Coordination between mitosis and apico-basal polarity in tissue organization and tumorigenesis

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Abbreviations

AB: apico-basal

AurA: Aurora A

AJ: Adherens junctions

APC/C: Anaphase promoting complex/ cyclosome

aPKC: atypical protein kinase C

Arm: Armadillo

Baz: Bazooka

C. elegans: *Caenorhabditis elegans*

Cdk: Cyclin dependent kinase

CENP: Centromere-associated Protein

Crb: Crumbs

DAPI: 4',6'- diamidino-2-phenylindole

DNA: Deoxyribonucleic acid

GFP: Green fluorescence protein

GMC: Ganglion Mother Cell

hsp70: Heat-shock promoter 70

Lgl: Lethal giant larvae

MDCK: Madin-Darby Canine Kidney

Mis12: Minichromosome instability 12

par: partitioning defective

RNAi: RNA interference

SAC: Spindle assembly checkpoint

SOP: sensory organ precursor cells

S2: Drosophila Schneider 2 cell line

TSGs: Tumour Supressor Genes

UAS: Upstream Activating Sequence

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Abstract

The ability to asymmetrically distribute components within a single cell, termed cell polarity, is an essential feature of most cell types. Loss of cell polarity and chromosome instability are hallmarks of cancer, however these defects alone cannot induce tumorigenesis. We aim to understand if mitotic defects cooperate with loss of cell polarity in tumorigenesis. We screened for genes involved in different aspects of cell division, whose simultanous inactivation with polarity genes results in tumorous phenotypes. Using the eye imaginal disc and the follicle epithelium as models of epithelial tissue, we observed that defects in cytokinesis have the ability to induce the overgrowth of disorganized tissue in a polarity defective background. Establishing cell polarity relies on the evolutionary conserved apical Par Complex, comprised by Par-3, Par-6 and aPKC proteins. In neuroblasts, polarization only occurs during division to determine the asymmetric segregation of determinants of the daughter cell fate. In contrast, epithelial cells are polarized along the apico-basal (AB) axis during interphase to localize functions and adhesive properties at distinct cortical domains. However, during mitosis epithelial cells depolarize to accommodate cell shape changes associated with division. Aurora A kinase (AurA) has an essential role in neuroblast polarization during mitosis. It phosphorylates Par-6, participating in aPKC activation and the consequent phosphorylation of Lgl. This cascade of events allows the exclusion of Lgl from the cell cortex, which determines the basal positioning of cell fate determinants. To understand how epithelial cells link cell polarity and mitotic events, we addressed the role of aurA in AB polarity in the follicular epithelium. Mosaic mutant clones of an aurA kinase dead allele revealed that aurA kinase activity is not required to maintain epithelial AB polarity. The Lgl complex controls epithelial polarization by inhibiting the Par Complex activity on the basolateral cortex. Given that Lgl exits from the cell cortex during neuroblast division, we tested if it exhibited the same dynamic behaviour in epithelial cell division. In fact, similarly to the neuroblast, Lgl exits from the cortex to the cytoplasm prior than Nuclear Envelope Breakdown. Importantly, exit from the cortex might depend on phosphorylation, as a triple serine mutant version of Lgl is maintained at the cell cortex during division. The mutated sequence includes the aPKC phosphorylation site on Lgl, suggesting that aPKC could participate in Lgl exclusion from the basolateral cortex. However, when comparing depolarization timings between Lgl protein and apical polarity markers in epithelial cells, we observed that Lgl exit from the cell cortex begins before aPKC complex depolarization. Therefore our data supports that the cascade of events determining changes in polarity during mitosis is inverted in epithelial cells.

Resumo

A capacidade de distribuir assimetricamente os constituintes de uma célula, polaridade celular, é uma característica essencial na maioria das células. A perda da polaridade e a instabilidade cromossómica são marcadores cancerígenos, sendo que estas anomalias sozinhas não são capazes de induzir tumorogénese. Propomo-nos a entender se anomalias a nível mitótico cooperam com a perda da polaridade celular de modo a iniciar a formação de tumores. Com este objectivo, procuramos genes envolvidos em diferentes aspectos da divisão celular, cuja inactivação, em simultâneo com a inactivação de genes envolvidos em polaridade, pudessem resultar em fenótipos tumurais. Usando o disco imaginal do olho e o epitélio folicular como modelos de tecido epitelial, observamos que defeitos em citocinese têm a capacidade de induzir o crescimento descontrolado de tecido desorganizado em cooperação com defeitos em polaridade. O estabelecimento da polaridade celular baseia-se no Complexo Par evolutivamente conservado, composto pelas proteínas Par-3, Par-6 e aPKC. Em neuroblastos a polarização só ocorre durante a divisão de modo a determinar a segregação assimétrica dos determinantes de diferenciação da célula filha. Em contraste, as células do epitélio são polarizadas ao longo de um eixo apico-basal (AB) durante a interfase para localizar funções e propriedades adesivas em domínios corticais distintos.

Contudo, durante mitose as células do epitélio despolarizam de modo a suportarem as mudanças na forma da célula associadas à divisão. A cinase Aurora A (AurA) tem um papel fundamental na polarização do neuroblasto durante a mitose. Fosforila o Par-6, participando na activação da aPKC e na consequente fosforilação do Lgl. Esta sucessão de eventos permite a exclusão do Lgl do córtex da célula, o que determina a posição basal dos determinantes de diferenciação. Para perceber de que modo o epitélio relaciona a polaridade celular e os diversos eventos mitóticos, fomos analisar qual o papel da AurA na polaridade AB das células epiteliais foliculares da *Drosophila*. Clones mosaicos de um alelo da cinase Aurora A "morto" revelaram que a actividade cinase não é necessária para a manutenção da polaridade AB. O complex Lgl controla a polarização epitelial ao inibir a actividade do Complexo Par no córtex basolateral. Dado que o Lgl sai do cortéx celular durante a divisão do neuroblasto, testamos se este exibia o mesmo comportamento dinâmico na divisão das células epiteliais. De facto, em semelhança com o neuroblasto, o Lgl sai do córtex para o citoplasma antes da ruptura do invólucro nuclear. Mais importante, a saída do córtex pode depender de fosforilação, já que uma versão mutante nas três serinas é mantida no córtex da célula durante a divisão. A sequência mutante inclui o local de fosforilação pela aPKC no Lgl, sugerindo que a aPKC pode participar na exclusão do Lgl do córtex

basolateral. Contudo, quando comparando os tempos de despolarização entre o Lgl e os marcadores de polaridade apicais na células epiteliais, observamos que a saída do Lgl do córtex celular começa antes da despolarização do complexo da aPKC. Portanto, os nossos resultados suportam a ideia de que a cascata de eventos que determina as mudanças na polaridade durante mitose está invertida nas células epiteliais.

CHAPTER 1 – INTRODUCTION

1. Cell division

To create a multicellular organism a single cell must divide multiple times. The sequence of events that enable cells to divide and generate two genetically identical daughter cells is known as cell cycle. Failure to maintain genomic stability has been associated with several diseases including cancer. Cell division comprises two major phases: interphase, during which the cell grows and accumulates nutrients required to duplicate its content; and the M-phase, during which the cell divides its content into two cells.

Interphase comprises three distinct phases: Gap phase 1 (G1), S phase and Gap phase 2 (G2). The DNA is faithfully replicated during S phase. This process is aided by both G phases ensuring that the cell is in favourable conditions to undergo division, giving time to the cell to accumulate all the macromolecules needed and checking for possible replication errors that would be catastrophic to the viability of the organism. If the extracellular conditions are not favourable, cells may enter in a specialized resting state known as Gap phase 0 (G0). Along with DNA replication, cells also replicate their centrosomes but these remain together until the onset of division. M-phase comprises two distinct stages: nucleic division also called mitosis, and cytoplasmic division or cytokinesis. Mitosis per se is also divided into five stages - prophase, prometaphase, metaphase, anaphase, and telophase – that must occur in this sequential order, while cytokinesis begins at the end of telophase and is concluded much later (Fig. 1) [2, 3].

The macromolecular components responsible for induction of all these sequential phases are the Cyclin-dependent protein kinases (Cdks), and their regulatory subunits called Cyclins. Different Cyclins are produced in each stage of the cell cycle, resulting in the formation of a series of Cyclin-Cdk complexes that regulate progression through each stage. After S phase, the cell contains a duplicated set of chromosomes each made up of two tightly associated sister chromatids. Prophase begins at the onset of chromosome condensation. At mid and late prophase, the centrosomes start to separate and the mitotic spindle begins to assemble in the cytoplasm, nucleated by the centrosomes [4]. Prometaphase begins abruptly with Nuclear Envelope Breakdown (NEBD) that is triggered by M-Cdk phosphorylation of the nuclear lamina. NEBD enables microtubules from the mitotic spindle to reach and interact with chromosomes (reviewed in [5]). Prometaphase continues until all sister chromatids are attached to the spindle and have migrated to the equatorial plane of the cell, a process known as chromosome

congression. At metaphase, all chromosomes are aligned at the center of the spindle (metaphase plate) waiting for the signal to separate (reviewed in [1]). The metaphase-to-anaphase transition is triggered by the activation of the anaphase promoting complex/ cyclosome (APC/C). The activation of APC/C leads to M-Cdk inactivation and Securin degradation by ubiquitination, thereby activating Separase – a protease that cleaves the cohesion complex that holds sister chromatids together, allowing their separation. In telophase, the final stage of mitosis, a nuclear envelope reassembles around each group of chromosomes to form the two daughter interphase nuclei (reviewed in [6]).

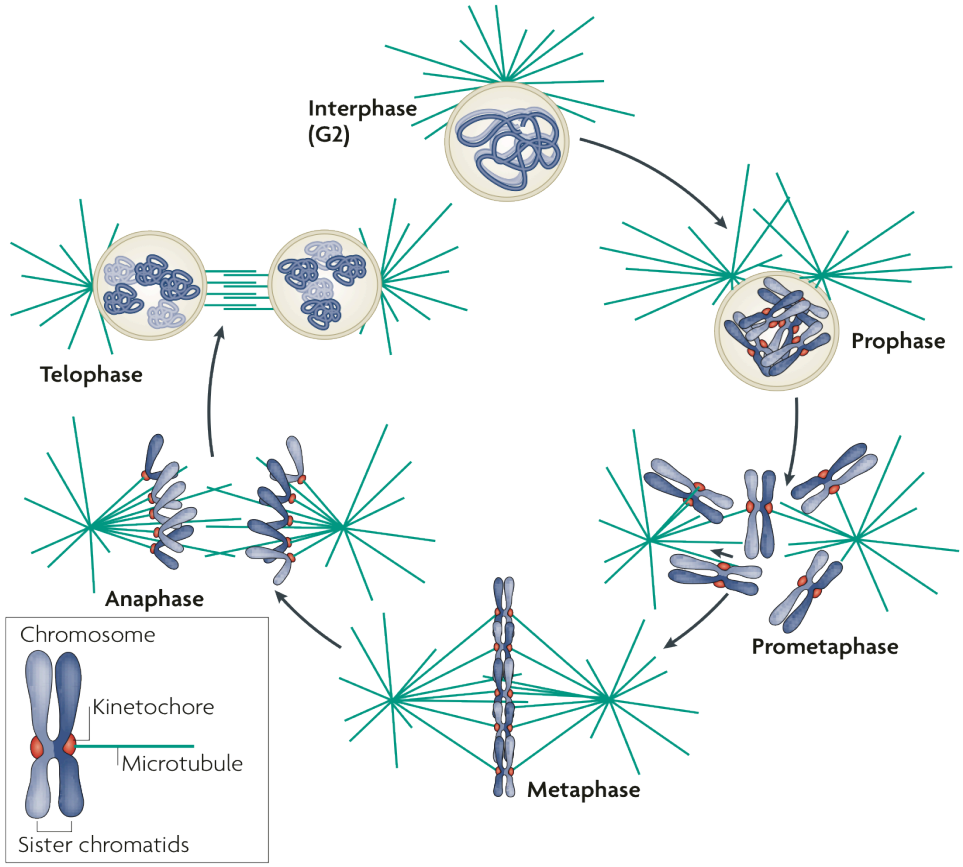


Figure 1 - Schematic representation of kinetochore-microtubule interactions during mitosis. As a highly dynamic process, mitosis comprises the occurrence of multiple processes: NEBD at prometaphase, chromosome bi-orientation on metaphase, and sister chromatids separation during anaphase. The correct interaction of microtubules with kinetochores is the key event to successful chromosome segregation. Adapted from ([1]).

Additionally to the precise separation of the DNA, the common cytoplasm and its contents must also be divided. The mechanical process that ensures the physical division of the cytoplasm is known as cytokinesis. This process in under tight temporal and spatial regulation to ensure that it only occurs after chromosome segregation and in a specific

orientation. Cytokinesis involves the establishment of a division plane and the fusion of the plasma membrane in both sides of the cleavage furrow, after contraction of the actomyosin ring. As any other type of cell shape change, cytokinesis requires the orchestration between several processes such as cytoskeleton remodelling, endocytic traffic of vesicles and the localization of several proteins involved in the process (reviewed in [7]). At the end of mitosis, after anaphase onset and chromosome segregation, the spindle microtubules in the midzone of the cell are enriched in several proteins that will boost cytokinesis (reviewed in [8]). One of these proteins is Anillin, which functions as a scaffold protein, binding to F-actin and myosin, and recruiting Septins – GTPases proteins - to the contractile ring. The contractile ability of this actomyosin ring is of extreme importance. It is this contraction that allows the physical separation of both daughter cells by promoting the ingression of the cleavage furrow that will merge at opposite sides of the membranes (reviewed in [9]). Anillin depletion in *Drosophila* and human cells causes abnormalities in the symmetry of the cleavage furrow and consequently raises the likelihood of cytokinesis failure ([10]). Failures in cytokinesis generally lead to tetraploidy creating genomic instability, which is known to be a mechanism that could lead to tumour development (reviewed in [8]).

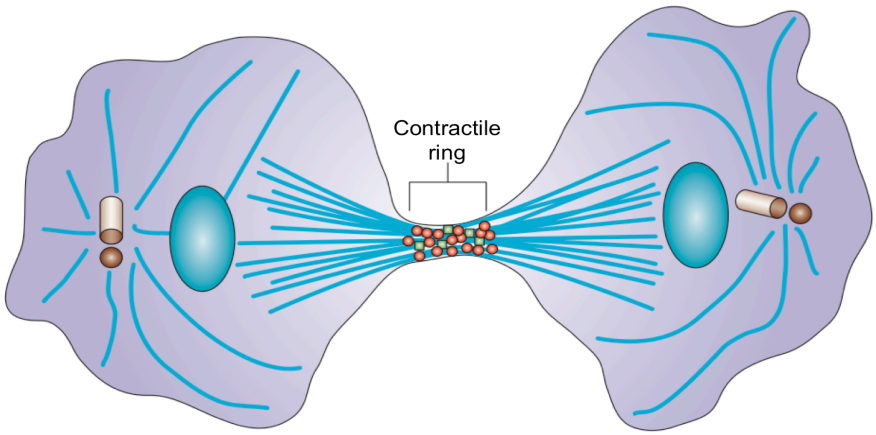


Figure 1.2 - Representation of cleavage furrow ingression during cytokinesis. The figure is showing the position of the contractile ring and its associated proteins. Adapted from ([11]).

The central purpose of a mitotic cell is to accurately segregate its chromosomes, generating two daughter cells with the same amount of DNA. Several strategies ensure the fidelity of this process in order to avoid errors that could compromise the viability of the organism. One of these strategies is the construction of a physical structure on specific regions of condensed chromosomes - the centromeres - that mediates and supports the

interaction between the microtubules of the mitotic spindle and the chromosomes. This proteinaceous structure is known as kinetochore (reviewed in [1]). If kinetochore formation fails, aberrant segregation occurs leading to cell death. Therefore the centromere plays an important role in cell cycle: it ensures that kinetochore assembles only in one single site, propagating this information through generations. Connecting the centromere to kinetochores there is CENP-A, which furnishes the bases to the assembly of the subsequent proteins (reviewed in [12]). CENP-C, a structural protein of kinetochores, allows the connection between the determinant of kinetochore identity- CENP-A – and the interface that will interact with microtubules – the KMN complex. This complex is composed by the KNL1 protein (also named Spc105 or Blinkin), and the Mis12 and Ndc80 sub-complexes. (reviewed in [1], [13]). The four subunit Mis12 complex - Mis12, Nnf1, Nsl1 and Dsn1 subunits – do not appear to interact directly with microtubules. However, when it binds to KNL-1, the Mis12 complex enhances the microtubule-binding activity of KNL-1 ([14]). The Ndc80 complex is composed by two heterodimers, Spc24/Spc25 and Ndc80/Nuf2 subunits, being the last heterodimer directly responsible for binding to microtubules [14-18]. The four-subunit Chromosomal Passenger Complex (CPC), that includes the Aurora B kinase, is the responsible for the correction of the errors in kinetochore-microtubule attachments. Lack of tension between sister kinetochores seems to be the principal factor that leads to Aurora B phosphorylation of the Ndc80 subunit, decreasing the microtubule-binding affinity of the Ndc80 complex, and eliminating incorrect kinetochore–microtubule attachments – Fig. 1.3 ([19]). If correction of erroneous kinetochore-microtubule attachments is a role for Aurora B kinase, the delay on mitosis that gives time to the cell to correct errors relies on the Spindle Assembly Checkpoint (SAC) [21-23]. This mitotic checkpoint is composed by several proteins that localize transiently in the kinetochore until the correct microtubule attachments are sensed. The MAD (mitotic-arrest deficient] proteins - MAD1, MAD2 and MAD3 [BUBR1 in humans) - the BUB (budding uninhibited by benzimidazole) proteins - BUB 1 and BUB3 - and also the MPS1 protein are all members of the SAC [24-26]. SAC negatively regulates the binding ability of Cdc20 - a co-factor of the ubiquitin ligase Anaphase-Promoting Complex/Cyclosome (APC/C) – preventing the polyubiquitylation by APC/C and consequent degradation of two key substrates, Cyclin B and Securin. As Securin is an inhibitor of Separase - a protease that cleaves the cohesin complex that holds sister chromatids together – and Cyclin B the activator of the mitotic Cdk, SAC signalling blocks cell exit from mitosis (Fig. 1.4).

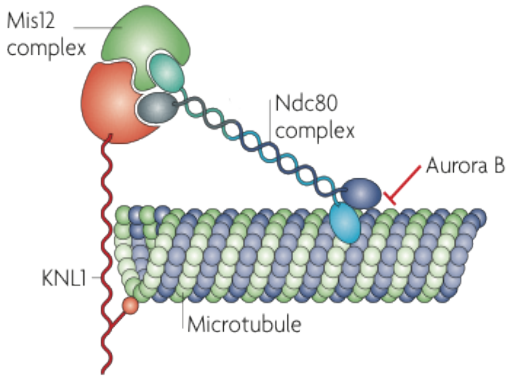


Figure 1.3 - KMN Network and Aurora B controling kinetochore-microtubule attachments. The CPC component Aurora B is the responsible for sensing incorrect kinetochore-microtubule attachments, phosphorylating the Ndc80 complex and allowing the reestablishment of bipolar attachments. Adapted from ([1]).

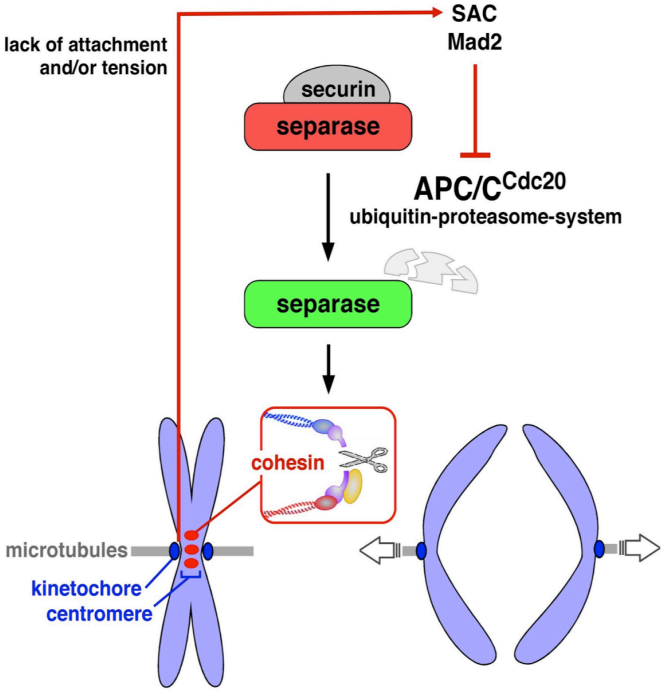


Figure 1.4 - Sister chromatid separation after activation of the APC/C^{Cdc20} complex . Correct kinetochore-MT attachments inactivate SAC signaling, that in turn allows the binding of Cdc20 to the APC/C. The activation of APC/C^{Cdc20} leads to the polyubiquitylation of securin and cyclin B, targeting them for destruction. Securin destruction releases separase that cleaves the cohesion complex, allowing anaphase completion. Adapted from department of Genetics, University of Bayreuth, Germany

Upon SAC inactivation, anaphase proceeds and the cell exits from mitosis (reviewed in [1]; [27, 28]; [29]) . “The Kinetochore is therefore a highly complex machine that does not merely bind and affects the dynamic behaviour of attached microtubules, but is involved in quality-control mechanisms that detect and correct defective or non-productive kinetochore-microtubule interactions” (reviewed in [30]).

2. Cell polarity

Multicellular organisms are though to result from the necessity to adapt to new environmental conditions and to utilize resources that a single-cell organism is unable to use. The cells within a multicellular organism are dependent on each other, such that each cell type is specialized in different and essential functions. These specialized cells constitute distinct tissues that work together within an organism. The epithelial tissue, shields us against pathogens, enables selective absorption and it also actively participates in the overall body secretion. In order to provide these functions, the epithelial tissue must form a selective barrier, so that signals between cells can be transmitted but harm signals from the external environment could be prevented to reach the organism. Epithelial cells developed a strategy to fulfil this propose: the formation of connections between cells. Cell-cell connections are a key feature of epithelial tissue, holding cells together in an organized monolayer that offers protection, and simultaneously allows transcellular transport. The Adherens Junctions are a type of connection only found in epithelium, considered by many as the closest junction in the living world. Besides its extracellular functions, Adherens Junctions participate in the definition of distinct domains within an epithelial cell by acting as an intracellular barrier to macromolecules, regulating cell polarity.

Cell polarity refers to all the asymmetry of macromolecules distribution that leads to differential localization of functions into particular regions of a given cell. Cell polarization is a highly complex process: coordination between microtubule and actin remodelling, vesicle trafficking and cell-cell junctions' establishment must be orchestrated to enable cells to carry out their functions properly. The importance of cell polarity is illustrated by several examples that rely on it: an axon must be polarized in order to transmit information to another neuron or an effector organ ([31]); leukocytes migrate towards a stimulus in a polarized-dependent manner ([32]); the establishment of asymmetric axes on vertebrate animals; the development of a fertilized egg or the growth of an unicellular organism ([33]). In conclusion, a cell from a multicellular organism must be specialized in a particular function, and in many cases to gain this specialization, that cell must become polarized to asymmetrically distribute its components. For instance, in epithelial cells, junctions must be formed at precise locations along the apical-basal axis.

2.1 Models of epithelial tissue in *Drosophila*

As previously mentioned, cell-cell connections and a remarkable polarization are common features of epithelial tissues. Giving their essentiality to the proper function of the tissue, these characteristics were conserved among several organisms, being *Drosophila* and mammals a well-known example of distant related eukaryotes where these features were retained. In spite of sharing common epithelial characteristics, vertebrates and invertebrates display some differences in what epithelial domains organization is concerned. Both eukaryotic classes share the existence of the Adherens Junctions. However while vertebrates have a Tight Junction above the previous referred one, in the invertebrate world such structure does not exist. Invertebrates have a Septate Junction that links epithelial cells just below the Adherens Junctions (Fig. 1.5). Additionally, there is high conservation among the signalling pathways that enables the formation of these features. *Drosophila* can therefore be used as a model to provide insights of how higher eukaryotes regulate their signalling pathways, (reviewed in [34]). Throughout this work two different epithelial models from *Drosophila* were used: the ovarian follicular epithelium and the eye/antennal imaginal discs. Description of the principal features of these models will be done in the following section.

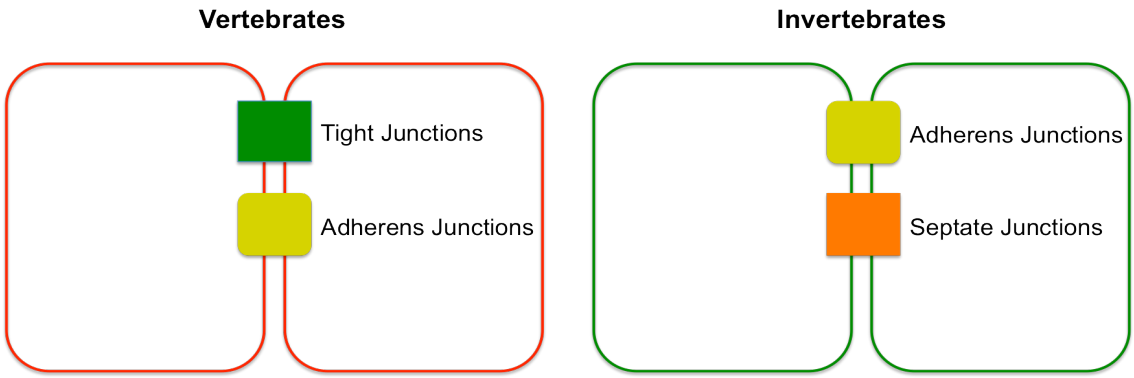


Figure 1.5 - Vertebrates and invertebrates epithelial domains organization. Both classes have the well-known Adherens junctions, however vertebrates have Tight Junctions instead of Septate Junctions that are presented by invertebrates.

2.1.1. Follicular epithelium

Each female has a pair of ovaries that are composed of a variable number of ovarioles (12 to 20). Each ovariole contains the germarium in the most anterior part, followed by progressively old egg chambers ([35]). The germarium accommodates the

germline stem cells that divide asymmetrically to give rise to other stem cell and a differentiated one, known as cystoblast. By undergoing four rounds of synchronous mitosis without complete cytoplasmic division, the cystoblast originates an oocyte and 15 nurse cells that remain connected by ring canals and therefore share the same cytoplasm. At the same time, somatic follicle cells surround the 16-syncytial germline cells, forming an epithelial monolayer, the follicular epithelium (reviewed in[36];[37]). The epithelial cells within the follicular epithelium show an unequal distribution of its proteins, being organized into apical and baso-lateral domains and so defining a striking apico-basal polarity (Fig. 1.6). As so this epithelial model can be used to understand a variety of biological aspects, as stem cell regulation or cell polarization. Moreover it offers the possibility of easily use molecular and genetic tools to manipulate gene expression, analysing the effects of the loss of a particular gene expression through division.

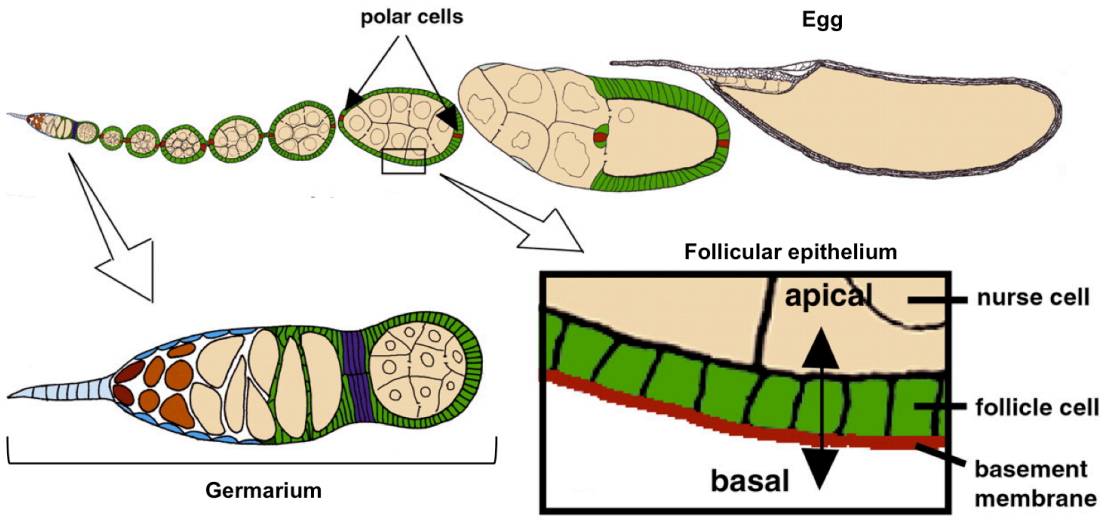


Figure 1.6 - Schematic representation of an ovariole and its specialized structures. The ovariole is composed of an anterior germarium that originates the posterior older egg chambers, ending in the formation of a mature egg. Surrounding each egg chamber is an epithelial monolayer called the follicular epithelium. Within this epithelium, the cells exhibit an apico-basal polarity, being the apical domain in intimate contact with the germline and the basal domain in contact with the extracellular matrix. Adapted from ([38]).

2.1.2. Eye/antennal imaginal discs

The adult *Drosophila* compound eye is another epithelial tissue commonly used to different studies. Primordially originated from the eye portion of the eye/antennal imaginal disc, the adult eye is constituted by more than 700 precisely arranged single unit eyes known as ommatidia. Tightly coordination between cell signalling, proliferation and cell death must be achieved in order to position and construct ommatidia correctly, enabling fly's vision. During *Drosophila* third instar larval stage, proliferation generates about 10,000 cells in the eye, setting the base where differentiation shall take place (reviewed in [39]). Proliferation ends when a physical constriction – the morphogenetic furrow (MF) – appears close to the posterior part of the eye/antennal disc. This morphogenetic furrow defines a barrier between proliferation - anterior to the morphogenetic furrow - and differentiation – posterior to this physical invagination. Within the furrow, cells arrest in G1-phase of the cell cycle. While it moves to the most anterior part of the eye portion of the disc, it allows the beginning of cell fate determination in its posterior side (reviewed in [40]). The differentiated cells will assemble in cores of 8 photoreceptors, cone cells and pigment cells, constituting each individual ommatidium (Fig. 1.7). As fly viability does not depend on the existence of an eye, the effect of specific genetic manipulations in the eye imaginal disc can be scored in the adult eye, which is particularly useful to test the role of essential genes.

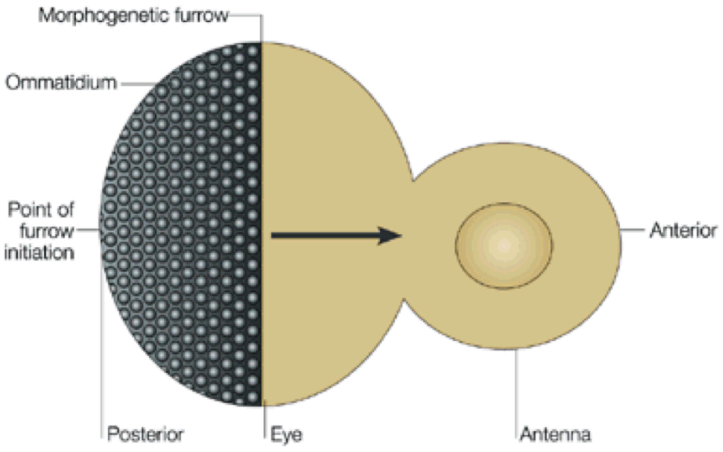


Figure 1.7 - Schematic representation of the eye/antennal imaginal disc in the third instar larvae stage. The morphogenetic furrow constriction initiates in the posterior part of the eye portion of the imaginal disc leaving a differentiation track as it moves to the anterior part of the eye disc. Adapted from ([41]).

2.2. Establishment of epithelial apico-basal polarity in *Drosophila*

Besides generating a huge number of cells to sustain the viability of a multicellular organism, these cells must acquire distinct and specific functions. So, different groups of cells must become specialized in a particular function to ensure that the organism takes the greatest advantage it can from the environmental resources. Cell specialization requires changes in the morphologic structure of the cell and in the distribution of its molecular contents. The asymmetric distribution of components within a cell that enable it to carry out distinct functions by distinct domains is known as cell polarity. Epithelial cells are segmented into four main specialized regions due to polarization: (1) an apical domain that orchestrates communication between the environment and epithelial cells; (2) specialized structures that promote connection of epithelial cells – adherens junction in *Drosophila* and tight junctions on vertebrates; (3) a lateral domain where Septate junctions are formed; (4) a basal domain that provides communication with the extracellular matrix (reviewed in [42]).

But which are the mechanisms that define cell polarization? In addition to my birth, key mediators of cell polarity were identified in *C.elegans during the eighties.* Ken Kemphues and Jim Priess aimed to identify proteins that interfere with the partitioning of C. elegans' embryo. During their genetic screen, six proteins, the Partition defective (PAR) proteins, were found to be involved in asymmetric cell division and distribution of proteins and RNAs essential for cell specification ([43]). This was the first insight that these proteins could coordinate an intracellular polarity pathway. The six identified PAR proteins have distinct biochemistry properties: PAR-1 and PAR-4 are serine-threonine kinases while PAR-5 is a 14-3-3 protein recruited to serines and threonines after phosphorylation. PAR-2 has a characteristic RING finger domain whilst PAR-3 and PAR-6 have PDZ domains (reviewed in [44]). Upon these findings in *C.elegans* the question was obvious: Do Par proteins also have related functions in other organisms? After cloning the six par genes, its sequences revealed that they were evolutionary conserved. Bazooka, a gene required in *Drosophila* cellular polarization, was found to be similar to PAR-3 ([45]). Posteriorly, in mammals, it was found that a PAR-3 homolog could bind to an atypical protein kinase C (aPKC) that would be sequentially identified in *C. elegans* as part of the group of proteins that causes defects in the partitioning of *C.elegans*' embryo ([46],[47]). PAR-3, PAR-6 and aPKC not only co-localize on the anterior part of the *C. elegans* zygote, but were also shown to form a complex in multiple systems, suggesting that they form a functional unit controlling cell polarization ([43];[48];[49]).

2.3. Interactions within the apical Par-6-aPKC-Par-3 Complex

It is now known that Par-6, aPKC and Par-3 form a complex involved in cell polarization in multiple biological contexts. By acting as a scaffold protein with multiple PDZ (Postsynaptic density 95, Discs large, Zonula occludens 1) domains, Par-3 (Bazooka in *Drosophila*) – sets the base for the association of Par-6 and aPKC, through self-association via its oligomerization CR1 domain in its N-terminal region. ([46];[50];[51];[52];[49];[53]). Par-6 also acts as a scaffold protein, binding to Par-3 through its C-terminal PDZ domain and to aPKC through its N-terminal Phox Bem1 (PB1) domain. In the middle of its sequence, Par-6 has a semi-CRIB (Cdc42 Rac Interacting Binding) motif that enables it to bind to GTP-bound Cdc42. It is known that when aPKC - the active component of the complex – interacts with Par-6, its kinase activity is suppressed. However the binding of GTP-bound Cdc42 to Par-6 semi-CRIB motif relieves Par-6 suppression of aPKC kinase activity. Thus, aPKC is correctly localized via PAR proteins, while its kinase activity is modulated via GTPase activity (Cdc42) (review in[54];[55];[52]). Mutation in any of the PAR Complex components leads to defects in the establishment of polarity, showing the importance of the interactions within the complex for cell polarization (reviewed in[34]).

2.4. Apical Polarity regulation

Adherens junctions (AJs) provide a cue to the establishment of different domains within an epithelial cell. They allow the communication between neighbouring cells while they generate an adhesive belt – the zonula adherens – that avoids the free passage of molecules from the external environment, improving this way epithelial selectivity. Its main constituent is a transmembrane protein, called E-cadherin, whose intracellular domain links to the cytoskeleton through β-catenin (Armadillo in *Drosophila*) that also has a role in AJs formation. By localizing themselves close to the apical region of the cell, they act like a barrier that is involved in the definition of the apical and the basolateral domains in *Drosophila* (reviewed in[56];[57]).

The formation of distinct domains relies on the existence of three different complexes: the above-mentioned Par Complex, the Scribble Complex, and also on the Crumbs Complex. This last complex is composed by 3 proteins: Crumbs (Crb) that is a transmembrane protein which localizes in the apical domain of a polarized cell; Stardust (Sdt) which binds to the intracellular region of Crb through its PDZ domains; and dPATJ that also interacts with Crb through its multiple PZ domains ([58];[59];[60],[61]). These

proteins have an essential role in establishing the apical domain, once that mutations in this complex result in loss of the apical identity. However they will not be further described because they were not studied on this work. The Scribble Complex will be described below.

Par-3, Par-6 and aPKC have been assumed to work as a complex to regulate polarity in many biological systems. However in epithelial cells, Baz (Par-3) was found to localize at the level of Adherens Junctions, being positioned below the Par-6 and aPKC proteins. ([62];[63];[64]). Consistent with this, Baz interacts with Armadillo and the Nectin-like protein Echinoid in Drosophila, and with JAM1-3 and Nectin in mammals, all components of Adherens and Tight Junctions, respectively, localizing in a more subapical region than the other members of the complex ([65];[66];[67]). In fact Bazooka have an essential role in the definition of the apical-lateral border by setting out the correct position of the AJs in *Drosophila* and the Tight Junctions in mammalian cells ([62]; [68];[69]). In the most apical region, Par-6, aPKC and Baz bind transiently to each other. However, Bazooka is phosphorylated on its serine 980 by aPKC. This action destabilizes the complex since phosphorylated Bazooka cannot bind to aPKC, disrupting Baz-aPKC interaction. ([46];[52];[49];[70]). Yet, Baz could still be maintained in the tripartite complex via its interaction with Par-6's PDZ domain. Nevertheless, the epithelial specific Crumbs Complex competes with Baz for the same PDZ domain of Par-6, breaking the interaction with Par-6 and excluding Baz from the apical domain ([70]). This exclusion leads to its localization on the subapical region where Baz defines the position of the Adherens Junctions. Evidences supporting this mechanism came from the fact that a non-phosphorylatable form of Bazooka – Baz^{S980A}, co-localizes with aPKC suggesting that phosphorylation by aPKC is necessary for the apical exclusion of Bazooka ([70]).

2.5. Interactions within the Scribble Complex and mutually inhibitory interactions with apical proteins

Drosophila provided the basis for the discovery of the Scribble Complex. Scribble was found in a screen with the purpose to identify maternal mutations involved in the disruption of cell adhesion and polarity. *scrib* mutations caused disorganization in the epithelial monolayer of *Drosophila* embryos ([71]). The Scribble protein belongs to the LAP (LRR and PDZ) protein family because it has 16 leucine-rich repeats (LRR) at its N-terminal region and four PDZ domains in the opposite region ([71]). Posteriorly, two other genes, Lethal giant larvae (Lgl) and Discs large (Dlg), were shown to present a similar phenotype when mutated, being all three known as tumour suppressor genes as they

cause neoplastic growth in larvae imaginal discs ([72]). Dlg is a 102-kDa protein that possess 3 PDZ domains, a Src homology 3 (SH3) motif and a guanylate kinase-like (GUK) domain, placing this protein as a member of MAGUK (membrane-associated with guanylate kinase domain) family. Lgl is a 130-kDa protein having short motifs (40 amino acids) of WD (Trp (W)-Asp (D)) repeats ([73];[74]). Due to their similar phenotype when mutated and their interdependent co-localization in multiple biological contexts, Scribble, Lgl and Dlg have been considered as a functional complex that regulates basal polarization on epithelial cells ([72];[73]). It was shown that Scribble indirectly interacts with Dlg in *Drosophila* neuromuscular synapses by both binding to GUK-holder protein (GUKH) ([75]). Furthermore, in mammalian epithelial cells, it was indicated that Scribble and Lgl2 had a physical interaction ([76]). None of these interactions have been identified in *Drosophila* epithelial cells so far. Despite of this, it is commonly accepted that these proteins function in a complex that orchestrate the basolateral polarity of epithelial cells. In fact, mutations in components of Scribble Complex result in the lateral extension of the apical domain and lateral disruption of the Adherens Junctions ([71]). As evidence, E-cadherin and Armadillo, both markers of the Adherens Junctions, are spread through plasma membrane in *scrib* mutants. It is therefore suggested that Scribble Complex participates in the exclusion of the apical proteins from the basolateral domain ([71]).

Both apical and basal complexes are involved in the correct positioning of AJs, once that Scribble Complex is essential to avoid AJs spreading through the lateral membrane, whereas the apical complex is required to exclude AJs from the apical domain. On the other hand, Armadillo prevents Dlg extension through the apical domain during follicular epithelium formation ([57]). AJs localization is then controlled by a mutually inhibitory mechanisms that comprise both apical and basolateral proteins. In fact, several lines of evidence suggest that apical and basal complexes restrict each other activity: (1) Loss of Scribble, Dlg or Lgl results in the extension of the apical proteins through the basolateral domain ([77]); (2) Both Lgl1 and Lgl2 bind Par-6 and suppress aPKC kinase activity in mammalian epithelial cells ([78];[79];[80]); (3) Lgl activity is restricted to the lateral cortex in an aPKC-phosphorylation dependent manner ([81]); (4) Crumbs overexpression has a similar phenotype as *scrib* mutant whilst *crb* mutant phenotype can be partially rescued by Scribble reduced levels ([77]); (5) it was found that Lgl2 and aPKC/Crb3 have an opposing function during *Xenopus* blastomeres polarization ([82]). All these data suggest that the separation between apical and basal domains requires the antagonism between the basal and the apical complexes that represses each other ectopic activity (Fig. 1.8).

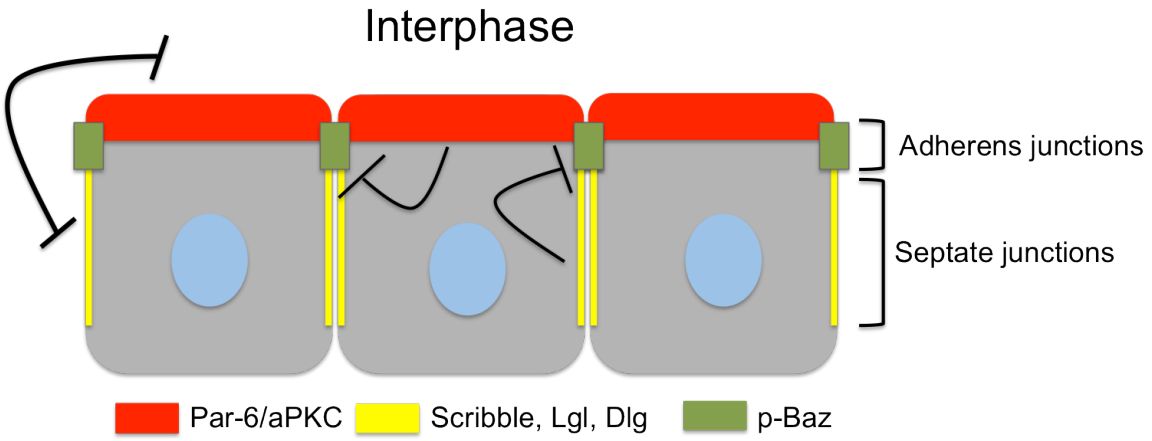


Figure 1.8 - Apico-basal interactions on the definition of epithelial cell polarity. Representation of the organization of the polarized domains within an epithelial cell. The main repressible interactions between Adherens Junctions and the apical a basal domains are represented by the inhibitory black curves.

3. Neuroblast polarity

While apico-basal polarity enables epithelial cells to organize specific functions within different cellular domains during interphase, the neuroblast makes use of the polarity machinery during mitosis to asymmetrically divide, creating two cells with different fates. Diversity is thus generated due to an asymmetric distribution of proteins that specifies two distinct regions: a self-renewal region, which will allow the maintenance of the neuroblast population, and a differentiation region that will lead to the formation of a differentiated cell, called Ganglion Mother Cell (GMC). This GMC will posteriorly divide originating two postmitotic cells that will become neurons or glial cells ([83]) (Fig. 1.9).

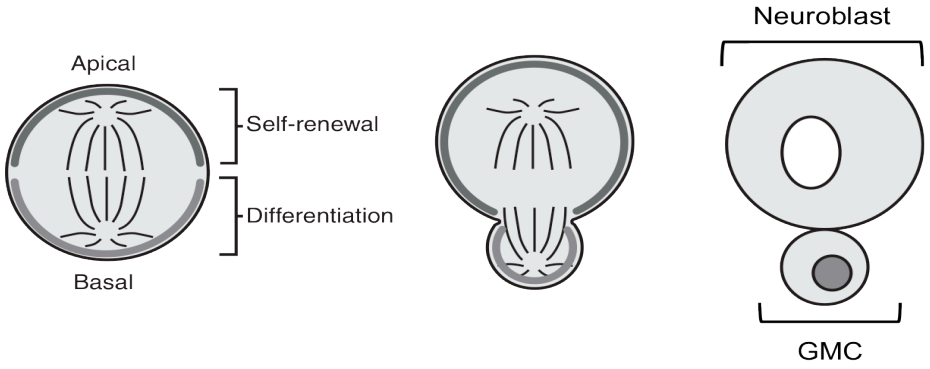


Figure 1.9 - Schematic representation of neuroblast division. Neuroblasts exhibit an apico-basal polarity during mitosis. After asymmetric division, two different cells are formed: a differentiated ganglion mother cell and a self-renewal neuroblast. Adapted from ([84]).

Polarization starts in prophase, when the GMC fate determinants start to be excluded from the apical cortex. By the time of metaphase the cell fate determinants are localized in the basal region of the dividing neuroblast, while the apical proteins occupy the upper domain; these two sets of distinct proteins will be segregated only into the GMC and in the self-renewal neuroblast, respectively ([85]).

The underlying mechanisms that enable the asymmetric distribution of proteins within the neuroblast have been extensively studied. Numb was the first identified cell fate determinant, being discovered in another cell type that divides asymmetrically, the SOP (sensory organ precursor) cells ([86]). Prospero (Pros) is a transcription factor that enables early identification of GMC ([87];[88];[89];[90]). Joining to the previous tumour suppressors, there is Brain tumour (Brat), which regulates miRNAs production ([91];[92];[93]). Either Pros or Brat are unable to bind directly to the cortex, requiring the adaptor protein Miranda ([94]). Partner of Numb (Pon) is a protein that enhances Numb association with the basal cortex ([95]). The mechanisms by which Numb and Miranda, in association with the other proteins – Pros, Brat, Pon – are cortically associated remain fairly unknown. Nevertheless, it is known that the apical proteins trigger the exclusion of the basal determinants to the basal domain, once that in the privation of an adequate polarized apical domain, the basal proteins have a cortical localization, which results in the loss of a differentiated daughter cell (reviewed in[84]). aPKC assumes an essential role in the exclusion of the basal determinants ([96];[97]). Wirtz-Peitz et al. in 2008 presented a very elegant mechanism by which the basal determinants are excluded in asymmetrically dividing cells, such as neuroblasts and SOP (sensory organ precursor) cells. These SOP cells are organized along an anterior-posterior axis, contrarily to the apico-basal axis arrangement on neuroblast. In SOP cells, during interphase, Lgl forms a complex with Par-6 and aPKC, and it is proposed to be an inhibitory substrate for aPKC ([98]). At the beginning of mitosis, Aurora A phosphorylates Par-6 reliving aPKC kinase activity from Par-6 suppression ([99];[98]). Thereby, upon activation, aPKC phosphorylates Lgl, solubilizing the protein and excluding it from the cell cortex, allowing it localization in the cytoplasm during mitosis in SOP cells ([98]). Lgl occupies the same binding site as Bazooka on aPKC and so Lgl exclusion allows Bazooka attachment to the Par-6-aPKC complex. Bazooka changes the substrate specificity of aPKC, recruiting Numb to be phosphorylated by the active component of the recent formed complex (Baz-Par-6-aPKC). Numb phosphorylation triggers its exclusion from that side of the cell, restricting its localization on the basal side ([100];[101]). Thus, Aurora A starts a cascade that ensures that Numb is phosphorylated in the correct time during mitosis, controlling neuroblast polarization ([98]). The same correlation between aPKC phosphorylation and Numb cortical exclusion was also found in mammals ([101]).

However, there are still many questions unanswered. An important question is how aPKC manages to restrict its activity to the apical domain of the neuroblast. As above-mentioned, these three proteins form a complex that is involved in the polarization of many cell types. Bazooka efficiently stabilizes both Par-6 and aPKC in the apical domain by directly binding to them, which is consistent with the fact that in *baz* mutants both Par-6 and aPKC are mislocalized ([96]). On the other hand, Cdc42 GTPase binds both the membrane and Par-6 through its semi-CRIB domain, having thereby a role in the recruitment of the Par Complex to the apical side of the neuroblast. Nevertheless, it has been shown that Cdc42 is mostly dispensable for Baz localization, suggesting that Baz could function upstream of Cdc42 ([99]).

Neuroblasts and epithelial cells share the key players on cell polarization. However, whereas neuroblasts become polarized during mitosis, epithelial cells are polarized during interphase. The polarity machinery of epithelial cells face therefore the challenge of accommodating large changes in cell shape during cell division. This fact lead us to investigate how the polarity machinery is re-organized during epithelial cell division and to address if the mitotic kinase Aurora A could contribute for this process.

4. Aurora A: A mitotic kinase with a role in cell polarity

Aurora A (AurA) gene was identified in a *Drosophila* screen for genes involved in the regulation of spindle-pole dynamics. Furthermore, it was shown that mutations in *aurA* lead to the formation of monopolar spindles by impairing centrosome separation ([102]). This gene encodes for a serine/threonine protein kinase. The Aurora gene family is known to include at least 3 genes, A, B and C in both invertebrates and vertebrates, however we will specifically focus on the functions of Aurora A. Aurora A associates with the centrosomes since their duplication in late S/early G2 until the next G1-phase ([102]). Moreover, proper centrosome maturation and separation requires Aurora A, whose recruitment to these organelles is Polo-like kinase-dependent ([103];[104];[105];[106];[107]). In *aur* mutants, the recruitment of several components of the pericentriolar material, such as γ-tubulin, is compromised. This fact leads to an increase in monopolar mitotic figures, and poliploidy (reviewed in[108]). It have also been observed that Aurora A localizes at the midbody, an “organelle” that is required for proper cytokinesis ([109];[110]). Finally, it was reported that Aurora A also has a role in the mitotic spindle assembly, which is consistent with its localization at the microtubules during mitosis. Aurora A targeting to the mitotic microtubules relies on its cofactor Tpx2, which promotes both Aurora A kinase activity and its autophosphorylation ([111];[112]). In

Drosophila aur mutants, misslocalization of mitotic spindle stabilizing proteins is observed, resulting in a reduced number of astral microtubules and a less lengthy bipolar spindle (reviewed in[113]).

Cell cycle progression is also dependent on Aurora A activity as it was shown among several organisms that mitotic entry is delayed when Aurora A function is impaired ([105];[114];[104]). The M-CDK major mitotic regulator is activated during prophase, triggering mitotic entry and being localized at centrosomes in this phase (reviewed in[115]). M-CDK localization in the centrosomes is Aurora A-dependent and coincides with centrosome maturation ([105]). Furthermore, activation of M-CDK at centrosomes relies on the phosphorylation of Cdc25B by Aurora A ([116];[117]). The role of Aurora A on mitotic entry was believed to be due to its function on centrosomes. However, it has been shown that Aurora A promotes mitotic entry independently of centrosomes in *Xenopus* egg extracts ([118]).

In addition to its roles in mitosis, Aurora A also performs functions on cell polarization, particularly in asymmetric cell division and cell fate determination. As described above, Aurora A initiates a cascade - by phosphorylating Par-6 and thereby allowing aPKC activation – that ends up with an asymmetric segregation of cell fate determinants during neuroblast division ([98]). Furthermore, a recent study in *Drosophila* S2 cells suggests that Aurora A may be also involved in spindle orientation during division. By inducing polarization in non-polarized S2 cells, it was found that Aurora A phosphorylates a serine within the $Pins^{LINKER}$ domain of Pins protein, a neuroblast apical component with a role on spindle orientation. Upon phosphorylation, this domain is able to bind and recruit Dlg, partially regulating spindle orientation ([119]). This relationship is also required for larval neuroblast asymmetric cells division in vivo. However, a non-phosphorylatable form of Pins ($Pins^{S436A}$), when expressed in neuroblast, gives a weaker spindle orientation phenotype than the one observed in aurora A null mutants ([120]; [121]). This result is still not surprising as Aurora A seems to regulate other processes that may influence spindle orientation, such as centrosome maturation. Through the wide range of functions described above, we can see that Aurora A has an important role by participating in both cell division and specialization in multicellular organisms.

5. The role of cell polarity and chromosomal instability in tumorigenesis

Cancer is known as a disease characterized by uncontrolled cell proliferation that could overflow nearby and distant tissues. In order to transform cells and allowing them to

form a tumour, several mechanisms within a cell must be affected. This is known as the multistage transformation process of cancer development. Below I will give an overview on how cell polarity and chromosomal instability can participate in transformation.

Aneuploidy is a term that concerns the changing in the normal number of chromosomes per cell, corresponding to the loss or gain of chromosomes. There are multiple mechanisms that can produce chromosome segregation errors, leading to aneuploidy. (1) Unequal distribution of the DNA content by failures in the spindle assembly checkpoint (SAC) response by delaying anaphase onset until all chromosomes are correctly attached (reviewed in [122]; [6]). (2) Defects on cohesion between sister chromatids or incorrect kinetochore-microtubules attachments can also lead to an abnormal number of chromosomes. This last condition occurs in the presence of merotelic attachments that are not solved by SAC signalling ([123]). (3) By the time of mitotic entry, if a cell possesses extra centrosomes, multipolar spindle can be formed and produce more than two cells with a defective quantity of DNA (reviewed in [124]). Different studies reported that abnormal levels of proteins involved in SAC signalling – MAD1, MAD2, BUB1, BUB3 and BUBR1 – and kinetochore structure – NDC80 - causes aneuploidy and chromosome instability ([125]; [126]; [127]; [128]; [129]).

Chromosome Instability and aneuploidy are often used as synonymous, however they do not represent the same phenomenon: while chromosome instability involves a dynamic behaviour, where the total chromosomes content within a cell population is changing through time, aneuploidy describes an abnormal number of chromosomes that resulted from chromosome instability in a precise point (reviewed in [130]).

Besides aneuploidy, other alterations in the total number of chromosomes per se are suitable to induce tumorigenesis. Proliferation of tetraploid cells was proven to also prompt tumour formation ([131]). There are several mechanisms whose disruption can lead to tetraploidy, as is the case of endoreduplication in *Drosophila* or cytokinesis failure induced by Aurora A overexpression. Nevertheless, tetraploidy may result in aneuploidy as uncontrolled proliferation of tetraploid cells accumulates structural and numerical chromosomal abnormalities (reviewed in [130]).

Whilst chromosome instability is directly related to cancer development, other mechanisms of tumour induction are not so obvious. Loss of cell polarization is a recently studied mechanism that has been associated to progression of tumours. Studies in *Drosophila* furnished the first evidences of the so-called tumour-suppressor genes (TSGs). The three current known fly TSGs – Lgl, Dlg and Scrib – presented a similar phenotype in the imaginal discs and larval brains suggesting that they may work together to control cell proliferation ([71]). In general, loss of TSGs in *Drosophila* results in uncontrolled cell proliferation and consequently neoplastic overgrowth and also failures in

the differentiation process, causing fly lethality before pupation (reviewed in [73]). In the follicular epithelium, loss of *dlg* causes invasion of germline by epithelial cells, occurring disorganization in the epithelial monolayer ([132]). Decreased expression of Scrib, Dlg and Lgl1 proteins is also found on human cancers, reinforcing the strategy of using *Drosophila* TSGs to easily provide insights about tumour development in mammals ([133]; [134]; [135]). However, as previously described, these TSGs are also implied in epithelial cell polarity regulation. Loss of *scrib*, *dlg* or *lgl* causes the spreading of the apical proteins and basolateral localization of AJs ([136]; [71]). Furthermore loss of cell polarity and tissue architecture has been implicated in various human cancers (reviewed in [73]). In summary (1) defects in several mitotic processes lead to tumorigenesis by promoting aneuploidy, tetraploidy and chromosome instability; (2) TSGs in *Drosophila* are both implied in tumour formation and loss of cell polarity; (3) loss of cell polarity is verified in several tumours as a mechanism that promotes progression during tumorigenesis. During this thesis, we will explore the link between mitosis, cell polarization and tumorigenesis.

6. Main goals

The work proposed in this project aimed to study further the relationship between cell polarity and tumour formation. For this we have used two highly polarized tissues of the developing *Drosophila* and asked two major questions:

1 – Identify possible interactors that would induce tumorigenesis in a polarity defective background of both epithelial models;

2 – Address the role of the mitotic kinase Aurora A in apico-basal polarity of follicle cells.

We hope to shed light on the conservation of cell polarity determination mechanisms between different tissues and then identify putative protein involved in tumour formation when cell polarity is compromised within a tissue.

CHAPTER 2 – MATERIALS AND METHODS

The principal *Drosophila* stocks used along this study are referred in table 2. The constructs non-mentioned in the table are described in Flybase (http:/flybase.bio.indiana.edu/). To manage the flies, standard procedures were used.

Table 2. Transgenes used into this study.

Construct	Source /Reference
anillin^{RNAi}	104674 – Vienna Drosophila RNAi Center (VDRC)
asp^{RNAi}	28741 - Bloomington Drosophila Stock Center (BDSC)
aur^{1}	Barros et. al, 2005
aur^{37}	Berdnik and Knoblich, 2002
aurA^{RNAi}	35763 – BDSC
aurA^{RNAi}	108446- VDRC
aurB^{RNAi}	28691 - BDSC
bub3^{RNAi}	21037 - VDRC
bubRI^{RNAi}	26109 - VDRC
cenp-c^{RNAi}	34692 – BDSC
cdc2^{RNAi}	36112 - BDSC
cnn^{RNAi}	35761 - BDSC
dlg^{RNAi}	25780 - BDSC
feo^{RNAi}	28926 - BDSC
kmn1^{RNAi}	106889 - VDRC
UASLgl^{3A}*-GFP*	From Jürgen Knoblich
UASLgl^{WT}*-GFP*	From Jürgen Knoblich
mad1^{RNAi}	43714 - VDRC
mad2^{RNAi}	106003 - VDRC
mis12^{RNAi}	19097 - VDRC
mitch^{RNAi}	104213 - VDRC
nuf2^{RNAi}	100235 - VDRC
orbit^{RNAi}	34669 - BDSC
par-6Par-6^{S34A}	From Jürgen Knoblich
pavi^{RNAi}	46137 - VDRC
peanut^{RNAi}	27712 - BDSC
polo^{RNAi}	33042 - BDSC
sak^{RNAi}	105102 - VDRC
sas4^{RNAi}	35049 - BDSC
scrib^{RNAi}	29552 - BDSC
septin1^{RNAi}	27709 - BDSC
septin2^{RNAi}	28004 - BDSC
smc1^{RNAi}	108922 - VDRC
smc5^{RNAi}	38969 - VDRC
tbw^{RNAi}	28982 - BDSC
UAS-AurA2	8377 - BDSC
UAS-AurA3	8378 - BDSC
UAS:Baz^{S980A}*-GFP*	Morais-de-Sá et al., 2010
UAS-polo	Mirouse et. al, 2006

2.1 The GAL4-UAS system

The GAL4-UAS system derived from yeast is a biochemical tool widely used to study the specific expression of a certain gene ([137]). It is composed by two main elements: the GAL4 driver and the Upstream Activating Sequence (UAS). The GAL4 protein is expressed under the control of a tissue-specific endogenous promoter. In turn the UAS sequence drives the expression of our transgene of interest, as the RNAi transgenes used into this study. After appropriate fly crossing and progeny selection, we have flies that carry concomitantly the GAL4 activator and the UAS-transgene as shown in Fig. 2.1. The GAL4 protein will therefore bind to the UAS-sequence, activating the expression of the gene that is under the control of the UAS. Therefore expression of our transgene of interest only occurs in the tissues that specifically express the GAL4 protein. Adaptations were made to this initially system. $GAL80^{ts}$ is a temperature-sensitive protein that binds and represses GAL4 protein activity. As so, by placing the $GAL80^{ts}$ under the control of the same enhancer of GAL4 promoting their concomitant expression, we are able to control the exact time of gene expression by placing the flies in a restrictive temperature that disrupts the $GAL80^{ts}$ protein.

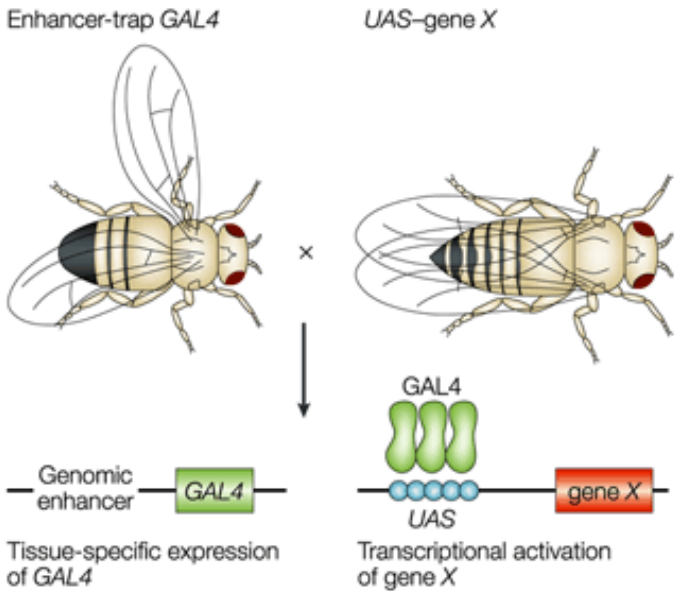


Figure 2.1 - Schematic representation of a crossing that enables the expression of a specific gene using the GAL4-UAS system. Addapted from ([138]).

2.2 The FLP/FRT system

The GAL4 system can be used in combination with other genetic tools to direct the expression of a certain gene to a restrict population of cell within a tissue. One of those tools is the “FLPout” system ([139]). The expression of the GAL4 protein is interrupted by the presence of a cassette flanked by Flipase recombination target (FRT) sequences, regardless the activation of its promoter. Temperature is the key controller of this system. By placing the flies at a restrictive temperature, the Flipase (FLP) protein expression is induced, once that it is under the control of a heat-shock promoter. Upon its expression, the Flipase protein will promote the recombination within the FLP recombination target (FRT) specific sites, promoting the excision of the cassette. After this excision, the GAL4 gene can be transcribed, allowing further activation of UAS sequences. Additionally to the UAS-transgene, this strategy concomitantly expresses a UAS-GFP transgene that positively marks the cells that are expressing the UAS transgenes, thereby creating GFP clones (Fig. 2.2).

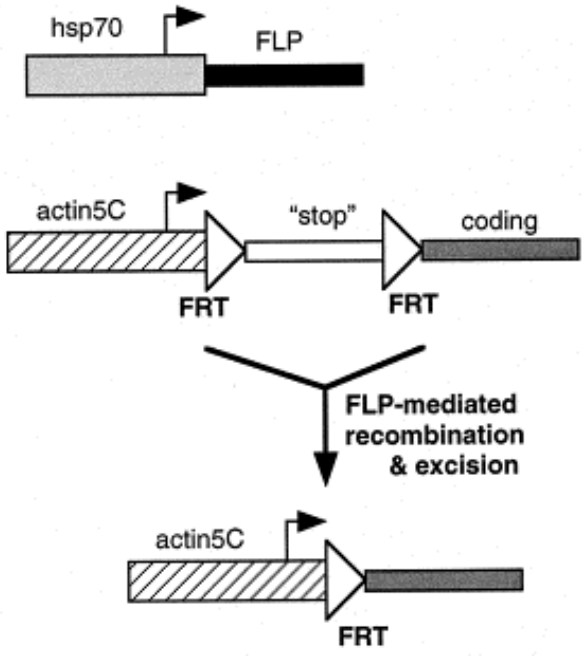


Figure 2.2 - Schematic representation of the combination of the FRT/FLP tool with GAL-UAS system. Actin5C is an ubiquitous promoter frequently used to drive the expression of the GAL4 protein. Adapted from ([140]).

2.3 The FRT system

In this strategy, the FRT sites are placed at identical positions within the chromosome arms of non-sister chromatids of the homologs ([141]). Flipase expression is induced by heat-shock, by placing the flies at a restrictive temperature. FLP will then recombine the FRT specific sites, which allows the exchange of the chromosome arms. If this recombination occurs after S-phase, two subsequent different populations of cells will be created as could be visualized in Fig. 3. One cell population will inherit both mutated alleles, becoming homozygous to the mutation. The other clonal population will be inherited both GFP genes, becoming wild-type relatively to the previous mutated allele. The wild-type clones are marked by GFP expression, while the mutated clones are marked by the absence of GFP, allowing clonal identification (Fig. 2.3).

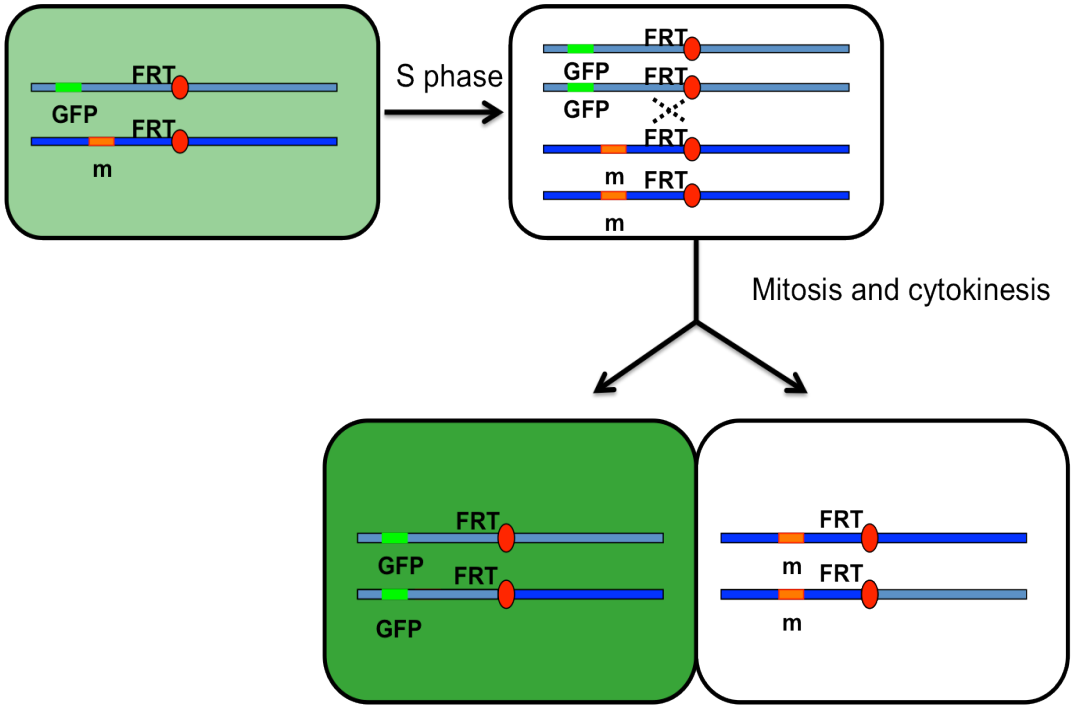


Figure 2.3 - Schematic representation of the FRT system operation during mitosis.

2.4 Immunofluorescence in *Drosophila* ovaries

Drosophila females were fed with yeast and when necessary placed at 29ºC to enhance the expression of the genes of interest. Ovaries were dissected in PBT (PBS +

0.2% Tween (Sigma-Aldrich)) and fixed for 20 minutes with a solution of 4% paraformaldehyde (Electron Microscopy Sciences) in PBT. Upon fixation the ovaries were washed 3x10 minutes with PBT, blocked with PBT-10% (PBT + 10% BSA) for 1 hour and incubated with the primary antibody in PBT-1% (PBT + 1% BSA) overnight at room temperature. 4x30 minutes washes with PBT+1% followed the removable of the primary antibody. Then the ovaries were incubated with the secondary antibody in PBT 0,1% (PBT + 0,1% BSA) during 2 hours. After the removal of the secondary antibody, the ovaries were washed 3x10 minutes with PBT and mounted in vectashield with DAPI (Vector Laboratories, Inc. Burlingame, CA84010). To perform the phalloidin (Molecular Probes) stainings, the ovaries were fixed for 30 minutes in the same solution and phalloidin was added during the fixation procedure. The subsequent procedure was the same.

2.5 Immunofluorescence on *Drosophila* eye/antennal imaginal discs

Larvae were picked at the third instar stage and the discs dissected in PBS (1x). Posterior fixation in 4% formaldehyde in PBS last for 20 minutes, upon which 3x10 minutes washes in PBT' (PBS+0,1%Triton (Sigma-Aldrich)) were performed. The primary antibodies were incubated in PBT' for 2 hours at room temperature or overnight at 4ºC. 3x30 minutes washes preceded the incubation with the secondary antibodies in PBT' during 1 hour and 30 minutes at room temperature or overnight at 4ºC. The discs were washed 3x10 minutes after removal of the secondary antibody and then vectashield with DAPI was added. The discs were mounted in 50% glycerol in PBS.

2.6 Clonal analyzes

In order to generate clones within the follicular epithelium that would allow the expression of specific alleles, female flies were placed at 37ºC for two hours during 3 days. This increase in temperature will allow the expression of the Flipase protein both in FRT and FRT/FLP systems, thereby generating the clones.

2.7 Drug-induced treatments

To induce an increase in the quantity of mitotic cells within the follicular epithelium, Drosophila females starved for 12 hours, upon which were fed with yeast in 500 μL of 20 μM colchicine (Sigma-Aldrich) during 17-to-19 hours. Ovaries were then dissected following the same procedure previously described.

2.8 Primary and Secondary antibodies

The primary antibodies used were: anti-aPKC (rabbit polyclonal;1/500) from Santa Cruz Biotechnology; anti-Arm (mouse monoclonal; 1/100) from Developmental Studies Hybridoma Bank (DSHB); anti-Discs large (mouse monoclonal;1/100) from DSHB; anti-Elav (rat; 1/100) from DSHB; anti-p-Baz (rabbit polyclonal from Eurico Morais-de-Sá et al., 2010); and pH3 (rabbit polyclonal; 1/500) from Upstate.

The secondary antibodies used were Alexa 488, Alexa 594, Alexa 647 from mouse, rabbit and rat (Molecular Probes).

2.9 Imaging

For time lapse imaging, ovaries were dissected into carbon oil and imaging at 25ºC using a spinning disk confocal microscope (Andor Revolution XD) with an electron multiplying charge-coupled device camera (iXonEM+; Andor) and a CSU-22 unit (Yokogawa) based on an inverted microscope (IX81; Olympus). A 63x objective was used and imaging was performed using two laser lines - 488 and 561 nm - for the excitation of GFP and RFP respectively. Z stacks were acquired every 30 seconds to all movies. Acquisition parameters, as exposure time or steps, were controlled by iQ software (Andor). Image processing and movie assembly was processed using Fiji.

To perform imaging of fixed tissues we used an inverted laser scanning confocal microscope (Leica TCS SP5 II) using a 40X water objective and the LAS 2.6 software. To the imaging four lasers were used: a 405nm Diode laser; the 488 nm Argon laser; the 561 nm DPSS; and the 633 nm HeNe. Images were preocessed using Fiji.

CHAPTER 3 – RESULTS

Cooperation between cell division and apico-basal polarity defects in tumorigenesis induction

3. Using the eye/antennal imaginal discs

3.1 Introduction

Drosophila has been used as a model to address the underlying mechanisms of cancer development and progression once that a high conservation of signaling pathways between *Drosophila* and mammals is observed (reviewed in [34]). In turn, epithelial cancers are the most common in humans (reviewed in [142]), which means that these highly polarized cells are susceptible to give rise to tumors. Furthermore, several studies related mutations in polarity proteins to mammalian tumor development (reviewed [143]). Besides loss of cell polarity, mitotic defects are also related to tumorigenesis as referred in the introductory chapter (reviewed in [130]). Tumor development relies in the acquisition of sequential defects that allow the development of each cancer hallmark. Thus, we aimed to determine if polarity and mitotic defects could cooperate in tumorigenesis, using the follicular epithelium and eye/antennal imaginal discs from *Drosophila* as working models.

Scribble (Scrib), Discs-large (Dlg) and Lethal giant larvae (Lgl) had been described as tumour suppressor genes in *Drosophila,* being also involved in epithelial cell polarity regulation ([73]). Studies reported that ectopic cell proliferation posterior to the morphogenetic furrow is observed in *scrib* mutants eye discs ([144]). Through interactions with the oncogenic Ras-Raf pathway, loss of *scrib* leads to the downregulation of the Hippo signaling pathway that is required to control tissue overgrowth ([145]; [144]). Furthermore, it was shown that *scrib* mutant cells are outcompeted by wild-type cells, inducing apoptosis in neighboring mutant cells. Therefore substantial overgrowth is only observed when all the tissue is mutated ([146]). The eye imaginal discs are widely used in cancer research. As previously referred, the eye/antennal imaginal discs develop in a short period of time. Thus we can observe the effect of interactions between altered levels of proteins on the tissue size and organization just in a few days. In addition, as we can induce errors on distinct mitotic processes, several divisions might be required to allow us to see the global effect on tissue organization, which is possible due to the high proliferation that occurs in the eye imaginal disc.

To carry out this study we induced the expression of a transgene expressing a Scribble inverted repeat (*scrib*^{RNAi}) that downregulates *scrib* levels by RNA interference, using the GAL4-UAS system. A specific driver - the eyeless promoter - drive the expression of the GAL4 protein, that in turn binds specifically to the UAS sequence upstream of the *scrib*^{RNAi} transgene activating its expression specifically on the eye imaginal disc. To search for interactions, several RNAis and protein overexpression transgenes that affect different mitotic processes were added to the system. The list of the different proteins that were under study is presented below (Table 3.1). It is important to note that the eyeless promoter was recombined with the Green Fluorescence Protein (GFP) allowing us to label the cells that express the different transgenes.

Table 3.1 List of the interacting proteins sorted by the mitotic process that they are affecting.

General Regulators	Cytokinesis	Centrosome related	Kinetochores related	SAC
Aurora A	Anillin	Abnormal-spindle	CENP-C	Bub3
Aurora B	Fascetto	Centrosomin	Kmn1	BubR1
Orbit	Pavarotti	SAK	Mis12	Mad1
Cdc2	Peanut	Sas-4	Nuf2	Mad2
Polo-like kinase	Septin1		Mitch	
UAS-Aurora A	Septin2			
UAS-Polo	Tumbleweed			
SMC1				
SMC5				

3.2 *scrib*^{RNAi} causes a reduction in the size of the *Drosophila* eye

Initially, we tested two RNAi constructs directed to the polarity proteins Scrib and Dlg, both driven by the *eyeless* promoter and raised at 25ºC. Although previous reports suggested a similar phenotype after Scribble and Dlg depletion, we did not observe that with the transgenes used, possibly due to different levels of the knockdown efficiency had a similar effect. While *dlg*^{RNAi} had no effect on the organization of the eye, expression of s*crib*^{RNAi} leads to smaller eyes with a reduced number of ommatidia when compared to the

wild-type (Fig. 3.1). This reduced eye size is probably due to cell death induced by normal cells that did not have a sufficient Scribble depletion. As the RNAi construct does not produce complete depletion of Scribble protein, this method generated a reduction in the efficiency of the polarity machinery. This weak phenotype allows therefore the identification of interactors that induce increased defects in eye organization.

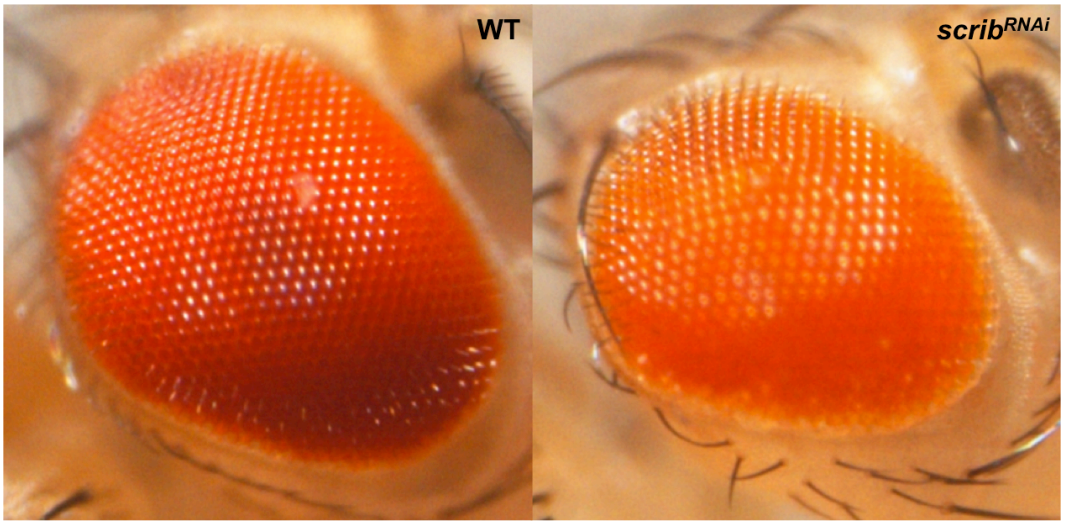


Figure 3.1 - *scrib*^{RNAi} reduces the normal *Drosphila* eye size.

3.3 Cytokinesis and Aurora A altered levels have an interaction with polarity defects to induce eye outgrowth

A fly stock containing both the driver and *scrib*^{RNAi} was constructed, which was then crossed with the several transgenes to identify potential interactors. Thus, flies expressing both *scrib*^{RNAi} and the modulator of the levels of the interacting genes could be chosen as indicated by the following scheme (Fig. 3.2). In addition, we also choose the sibling progeny that had no *scrib*^{RNAi} as internal controls to check if the altered levels of the tested interactors could produce phenotypes on their own. The phenotypes presented by the progeny were then classified into the categories that are shown in Figure 3.3. *Drosophila*'s head is essential to the hatching process. We observed that not all the progeny were viable revealing that the crucial role of some proteins impairs cell survival, leading to partial or complete loss of the head. In fact, we observed the existence of headless dead flies in their pupae. The table below summarizes our data relatively to progeny lethality (Table 3.2). Previous studies suggested that differences exist between males and females replicative times during S-phase, showing that differences between males and females are beyond the sexual determinants ([147]). Indeed, we also verified

that the expressivity of the phenotypes for the same transgene were different in males and females. In this screen, males seem to be more susceptible to tumor development than females since the same interaction could result in no phenotype in females while males show a strong phenotype. Therefore, we quantified males and females phenotypes individually.

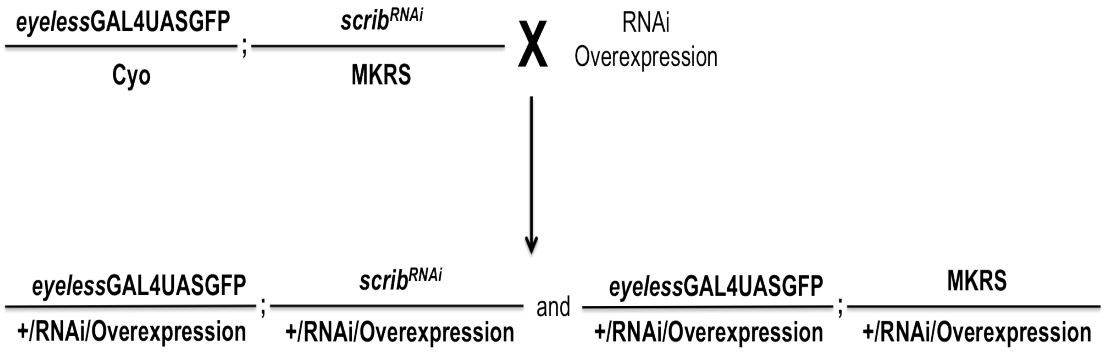


Figure 3.2 - Crossing scheme for the eye screen for interactions between *scrib*^{RNAi} and mitotic defects.

Table 3.2 Progeny lethality in males and females. Semi-lethal phenotype includes the progeny that have 5 or less viable adults expressing the indicated RNAi transgenes per cross. The other tested transgenes were all viable with more than 5 flies [10 eyes] per cross. Differences were found between males and females in what concerns to the lethality of certain transgenes.

Males		Females	
Lethal	Semi-lethal	Lethal	Semi-lethal
orbit	*aurora B*	*aurora B*	*mis12*
cdc2	*bub3*	*orbit*	*smc1*
cenp-c	*mad1*	*cdc2*	*tumbleweed*
kmn1	*mis12*	*cenp-c*	
peanut	*mitch*	*kmn1*	
polo	*pavarotti*	*peanut*	
smc1	*sas-4*	*polo*	
	tumbleweed		

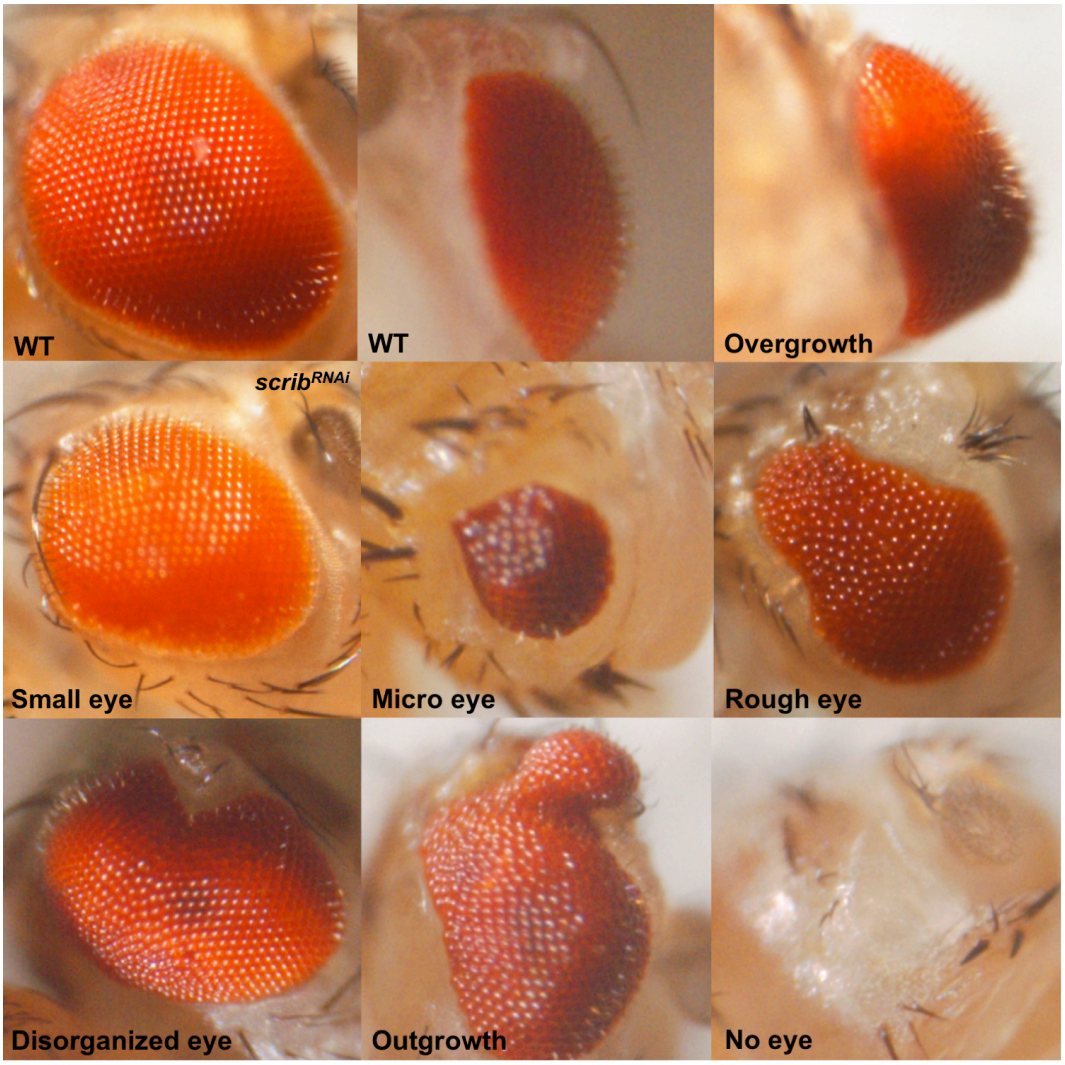


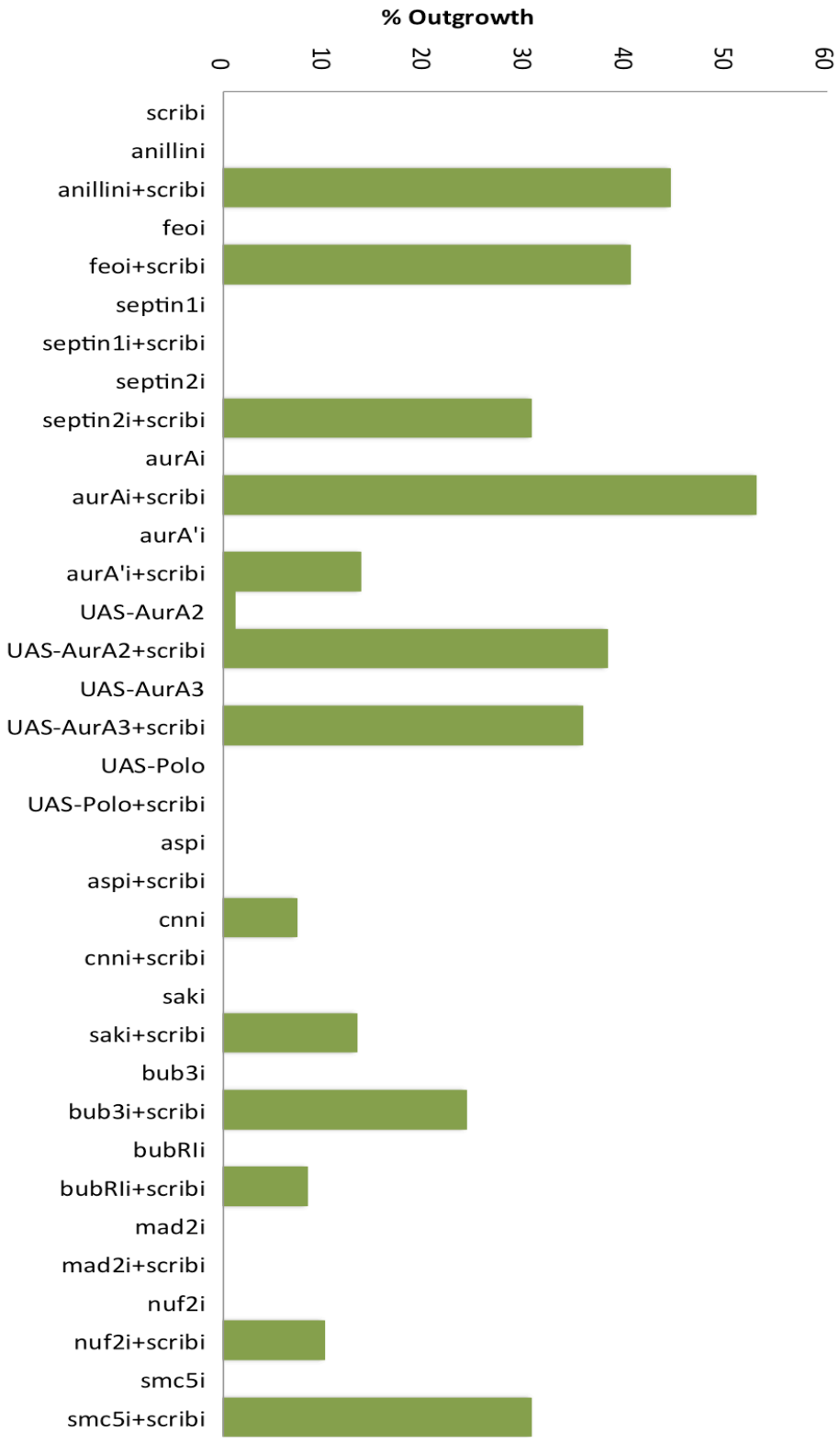
Figure 3.3 Representative pictures of each category used to score the eye phenotype. For a more precise quantification, an increase in the number of ommatidia maintaining the normal structure of the eye was classified as overgrowth, whereas eye masses popping out of the eye were classified as outgrowth.

As the main interest of this study was to test the development of tumor like structures due to depletion of interacting genes, we present a graph of the outgrowth phenotype observed for each genetic background (See Fig. 3.4, Graph. 3.1 and 3.2). A table with all scored phenotypes is presented in the appendix. In general, we observed that depletion of proteins with functions on cytokinesis lead to outgrowth phenotypes when Scribble protein was simultaneously depleted. In addition, we also found a high percentage of outgrowths when we disturbed Aurora A protein levels in a polarity defective background.

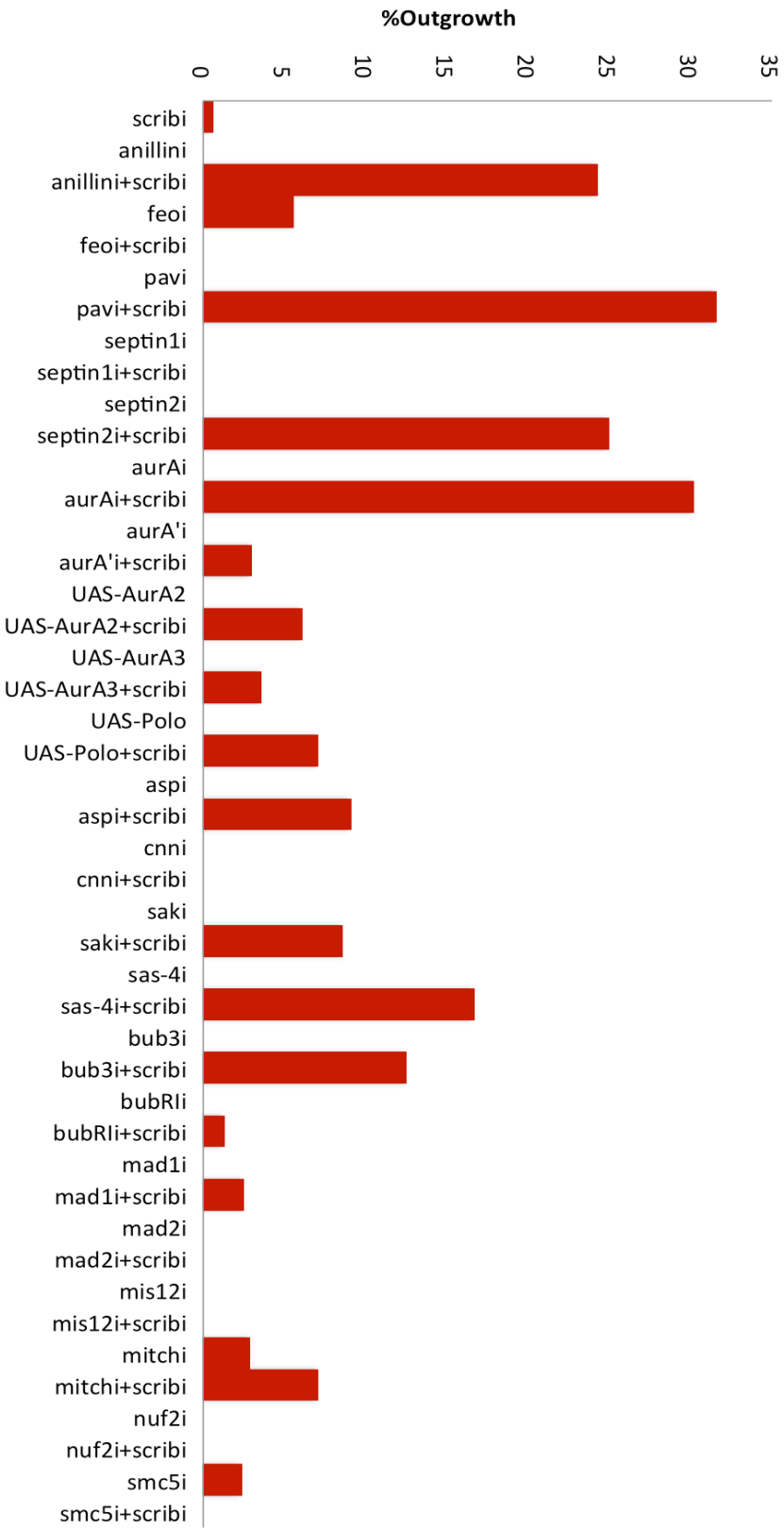
3.3.1. Cooperation between cytokinesis and polarity defects in tumorigenesis does not depend on centrosome amplification

As referred in the introductory chapter, failures in the completion of cytokinesis eventually leads to polyploidy – increased number of chromosomes per cell - and consequently genetic instability ([148]). These two characteristics are widely identified in several human cancers, including cervical carcinomas and Barrett's esophagus, just two examples where tetraploid cells were found ([149]; [150]). Besides the accumulation of all DNA content after replication just into one cell, failures in cytokinesis also leads to the accumulation of the replicated organelles. As so, cytokinesis failures lead to centrosome amplification, which is frequently correlated with malignancy of tumors. A cell that possesses more than two centrosomes will eventually have multipolar mitotic spindles, resulting in chromosome missegregation that probably ends in aneuploidy (reviewed in [151]). Therefore, we planned determine which was the process, resulting from cytokinesis failure, that was driving tumor development by interacting with polarity defects. With this purpose, we tested if the resulting outgrowth phenotype was due to polyploidy, centrosome amplification or the cooperation between both defects.

Sas-4 was shown to be essential for centriole replication in *Drosophila*, although cells lacking of centrosomes are able to divide ([152]). Therefore, by depleting *sas-4* we were able to provide the acentrosomal background needed for the study. To test if centrosome amplification was the underlying cause of tumorigenesis in a polarity and cytokinesis defective background, we generated flies that simultaneously lacked centrosomes by doing simultaneous depletion of *scrib*, *anillin* and *sas-4* (Fig. 3.5). However these flies were not viable at 25ºC, probably because the combination of the three RNAi constructs was causing too many defects within the cells, leading to headless flies and consequently their death. To overcome this technical hurdle, we raised the flies at 18ºC once that at this temperature the expression of the GAL4 protein and so the expression of the RNAi constructs was reduced. At this temperature, flies expressing the three RNAi are viable, enabling us to quantify the outgrowth phenotype. However, this approach also results in a less pronounced depletion of the respective proteins, it required a re-quantification of the phenotypes for the single RNAi depletions.



Graph 3.1 Percentage of outgrowth phenotype in males. As can be seen in the graphic, proteins that interfere with cytokinesis have a higher percentage of tumour-like phenotype as well as the Aurora A altered levels.



Graph 3.2. Percentage of outgrowth phenotype in females. Once more cytokinesis failures and Aurora A induced the higher percentage of overgrowth among the several proteins tested.

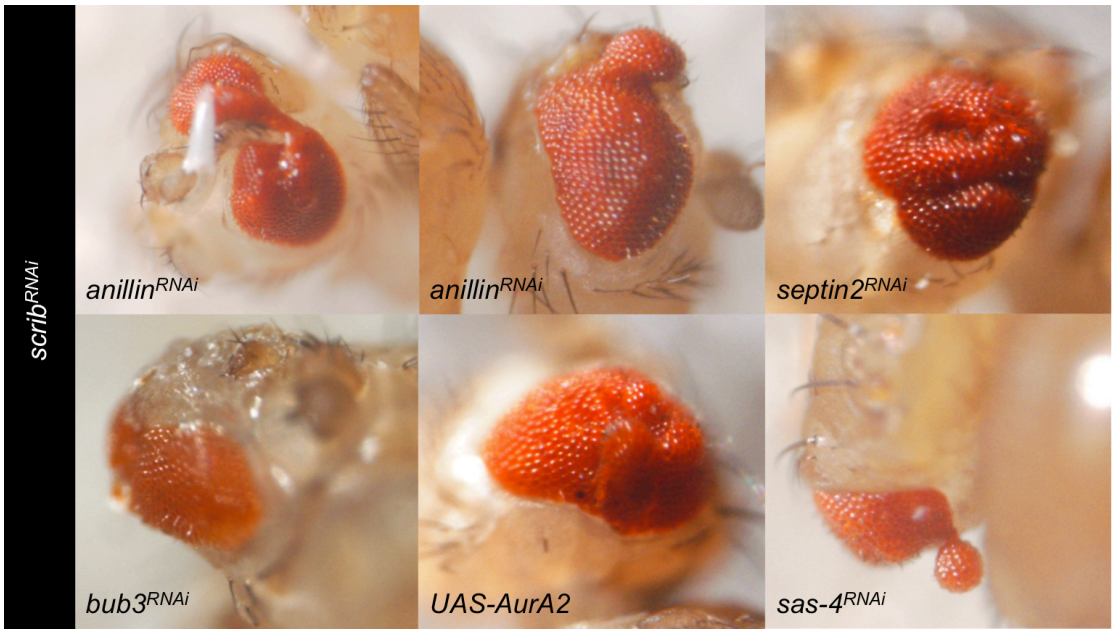


Figure 3.4 Examples of outgrowth phenotype observed. These phenotypes were all found in the presence of defective levels of Scribble protein.

As can be seen in Graph 3.3, *anillin*^{RNAi} and *scrib*^{RNAi} are still interacting in the absence of centrosomes as the outgrowth phenotype is still observed in the presence of *sas4*^{RNAi}. As expected, the phenotype is not as strong as it is at 25ºC. It also should be noted that *sas-4*^{RNAi} by itself did not originate tumors at this temperature. Thus, this data supports that the underlying mechanism of tumorigenesis induction in a cytokinesis and polarity defective background is polyploidy and not centrosomal amplification (see appendix and graphic 3.3).

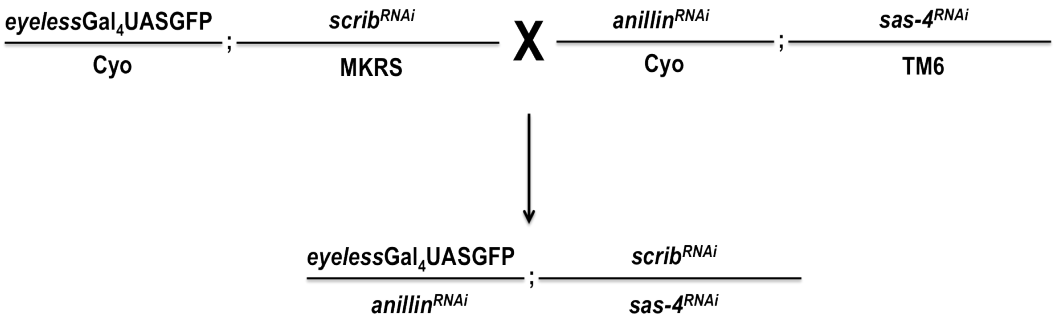
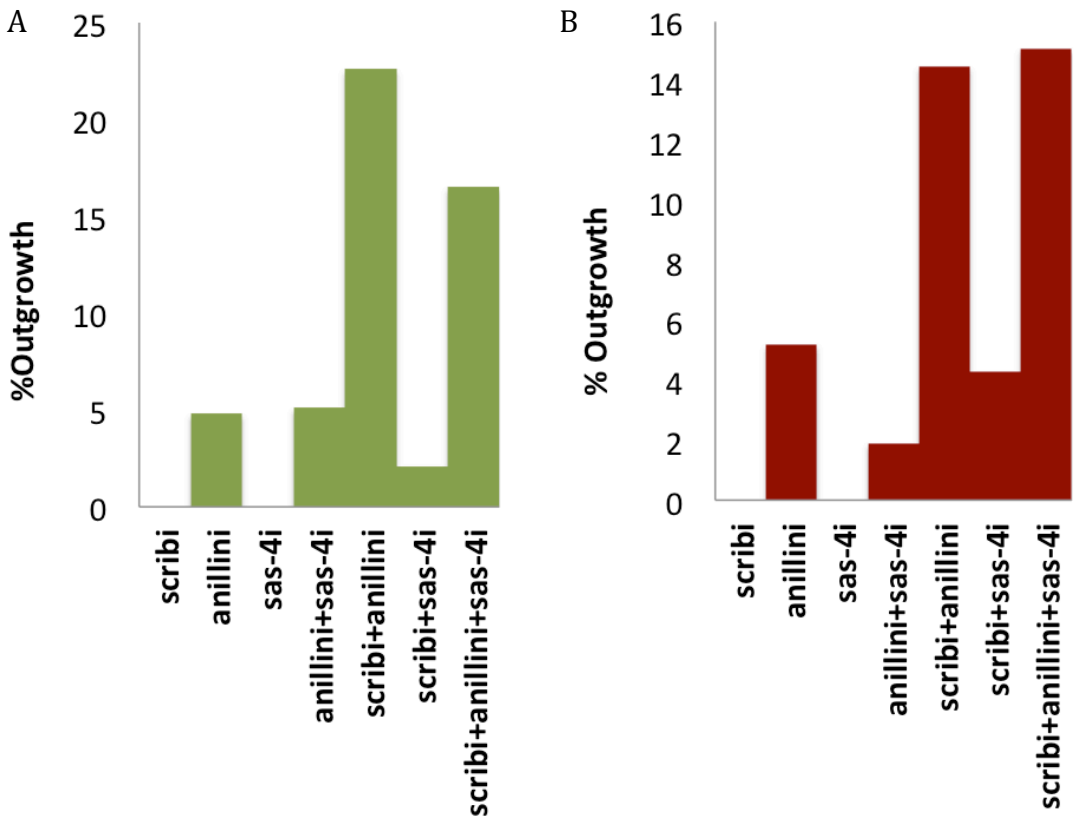


Figure 3.5. Crossing scheme to generate adults that simultaneously expressed *anillin* , *scrib* and *sas4* RNAi's in the eye imaginal discs.



Graph 3.3. Centrosome amplification is dispensable to outgrowth formation in the cytokinesis/polarity defective background. Representation of the outgrowth phenotype observed in males (A) and in females (B). There were no significant differences between the percentage of outgrowth phenotype caused by *anillin*^{RNAi} and *scrib*^{RNAi} when *sas-4*^{RNAi} was or not present in the system.

3.4 Anillin and Scribble defective levels causes dramatic morphologic changes in the eye/antennal imaginal discs

The idea behind this study was to find possible interactions between proteins whose depletion causes defects both in mitotic processes and in apico-basal polarity to induce tumorigenesis. Upon the observation of the phenotypes derived from those interactions, we concluded that cytokinesis and Aurora A were the best candidates as interactors to proceed with the studies on the eye/antennal imaginal discs level. Thus we attempted to observe the effect of diminishing the levels of the above-mentioned proteins in the morphologic organization of the discs. One of the features of these imaginal discs is the formation of the morphogenetic furrow by the end of the second instar larvae. This morphogenetic furrow (MF) is characterized by dividing the eye disc into two different cell populations. By moving in a posterior to anterior axis, the MF promotes cell differentiation

and organization of the photoreceptors in the most posterior part of the disc, within which cells are arrested in G1. In the anterior part, cells are undifferentiated and continue to proliferate until the Morphogenetic furrow (reviewed in [153]). Scribble has long been used in different organisms and tissues to provide insights of how different signaling pathways can be related to tumor progression ([154]; [146]; [144]). It was shown that loss of both *scrib* alleles resulted in cell overproliferation and polarity disruption, resulting in neoplastic tumors in larval wing imaginal discs ([71]). Particularly in the case of eye imaginal discs, clonal analyzes suggested that in homozygous *scrib* mutants, ectopic cell proliferation is observed posterior to the MF ([145]). In this study we used the same scheme presented in Fig. 3.2, but the balancer used on the third chromosome was TM6, since that would enable us to see the larvae that had no $scrib^{RNAi}$. Then we raised the flies at 25ºC and the collection of the larvae was done 7 days after crossing. At this time larvae were in their third instar stage just before pupation, allowing us to examine the eye imaginal discs at a time that cells would already be differentiated and before metamorphoses. Immunofluorescence was performed using anti-Elav - that marks the differentiated cells, in this case the photoreceptors – and anti-Phospho-Histone-3 (pH3) – that labels mitotic cells. As can be seen in Fig. 3.6, in the wild-type discs the morphogenetic furrow separates the differentiated photoreceptors from the cells that are still on division, and no labeling by pH3 is found within the MF (Fig. 3.6A). However ectopic cell proliferation is found in a band just posterior to the MF, once that cells immediately after the MF undergoes a synchronous S phase ([145]). When $scrib^{RNAi}$ is expressed in the discs, the MF constriction is no longer correctly aligned in a dorsal-ventral axis as in the wild-type (Fig. 3.6B). We also observed disorganization in the normal pattern of distribution of photoreceptors and an increased number in pH3 positive cells randomly distributed between the differentiated photoreceptors. Although this suggests that some cells have differentiation problems, this was not generalized. $sas\text{-}4^{RNAi}$ did not affect discs morphology or photoreceptor organization per se (Fig.3.6C), and when combined with $scrib^{RNAi}$ the phenotype observed resembles the one of $scrib^{RNAi}$ alone, presenting an unaligned MF and photoreceptor disorganization when compared to the pattern observed in the wild-type discs (Fig.3.6D). Although no significant changes in the morphology of the disc are observed in $anillin^{RNAi}$ per se (Fig. 3.6E), when combined with $scrib^{RNAi}$ dramatic morphologic problems occur: the morphology of the whole disc is affected an the morphogenetic furrow can not be distinguished by the absence of pH3 labeling. Additionally we found that photoreceptors fail to differentiate once that few cells are marked with Elav and pH3 labels random cells within the posterior part of the eye disc (Fig.3.6F,G).

3.5 Aurora A overexpression induces tumorigenesis by promoting photoreceptor differentiation failure

Aurora A has several reported functions including involvement in centrosome maturation and separation, establishing of spindle bipolarity and a role in the organization of apical polarity in *Drosophila* neuroblasts ([102]; [120]; [98]). As a consequence, its absence causes many defects, such as monopolar spindles due to its role on centrosome separation and consequently the production of tetraploid cells ([113]). As previously referred, tetraploidy is frequently found in tumors, a fact that might provide insights on how Aurora A causes the outgrowth phenotype previously observed (see graphics 3.1 and 3.2). Additionally Aurora A overexpression was also implied in the generation of multinucleated cells and aberrant mitosis in other organisms ([155]; [156]). Aurora A altered levels could therefore lead to tumor development. Indeed, our observations suggested that when the polarity machinery is weakened, both $aurA^{RNAi}$ and *UAS-AurA* causes tumorigenesis. Therefore, we also performed immunofluorescence analyzes in $aurA^{RNAi}$ and *UAS-AurA* transgenic discs, in the presence and absence of $scrib^{RNAi}$, using both Elav and pH3 antibodies. $aurA^{RNAi}$ per se did not result in significant alterations in the organization of the disc when compared with the wild-type, but some photoreceptor disorganization could be seen (Fig. 3.7A). However, when in a defective polarity background, $aurA^{RNAi}$ eye imaginal discs show an increased number of cells labeled with pH3, indicating that some cells that should be already differentiated are undergoing mitosis (Fig. 3.7B). In the case of *UAS-AurA,* it shows no differences in comparison with the wild-type cells (Fig. 3.7C). Yet, when $scrib^{RNAi}$ is added to the system, the normal morphology of the discs is lost. It should be noted that we observed a correlation between high expression of the transgenes (as indicated by the higher levels of GFP) and absence of Elav labeling, indicating that the high levels of AurA block cell differentiation (Fig. 3.7D). Furthermore we observe multiple layers of cells, which could be an indicative of cell overproliferation, explaining the observed outgrowth phenotypes (Fig. 3.7E). In addition, pH3 labeling shows random proliferating cells throughout the entire eye/antennal imaginal disc suggesting that the MF is not correctly formed (Fig. 3.8).

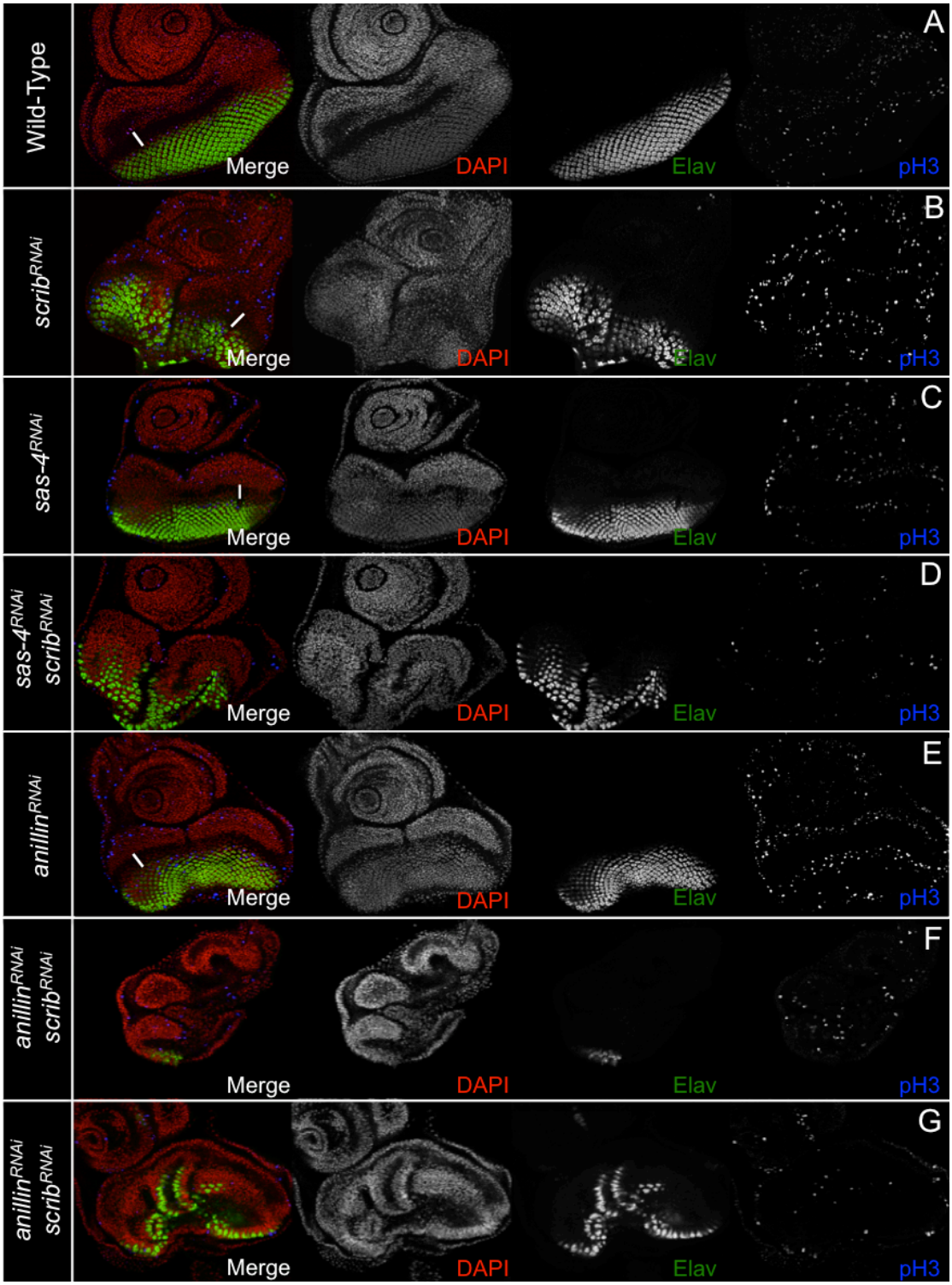


Figure 3.6 Simultaneous *anillin* and *scribble* downregulation leads to reduced photoreceptor differentiation. Third instar larvae eye discs with the most posterior part at the bottom of the figure in all discs. DAPI labels the DNA, anti-Elav the differentiated cells and pH3 the cells that are undergoing mitosis. The white bar marks the morphogenetic furrow.

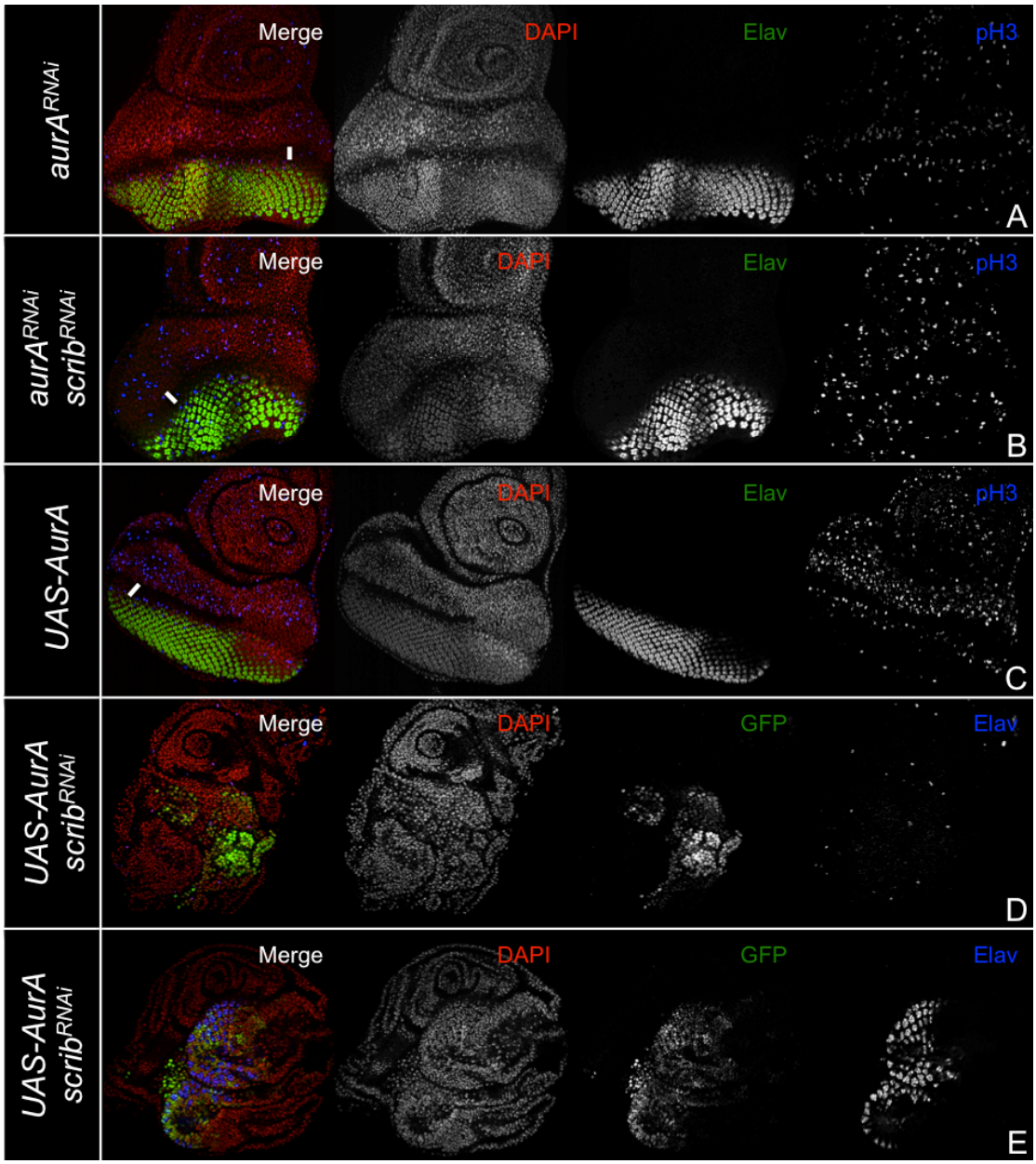


Figure 3.7 *UAS-AurA* causes differentiation failure. Third instar larvae eye discs with the most posterior part at the bottom of the figure in all discs. DAPI labels the DNA, anti-Elav the differentiated cells and pH3 the cells that are undergoing mitosis. GFP indicates the level of expression of the transgenes. The white bar marks the morphogenetic furrow.

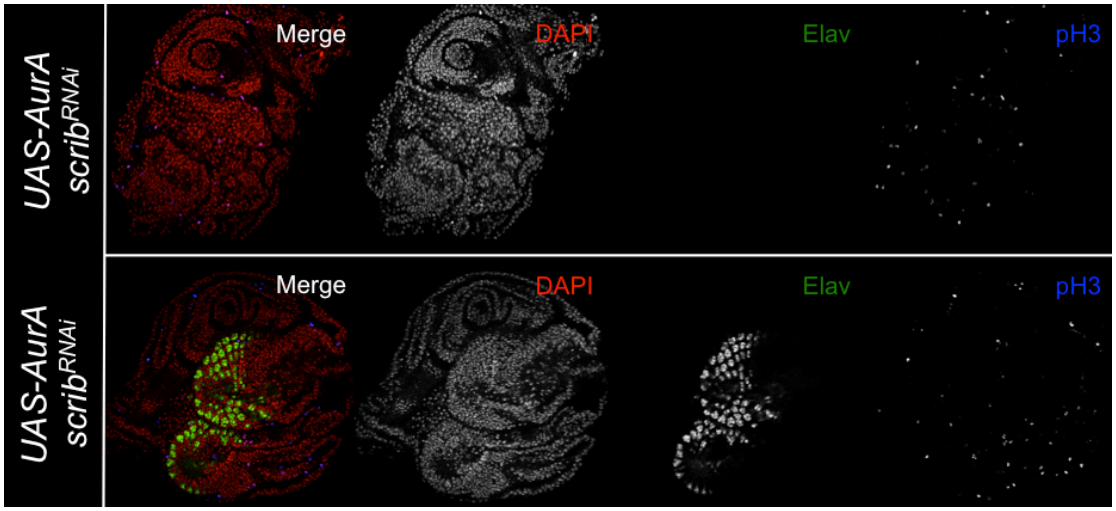


Figure 3.8 *UAS-AurA, scrib*^{RNAi} causes random proliferation through the entire imaginal disc. In the previous present *UAS-AurA* transgenic discs, anti-pH3 was also used to label cells that were undergoing mitosis. Proliferation is observed randomly distributed within the imaginal disc. DAPI marks the DNA while Elav labels the differentiated cells.

4. Using the follicular epithelium from Drosophila ovaries

4.1 Background

Drosophila oogenesis is a complex process that involves the establishment of anterior-posterior and dorsal-ventral axis of *Drosophila* by localizing RNAs specifically within the oocyte ([157]; [158]). As described in the introductory chapter, the ovaries are divided in functional "subunits", the ovarioles, within each the egg is produced. Each ovariole contains multiple egg chambers at different stages of oogenesis. Each egg chamber develops along 14 stages, classified as early (1 to 6), mid (7 to 10) or late (11 to 14) stages of oogenesis (reviewed in [36]). The early stages of *Drosophila* oogenesis have particular importance to this work, since it is within these stages that the follicle cells chamber are undergoing mitosis. Follicle cells cease dividing at the end of the sixth stage. Thus, it possible to perform live imaging studies of division in follicular epithelial cells only until stage 6 of oogenesis.

Using another epithelial system, the follicular epithelium, we could validate the previously found interactions in the initial eye screen. In comparison with the eye imaginal discs, the follicular epithelium offers us several advantages, including the possibility of observing invasion of the tumour-like cells, which is not possible in the eye imaginal disc.

Since the epithelial cells are in intimate contact with the germline, when mutations leading to the disorganization of the epitelial monolayer occur, we could observe epithelial cells streaming within the germline environment. Furthermore, imaging of the apico-basal polarity is straightforward in the follicular epithelium, which is compatible with the use of a variety of genetic and molecular tools. Thus, it is a good epithelial model to understand the underlying mechanisms of tumour development, such as factors that can result in tissue disorganization.

4.2 *dlg*^{RNAi} causes tissue disorganization in the follicular epithelium

To start the screen, the phenotypes present by RNAi depletion of the polarity proteins Scribble and Dlg were addressed. Both RNAis transgenes were driven by the actin promoter, however as the loss of TSGs leads to lethality, the flies were raised at 18ºC using the GAL4/UAS system coupled with the repressor of GAL4, the GAL80^{ts} protein. To induce the expression of the RNAi transgenes, flies were placed during 2 days at 29ºC, so that GAL80 repression was blocked. While *scrib*^{RNAi} presented a weak phenotype, showing just two or more individual cells out of the monolayer, *dlg*^{RNAi} shows groups of cells forming a double layered and disorganized tissue. This was classified as an intermediate phenotype (Fig. 4.1). This phenotype is adequate for the propose of the study since that enables us test if for interactions that can increase the aggressiveness of the phenotype.

To confirm that the RNAi against Dlg is being effective, we used the Flpout system to make clones within the follicular epithelium where the *dlg*^{RNAi} construct driven by the actin promoter is exclusively expressed. The clones expressing the RNAi transgene are marked by the concomitant expression of the GFP protein. As can be seen in Fig. 4.2, Dlg is highly down regulated in clonal cells when compared with the wild-type cells marked by the absence of GFP, showing that the *dlg*^{RNAi} construct is depleting the Dlg protein.

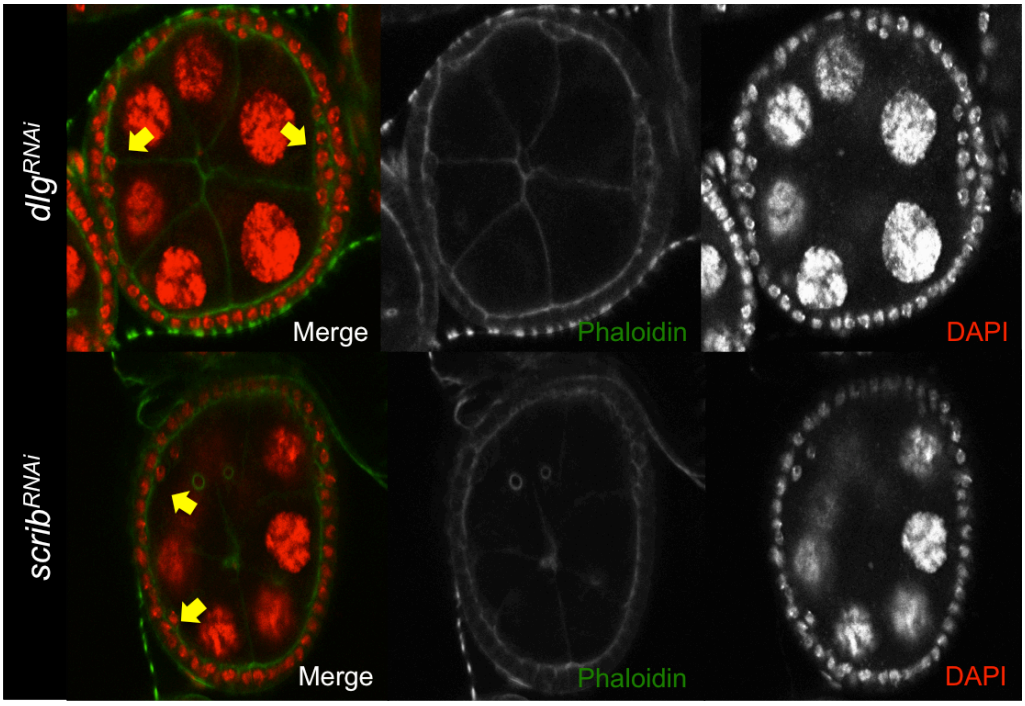


Figure 4.1 Weak and intermediate phenotype present by *scrib*^{RNAi} and *dlg*^{RNAi}, respectively. After two days at 29ºC to inactivate GAL80^{ts} supressor, *scrib*^{RNAi} and *dlg*^{RNAi} present a weak and intermediate phenotype, respectively. DAPI marks DNA, while phalloidin labels the actin.

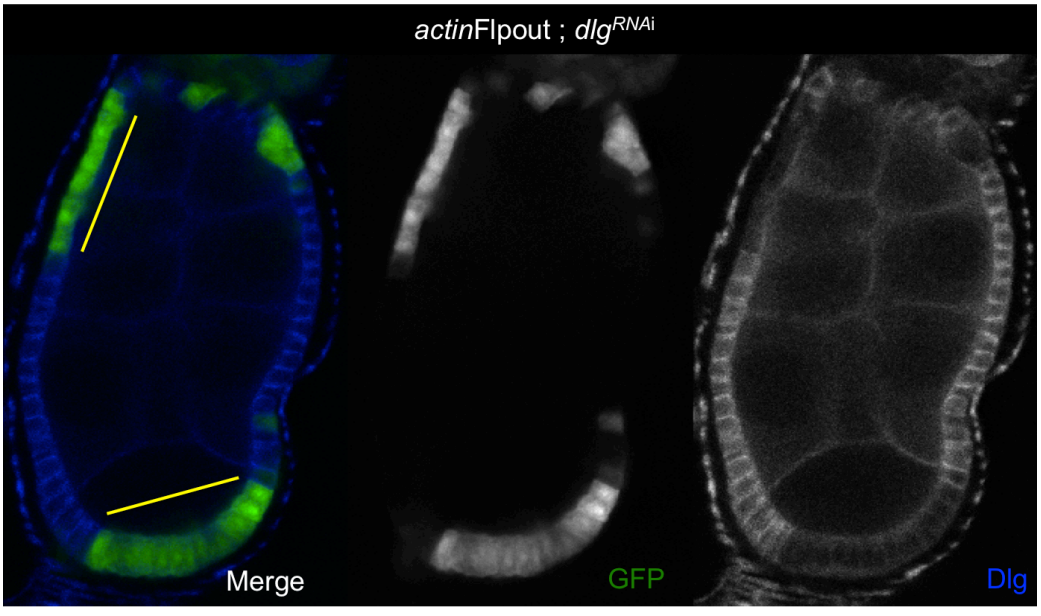


Figure 4.2 *dlg*^{RNAi} depletes efficiently the endogenous Dlg protein. The yellow lines mark the clones were the anti-Dlg antibody detects very low amounts of protein. This can be compared with the wild-type cells marked by GFP absence.

4.3 Anillin depletion and Aurora A overexpression enhance the intermediate phenotype presented by *dlg*^{RNAi}

The same proteins involved in the mitotic processes mentioned in the eye/antennal imaginal discs screen were also tested in the follicular epithelium (Table 3.1). The *dlg*^{RNAi} construct was driven by the actin promoter using the system GAL4/UAS system in combination with its repressor, the GAL80^{ts} protein that enables flies to born. A stock containing the actin promoter and *dlg*^{RNAi} was constructed and the progeny was chosen as indicated in Fig. 4.3.

The phenotypes observed were classified into 4 main categories. The "None" phenotype means that no disruption of the epithelial monolayer was observed. The "Weak" phenotype was assigned when one or a few individual cells were seen away from the monolayer. "Intermediate" phenotype corresponds to egg chambers where a group of cells form a second layer. "Strong" phenotype is observed when more than two layers of epithelial cells are seen invading the germline. Examples of the observed phenotypes are shown in Fig. 4.4.

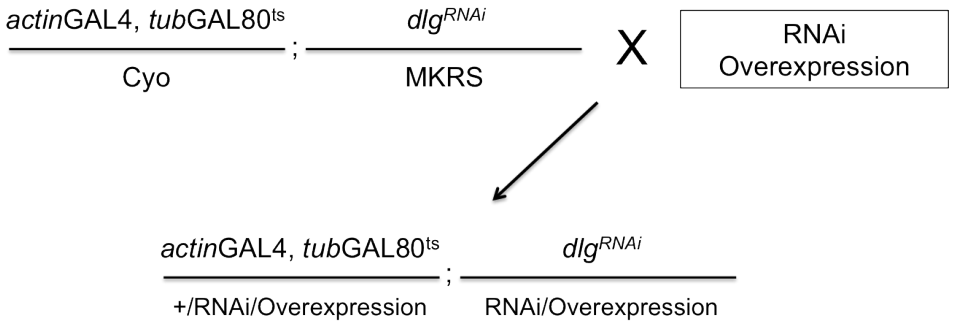


Figure 4.3 Schematic representation of the progeny selection for the follicular epithelium screen. The flies were raised at 18ºC once that expression of the RNAi construct in the whole organism is lethal. Upon progeny selection, the flies were placed for four days at 29ºC to disrupt the GAL4 inhibitor - GAL80^{ts}.

This screen revealed that depletion of a few mitotic genes is occasionally able to enhance the phenotype of *dlg*^{RNAi} (Fig. 4.5). However, as these results are still preliminary, we will just focus on the protein interactions that were shown to give a strong phenotype in the eye/antennal imaginal disc screen. Therefore, Aurora A and Anillin were the proteins chosen to proceed to quantification. Flies with RNAi constructs against these proteins show no tumour-like phenotypes per se. The same was tested in relation to *UAS-AurA* and no invasion was observed. As observed in Fig. 4.6, only two of the tested conditions -

UAS-AurA2 and *anillin*^{RNAi} - seem to enhance the tissue disorganization of the polarity defective background tested (see Fig. 4.4). Double *anillin*^{RNAi} ,*dlg*^{RNAi} resulted in the strongest phenotype observed in the follicle epithelium, resulting frequently in multiple layers of cells within the germline (Fig. 4.6).

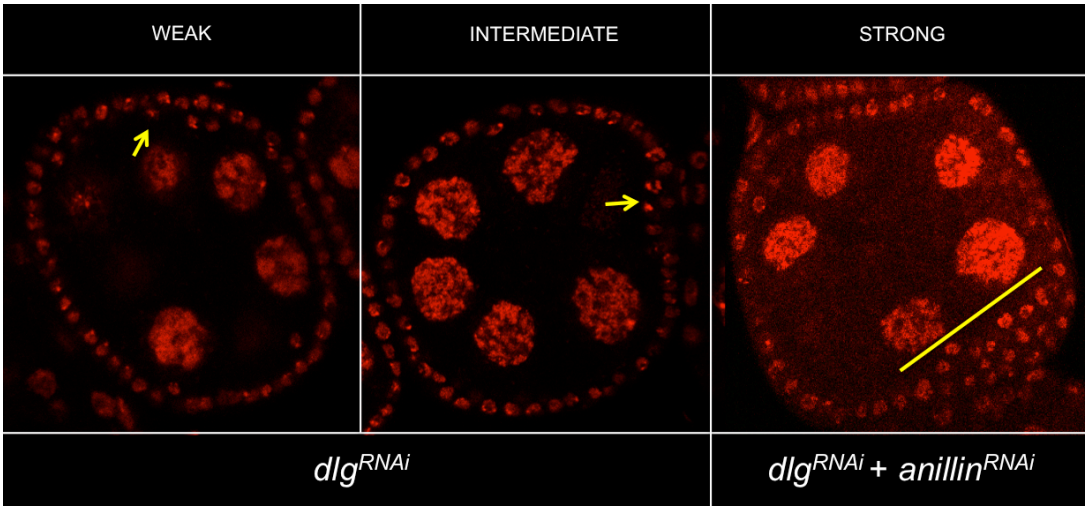


Figure 4.4 Representation of the three types of phenotype that were classified in the follicle epithelium. We show the effect of the combination of defective levels of both Anillin and Dlg as an example of a strong phenotype. Yellow arrows show the localization of invasions as well as the bar. DAPI is labelling the DNA in red.

On the other hand, depletion of AurA does not seem to interfere with the phenotype of *dlg*^{RNAi}. As can be seen in Graph 4.1, *aurA*^{RNAi}, *dlg*^{RNAi} have a similar penetrance of the intermediate phenotype relative to *dlg*^{RNAi} alone. In turn, expression of *UAS- AurA* leads to a larger frequency of intermediate phenotypes in the presence of *dlg*^{RNAi}, comparing with *dlg*^{RNAi} per se. Thus, overexpression of Aurora A seems to be inducing higher levels of epithelial cell invasion when the follicular epithelium presents defects in the polarity machinery. *anillin*^{RNAi}, *dlg*^{RNAi} revealed the presence of several strongly affected ovarioles, confirming that also in this ovarian epithelial context, cytokinesis cooperates with defects in epithelial polarity to induce tissue disorganization, which is a characteristic of tumor malignancy (Graph. 4.1).

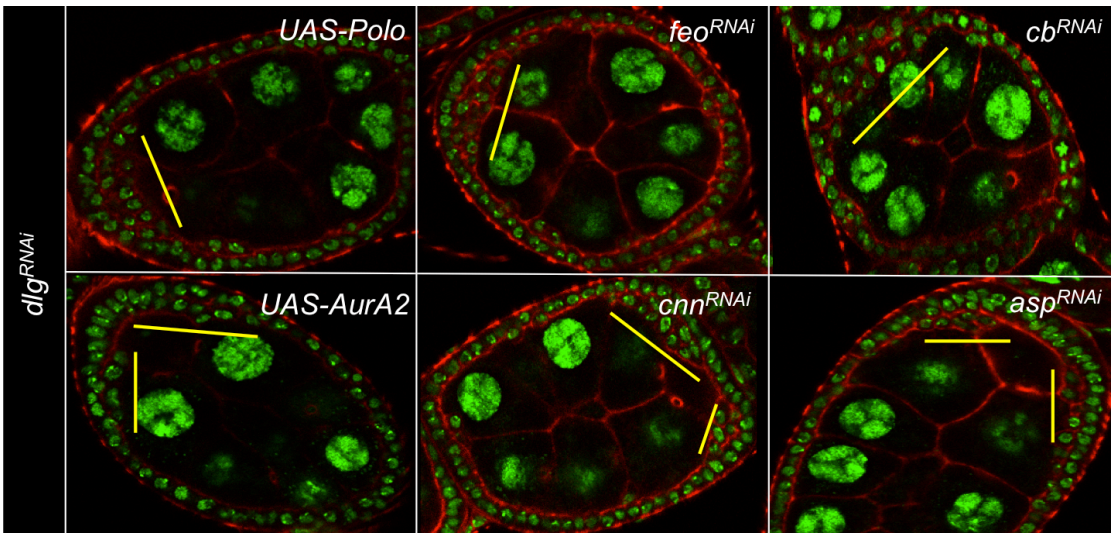


Figure 4.5 Examples of the strongest phenotypes observed after alteration of the levels of a mitotic protein in a polarity defective background. The yellow lines are indicating the most affected areas. Phalloidin is labelling the actin filaments while DAPI marks the DNA.

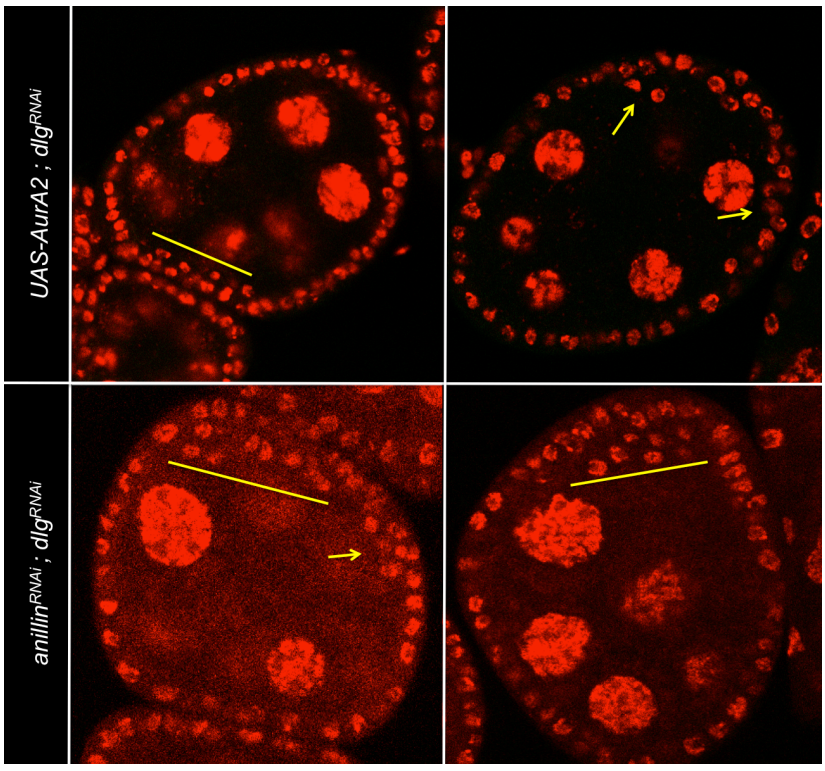
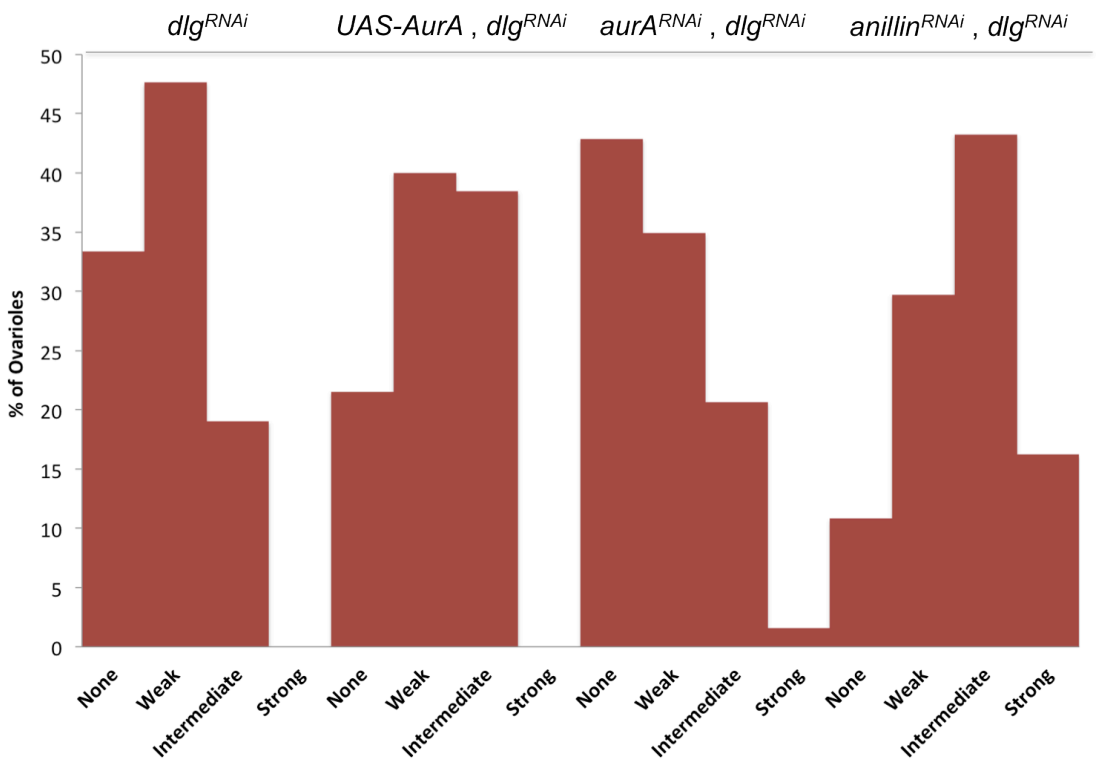


Figure 4.6 Examples of the observed phenotypes of *anillin*^{RNAi} and Aurora A overexpression when combined with *dlg*^{RNAi}. When Aurora A is overexpressed in a polarity defective background, we observe an increase in the levels of intermediate phenotypes. In turn, *anillin*^{RNAi} , *dlg*^{RNAi} shown an average percentage of strong phenotypes in the total quantification.



Graph 4.1 Quantification of the invasion phenotype per ovariole. *anillin*^{RNAi} gave the strongest interaction with *dlg*^{RNAi} while Aurora A defective levels seems to have no interaction with defective levels of Dlg.

5. Dissecting the role of AurA in the follicular epithelium

5.1 Background

Aurora A is a mitotic kinase that was shown to have a function on neuroblast polarization during mitosis. It phosphorylates Par-6, thereby inducing aPKC activation and therefore allowing the inclusion of cell fates determinants into the basal domain of *Drosophila* neuroblasts ([98]). Besides its roles on mitosis and neuroblast polarization, Aurora A defects were also associated with several human cancers. Both reduction and overexpression of Aurora A induces the formation of tetraploid cells, which promotes genomic instability which is known as a hallmark of cancer (reviewed in [159]; [160]). Furthermore, our screens that searched for possible mitotic interactors to induce tumorigenesis within a polarity defective background, suggest Aurora A as a possible candidate. Taking these facts into account, we proposed to determine if Aurora A has a role in the establishment of epithelial polarization of the follicle epithelium and if, by interacting with defective levels of components of Scribble Complex, it induces tumorigenesis.

5.2 Aurora A overexpression delays anaphase onset

As previously referred, Aurora A overexpression was found in the root of tetraploid cell formation within several cancers. Besides, Aurora A has several roles during mitosis, as centrosomal maturation/separation and spindle bipolarity organization, that were described in other systems. Therefore, we addressed if Aurora A overexpression could alter the dynamics of mitosis within follicle cells. We used flies expressing simultaneously His-RFP and Aurora A-GFP (UAS-AurA-GFP) transgene, in order to follow Aurora A localization in epithelial cells during mitosis. Not surprisingly, we found that the mitotic spindle was unstable for a long time during early stages of mitosis before stabilizing prior to anaphase onset. This made timing quantifications difficult since we could not determine in many cases the exact moment of anaphase onset. We also found that Aurora A overexpression causes a delay in anaphase onset (AO) when compared with the wild-type (Fig. 5.1). The timing from NEB to AO was however extremely variable. This might be explained by the fact that we used the GR1 promoter, which induces variable mosaic UAS-AurA-GFP expression within the epithelial monolayer. Thus, different NEB-to-AO timing could result from different levels of transgene expression. Quantifications of NEB-

to-AO of Aurora A overexpression have shown that Aurora A delays anaphase onset in about 9 minutes in relation to the wild-type average (Graph. 5.1).

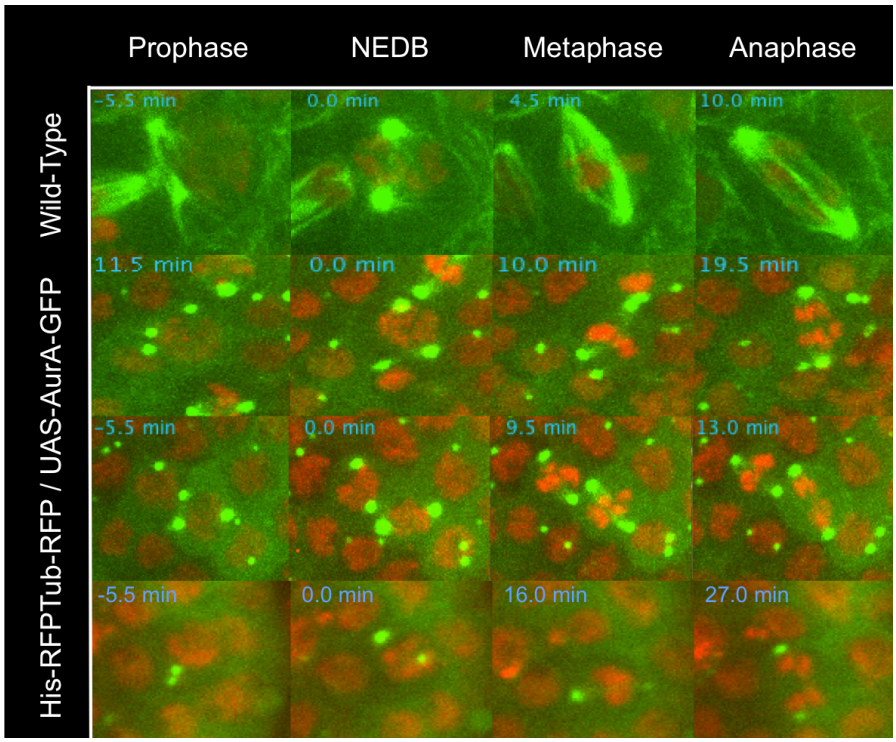
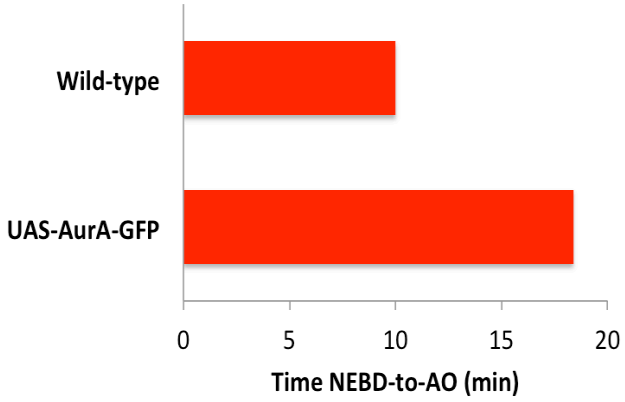


Figure 5.1 - Aurora A overexpression cause a delay in anaphase onset. Frames from movies showing UAS-AurA-GFP localization during mitosis. The expression of the His-RFP construct concomitantly with UAS-AurA-GFP enabled us to visualize NEB and follow Aurora A dynamics during the mitotic process. In the wild-type frames, Tubulin was tagged with GFP and Histone with RFP (top panel).



Graph 5.1 - NEBD-to-AO mitotic timing. The average time that a wild-type cell takes from NEBD to anaphase onset is about 9 minutes. When Aurora A is overexpressed it takes 18.4 ± 6 min (N=4) to progess between the same mitotic processes. The wild-type takes 9.0 ± 1.4 min (N=10) since NEB to AO. [Eurico Morais-de-Sá and Claudio Sunkel, unpublished data]

5.3 Low levels of Aurora A do not cause any defects in apico-basal polarity

To address if Aurora A has a role on apico-basal polarity we first expressed a RNAi construct against Aurora A (*aurA*^{RNAi}) in the follicular epithelium. *aurA*^{RNAi} was expressed with the GR1 promoter, a specific promoter only expressed in the follicle cells. Flies were placed at 29ºC to enhance the expression of the RNAi construct to further reduce the levels of Aurora A. As can be seen in Fig. 5.2C, defective levels of Aurora A did not cause any defects on apico-basal polarity. Both aPKC and Armadillo are properly localized in the apical and at the AJs, respectively, similarly to wild-type cells (Fig. 5.2B). To directly compare cells depleted of *aurA* with wild-type cells within the same egg chamber, we used the Flpout system. With this system, *aurA*^{RNAi} is only expressed in clones of cells that also express GFP, thereby marking the cells that were expressing the construct. Once again, we did not detect any defects on the localization of the previously referred proteins when compared with the internal control - cells marked with the absence of GFP (Fig. 5.2D). Although Aurora A does not seem to affect aPKC localization, we considered that it could be required for its kinase activity in epithelial cells. Therefore, anti-Phospho-Bazooka antibody (p-Baz), which is a marker for functional aPKC kinase activity was used to detect aPKC kinase activity in clones from same FRT/Flpout system. This antibody detects Bazooka phosphorylated by aPKC in its serine 980 thereby indicating whether aPKC is active or not. The results showed that Bazooka phosphorylation is unaffected by AurA depletion (Fig. 5.2E). These results indicate that Aurora A does not regulate apico-basal polarity in epithelial cells from the follicular epithelium. However, low Aurora A levels resulting from RNAi depletion could still be enough to fulfil AurA function. Thus, we tested the effect of the same RNAi construct but in a background that was heterozygous for a deficiency that removes *aurA* locus. We used this strategy, to decrease the levels of Aurora A even further. Immunofluorescence assays were performed using antibodies against aPKC and Dlg. It can be seen that the localizations of both proteins is similar to that of the wild-type in this condition (Fig. 5.2F). Following the same strategy, we combined the deficiency covering *aurA* locus with a hypomorphic allele for the same protein ([161]). A hypomorphic allele results in a protein that has a partial loss of function. This combination would lower even more the levels of functional Aurora A. Upon testing aPKC, Arm and Dlg antibodies, we found no alterations in the apico-basal polarity in the epithelium (Fig. 5.2G). These results confirm that lower levels of Aurora A do not cause any perturbation in the apico-basal polarity of epithelial cells and therefore AurA does not appear to have a major role in apico-basal polarity within the follicle epithelium.

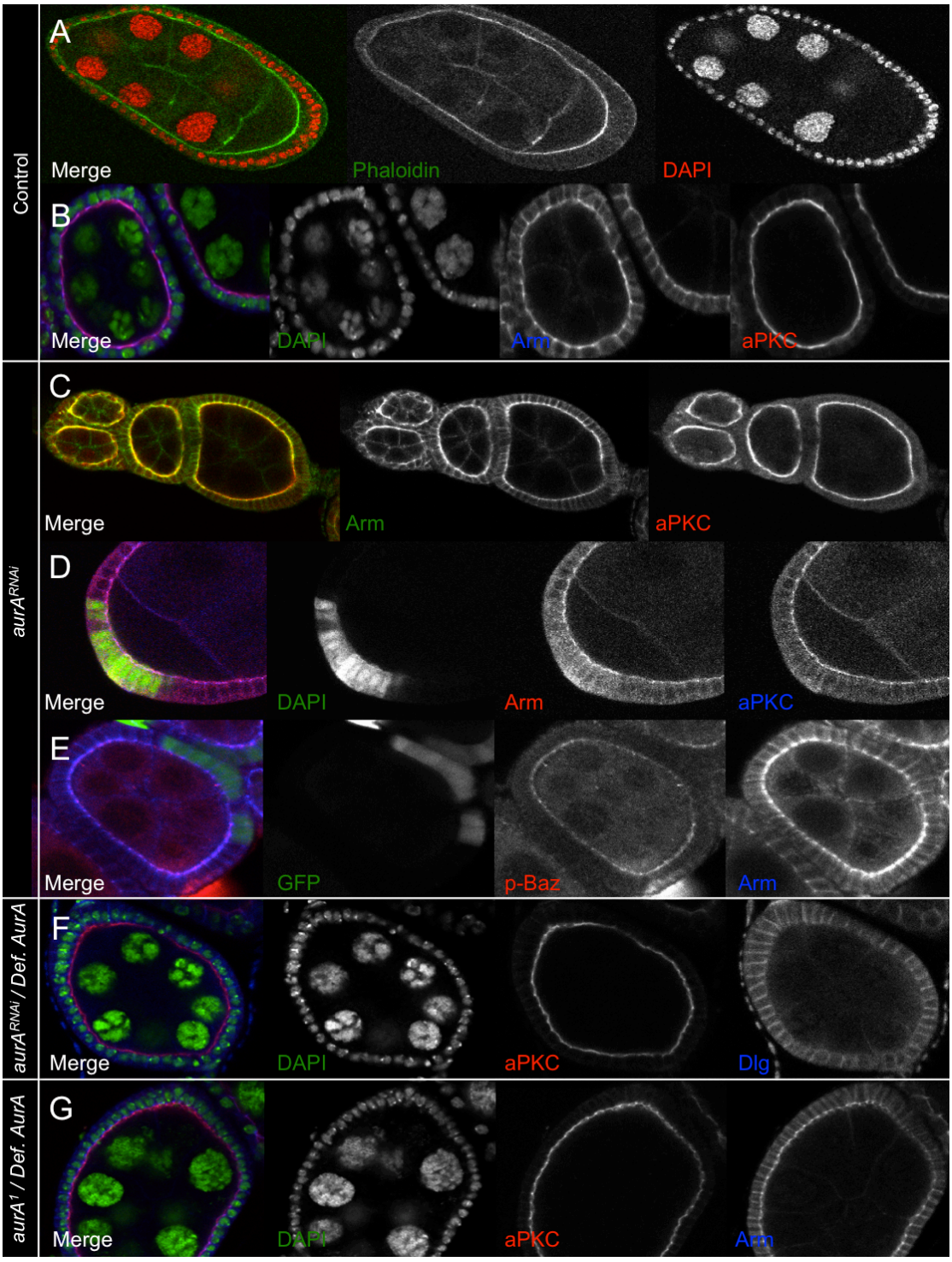


Figure 5.2 - Low levels of Aurora A have no effect on apico-basal polarity of follicle cells. Anti-aPKC, anti-Arm, anti-Dlg and anti-p-Baz antibodies were tested and no changes in the localization of the referred proteins were found. DAPI marks the DNA and phalloidin the actin filaments in A.

5.4 Aurora A kinase activity is not required to maintain apico-basal polarity in epithelial cells

In SOP cells, Aurora A kinase dead mutants (*aurA*^{37}) failed in Lgl cortical exclusion during mitosis once that Aurora A kinase activity is required for aPKC activation, and the consequent Lgl phosphorylation and exclusion from the cell cortex ([98]). Therefore, we decided to test if apico-basal polarity of epithelial cells also require Aurora A kinase activity. We selected the FRT system to induce clones that express in both chromosomes the *aur*^{37} allele. The kinase dead allele presents an arginine-to-histidine transition within the catalytic site impairing Aurora A substrates phosphorylation ([103]). Anti-aPKC, anti-p-Baz and anti-Arm antibodies were used and they show that the localization of the proteins was the same both in clones marked by GFP absence and in wild-type cells (Fig. 5.3). These results suggest that Aurora A has no major role on the establishment of apico-basal polarity in the epithelial cells of the follicular epithelium.

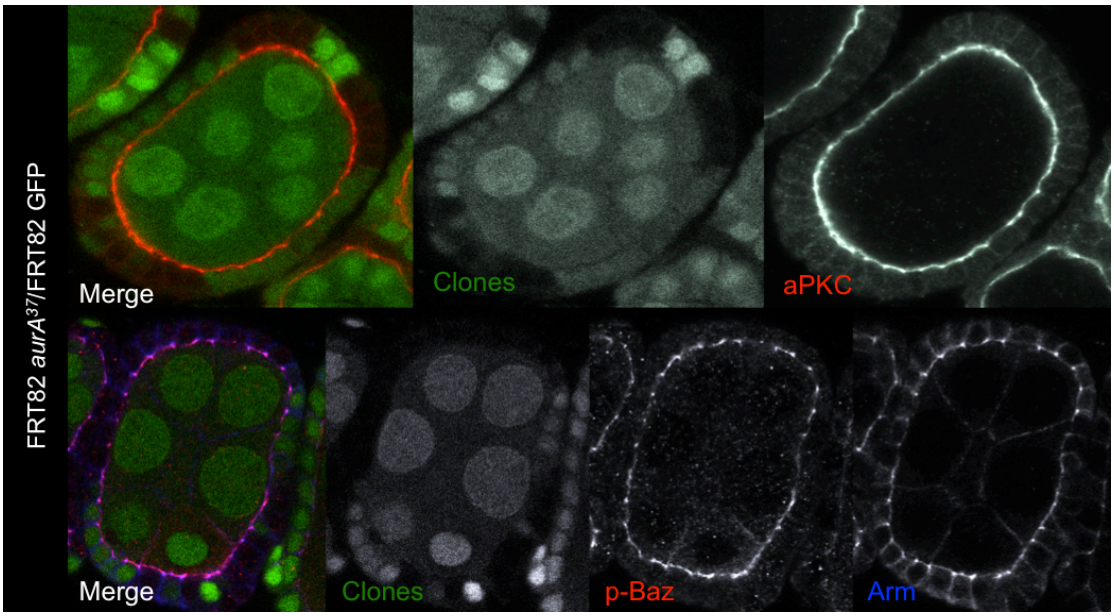


Figure 5.3 - Aurora A kinase activity is not required to maintain apico-basal polarity of follicle cells. Clones homozygous to the *aurA*^{37} kinase dead allele show no disruption of apico-basal polarity. Homozygous mutant cells are marked by GFP absence.

5.5 Par-6 phosphorylation on Ser34 is dispensable for apico-basal polarity

As previously mentioned, Par-6 is phosphorylated by Aurora A to initiate the establishment of the apical domain of neuroblasts ([98]). Although our previous results suggest that Aurora A does not have a role in the establishment of apico-basal polarity of the follicle epithelium, we wanted further confirmation to support this conclusion. We created flies that were homozygous for a *par-6* null mutation, but that were expressing the Par-6^{S34A} transgene under the control of the endogenous *par-6* promoter (Fig. 5.4).

$$\frac{\textit{par-6}}{\textit{par-6}} ; \frac{\text{Par-6}^{S34A}}{\text{Par-6}^{S34A}}$$

Figure 5.4 - Stock created to test if Par-6^{S34A} is able to rescue apical domain disruption in *par-6* mutans. A non-phosphorylatable form of Par-6 on its serine 34 (Par-6^{S34A}), the conserved residue of Aurora A phosphorylation, was expressed in *par-6^{D226}* mutant flies.

To confirm that *par-6* causes apical proteins mislocalization, immunofluorescence analyses were performed. Anti-aPKC and anti-Arm antibodies show mislocalization of both proteins when compared with the internal control, marked by the presence of GFP (Fig. 5.5A). Par-6^{S34A} rescues this phenotype as can be seen by the presence of aPKC in the apical domain (Fig. 5.5B). Thus, Par-6 phosphorylation in Ser34, which is dependent on Aurora A kinase on the neuroblast, is also not required for apical-basal polarity further demonstrating the AurA has no role in setting up or maintaining apico-basal polarity of the follicle epithelium.

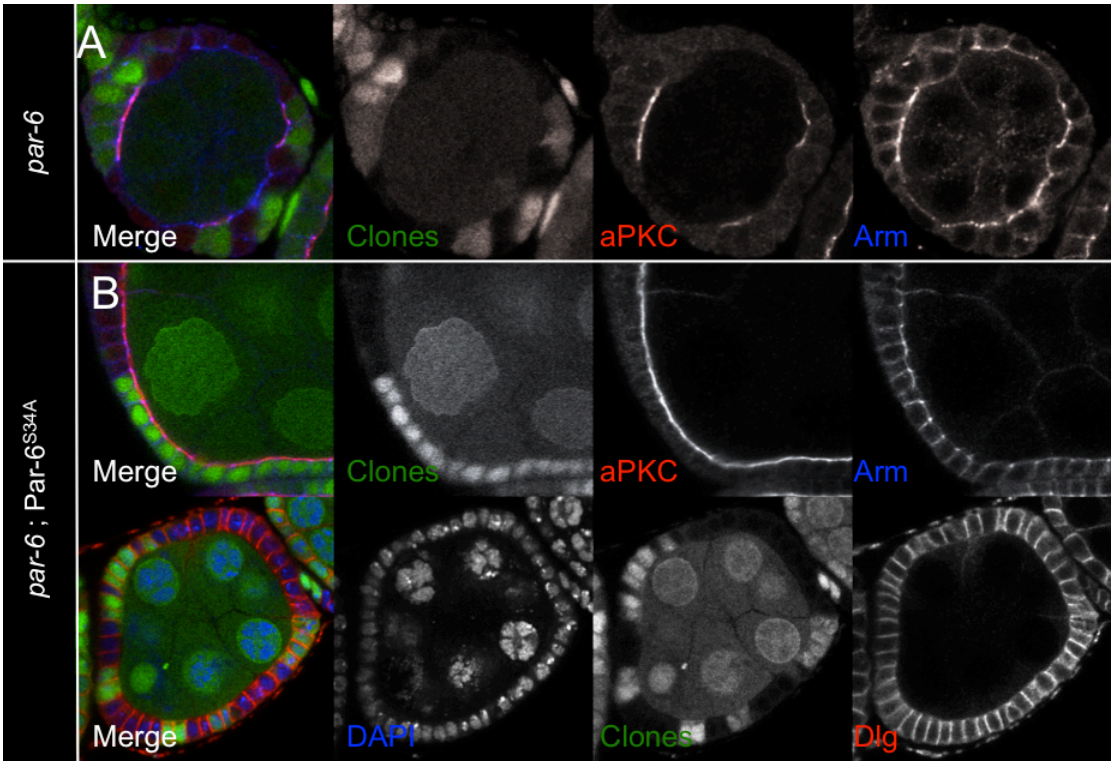


Figure 5.5 - Par-6 phosphorylation is dispensable for the maintenance of apico-basal polarity in follicle cells. The non-phosphorylatable form of Par-6 rescues mislocalization of the apical proteins in *par-6* mutants.

5.6 Lgl dynamics during mitosis on the follicular epithelium

The conservation among the proteins that govern cell polarity is well known. Lgl exclusion from the posterior cortex in SOP cells is essential for cell fate determination since that it exclusion allows the association of Bazooka with the PAR Complex and thereby Numb phosphorylation ([98]). Our results suggested an independence of Aurora A kinase activity in epithelial polarization. However, given the conservation of the proteins among different organisms, it is important to determine if the underlying mechanism of Lgl dynamics is also essential for epithelial cell polarization, given that Aurora A kinase activity is not. The behaviour of Lgl in SOP cells during mitosis is well known: Lgl starts to be excluded from the cortex in early prophase, remaining in the cytoplasm during mitosis ([98]). A form of Lgl that is non-phosphorylatable by aPKC - the Lgl^{3A} – remains localized in the cortex during cell division, suggesting that Lgl exits from the cortex in a phosphorylation-dependent manner ([98]). Accordingly, we wanted to know how Lgl behaves during mitosis in epithelial cells. We followed cell division using His-GFP to mark DNA, together with Lgl-RFP. We concluded that Lgl starts to exit from the cell cortex

around 21 minutes prior to Nuclear Envelope Breakdown (NEBD) remaining in the cytoplasm during division and returning to the cortex after cytokinesis (Fig. 5.6 and Graph. 5.2).

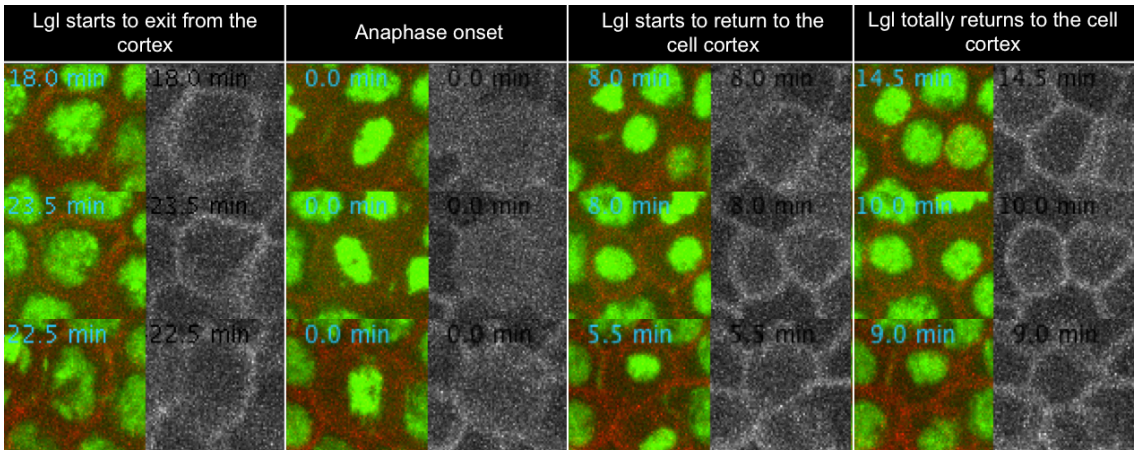
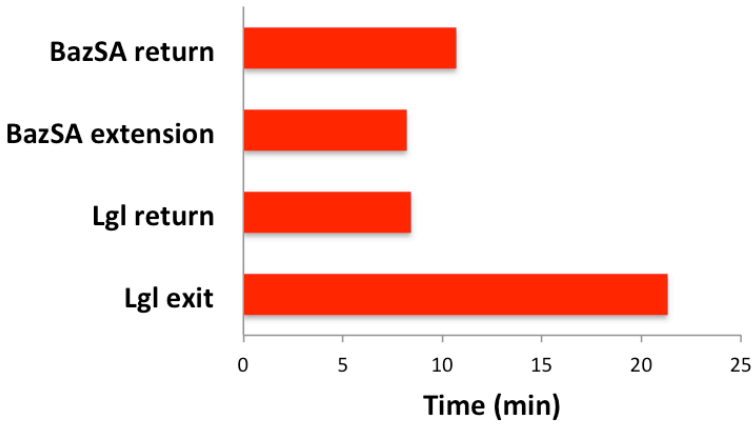


Figure 5.6 - Lgl^{WT}-GFP behaviour during mitosis in epithelial cells. Frames were taken from movies that show Lgl:RFP dynamics during mitosis. His:GFP enables us to determine the exact moment of anaphase onset. We took this time as a start point to quantify when Lgl exits and then returns to the cortex.



Graph 5.2 - Lgl and Baz^{S980A} dynamics during mitosis. Lgl starts to exit from the cell cortex 21.3 ± 2 min (N=6) prior than anaphase onset and returns 8.4 ± 2 min (N=6) after anaphase onset. In turn, Baz^{S980A} starts to depolarize in average 8.1 ± 3 min (N=4) prior than anaphase onset while it fully returns to the apical mesh in 10.7 ± 2 min (N=6) after AO. This results shows that Baz^{S980A} depolarization starts after Lgl cortical exclusion.

Accordingly, to determine whether Lgl cortical exclusion is phosphorylation dependent, we performed immunostaining in cells from flies with Lgl^{WT}-GFP and Lgl^{3A}-GFP using anti-pH3 to mark the cells that were undergoing mitosis. Our observations

show that Lgl^{WT}-GFP localizes at the cell cortex during interphase, however, in cells that were labelled with pH3, Lgl^{WT}-GFP was diffused in the cytoplasm (Fig. 5.7A). In turn, the non-phosphorylatable form (Lgl^{3A}-GFP) remained in the cortex during interphase and mitosis (Fig. 5.7B). These results suggest that during mitosis, Lgl has a similar behaviour in SOP cells and in epithelial cells, being excluded from the cortex in an aPKC phosphorylation dependent manner.

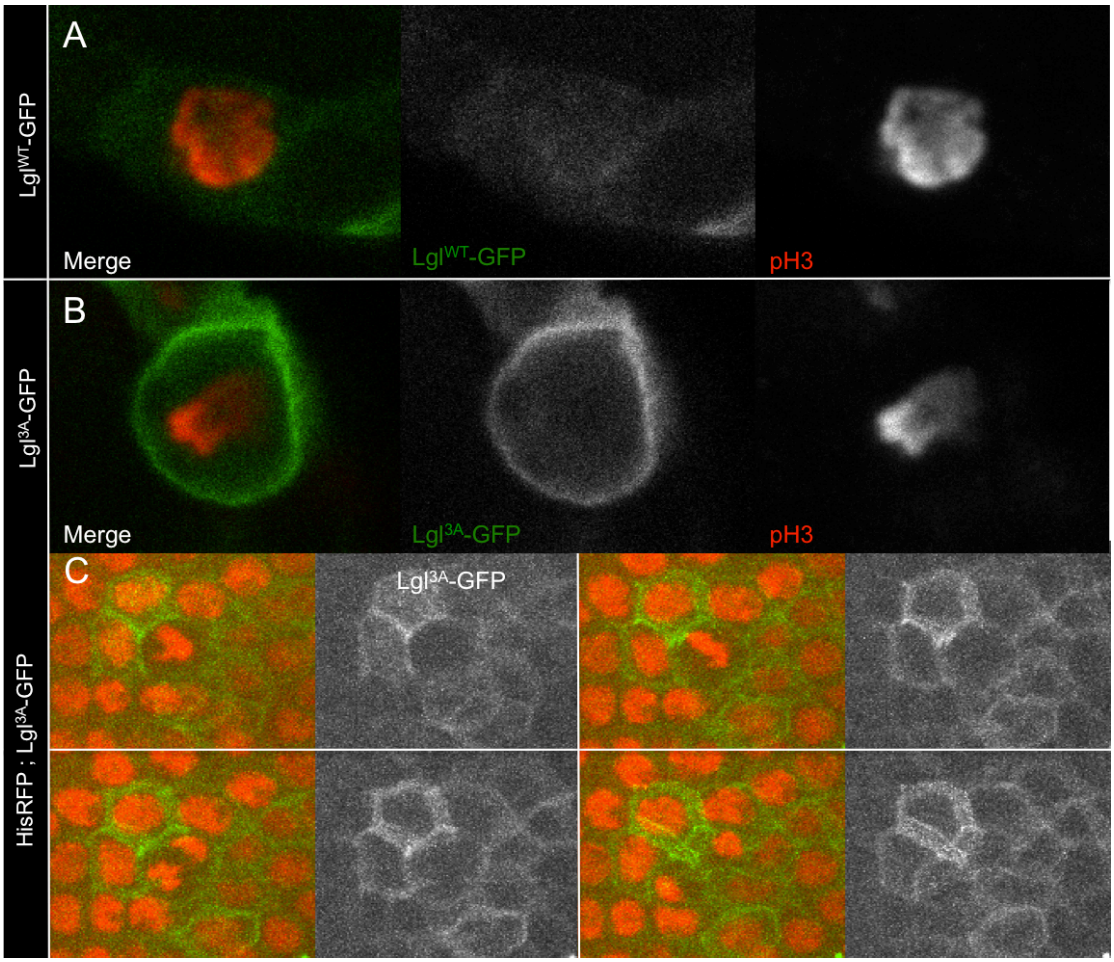


Figure 5.7. Lgl is excluded from the cortex in a phosphorylation dependent manner. The non-phosphorylatable form of Lgl remains cortically during all cell cycle (B) when comparing to the wild-type (A). Anti-pH3 labels cells that are undergoing mitosis. Frames from movies of Lgl^{3A}-GFP show that this mutant variant remains in the cortex during mitosis (C).

5.7 Using Baz^{S980A} to address the dynamic behaviour of apical proteins during mitosis in epithelial cells

After characterizing the dynamics of Lgl during mitosis, we asked how would the apical domain behave. Given that Lgl cortical exclusion occurs in a phosphorylation-

dependent manner, lateral exclusion of Lgl would be expected to be preceded by a re-organization of apical proteins. In order to determine the localization of apical proteins during mitosis, we used colchicine to increase the percentage of mitotic cells within the follicular epithelium. We performed immunostainings using flies that expressed Par-6-GFP, to address the localization of the apical complex during mitosis. As can be seen in Fig. 5.8, the nucleus of the follicle cells are labelled with pH3, indicating that many cells are undergoing mitosis. Both the anti-aPKC antibody and the endogenous Par-6-GFP revealed that the apical proteins present different localizations in the cells that were labelled with pH3. pH3 marks the phosphorylation of the Histone 3 since the beginning of mitosis – prophase – which means that all the cells that are in prophase or the subsequent phases are labelled with pH3. Since, we observe that in some cells, indicated by the yellow arrow, aPKC and Par-6 are distributed essentially through the cytoplasm while in others (marked by the green arrow) aPKC and Par-6 remain in the apical mesh. This result suggests that the apical complex has a specific time during mitosis within which depolarization starts.

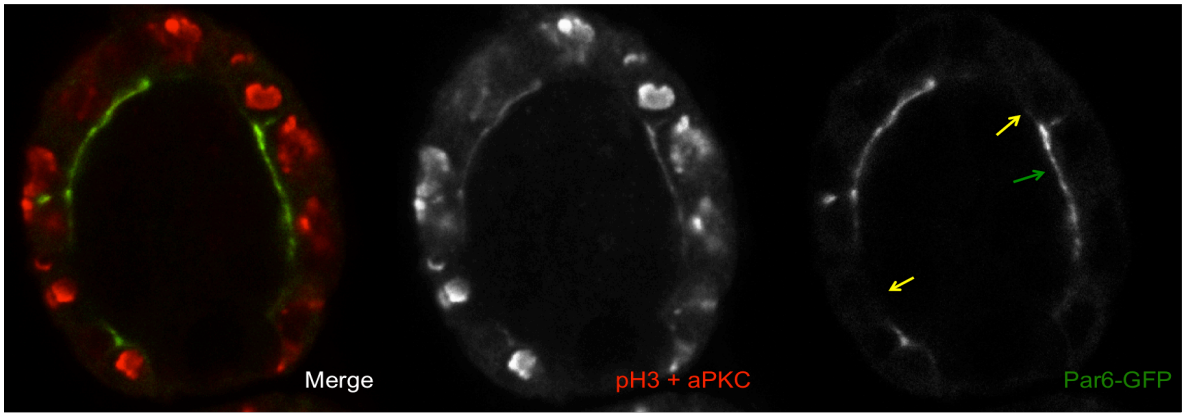


Figure 5.8 - Apical Complex localization in a mitotic enriched follicular epithelium. Females were fed with colchicine, which blocks microtubules depolymerisation, thereby arresting cells in metaphase. pH3 staining (red) shows cells that are undergoing mitosis. The Par-6-GFP (green) construct and the anti-aPKC antibody (red) show the subcellular localizations of the apical complex.

To characterize the dynamic of depolarization of the apical complex during mitosis, we performed live imaging. In the absence of a functional fluorescent tagged aPKC protein, we used the non-phosphorylatable form of Bazooka (Baz^{S980A}-GFP) to mark the localization of apical proteins throughout mitosis. Since it cannot be phosphorylated by aPKC, Baz^{S980A} is not excluded from the apical complex. It was surprising to find that Baz^{S980A} starts to extend through the cortex around 8 minutes before NEBD (Graph. 5.2).

Furthermore, complete re-localization of Baz^{S980A} to the cortex occurs after cytokinesis around the same time that Lgl^{WT}-GFP relocalizes to the cell cortex (Fig. 5.9). Differences were seen between Par-6-GFP and Baz^{S980A} localization during mitosis. While Par-6-GFP presents a cytoplasmic localization, Baz^{S980A} extends through the cell cortex.

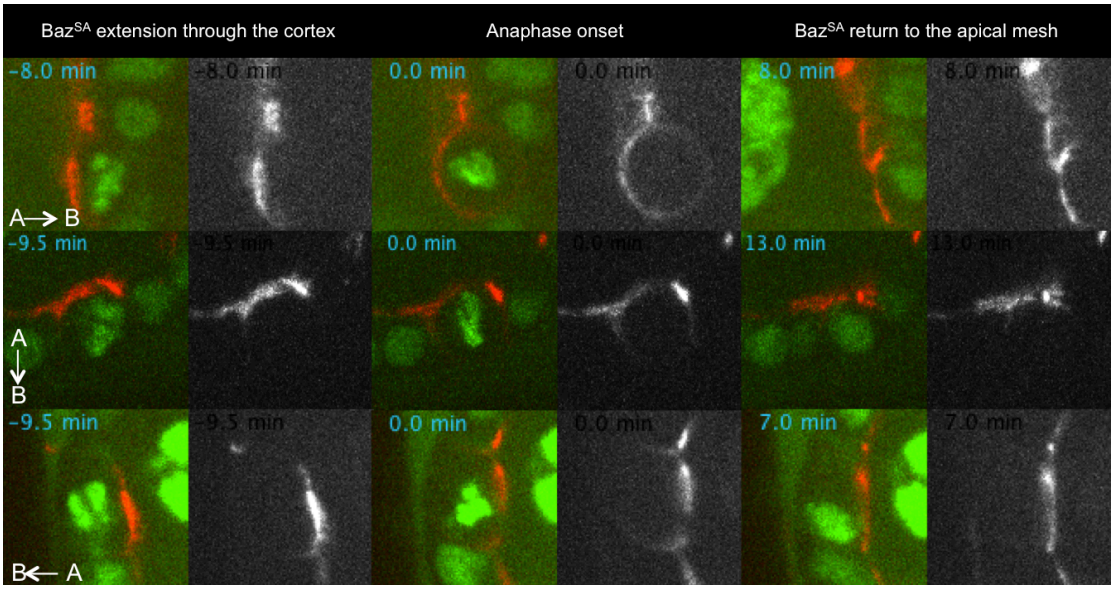


Figure 5.9 - Baz^{S980A}-GFP extends through the cortex during mitosis. Frames from movies of Baz^{S980A}-GFP (that is shown in red) showing its cortical extension occuring after Lgl^{WT} cortical exclusion. The apical-basal axis orientation is shown to each figure. This extension occurs after Lgl^{WT} cortical exclusion. Baz^{S980A} dynamic times during mitosis are shown in Graph. 5.2.

CHAPTER 4 – DISCUSSION AND CONCLUSION

Several mitotic defects have been associated with the induction of cellular transformation presumably by promoting aneuploidy, a chromosomal defective condition that is frequently found in tumors ([130]). On the other hand, loss of cell polarity was also associated with cancer progression since it causes tissue disorganization and cell overproliferation ([162]). These observations provide the basis for the main aim of this work: to search for mitotic interactors that would enhance tumorigenesis in a polarity defective background. To accomplish this, we used two different epithelial models from *Drosophila*: the eye/antennal imaginal discs and the ovarian follicular epithelium. As described in the results chapter, two proteins, Anillin and Aurora A, were identified as possible interactors that severely enhanced the tumor-like phenotype of these cells when cell polarity is compromised.

6.1 Cytokinesis failures can drive tumorigenesis in a polarity defective background

Anillin is a scaffold protein, whose depletion results in cytokinesis failure both in *Drosophila* and humans, resulting in tetraploidy ([10]). The expression of $anillin^{RNAi}$ within a polarity defective background in both epithelial models revealed an enhancement of the tumor-like phenotype when compared with the respective controls. This result suggests that cytokinesis failure cooperates with polarity defects to induce tumorigenesis. However, incomplete cytokinesis also leads to the accumulation of centrosomes within a single cell. The increasing number of centrosomes could generate multipolar spindles at the time of division, resulting in the formation of aneuploidy cells (reviewed in [130]). Therefore, anillin depletion could be enhancing tumorigenesis due to the formation of multipolar spindles and not due to its role in cytokinesis. To address which was the driving force that was enhancing tumorigenesis after anillin depletion, we depleted sas-4 in the context of scribble and anillin depletions, providing an acentrosomal background to these cells. Our observations suggest that anillin depletion induces tumorigenesis independently of centrosome accumulation, since the reduction of the levels of sas-4, and consequently the absence of centrosomes, was irrelevant to the enhancement of the tumor-like phenotype. However, the levels of protein depletion caused by using $sas4^{RNAi}$ need to be verified to ensure that centrosome duplication is indeed impaired. Furthermore, studies using *sas4 null* mutants associated with the deficiency for the sas4 locus would be required in order

to ensure that centrosomes are completely absent from the epithelial tissue. Nevertheless, our results indicate that, polyploidy is most likely the cause underlying the enhancement of tumor-like phenotypes produced by anillin RNAi. Consistent with this, the direct tumorigenic potential of tetraploid cells has been previously documented. Induction of cytokinesis impairment into $p53^{-/-}$ mouse mammary epithelial cells (MMECs) leads to the formation of tetraploid cells ([131]). Carcinogenic experiments *in vitro* have shown that tetraploid cells were able to be transformed and to induce malignant pathologies after removal of the carcinogen. Consistently, other studies provided evidences that although extra centrosomes can induce tumorigenesis in specific fly tissues, they do not always lead to aneuploidy and genetic instability, given that extra centrosomes appear to coalesce, allowing the formation of mostly bipolar spindles in these abnormal cells ([163]).

Although mutations on Aurora A gene seem to be insufficient to initiate the malignant pathology, Aurora A has long been associated with tumour formation in different organisms ([164]; [165]; [156]). The reduction of Aurora A levels causes defects on the mitotic spindle due to the impairment of centrosome maturation and separation, leading to the formation of tetraploid cells. In turn these tetraploid cells are frequently found in cancers suggesting that tetraploidy promotes genomic instability, which is a well-known hallmark of cancer (reviewed in[159]). Consistently, we observed that the expression of $aurA^{RNAi}$ induced outgrowth phenotypes within the *Drosophila* eye. However, the observation of larvae imaginal discs did not allow us to conclude what happens at the cellular level, since the morphology of the disc was not significantly altered when compared with the wild-type, regardless an increase in cell proliferation throughout the whole disc. Further studies will aim to elucidate if downregulation of AurA resulted in tetraploidy in the larvae imaginal discs. On the other hand, the expression of $aurA^{RNAi}$ in the follicle cells whose polarization is compromised, did not result in a significant increase of invasion into the germline, although some intermediate phenotypes are observed. One possible explanation is that the depletion by expression of RNAi constructs is variable. Additionally, in *Drosophila* early stages of oogenesis, the expression of the RNAi constructs is low and epithelial cells divide just until stages 6/7. Therefore, a possible explanation for these results relies on the weak depletion of the protein levels by $aurA^{RNAi}$ with the few divisions of the epithelial cells that did not allow the accumulation of errors and consequently cell transformation and invasion. The imaginal discs are dissected around 6/7 days after egg laying but the *Drosophila* eye can only be seen after the fly hatches, which means about 10 days after fertilization. As the RNAis have a variable expression and as efficient protein depletion takes several days to be achieved, it is possible that the effect seen in the adult eye is stronger than in the imaginal discs. Furthermore, as the drivers expressing the RNAis constructs in both epithelial models are

different, the level of depletion of Aurora A achieved within both systems is different. Moreover, the expression of the RNAi construct in the eye is longer than in the follicular epithelium due to the large development period of the eye. This may explain why we observe that Aurora A cooperates with compromised polarity in the eye but not in the follicular epithelium.

During both screens we also noticed that *UAS-AurA* caused an enhancement of the tumour-like phenotype in cooperation with defects in the polarity machinery. Although differences between males and females were significant in the eye, the overexpression of Aurora A lead to a substantial outgrowth in association with a high percentage of overgrowth phenotypes in the adult flies eyes. Further analyses of the larvae imaginal discs have shown that overexpression of Aurora A induced a failure in photoreceptor differentiation along with a random cell proliferation throughout all disc regardless MF constriction. Likewise, overexpression of this mitotic kinase regulator promoted an increase in the intermediate phenotype on the follicular epithelium when compared with the polarity defective control. However, this phenotype was not as aggressive as in the eye. This could be explained by fewer divisions of the epithelial monolayer, which decrease the accumulation of errors that would be required to allow cell transformation. It has been described that Aurora A overexpression induces cytokinesis failures and p53 inactivation and degradation ([118]; [166]). By promoting cytokinesis failure, Aurora A promotes the formation of tetraploid cells that would normally arrest in the next G1-phase ([167]; [168]; [169]; [170]). However, by destabilizing p53 through phosphorylation, the p53-dependent checkpoint that prevent tetraploid cell division is weakened, enabling tetraploid cells to divide and proliferate ([155]; reviewed in [160]). Therefore, the underlying mechanism by which *UAS-AurA* cooperates with polarity defects probably relies on the ability of Aurora A (when overexpressed) to promote cytokinesis failure resulting in tetraploidy. Phosphorylation and consequently degradation of p53 induced by Aurora A is also observed at physiological levels, however this effect is increased upon Aurora A overexpression. This may explain why cells depleted of Aurora A have a less consistent tumour-like phenotype, since the p53-checkpoint is not weakened as a result of decreased levels of Aurora A phosphorylation ([166]).

6.2 Aurora A is not required to the establishment of apico-basal polarity in epithelial cells

Studies performed in *Drosophila* neuroblasts have shown that Aurora A kinase activity is essential to differentially segregate Numb into the GMC. Aurora A phosphorylation on Par-6 relieves aPKC suppression, and therefore activates it. aPKC

activation is essential to neuroblast polarization during its asymmetric division to phosphorylate Numb, allowing its specific localization into the GMC and therefore cell differentiation ([98]). Our results show that Aurora A cooperates with polarity defects to induce tumorigenesis, suggesting that it might also have a role in the establishment of epithelial polarity. Therefore, we wanted to determine whether Aurora A is also involved in epithelial polarization.

We started by expressing a specific RNAi construct against Aurora A, thereby decreasing its levels in the follicular epithelium. As no defects of the apical-basal polarity were observed, we used a hypomorphic allele combined with the deficiency for the locus of Aurora A to decrease even further its protein levels. Our results suggested that decreased levels of Aurora A had no influence on the establishment of epithelial apico-basal polarity. However, it was still possible that incomplete depletion of Aurora A by the RNAi transgene was enough to support the normal functions of the mitotic kinase. Therefore, we used a kinase dead allele to address if its kinase activity was required for the maintenance of epithelial polarity. Immunostainings using phospho-specific antibodies revealed that Aurora A kinase activity was not required for aPKC apical localization or activity, as shown by the wild-type localization of anti-p-Baz antibody within the GFP clones. Therefore, our results suggested that Aurora A does not have a major role in the establishment of follicle cell polarity.

Several hypotheses could be raised to explain why Aurora A activity is dispensable for follicle cell polarity. Looking at aPKC kinase activity essentiality along the establishment of apico-basal polarity during epithelial interphase, it is reasonable to propose that at some point its kinase activity would be activated to carry out aPKC specific functions. This activation could be performed by a kinase other than Aurora A, thereby maintaining part of the mechanism identified in neuroblasts. Other possibility could rely on activation by Cdc42 during interphase, since it was described in mammals that although Par-6 is the responsible for aPKC localization, Cdc42 modulates it kinase activity ([171]; [55]; [52]). Finally, aPKC could be constitutively active throughout the cell cycle and thereby any activation would be dispensable. Nevertheless, follicle cells seem to undergo depolarization during mitosis, and therefore it is possible that the activity of aPKC is dispensable at this stage. Given that the apical and basolateral domains are depolarized during mitosis, aPKC activity may not be needed to restrain their locations during interphase.

6.3 Possible role of Lgl in driving mitotic depolarization of epithelial cells

To understand if the mechanisms triggered by Aurora A phosphorylation in neuroblasts/SOPs were also conserved in epithelial cells, we analysed the dynamic behaviour of Lgl during mitosis. As in SOP cells, Lgl is cortically excluded during mitosis in a phosphorylation-dependent manner as a form of Lgl (Lgl^{3A}-GFP) that is non-phosphorylatable by aPKC remains cortical during all cell cycle. In SOP cells, Aurora A phosphorylation induces aPKC activation whose kinase activity is responsible for Lgl exclusion from the cortex ([98]). However, despite our results demonstrating that Aurora A function is not required in epithelial cells, aPKC still phosphorylates and excludes Lgl from the apical domain during interphase, whereas it is known that Lgl is able to repress aPKC activity if stably bound to it ([81]; [79]; [96]). Therefore, we wanted to investigate how the apical domain behaves during mitosis in epithelial cells. It has been previously found in the lab [Eurico Morais de Sá and Claudio Sunkel, unpublished data] that the proteins Par-6 and aPKC lose their polarized apical distribution during mitosis. Increasing the frequency of mitotic follicle cells (using colchicine) in flies that carry a Par-6-GFP construct, we were able to see that epithelial cells labelled with pH3 had different subcellular localizations of both anti-aPKC and Par-6-GFP, thereby suggesting a dynamic depolarization during mitosis. Therefore we used Baz^{S980A} as a surrogate marker of the apical complex to determine in detail the dynamics of the apical complex. Our data supports that the apical complex starts to extend throughout the cortex after Lgl cortical exclusion. However, while Par-6-GFP shows a cytoplasmic localization, Baz^{S980A} depolarization was restricted to the extension through the cortex. Baz^{S980A} cortical localization could partially reflect its ability to bind Adherens Junctions components. Given that Par-6-GFP is expressed at endogenous levels, its localization is more reliable in reflecting the localization of the apical Complex. Previous results in the lab show that the depolarizing times in Par-6-GFP expressing cells, are nevertheless similar to the ones obtained with Baz^{S980A}. A timeline showing and overview of the respective timings is shown in Fig 6.

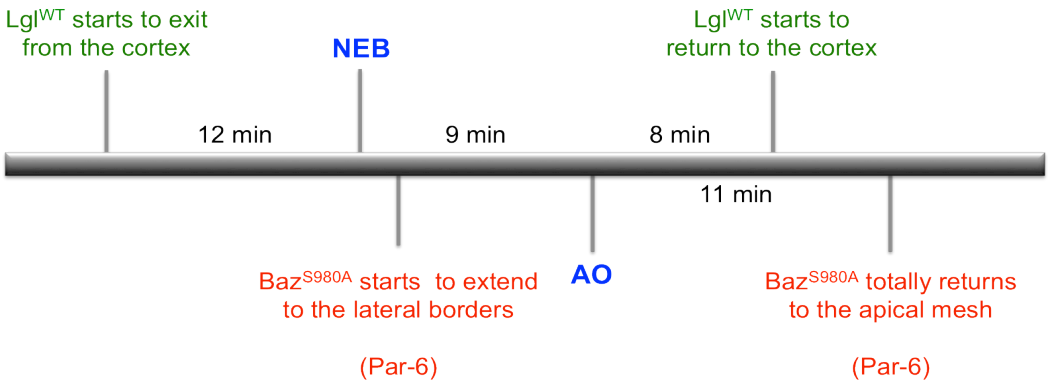


Figure 6 - Relative timings of the re-organization of the polarity proteins during cell division in follicle cells. NEB – Nuclear Envelope Breakdown. AO – anaphase onset. It is worth to note that similar timings were observed for exclusion of Par-6 from the apical cortex [Eurico Morais de Sá and Claudio Sunkel, unpublished data].

These results are somewhat paradoxical. On the one hand, Lgl cortical exclusion is phosphorylation-dependent. The mutation of the three phosphorylatable sites by aPKC within Lgl leads to a retention in the cortex during all cell cycle, thereby suggesting that aPKC could be the kinase responsible for it exclusion. On the other hand, we have results indicating that Lgl exclusion from the lateral cortex occurs previously to the lateral extension of apical proteins. Therefore, how can aPKC be responsible for Lgl removal from the cortex if Lgl is already in the cytoplasm at that time? To resolve the issue we propose a model that places Lgl as a controller of epithelial depolarization during mitosis by promoting the apical exclusion of the PAR complex. This model is based in the particular features relative to the timing and purpose of polarization that vary between the neuroblast and the follicle cell systems. (1) For the correct asymmetric division of the neuroblast, polarization must occur during mitosis in order to accurately segregate the cell fate determinants. Instead, epithelial cells are already polarized during interphase, and contrary to the neuroblast, there is depolarization during mitosis. (2) Aurora A triggers a phosphorylation cascade that leads to aPKC activation and consequently to Lgl exclusion from the PAR Complex. Contrarily, Aurora A kinase activity is unnecessary for the activity of aPKC in epithelial cells. This is in agreement with the idea that aPKC does not need to be polarized during epithelial cell division. However conserved features are also observed, since Lgl presents the same dynamic behaviour during mitosis in both systems meaning that it is excluded in a phosphorylation-dependent manner. Three phosphorylation residues within the Lgl are conserved between humans and *Drosophila*, and only the mutation of the three residues blocks cortical exclusion during mitosis ([81]). Therefore,

we propose that Lgl could be excluded by other kinase rather than aPKC. After activation and consequent lateral exclusion, Lgl would be able to bind aPKC on the apical domain, inhibiting its activity and exerting a depolarizing effect over the apical domain. Indeed, Par-6-GFP shows a cytoplasmic localization during mitosis that would be consistent with Lgl binding to the apical complex and sequestering it in the cytoplasm. Thus, we could hypothesize that Lgl could be the driver for depolarization of epithelial cells during mitosis, while aPKC is the driver of polarization during neuroblast division. Supporting this idea is the previous mentioned observation that Lgl is able to bind and inactivate aPKC, which also agrees with a depolarization model of epithelial mitosis that does not require an activated aPKC. Accordingly, the formation of a complex formed by Lgl, Par-6 and aPKC is increased during mitosis in HEK293 cells ([80]). Furthermore, other studies using MDCK cells, that are also epithelial cells, suggested a comparable model to the one we propose for follicle cells. During depolarization of MDCK cells, it has been found that Lgl induces the disassembling of the apical proteins, Par-6 and aPKC. Through Ca2+ depletion, MDCK cells undergo depolarization that is mediated by Lgl suppression of the apical proteins by impairing Par-6-aPKC interaction with Par-3 or Cdc42 ([80]). Moreover, a recent study proposed that Lgl is able to stimulate the endocytosis of the apical transmembrane protein Crumbs, blocking the spreading of the apical proteins into the basolateral membrane. On the other hand Crb-Crb interactions stabilize the localization of the apical proteins Par-6/aPKC into the apical domain ([172]). Thus, Crumbs endocytosis mediated by Lgl could participate in the destabilization of apical pool of Crb-Par-6-aPKC. This mechanism could alternatively explain how Lgl drives apical depolarization during mitosis in epithelial cells, as proposed by our model. Although depolarization during mitosis might be important to allow symmetric cell division of epithelial cells, the question of how it is regulated is unclear. Determining if the apical domain remains polarized in cells that are unable to recruit any Lgl to the cytoplasm during mitosis will start answering this question in the future.

During this work we also found that cytokinesis impairment cooperates with polarity defects to induce tumorigenesis in flies, providing therefore a starting point to dissect interactions between mitotic and polarity proteins in tissue architecture and proliferation. It was also found that Aurora A is dispensable for epithelial polarity suggesting that a different regulatory mechanism directs the polarity machinery during epithelial cell division. Although this finding presents to be different from neuroblasts, some similar features between neuroblast and epithelium polarity are conserved, as is the case of Lgl dynamic behaviour during mitosis. Our results suggest that Lgl could act as a driver of depolarization in epithelial cells, however further evidences need to be found in order to support our model.

Appendix

WT- Wild-type; S-eye – Small eye; M-eye – Micro eye; R-eye – Rough eye; D – eye – disorganized eye; T – eye – Outgrowth; O- eye – Overgrowth; N – eye – No eye

Table 1. Frequency of phenotypes shown in male eyes in the absence of scrib^{RNAi}.
WT- Wild-type; S-eye – Small eye; M-eye – Micro eye; R-eye – Rough eye; D – eye – disorganized eye; T – eye – Outgrowth; O- eye – Overgrowth; N – eye – No eye

RNAi/O.e.	WT	S-eye	M-eye	R-eye	D-eye	T-eye	O-eye	N-eye	N
anillin	40	27	3	16	7	0	0	0	93
asp	4	30	0	0	0	0	0	0	34
aurA	27	35	0	2	0	0	0	0	64
aurA'	76	14	0	0	0	0	0	0	90
bub3	20	48	0	0	0	0	0	0	68
bubRI	40	0	0	0	0	0	0	0	40
cnn	14	22	0	2	0	3	0	0	41
feo	43	47	0	4	0	0	3	0	97
mad1	6	2	0	0	0	0	0	0	8
mad2	22	0	0	0	0	0	0	0	22
mis12	0	1	2	0	0	0	0	3	6
mitch	8	17	0	7	8	4	0	0	44
nuf2	58	0	0	0	0	0	0	0	58
pavi	0	0	0	0	0	0	0	2	2
sAK	8	38	0	0	0	0	0	0	46
sas-4	130	6	0	0	0	0	0	0	136
scrib	2	18	0	1	1	0	0	0	22
sept$_1$	26	12	0	0	0	0	0	0	38
sept$_2$	48	25	0	1	0	0	0	0	74
smc1	14	4	0	0	0	0	0	0	18
smc5	32	12	0	0	0	0	0	0	44
UAS-AurA^2	52	36	0	0	1	1	0	0	90
UAS-AurA^3	20	9	0	0	0	0	5	0	34
UAS-Polo	28	30	0	0	0	0	0	0	58

Table 2. Frequency of phenotypes shown in female eyes in the absence of scrib^{RNAi}.

RNAi/O.e.	WT	S-eye	M-eye	R-eye	D-eye	T-eye	O-eye	N-eye	N
anillin	123	19	1	16	6	0	0	0	165
asp	8	30	0	0	0	0	0	0	38
aurA	91	11	0	2	0	0	0	0	104
aurA'	100	0	0	0	0	0	0	0	100
bub3	14	58	0	1	0	0	0	0	73
bubRI	56	2	0	0	0	0	0	0	58
cnn	59	17	0	0	0	0	0	0	76
feo	98	34	0	1	0	8	3	0	144
mad1	12	4	0	0	0	0	0	0	16
mad2	30	0	0	0	0	0	0	0	30
mis12	2	9	8	1	3	0	0	3	26
mitch	33	18	4	3	7	2	0	1	68
nuf2	52	8	0	0	0	0	0	0	60
pavi	0	0	0	1	1	0	0	0	2
sAK	8	28	0	0	0	0	0	0	36
sas-4	146	0	0	0	0	0	0	0	146
scrib	38	123	0	2	5	1	0	0	169
sept$_1$	50	14	0	0	0	0	0	0	64
sept$_2$	52	0	0	0	0	0	0	0	52
smc1	18	16	0	1	1	0	0	0	36
smc5	48	32	0	0	0	2	0	0	82
UAS-AurA^2	58	44	0	0	0	0	0	0	102
UAS-AurA^3	39	27	0	0	0	0	0	0	66
UAS-Polo	80	6	0	0	0	0	0	0	86

Table 3. Frequency of phenotypes shown in male eyes expressing scrib^{RNAi}.

RNAi/O.e.	WT	S-eye	M-eye	R-eye	D-eye	T-eye	O-eye	N-eye	N
anillin	2	4	0	0	2	8	1	1	18
asp	0	28	0	0	0	0	0	0	28
aurA	1	6	0	2	0	18	7	0	34
aurA'	10	12	0	1	0	6	15	0	44
bub3	0	28	0	5	5	13	3	0	54
bubRI	5	37	0	0	2	4	0	0	48
cnn	0	14	0	0	0	0	0	0	14
feo	0	17	0	4	0	21	10	0	52
mad1	6	2	0	0	0	0	0	0	8
mad2	5	17	0	6	2	0	0	0	30
mis12	0	2	0	0	0	0	0	0	2
mitch	5	0	2	0	2	1	0	0	10
nuf2	4	14	0	0	0	2	0	0	20
pavi	0	0	0	0	0	1	1	2	4
sak	0	32	0	1	0	5	0	0	38
sas-4	6	0	0	0	0	0	0	0	6
sept1	2	36	0	0	0	0	0	0	38
sept2	16	6	0	0	3	11	0	0	36
smc1	0	0	0	0	0	0	0	0	0
smc5	14	5	0	6	0	11	0	0	36
UAS-AurA2	5	13	2	4	1	16	1	0	42
UAS-AurA3	0	3	0	1	0	5	5	0	14
UAS-Polo	18	47	0	0	1	0	4	0	70

Table 4. Frequency of phenotypes shown in female eyes expressing scrib^{RNAi}.

RNAi/O.e.	WT	S-eye	M-eye	R-eye	D-eye	T-eye	O-eye	N-eye	N
anillin	20	23	0	6	5	18	1	1	74
asp	0	20	0	0	0	2	0	0	22
aurA	5	10	0	10	0	16	12	0	53
aurA'	17	40	0	2	0	2	5	0	66
bub3	5	20	3	6	1	5	0	0	40
bubRI	34	35	0	0	8	1	0	0	78
cnn	0	11	0	1	0	0	0	0	12
feo	8	30	0	11	0	0	7	0	56
mad1	11	27	0	1	0	1	0	0	40
mad2	7	27	0	0	0	0	0	0	34
mis12	3	4	2	1	0	0	1	1	12
mitch	11	6	0	4	4	2	0	1	28
nuf2	23	17	0	1	0	1	0	0	42
pavi	0	9	3	5	5	12	1	3	38
sak	0	49	0	4	0	5	0	0	58
sas-4	11	1	0	4	4	4	0	0	24
sept1	4	12	0	0	0	0	0	0	16
sept2	6	10	1	1	1	7	2	0	28
smc1	0	2	0	0	0	5	1	0	8
smc5	25	34	0	2	0	0	0	0	61
UAS-AurA2	23	32	0	0	5	4	1	0	65
UAS-AurA3	9	13	0	3	0	1	2	0	28
UAS-Polo	26	35	0	4	0	5	0	0	70

Table 5. Relative percentage of the frequency of phenotypes shown in male eyes expressing scrib^{RNAi}.

RNAi/O.e%	WT	S-eye	M-eye	R-eye	D-eye	T-eye	O-eye	N-eye
anillin	11,1	22,2	0	0	11,	44,4	5,6	5,6
asp	0	100	0	0	0	0	0	0
aurA	2,9	17,6	0	5,9	0	52,9	20,6	0
aurA'	22,7	27,3	0	2,3	0	13,6	34,1	0
bub3	0	51,9	0	9,3	9,3	24,1	5,6	0
bubRI	10,4	77,1	0	0	4,2	8,3	0	0
cnn	0	100	0	0	0	0	0	0
feo	0	32,7	0	7,7	0	40,4	19,2	0
mad1	75	25	0	0	0	0	0	0
mad2	16,7	56,7	0	20	6,7	0	0	0
mis12	0	100	0	0	0	0	0	0
mitch	50	0	20	0	20	10	0	0
nuf2	20	70	0	0	0	10	0	0
pavi	0	0	0	0	0	25	25	50
sak	0	84,2	0	2,6	0	13,2	0	0
sas-4	100	0	0	0	0	0	0	0
sept1	5,3	94,7	0	0	0	0	0	0
sept2	44,4	16,7	0	0	8,3	30,6	0	0
smc1	0	0	0	0	0	0	0	0
smc5	38,9	13,9	0	16,7	0	30,6	0	0
UAS-AurA2	11,9	31,0	4,8	9,5	2,4	38,1	2,4	0
UAS-AurA3	0	21,4	0	7,1	0	35,7	35,7	0
UAS-Polo	25,7	67,5	0	0	1,4	0	5,7	0

Table 6. Relative percentage of the frequency of phenotypes shown in female eyes expressing scrib^{RNAi}.

RNAi/O.e%	WT	S-eye	M-eye	R-eye	D-eye	T-eye	O-eye	N-eye
anillin	27,0	31,1	0	8,1	6,8	24,3	1,4	1,4
asp	0	90,9	0	0	0	9,1	0	0
aurA	9,4	18,9	0	18,9	0	30,2	22,6	0
aurA'	25,8	60,6	0	3,0	0	3,0	7,6	0
bub3	12,5	50	7,5	15	2,5	12,5	0	0
bubRI	43,6	44,9	0	0	10,3	1,3	0	0
cnn	0	91,7	0	8,3	0	0	0	0
feo	14,3	53,6	0	19,6	0	0	12,5	0
mad1	27,5	67,5	0	2,5	0	2,5	0	0
mad2	20,6	79,4	0	0	0	0	0	0
mis12	25	33,3	16,7	8,3	0	0	8,3	8,3
mitch	39,3	21,4	0	14,3	14,3	7,1	0	3,6
nuf2	7,1	9,5	4,8	2,4	0	0	2,4	2,4
pavi	0	23,7	7,9	13,2	13,2	31,6	2,6	7,9
sak	0	84,5	0	6,9	0	8,6	0	0
sas-4	45,8	4,2	0	16,7	16,7	16,7	0	0
sept1	25	75	0	0	0	0	0	0
sept2	21,4	35,7	3,6	3,6	3,6	25	7,1	0
smc1	0	25	0	0	0	62,5	12,5	0
smc5	41,0	55,7	0	3,3	0	0	0	0
UAS-AurA2	35,4	49,2	0	0	7,7	6,2	1,5	0
UAS-AurA3	32,1	46,4	0	10,7	0	3,6	7,1	0
UAS-Polo	37,1	50	0	5,7	0	7,1	0	0

Table 7. Relative percentage of the frequency of phenotypes shown in male eyes in the absence of scrib^{RNAi}.

RNAi/O.e%	WT	S-eye	M-eye	R-eye	D-eye	T-eye	O-eye	N-eye
anillin	43,0	29,0	3,2	17,2	7,5	0	0	0
asp	11,8	88,2	0	0	0	0	0	0
aurA	42,2	54,7	0	3,1	0	0	0	0
aurA'	84,4	15,6	0	0	0	0	0	0
bub3	29,4	70,6	0	0	0	0	0	0
bubRI	100	0	0	0	0	0	0	0
cnn	34,1	53,7	0	4,9	0	7,3	0	0
feo	44,3	48,4	0	4,1	0	0	3,1	0
mad1	75	25	0	0	0	0	0	0
mad2	100	0	0	0	0	0	0	0
mis12	0	16,7	33,3	0	0	0	0	50
mitch	18,2	38,6	0	15,9	18,2	9,1	0	0
nuf2	100	0	0	0	0	0	0	0
pavi	0	0	0	0	0	0	0	100
sak	17,4	82,6	0	0	0	0	0	0
sas-4	95,6	4,4	0	0	0	0	0	0
sept1	68,4	31,6	0	0	0	0	0	0
sept2	64,9	33,8	0	1,4	0	0	0	0
smc1	77,8	22,2	0	0	0	0	0	0
smc5	72,7	27,3	0	0	0	0	0	0
UAS-AurA2	57,8	40	0	0	1,1	1,1	0	0
UAS-AurA3	58,8	26,5	0	0	0	0	14,7	0
UAS-Polo	48,3	51,7	0	0	0	0	0	0

Table 8. Relative percentage of the frequency of phenotypes shown in female eyes in the absence of scrib^{RNAi}.

RNAi/O.e%	WT	S-eye	M-eye	R-eye	D-eye	T-eye	O-eye	N-eye
anillin	74,5	11,5	0,6	9,7	3,6	0	0	0
asp	21,1	78,9	0	0	0	0	0	0
aurA	87,5	10,6	0	1,9	0	0	0	0
aurA'	100	0	0	0	0	0	0	0
bub3	19,2	79,4	0	1,4	0	0	0	0
bubRI	96,6	3,4	0	0	0	0	0	0
cnn	77,6	22,4	0	0	0	0	0	0
feo	68,1	23,6	0	0,7	0	5,6	2,1	0
mad1	75	25	0	0	0	0	0	0
mad2	100	0	0	0	0	0	0	0
mis12	7,7	34,6	30,8	3,8	11,5	0	0	11,5
mitch	48,5	26,4	5,9	4,4	10,3	2,9	0	1,4
nuf2	86,7	13,3	0	0	0	0	0	0
pavi	100	0	0	0	0	0	0	0
sak	22,2	77,8	0	0	0	0	0	0
sas-4	100	0	0	0	0	0	0	0
sept1	78,1	21,9	0	0	0	0	0	0
sept2	100	0	0	0	0	0	0	0
smc1	50	44,4	0	2,8	2,8	0	0	0
smc5	58,5	39,0	0	0	0	2,4	0	0
UAS-AurA2	56,9	43,1	0	0	0	0	0	0
UAS-AurA3	59,1	40,9	0	0	0	0	0	0
UAS-Polo	93,0	7,0	0	0	0	0	0	0

Table 9. Frequency of phenotypes shown in male eyes.

RNAi	WT	S-eye	M-eye	R-eye	D-eye	T-eye	O-eye	N-eye	N
anillin	15	4	3	14	0	2	0	4	42
sas-4	22	20	0	0	0	0	0	0	42
anillin+aas-4	11	9	8	80	18	7	3	0	136
scrib+anillin	4	0	4	12	2	7	2	0	31
scrib+sas-4	12	30	0	0	2	1	3	0	48
scrib+anillin+sas-4	29	19	3	23	18	20	7	2	121

Table 10. Frequency of phenotypes shown in female eyes

RNAi	WT	S-eye	M-eye	R-eye	D-eye	T-eye	O-eye	N-eye	N
anillin	47	7	5	10	3	4	1	0	77
sas-4	39	9	0	0	0	0	0	0	48
anillin+aas-4	14	7	2	106	16	3	7	1	156
scrib+anillin	20	6	1	18	3	9	4	1	62
scrib+sas-4	22	19	0	1	0	2	2	0	46
scrib+anillin+sas-4	14	12	6	55	26	23	16	0	152

Table 11. Relative percentage of the frequency of phenotypes shown in male eyes

RNAi	WT	S-eye	M-eye	R-eye	D-eye	T-eye	O-eye	N-eye	N
anillin	35,7	9,5	7,1	33,3	0	4,8	0	9,5	100
sas-4	52,4	47,6	0	0	0	0	0	0	100
anillin+aas-4	8,1	6,6	5,9	58,8	13,2	5,1	2,2	0	100
scrib+anillin	12,9	0	12,9	38,7	6,5	22,6	6,5	0	100
scrib+sas-4	25	62,5	0	0	4,2	2,1	6,25	0	100
scrib+anillin+sas-4	24,0	15,7	2,5	19,0	14,9	16,5	5,8	1,7	100

Table 12. Relative percentage of the frequency of phenotypes shown in female eyes

RNAi	WT	S-eye	M-eye	R-eye	D-eye	T-eye	O-eye	N-eye	N
anillin	61,0	9,1	6,5	13,0	3,9	5,2	1,3	0	100
sas-4	81,3	18,7	0	0	0	0	0	0	100
anillin+aas-4	9,0	4,5	1,3	68,0	10,3	1,9	4,5	0,6	100
scrib+anillin	32,3	9,7	1,6	29,0	4,8	14,5	6,5	1,6	100
scrib+sas-4	47,8	41,3	0	2,2	0	4,3	4,3	0	100
scrib+anillin+sas-4	9,2	7,9	3,9	36,2	17,1	15,1	10,5	0	100

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