Université de Montréal

Asymmetric Cell Division Intersects with Cell Geometry: a method to extrapolate and quantify geometrical parameters of sensory organ precursors

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Ce mémoire intitulé:

Asymmetric Cell Division Intersects with Cell Geometry: a method to extrapolate and quantify geometrical parameters of sensory organ precursors

Présenté par :
Arturo Papaluca

A été évalué par un jury composé des personnes suivantes :

Jean-Claude Labbé, président-rapporteur
Gregory Emery, directeur de recherche
Gilles Hickson, membre du jury

Résumé

La division cellulaire asymétrique (DCA) consiste en une division pendant laquelle des déterminants cellulaires sont distribués préférentiellement dans une des deux cellules filles. Par l'action de ces déterminants, la DCA générera donc deux cellules filles différentes. Ainsi, la DCA est importante pour générer la diversité cellulaire et pour maintenir l'homéostasie de certaines cellules souches. Pour induire une répartition asymétrique des déterminants cellulaires, le positionnement du fuseau mitotique doit être très bien contrôlé. Fréquemment ceci génère deux cellules filles de tailles différentes, car le fuseau mitotique n'est pas centré pendant la mitose, ce qui induit un positionnement asymétrique du sillon de clivage.

Bien qu'un complexe impliquant des GTPases hétérotrimériques et des protéines liant les microtubules au cortex ait été impliqué directement dans le positionnement du fuseau mitotique, le mécanisme exact induisant le positionnement asymétrique du fuseau durant la DCA n'est pas encore compris. Des études récentes suggèrent qu'une régulation asymétrique du cytosquelette d'actine pourrait être responsable de ce positionnement asymétrique du faisceau mitotique. Donc, nous émettons l'hypothèse que des contractions asymétriques d'actine pendant la division cellulaire pourraient déplacer le fuseau mitotique et le sillon de clivage pour créer une asymétrie cellulaire. Nos résultats préliminaires ont démontré que le blebbing cortical, qui est une indication de tension corticale et de contraction, se produit préférentiellement dans la moitié antérieure de cellule précurseur d'organes sensoriels (SOP) pendant le stage de télophase.

Nos données soutiennent l'idée que les petites GTPases de la famille Rho pourraient être impliqués dans la régulation du fuseau mitotique et ainsi contrôler la DCA des SOP. Les paramètres expérimentaux développés pour cette thèse, pour

étudier la régulation de l'orientation et le positionnement du fuseau mitotique, ouvrirons de nouvelles avenues pour contrôler ce processus, ce qui pourrait être utile pour freiner la progression de cellules cancéreuses. Les résultats préliminaires de ce projet proposeront une manière dont les petites GTPases de la famille Rho peuvent être impliqués dans le contrôle de la division cellulaire asymétrique in vivo dans les SOP. Les modèles théoriques qui sont expliqués dans cette étude pourront servir à améliorer les méthodes quantitatives de biologie cellulaire de la DCA.

Mots-clés : Précurseurs d'organe sensoriel (SOP), mécanisme du fuseau mitotique, cellule sort déterminants, petites GTPases, blebbing cortical.

Abstract

Asymmetric cell division (ACD) consists in a cellular division during which specific cell fate determinants are distributed preferentially in one daughter cell, which then differentiate from its sibling. Hence, ACD is important to generate cell diversity and is used to regulate stem cells homeostasis. For proper asymmetric distribution of cell fate determinants, the positioning of the mitotic spindle has to be tightly controlled. Frequently, this induces a cell size asymmetry, since the spindle is then not centered during mitosis, leading to an asymmetric positioning of the cleavage furrow.

Although small small GTPases have been shown to act directly on the spindle, the exact mechanism controlling spindle positioning during ACD is not understood. Recent studies suggest that an independent, yet uncharacterized pathway is involved in spindle positioning, which is likely to involve an asymmetric regulation of the actin cytoskeleton. Indeed, actin enables spindle anchoring to the cortex. Hence we hypothesize that asymmetric actin contractions during cytokinesis might displace the mitotic spindle and the cleavage furrow, leading to cell size asymmetry. Interestingly, from our preliminary results we observed that cortical blebbing, which is a read-out of cortical tension/contraction, preferentially occurs on the anterior side of the dividing sensory organ precursor (SOP) cells at telophase.

Our preliminary data support the idea that Rho small GTPases might be implicated in regulation of the mitotic spindle hence controlling asymmetric cell division of SOP cells. The experimental settings developed for this thesis, for studying regulation of the mitotic spindle orientation and positioning will serve as proof of concept of how geneticist and biochemist experts could design ways to control such process by different means in cancerous cells. The preliminary results

from this project open novel insights on how the Rho small GTPases might be implicated in controlling asymmetric cell division hence their dynamics *in vivo* of such process during SOP development. Furthermore, the assays and the theoretical model developed in this study can be used as background that could serve to design improved quantitative experimental methods for cell biology synchronizing sub-networks of ACD mechanism.

Keywords: Sensory organ precursors (SOP), mitotic spindle mechanism, cell fate determinants, small GTPases, cortical blebbing.

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Dedicated to my Mother and Father for everything!

To those who are longing to change and ready to change to a meaningful and successful life of abundance and happiness.

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As always,
May the Beer in your glasses never end!
May the Rock N' Roll in your lives always be loud!
May Eternal Peace never leave your soul!
Cheers!

1. Introduction

1.1. Preamble : Subject Situation

The tight control of cellular self-renewal, cell fate diversity and daughter cells differentiation is orchestrated by extrinsic and intrinsic asymmetric cell divisions[1,2]. Understanding this mechanism of generation of distinct cell fates is pivotal in specific areas of research such as drugs development and cancer medical therapy. Several research works have reported a strong connection between polarity proteins and small GTPases, showing that activation of these proteins requires physical interactions with constitutively active small GTPases[3,4]. The small GTPases family of proteins have been the focus of cancer research since the discovery of the isoform *H*-Ras p21 mutant in human tumour cells [5]. This discovery highlighted the importance of the implication of proteins from the small GTPases family in different types of diseases and cellular processes. Therefore, they represent important targets for symmetric and asymmetric cell division pathway activation and control, since they can regulate a wide variety of related functions[6,7] serving as excellent candidates to shed more light into the regulation of asymmetric cell division.

Previous work done by Cabernard *et al*.[8] demonstrated a spindle-independent mechanism for cleavage furrow positioning in *Drosophila melanogaster* neuroblasts. They identified that furrow specific proteins are localized at the basal cortex at anaphase onset and can induce furrow displacement in the total absence of the mitotic spindle. The authors showed a very interesting mechanism for asymmetric cell division leading to the hypothesis that another regulatory pathway possibly involving small GTPases might act through the actin cytoskeleton. Such mechanism raises several questions: (i) Whether Rho small GTPases and their connection to polarity proteins can regulate mitotic spindle orientation? (ii) If they regulate the stability of the cleavage furrow's position? (iii) Whether they influence

the balance of polar forces and tension release that define asymmetric division? (iv) How spindle positioning can be quantified in order to statistically differentiate between wild type and abnormal conditions? To start answering such questions, we used *Drosophila melanogaster* sensory organ precursors (SOP) as a model system since it allows the use of genetic tools and advanced *in vivo* 4D time-lapse microscopy techniques. The aims of this master thesis are (i) To determine geometrical parameters of SOP division, (ii) Determining the sub-cellular activation of Rho small GTPases during asymmetric cell division (ACD) and (iii) Assess the effect of perturbing the actin cytoskeleton on SOP division.

Using high-resolution 4D confocal microscopy techniques, we developed a simple method that uses available geometrical parameters to assess perturbations of ACD in SOPs due to abnormal Rho small GTPase activity, which allows us to determine spindle positioning over time regarding to other axes of polarity. Our approach will help to better understand the mechanism of mitotic spindle positioning and how it can be regulated by influential polarity proteins and individual Rho small GTPases during ACD. Moreover, our method could be implemented for other investigations to extract parameters in order to differentiate important observations in the asymmetrically dividing cells of the sensory organ precursors; such as mitotic spindle positioning, bleb formation and polarity crescent formation-expansion at metaphase, to mention a few.

1.2. Asymmetric Cell Division

The process of asymmetric cell division was first described a century ago by American biologist Edwin Conklin. Using ascidian embryos, he observed that during early division, an area of yellow cytoplasm was always co-segregating with cells that will eventually differentiate from the others and become muscle cells[9]

(**Figure 1**). This observation opened the field of study of asymmetric cell division until today. The process of asymmetric cell division has been fascinating scientists for more than a century, leading research in the field using various model organisms such as the worm *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster* and mammalian systems like *Mus musculus*[1,2]. Cellular diversity is generated by the processes of symmetric and asymmetric cell divisions. Following symmetric cell divisions, daughter cells can acquire different fates depending on the cellular environment. Hence, this type of cell diversity is known to be extrinsic. Cellular self-renewal is also orchestrated by the asymmetric distribution of different cell fate determinants occurring in several steps processes recognized as intrinsic asymmetric cell divisions[1]. Although asymmetric cell division has fascinated scientists for over a century, a thorough understanding of the underlying mechanisms has only recently emerged. Much of this increased knowledge has come from studies in *Drosophila melanogaster* and *Caenorhabditis elegans* that have led to the identification of conserved cellular principles and molecular players that govern asymmetric cell division.

Intrinsic asymmetric cell division occurs in simple step processes. First, after –mother cell- symmetry breaking, the mother cell becomes polarized. Second, cell-fate determinants are segregated towards both distinct poles of the mother cell. Third, the mitotic spindle is aligned so in turns the cleavage furrow results in the proper inheritance of cell-fate determinants to the daughter cells. Fourth, during mitosis following by cytokinesis, different fates for the daughter cells are established. As a result of these crucial steps, the generation of two daughter cells born at the same time, are not identical[2,10]. Therefore, asymmetric cell division is pivotal for generating cell diversity. In this thesis we focus on intrinsic asymmetric cell division using the well known biological model system *Drosophila melanogaster.*

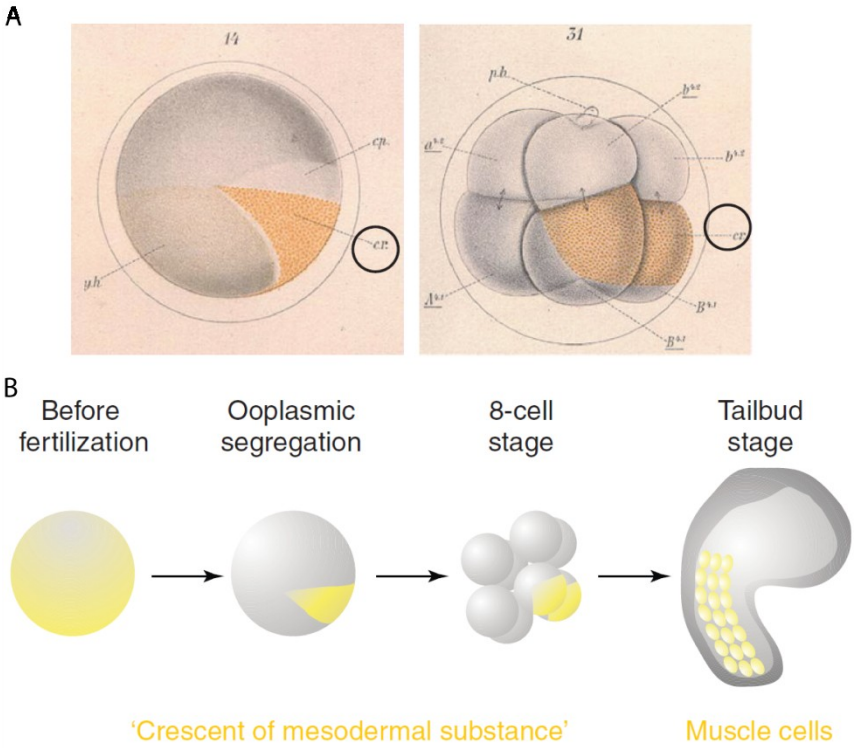
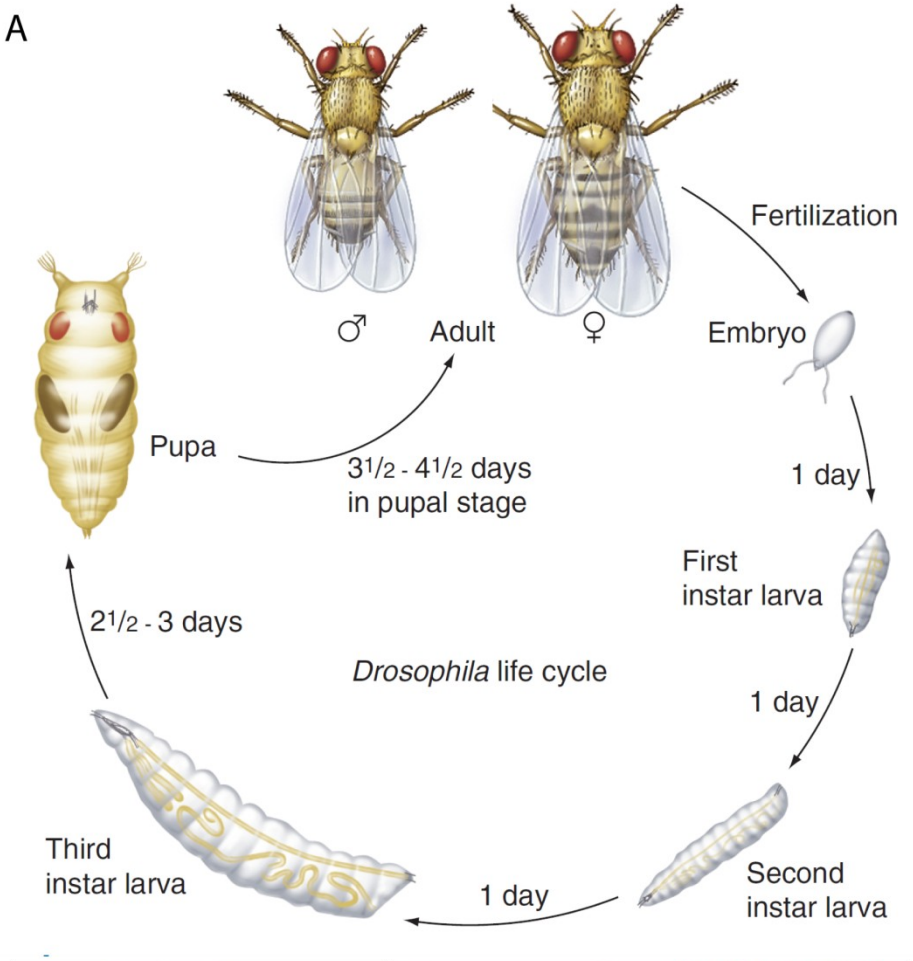


Figure 1: Discovery of Asymmetric cell division with the Ascidian embryo. Depicted is Edwin Conklin's original drawings of a one-cell stage (left) and eight cell stage (right) embryo **(A)** Yellow pigment representing the crescent (cr) of mesodermal substance (marked by black circles) co-segregating with muscle cells of the tadpole. **(B)** Schematic representation of Edwin Conklin's observations pinpointing the asymmetric segregation and localization of the yellow pigment forming muscle cells (Adapted from [9,11]).

1.3. *Drosophila melanogaster* as a model system

The fruit fly *Drosophila melanogaster* serves as one of the most studied biological systems and is a splendid model for studies towards understanding cellular processes and development of multi-cellular organisms. Used in physiology and genetics studies, *Drosophila melanogaster* contributes to the development of a broad variety of genetics and microscopy tools which have been carefully designed and optimized to study any specific gene function in this wonderful organism [12, 13]. For the purpose of this master's thesis, live imaging is a crucial tool in order to understand *in vivo* processes occurring throughout the entire cell cycle. Using the fruit fly *Drosophila melanogaster* as a model, we are able to fulfill such a requirement since it allows *in vivo* tracking of each step of the dynamic process of development at both tissue and cellular levels[14, 15, 16].

The approach taken in this research project consists in following asymmetric cell divisions linked exclusively to the proper and tight alignment of the mitotic spindle. *Drosophila melanogaster* provided us with the working model of choice, the Sensory Organ Precursors. These particular cells display a wide range of asymmetric morphologies such as daughter cells size and asymmetric division variants like cell fate determinants, which helped the development of this project. Moreover, this multi-cellular organism allows the *in vivo* study of developmental processes like the cell cycle, actin cytoskeleton organization, cellular trafficking, memory systems, metabolic regulation, signalling processes, chromosome recombination and receptor behaviour occurring during development [17,18].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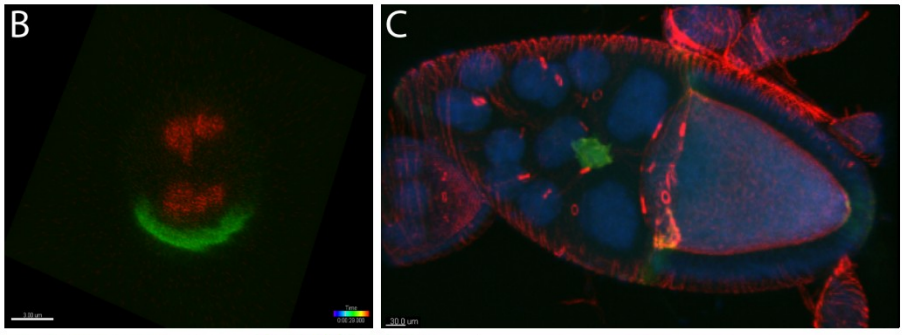
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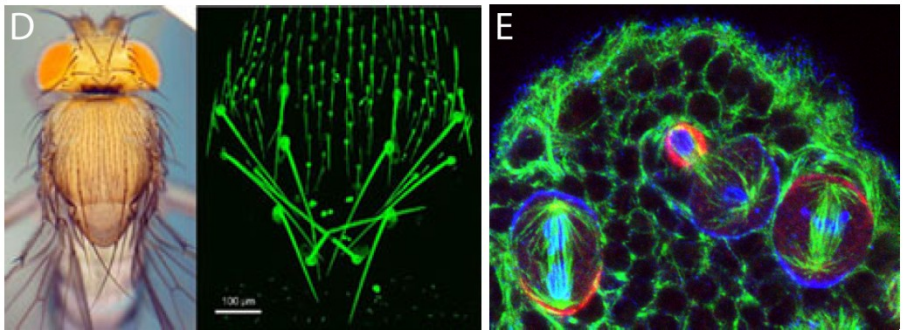
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Figure 2 : *Drosophila melanogaster* as a model system. **(A)** The Drosophila life cycle. The transition from an embryo to a first instar larva is called hatching. The transitions between larval instars are molts. The process that converts a third instar larva to a pupa is pupariation. Emergence of the adult from the pupal case is called eclosion (Adapted from Genetics: From Genes to Genomes Book [19]). (B, C, D, E) Examples of live imaging using diverse *Drosophila* cell lineages **(B)** Sensory Organ Precursor cell at anaphase onset during asymmetric cell division. Cell fate determinants (green) co-segregate with anterior PIIB daughter cell. DNA (red) **(C)** Egg chamber with migrating border cells cluster (green) **(D)** An adult fly expressing GFP-actin in bristles and socket cells (Guild Lab, University of Pennsylvania) **(E)** Regulation of cell fate within neuroblast cell lineages (Doe Lab, Institute of Neuroscience, University of Oregon). Images in B and C were acquired at the IRIC Bio-imaging facility, Emery Lab.

1.3.1. Asymmetric cell division in *Drosophila melanogaster*

Asymmetric cell division of somatic cells was first described in *Drosophila melanogaster* by Rhyu *et al*. [20]. They characterized the function of asymmetrically distributed cell fate determinant Numb. Rhyu *et al.* observed that during mitosis, the fate determinant protein Numb was always segregating towards one of the two daughter cells [20]. They observed that Numb localized on one side of the cell forming a crescent during early metaphase. This observation became a characteristic behaviour of cell fate determinants which also led the identification of others. Furthermore, it was shown that Numb is implicated in the regulation of external sensory organs [21]. Partial or total loss of Numb leads to abnormal development of external sensory organs supporting the importance of proper cell fate determinants inheritance amongst daughter cells during asymmetric cell division.

1.3.2. Strength of Sensory Organ Precursors to study asymmetric cell division

Asymmetric cell division of Sensory Organ Precursor (SOP) cells occurs along the anterior-posterior axis of the fly notum. Single SOP cells "PI" are able to generate two daughter cells of different sizes and fates. The anterior "PIIB" cell gives rise to neurons and sheath cells and the posterior "PIIA" cell gives rise to socket and hair cells[11,22] (**Figure 3**). More specifically, this cell fate differentiation comes from complex signaling cues between PIIA and PIIB. This mechanism of differentiation requires the ligand Delta in PIIB and the receptor protein Notch in PIIA cells. This is one of the mechanism responsible for different cell fate distribution at the moment of division[22,23]. Directional signalling between PIIA and PIIB is in part established through the asymmetric distribution of Numb and Neuralized (Neur). Numb and it's anchor protein Partner of Numb (Pon) act as cell fate determinant markers during asymmetric cell division being unequally localized

in SOP cells[24]. Numb is inherited by the anterior PIIB cell where Notch signal is shut down, and is absent in the PIIA cell where Notch signal is active (**Figure 4**). Numb and its partner Sanpodo play a role in establishing Notch signaling at cytokinesis onset[20,25]. Numb regulate Notch trafficking and establishes directional signaling during cytokinesis[25]. In neuroblasts and SOP cell lineages, Numb's localization at the pole is facilitated by its anchor protein Pon[2]. These two proteins have been very instrumental for live cell imaging studies of asymmetric cell division in *Drosophila melanogaster*.

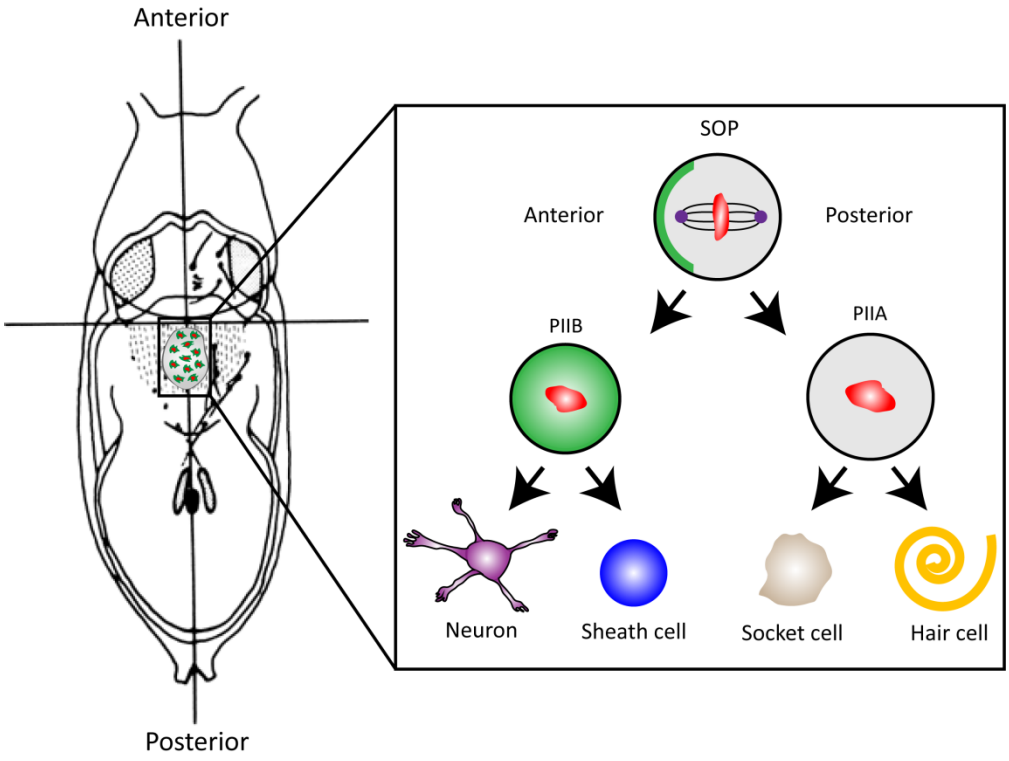


Figure 3: Model for asymmetric cell division in *Drosophila melanogaster* sensory organ precursor (SOP). All SOP cells divide along the anterior-posterior axis of the pupa. Cell fate determinants (green) are segregated into the smaller anterior daughter cell (PIIB), making it different from its posterior sibling (PIIA). PIIB gives rise to neurons and sheath cells, whereas PIIA gives rise to socket and hair cells.

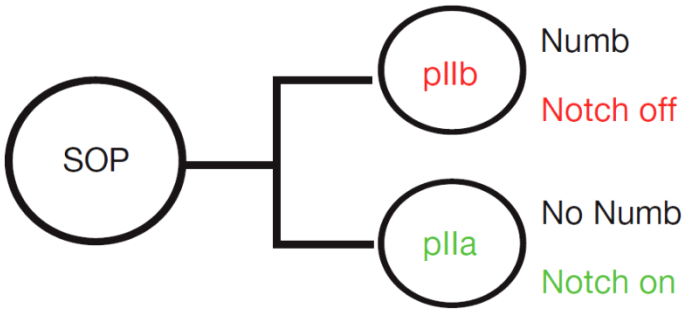


Figure 4: Numb-Notch activation during asymmetric cell division of SOP cells. (Adapted from Couturier *et al*.[25])

1.4. Molecular regulators of asymmetric cell division in sensory organ precursors

1.4.1. Polarization

The Par protein complex has a conserved function in establishing proper cell polarity during asymmetric cell division in *C. elegans* and *Drosophila melanogaster*[10]. This occurs by a series of phosphorylation events, which has been proposed to also take place in SOP cells. At mitosis, activation of the mitotic kinase Aurora-A promotes a phosphorylation cascade. When phoshorylated, Aurora-A in turns phosphorylates and thus activates a aPKC's regulatory subunit Par6. During interphase, Lgl gets phosphorylated leading to its release from the cell cortex. It is then released from aPKC, which contributes to the dissociation of the Par6/ aPKC /Lgl complex. Next, Baz is recruited to form the Par6/aPKC/Baz complex, allowing aPKC to phosphorylate Numb leading to its localization at the anterior pole the cell cortex hence being inherited by PIIB[26]. This mechanism reveals how Numb is

localizing asymmetrically and demonstrates how cell polarity can be linked to the cell cycle. Moreover, loss of the Par polarity complex at the cortex abrogates the mitotic spindle positioning during anaphase, resulting in the formation of daughter cells of equal sizes[27,28]. Therefore, both the Par complex and cell shape changes contributes to the regulation of the orientation and position of the mitotic spindle demonstrating the importance of the Par complex.

This cell polarity model can be summarized in four simple steps. (i) During mitosis, the Par proteins along with cell fate determinants set up a polarity axis. (ii) This axis is used for mitotic spindle positioning and for asymmetric localization of cell fate determinants at the cell poles. (iii) During the transition from anaphase to telophase, this tightly controlled orientation, positions the mitotic spindle ensuring proper asymmetric localization of cell fate determinants (iv) At two cell stage, cell fate determinants are inherited by only one daughter cell[2,24,29]. Our study focuses on the possible mechanisms that regulate the orientation and position of the mitotic spindle as the driving force for asymmetric cell division in SOP cells.

1.4.2. Segregation

Several mechanisms of unequal protein segregation have been proposed to occur through a phosphorylation cascade[26,30]. One of them occurs in the *Drosophila* neuroblast cell lineage, Partition defective (Par) proteins Par6, Baz and aPKC form a complex and localize at the apical pole guiding the localization of the cell fate determinants Prospero (Pros), Numb, his anchor protein Pon and the adaptor protein Miranda (Mira) to the basal pole. This tight localization ensures proper segregation into the basal daughter cell[2,11,30]. Next, the Par complex phosphorylates the cytoskeletal protein Lethal (2) giant larvae (Lgl) recruiting cell fate determinants to the cortex. aPKC phosphorylates Lgl leading the release from the cortex and the actin cytoskeleton, prohibiting the localization of cell fate determinants to the apical

pole and excluding Numb and Neuralized from the posterior pole. This phosphorylation event restricts Lgl activity and Miranda localization to the basal pole of the cell[30]. Despite this precise phosphorylation mechanism, an over expression of a non-phosphorylatable version of Lgl (Lgl3A) is sufficient to disrupt cell fate determinants destiny.

1.4.3. Spindle Orientation

During cell fate diversity generation, the mitotic spindle plays a pivotal role, orchestrating the mechanisms for unequal segregation of cell fate determinants, influencing the proper inheritance by the two daughter cells and assuring appropriate cell size. The mitotic spindle is a conserved cell division structure from yeast to humans[28]. This machinery features two spindle poles from which emanate three classes of microtubules from their minus-ends, (i) kinetochores, that are attached to chromosomes, (ii) interpolar microtubules, that are structured in an antiparallel fashion in the middle of the spindle poles and (iii) astral microtubules, that diverge towards the cell cortex from the spindle poles and use their plus-ends to attach the spindle to the cell cortex[28,31]. The tight coordination of these structures orchestrates cell division, serving as a pulling force for chromosome segregation.

Mitotic spindle positioning depends on subtle interactions between astral microtubules and the cell cortex[32]. The dynein-dynactin complex is the main player responsible for mitotic spindle alignment along the anterior-posterior axis of the pI cell in *C. elegans* embryos[33]. The action of the dynein-dynactin complex is a conserved spindle alignment and pulling force mechanism across species[28]. Dynein associates with the dynactin complex which foster dynein to its cargo proteins allowing the complex moving the spindle towards the cortex[34,35]. In *D. melanogaster*, several proteins are needed for polarity and spindle position during asymmetric cell division. Such proteins Numb and Pon co-localize with Partner of

inscuteable (Pins), Locomotion defects (Loco), Mushroom body defective (Mud) and Gαi forming the Mud-Pins-Loco-Gαi complex at the anterior side of neuroblasts and SOP cells[10,36]. Par proteins Par6, Baz and aPKC and smallGTPase Cdc42 associate with the Mud-Pins-Loco-Gαi complex through the dynein-dynactin complex allowing proper orientation and positioning of the mitotic spindle during asymmetric cell division[28]. The control of mitotic spindle orientation and positioning in developmental systems is based on the full coordination of the previously mentioned mechanism and the activity of cortical blebbing.

1.4.4. Mitosis and Cytokinesis

During mitosis, following by cytokinesis, several components are pivotal for proper cell-fate inheritance by the two daughter cells. Time-lapse quantitative experiments demonstrated that Pon-GFP is recruited to the cortex of the PI cell upon progress into mitosis becoming enriched on the anterior pole of the SOP[11]. Proteins like actin and Myosin II are required for –anterior pole- enrichment of Numb and its anchor protein Pon, suggesting a mechanism that drives asymmetric segregation of cell-fate[10]. In turns, Pon and Numb are inherited only by the PIIB daughter cell. In SOP cells, polarity proteins Par6, Baz and aPKC interact with each other forming the Par complex which localizes to the posterior pole cortex. The posterior localization of the Par complex along with the anterior localization of Numb, Pon and Neuralized and spindle proteins Mud, Pins, Loco and Gαi, establish the axis of polarity, essential for spindle orientation and asymmetric protein localization during mitosis. Finally, at cytokinesis, cell-fate determinants are inherited respectively by only one daughter cell (**Figure 5**).

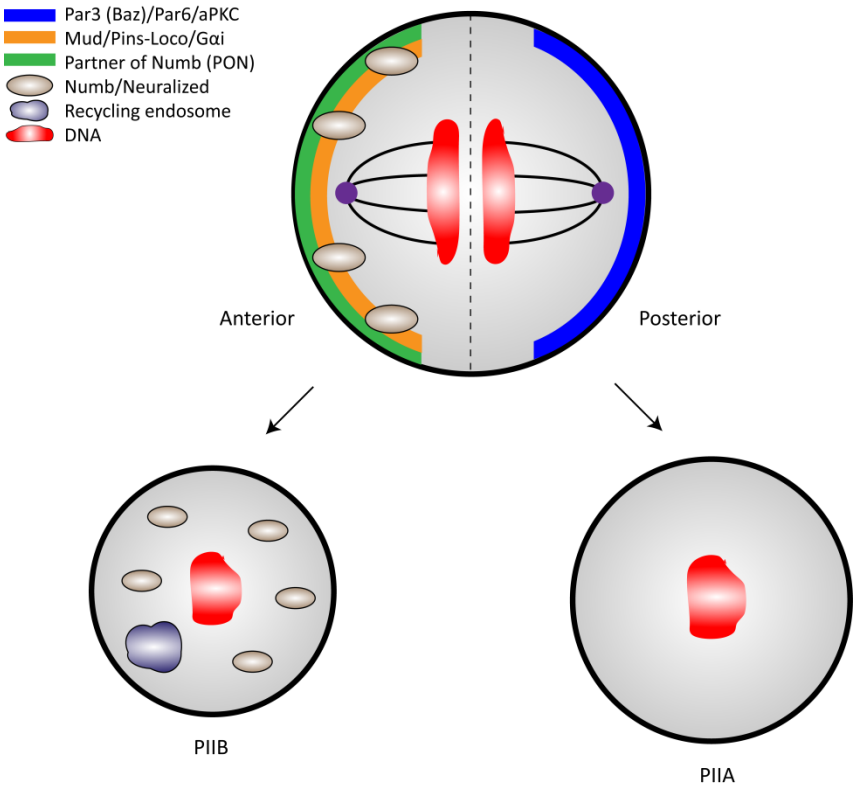


Figure 5: Proper spindle orientation leads to proper segregation of cell-fate determinants. First: spindle orientation controls the axis of cell division and determine cell-fate determinants segregation in an asymmetric fashion. Second: position of the spindle within the dividing cell determine the relative size of the two daughter cells.

1.5. The small GTPases family of proteins

Developmental processes in multi-cellular organisms are controlled by specific proteins, which are part of a wide variety of complex signaling networks. Amongst those regulatory proteins is the Ras family of guanosine triphosphates (small GTPases). The small GTPases family is constituted of the Ras, Rho, Rab, Ran and Arf subfamilies. Each of these subfamilies is found in different functional branches across species[37]. These proteins are of special interest because they regulate intracellular signal transduction pathways in response to external and internal stimuli. They act as molecular binary switches that are either turned on or off depending on the cell's needs (**Figure 6**). Small GTPases are known to be involved in various coordinated processes such as cell polarity [3,38], polarized growth [39], collective cell migration [40], vesicle trafficking [41,42], actin and septin organization and development [43], cell cycle regulation and cell survival [44]. This conserved family of proteins has been well studied in humans, budding yeast *Saccharomyces cerevisiae*, the fruit fly *Drosophila melanogaster* and the worm *Caenorhabditis elegans*[44,45]. Thus, small GTPases serve as an excellent working mechanism for the development of complex signaling processes established at both levels of functional and structural levels [46].

1.5.1. The smallGTPases function as molecular switches

The small GTPases family of proteins features a GDP-GTP cycle mechanism which is similar among small GTPases subfamilies such as Ras, Rho, Rab, Ran and Arf. The small GTPases cycle of activation and inactivation is controlled by GEFs (Guanine Exchange Factors) that stimulate the exchange of GDP into a GTP. The inactivation is controlled by GAPs (small GTPase activating proteins) that promote hydrolysis from GTP into GDP. The mechanism of small

GTPases activation and inactivation relies on specific membrane receptors, which sense extracellular signals, leading to the initiation of complex signal transduction pathways. This particular mechanism leads to the recruitment of a specific GEF for the activation of a small GTPase through binding to GTP (GTP-bound state). These GEF proteins can act specifically on one small GTPase or on several of them. This active signal is controlled when GTP gets hydrolyzed ending in a GDP-bound state. Hence, cellular behaviours can be determined by single or multi small GTPase specificity [47].

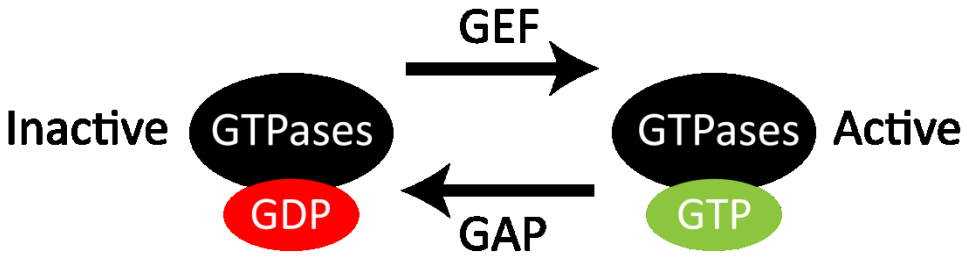


Figure 6: The GDP-GTP cycle of small GTPases. These proteins are in their active state when bound to a GTP molecule and are inactive when bound to a GDP molecule. Small GTPases activation is controlled by GEF (Guanosine Exchange Factors) that stimulates the exchange of GDP into a GTP and inactivation is controlled by GAP (small GTPase Activating Proteins) that hydrolyse the GTP into GDP.

1.5.2. The small GTPases function in *Drosophila melanogaster*

In the past years, the fruit fly *Drosophila melanogaster* has risen as a wonderful genetic system for the study of small GTPase proteins in developmental and molecular processes. We are interested in better understanding how the particular family of Rho small GTPases is implicated in the actin cytoskeleton, cell polarity and asymmetric cell division.

1.5.2.1. The Rho small GTPases activity

The Rho subfamily of small GTPases was found to be evolutionary conserved across species[37,48]. This particular subfamily of proteins is in charge of maintaining the appropriate cell morphology and coordinates migratory movements, which are essential for homeostasis and dynamic processes [49]. The principal members of this family are Rho, Rac and Cdc42. These proteins function as molecular binary switches changing from a GTP-bound active state to a GDP-bound inactive state, depending on intra or extra-cellular signals (Section 1.5.1). Rho small GTPases are also regulated by third class of regulatory protein called Guanine Nucleotide Dissociation Inhibitors (GDIs). These regulators not only prevent the GDP ↔ GTP exchange cycle, but also maintain proteins in their GDP inactive state and prevent their localization at the membrane [6,7,50].

The fruit fly *Drosophila melanogaster* undergoes several morphological changes during development [19]. Therefore this subfamily of proteins participates actively in many processes including regulation of the actin cytoskeleton, cell growth, cell fates, cell survival and differentiation, axonal guidance, cell-cell interaction and cell proliferation, which implicates control of the cell cycle [7]. These are fundamental processes that are essential for development in higher organisms including *Drosophila*. The small GTPases Rac1 and Cdc42 are the most

investigated since they are involved in regulating many cellular functions through protein-protein interaction dynamics. This diverse regulation happens via a number of effector molecules which have been well characterized in structure and functions[7,51]. Moreover work done by members of the Emery lab, demonstrated that Rac1 activity and polarization during collective cell migration is regulated by members of the Rab small GTPases family[52]. The actin cytoskeleton organization plays an important role in determining cell polarization and proper distribution of cell fate determinants. This section describes such processes and the involvement of small GTPases Rac1 and Cdc42 in the generation of several cell lineages and specific functions in fruit flies.

1.5.2.1.1. General roles of Rac1 and Cdc42 on the actin cytoskeleton

The small GTPase Rac1 is able to control through a series of complex signaling pathways, some of the most important processes of cell morphology. The principal roles of Rac1 include regulation of the actin cytoskeleton, epithelial morphogenesis and axon growth and guidance [53]. This particular small GTPase has two homologs, Rac2 and Mtl, having overlapping roles in the control of *Drosophila* development [54]. To this date, not much information is known about Rac1 being involved in regulating asymmetric cell division in SOP cells. Our focus on Rac1 relies particularly on evidence suggesting it has one of the principal roles in regulating the actin cytoskeleton. Rac1 is present in almost all eukaryotic systems, conserved from yeast to humans [55]. Such conservation suggests that basic mechanisms involved in cell morphology were conserved during evolution. These mechanisms have designated the finest tasks in development and maintenance of the actin cytoskeleton. In *Drosophila,* mammalian systems and other organisms, rearrangement of the actin cytoskeleton is necessary for cell shape changes driving

cell movements and migration. These dynamic changes are responsible for cell migration in higher organisms[48,56].

Cell migration features a series of subsequent dynamic actions, including lamellipodia extension, formation of focal adhesions and contractions, all requiring tight control of the actin cytoskeleton and it's downstream effectors[57]. These include WAVE/Scar, Sra1, PAK and Plexin-B1 amongst the most notorious effectors involved in actin cytoskeleton regulation [7]. Rac1 is able to induce localized actin branches formation, which leads the generation of polarized morphological changes known as protrusions. These protrusions, in cooperation with other mechanisms help the cell to control the direction of migration [58]. Also, FRET biosensors data revealed that Rac1 localizes at the leading edge of these protrusions *in vivo*, suggesting a strong influence on remodelling of the actin cytoskeleton for cell movement[59].

The multiple roles of Rac1, Cdc42 and other small GTPases became evident when extensive studies began implementing ectopic expression of constitutively active and dominant negative mutant of these proteins [60]. For small GTPases, a constitutively active mutant is when they are unable to hydrolyze GTP and a dominant negative mutation is when they are unable to remove GDP [61]. The most common constitutively active mutants are found in the P-loop, when the catalytic Glycine (G) residue at position 12 is exchanged by Valine (V) (G12V) and in the catalytic residue, when Glutamine (Q) at position 61 is exchanged by Leucine (L) (Q61L) resulting in a GTP-lock state [44,61,62]. As well, the most notorious dominant negative mutants are based on the Ras S17N, founded as Serine (S) at position 17 is exchanged by Asparagine (N) (S17N) and Aspartic Acid (D) at position 119 is exchanged by Asparagine (D119N)[63,64]. These single nucleotides substitution lead to a GDP-lock state unable to interact with downstream effectors. Such mutations

have been found in cancerous cells when small GTPases functions have been completely abrogated[5].

Recently, a few examples of altered Rac1 and Cdc42 small GTPase's function have been reported in lymphocytes development, differentiation, activation and migration. It has been demonstrated that Rac1 has a pivotal role in B cell development, where loss of Rac1 blocks migration processes. This leads to an arrest in B cell development in the spleen, showing that Rac1 is required during the earlier stage of transitional B cells in mammalian systems[65]. Rac1 and Cdc42 coordinate actin polymerization and hence cell motility *in vivo*. This dynamic coordination is completely loss when known dominant negative and constitutively active versions of Rac1 and Cdc42 are expressed[66]. Data regarding actin cytoskeleton regulation also suggest a coordinated task between Rac1 and Cdc42[67]. This spatiotemporal coordination between Rac1 and Cdc42 has been observed using FRET biosensors, such as when activation between these small GTPases overlap, it results in a protrusion-retraction cycle[68]. Moreover, the use of a dominant negative version of Rac1 leads to memory deregulation acting through remodelling of the actin cytoskeleton[69]. All these results confirm the implication of Rac1 and Cdc42 in coordinating the actin cytoskeleton in higher organisms.

Cdc42 is known as the master regulator of cell polarity[70], is a highly conserved small GTPase essential for establishment and maintenance of cell homeostasis from yeast to humans[37]. It acts as a molecular binary switch modulating a wide range of signalling processes. Mutant versions of Cdc42 show defects in the organization of actin cytoskeleton and septins, which have pivotal roles during progression of the cell cycle. This main regulator is known to be involved in processes like actin patch polarization[38] and controlling the formation of actin bundles containing filopodia at the cellular periphery. Furthermore, Cdc42 regulates the pheromone response pathway[71], actin cable nucleation and septin organization,

which are implicated in the maintenance of cell morphology[72]. Cdc42 functions at the plasma membrane, localized at specific domains, and coordinating polarized organization of the actin cytoskeleton during cell migration. In the next section I will describe the link between Cdc42 and polarity proteins and their involvement in the regulation of asymmetric cell division in *Drosophila* neuroblast cell lineages and the worm *Caenorhabditis elegans*. These are the major reasons why we chose the small GTPases Rac1 and Cdc42 and to study their involvement in the regulation of the mitotic spindle during asymmetric cell division. They are very efficient at inducing different phenotypes through coordination of actin cytoskeleton dynamics. Moreover, they can induce malignant cells forming tumours in humans.

1.5.3. Cdc42, Rac1 and their relation with Par proteins

As described above, the small GTPases Rac1 and Cdc42 play key signaling roles in regulating cell polarity and the actin cytoskeleton. The two smallGTPases share 70% sequence conservation and identity among them and with human homolog Rac1 and Cdc42[73,74]. This suggests that non-conserved sites might be defining different specificities and thus specific functions[75]. Since there is a strong link between Cdc42 and Par proteins as well as sequence similarity between Cdc42 and Rac1, we wondered whether Rac1 could contribute to the regulation of polarity cues. The first link between Rho smallGTPases, polarity proteins and asymmetric cell division came from research done in *Caenorhabditis elegans* and *Drosophila melanogaster.* In these eukaryotes, the unequal distribution of polarity proteins and cell fate determinants coordinates local differences regarding the actin cytoskeleton and actomyosin meshwork[10].

The Par proteins were first indentified in a genetic screen using the embryos of the worm *C. elegans*. It encodes six different proteins all required for proper asymmetric cell division[76]. After fertilization, *C. elegans* embryos divide

asymmetrically along the anterior-posterior axis of the cell. Par3 and Par6 proteins segregate to the anterior pole, whilst Par1 with Par2 segregate to the posterior pole. This coordinated localization of Par proteins leads to an actomyosin meshwork restricted to the anterior pole which promotes contractility while the posterior pole remains non-contractile[77,78]. The small GTPase Cdc42 provides the link and regulates the actomyosin complex with Par proteins through a series of protein-protein interactions[77,79].

Similar to *Drosophila* sensory organ precursors, the neuroblast cell lineages divide asymmetrically, but along the apical–basal polarity plane following a similar protein segregation mechanism in both SOPs and *C. elegans*. During asymmetric cell division of neuroblasts, the polarity proteins Bazooka (Baz, Par3 homolog in *Drosophila*), aPKC and Par6 segregate to the apical pole. Alignment of the mitotic spindle along the apical–basal axis is controlled by Scribble (Scrib), Discs large (Dlg) and Lethal giant larvae (Lgl)[2]. Cell fate determinants Prospero, Brat and Numb localize at the basal pole and hence segregate into the basal daughter cell[2,11]. The basal localization of cell fate determinants depends on the asymmetric actomyosin contraction at the apical pole of neuroblasts[80]. This regulation of actomyosin occurs by apical restriction of Myosin II (Squash (Sqh) in *Drosophila*) by Lgl. This mechanism takes place when Lgl gets phosphorylated by aPkc, which restricts Lgl at the apical pole[30]. Par6 and aPkc establish polarity by localizing on the apical pole of the daughter cell. It has been reported that apical localization of Par6 requires physical interaction with Cdc42, which acts downstream of Baz to establish polarity[81]. Dominant negative and constitutively active versions of Cdc42 are able to dislocate such epithelial polarity[3], demonstrating the role of a small GTPase in the regulation of epithelial polarity during asymmetric cell division of *Drosophila* neuroblasts.

Despite the sequence similarities between *Drosophila* small GTPases Rac1 and Cdc42, *in vitro* binding assays between *Drosophila* Par6 and Rac1 does not show a physical interaction, with neither wild type, dominant negative nor constitutively active versions of Rac1[3]. However, a physical interaction between *Drosophila* Par6 and Rac1 has been detected in a yeast two-hybrid assay screen[82]. Also, a physical interaction between mammalian Rac1 and Par6 has been detected, suggesting a possible role in coordinating polarity of asymmetric cell division in mammals[83]. These overlapping results led us to investigate protein-protein interactions between Par proteins and the small GTPases Rac1 and Cdc42.

Cdc42 and Rac1 interact with proteins that feature a short conserved motif named CRIB (Cdc42/Rac1 interacting binding) [84]. Remarkably, Par6 possesses a semi-CRIB motif and an adjacent PDZ domain required for its biological regulation and interaction with Cdc42 and Rac1[4,85]. The crystal structure of a complex between Cdc42 and Par6 inducing a conformational change upon direct binding has been reported. As well for Rac1[4] but with lower affinity due to two overlapping residues in sequence with Cdc42[86]. To this point, published data suggests connection between Rac1 and polarity cues. However, neither physical, genetic nor molecular evidence link Rac1 to the control of the mitotic spindle and hence regulation of asymmetric cell division. Therefore we are aiming to unveil whether or not there exists a pathway that involves this small GTPase.

1.6. Blebs: Possible implication with the mitotic spindle

In living cells cortical tension is the combined result of the physical properties of the membrane and the cortical network[87]. Cellular tension release relies on a very efficient cortical mechanism inducing cortex deformation around the cell surface. Such mechanism in living cells is known as the process of membrane blebbing[88,89]. Blebs have been well studied during cell division in mammalian systems and regularly observed from anaphase to cytokinesis[88]. They also play a role in cell migration and tissue morphogenesis[90]. Although bleb studies have reported stabilization of cell shape during cytokinesis[91], the role in asymmetric cell division still remains unclear. In non-motile cells, several membrane-associated proteins have been shown to coordinate the sequential recruitment-expansion cycle of blebs. Such proteins include actin cytoskeleton membrane linker proteins, actin bundling proteins and contractile proteins[88]. Some commons proteins involved in expansion are the, small GTPase RhoA, ROCK, Myosin II and Src. The most notorious proteins involved in retraction include, Ezrin, Actin, Moesin and Myosin II[88,92].

Research on blebbing have shown that during asymmetric cell division of *C. elegans* Q neuroblasts cells, unbalanced contraction at the anterior pole directly through Myosin II action generates daughter cells of different sizes. This suggests a Myosin II driven mechanism on the anterior-posterior poles of a dividing cell helping to regulate the size and fate of the daughter cells[93]. In *Drosophila* neuroblasts Myosin II regulates asymmetric cell division by excluding cell fate determinants from the apical pole. From prophase to metaphase, Myosin II restricts these cell fate determinants from the apical pole. From anaphase to telophase, Myosin II concentrates at the cleavage furrow of the contractile ring to promote

cytokinesis and complete the proper distribution of cell fate determinants from the neuroblast to its daughter cells[94]. Also Myosin is required for proper orientation and position of the mitotic spindle in *Drosophila* neuroblasts[95]. Myosin helps orient the mitotic spindle by 90° alignment along the apical-basal axis and by localizing the adaptor protein Miranda at the basal pole. This suggests a mechanism where a higher actin contraction at the basal pole of neuroblasts is required for proper mitotic spindle positioning during asymmetric cell division. The interaction of the mitotic spindle with the cell cortex is one of the principal regulators of the spindle alignment. It is thought that actin contraction forming blebs can orchestrate the orientation of the mitotic spindle. Therefore, unbalance of this process could randomize the proper orientation. These proposed mechanisms in *Drosophila* and *C. elegans* are important in order to better understand unbalanced contraction differences and the role of blebs around the cell cortex. These studies led us to question how asymmetric actin contractions of SOP cells occur during cytokinesis, and whether they play a role in the positioning of the mitotic spindle.

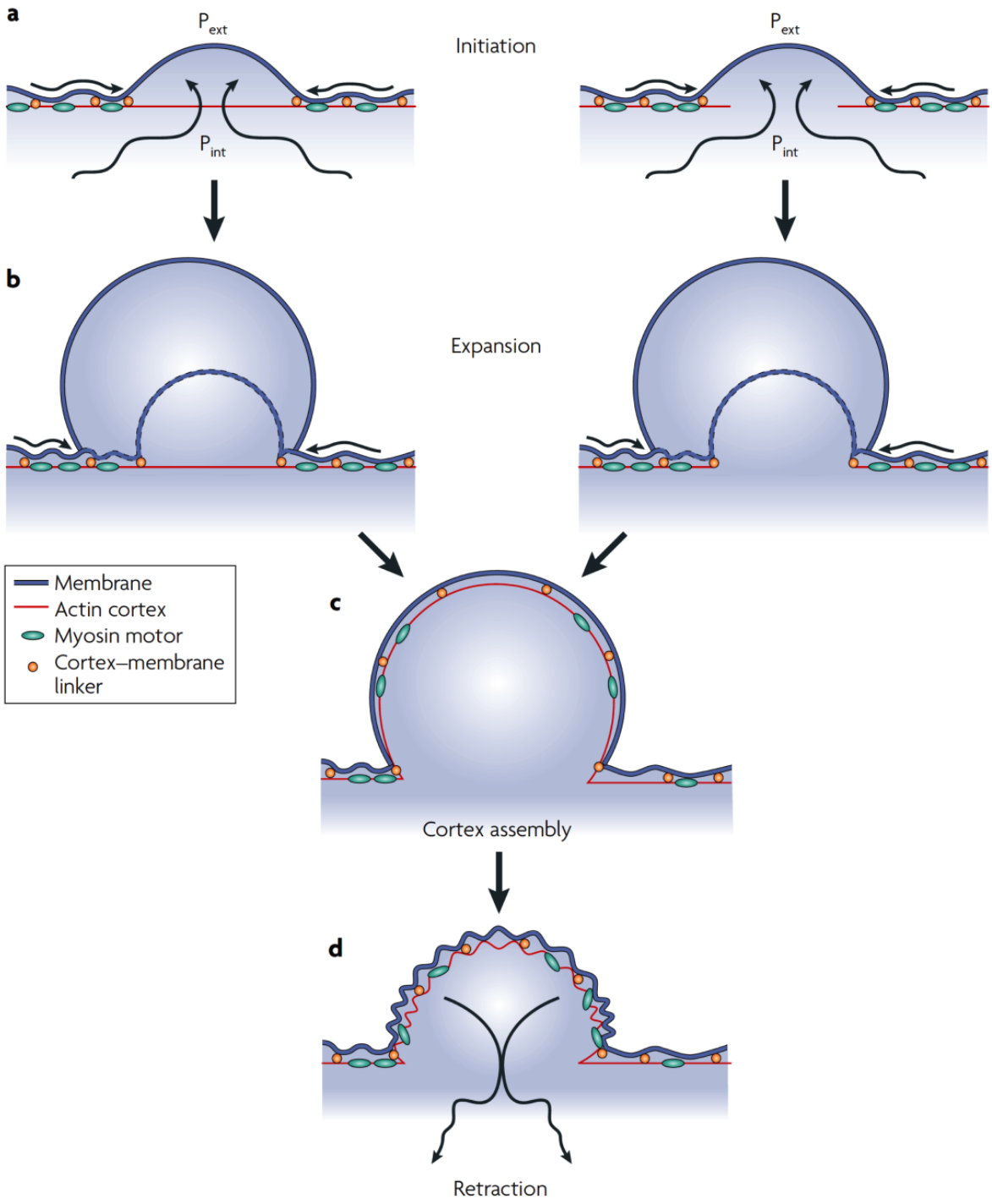

a
P_{ext}
P_{int}
Initiation
P_{ext}
P_{int}
b
Expansion
Membrane
Actin cortex
Myosin motor
Cortex–membrane linker
c
Cortex assembly
d
Retraction

Figure 7: Bleb life cycle. The bleb expansion-retraction cycle can be subdivided into three phases: bleb initiation (nucleation), expansion and retraction. **(A)** Bleb initiation can result from a local detachment of the cortex from the membrane (left model) or from a local rupture of the cortex (right model). **(B)** Hydrostatic pressure in the cytoplasm (P_{int}) then drives membrane expansion by propelling cytoplasmic fluid through the remaining cortex (left model) or through the cortex hole (right model). Concomitantly, the membrane can detach further from the cortex, increasing the diameter of the bleb at the base (dashed line). **(C)** As bleb expansion slows down, a new actin cortex reforms under the bleb membrane. **(D)** Recruitment of myosin to the new cortex is followed by bleb retraction. P_{ext}, extracellular hydrostatic pressure (Adapted from Charras and Paluch [88]).

1.7. Geometry of asymmetric cell division

Accurate asymmetric cell division requires precise coordination of the mitotic spindle. Complex signaling pathways respond to cortical tension generated by both, internal and external environmental changes to control the mitotic spindle[28]. The orientation and position of the mitotic spindle in SOP cells determines the relative size of the PIIA and PIIB daughter cells, as well as determines the proper distribution of cell fate determinants[96]. The mechanism that controls asymmetric cell division of SOP cells copes with subtle geometrical changes in the orientation of the mitotic spindle, and understanding the geometry that leads to proper asymmetric cell division is a difficult task.

Cell geometry plays an important role in controlling the cell cycle in different animal cells[97]. Research on *C. elegans* and different *Drosophila melanogaster* cell lineages including neuroblasts, SOP cells, intestinal stem cells[98], epidermoblasts[99], and many others, feature specific cell components like cortical cell polarity proteins and polarized cortical pulling forces[28] that can monitor cell geometry. These specific components are used to control the mitotic spindle and cell fate determinants inheritance[28]. It is thought that geometry sensing mechanisms control decisions ensuring proper cell division[100] in living organisms. These observations converged into the key idea that the majority of signaling cues plays an important role in determining the cell geometry, which controls the mitotic spindle, cell fate determinant localization, crescent formation and inheritance, chromosome segregation and daughter cells of different sizes. These geometrical parameters become interesting to determine and quantify the mitotic spindle orientation and other parameters during asymmetric cell division of SOP cells. One of the principal aims of this research work relies on the proper acquisition and quantification of such geometrical parameters.

1.8. Hypothesis

We are interested to investigate whether the small GTPases control the positioning of the mitotic spindle during ACD in SOP cells. I want to establish a method to visualize SOP cells at different stages of the ACD cycle and quantify various parameters of mitotic spindle along the course of ACD. Therefore, we hypothesize that **"Asymmetric actin contractions during cytokinesis might displace the mitotic spindle and the cleavage furrow, leading to cell size asymmetry"**. In order to test this hypothesis, I propose, first to implement different quantification methods to assess the subtle dynamics of a normal ACD. After, this will serve to detect subtle movements of the mitotic spindle after perturbing the asymmetric cell division mechanism of SOP cells. Simultaneously, this study will shed more light regarding the sub-cellular activation of certain proteins like small GTPases, which might be involved in regulating mitotic spindle dynamics in SOP cells.

1.9. Specific aims

1.9.1. Determining geometrical parameters of SOP asymmetric division:

By using *Drosophila* lines expressing markers for the asymmetrically distributed protein Pon (Pon-GFP and Pon-RFP), for DNA (Histone-RFP) and for centrosome (Aurora-GFP), I will determine the exact geometry of ACD of SOP cells. Using 4D confocal microscopy, I will perform tridimensional, multichannel, high-speed acquisition of dividing SOP cells. Since the DNA, as well as the centrosomes and Partner of Numb do not overlap, I can image these proteins simultaneously. From these experiments, I will determine several parameters regarding the geometry of SOP division, such as the exact positioning of the spindle

and the cleavage furrow, the crescent formation-expansion and diameters of the two daughter cells. This will allow us to construct a precise mathematical model of SOP division and to measure subtle alteration of ACD as described in aim#3.

1.9.2. Determining the sub-cellular activation of Rho small GTPases during ACD:

From previous work done on collective cell migration, the laboratory has acquired and developed tools like Rac1-FRET and Cdc42-FRET, to determine where Rho small GTPases are active *in vivo*. Here, we will take advantage of such FRET biosensors, in order to determine where Rho small GTPases are activated during SOP division.

1.9.3. Assessing the effect of perturbing the actin cytoskeleton on SOP division:

Here, I will perturb the actin cytoskeleton by different means to determine its role in positioning the mitotic spindle. Initially, I will focus on Rho small GTPases. I will express DN and CA forms to determine their effect on the geometry of SOP division. As certain experiments are out of the scope for this master thesis, future work could be done. Taking advantage of photoactivatable versions of Rho small GTPases and FRET probes, the dynamics of Rho small GTPases can be further explored.

2. Materials and Methods

The experiments of this project consisted of four important steps: basic genetic crossing of Drosophila virgin females with young males, efficient dissection of flies at pupal stage, high definition four-dimensional (4D) confocal microscopy and the image analysis. We built custom homemade image analysis and quantification tools. Together, these procedures led to efficient analyses of the changes in asymmetric cell division of SOP cells.

2.1. *Drosophila* fly stocks and genetic crosses

The *Drosophila* fly stocks were maintained at 18°C and 25°C. All genetic crosses were performed at 25°C. The stocks used in this study are listed below along with the relevant reference (the laboratory and Bloomington (BL) stock number). Fly crosses were performed before image acquisition through confocal microscopy.

2.1.1. Fly Stocks:

w; Neuralized Gal4 / Tm6b, Tb (G019)
w; UAS H2A::RFP, UAS Pon.LD::GFP, Neuralized Gal4 / Tm6b, Tb on III (This study)
w; Neuralized Gal4, UAS H2A::RFP / Tm6b (This study)
y[1] w[*]; P{w[+mC]=UAS-Rac1.T17N}1 on III (BL6292) (O017)
y[1] w[*]; P{w[+mC]=UAS-Cdc42.T17N}3 on II (BL6288) (O054)
w[1118]; P{w[+mC]=UAS-RhoL.T25N}AM / Cyo on II (BL4849) (O073)
y[1] w[*]; P{w[+mC]=UAS-Rac1-FRET} / Cyo ; MKRS / Tm6b (From Ramel *et al.* [52])

w[1118]; P[mW, UAS Pon.LD::RFP], Neuralized Gal4, P[mW, UAS Aurora::GFP] / Tm6b, Tb on III (396) (From Schweisguth Lab Gomes *et al.* [101])

w[1118]; P[mW, pNeuralized, H2B::RFP] on I (619) (From Schweisguth Lab published by Gomes *et al.* [101])

w[1118]; P{w[+mC]=UAS-Moesin.GFP} on III (O046)

w[1118]; P{w[+mC]=UAS-Lgl3A.GFP}/ Tm3 on III (O578) (From Wirtz-Peitz *et al.* [26])

- The small GTPase fly lines and the UAS-Rac1-FRET probe were balanced on the II and III chromosomes using the double balancer line:
  w; If / Cyo ; MKRS / Tm6b, Tb (T033)

2.1.1.1. Definitions:

- Neuralized Gal4: Tissue Gal4 driver used for specific gene expression in the sensory organ precursor cells.
- Partner of Numb (Pon): Cell fate determinant marker used to follow asymmetric cell divisions of SOP cells. Tagged with a green fluorescence protein (GFP) and a red fluorescence protein (RFP).
- Histone 2 A and Histone 2 B: DNA marker tagged to a red fluorescence protein.
- Aurora A (AurA): Centrosomes marker tagged to a green fluorescence protein (GFP).
- Moesin (Moe): Actin binding moesin used for cortex marker tagged to a green fluorescence protein (GFP).
- Lgl3A: Lethal (2) Giant Larvae, triple alanine mutant, prevents the release of Lgl from the cortex during ACD. This allows the visualization of blebs formation until division. Tagged to a green fluorescence protein (GFP).

- If: Irregular facets, dominant mutation that results in small white eyes with fused ommatidia. Marker used to follow the second chromosome.
- CyO: Curly of Oster, which wings are curled at the end. Marker used to follow the second chromosome.
- MKRS: Minute-Karmoisin-Rosy-Stubble, which flies that, features short bristles. Marker used to follow the third chromosome.
- Tm6b: Tubby, marker used to follow the third chromosome and to differentiate crosses at pupal stage prior to dissection.

2.1.2. Genetic crosses performed at 25°C:

Table 1: The following table enlists the genetic crosses we performed in this study, indicating the female and male fly stocks used and the experimental purpose. All crosses were performed at 25°C.

Cross ID	♀ Female	♂ Male	Purpose
1	w; UAS H2A::RFP, UAS Pon.LD::GFP, NeurGal4 / Tm6b,Tb	w; UAS H2A::RFP, UAS Pon.LD::GFP, NeurGal4 / Tm6b,Tb	Control
2	w[1118]; P[mW, UAS Pon.LD::RFP], NeurGal4, P[mW, UAS Aurora::GFP] / Tm6b,Tb	pNeuralized, H2B::RFP	Extraction of geometrical parameters of the spindle. Control
3	w; UAS H2A::RFP, UAS Pon.LD::GFP, NeurGal4 / Tm6b,Tb	If / Cyo ; UAS-Rac1 T17N / Tm6b	Test for spindle positioning

4	w; UAS H2A::RFP, UAS Pon.LD::GFP, NeurGal4 / Tm6b,Tb	UAS-Cdc42 T17N / Cyo ; MKRS / Tm6b	Test for spindle positioning
5	w; UAS H2A::RFP, UAS Pon.LD::GFP, NeurGal4 / Tm6b,Tb	UAS-RhoL T25N / Cyo ; MKRS / Tm6b	Test for spindle positioning
6	w; NeurGal4 / Tm6b,Tb	UAS-Rac1-FRET/ Cyo ; MKRS / Tm6b,Tb	FRET
7	w; NeurGal4, UAS H2A::RFP / Tm6b	UAS Moesin GFP	Blebs quantification
8	w; NeurGal4, UAS H2A::RFP / Tm6b	UAS Lgl3A GFP / Tm3	Blebs quantification

2.2. Procedure to dissect *Drosophila* pupae

2.2.1. Required materials:

- Zeiss Stereo Discovery V8 microscope (Carl Zeiss, Oberkochen, Germany)
- Custom microscope plastic slide (feature a small canal where pupae are placed)
- Micro cover glass (No.1.5 mm 22 x 40 mm) (VWR, Radnor, United States)
- Dissection forceps (size 5 or 5.5) and scissors
- Thin paint brush
- 5 cc syringe for oil distribution
- Rubber glue
- Halocarbon oil 27 (Sigma-Aldrich, St. Louis, Missouri, United States)

- Confocal microscope with digital camera and image acquisition software

2.2.2. Procedure:

- Set up the fly crosses or place flies from a stock you wish to image in several fresh vials at 25°C.
- SOP cells generally begin to proliferate on the pupae thorax at fifteen hours after the onset of pupariation, we therefore select pupae at 0 hour after pupae formation, which can be recognized by their white color.
- Incubate the pupae at 25°C for 15 hours.
- Collect pupae and place them into a rubber petri dish with the ventral side down. Grasp the edge of the operculum (the circular hatch on the anterior dorsal tip of the pupae case) with special forceps and carefully cut slowly with scissors. (**Figure 8** A)
- Gently lift, remove, and discard the operculum, revealing the head along with the notum of the pupa.
- After the cut, use the forceps to begin tearing along the side of the pupal case. Lift the midsection of the pupae case from the torn side and bring it over to the opposite side. Pupal case can be removed completely or partially. (**Figure 8** B)

2.2.3. Pupae mounting:

- Isolated pupae have to be placed on the center of the custom plastic slide dorsal side up with head facing the anterior side. (**Figure 8** C, D)
- Using a 5 cc syringe filled with halocarbon oil, apply a thin uniform layer in the middle of the micro cover glass. An oil overload can cause asphyxia to flies preventing proper asymmetric division of SOP cells.
- Place a small drop of water (1 μl) on the sides of a 22 x 40 mm micro cover glass and place it on the above preparation such that the small halocarbon

oil contacts the surface you want to image, full notum in this case). Compress gently to form a complete seal and flat contact surface between the micro cover glass and pupae cuticle.

- Sample can then be imaged on an inverted or upright confocal microscope fitted with laser scanning or spinning disk, as well two-photon confocal abilities. (**Figure 8** E)
- Being careful enough might allow adult flies to be recovered after several days.

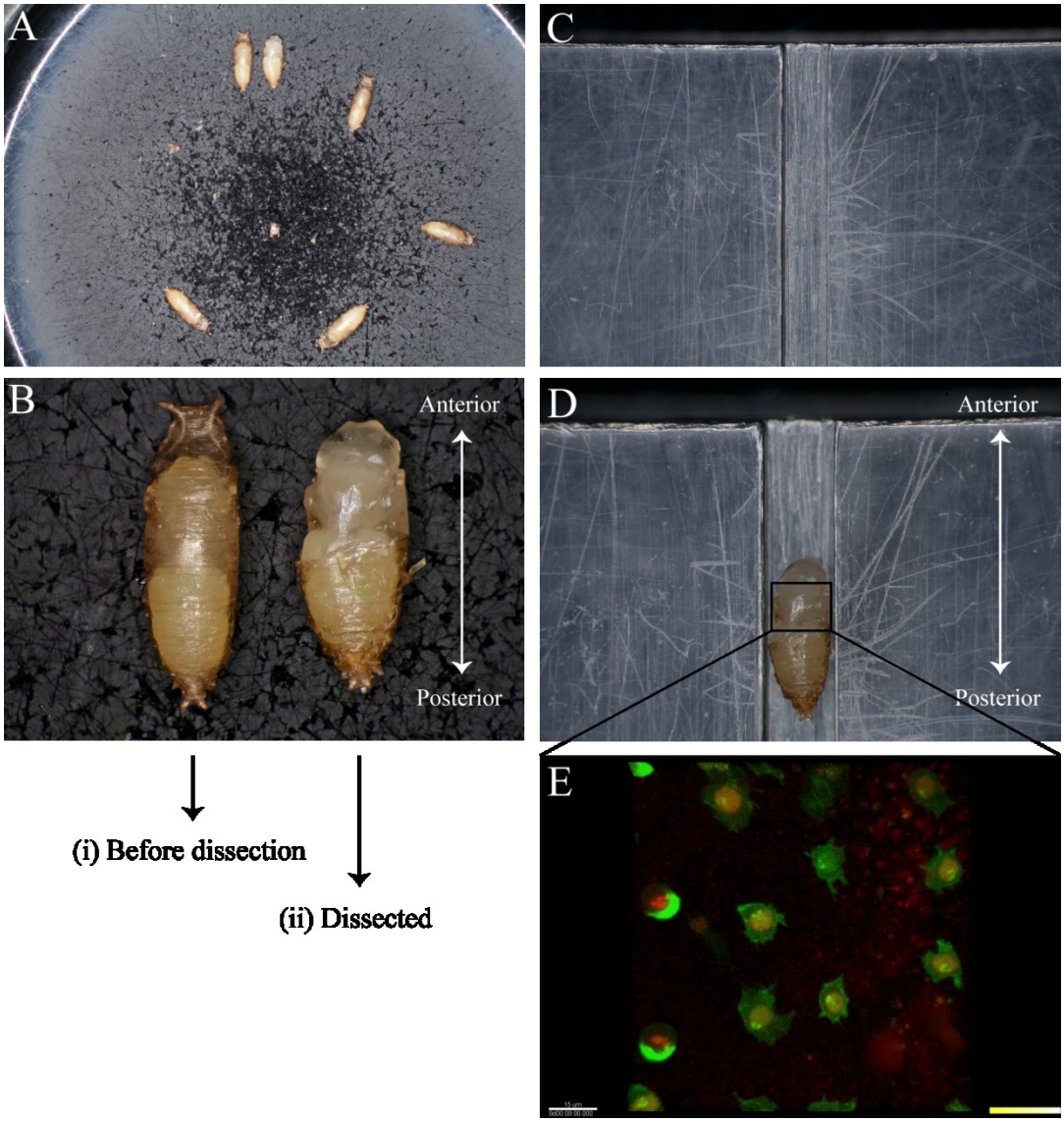


Figure 8: Step by step dissection procedure showed in images for live cell imaging of SOP cells. **(A)** Few pupae placed in rubber petri dish after being incubated for 15 hours at 25°C ready for dissection. **(B)** A pair of pupae before (i) and after dissection (ii). Head is toward anterior and abdomen is toward posterior **(C)** Empty custom made plastic slide featuring pupa fitting groove **(D)** Slide featuring a pupa placed on groove ready for live imaging **(E)** An array of few SOP cells on the notum of a fly pupa. Asymmetric segregation of cell fate determinants can be

visualized by Partner of Numb tagged to GFP protein. H2A is fused to RFP protein for DNA visualization. All SOP cells divide asymmetrically along the anterior-posterior axis of the fly pupa, where anterior PIIB gives rise to neurons and sheath cells, whereas posterior PIIA give rise to hair and socket cells.

2.3. Microscopy, image acquisition and processing

Images from sensory organ precursor cells were acquired using an inverted confocal microscope Nikon A1R (Shinjuku, Tokyo, Japan) using a 63X oil immersion objective. Images were acquired by sequential multi-channel scans using red and green channels. For acquired 4D movies, Imaris software (Bitplane Scientific Solutions Belfast, United Kingdom) was used. For figures assembly, Adobe Illustrator CS6 (Adobe Systems, San José, California, United States) was used. Spindle positioning and quantifications were performed on the original images using the Image J program (National Institutes of Health (NIH), Bethesda, Maryland, United States) for angle manual measurements and data extraction. For compass plot representation, angle measurement data were processed using Matlab software (Mathworks, Massachusetts, United States).

2.4. Time-lapse imaging and quantification

In order to study and extract geometrical parameters of asymmetrically dividing SOP cells, and to classify and identify spindle positioning in different conditions, we implemented the following procedure.

For time-lapse 4D microscopy of sensory organ precursor cells, *Drosophila melanogaster* pupae were incubated for 15 hours prior to imaging, dissected and imaged as previously described [42], [96]. The GFP and RFP channel movies were acquired using a Nikon A1R 60X N.A. 1.4 oil immersion objective confocal microscope (Shinjuku, Tokyo, Japan), using a 488 Argon laser for green channel and 561 Diode laser for red channel. Movies were acquired with no delay time interval in resonant mode (high-speed acquisition scanner). The 4D movies were rendered and processed for visualisation using Imaris software (Bitplane Scientific Solutions Belfast, United Kingdom). Pon-GFP, H2A-RFP and Aurora-GFP image stacks were acquired with no delay at intervals ranging from 0.15 to 0.5 μm, 27 to 32 steps using high-speed piezo objective-positioning Z stage system.

2.5. Assembling a procedure to extract geometrical parameters from SOP cells

In order to quantify, extrapolate and visualize geometrical parameters of asymmetrically dividing sensory organ precursor cells; we developed a protocol that allows for quantification and visualization of spindle positioning in an easily accessible process. In this procedure, it is very important to remember the interpretation of the X and Y axes of an asymmetrically dividing SOP cell. Below is an outline of these important interpretations.

Considering the cell as a circle, the X axis defines the DNA and the Y axis defines the Spindle and the intersection of both forms a 90° angle when aligned at metaphase onset. At this stage of the cycle, asymmetric segregation of cell fate determinants can be visualized by Pon-GFP protein which forms a crescent positioned at the anterior side of the SOP. This crescent serves as a pivotal reference

for angle formation and its measurement when the DNA (X) is horizontal and the Spindle (Y) is vertical with respect to the crescent (**Figure 9**). All these angles have been manually measured using Image J (National Institutes of Health (NIH), Bethesda, Maryland, United States). In our procedure, all these angles have been manually measured using Image J and the data obtained have been processed using a custom built software tools in Matlab software (Mathworks, Massachusetts, United States).

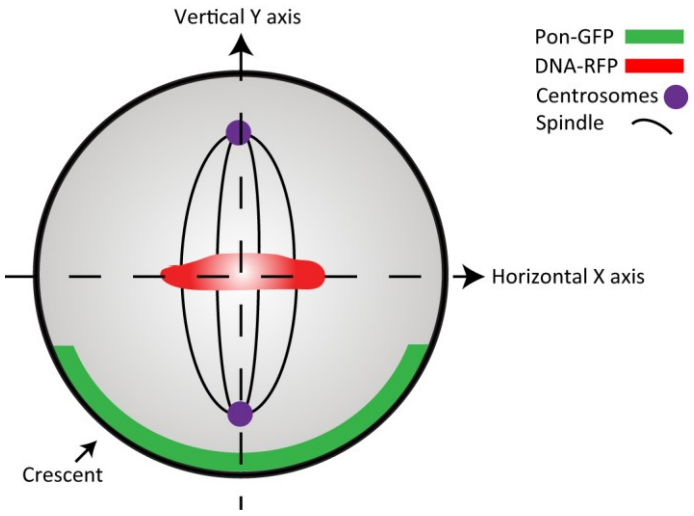


Figure 9: Cartoon depicting horizontal and vertical axis of polarity. Horizontal "X" axis represents DNA which in wild type conditions is aligned horizontally with respect the crescent (Pon-GFP). The vertical "Y" axis represents spindles which in wild type conditions are aligned vertically with respect to the crescent.

2.5.1. Detailed procedure to quantify spindle positioning

Before starting the procedure, the acquired movies should be processed with Imaris software (Bitplane Scientific Solutions Belfast, United Kingdom) and exported as time-lapse frames into a separate folder which can be named as desired depending on the user. Time-lapse frames of SOP cells at metaphase onset must be selected for angle measurements. Angle measurement is a critical step of our process. Considering an SOP cell as a circle and measuring the angle, a vertex is formed. Such a vertex is formed at the center where DNA aligns (**Figure 11**). At metaphase, while the DNA alignment occurs in a horizontal manner (X to X'), the spindles are always aligned in a vertical manner to the crescent (Y to Y') (**Figure 12**, A). As the cells exit from the metaphase, DNA separation occurs due to the pulling forces produced by the mitotic spindles. These forces induce dynamic changes to the DNA arrangement pattern and the spindle lengths. This pivotal event arises as a result of the alignment (or the effective angle) between DNA and spindles, with respect to the crescent, which has to be tight and highly precise. By measuring the alignment angle between DNA and spindles, with respect to the crescent, and at successive time points starting at the metaphase, we can monitor the cell division. Thus, by measuring the changes in these geometrical patterns as SOP cells goes through division, we can monitor the cells behavior with quantitative measurements. Hence the development of such method.

(i) Take a time-lapse frame of an SOP cell at metaphase onset. At this stage of the asymmetric division cycle, in wild type conditions, the asymmetric cell fate determinant Partner of Numb (Pon-GFP) forms a crescent on the anterior side of the SOP cell. With respect to the crescent, DNA aligns horizontally (X to X') whereas the spindle aligns vertically (Y to Y'). When measured with the crescent as reference, these two axes perfectly intersect orthogonally to result in a 90° angle (**Figure 12**, A). When the mitotic spindle is misaligned, measured with respect to

the crescent these two axes intersect orthogonally to result in a 180° angle (**Figure 12**, B), suggesting an opposite mechanism to wild type conditions.

(ii) In the second step, angle measurements are converted into Cartesian coordinates in order to represent them in a compass plot for better visualization. The compass plot can be generated using the "compass function" in Matlab (Mathworks, Massachusetts, United States). The function takes Cartesian coordinates and plots them on a circular grid. Convert the DNA / Spindle angles, into radians before converting into Cartesian coordinates. Better details are provided below.

Step #1: Angle measurement using Image J

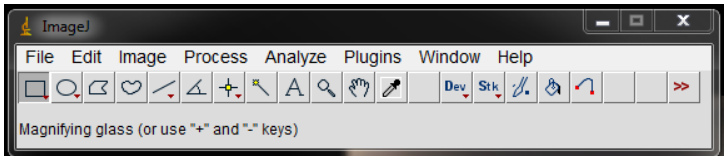


Figure 10: Image J layout with angle tool

→Select angle tool 

→Measure → Values will be displayed in external table label "parameters"

→Copy the angle value in an Excel file

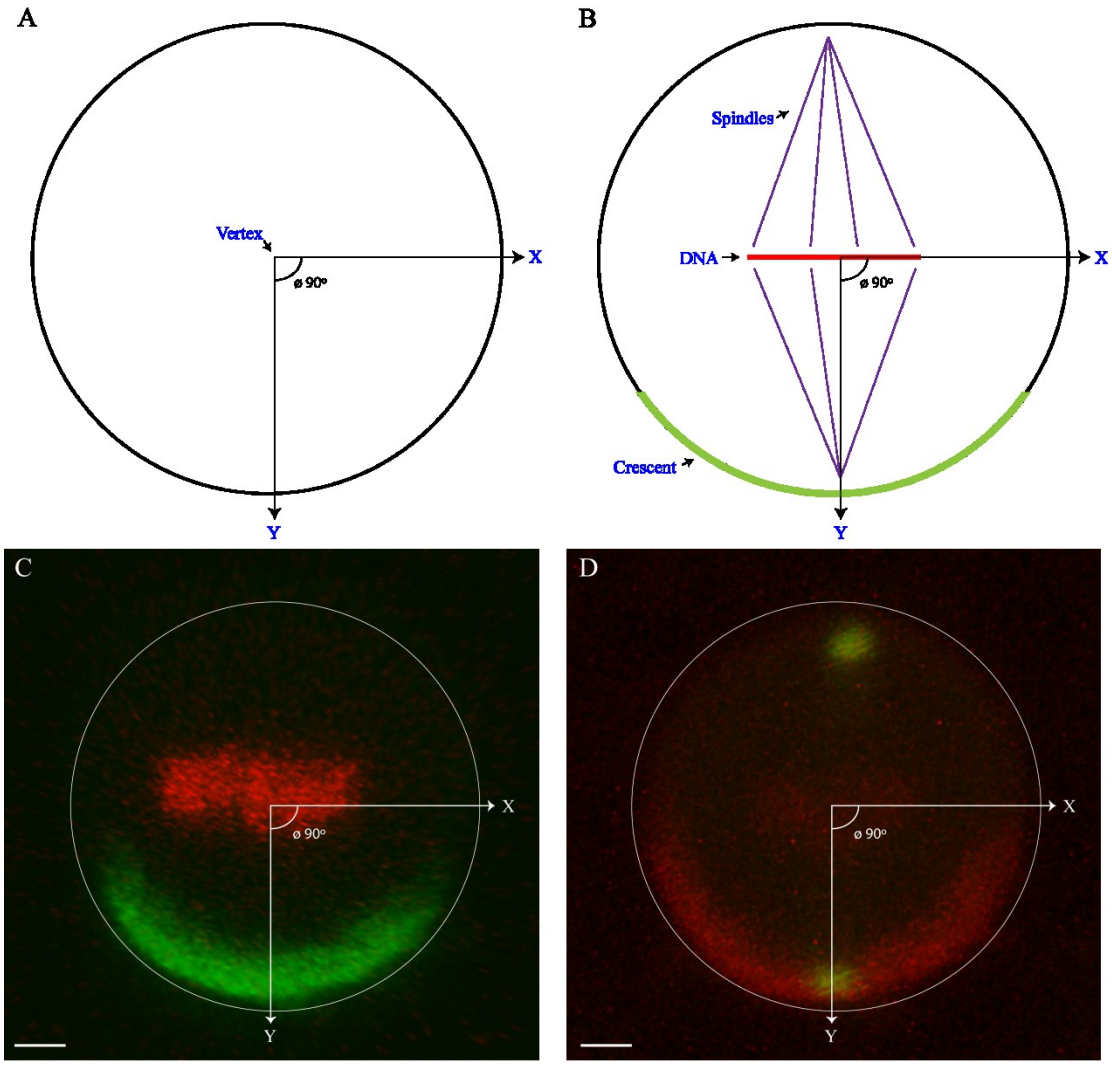
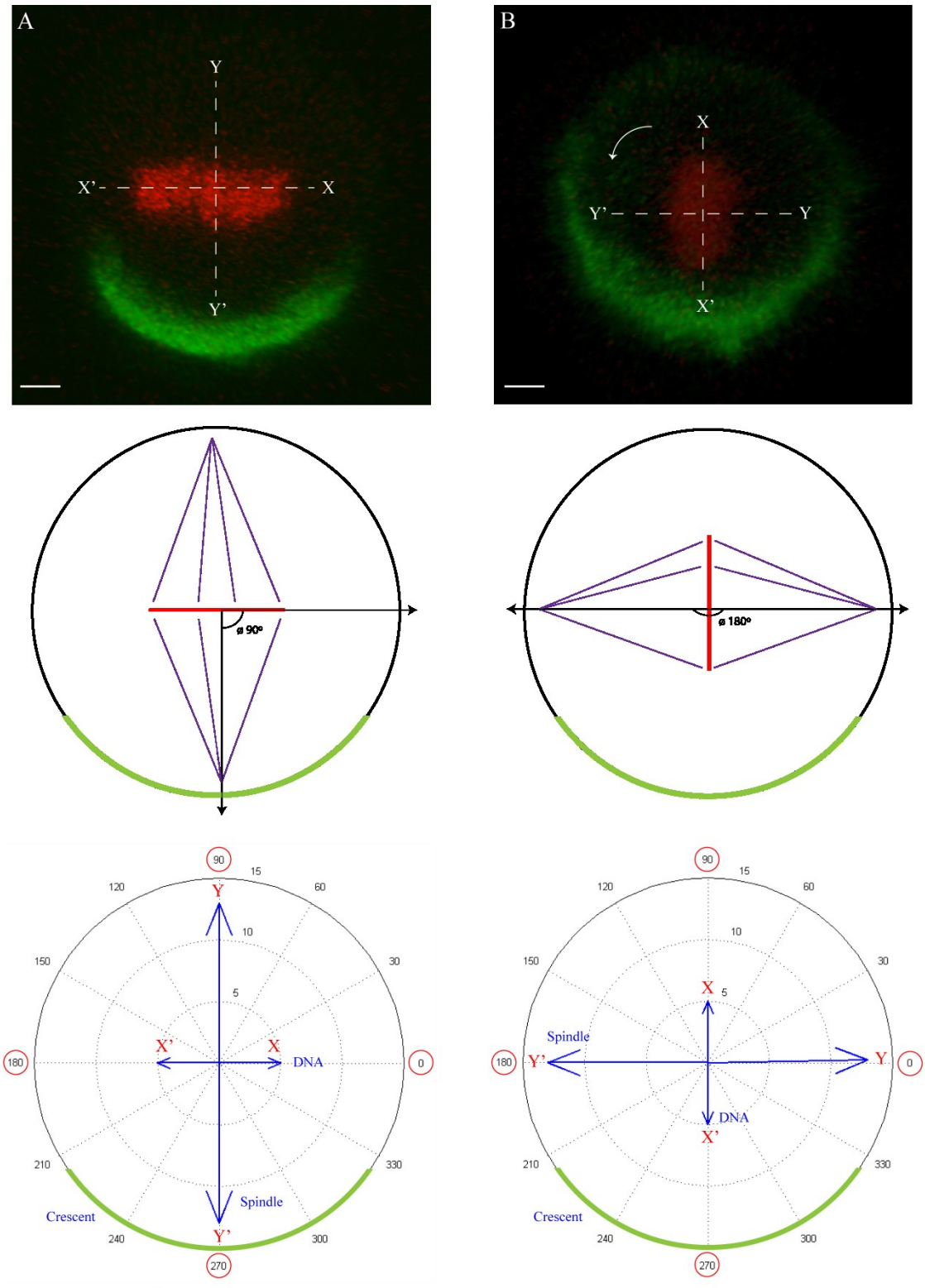


Figure 11: Modelization of an SOP cell as a circle. **(A)** A circle featuring an X – Y 90° angle in which the vertex is found exactly in the middle of the circle. **(B)** A SOP cell considered as a circle. DNA is aligned horizontally and spindles are aligned vertically with respect to the crescent. X and Y lines intersect orthogonally and hence form a vertex at the centre of SOP and with a 90° angle between DNA and spindles. **(C - D)** A time-frame of an SOP cell at metaphase onset with a 90° angle between DNA and spindles with respect to the crescent. The spindles are represented in **(D)** by a centrosome marker (green). Scale bars = 2 µm.



When Angle = 90°

When Angle = 180°

Figure 12: Angle measurement comparisons in SOP cells modelized as circles. Time-frames examples of SOP cells at the onset of metaphase. **(A)** When a 90° angle is formed. **(B)** When a 180° angle is formed. Underlying is their respective compass plots, for which the procedure is explained in the text (Step #3). The mitotic spindles are represented by Y - Y' in both images. The SOP cells are expressing Pon-GFP (cell-fate determinant marker, green) and H2A-RFP (DNA marker, red). Scale bars = 2 μm.

Step #2: X to X' axis / Y to Y' axis combination values

Table 2: Combination of values for corresponding DNA and Spindle positions. How values are they positioned on the Cartesian plane, are shown in **Figure 13**, depicted as compass plots. Such table features 180 combinations in the Cartesian plane. These values are detailed for easy localization in the compass plot and for comparison purposes.

Angle	X – X' axis / DNA	Y – Y' axis / Spindle
When angle = 90°	1 – 180	90 – 270
When angle = 180°	90 – 270	1 – 180
When angle increase	90 + x ; 270 + x	(1 + x) % 359 ; 180 + x
When angle decrease	90 - x ; 270 - x	(-1 - x) % 359 ; 180 - x

Step #3: Compass plots generation

To generate our compass plots we used the compass (U, V) function of Matlab. This function takes two vectors as inputs (U and V), and plots *n* arrows into a circle, where *n* is the number of elements in U or V. The location of the base of each arrow is the center of the circle, while the location of the tip of the *ith* arrow is a determined by both the *ith* elements of U and V: U (i), V (i) (Referenced from http://www.mathworks.com/help/matlab/ref/compass.html).

As well, this function shows vectors emanating from the origin of a graph. This function takes Cartesian coordinates and plots them on a circular grid for easy visualisation. How does it work? Two vectors:

(i) X and X' defines the DNA position. Y and Y' define the spindle position.

(ii) The combination or the intersection of both defines an angle. This function converts the DNA / Spindle positions, given as angles, into radians before converting such positions into Cartesian coordinates (**Figure 13**).

(iii) The generation of these different compass plots can be done by simply running the following custom script in Matlab:

→Launch Matlab software 

→Set up new work directory using the "mkdir" and "cd" functions.

mkdir creates a new work directory and cd changes the current folder to the desire working folder. In this example, the working folder is assigned as "DNA / Spindle quantification".

Open script by:

→File

→Open

→Choose desktop

→Open DNA / Spindle quantification folder

→Choose the M-file DNA / Spindle positioning script (DNA_Spindle.m) (Appendix).

Another alternative is to simply copy / paste the script from a notepad directly into the Matlab command window. This should display the plots as well.

→Refer to the table to carefully select the combination values for X and Y axes that correspond to the angle measured from the time-frames (Appendix).

In order to visualize changes in axes, combination values have to be changed in the script before running (Appendix).

(iv) Replacing the values: In sdir brackets the 90 270 [Y – Y'] is a combination for spindle position and 180 1 combination for DNA [X – X']. The [90 270 180 1] is equal to an angle of 90° in the table. When the angle values changes, those numbers have to be replaced by the corresponding ones that can be found in the table (Appendix).

```
%% Example script "wild type condition" %%

sdir = [90 270 180 1];              % X - X' and Y – Y' axes positions

knots = [13 13 5 5];                % spindle position distance from to the cortex

rdir = sdir * pi/180;               % convert to radians

[x y] = pol2cart(rdir, knots);      % polar to cartesian coordinates

compass(x,y)

hline = findobj(gca, 'Type', 'line');

set(hline, 'LineWidth', 2, 'color', 'b')

label = {'DNA/Spindle position at metaphase', 'Ng4, H2A-RFP, Pon-GFP / Tm6B',
...

    'Wild type' 'N=20'};

text(-30, 15, label)                % Figure label
```

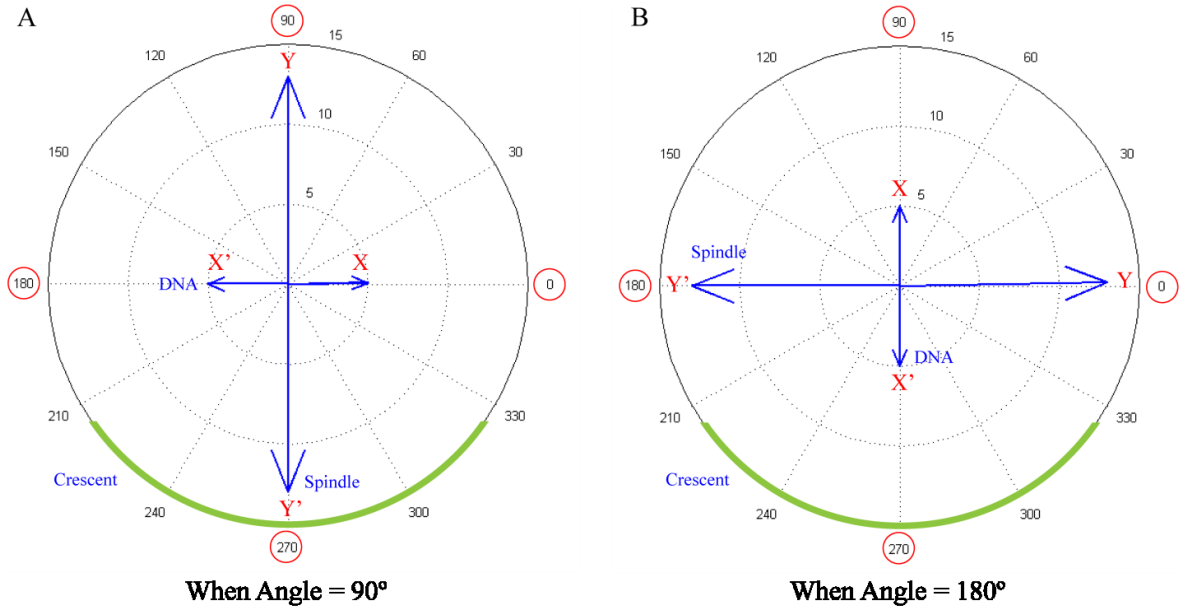


Figure 13: Compass plots showing Spindle / DNA positions. This function serve as an alternative to visualize and compare between SOP cells in different conditions. **(A)** When angle is equal to 90°, it represents wild type condition. **(B)** When angle is equal to 180°, it represents abnormal condition. In wild type conditions, for proper asymmetric cell division, spindle aligns from 90 to 270 (X – X'), and DNA aligns from 1 to 180 (Y – Y') (encircle in red) in the cartesian plane being equal to and angle of 90°. In abnormal condition, spindle and DNA are aligned otherwise, being equal to an angle of 180°.

2.6. Crescent formation-expansion

In wild type SOP cells, Pon-GFP accumulates at the anterior side of the cell building a polarized crescent, which faces the horizontal alignment of DNA. Such crescent starts polarization at prophase, band it is completely polarized by the onset of metaphase. We wanted to better understand whether the positioning of the mitotic spindle will influence the polarization forming crescent of cell fate determinant at the anterior side. For this we measured the crescent formation-expansion at metaphase onset. For this end, again using Image J (National Institutes of Health (NIH), Bethesda, Maryland, United States) angle tool and considering the cell as a circle. We measured the angle formed between the two ends of the crescent –visually determined- and the horizontal DNA, as shown in (**Figure 14**).

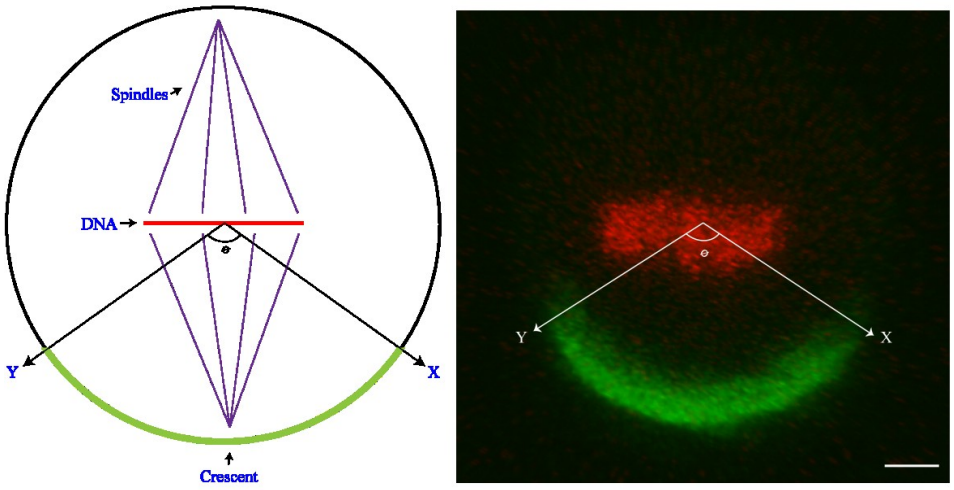


Figure 14: Modelization of crescent formation-expansion. Depicted is an SOP cell at metaphase onset where the crescent (green) is completely built having an "x" expansion (or width) from X to Y. The expansion is measured by an angle. Scale bars = 2 µm.

2.7. Blebs quantification

Blebs quantification has been manually done from interphase to two cell stage for anterior and posterior sides of SOP cells. From each acquired time-lapse movie, we went through each time-frame in order to be as precise as possible. This allowed us to statistically compare the number of blebs at both sides of the SOP cells until division occurs. To better visualize blebs, besides using Pon-GFP as cell fate determinant marker, we utilized two other *Drosophila* fly lines. In one the actin binding domain Moesin is tagged to GFP (UAS Moesin-GFP) and in the other, Lethal Giant Larvae triple alanine mutant is tagged to GFP (UAS Lgl3A-GFP) and is driven specifically in SOP cells under the control of Neuralized Gal4 (**Table 1**, Cross ID 7 and 8).

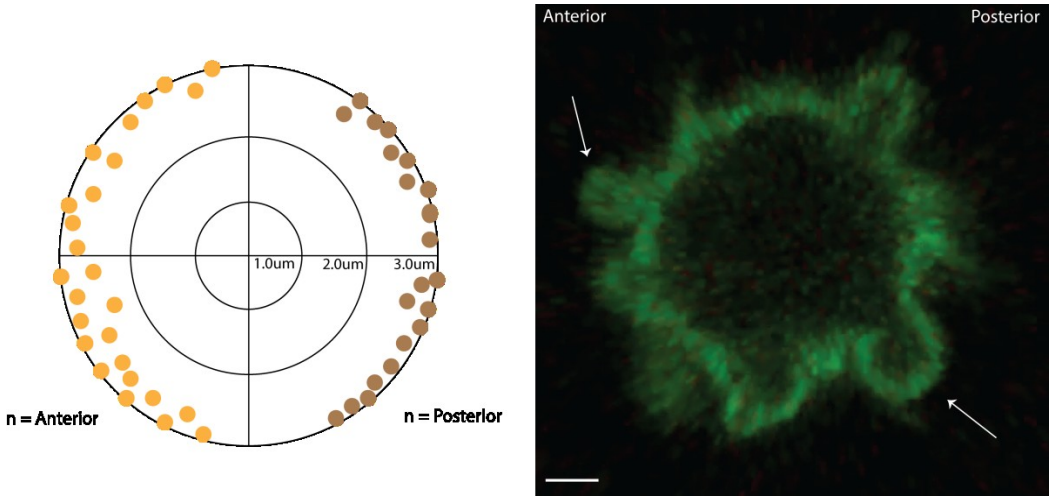


Figure 15: Blebbing quantification. A blebbing SOP cell with quantification circle. Anterior yellow dots and posterior brown dots. In time-frame image, anterior is oriented towards left. Scale bars = 2 µm.

3. Results

3.1. Extracting geometrical parameters

The asymmetric cell division of SOP cells have been the subject of intense research as it serves as a perfect working model to understand controls of cellular diversity and homeostasis [1]. For our first aim, we wanted to better understand how the positioning of the mitotic spindle controls the asymmetric cell division cycle of SOP cells, it is necessary to monitor subtle dynamic movements behind such mechanisms. To do so, we chose to carefully extract geometrical parameters of SOP cells by using the methods described above.

3.1.1. Using DNA as reference for the positioning of the mitotic spindle

First, we started to use a *Drosophila* line that expresses the cell fate determinant marker Pon fused to GFP in combination with DNA marker H2A-RFP driven by Neuralized Gal4 for specific expression in SOP cells (**Table 1** Cross ID 1). The fly line name can be resumed as Ng4, UAS Pon-GFP, H2A-RFP / Tm6b. Due to a lack of spindle and centrosomes marker at the time, this line was the most appropriate to start with for such purposes. As described in Section 2.5, DNA aligns horizontally with respect to the crescent at the metaphase onset. This observation serves as reference for the positioning of the mitotic spindle which is aligned vertically with respect to the crescent at metaphase. Observing the precise positions of DNA and Spindles and their alignment with respect to the crescent can provide insights into the positioning of mitotic spindle during asymmetric cell division of SOP cells (**Figure 16**).

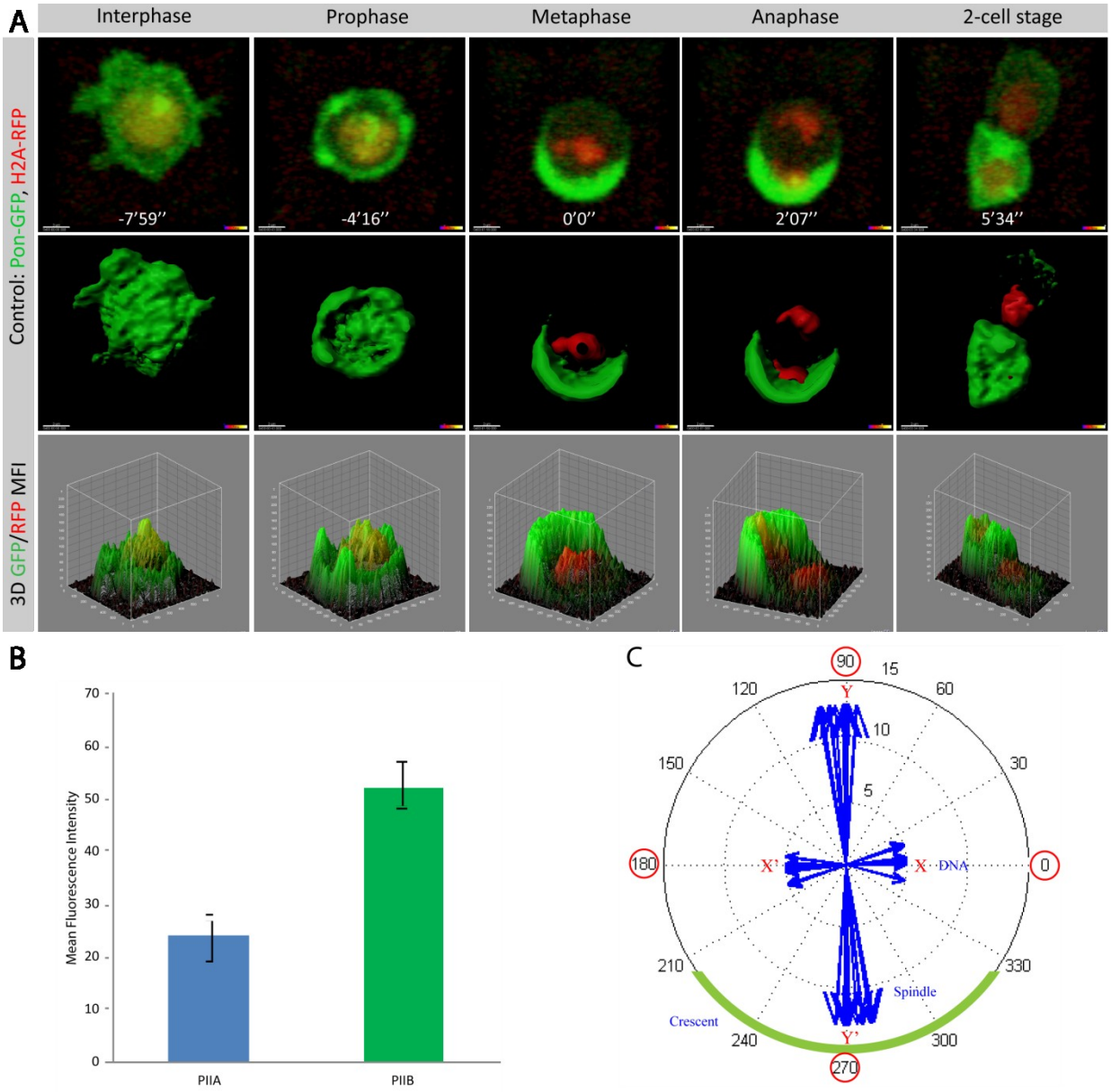

A
Interphase
Prophase
Metaphase
Anaphase
2-cell stage
Control: Pon-GFP, H2A-RFP
-7'59''
-4'16''
0'0''
2'07''
5'34''
3D GFP/RFP MFI
B
Mean Fluorescence Intensity
70
60
50
40
30
20
10
0
PIIA
PIIB
C
90
15
60
30
0
330
300
270
240
210
180
150
120
10
5
Y
Y'
X
X'
DNA
Spindle
Crescent

Figure 16: Quantification of geometrical parameters of wild type SOP cells. Partner of Numb – Pon-GFP and Histone-RFP were coexpressed in SOP cells. Metaphase onset is t = 0. Anterior is oriented toward bottom of SOP. **(A)** Pon-GFP is recruited to the cortex in prophase and is asymmetrically localized at metaphase returning to the cytoplasm after mitosis. **(B)** Mean fluorescent intensity (MFI) were measured and shown as 3D distribution plots in order to differentiate Pon-GFP distribution in PIIB from PIIA. **(C)** Spindle angle position is oriented at 90° compared to the Pon-GFP crescent, N=10 SOP. Spindle position angles were calculated in the Cartesian plane and shown in a compass plot. Angles were measured at metaphase onset using DNA as reference. Combination values are shown in **Table 3**. Scale bars = 3 μm. Time is shown in minutes: seconds before and after metaphase onset.

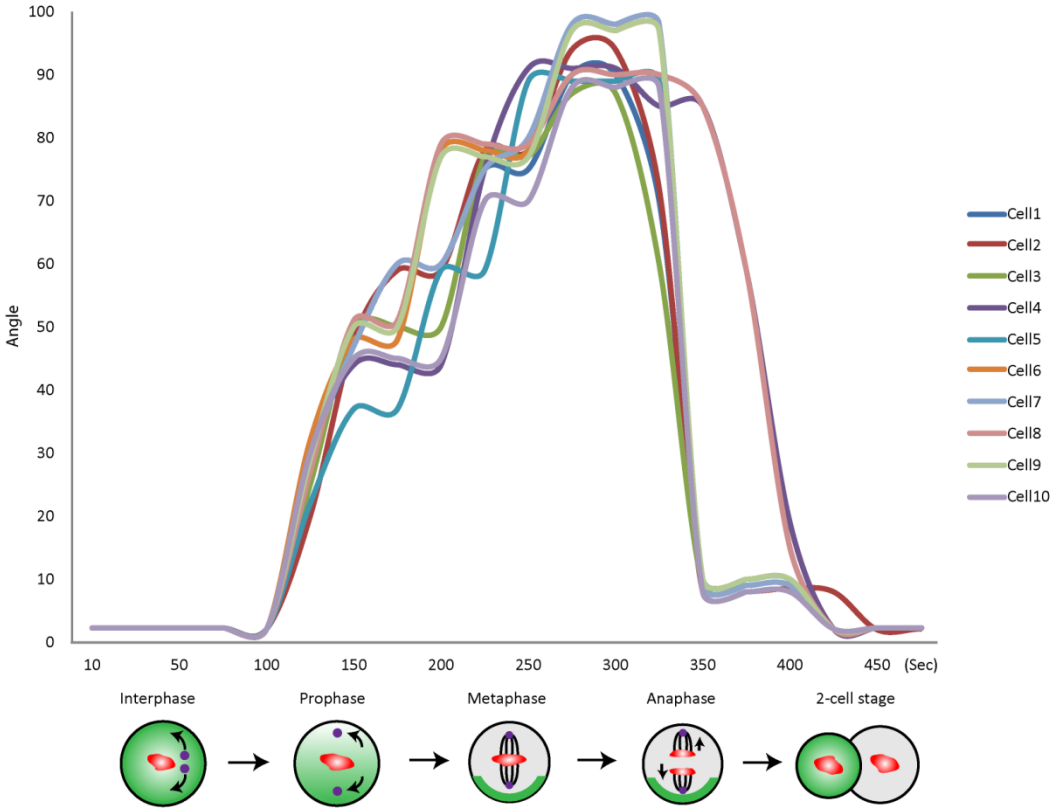


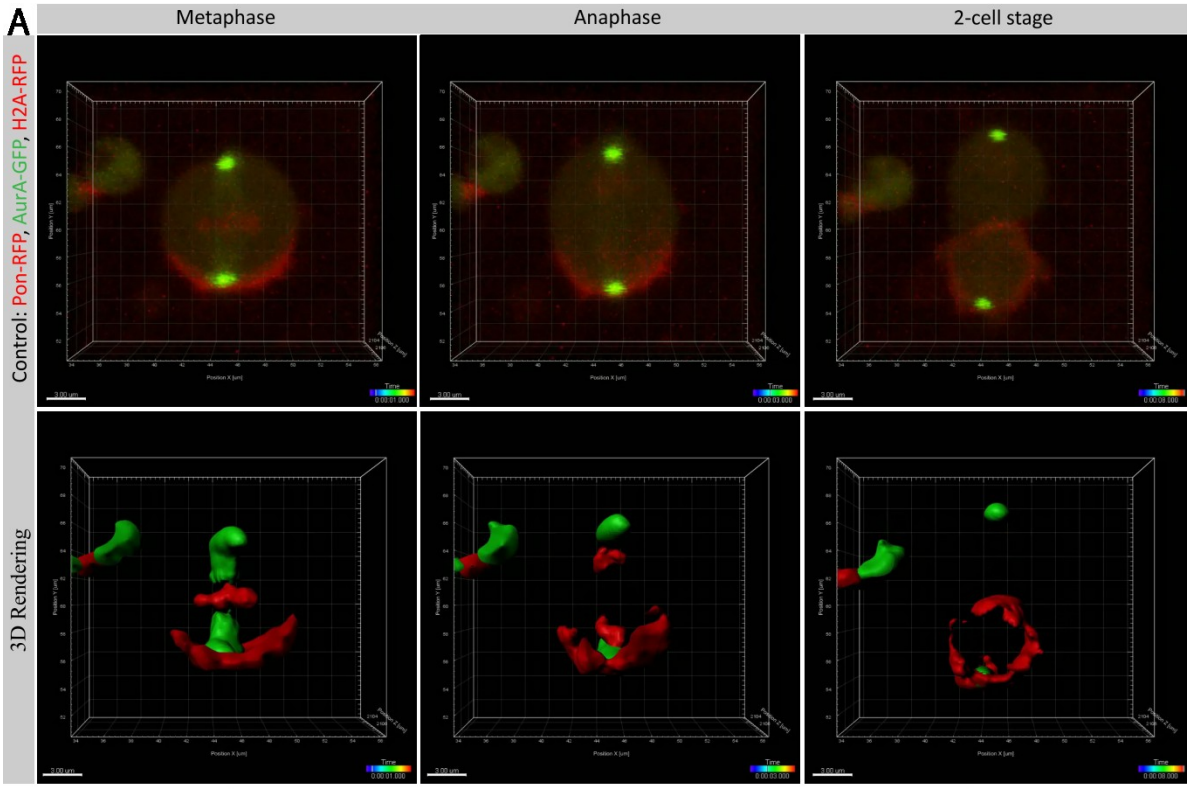
Figure 17: Mitotic spindle positioning during a time-course. Measurement of the spindle orientation of 10 individual wild type SOP cells extrapolated from the DNA position. Ng4, Pon-GFP, H2A-RFP / Tm6b serves as a control. Angle positioning were registered in time according to metaphase onset (t = 0). The cartoon shows spindle assembly from interphase to 2-cell stage. It is not according to time.

3.1.2. Using Aurora for the positioning of the mitotic spindle

In order to obtain better results, the quantification of positioning of the mitotic spindle needs to be as accurate as possible. To this end, we requested *Drosophila* fly lines developed by the Schweisguth Lab [101]. Aurora-A is an excellent centrosomes marker, which serves remarkably for precise quantification of the mitotic spindle. Visualising the positioning of the mitotic spindle with Aurora-A marker is very superior and precise. By having Aurora-GFP as centrosomes / spindle marker, Histone-RFP for DNA and Pon-RFP for crescent, allowed us to develop improved methods for extraction of precise geometrical parameters on the mitotic spindle of SOP cells. The genetic cross can be found in (**Table 1** Cross ID 2). However, measuring the mitotic spindle positioning angle with Aurora-GFP as a reference, gave us similar results than measuring with DNA as reference angle which are explain below.

3.1.3. Comparing quantification of the mitotic spindle in SOP cells with DNA and Aurora as references

Using DNA as reference for quantification of the positioning of the mitotic spindle is, we quantified the mitotic spindle positioning using Aurora-GFP as centrosomes / spindle marker along with DNA. We obtained similar results as using only DNA as reference. The combination of both results allows us to confirm our first quantification results. This step led us to set a threshold which serve for future detection of subtle movements of the mitotic spindle in different conditions. The results for both quantifications are placed in **Table 3**. This table contains the X – X' (DNA) and Y – Y' (spindle) combination values along with their corresponding angle measures which serve for comparison purposes. These results allow us to set an arbitrary threshold in order to detect subtle movements of the mitotic spindle.


A
Metaphase
Anaphase
2-cell stage
Control: Pon-RFP, AurA-GFP, H2A-RFP
3D Rendering

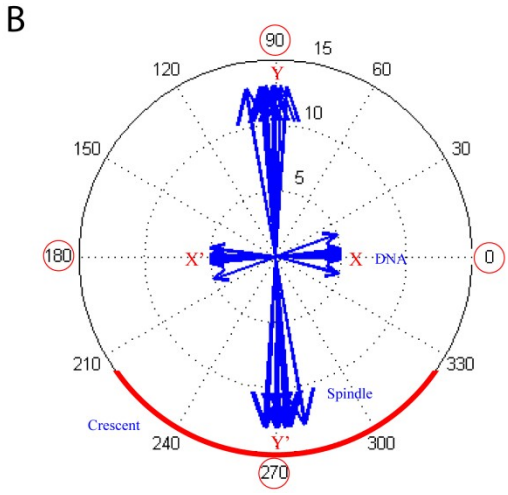

B
90
15
60
120
10
30
150
5
180
X'
X
DNA
0
210
330
Spindle
Crescent
240
Y'
300
270

Figure 18: Quantification of geometrical parameters of wild type SOP cells with Aurora-GFP. High definition and 3D rendered images with SOP featuring Partner of numb – Pon-RFP, Aurora-GFP and Histone-RFP. Images were recorded from prometaphase to anaphase to measure centrosomes and Pon-RFP positions. **(B)** Spindle angle position is oriented at 90° compared to the Pon-RFP crescent, N=10 SOP. Spindle position angles were calculated in the Cartesian plane and shown in a compass plot. Angles were measured at metaphase onset using centrosomes and DNA as references. Scale bars = 3 μm.

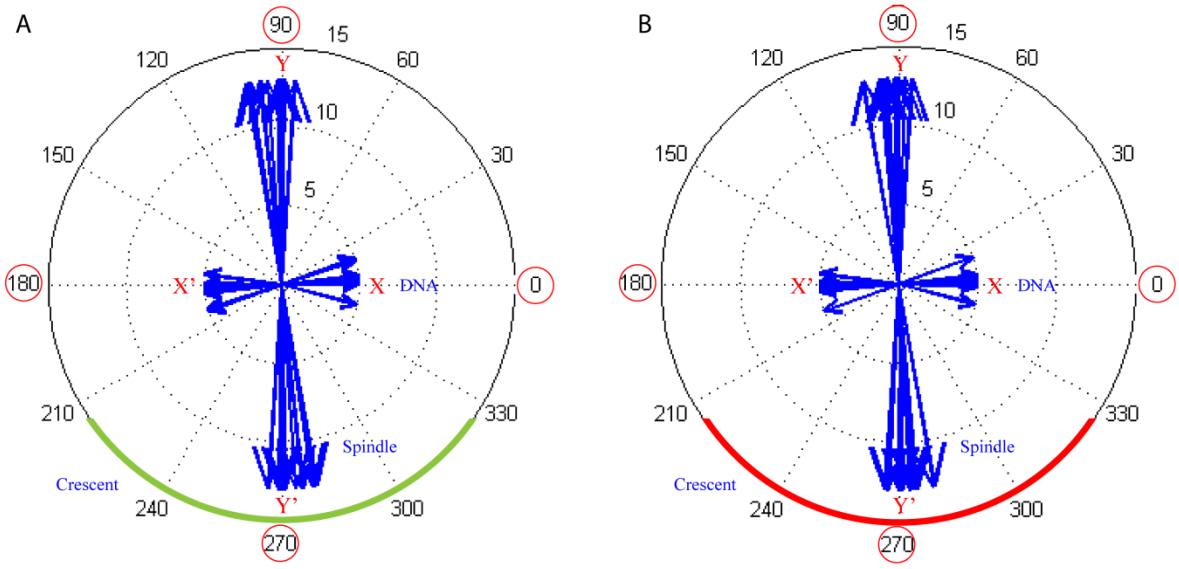


Figure 19: Mitotic spindle comparison using DNA and Aurora. **(A)** Pon-GFP (crescent) and H2A-RFP (DNA) used as references for mitotic spindle positioning **(B)** Pon-RFP (crescent), H2A-RFP (DNA) and Aura-GFP used as references for mitotic spindle positioning. Two vectors defined the positions: (Y – Y') spindle / centrosomes and (X – X') Pon-GFP / RFP crescent. Spindle position is calculated using angle intersection between the crescent (vector X) and centrosomes (vector Y)). Angle measurements were converted into Cartesian coordinates where all possible angle combinations were calculated and exported as compass plots. Corresponding values were place in **Table 3** for comparison.

Table 3: Angle measurements with DNA and Aurora as references. **(A)** Results of the mitotic spindle positioning in 10 wild type SOP cells using only DNA as reference. **(B)** Angle results of the mitotic spindle positioning in 10 wild type SOP cells using DNA and Aurora as references. In both cases, the combination for the Cartesian plane and posterior compass plot visualization can be seen in the X – X' panels for DNA and Y – Y' panels for spindle. Such combination values are for easy visual localization and comparison in the Cartesian plane.

(A) **Ø DNA**	**X - X' axis**	**Y - Y' axis**	**(B)** **Ø Aurora**	**X - X' axis**	**Y - Y' axis**
90°	1 - 180	90 - 270	91°	2 - 181	91 - 271
94°	5 - 184	94 - 274	95°	4 - 183	95 - 275
87°	357 - 177	87 - 266	90°	1 - 180	90 - 270
91°	2 - 182	91 - 271	97°	8 - 187	97 - 277
89°	359 - 179	89 - 268	89°	359 - 179	89 - 268
97°	8 - 187	97 - 277	91°	2 - 182	91 - 271
98°	9 - 188	98 - 278	98°	9 - 188	98 - 278
90°	1 - 180	90 - 270	90°	1 - 180	90 - 270
88°	358 - 178	88 - 267	98°	9 - 188	98 - 278
92°	3 - 182	92 - 272	87°	356 - 176	86 - 266

3.1.4. Setting a threshold for the mitotic spindle positioning

From the obtained angle results from both DNA and centrosomes as references, we increased the number of SOP cells in order to set up a threshold based on wild type conditions. Using again Image J, we measured the angle of up to 100 random wild type SOP cells at the onset of metaphase. These results have being normalized prior to calculate the angle mean, and are represented in a histogram plotted as a function of the angle mean. Using the minimum and maximum angle values, the threshold for the positioning of the mitotic spindle were chosen arbitrary, where everything outside the range of <84°>98° will be considered as misaligned spindles. These results support the observation that in SOP cells, the mitotic spindle forms an angle with very little variations, which is tightly aligned with respect to the crescent at the onset of metaphase.

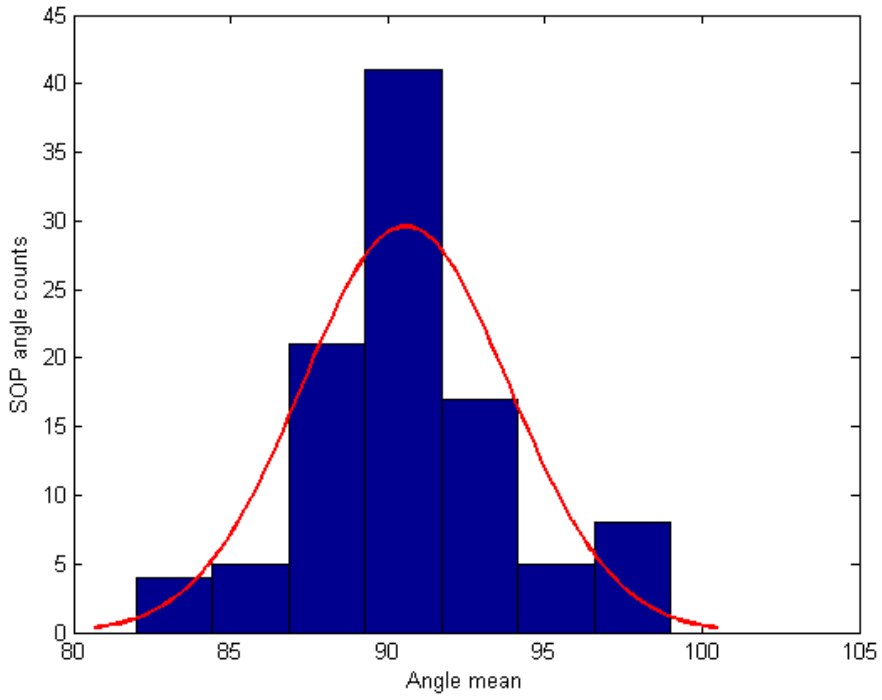


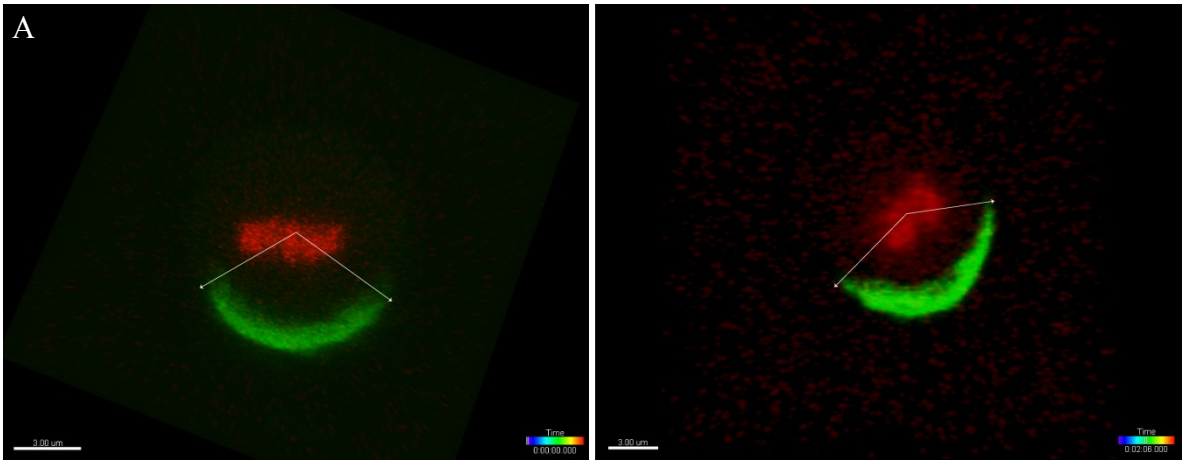
Figure 20: Probability histogram of Gaussian-distributed of angle measurements of the mitotic spindle of 100 random wild type SOP cells at the onset of metaphase. Mean = 91°, Standard deviation σ = ± 3.3. Arbitrary threshold between 84° and 98°. <84°>98° will be considered as misaligned spindles.

3.2. Crescent formation-expansion

Following our aim of extracting all possible geometrical parameters of SOP cells, another parameter that we quantified was the crescent formation-expansion (width). In SOP cells, cell fate determinants like Numb and its anchor protein Partner of Numb (Pon) localize at the anterior side of the SOP building up a crescent [96]. The crescent is aligned within the anterior-posterior axis of the pupae. The proper formation and expansion of the Pon-GFP crescent depends on the tight and precise alignment of the mitotic spindle with respect to the crescent. Whether a mitotic spindle misalignment occurs, segregation of cell-fate determinants at the 2 cell-stage might fail, therefore the daughter cells PIIA and PIIB will give rise to the same cell fates. Hence another reason why the positioning of the mitotic spindle has to be tightly controlled.

As described in the methods (Section 2.6), we measured the expansion (width) of the crescent at the onset of metaphase. We took a group of 10 SOP cells expressing Pon-GFP and another 10 expressing Pon-RFP in order to explore whether there is variation between expressing different fluorophores. We found that when SOP cells express Pon-GFP there is less variation in crescent formation (**Figure 21**). Whereas when SOP cells express Pon-RFP, they show more variation in crescent expansion (**Figure 22**). This might be due to the fluorophore stability during the process of live-imaging, suggesting that Pon-GFP might be a more

suitable candidate for detection and quantification of subtle movements of the mitotic spindle during asymmetric cell division. Afterwards, we compared among both fluorophores quantification and we found that -despite difference in crescent expansion-, they show no significance among crescent width (angle measurement *P*-value = 0.3308) (**Figure 23**).



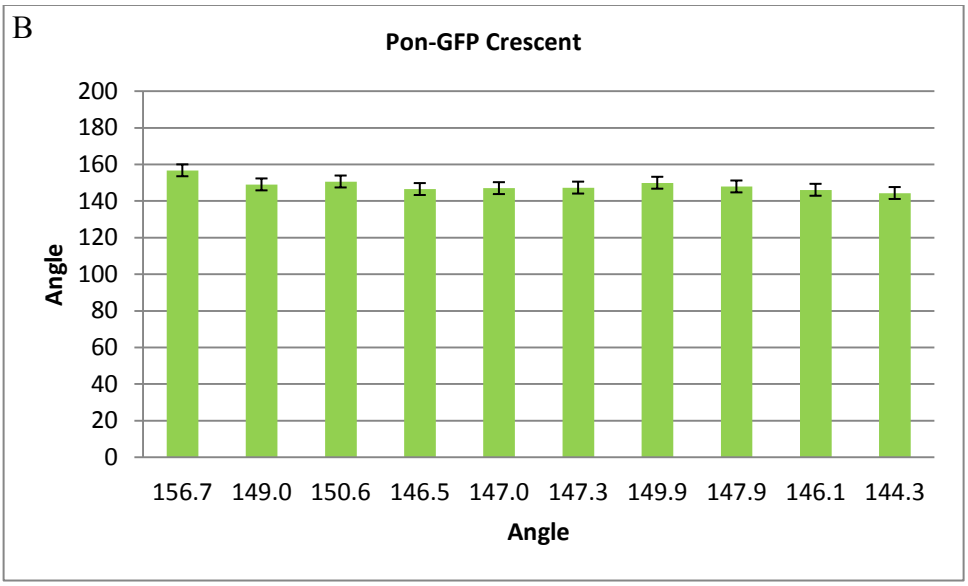
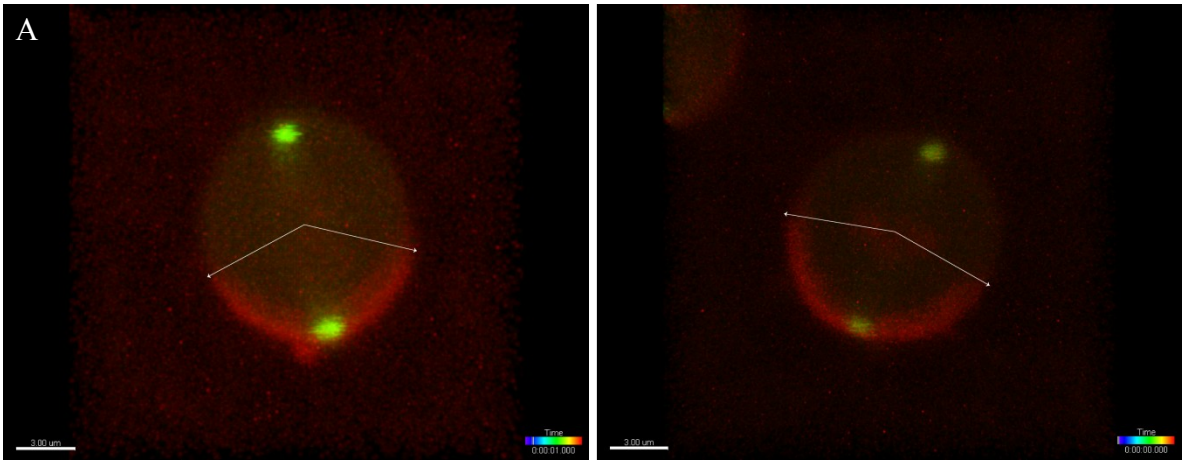


Figure 21: Quantification of Pon-GFP crescent expansion. **(A)** A pair of confocal time lapse images of wild type SOP cells showing complete built-expanded crescent at metaphase onset. In both cases, Pon-GFP expansion is similar. **(B)** Quantification of crescent expansion of 10 SOP cells. Pon-GFP expansion shows little variance. The X axis shows angle results for each cell. Error bars is standard deviation = 3.3. Scale bars = 3 μm.



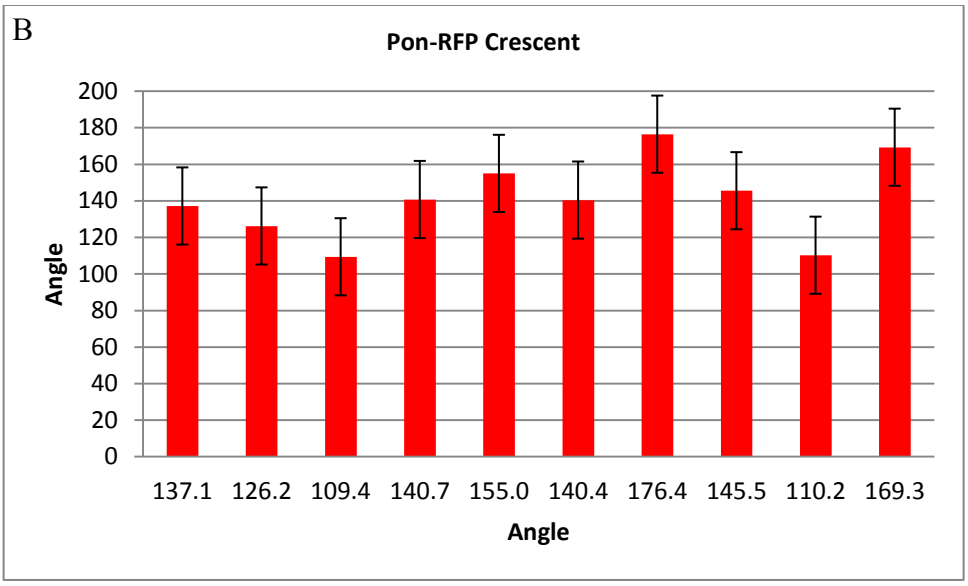


Figure 22: Quantification of Pon-RFP crescent expansion. **(A)** A pair of confocal time lapse images of wild type SOP cells showing complete built-expanded crescent at metaphase onset. In both cases, Pon-RFP expansion shows some variance. **(B)** Quantification of crescent expansion of 10 SOP cells. Pon-RFP expansion shows significance variance among SOP cells. The X axis shows angle results for each cell. Error bars is standard deviation = 18.2. Scale bars = 3 μm.

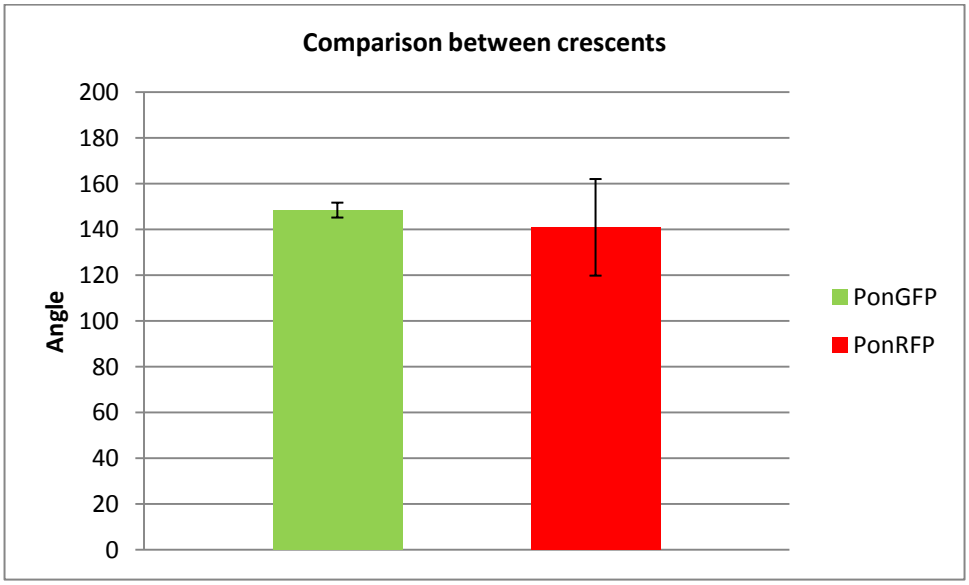


Figure 23: Quantification of Pon-GFP and RFP crescent expansion. Graph showing a comparison between expression of Pon-GFP and RFP in SOP cells. Angle mean for Pon-GFP is 148.5 and for Pon-RFP are 142.8. Pon-GFP shows less variation in expansion, whereas RFP results shows otherwise. Standard deviation: Pon-GFP = 3.3 / Pon-RFP = 18.2. *P*-value: 0.3308.

3.2.1. Setting a threshold for crescent expansion (width)

From the obtained angle results from measuring the crescent expansion (width), again, we increased the number of SOP cells in order to set up a threshold based on wild type conditions. Using Image J, we measured the angle of the crescent width of up to 100 random wild type SOP cells. Also at the onset of metaphase. These results are represented in a probability histogram of Gaussian-distribution. Using the minimum (128.2°) and maximum (161.2°) angle of expansion (width) values, the threshold for the crescent formation-expansion was chosen arbitrary. These results, that SOP cells in wild type conditions at the onset of metaphase have variation in crescent expansion, suggest the idea that the

mechanism of cell fate determinant recruitment to the cortex can be independent of the mitotic spindle alignment in SOP cells. The crescent expansions that fall outside the range of <128.2°>161.2° will be considered as abnormal crescent expansion (width).

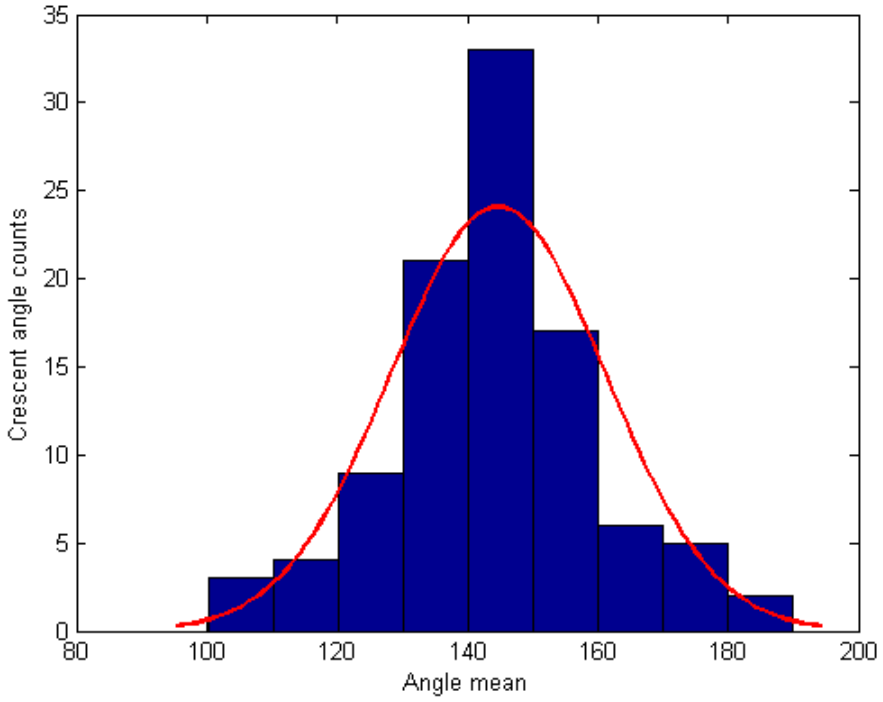


Figure 24: Probability histogram of Gaussian-distributed of angle measurements of the crescent expansion (width) of 100 random wild type SOP cells at the onset of metaphase. Mean = 144.7°, Standard deviation σ = ± 16.5. Arbitrary threshold between 128.2° and 161.2°. <128.2°>161.2° will be considered as abnormal crescent expansion (width).

3.3. Bleb dynamics in SOP cells

It is well known that blebbing is a read-out of cortical tension release. It arises when the membrane detaches from the actin cytoskeleton conceiving an expansion-retraction cycle [88]. Very little is known about the blebbing mechanism in SOP cells that we decide to explore blebs behaviour during asymmetric cell division. We have hypothesize that blebbing occurs at the posterior side of the dividing SOP cells at telophase. We manually quantified time-lapse images of SOP cells and observe that blebs tend to occur more at the anterior side of SOP suggesting high anterior actin contractions. Our obtained results disproved the hypothesis that blebs occurs at the posterior pole of SOP cells.

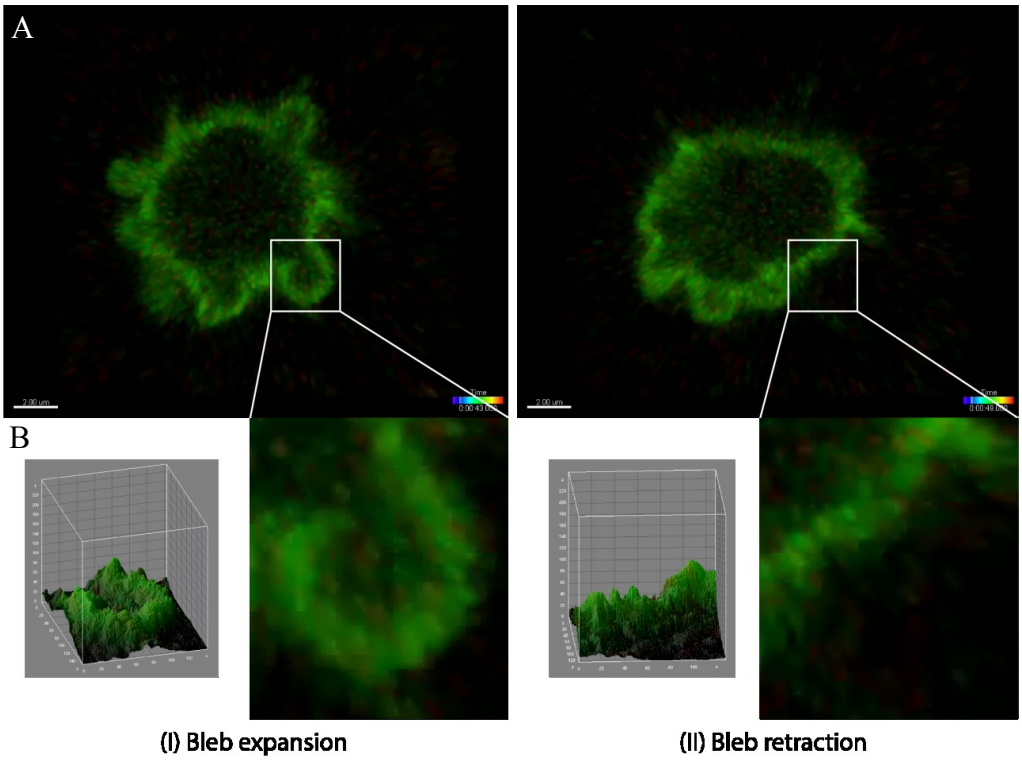
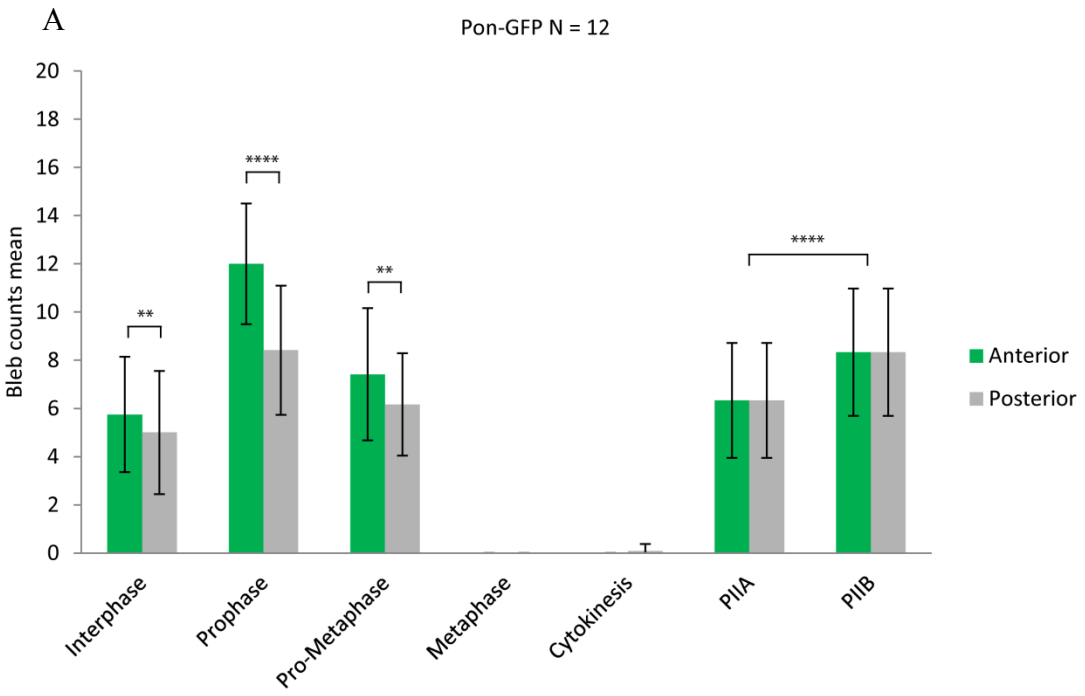
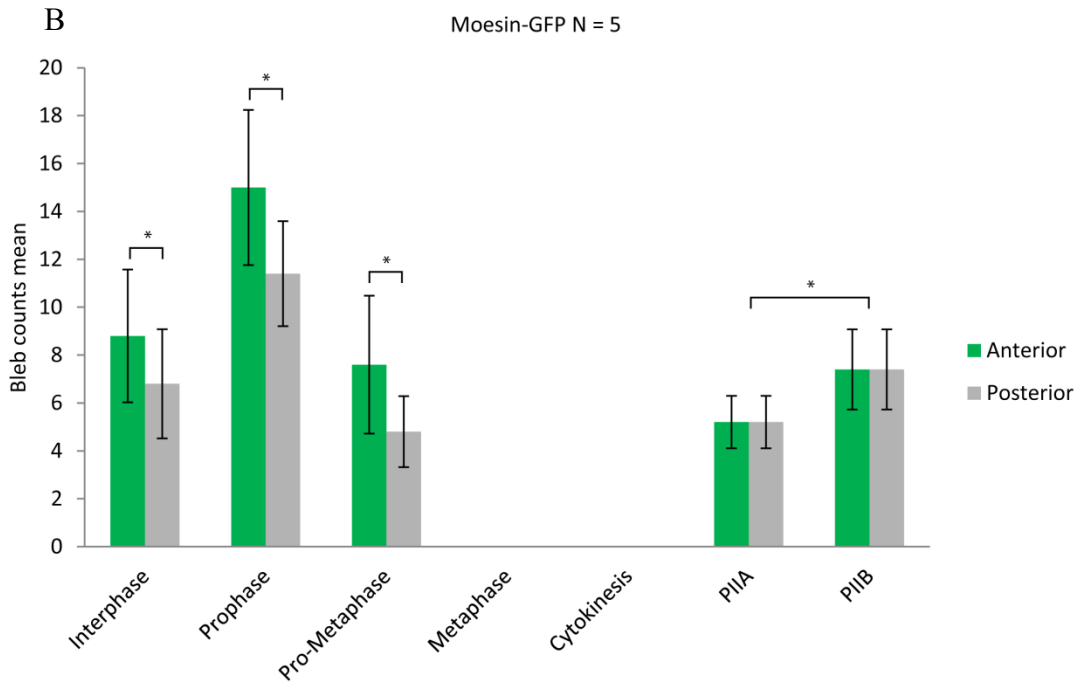


Figure 25: Blebbing SOP cell. **(A)** Time-lapse images showing blebs expansion-retraction cycle. As cells enters prophase, cortical tension increase hence increasing the number of blebs **(B)** High magnification of assembly and disassembly of the actin cortex. GFP intensity of blebs is shown as 3D distribution plots. Anterior is oriented towards left. Scale bars = 2 μm.

3.3.1. Quantifying blebs with Pon-GFP, Moesin-GFP and Lgl3A-GFP




B
Moesin-GFP N = 5
Bleb counts mean
20
18
16
14
12
10
8
6
4
2
0
*
*
*
*
Interphase
Prophase
Pro-Metaphase
Metaphase
Cytokinesis
PIIA
PIIB
Anterior
Posterior

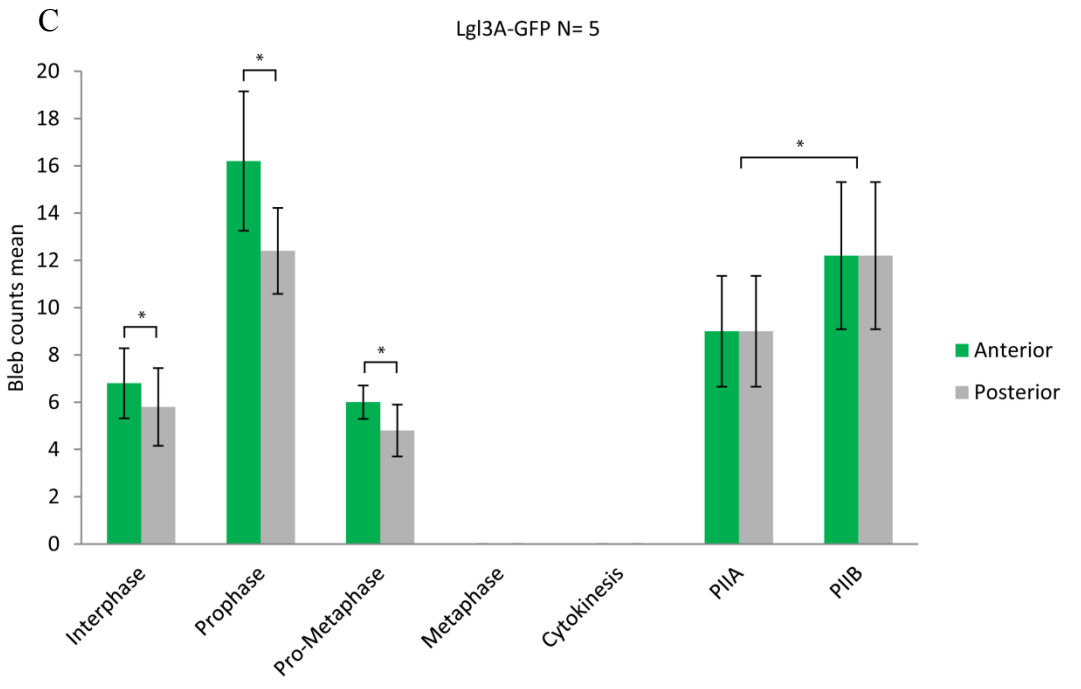

C
Lgl3A-GFP N= 5
Bleb counts mean
20
18
16
14
12
10
8
6
4
2
0
*
*
*
*
Interphase
Prophase
Pro-Metaphase
Metaphase
Cytokinesis
PIIA
PIIB
Anterior
Posterior

Figure 26: Bleb quantification during the asymmetric cell division cycle. Quantification of blebs of time-lapse imaging of SOP cells from interphase to division into PIIA and PIIB **(A)** Pon-GFP **(B)** Moesin-GFP **(C)** Lgl3A-GFP. In all conditions blebs are increased at the anterior pole from interphase until division. These results suggest high actin contractions at the anterior pole. Error bars represent SD. Asterisks denote significant differences in bleb quantification between anterior-posterior SOPs during time at different stages of the cycle. *P*-values can be observed in (**Table 4**).

	Interphase	**Prophase**	**Pro-metaphase**	**Metaphase**	**Cytokinesis**	**PIIA**	**PIIB**
Pon-GFP N = 12	0.0015**	<0.0001 ****	0.0063**	0	0	<0.0001 ****	<0.0001 ****
Moesin-GFP N = 5	0.0474*	0.0288*	0.0189*	0	0	0.0109*	0.0109*
Lgl3A-GFP N = 5	0.0341*	0.0270*	0.0327*	0	0	0.0349*	0.0349*

Table 4: Table of *P*-values for bleb counts for corresponding stages during asymmetric cell division cycle. *P*-values were calculated from anterior-posterior bleb counts mean.

4. Discussion and Conclusion

4.1. Mitotic spindle orientation, small GTPases and blebbing

Cellular self-renewal, cell fate diversity and daughter cell differentiation is often generated by asymmetric cell division[1,2]. Our time-course imaging show that both Pon-GFP and Pon-RFP localize at the anterior pole of the PI SOP during prophase before the formation of the mitotic spindle (**Figure 16**, **Figure 17** and **Figure 18**). In addition to Pon, while other proteins such as Numb, Pins, Mud and Gαi localize at the anterior pole. Par proteins such as Baz, Par6 and aPKC localize at the posterior pole[2,28]. The tight cooperation of these network of proteins at the anterior-posterior poles helps the orientation and position of the mitotic spindle relative to the cell polarity axis during asymmetric cell division[8,96]. Previous work published by Cabernard *et al*. has shown a spindle-independent mechanism for cleavage furrow positioning in *Drosophila melanogaster* neuroblasts. They showed a unique mechanism for asymmetric cell division by which furrow specific proteins are localized at the basal cortex at anaphase onset and can induce furrow displacement in the total absence of the mitotic spindle. These observations led to the hypothesis that another regulatory pathway possibly involving small GTPases might act on the mitotic spindle through the actin cytoskeleton.

Previous works have reported connections between polarity proteins Baz, Par6 and small GTPases, showing that activation of these proteins requires physical interactions with constitutively active forms of small GTPases[3,4,81,86]. These works highlighted the importance of the association of small GTPases with polarity proteins suggesting a role of these small GTPases at the cellular poles. Therefore, they represent important targets for the regulation of asymmetric cell division and possible implication on the mitotic spindle regulation.

Mitotic spindle orientation and position is an important mechanism to line up the spindle with asymmetrically localized cell-fate determinants in organisms like *C. elegans* and *D. melanogaster*[10]. In the P1 SOP cell, centrosomes separate at a random position around the cytoplasm to start aligning the mitotic spindle as the cell enters prophase. As the cell enters metaphase, each centrosome localizes at the anterior and posterior poles of the SOP respectively forming the whole structure with the spindles and DNA. This structure aligns towards the anterior crescent positioning of the DNA and mitotic spindle with a 90° angle relative to the SOP in the cell polarity axis (**Figure 16**, **Figure 17** and **Figure 18**).

We set out to accurately quantify the mitotic spindle angle in order to detect subtle movements of the spindle. Indentifying these components in a time-course of the asymmetric cell division cycle was pivotal. Using SOP cells in wild type conditions, we extracted and quantified the geometry of the mitotic spindle, the DNA and the anterior crescent formation-expansion. We performed this in up to 100 SOPs in order to be statistically significant. After obtaining the angle results from both the DNA and the centrosomes as reference, we increased the number of SOP cells up to 100 in order to set up a threshold based on wild type conditions. We measured the angle of random wild type SOPs at the onset of metaphase. By using the minimum and maximum angle values, the threshold for the positioning of the mitotic spindle were chosen arbitrary, where everything outside the range of <84°>98° will be considered as misaligned spindles. Our data supports the observation that in SOP cells, the mitotic spindle forms an angle with very little variations, which is tightly aligned with respect to the crescent at the onset of metaphase (**Figure 20**).

In SOP cells, cell fate determinants like Numb and his anchor protein Partner of Numb (Pon) localize at the anterior side of the SOP building up a crescent[96]. We quantified the crescent formation-expansion (width) of SOPs at the onset of

metaphase. The crescent is aligned along the anterior-posterior axis of the pupae plane. It is important to measure the proper formation-expansion process of the crescent since it depends on the precise alignment of the mitotic spindle with respect to the crescent. Failure on the mitotic spindle alignment will be reflected on the crescent formation. The crescent will not form and instead be observed to be distributed all along the SOP due to abnormal spindle positioning, leading to equal inheritance of cell-fate determinants by PIIA and PIIB.

In addition, we asked whether there are variations between expressing different fluorophores, Pon-GFP and Pon-RFP. After measuring a set of SOPs expressing individually both fluorophores, we found that when SOP cells express Pon-GFP there is less variation in crescent formation (**Figure 21**) than SOP cells expressing Pon-RFP, which shows more variation in crescent expansion (**Figure 22**). One can argue that this behaviour might be due to the fluorophore stability during the process of *in vivo* imaging SOPs, suggesting that Pon-GFP might be a more suitable candidate for detection and quantification of subtle movements of the mitotic spindle during asymmetric cell division. Despite difference in crescent expansion, they show no significance among crescent width (**Figure 23**). Further, we set up a threshold based on wild type conditions by measuring the angle of the crescent width of up to 100 random wild type SOP cells at the onset of metaphase.

These results, that SOP cells in wild type conditions have variation in crescent width, suggests the idea that the mechanism of cell fate determinant recruitment to the cortex can be independent of the mitotic spindle alignment in SOP cells, meaning that cell fate determinant recruitment serves as starting point for the spindle alignment. In our measurement threshold, the crescent width that fall outside the range of <128.2°>161.2° will be considered as abnormal crescent width, thus defect in formation.

Another important behaviour to further understand asymmetric cell division is membrane blebs. Blebbing is a read-out of cortical tension release arising when the membrane detaches from the actin cytoskeleton conceiving an expansion-retraction cycle in different cellular organisms[88]. We had hypothesized that blebs tend to happen at the posterior side of the dividing SOP cells at telophase. On the contrary, our results disproved such hypothesis. We manually quantified time-lapse images of SOP cells and observe that blebs occur at the anterior side of SOP cells suggesting high anterior actin contractions at the anterior pole (**Figure 26**). These result led us to argue that perhaps higher actin contractions at the anterior pole of SOPs, have an influence on the mitotic spindle positioning, as well as on the size difference between PIIA and PIIB.

In the model presented by Bastos *et al.*[102] CYK4 GAP (Tum in *D. melanogaster*) for Rac1 regulates its activity at the onset of anaphase. Rac1 is inactivated at the cleavage furrow by CYK4 GAP, a component of central spindle complex. These events create an area where Rac1 shows low activity. However, this process shows otherwise at both poles of HeLa cells. Therefore, Rac1 activity is higher in these regions suggesting a possible role for Rac1 in regulating central spindle microtubules activity during cell division. Proteins Map205 and Clasp/Chb are involved in regulating astral microtubules and generation of pushing-pulling forces to maintain spindle position and proper division axis in mammalian systems[103]. Affinity capture-western experiments reveal physical interaction with small GTPase Rac1. With this data we could hypothesize that "Anterior cortical actin contractions clout the capture of astral microtubules at the anterior pole regulating the precise positioning of the mitotic spindle during asymmetric cell division". These provide a possible pathway through which Rac1 might regulate the mitotic spindle through actin cytoskeleton.

4.2. Geometry of the Sensory Organ Precursor

For this thesis, to study whether small GTPases act on the mitotic spindle, we have performed accurate quantification of the mitotic spindle positioning to detect subtle geometrical displacements of this mechanism. Computer-based image analysis allows collecting precise quantitative results. However, despite immense progress in image analysis and computer vision, such approaches fail to address many key aspects of cell division. This included the mitotic spindle that drives asymmetric cell division of SOP cells.

We built a simple platform to study the precise positioning of the mitotic spindle during asymmetric cell division of SOPs. This platform is based on accurate quantification whose strength is statistical significance. We have integrated geometric information that includes the mitotic spindle positioning, the crescent width and cortical blebbing. Understanding the behaviour of these parameters is pivotal to study asymmetrically dividing SOPs. Generally, the mitotic spindle of SOPs form an angle of about 90° in wild type conditions. This angle aligns along the anterior-posterior axis yielding asymmetric distribution of cell fate determinants and daughter cells of different sizes. Correct inheritance of cell fate determinants by the daughter cells depends on the proper position of the mitotic spindle. Subtle alteration in the positioning of the mitotic spindle leads to symmetric cell divisions. Also, measuring the width of the crescent can tell us whether the segregation of cell fate determinant drives the mitotic spindle formation or the formation and alignment of mitotic spindle that drives the proper segregation of cell fate determinants. Another parameter measured was blebs which is a read-out of how cells release cortical tension during and after division.

We have characterized a tendency of blebs to occur at the anterior side of the SOP. It is known that at the anterior side of SOPs, cell fate determinants like Numb and Pon and spindle orientation proteins Mud, Pins and Gαi[28] are localized. With our observations and the ones in the scientific literature, we can hypothesize that the high cortical tension at the anterior pole, the sub-network of protein interactions between Numb, Pon, Mud, Pins and Gαi clout the proper formation of the mitotic spindle and the generation of asymmetry thus cell size difference between PIIA and PIIB. However, the lack of control over where and when (which assemble first and which assemble last) this whole structure is formed makes the precise spatiotemporal monitoring of mitotic spindle positioning and cortex behaviour a difficult task.

4.3. Perspectives and future approach

Taking together, the method discussed above may help to reveal important insights into how the entire geometry of asymmetric cell division can impact a variety of cell fates. The method established in this study can help to detect subtle movements of the mitotic spindle and how it orchestrates cell fates. The experimental settings developed in this thesis, to study regulation of the mitotic spindle orientation and positioning will serve as proof of concept for how geneticist and biochemist experts could design ways to control such processes through interdisciplinary methods in for example cancerous cells. Furthermore, the assays and the theoretical model developed in this study can be used as background that could serve to design improved quantitative experimental methods for cell biology such as synchronizing sub-networks of ACD mechanism. Moreover, due to the nature of this project, I programmed a theoretical model that can represent the asymmetric cell division in the SOP of *D. melanogaster* and generated testable

hypothesis using this model. This method can be easily used to extract, extrapolate and visually compare geometrical parameters of *in vivo* SOPs. With this method, it is now easier to quantify asymmetric cell division of a SOP population taking into account the statistical significance. At the moment this approach is under optimization to be extended towards *in vivo* 3D analysis. And to be more sensitive to slightly discriminate angle measurements in 3D of the DNA positioned towards the crescent formed by cell fate determinants.

This work provides an insight into how one should design and calculate the experimental strategies to visualize functional mitotic spindle orientation and position *in vivo* at a population scale of SOPs. With this, we can further explore the aspects of adaptive dynamics of the geometry of SOPs and distinct signaling pathways that controls the mitotic spindle. For instance, studying the protein-protein interaction networks at the anterior and posterior poles that control these processes. Observations from such studies can be used to analyze whether the fitness of such networks are dependent on the combination of the whole or on simple independent cues that control this wonderful process during a specific cell fate choice. Our work opens new avenues to study the importance of subtle alterations that can drive the whole cellular system and to take control of such behaviour towards controlling diseases such as cancer.

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6. Appendix

6.1. Table of angle combinations

Angle	X – X' axis / DNA	Y – Y' axis / Spindle
When angle = 90°	1 – 180	90 – 270
When angle = 180°	90 – 270	1 – 180
When angle increase	90 + x ; 270 + x	(1 + x) % 359 ; 180 + x
When angle decrease	90 - x ; 270 - x	(-1 - x) % 359 ; 180 - x

6.2. Matlab script

```
########################################################################
##          DNA          /          Spindle          position          quantification
########################################################################

%% Create new folder

mkdir('DNA_spindle')                    % Asign folder name as "DNA_spindle"
mkdir('parentFolder','DNA_spindle')     % "parentFolder" asign a new working path
E.g: C:\Users\Art\Desktop\MatLab Directory\DNA_spindle

status = mkdir(___)                     % Display the directory you are in

%% Change current folder
```

```
cd(newFolder)                    % Changes the current folder to the new created folder
oldFolder = cd(newFolder)        % Specify current folder

cd

##########################################################################

%% Ng4, H2ARFP, PonGFP / TM3 %% Wild Type

sdir = [90 270 94 274 86 266 90 270 89 269 99 279 100 280 90 270 96 276  91 271
180 1 184 4 172 345 180 1 179 360 198 18 200 20 180 0 186 6 181 1];

knots = [13 13 13 13 13 13 13 13 13 13 13 13 13 13 13 13 13 13 13 5 5 5 5 5 5 5 5
5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5];   % spindle position distance to the cell

cortex or "cell surface"

rdir = sdir * pi/180;                % convert to radians

[x y] = pol2cart(rdir, knots);  % polar to cartesian coordinates

compass(x,y)

hline = findobj(gca, 'Type', 'line');

set(hline, 'LineWidth', 2, 'color', 'b')

label = {'DNA/Spindle position at metaphase', 'Ng4,H2ARFP, PonGFP / TM3', ...

      'Wild type' 'N=10'};

text(-30, 15, label)

##########################################################################

%% Ng4, H2ARFP, PonGFP / Rac1T17N %% Mutant
```

```
sdir = [182 2 92 272 145 319 61 183 74 222 76 228 49 147 56 168 45 135 63 189 137
301 122 305 290 130 122 302 148 332 152 328 98 278 112 268 90 270 126 254 274 94
244 64];

knots = [13 13 13 13 13 13 13 13 13 13 13 13 13 13 13 13 13 13 13 13 13 13 13 5 5
5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5];   % spindle position distance to


the cell cortex or "cell surface"

rdir = sdir * pi/180;                  % convert to radians

[x y] = pol2cart(rdir, knots);  % polar to cartesian coordinates

compass(x,y)

hline = findobj(gca, 'Type', 'line');

set(hline, 'LineWidth', 2)

label = {'DNA/Spindle position at metaphase', 'Ng4,H2ARFP, PonGFP / Rac1T17N',
...

      'mutant' 'N=10'};

text(-30, 15,  label)

##########################################################################

%% Ng4, H2ARFP, PonGFP / Cdc42T17N %% Mutant

sdir = [90 270 114 285 128 320 140 308 41 221 130 310 136 316 92 272 90 270 80
260 180 0 228 48 256 76 280 100 220 40 32 212 36 216 182 2 180 0 165 345];

knots = [13 13 13 13 13 13 13 13 13 13 13 13 13 13 13 13 13 13 13 5 5 5 5 5 5 5 5
5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5];   % spindle position distance to the cell
```

```
cortex or "cell surface"

rdir = sdir * pi/180;                  % convert to radians

[x y] = pol2cart(rdir, knots);  % polar to cartesian coordinates

compass(x,y)

hline = findobj(gca, 'Type', 'line');

set(hline, 'LineWidth', 2)

label = {'DNA/Spindle position at metaphase', 'Ng4,H2ARFP, PonGFP / Cdc42T17N',
...

      'mutant' 'N=10'};

text(-30, 15, label)

##########################################################################

%% Ng4, H2ARFP, PonGFP / RhoLT25N %% Mutant

sdir = [78 234 177 357 94 274 87 263 90 270 54 164 109 289 79 259 96 276 86 266 95
275 154 326 87 267 188 8 174 354 180 0 110 160 19 199 169 349 186 6 176 356 185
5];

knots = [13 13 13 13 13 13 13 13 13 13 13 13 13 13 13 13 13 13 13 13 13 5 5 5 5 5
5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5];   % spindle position distance to the

cell cortex or "cell surface"

rdir = sdir * pi/180;                  % convert to radians

[x y] = pol2cart(rdir, knots);  % polar to cartesian coordinates
```

```
compass(x,y)

hline = findobj(gca, 'Type', 'line');

set(hline, 'LineWidth', 2)

label = {'DNA/Spindle position at metaphase', 'Ng4,H2ARFP, PonGFP / Rho1G14V',
...

      'mutant' 'N=10'};

text(-30, 15, label)
```