some ESCRT-III components for example, often does not cause a large increase in multinucleation.”

We have added experiments to this manuscript that demonstrate that a Rab11 CRISPR line has a significantly higher percentage of binucleate cells compared to controls. Additionally, we have provided time-lapse images of these binucleate cells forming following the formation of a cytokinetic bridge (new Supplementary Figure 5c-f). This quantification is very similar to the results of the optogenetic clustering of Rab11 (Figure 5c). From these images, we hope you can appreciate that the process of cytokinesis occurs as normal, however abscission fails and the dividing cell transforms into a binucleate cell well after the formation of the cytokinetic bridge.
Reviewer #3 (Remarks to the Author):

“The revised manuscript from Rathbun and colleagues provides significant improvements. The study now comes with more quantifications of the authors observations, some control experiments and with a new functional assay to support the authors’ hypothesis. I have some questions regarding these additions, which I am convinced the authors should be able to address, and I remain very skeptical about the benefit of including the theoretical model in this study.

Major concerns:

1 – The authors include a modified theoretical model of the function that cytokinetic bridges (CB) could have in cell packing. This is mentioned in the main text in a brief paragraph and more detailed in supplementary. The model is not mentioned in the rest of the text, which questions its relevance to the study.

The authors claim the model is “testable” but I fail to see where they describe how they test it. In addition, I fail to see what predictions come out of the model that would be relevant to the rest of the study.

The model hypothesis would be that CB could provide a line tension that would help packing cells. I see how a leash connecting sister cells could prevent them from going too far apart and essentially tether cells to one another. I find it more difficult to conceive how this would pack cells into a compact structure in the same way as actomyosin contractility and/or adhesion could do, as described in tens (if not hundreds) of studies.

The authors then plug in values found in the literature to compare the line tension
applied by contractility onto the CB to speculate that CB could withstand this tension thanks to a line tension that would be between twice and 20 times the one of contractility. This is based on values found in the literature in different cells and experimental settings. In addition to their relevance to the KV, the uncertainty on these values makes the conclusion of the model weak in my opinion. Besides, this would explain how sister cells stay together but not how the entire KV packs itself. Finally, the laser ablation experiment disproves the authors speculations. After ablating the midbodies (MBs), the KV doesn’t form a lumen and nevertheless stays nicely packed, arguing that CBs are dispensable for KV packing. In conclusion, I think the model lacks relevance and consistency with the study. As such, it is detrimental to the study and should be removed.”

We thank Dr. Maître for his careful analysis of the model included in this manuscript. We agree with quite a bit of what is said above.

Very briefly, we intended the model to provide a null hypothesis about the magnitude of the mechanical work involved in forming a rosette structure, under a strong assumption that the cortical actomyosin is not significantly remodeled during rosette formation. This was to help us understand whether there could be a mechanical role for the cytokinetic bridges. As Dr. Maître correctly highlights, in the end the experimental data contradicted our null hypothesis: our data suggest cytokinetic bridges are not mechanically necessary for the packing, just the lumen formation. We should have been more clear about this in the original version of this manuscript. As teasing
apart these details is neither necessary or important for this manuscript, and we have now removed the modeling description entirely from the new revision.

We hope to revisit the question in future work.

2 – “The authors have added quantifications to some of their experiments but some experiments remain unquantified and some quantifications are unclear. For example, the data shown in figures 2 and 3 are not quantified and it is therefore difficult to appreciate how frequent the phenomena are. The authors could count the MBs or CBs positioned apically over the total number of MBs and CBs in the KV.”

To address this comment and to further demonstrate the frequency of cytokinetic bridge placement towards the center of developing KV, we have quantified midbody localization within KV as requested. We determined the percentage of midbodies located within the inner 50% of the KV volume, termed “apical midbodies”, during pre-rosette, rosette, and lumen stages of KV development (diagram describing quantification found in Supplementary Fig. 2h). We found that in both fixed (new Fig. 2d-e) and live samples (new Supplementary Fig. 3c-d), there was a significant increase in the percentage of midbodies located in the center of KV.

“When assessing the size of the lumen, the authors measure the projected area. Because the KV is not spherical (it is more of a flat asymmetric capsule), the projected area is going to depend on the orientation of the imaging (and this orientation will change with the migration of the KV over the spherical embryo). I think this readout is
not ideal. From the methods, it is unclear to me if the authors correct for the orientation of the imaging (dynamically that is) to measure the KV projected area facing its equatorial plane. Based on their images, I think the authors may be better off measuring the volume of the KV. There are quick and efficient software nowadays that can do the job fairly easily. I could recommend Limeseg, which is a FIJI plugin that is very easy to use and will give a more accurate measurement than what the authors currently show.”

When mounting zebrafish embryos in agarose, every effort is made to ensure Kupffer’s vesicle is close to the coverglass and as flat as possible to ensure the area measurement is representative of the true KV lumen structure. All KVs were processed and lumen area quantified in Imaris software to ensure lumen was facing its equatorial plane when measured. Imaris is ideal for 3D imaging as it allows the 3D rendering to be turned (see Rebuttal Figure 2). We’ve carefully detailed this into our revised methods section (page 24, lines 516-525).
**Rebuttal Figure 2.** Representative image of KV lumen before (left) and after (right) reorientation in Imaris. KV images were turned in Imaris to orient the KV along its equatorial axis for proper lumen area measurement.
“In Figure 6b, the authors normalize the projected lumen area to the mean control area. It is unclear to me why the authors decided to normalize the area for this experiment specifically. It may be linked to my previous point about the inaccuracy of the area measurement. Nevertheless, the absolute values should be given to unambiguously report the authors’ findings.”

Our optogenetic experiments were completed through the use of a NightSea fluorescent lamp to blanket the embryos in blue light. Therefore, many of these experiments were completed at “room temperature”, which fluctuated slightly based on season and building parameters. This slight temperature variability may affect developmental timing, as slight changes in temperature have been shown to either speed up or slow down zebrafish development. In order to control for this possibility, we normalized the lumen area data from each independent experiment to the mean of the uninjected control group for that experiment. This would ensure that differences we noted between the experimental group and controls of the amalgamated data set were in fact due to the variables being tested, instead of differences in ambient room temperature and subsequent zebrafish developmental speeds between independent experiments.

Because embryos were ablated at a specific developmental timepoint and followed through live microscopy, data normalization wasn’t necessary for ablation experiments. However, a large population was used for the optogenetic experiments where developmental stages varied slightly from embryo to embryo within the population. Since these embryos were exposed to blue light in bulk and differences in
room temperature and clutch developmental speed would alter their exact developmental stage, data normalization was necessary to be able to compare results between separate independent experiments. We have included a raw data set to demonstrate that while the trend remains the same as in the normalized data set, this normalization accounts for differences in lumen area due to slight variations in clutch developmental timing to focus on the effects from the optogenetic experiment specifically (see new Supplemental Figure 6b where clutches are individually color coded).

“When reporting the effects of the ablation and optogenetic experiments on KV formation, the authors give the area under the curve (AUC) of the lumen projected area over time, the final projected area and the growth rate. The first two parameters are very sensitive to the timing of lumen opening time relative to the time of imaging (that’s what I gathered this time indicates, it is unclear from the description and, by the way, a time indicated as hour post-fertilization would be much more useful and appropriate to be able to reproduce these experiments). Any delayed embryos (and this seems to be frequent in the reported experiments) will see these parameters affected in an unspecific manner. Indeed, the growth of the KV does not seem to reach a plateau during the time of imaging shown by the authors. When is the final area value taken then? What is the time period taken for the AUC measurement? The growth rate on the other hand seems more robust to these experimental biases.”
These are valid points and we agree that perhaps AUC is not the simplest way to quantify the differences we’re seeing. Therefore, we are simplifying our analyses to only include graphs depicting lumen area over time and lumen growth rate.

For these experiments, laser ablations were performed at midbodies within or outside KV, at KV cell-cell interfaces or cytosol (experimentally modeled in new Supplemental Figure 4a). From here, embryos were imaged for approximately two hours post-ablation to track lumen growth. Since timepoint duration differed in a few of the experimental setups due to the number of embryos being imaged, lumen areas were binned and plotted every thirty minutes to depict the averaged lumen growth over time for each of the control and experimental groups (see updated Figure 4d-e). Lumen growth rate was determined by dividing the final lumen area by the timepoint of the last image to calculate the growth in m²/ min. These values were then plotted in the bar graph shown in updated Figure 4f. n-values were increased for all treatments.

3 – “The authors have added some controls to their previous experiments but some controls for the newly added experiments are missing or would need clarifications. Regarding the ablation experiments, the Figure 4a shows a control where no ablation is performed. This is not the appropriate control to show. The authors would need to show a case in which a contact or a cell has been ablated.”

We have included example images of ablation controls in our revised manuscript. The unablated control, MB outside lumen ablation, cell-cell interface ablation, cell cytosol ablation, and MB within KV ablation are represented in updated Figure 4a-c.
“Along this line, how are contacts and cells chosen for the control ablation experiments? These control cells should be chosen for having an apical MB or CB. I could not find this information and this needs to be described clearly whether it is the case or not. “

We have included a diagram to describe the relative locations of ablations in the developing KV (new Supplementary Figure 4a), where we have designed our controls to test how KV development as an organ is effected by the premature severing of a bridge compared to other locations in the tissue. When choosing cell-cell interface and cell cytosol ablation locations, we chose regions that we could clearly focus on and ablate. Control conditions did not have a cytokinetic bridge attached, only a clearly identifiable region that was either a cell-cell interface or cytosol (cell-cell interface inset shown in updated Figure 4c). MBs outside KV were ablated within the same microscope field as KV. MBs inside KV (experimental group) were located at the center of KV, colocalizing with the central rosette structure just prior to lumen formation. When we do laser ablations we work in a single z-plane for the ablation (limitations of our ablation set up), this makes it very difficult to resolve the edges of the cells that are associated with the bridge. Cell edges are only clearly identified when we do 3D image analysis post acquisition (Figure 3). Following the short 2D ablation time-course, we then do 3D imaging over time to assess defects in lumen formation. Therefore, due to our experimental limitations, we picked structures/locales that were not cytokinetic bridges to ablate and compare to bridge ablation. This allowed us to control for the effects of laser ablation upon the KV organ as a whole compared to the targeted ablation of a cytokinetic midbody at the site of future lumen formation. Under these conditions, we
saw significant defects in lumen formation when ablating cytokinetic bridges and not with cell-cell interfaces or cytosolic regions. This is now clearly described on page 11, lines 234-238.

“Also, the authors indicate that the ablation of MBs is ensured by the lack of fluorescent recovery. I think such example would be good to show as a control of when ablation is incomplete, if the KV can form.”

We added a new figure panel to distinguish a successful laser ablation from an unsuccessful laser ablation. In new Supplemental Figure 4b, we depict the lack of fluorescence recovery noted during a successful laser ablation (top panel) compared to the subsequent fluorescence recovery noted after an unsuccessful laser ablation. Typically, the fluorescence recovery of an unsuccessful laser ablation is noted less than a second or two later as shown in Supplemental Figure 4c.

Furthermore, we included an example of a successful laser ablation where the cleavage of the cytokinetic bridge can be observed with improved microscopy parameters (see new Supplementary Figure 4c). The retraction of the cytokinetic bridge can be observed post-ablation in the time lapse included, demonstrating the result of a successful ablation.

“Regarding the optogenetic experiments, the failed division described in figure 5 are quite difficult to see. Are the images of Hela cells shown in 5a,b the images used for counting binucleated cells? Are the movies more convincing than the still images? As
far as KV cells are concerned, I am not convinced that the image shown in 5d is of a binucleated cell but could rather be of a cell with lobed nucleus. Can the authors film a failed division instead or can they obtain more convincing images of multiple nuclei in a cell? Since the authors express their constructs in the entire embryo, this could maybe be observed in a different tissue where imaging is easier.”

We have included additional images in updated Figure 5d-e to represent the binucleate and multinucleate cells we observe in Rab11-disrupted embryos. We have also outlined the boundaries between the multiple nuclei for clarity. In addition, we included examples of cells failing abscission and forming binucleates when Rab11 expression is removed from HeLa cells in new Supplementary Figure 5c-f, which also contains examples of binucleates (new Supplementary Figure 5d) that are very similar to the results noted when Rab11 function is disrupted with optogenetics (Figure 5d).

“Following up on this, the effect of the inhibition of Rab11 in the surrounding tissue should be assessed to know whether what the authors report is autonomous to the KV (as they explicitly assume) or not. This should be at least discussed explicitly or tested by doing late yolk injections, which would lead the mRNAs to be up-taken by the YSL and the forerunner cells, which will become the KV cells.”

The advantage to optogenetics is that we can acutely expose embryos to 488nm light specifically during the development of KV. While this is still ~4-6 hours, we find that embryo death rates were similar and that the loss of embryos noted was consistent across injection control and experimental groups, suggesting that embryo death was
due to injection trauma (updated Supplemental Figure 5h). Additionally, we quantified heart looping defects in the embryos at the end of the trials and found that while defects were present in heart looping in the experimental groups, the fish appeared to have developed normally otherwise. This indicates that the Rab11 clustering during that 4-6 hour window was mainly specific to KV development (producing left-right asymmetry defects and little else in terms of morphological defects). One reason for this is that defects that occur in non-KV cells could recover after light was removed. This 4-6 hour window of 488nm light exposure occurs when the KV sets up a left-right body axis, and is likely unable to recover. We would like to follow up on these studies in a later manuscript.

We attempted the late injection experiment that Dr. Maître mentioned in his comment, however we were unable to obtain mRNA expression specifically in KV using this tactic (see Rebuttal Figure 3). While we were unable to directly test this as requested, we would like to add that the mosaic expression of optogenetic constructs in regions surrounding KV but excluding KV resulted in lumen areas similar to those in uninjected or single injected embryos (see updated Figure 6b), suggesting that Rab11 disruption in the tissue surrounding KV has no discernable effect on KV development. This result is in stark contrast to our experimental group that includes embryos with KV expression of the optogenetic constructs under 488nm light conditions, where severe lumen defects were noted. We have expanded upon this section in the manuscript text (see page 15, lines 314-323).
Rebuttal Figure 3. Representative images from a late-injection optogenetic experiment. KV marker (CFTR-GFP, grayscale/magenta) and CIB1-mCherry-Rab11 (grayscale/cyan) expression shown. Uninjected (top), CIB1-mCherry-Rab11-only injection, and CRY2 + CIB1-mCherry-Rab11 double injection groups displayed. Embryos were injected between the 500-1000-cell stages of development.
“Following up on this, the time window of illumination used by the authors is very broad and will affect the precursors of the KV, namely the forerunner cells. How can the authors ascertain that the effects they observe on lumen opening comes specifically from the inhibition of Rab11 function in CB related trafficking? Rab11 related defects could arise, not only from the surrounding tissue, as mentioned previously, but also from defects inherited from the precursors of the KV cells. This is not discussed explicitly by the authors and could be tested by narrowing down the time window of light exposure to, as the authors claim, “acutely” inhibit CBs polarizing functions.”

In order to test this, we narrowed the time frame of light exposure by two hours, beginning blue light exposure at the 75-90% epiboly stages versus the 50-60% epiboly. We carefully quantified the lumen area of these embryos and found a similar trend, where lumen area is significantly decreased in embryos injected with both optogenetic mRNA constructs and blue light treatment. We have added this late light exposure experiment into updated Figure 6b of the manuscript.

“The functions of Rab11 in cell trafficking are broader than just the ones associated to the CB (this should be explicitly mentioned). It is nice to see the effect on CFTR trafficking upon Rab11 inhibition. The same readout would be important to see after laser ablation, which is more specifically targeting the MB and more acute. It would therefore nicely complement these observations.”

To address this, we ablated embryos expressing CFTR-GFP and followed the fate of CFTR localization. We were unable to resolve the trafficking of CFTR to the
cytokinetic bridge within the single ablated cell due to photobleaching of the ablated area. We have added this experiment to new Supplementary Figure 4b-c.

“Minor concerns:

1 – I wonder how the authors explain that inhibiting cell division in Figure 1 results in a graded effect on lumen size when they propose that cell division would have more of an on/off or permissive function.”

PLK1 inhibition can halt cells in G2, prometaphase, metaphase, or cytokinesis. This inhibition is leaky and can therefore results in this wide variety of mitotic delays, as well as allowing for cells to exit mitosis on occasion. Additionally, mitotic delay from nocodazole inhibition is also leaky, allowing cells to either sync in prometaphase or progress through mitosis on occasion. We attribute these phenomena to the dosage dependent inhibition with both chemicals. We have changed the language in the revised manuscript accordingly (refer to page 6, lines 111-131).

2 – “The nocodazole experiment is quite brutal as all intracellular trafficking will be affected, not only the spindle formation. This should be stated explicitly.”

This has been updated in our revised manuscript. Please refer to page 6, lines 116-119.

3 – “The observation of MB apical localization in MDCK cysts is already known and not particularly relevant to the study. I think it is sufficient to show the KV data.”
We have moved this panel from the main figure to Supplementary Figure 2g to support previous relevant studies.

4 – “Figure S5 shows 75% survival of the control embryos. This seems low.”

We have added data from three independent experiments with our wildtype zebrafish line to the graph in Supplementary Figure 5h. We attribute the low control survival rate to the transgenic line, Sox17:GFP-CAAX, when compared to a wild-type TAB line.

5 – “The time stamps are missing in Figure S2c-f.”

These panels were fixed zebrafish embryos with mRNA expression and/or immunostaining. Still images from live embryo movies are in Supplemental Figure 2a-b and now are clearly labeled with time stamps.

Best wishes,

Jean-Léon Maître
A.3. The following Response to Reviewers is from the 07/22/2019 resubmission:

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

“In the manuscript by Rathbun et al the authors examine the role of cytokinetic bridges (CKBs) in lumen formation in the zebrafish organ of laterality (KV). They first use modeling to explore the potential role of CKBs in the organization of rosettes in the KV primordium and take published tension values to estimate the relative strength of CKBs. They argue CKBs withstand much greater tensions (up to one order of magnitude) than that of acto-myosin along cell boundaries. They then examine the formation of CKBs and the localization of midbodies during KV development and monitor the cell division angles between KV cells. They observed that CKBs are unusually long-lived, that midbodies localize to the apical domains before lumen opening and that, unlike in vitro results, cell divisions are not oriented perpendicular to the prospective lumen. Importantly, they observe that the position of the CKBs correlates with the site of lumen formation, which occurs following CKB cleavage. Finally, they test the importance of timing of CKB cleavage in lumen formation using laser ablation and conclude premature cleavage impairs lumen formation.

Overall, this manuscript addresses an interesting question from a novel angle. While much of the data is descriptive, it does provide new insights. However, further experimental evidence is needed to support their conclusions. The major points are:
1-It is unclear whether midbody localization to the prospective apical membrane precedes apical polarization and results mainly from the site of CKB cleavage or whether this simply reflects centrosome localization and microtubule organization. That is, midbody localization would be downstream of cell polarization and depend on centrosome localization rather than how it is proposed.”

Whether midbody formation precedes or follows apical polarity is an interesting point, specifically whether the centrosome localizes at the apical membrane before cleavage. To address this point we monitored the placement of the cytokinetic midbody in relation to the mitotic centrosomes in live embryos by assembling a video montage, along with visualizing fixed embryos where we focused on dividing cells. To do this we had to find and then utilize several mitotic markers that would work in zebrafish embryos (New Supplementary Fig. 2). One of these markers, the mitotic kinase Polo-Like Kinase-1 (PLK1) labels both the midbody and mitotic centrosomes. Here we find that when a midbody forms, the mitotic centrosomes are still on the side of the nucleus furthest away from the cytokinetic bridge, yet to orient towards the cytokinetic bridge where an apical membrane forms (Supplementary Fig. 2a-c, late abscission, green arrows). With this, we argue that formation of the cytokinetic bridge and associated midbody precedes cell polarity formation and centrosome positioning to the apical membrane.

However, we do not intend to propose that the midbody drives apical polarity formation, but that the process of abscission is required for establishment of a de novo
lumen and robust apical polarity. The midbody serves as a landmark for our studies to follow the cytokinetic bridge. A long-term goal of my lab is to examine whether the centrosome directs Rab11-endosome transport into the cytokinetic bridge to initiate polarity formation. However, we argue that this is beyond the scope of this paper. This study is important in that it first demonstrates that KV morphogenesis requires division (updated Fig. 1), and that the process of abscission is involved in the initiation of the KV lumen formation (new Fig. 5-6).

“2-The ablation experiment lacks controls and it is impossible to determine whether lumen formation is impaired due to premature CKB cleavage, due cell damage or any number of side effects. 2A-What happens if the ablation is performed at a different time? 2B-Can the role of CKB cleavage be explore using genetic tools? An alternative method is clearly needed.”

We were able to address your concerns and perform additional ablation experiments and additional controls (updated Fig. 4, and Supplementary Fig. 4). In addition, we utilized alternative approaches to examine the general role of the cell cycle in lumen formation (new Fig. 1d-e, Supplementary Fig. 1d) and a genetic tool to examine the role of cytokinetic bridge abscission in de novo lumen formation (new Fig. 5 and 6).

For our ablation experiments, we observe little to no cell death over a two-hour period following the ablation. This is either when we ablate a midbody inside the KV (experimental), a midbody outside the KV (control), a site of cell-cell contacts within KV
(control), and within the cell body of a cell within KV (control) (updated Fig. 4). With the three control groups, we hope to control for the additional side effects that can no doubt occur with ablation techniques. We analyzed our control and experimental conditions by live cell imaging where cell membranes and cell bodies could be monitored. We observed no obvious signs of cell death such as severe loss in overall cell number during this two-hour period. Under control ablation conditions, normal lumen formation suggests that the process of applying an ablation laser is not causing severe effects to the embryo that would inhibit lumen formation. It is only when we apply the ablation laser to the cytokinetic bridge-associated midbody that we prevent lumen formation (updated Fig. 4).

We have strategically focused on ablating midbodies during apical clustering because during this time, KV is towards the outside of the embryo and can be placed closer to the cover glass to optimize ablation conditions. Also, this developmental stage precedes the de novo formation of the lumen. Once the lumen starts to form, the KV dives into the cell mass of the embryo (>200 nm away from the coverglass). Once this occurs, it is difficult to accurately ablate a midbody.

We have now utilized two alternative methods to test the role of cell division in KV lumen formation. First, we took a pharmacological approach and treated cells with two different concentrations of nocodazole, a microtubule destabilizing drug that can synchronize cells in prometaphase (100 nM and 1µM), and BI2536, a PLK1 inhibitor (100 nM and 1µM). Both of these drugs resulted in cells that were unable to exit metaphase, leading to an increased mitotic index within the KV (Fig. 1d-e,
Supplementary Fig. 1d). Strikingly, our findings demonstrate that if cells are not allowed to progress through mitosis, a KV lumen cannot fully form (new Fig. 1d-e) suggesting that the process of cell division is required for KV lumen formation.

A second new alternative approach to our ablation studies that prematurely severed the cytokinetic bridge (Fig. 4) was to test a scenario where we could prevent abscission while also inhibiting the delivery of apical polarity proteins to the cytokinetic bridge (new Figs 5 and 6). Previous work in an in vitro model has identified that apical-targeted endosomes containing the Par3/aPKC polarity complex assemble adjacent to the cytokinetic midbody. These endosomes contain a small monomeric GTPase, Rab11, required for late cytokinesis and to initiate abscission. To determine the role of these Rab11-associated vesicles in lumen formation, we acutely inhibited Rab11-associated membrane vesicles through an optogenetic oligomerization approach (modeled from 9, new Supplementary Fig. 5a). We first tested the efficacy of this system in HeLa cells (new Fig. 5a) where we can promote a blue-light-inducible hetero-interaction between CRY2 and CIB1. To examine whether the cellular aggregation of Rab11-associated membranes disrupts function, HeLa cells in the early stages of abscission were treated with and without blue light. These cells co-expressed CRY2-mCherry and CIB1-Cerulean-Rab11 (new Fig. 5a-b). Under control conditions, cells can progress through the final stages of cytokinesis/abscission within approximately 90 min. (new Fig. 5a). When cells are exposed to 488nm light throughout the 90 min. time course, Rab11-associated vesicles are unable to move into the cytokinetic bridge and remain clustered within the cell body, inhibiting the ability of this cell to abscise (new
Fig. 5b) resulting in an increased number of binucleated cells (new Fig. 5c). Based on these studies, we next utilized this system in vivo to inactivate Rab11-associated vesicles during KV formation and found that blue light-induced clustering of Rab11-associated endosomes also resulted in a significant increase in binucleated KV cells (new Fig. 5d-e). These studies strongly suggest that the optogenetic clustering of Rab11 causes abscission inhibition.

By utilizing the CIB1-mCerulean-Rab11 and CRY2-mCherry optical clustering system outlined in Supplementary Fig. 5a, we examined whether disrupting Rab11 caused defects in KV formation (experimentally modeled in Supplementary Fig. 5c). In control embryos (uninjected, injected with CRY2-mCherry only, or injected with CIB1-mCerulean-Rab11 only), a robust lumen was able to form by the 4-somite stage (new Fig. 6a-b). Lumen area was calculated across multiple embryos. No significant difference in lumen area was noted between uninjected and injected control embryos exposed to blue light (new Fig. 6b). In double injected embryos exposed to blue light where KV cells have clustered Rab11-associated membranes, significant defects in KV lumen formation occurred such as decreased lumen area or an inability to form a lumen at all (new Fig. 6b). These findings suggest that acute inhibition of Rab11-associated vesicles within KV-destined cells during early KV development disrupts KV formation.

"3-The simulations are used to argue about tissue tension and the potential role of CKBs. 3A-Do membranes recoil upon CKB ablation? 3B-if so, is the speed greater than that of cell-cell adhesions? 3C-Is rosette organization affected by CKB ablation?"
We don’t observe significant or measurable recoil (Refer to updated Fig. 4b, Supplementary Fig. 4a). We believe this to be the case because we are ablating a very small cytokinetic bridge instead of an area that is composed of multiple cell-cell interfaces. This makes it difficult to resolve the recoiling of the bridge in the mass of membranes projecting towards the center of the apical cluster.

Since we ablate when KV is in a rosette structure, we see some moderate changes in cell geometry but the rosette itself remains intact. We want to examine this in future studies.

Additional points:

“4-The findings on cell division angles presented in Fig.2 are surprising given the published literature, which includes other refs not cited (e.g. PMID 19001128, PMID 24421325, PMID 22965908). 5A-Are the results comparable? i.e. in the published literature the angle of cell division affects single lumen maintenance rather than establishment.”

PMID19001128 is a study from Alan Hall’s lab that examines the role of CDC42 in spindle orientation in 3D MDCK cells, PMID 24421325 and 22965908 are similar studies from the Martin-Belmonte group that uses the same model system to show the role of spindle orientation in epithelial morphogenesis. While these studies are compelling in creating a testable model for the role of spindle orientation in epithelial morphogenesis, this is an in vitro model system (MDCK cells). While there are striking similarities between 3D MDCK cells and KV lumenogenesis, such as the organization of
cytokinetic midbodies at sites of apical polarity (refer to new Fig. 2a that uses MDCK cells and compare to Fig. 2b-d that demonstrates midbodies at the sites of apical clustering in the zebrafish KV), during the formation of KV, spindle orientation is randomized and doesn’t seem to be essential for placing daughter cells in the correct position preceding lumen formation (Fig. 3, Supplementary Fig. 3).

“5-Do the signals from antibody staining and tagged construct for MKLP co-localize?”

We now have included Supplementary Figure 2 with corresponding Supplementary Movies 2-4, where we utilize multiple mitotic markers to monitor cytokinesis and demonstrate that MKLP1, RacGAP, and PLK1 all localize to the midbody. This localization was also confirmed by immunostaining. These studies are the first to examine these markers in the developing zebrafish embryo.

“6-The mitotic index for non-KV cells is reported at ~2%, isn’t this too low for an early embryo?”

We have added an additional study where we have carefully calculated the mitotic index of a developing zebrafish embryo during the cleavage period, blastula period, gastrula period, and segmentation period (new Supplementary Fig. 1a-c). During the cleavage period the mitotic index is 100% and steadily decreases to ~3% during the subsequent periods (new Supplementary Fig. 1a-c, refer to new Supplementary Movie 1).
Due to reviewer 1’s comments we have extensively reworked the paper to focus on specific conclusions per figure, which we hope will bring added clarity. Due to this expansion of figures we have rewritten the text in hopes of clarifying the studies being presented.

**Reviewer #2 (Remarks to the Author):**

“The midbody is an organelle that forms at the center if the intercellular bridge during cytokinesis. Recent studies have indicated that the midbody is not only required for the final abscission of the two daughter cells, but it is also involved in establishing cell fate, apical-basal polarity, lumen and cilium formation. In this manuscript, Rathbun et al. investigates the correlation between midbody positioning and lumen formation in the Kupffer’s vesicle (KV) of zebrafish embryos. The authors show that KV cells assemble their intercellular bridges at the apical site and that spindle orientation is not required for KV organization. They then report that intercellular bridges are positioned at the site of apical clustering and that abscission seems associated with lumen formation. Finally, they show that prematurely severing the intercellular bridge by laser-mediated midbody ablation prevented lumen formation.

This a very descriptive paper that does not provide any novel insights into the role of the midbody in lumen formation, which was already described in a different system by the Prekeris lab (Magan et al, 2016, Nat Commun., PMID: 27484926).
Moreover, the only evidence that the midbody is required for lumen formation is based on a single ablation experiment (Fig. 4). For these reasons, in my opinion this paper does not meet the standards for publication in Nature Communications.”

The study mentioned above (Mangan et al, 2016) did not examine this process in vivo, but instead used a 3D in vitro tissue culture system. There are some key findings that our study highlights that are unique from an in vitro 3D culture system: (1) unlike MDCK 3-D acini, zebrafish KV do not utilize spindle orientation in expanding a lumen. (2) Uniquely, zebrafish KV utilizes a prerequisite temporary rosette structure before evolving into a sphere. Our study, for the first time, has identified that rosette formation incorporates cell division. Unlike zebrafish, in vitro MDCK 3D acini do not utilize a rosette scenario. Instead one cell divides, forms two daughter cells, and then a lumen forms between the two cells where the cytokinetic bridge was once placed, bypassing the formation of a multicellular rosette. While this system can identify many molecular players that are involved in lumen formation, it does not define the steps of forming a de novo lumen in vivo, which we have done in our study in regard to KV. This suggests that there are differences between these two systems, and the importance of identifying what is occurring in vivo. These two points make our study broadly applicable to both developmental and cellular biologists.

“I have no major technical comments, as I am not very familiar with the system used by authors, but I can say that the images are not very clear and the number of markers used in this study is very limited. It is also unclear to me why the authors did not use the
zebrafish KIF23/MKLP1 gene instead of the human homolog in their live-imaging experiments in Fig. 3.”

We have increased the number of mitotic markers used in this study to denote the cytokinetic bridge and/or mitotic stages (please refer to new Supplementary Fig. 2). We think that this comment has increased the significance of our manuscript in that none of the sub-cellular localization patterns of these markers have been previously described during zebrafish development. The new data and markers we have used for this resubmission include acetylated tubulin, RacGAP, PLK1, and MKLP1 (highlighted in our new Supplementary Fig. 2, Fig. 2 and 3). These studies were completed through immunostaining or fluorescently tagged constructs. We also have confirmed that the localization of the expressed human MKLP1 localizes in the same locale as two zebrafish midbody proteins, MKLP1 and RacGAP, by immunohistochemistry (Supplementary Fig. 2). All of this is included in new Fig. 2, and new Supplementary Figure 2.

Reviewer #3 (Remarks to the Author):

“In “Cytokinetic bridge triggers de novo lumen formation in vivo”, Rathbun, Colicino and colleagues describe the cellular events following cytokinesis of Kupffer’s Vesicle cells as they initiate lumen formation during zebrafish gastrulation. They propose that cytokinetic bridges are essential for bringing cell apices together into a rosette so that lumen formation can be initiated where midbodies are being released. Although, they become more frequent, this is one of the few in vivo studies of lumen formation, which moreover
takes advantage of live imaging possibilities of the zebrafish embryo. This study
remains largely descriptive, sometimes relying on anecdotal observations. The single
functional experiment of the study is based on laser ablation, which should be taken
with more caution than the authors did. Finally, there is a theoretical model included in
the study. It is unclear to me what this model brings to the study and what the study
brings to the model.”

We thank the reviewer for acknowledging that this is one of the few in vivo
studies that examines lumen formation. To address the reviewers concerns on our
laser ablation studies we have added additional parameters, and discussed more
thoroughly controls that were used, new controls performed, and additional results
obtained (updated Figure 4). We have also developed a genetic tool to analyze the role
of Rab11-vesicle trafficking, an essential process for abscission, in forming a lumen
(new Fig. 5 and 6). In regard to the theoretical model, we used this model to present a
testable hypothesis that an intercellular connection is likely present to explain for the
discrepancy in force. We have now presented the model to frame a question for
examining whether cells were remaining interconnected by a cytokinetic bridge during
KV formation (Supplementary Equations and Model). Specific concerns are addressed
below.

**Major concerns:**

“1 - The manuscript begins with a theoretical model which concludes that cell-cell
adhesion and cell contractility, two major morphogenetic force generators, are too weak
to bring cells together during KV cells gathering before lumen opening. This claim is based on values of abscission, adhesion and contractile forces that have been taken is vastly different contexts, none of them being in the system that is studied here. Also, there is no attempt from the authors to actually test any of the assumptions of the model. Finally, it is unclear what this conclusion brings to the study. Altogether, this is far from convincing if the authors want to make their point that adhesion would not be sufficient to keep sister cells together while they regroup with other KV cells.”

We apologize for the confusion, but we did not want to state that adhesion was not sufficient. Instead, we hoped to propose a scenario where adhesion is likely not the only force driving this process and that cytokinetic bridges may provide additional support for packing geometries seen in KV. Due to your concern, we have rewritten this section where we present this theoretical model as a model for why cytokinetic bridges may be important, which sets precedence for our ablation studies. Our future directions are to examine aspects of this model, such as differences in forces at cell-cell interfaces compared to the bridge. We argue that our study highlights the novelty that cytokinetic bridges are present during this developmental process and are likely contributing to tissue morphogenesis that requires further consideration.

“2 - The authors use live imaging to follow the movements of the cells, cytokinetic bridges and midbodies during KV formation. They quantify division rates, angles of divisions, velocities and fluorescent intensities. However, several experiments are not backed up with quantifications, making some observations rather anecdotal. For
example, the laser ablation experiment is not quantified. Another example is the description of the cell movements in Fig 3a,b which is not quantified either. How common are these phenomena?”

We have significantly expanded our ablation studies to address your concerns. We ablated a midbody inside the KV (experimental), a midbody outside the KV (control), a site of cell-cell contact within KV (control), and within the cell body of a cell within KV (control) (updated Fig. 4). With our three control groups, we hope to control for the additional side effects that can occur with ablation techniques. We measured lumen area over time following the apical clustering stage. In non-ablated embryos, lumens form in ~20 min (new Fig. 4c). When midbodies are ablated outside KV a delay in lumen formation occurs, but the lumen can still reach maximum area (Fig. 4c). Here we predict application of the ablation laser outside KV causes trauma to the embryo resulting in a slight but significant delay in KV lumen formation. We found a severe inhibition or delay in lumen formation when midbodies are ablated in KV during apical clustering (Fig. 4d-g). In KVs that do form a lumen, we find that lumen area is decreased at least by half (Fig. 4g).

We monitored the movements of 17 pairs of daughter cells across 7 embryos in updated Supplementary Fig. 3b. Here we monitored daughter cell distances (nucleus to nucleus) from cytokinesis into abscission and found that this distance decreases as cells pack together. Thus, we suggest that this is a common occurrence where daughter cells remain interconnected to pack next to each other into the forming KV.
“3 - The laser ablation experiment is key to the claim of the authors that cytokinetic bridges and midbodies would be essential for lumen formation. Laser ablation is generally a crude experiment which, if badly done, can have many unwanted consequences, like the death of the sample for example or wound response. The fact that the authors see no more development of the tissue (no lumen formation) after ablation is not reassuring. After ablation, are the cells still dividing? Is the embryo still developing? Are their left/right asymmetry affected (which is a standard readout for KV related phenotypes)? Do KV cells still polarize apico-basally? What is it that goes wrong so that they do not form the lumen? This clearly needs to be better described.

Also, the control experiment is only shown (without quantification either) in supplementary material and is not mention in the main text, as far as I could read. Controls are essential, this should be displayed in the main figure, quantified and mentioned in the main text.”

Similar to Reviewer 1 comment 2, we believe Reviewer 3’s main concerns were what controls we were using, and what are the downstream consequences of disrupting the timing of abscission. To address these concerns, we have added additional controls for our ablation experiments, and utilized alternative models to examine the general role of the cell cycle in lumen formation (Fig. 1d) and a genetic tool to examine the role of the cytokinetic bridge abscission in de novo lumen formation (new Fig. 5 and 6).

For our ablation experiments, we observe little to no cell death over a two-hour period following the ablation. This is either when we ablate a midbody inside the KV (experimental), a midbody outside the KV (control), a site of cell-cell contact within KV
(control), and within the cell body of a cell within KV (control) (updated Fig. 4). However, we did not notice any additional divisions during this time. We only note about 1-2 metaphase cells within this time frame and think the majority are in cytokinesis/abscission. This point has been difficult to quantify due to our mosaic labeling of MKLP1 to resolve the cytokinetic bridge. When we do ablation studies, embryos are dechorinated and mounted in low-melting point agarose on MatTek cover-glass bottom dishes. Imaging occurs on an inverted Leica DMI8 microscope. Following ablation, the embryos are very difficult to extract from the agarose and both unablated and ablated embryos end up falling apart. Thus, it’s very difficult with our ablation experiments to examine whether there are left/right asymmetry defects. What we can conclude from our ablation studies, especially now with our additional controls, is that the cytokinetic bridge with its associated midbody is a structure involved during apical clustering to initiate lumen formation in vivo.

Based on Reviewer 3’s concerns, we developed an alternative genetic approach to test a scenario where we could prevent abscission while also inhibiting the delivery of apical polarity proteins to the cytokinetic bridge (as described in Reviewer 1 comment 2). In brief, we acutely inhibited Rab11-associated membrane vesicles through an optogenetic oligomerization approach (modeled from 9, Supplementary Fig. 5a). We first tested the efficacy of this system in HeLa cells (new Fig. 5a-b) where we can promote a blue-light-inducible hetero-interaction between CRY2 and CIB1. HeLa cells co-expressing CRY2-mCherry and CIB1-Cerulean-Rab11 (Fig. 5a-c) were treated with and without blue light in the early stages of abscission. Under control conditions,
cells progress through abscission within approximately 90 min. (Fig. 5a), but when cells are exposed to blue light, Rab11-associated vesicles are unable to move into the cytokinetic bridge and remain clustered within the cell body. Under these conditions abscission is inhibited (Fig. 5b) and results in an increased number of binucleated cells (Fig. 5c). Based on these studies, we next utilized this system in vivo to inactivate Rab11-associated vesicles during KV formation.

By utilizing the CIB1-mCerulean-Rab11 and CRY2-mCherry optogenetic clustering system, we examined whether Rab11-associated membranes were required for KV formation. In control embryos (uninjected, injected with CRY2-mCherry only, or injected with CIB1-mCerulean-Rab11 only), a robust lumen was able to form by the 4-somite stage (Fig. 6b). No significant difference in lumen area was noted between uninjected and injected control embryos exposed to blue light (Fig. 6b). In double injected embryos exposed to blue light (experimental group) where KV cells have clustered Rab11-associated membranes, significant defects in KV lumen formation occurred such as decreased lumen area or an inability to form a lumen at all (Fig. 6b), along with similar defects in assembling an apical membrane (Fig. 6c). These findings suggest that acutely inhibiting Rab11-associated vesicles within KV-destined cells starting at the migratory stage disrupts KV formation.

Under these conditions, we do not calculate a significant difference in embryo death between control and experimental groups over a 42 hour period (Supplementary Fig. 5d). However, we note an increase in rightward heart looping under conditions where embryos co-expressed CRY2-mCherry and CIB1-Cerulean-Rab11 and were
exposed to blue light, but not under controls conditions (data not shown). This finding suggests that disrupting Rab11 vesicle transport, which leads to defects in abscission (Fig. 5, 8) and apical polarity (Fig. 6c-e) specifically during KV development results in left/right asymmetry defects. We chose not to include this data in this study because we wanted to focus on the cellular mechanisms in KV morphogenesis.

“Finally, I have difficulties following the rational of the authors in the interpretation of this experiment. Their working model is that midbodies help lumen formation and they observe multiple midbodies in each KV before they form their lumen. However, they claim to be precisely ablating only one midbody. Why aren’t the remaining midbodies able to trigger lumen formation? How many midbodies are left after ablation?”

We are sorry for the confusion. First, we would like to clarify that we utilize midbodies as a marker for the cytokinetic bridge locale. We argue that the cytokinetic bridge is a locale where directed membrane transport can occur towards the cytokinetic midbody where vesicles can deliver apical polarity proteins to the newly forming apical membrane. Rab11-associated vesicles have been identified to carry apical proteins that include CFTR, which is essential in lumen expansion10. CFTR is a master regulator of fluid secretion by controlling the transport of chloride to generate osmotic gradients that drive the movement of water through a tissue11. If the cytokinetic bridge is prematurely removed or if its abscission is inhibited through blocking Rab11-membrane transport and thus blocking CFTR delivery, then lumens are unable to form properly (Fig. 6). From this we present a model where premature abscission or clustering of
Rab11-associated vesicles carrying CFTR results in a decrease of CFTR at the apical membrane (new Fig. 6c-e), causing a loss of fluid flow and defects in lumen formation and/or expansion.

We have clarified the text to state that we are attempting to ablate one midbody. A particular experiment where we attempt to ablate one midbody is in updated Fig. 4b. A time series demonstrates that one midbody (marking a cytokinetic bridge) is lost after application of an ablation laser and the subsequent result was an inability to form a lumen. We also have shown the membrane (GFP-CAAX) during this series (updated Fig. 4b). It's difficult to determine whether an additional cytokinetic bridge could be in the vicinity of the ablation laser. Thus, we performed this experiment over a number of embryos when we ablate a midbody during apical clustering. We find that some lumens are unable to form, and some are able to form but are significantly smaller (refer to Fig. 4e-g). This could be due to a number of possibilities that include: 1) some cytokinetic bridges are more important in providing a landmark for initial lumen formation, 2) more than one cytokinetic bridge could have been accidentally ablated, or 3) adjacent cell-cell interfaces where adhesions could be important may have become disrupted. This last point we do not believe to be the case, because one of our controls is ablating cell-cell interfaces where no defects in lumen formation are calculated (Fig. 4d-g).

The question “How many midbodies are left after ablation?” is technically challenging to answer. In our experiments we try to only ablate one midbody (Fig. 4b) and if you refer to Supplementary Movie 6, the midbody is released into the expanding lumen following abscission and is very difficult to follow after that point. Thus, with our
current approaches it is difficult to monitor midbody number throughout the development of KV.

“Altogether, this is the only functional experiment of the study that is backing up the working model of the authors. It needs to be done carefully and ideally an alternative strategy should complement it.”

Based on your concern we added additional controls for the ablation studies (Fig. 4), an additional optogenetic system (Fig. 5 and 6), and small molecule inhibitors to block the cell cycle in general (Fig. 1d-e). We argue that these studies for the first time highlight the importance of cell division during KV development and de novo formation of its lumen.
Appendix References:


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Education and Training:

**B.S. Biology** State University of New York at Geneseo
Graduated: May 2015

**PhD Biology** Syracuse University
Hehnly Lab, Department of Biology
Successful Defense Date: 20 March 2020

Personal Statement:

My graduate research in Dr. Heidi Hehnly’s lab (Syracuse University) focuses on how cell division is used to create more complex, three-dimensional structures during development. To test this, I use the vertebrate developmental model *Danio rerio* (zebrafish) to uniquely address aspects of cell biology such as cell division and membrane trafficking. My studies have examined the mechanisms involved in spindle formation and subsequent placement in both *in vitro* cell culture (Colicino et al 2019 *Molecular Biology of the Cell*, Bucko et al 2019 *eLife*, Rathbun et al *Nature Communications*, *in press*, Taneja et al 2016 *Scientific Reports*, Taneja et al 2019 *Current Opinions in Cell Biology*), and in an *in vivo* developmental model (Colicino et al 2019 *Molecular Biology of the Cell*, Bucko et al 2019 *eLife*, Rathbun et al *Nature Communications*, *in press*). Additionally, my first author publication from Dr. Hehnly’s lab identified for the first time *in vivo* that the cleavage of the cytokinetic bridge is associated with lumen formation of the zebrafish organ of asymmetry, Kupffers Vesicle (KV, Rathbun et al *Nature Communications*, *in press*).

- Taneja N, Rathbun L, Hehnly H, Burnette DT. The balance between adhesion and contraction during cell division. *Current opinion in cell biology*. 2019 Feb 1;56:45-52. PMID: 30268802
Professional Memberships and Experiences
2016-present  American Society for Cell Biology (ASCB)
2017-present  Central New York Zebrafish Group
2018  Search Committee for SUNY Upstate Reference and Research Services Librarian
2018  Selection Committee for SUNY Upstate President’s Award for Research
2018-present  International Zebrafish Society (IZFS)
2018-present  Women in Science and Engineering (WiSE) Future Professionals Program at Syracuse University

Awards and Honors
- Travel Award to attend ASCB/EMBO 2019 conference (December 2019)
- Inaugural Summer Dissertation Fellowship (Summer 2019)
- 3rd Place Poster Award at International Zebrafish Society (IZFS) Meeting (June 2018)
- Multiple Travel Awards from SUNY Upstate to attend IZFS International Meeting (June 2018)
- Multiple Travel Awards from SUNY Upstate to attend the American Society for Cell Biology (ASCB) international conference (December 2016, 2017, 2018)
- Research Presentation Award for Best Poster (SUNY Upstate, Spring 2017)

Invited Seminars
November 2019  Developmental Biology New York (DBNY), Ithaca College, NY
Title: “The role of cell division during lumen formation”
July 2019  Donut Talk Seminar, Biology Department, University of Rochester, Rochester, NY
Title: “Shaping tissues through cell division”
April 2019  Central New York Zebrafish Meeting, Syracuse, NY
Title: “Optogenetic clustering of Rab11 vesicles during Kupffer’s Vesicle development”
June 2018  International Zebrafish Society Meeting, Madison, WI
Title: “Cytokinetic bridge triggers de novo lumen formation in vivo”
May 2017  Developmental Biology Interest Group of Syracuse, NY
Title: “Mechanisms regulating tissue expansion”
April 2017  Central New York Zebrafish Meeting, Syracuse, NY
Title: “The role of spindle orientation in embryonic patterning”
December 2016  American Society for Cell Biology (ASCB) International Meeting, San Francisco, CA
Title: “The role of division orientation in tissue patterning”
Contributions to Science

In collaboration with Dr. Dylan Burnette’s laboratory (Vanderbilt University, Nashville, TN), we found that focal adhesions asymmetrically assemble following anaphase underneath the oldest spindle pole to direct the placement of the mitotic spindle. These studies resulted in a publication in *Scientific Reports* (Taneja et al. 2016), and a review article in *Current Opinions in Cell Biology* (Taneja et al 2019).

- Taneja N, Rathbun L, Hehnly H, Burnette DT. The balance between adhesion and contraction during cell division. Current opinion in cell biology. 2019 Feb 1;56:45-52. PMID: 30268802

To further explore the mechanisms driving spindle placement in vivo, the zebrafish embryo was used. These studies were done either immediately post-fertilization or during the development of the zebrafish organ of asymmetry, Kupffer’s vesicle (KV). Our studies identified that during the first five rounds of cell division, the spindle synchronously orients with the spindles in the neighboring cells to set up the first monolayer of cells on top of the yolk (Rathbun et al., *in preparation*). Later in development, we found that spindle orientation is dispensable during KV development, but in this case placement of the cytokinetic bridge plays an important role in organizing cell placement post-mitosis in this developing tissue (Rathbun et al., 2020 Nat. Comm.).


Through work with Erica Colicino (graduate student in Dr. Hehnly’s laboratory), I utilized zebrafish embryos that expressed fluorescently labelled PLK1 to document its localization and asymmetry across the two mitotic centrosomes, and correlated this with the presence of missegregated or lagging chromosomes during mitosis. This project resulted in a publication at *Molecular Biology of the Cell* (Colicino et al 2019). Additionally, in a collaborative project with John Scott’s lab at University of Washington at Seattle, I utilized a centrosome-targeted version of the PLK1 inhibitor BI2536 to investigate the function of PLK1 specifically at zebrafish centrosomes.

Student Outreach

I am an active participant in the Skype a Scientist program, where I have been matched with classrooms around the country to video conference with K-12 students to discuss scientific topics and STEM as a potential career choice. Additionally, I have helped to organize a microsymposium with the high school biology class at Naples High School (Naples, NY) where we discussed the day-to-day activities in a graduate-level research lab, our paths to college/graduate school and the possible career options afterwards.

Community Outreach

I participate as a student organizer for the BioArt Mixer seminar series that serves as a bridge and conversation starter between the science and art communities of Syracuse University, SUNY Upstate, and the greater Syracuse area. Additionally, I volunteer with other graduate students at the Westcott Community Center in Syracuse, NY, where we lead students in scientific activities afterschool.

Mentor to Graduate/Undergraduate Students

Nicole Hall
Syracuse University undergraduate, September 2019-present

Abrar Aljiboury
Syracuse University graduate student, Spring 2019-present

Julie Manikas
Syracuse University undergraduate, Fall 2018-Spring 2019
Post-baccalaureate technician, Fall 2019 - present

Erin Curtis
Syracuse University post-baccalaureate technician, Nov. 2018-May 2019,
Current graduate student at Duke University

Shannon Coyne
SUNY Geneseo summer student, June 2017 - August 2017
Current medical student at Tuoro School of Osteopathic Medicine

Shelby Helwig
Lock Haven University summer student, June 2016 - August 2016
Current graduate student at University of Maine

Alice Garrastegui
Syracuse University undergraduate, August 2016 - May 2018
Current medical student at State University of New York University at Buffalo, Jacobs School of Medicine

Paul Lovell
Syracuse University undergraduate, August 2015 – May 2017
Current graduate student at University of Nebraska