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# Regulation of Abscission in Female *Drosophila* Germ Cells

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**THESE DE DOCTORAT DE L'UNIVERSITE PARIS SUD**

Ecole Doctorale Gènes Génomes Cellules

Présentée par

**Mlle Neuza Reis MATIAS**

Pour obtenir le grade de

**DOCTEUR de l'UNIVERSITE PARIS SUD**

**Regulation of Abscission in Female Drosophila Germ Cells**

dirigée par Dr Jean-René HUYNH

à l'Institut Curie, U934/UMR3215, 26 rue d'Ulm, 75005, Paris, France

soutenue le 22 Septembre 2015, devant le jury composé de:

Pr Laurent THÉODORE.....Président

Dr Barry THOMPSON.....Rapporteur

Dr Arnaud ECHARD.....Rapporteur

Dr Stéphane RONSSERAY.....Examineur

Dr Roland LE BORGNE.....Examineur

Dr Jean-René HUYNH.....Directeur de these

Dr Juliette MATHIEU.....Co-directeur de these

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# TABLE OF CONTENTS

ABSTRACT .....	6
INTRODUCTION .....	7
CHAPTER I – GERMLINE CYSTS .....	8
1- Germline cysts formation.....	12
2- Mice pre-adult cysts.....	14
3- <i>Drosophila</i> female germline cysts.....	18
4- The fusome.....	21
CHAPTER II - GERMLINE STEM CELL MAINTENANCE AND CYSTOBLAST DIFFERENTIATION	28
1- Germline Stem Cell maintenance .....	33
2- Cystoblast differentiation .....	40
3- Regulation of cyst divisions.....	45
CHAPTER III - AURORA B AND CYCLIN B CONTROL THE TIMING OF GSC ABSCISSION .....	48
CHAPTER IV - ABSCISSION: THE LAST STEP OF CYTOKINESIS .....	54
1- Cleavage plane specification.....	56
2- Assembly and constriction of the actomyosin ring.....	57
3- Abscission.....	58
4- Abscission timing regulation .....	65
CHAPTER V - OTHER FUNCTIONS OF THE ESCRT PROTEINS.....	71
1- ESCRT in multivesicular bodies biogenesis .....	71
2- ESCRT in signaling regulation .....	75
3- ESCRT and Lethal (2) giant discs (Lgd).....	80
4- ESCRT in plasma membrane fission .....	84

5- ESCRT in surveillance of nuclear pore complexes formation .....	84
6- ESCRT in nuclear envelope sealing.....	85
INTRODUCTION TO MY PhD PROJECT .....	88
RESULTS.....	91
ARTICLE.....	92
SUPPORTING INFORMATION .....	93
SUPPLEMENTARY RESULTS .....	97
1- Transheterozygous <i>shrb<sup>G5</sup>/lgd<sup>d7</sup></i> rescued 32-cell cyst phenotype due to abscission in late cysts.....	97
2- Lgd function in the mitotic cysts seems to be cell autonomous .....	99
3- Is Lgd function in GSC abscission regulated by Cdk1 phosphorylation?.....	100
DISCUSSION .....	106
1- Shrb promotes GSC abscission.....	107
2- Shrb localizes at the midbody .....	111
3- The Chromosomal Passenger Complex may inhibit Shrb during GSC abscission .....	114
4- GSC abscission is different in females and males .....	115
a) Cell autonomous regulation.....	115
b) Non-autonomous regulation .....	118
5- Lethal (2) giant discs (Lgd) promotes GSC abscission and localizes at the midbody.....	119
6- How does lgd loss of function induce ectopic abscission in the cysts.....	121
a) Lgd has opposite functions in the GSC and in the cysts.....	121
b) Lgd downregulation induce abscission through cell fate modulation.....	122
7- Lgd regulates the activity of the BMP/Dpp signalling pathway during <i>Drosophila</i> oogenesis (Morawa, 2015).....	127
8- How is abscission blocked in the dividing cysts? .....	129
ANNEXES.....	131

ANNEX 1: AURORA B IN POSITION EFFECT VARIATION.....	132
INTRODUCTION .....	132
RESULTS AND DISCUSSION.....	133
ANNEX 2: AURORA B IN BORDER CELL MIGRATION .....	142
INTRODUCTION .....	142
RESULTS AND DISCUSSION.....	145
ANNEX 3: REVIEW (Molla-Herman, 2014).....	155
REFERENCES .....	156

## TABLE OF FIGURES

Figure 1: Germline cysts .....	9
Figure 2: Somatic cysts .....	11
Figure 3: Male germline stem cell lineage .....	13
Figure 4: Mice pre-adult cysts .....	16
Figure 5: TEX14 regulates mouse germ cells abscission .....	17
Figure 6: Drosophila early oogenesis .....	19
Figure 7: Drosophila ovarian meiotic ring canal formation .....	20
Figure 8: Divisions of female <i>Dytiscus</i> germline cyst .....	22
Figure 9: Formation of the fusome .....	23
Figure 10: The fusome.....	26
Figure 11: Extrinsic and intrinsic factors work concertedly to control germline stem cell (GSC) self-renewal and cystoblast (CB) differentiation .....	28
Figure 12: The fate of germline cells depends on the regulation of BMP signaling .....	30
Figure 13: BMP signaling pathway .....	32
Figure 14: Function of Cap Cells .....	34
Figure 15: Escort cell cytoplasmic protrusions.....	35
Figure 16: Rab11 is enriched on the fusome of GSCs and germline cysts .....	36
Figure 17: Mutants for nos, pum and pelo result in GSC loss.....	37
Figure 18: Distribution of GSCs displaying specific fusome morphologies relative to cell cycle phases.....	39
Figure 19: Dynamics of Escort cells cytoplasmic processes .....	41
Figure 20: Escort cells control cysts differentiation through regulation of BMP signaling .....	42
Figure 21: Fu acts in concert with Smurf to regulate the ubiquitination of Tkv in the CB .....	43
Figure 22: Expression patterns of key regulators in germaria .....	44
Figure 23: Bam controls the number of cyst divisions in males .....	46
Figure 24: The level of cyclins control number of cyst divisions in female.....	47
Figure 25: Chromosomal passenger complex localization and function during mitosis .....	49
Figure 26: The CPC and CycB/Cdk1 control the timing of germline abscission .....	52
Figure 27: Schematic model of the interactions between the CPC and CycB/Cdk1 during early and late cytokinesis .....	53

Figure 28: Overview of Animal Cell Cytokinesis.....	55
Figure 29: Central spindle formation and signaling .....	57
Figure 30: Maturation of the Intercellular bridge.....	60
Figure 31: Assembly of ESCRT-III filaments.....	61
Figure 32: Localization of ESCRT proteins during abscission .....	63
Figure 33: Vps4 structure and localization during abscission.....	64
Figure 34: ESCRT-III assembly is regulated in time by forces exerted on the bridge .....	66
Figure 35: CHMP4C localization during abscission .....	68
Figure 36: A model for the ANCHR-mediated regulation of VPS4 on abscission checkpoint activation (NoCut) .....	70
Figure 37: Composition and molecular interactions of the ESCRT machinery .....	73
Figure 38: Ultrastructural characterization of ESCRT-mutants.....	74
Figure 39: EGFR accumulation and EGFR signaling is overactivated in ESCRT mutants .....	76
Figure 40: Role of Hrs in Dpp signalling and Tkv trafficking in follicle cells .....	77
Figure 41: Notch accumulates in endosomes of ESCRT mutant cells leading to overactivation of signaling .....	79
Figure 42: Model of activation of Notch in lgd cells .....	83
Figure 43: ESCRT-III is transiently recruited around chromatin discs during nuclear envelope reformation .....	87

## ABSTRACT

At the end of cytokinesis, a thin cytoplasmic intercellular bridge is cleaved to allow physical separation of the two daughter cells. This process is called abscission, and its cellular and molecular events have been extensively explored in yeast and isolated mammalian cells. However, how abscission is regulated in different cell types or in a developing organism remains poorly understood.

*Drosophila* oogenesis is a great model to study how abscission is regulated developmentally, as within the same developmental unit, the germarium, we find cells undergoing abscission next to others where this process is blocked. Indeed, the germline stem cell (GSC) divides asymmetrically to give rise to another GSC and to an individualized cystoblast. This cell then enters a well-studied process of differentiation, where through four rounds of mitosis with incomplete cytokinesis, gives rise to a cyst of 16 interconnected cells. The duration of abscission, seems to be tightly regulated and dependent on the developmental context. Our lab has recently discovered that AurB and CycB/Cdk1 function as abscission timers in *Drosophila* GSC and isolated mammalian cells. Thus, my work consisted in exploring how this process is regulated in the *Drosophila* female germline.

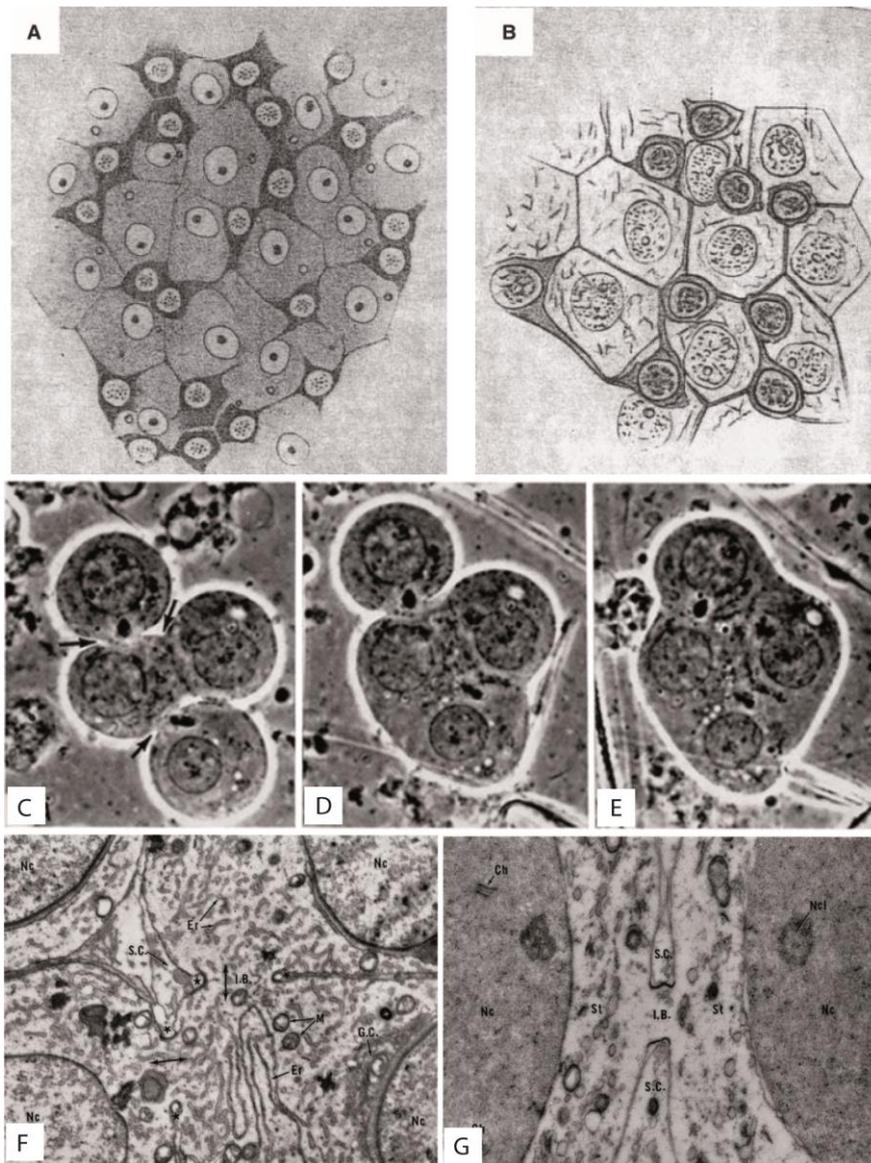
We showed that the ESCRT-III protein Shrb localizes to the midbody of the dividing GSC, functioning to promote abscission. Indeed, we found that reduced levels of Shrb resulted in the blockage, or strong delay, of abscission in the GSC and formation of a structure similar to a cyst. In these so called stem-cysts, the GSC keeps dividing while interconnected to its daughter cells. As a consequence, we saw the appearance of egg chambers formed of 32 cells, instead of 16. Furthermore, Shrb function in abscission seems to be counteracted by AurB, as reducing AurB levels in Shrb heterozygous resulted in decreased stem-cysts and 32-cell cysts. Finally, Lethal giant discs (Lgd), required for Shrb function in the endosomal pathway, was also seen localizing at the midbody and regulating abscission in GSCs. Removing one copy of Lgd from Shrb heterozygous increased the number of stem-cysts, but surprisingly the number of 32-cell cysts was reduced. This paradoxical result was explained with the observation of late abscission events in mitotic cysts, which divided the 32-cell cysts in the middle, leading to the formation of two cysts of 16 cells.

# **INTRODUCTION**

## CHAPTER I – GERMLINE CYSTS

Among all types of intercellular communication, formation of cysts is probably the most efficient process to allow cell coordination during development. The cells in a cyst are connected, forming a syncytium, and are able to share several molecules and organelles. Such organization was first observed in 1865 by La Valette St. George, when male germ cells appeared to be connected in a chain. This observation was confirmed in 1877 by Sertoli and later in 1888 by von Ebner with the description of mammalian spermatogonia connected by processes (Fig1A-B). However, it was only in 1955 that the nature of cyst intercellular connections was described (Burgos and Fawcett, 1955). Electron microscopy allowed the observation of cytoplasmic channels connecting neighboring cat spermatids. These intercellular bridges were then found connecting male germ cells of several other species: *drosophila*, opossum, pigeon, rat, hamster, guinea pig, rabbit, monkey, and human (Fawcett et al., 1959) (Fig1C-G).

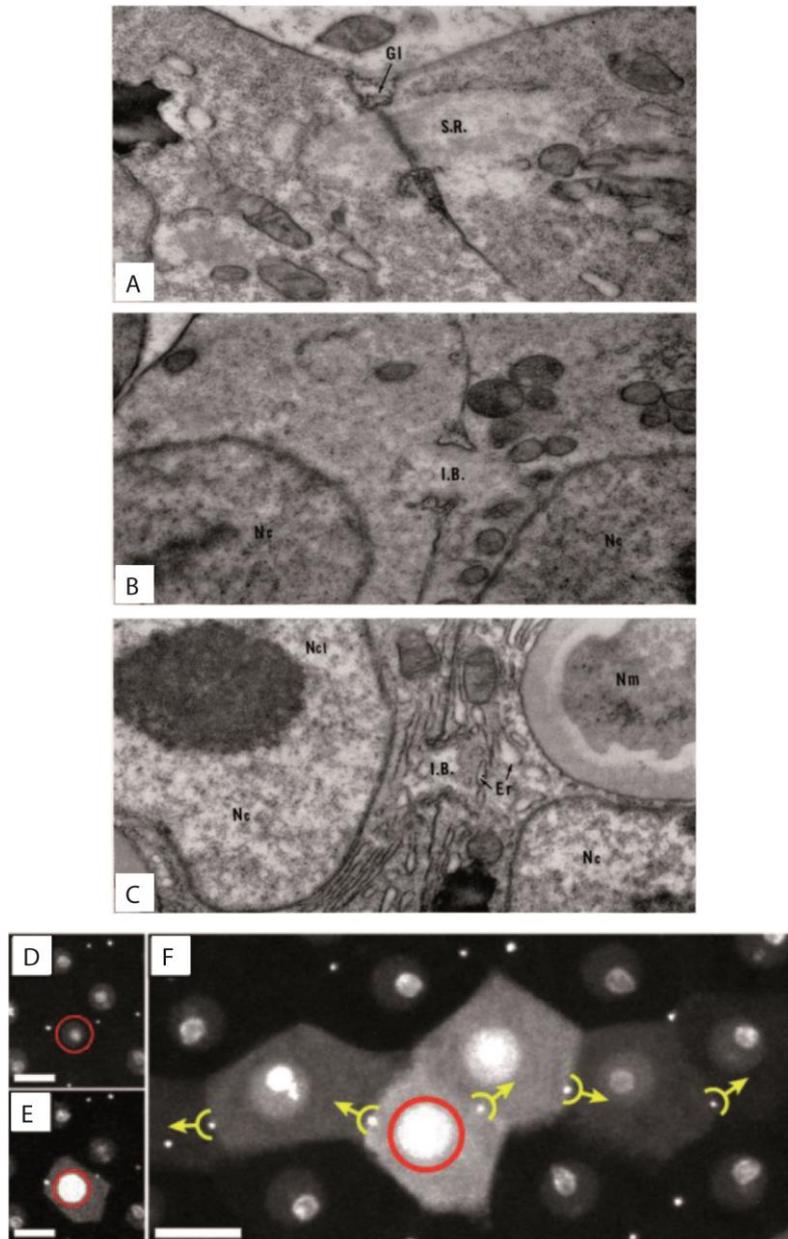
Although cysts were also found in adult females of higher insects, including *Drosophila*, adult female vertebrates do not have interconnected germline cells. However, cysts were very recently observed to be formed in the pre-adult gonad in both male and female mice (Lei and Spradling, 2013).



**Figure 1: Germline cysts (A-B)** Sketches of mammalian intercellular bridges. Observations by Sertoli (1877) **(A)** and von Ebner (1888) **(B)** show darker staining spermatogonia connected by intercellular bridges. The lightly staining cells are Sertoli cells (Greenbaum et al., 2011). **(C-E)** Phase contrast photomicrographs of a group of rat spermatids illustrating the progressive changes after isolation from the germinal epithelium. X 1000. **(C)** Four spermatids are joined by intercellular bridges at the sites indicated by arrows. **(D)** A few minutes later, the constrictions between three of the cells have disappeared and the bridge connecting them with the fourth is distinctly wider. **(E)** All four have coalesced to form a quadrinucleate mass (Fawcett, 1959). **(F)** Portions of four guinea pig spermatids. The bridges (I.B.) connecting three of them are included in this field (arrows). The endoplasmic reticulum (Er) of one cell is in communication with that of the adjacent cell through the bridges (Fawcett et al., 1959). **(G)** Two human primary spermatocytes (St) joined by a bridge (I.B.). The cells are recognizable as primary spermatocytes by their size and by the presence in the nucleus (Nc) of chromosomal cores (Ch) that are found only in prophase of meiosis. Notice the processes of the Sertoli cell (S.C.) extending deep into the cleft between the two cells (Fawcett et al., 1959).

Several hypotheses have been already proposed to explain the importance of germline cysts for fertility. 1) Intercellular communication would allow the coordination into the entry in meiosis of all cells in the cyst simultaneously. 2) Gene products could be shared by meiotic haploid cells in the cyst, which would be maintained in a “diploid phenotypic state”. This would be particularly important in males, as half of the spermatids in a cyst inherit the Y-chromosome and do not possess crucial X-linked genes. An example is the *Akap4* gene expressed from the X-chromosome, important for sperm motility and fertility. It was shown that its mRNA was able to pass through the intercellular bridges and even the protein dose among all the cells in the cyst (Morales et al., 2002). 3) Heterogeneity, which could originate from stochastic gene expression during germline development, would be eliminated through cytoplasmic share and give rise to relatively uniform gametes (Guo and Zheng, 2004). 4) Healthy cells from the cyst would be able to detect newly arising defects of their sisters and promote cyst elimination through apoptosis (LeGrand, 2001).

Even though cyst formation is usually thought to be a characteristic unique to the germline, few examples of somatic cysts have been reported. In *Hydra*, interstitial cells in the ectoderm divide to give rise to cysts of interconnected cnidoblasts, which differentiate into poisoned nematocysts (Fawcett et al., 1959) (Fig2A-C). Somatic tissues in *Drosophila*, including ovarian follicle cells, larval imaginal discs and pupal legs, were also reported to contain canals connecting neighboring cells (Giorgi, 1978; Kramerova and Kramerov, 1999). It was recently shown that the epithelial follicular cells of the fly ovary diffuse rapidly many cytoplasmic proteins within the cyst (Fig2D-F). The authors proposed that equilibration of protein levels could be a way of counteracting any possible transcription variations between the cells in the cyst (McLean and Cooley, 2014).

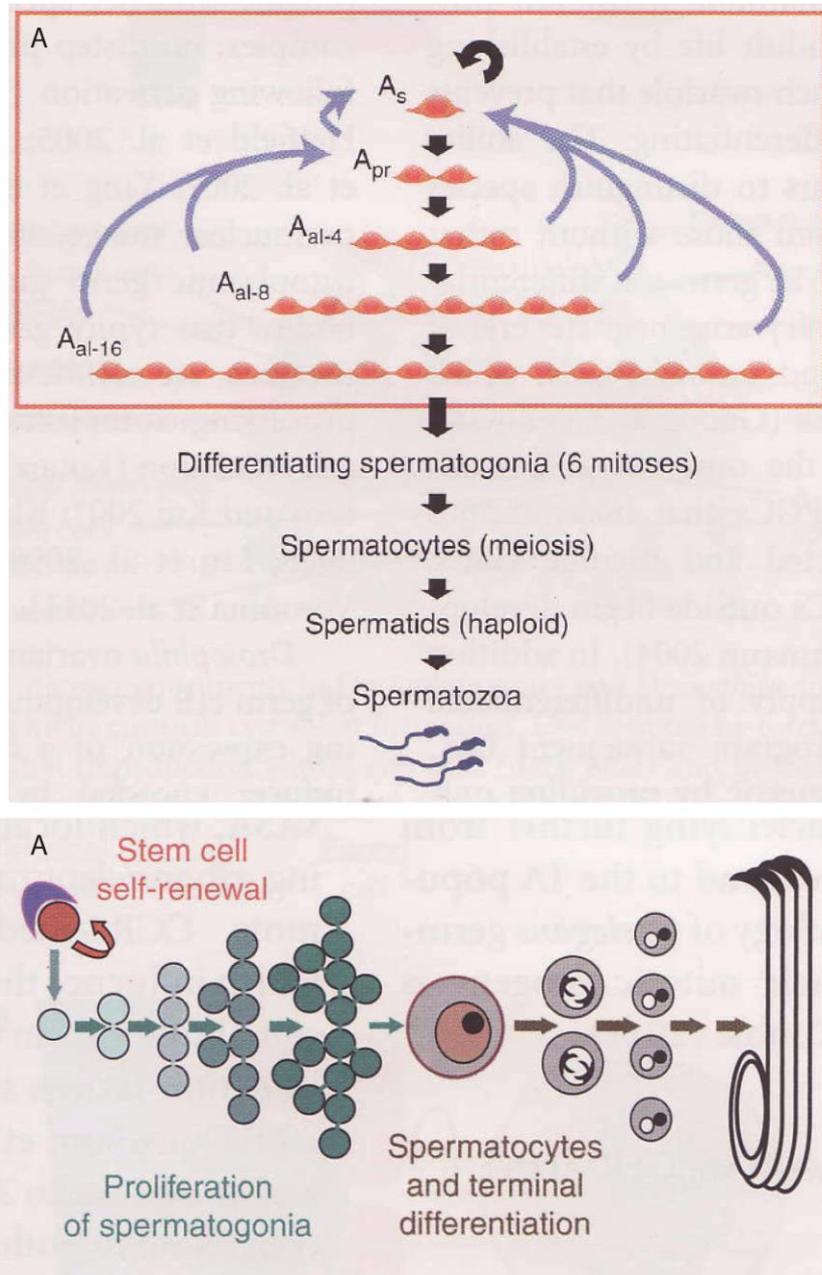


**Figure 2: Somatic cysts (A-C)** Formation of the bridge between early cnidoblasts of Hydra. **(A)** The spindle filaments (S.R.) have been largely resorbed or have dissolved in specimen preparation. In their place is a light area devoid of granules. The dense band across the middle of the bridge persists. The surrounding membrane bends outward opposite this band to form a ridge that encircles the bridge at its midpoint. **(B)** A later stage in the evolution of a bridge (I.B.). All traces of spindle filaments have disappeared and the interior of the bridge is filled with cytoplasm only slightly less dense than that of the adjacent cell bodies. **(C)** Two cnidoblasts at an advanced stage of differentiation still joined by a bridge containing elements of the endoplasmic reticulum (Er) that probably pass from one cell to the other (Fawcett, 1959). **(D-F)** Follicular cells expressing GFP::Pav (Pavarotti marks the bridge) and Tub>mC3PAGFP (photo-activable) were individually photoactivated (red circle) and images were collected pre-activation **(D)**, post-activation **(E)**, and 18 minutes after activation **(F)**. PAGFP moved only into cells connected by RCs. Yellow markers, arrows indicate direction of movement (McLean, 2013).

## 1- Germline cysts formation

The presence of intercellular bridges connecting the cells of the germline cysts is conserved from insects to humans, more than a billion years of evolution. These are structures formed due to programmed incomplete process of cell division. Cytokinesis, the last step of cell division that leads to the physical separation of the two daughter cells, is blocked during furrow ingression and stable channels between the daughter cells are formed. Because communication between cells is maintained, the cell cycle factors are coordinated in each cell and divisions occur in a synchronous fashion. Thus, the number of cells per cyst, all derived from a single founder, is a power two ( $2^n$ ), with n being the number of divisions. The characteristic number of cells per cyst (2, 4, 8, 16..), synchrony of cell divisions and the observation of connecting cytoplasmic bridges between the cells, allowed cyst identification across several species and stages of development.

Cysts organization varies among species. In male adult mice the cells in the cyst are disposed linear, being each cell connected to in maximum two other cells in the cyst (Fig3A). This disposition is thought to facilitate the communication between cells and synchronize their development. In male *Drosophila* testis, and the same is observed in ovaries, the cysts adopt a branched organization, with the cells being connected up to 4 other cells (Fig3B). For the cells in these cysts to be synchronous, a specialized organelle called fusome needs to be present (discussed later).



**Figure 3: Male germline stem cell lineage (A)** Male germline stem cell lineage in the mouse. In mice the prevailing model (black arrows) assumes that the single isolated spermatogonia ( $A_s$ ) is the predominant stem cell during steady-state spermatogenesis. However, of all the cells shown in the box, the  $A_s$ ,  $A_{pr}$ , and  $A_{al}$  spermatogonia, have stem cell potential. At the 16 cell-cyst stage, the  $A_{al-16}$ , transforms without cell division into differentiating spermatogonia, which subsequently undergo six rounds of mitosis before entering meiosis. **(B)** Male germline stem cell lineage in *Drosophila*. A stem cell (red) at the tip of the testis divides assymetrically, producing a stem cell and a gonialblast, which initiates four rounds of synchronous spermatogonial mitotic TA (trans-amplification) divisions with incomplete cytokinesis. The resulting 16 interconnecting cells undergo premeiotic DNA replication in synchrony and switch to spermatocyte program of cell growth, meiotic prophase, and transcription of terminal differentiation genes. (all 16 cells become oocyte, but only one is shown for simplicity) (Fuller, 2011).

## **2- Mice pre-adult cysts**

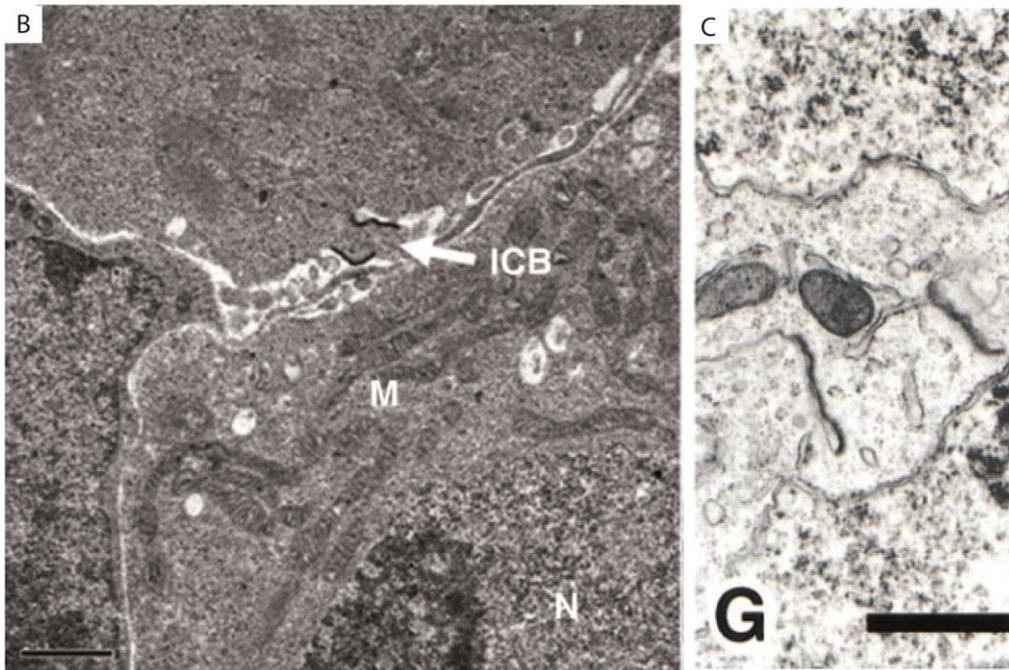
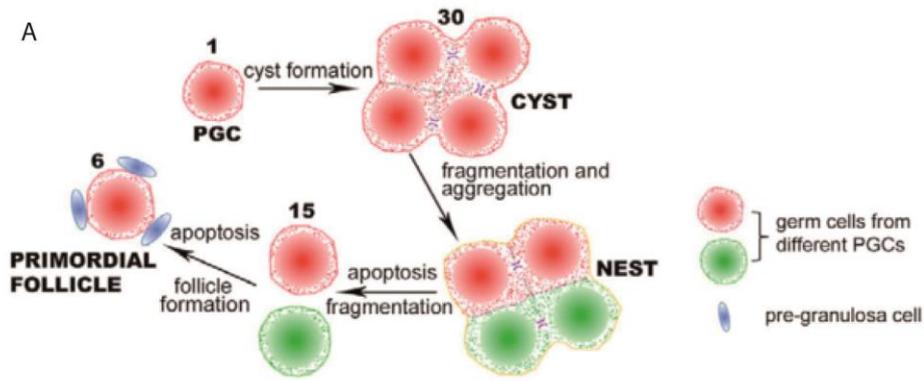
Germline cyst formation is thought to happen before meiosis entry. Thus, because meiosis occurs throughout adult life in vertebrate males and higher insects, these have germline cysts in their adult gonads. However, in vertebrate females, which start meiosis as embryos, cysts were observed to be formed only in the pre-adult gonad (Pepling and Spradling, 2001) (Fig4B).

A recent study in mouse germline followed the development of the newly arrived primordial germ cells, by lineage tracing (Lei and Spradling, 2013). To mark single germ cells, the authors utilized low-level Tamoxifen (Tmx) to mediated induction of Cre recombinase activity to activate heritable R26R-YFP expression in just a few isolated, random cells within all tissues. With these experiments, they showed that once female primordial germ cells (PGCs) colonize the pre-adult gonads, they divide with incomplete cytokinesis forming cysts of 2, 4 or 8 interconnected cells. These early cysts however fragment through the breakage of one or more intercellular bridges, which complete cytokinesis. The fragmented cysts then aggregate with neighboring cysts forming nests. Cells initially part of the same cyst keep their coordination and start meiosis synchronously, independently of other groups of interconnected cells in the same nest. The cysts continue to fragment and by the time of birth are reduced to single cells. Massive germline apoptosis then leads to primordial follicle formation (Fig3A). In males the initial process of gametogenesis is very similar, with the formation of cysts in pre-adult gonads. These cysts also go through fragmentation and aggregation into nests, but meiosis is not initiated. The arrested germ cells continue to fragment, without apoptosis, and form the single cell population of stem cells, found shortly after birth. In the adult male mouse, germline stem cells divide giving rise to cysts of interconnected cells.

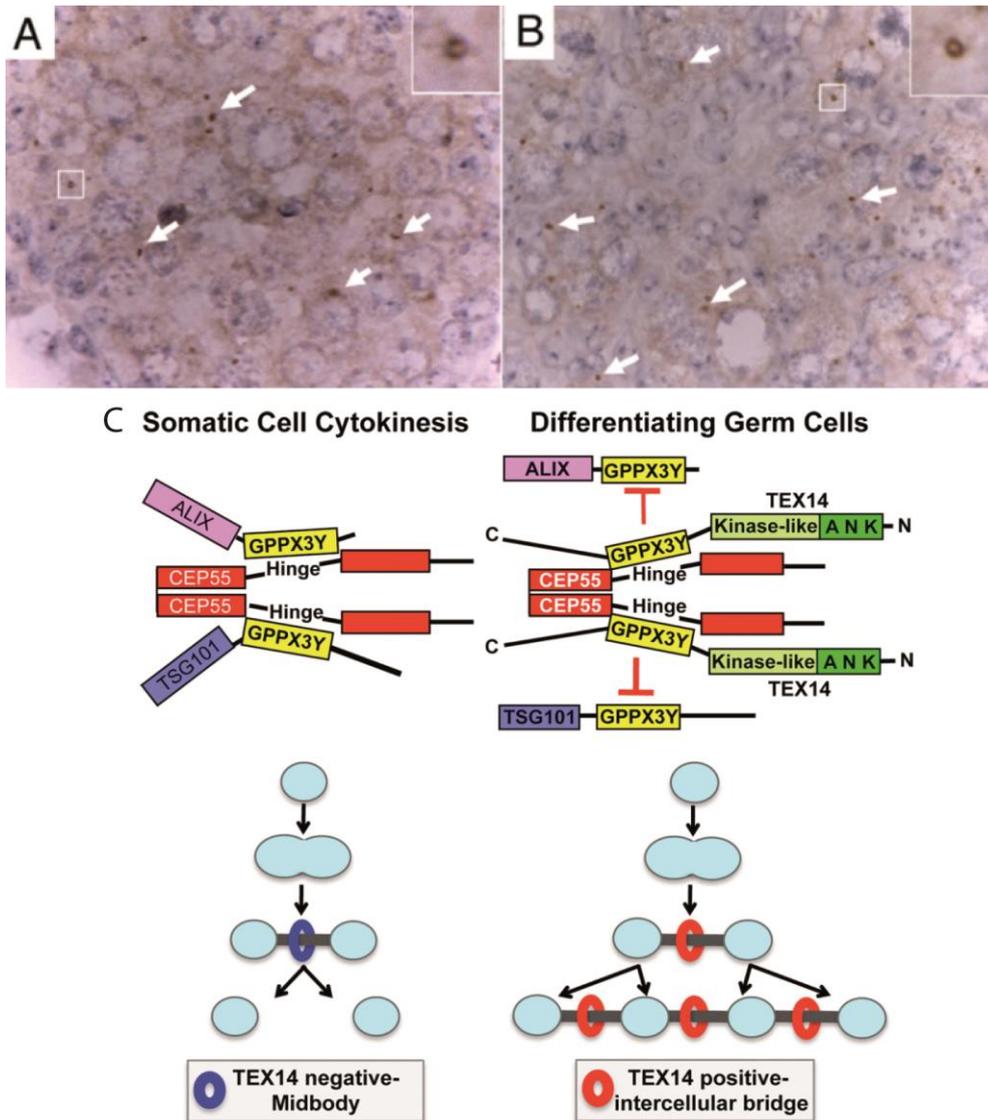
The fetal germ cells are in contact with somatic cells. This contact gets straighter at the time of cyst fragmentation. Electron microscopy showed that somatic processes are closely associated with the intercellular bridges of the germ cells. This suggests that the somatic cells may contribute for the breakage of the cyst (Pepling and Spradling, 2001).

Another interesting observation, looking at electron microscopy images, was the great reorganization of mitochondria and ER membranes prior to cyst fragmentation (Fig4C). Mitochondria were seen within the cytoplasmic bridges, suggesting active transport of these organelles throughout the cyst cells (Pepling and Spradling, 2001). This process could ensure that individualized oocytes, selected to form primordial follicles, acquire functional mitochondria, while defective mitochondria would trigger the apoptotic program of sister cells.

Very surprisingly, it was observed that cysts formation in pre-adult stage is not necessary for fertility in mice females (Greenbaum et al., 2006). Tex14 localizes early to the site of furrow ingression during cyst cells divisions, and stabilizes the intercellular bridge by interaction with components of the bridge, inhibiting the final step of membrane fission (Fig5) (discussed later) (Greenbaum et al., 2009; Greenbaum et al., 2006; Iwamori et al., 2010). Mutants for this protein fail to maintain the bridges and cysts are no longer formed. Although females null for tex14 can still form few oocytes, males are sterile with incomplete meiosis.



**Figure 4: Mice pre-adult cysts (A)** Model of female mouse PGC development. Each PGC (red) initially forms a cyst, which fragments into smaller cysts and aggregates with cysts from different PGCs (green) to form a nest. Further fragmentation and apoptosis leads to primordial follicle formation. The average number of germ cells derived from an initial PGC at each step of this process is indicated (Lei, 2013). **(B)** Two oocytes in a mouse germ cell cyst connected by an intercellular bridge (ICB). M, mitochondria; N, nucleus (Greenbaum et al., 2009). **(C)** Mitochondrion and ER-like membranes within a ring canal in mouse female pre-adult cyst (Pepling and Spradling, 2001).



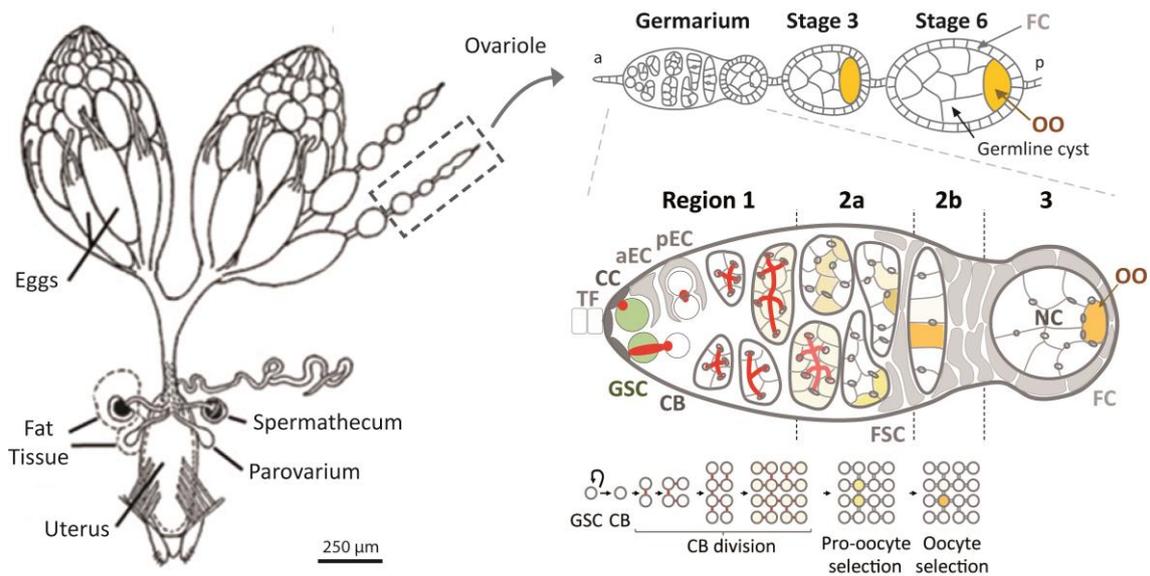
**Figure 5: TEX14 regulates mouse germ cells abscission (A-B)** TEX14 localizes to intercellular bridges in embryonic and newborn gonocytes and oogonia. TEX14 is immunohistochemically stained with diaminobenzidine, and nuclei are counterstained with hematoxylin. TEX14 localizes to the intercellular bridge in E14.5 testis (**A**) and E14.5 ovary (**B**) (Greenbourn, 2009). (**C**) Models for cytokinesis and intercellular bridge formation. (Left) Model of somatic cell abscission. CEP55 is essential in the recruitment of additional proteins (TSG101 and ALIX) that are required for abscission of the midbody. The regions of TSG101 and ALIX containing GPPX3Y motifs interact with the hinge region of CEP55 to complete cytokinesis. (Right) Model of the intercellular bridge in differentiating germ cells. The conserved GPPX3Y motif of TEX14 interacts strongly with the hinge region of CEP55 in differentiating germ cells to block CEP55 interactions with TSG101 and ALIX, resulting in formation of a stable intercellular bridge (Iwamori et al., 2010) (This will be discussed later).

### 3- *Drosophila* female germline cysts

The process of germline cyst formation has been extensively studied in *Drosophila*. The *Drosophila* female has a pair of ovaries, each composed of 16-20 ovarioles. These are chains of progressively maturing egg chambers, which are produced throughout adult life time (FIG). The continue process of gametogenesis is maintained by the divisions of the germline stem cells (GSCs), localized in a specialized anterior structure called germarium (Figure 1A). The germarium is organized into 4 morphological regions. The region 1 is the mitotic region of the germarium, where the cystoblast (CB), originated from the GSC division, divides 4 times giving rise to a cyst of 16 inter-connected cells ( $2^4$ ). Then, in region 2a, the cyst cells enter meiosis synchronously and by region. In most organisms, germ cells first amplify their number through mitosis and only then start meiosis. However, in female *Drosophila* it appears that meiosis “starts” during mitosis (Christophorou et al., 2013). In this study, the authors observed mitotic cells express components of the synaptonemal complex and start clustering their chromosomes, characteristics only observed in meiotic cells. In region 2b meiosis gets restricted to one cell, which is selected to be the oocyte. In region 3 (or stage 1), somatic follicular cells form an epithelial monolayer surrounding the cyst, which adopts a round conformation, and the oocyte repolarizes at the posterior side on the cyst. Then the cyst buds out from the germarium and proceeds to vitellogenesis. The oocyte is fed by the other 15 sister nurse cells and grows throughout 14 different stages of development, until it forms the egg (Fig6).

In female *Drosophila*, the cellular bridges connecting each cell in the cyst are called ring canals (RCs). The number of RCs of each cyst cell directly correlates with the number of division that the cell went through. Thus, the first two cells to be formed will each have four RCs, their two daughter cells will have three, the next four cells will have two, and the final eight cells have only one RC. Ring canals are maintained throughout oogenesis, however their constitution changes during development (Fig7). After the four mitotic divisions, the RCs mature and get further stabilized by the actin bundling protein Kelch (Kelso et al., 2002). Females mutant for *kelch* are sterile, as the transport through the RC is impaired due to a

disorganization of actin filaments (Robinson et al., 1994). Cytoplasmic transfer of the nurse cells to the oocyte does not happen and the oocyte does not grow.



**Figure 6: *Drosophila* early oogenesis.** The female *Drosophila* has two ovaries, which are composed of several ovarioles (left, (Miller, 1950)). Each ovariole (top right) is made of a chain of progressively more mature egg chambers toward the posterior (p) (anterior (a)). An egg chamber comprises 16 germline cells (cyst) surrounded by a monolayer of follicle cells (FC). In region 1 of the germarium (bottom right), germline stem cells (GSCs) divide asymmetrically giving rise to another GSC and to a cystoblast (CB). The CB goes through 4 rounds of mitosis, with incomplete cytokinesis, forming a cyst of 16 cells. These are interconnected by the fusome (red) that passes through their ring canals. In region 2a only two cells of the cyst become pro-oocytes (light yellow) and, by region 2b, only one is selected as the oocyte (OO, yellow). The other 15 cells become polyploid nurse cells (NC). In region 3 (or stage 1), the follicular stem cells (FSC) divide to give rise to follicular cells (FC). The cyst becomes spherical and buds out from the germarium. Somatic cells from the germarium: Terminal filament cells (TFCs), cap cells (CC), anterior escort cells (aEC), posterior escort cells (pEC).

### Ovarian meiotic ring canal formation

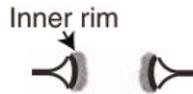
Arrested cleavage furrow



Outer rim:  
Contractile ring actin  
Anillin  
PY protein  
*cheerio* or *hts* mutation



Partly assembled ring canal



Outer rim:  
Anillin  
PY protein

Inner rim:  
PY protein  
Actin  
HTS



Completely assembled ring canal



Outer rim:  
PY protein

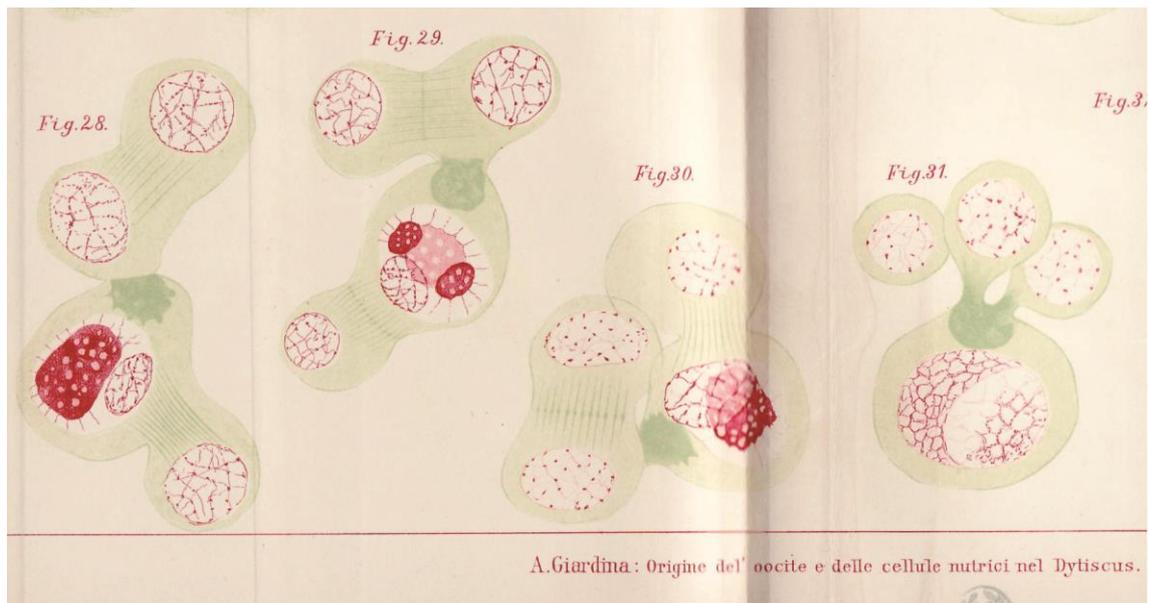
Inner rim:  
PY protein  
Actin  
HTS  
KELCH

**Figure 7: *Drosophila* ovarian meiotic ring canal formation.** Female *Drosophila* intercellular bridge formation begins with an increase in phosphotyrosine epitopes (PY). Cleavage furrows form an inner ring with Hts and Kelch and lose anillin (Greenbaum et al., 2011).

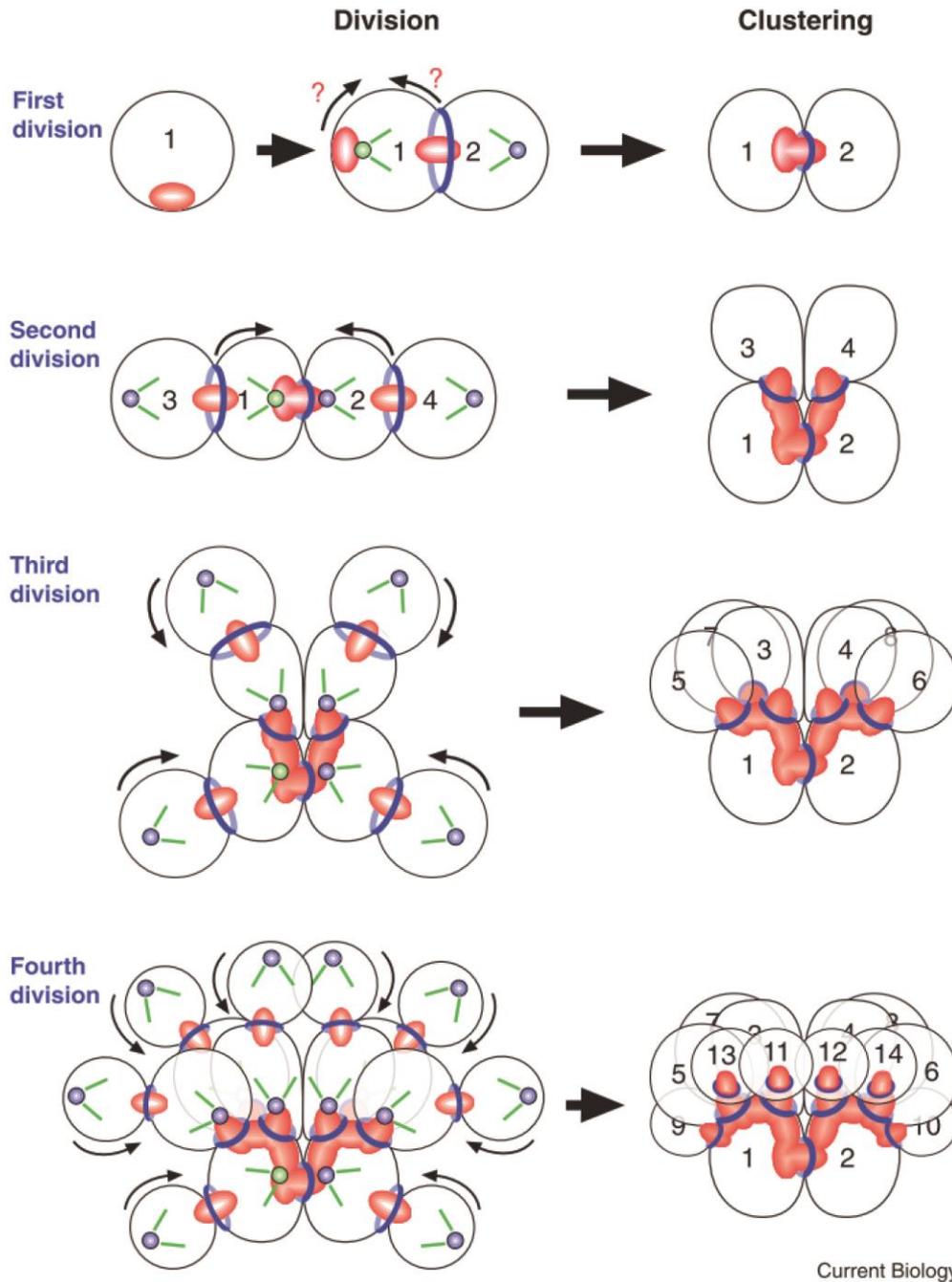
#### 4- The fusome

A very important feature of *Drosophila* cysts is the presence of a large intracellular organelle, called the fusome (Huynh, 2005). This is mainly composed by ER (endoplasmic reticulum) vesicles that localize to the intercellular bridges, linking all cells through their ring canals. This structure, first recognized by Telfer in 1975, was already part of Giardina drawings of female germline cyst of the diving beetle *Dytiscus*, dating 1901 (Fig8). Several studies focused on understanding the function and organization of this organelle, as it seems to be crucial for oogenesis.

The fusome appears during the first division of the CB, but it arises from a similar structure present in the GSC called spectrosome. The GSC localizes to the most anterior tip of the germarium and is in straight contact with somatic cells (cap cells, CC) responsible to maintain its stemness. The spectrosome is localized in the anterior side of the GSC that contacts the CC, and during mitosis it anchors the spindle pole, orienting the division along the antero-posterior axis. The renewed GSC stays at the anterior, while the CB is produced towards the posterior. At the end of telophase, a new fusome plug is formed at the transient ring canal. The spectrosome elongates and fuses with the plug. When cytokinesis is completed, one third of it is inherited by the CB. Similar to the GSC division, during the 4 divisions of the cyst, one pole of each mitotic spindle is anchored to the fusome. A new fusome plug forms at the stabilized ring canal and the first plug fuses with the new one (Fig9).



**Figure 8: Divisions of female *Dytiscus* germline cyst.** The divisions are asymmetric and the future oocyte inherits more fusome (dark green) and more ribosomal DNA (dark red). Its size is also bigger, comparing with the other cells from the cyst (Giardina, 1901).



**Figure 9: Formation of the fusome.** During the first incomplete division, the spectrosome (red) of the cystoblast interacts with one of the centrosomes (green and blue spheres) to anchor one pole of the mitotic spindle (green lines). A fusome plug (red) forms in the arrested furrow or ring canal (blue). The spectrosome and the fusome plug come together to fuse. The same mechanism is repeated for the second, third and fourth division: first, one pole of each mitotic spindle is anchored by the fusome and a new fusome plug forms in each ring canal. Then the ring canals move centripetally for the fusome plugs to fuse with the central fusome (black curved arrows) (Huynh and St Johnston, 2004).

Unlike mammalian germline cysts that form linear clusters, in *Drosophila*, the anchorage of one pole of each mitotic spindle to the fusome gives rise to a “maximal branched” pattern of interconnections. Another characteristic of *Drosophila* cysts is the creation of rosette-like structures. This happens because, at the end of each division, the new fusome plugs and the ring canals move centripetally to fuse with the central fusome. These positions are then stabilized by the formation of adherent junctions around the ring canals. This shape of the cyst could be a way of improving inter-cell communication, by minimizing the distances between the cells.

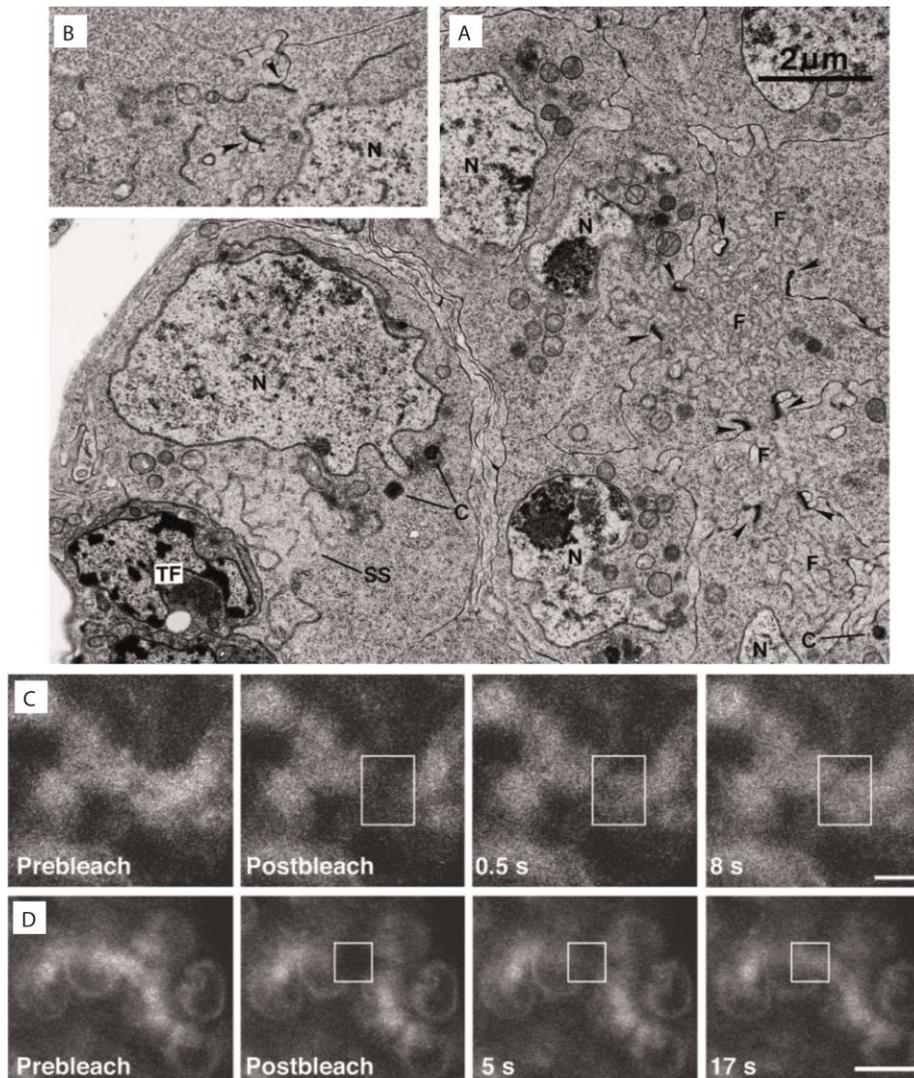
Interestingly, it seems that this polarized patterning is important for the specification of the oocyte, as it always arises from one of the oldest cells, the central cells with more fusome (de Cuevas and Spradling, 1998; Huynh and St Johnston, 2004).

Work done by the group of Mary Lilly explored how the fusomal endomembranes were organized (Snapp et al., 2004) (Fig10A). Confocal live cell imaging of germaria showed that the fusome was mainly formed by ER vesicles, as the ER luminal protein, Lys-GFP-KDEL, and the ER membrane protein, Sec61 $\alpha$ -GFP, labeled the branching fusomes. To understand whether the ER consisted in discrete vesicles or formed a continuous network of interconnected tubules, they proceeded to photobleaching experiments comparing the mobility of Lys-GFP-KDEL versus Sec61 $\alpha$ -GFP (Fig10C-D). If the ER was composed of several individualized vesicles, one would expect that lumen proteins would move with the same dynamic of membrane proteins. However, the lumen protein Lys-GFP-KDEL recovered faster than the membrane protein Sec61 $\alpha$ -GFP after photobleaching. It was thus concluded that ER membranes of the fusome are formed by a network of continuous tubules passing through all the cells of the cyst. The authors also showed that these tubules were connected to the cytoplasmic ER of all the cells in the cysts. These results suggest that the fusome could act as a direct channel of communication, synchronizing the development of the cyst cells. In agreement, they observed that the ER continuity between the cells was lost prior to entry in meiosis in region 2a, time point at which the cells in the cyst lose their developmental synchrony (Snapp et al., 2004).

Other important components of the fusome are the membrane associated skeletal proteins. Hu-li tao shao (Hts), the *Drosophila* homologue of adducin known to bind actin and

spectrin, was observed to localize at the fusome (Yue and Spradling, 1992). Accordingly, both  $\beta$ -spectrin and  $\alpha$ -spectrin were also enriched in the fusome (de Cuevas and Spradling, 1998; Lin et al., 1994).

In *hts* mutant cysts (*hts*<sup>1</sup>) no fusome material can be detected, by both immunofluorescence ( $\alpha$ -spectrin antibody) and by electron microscopy (Fig10B) (Lin et al., 1994). The egg chambers contain less than 16 cells, and their number often does not follow the 2<sup>n</sup> rule. This suggests that the cyst cells went through less than 4 divisions, and that these were asynchronous (Yue and Spradling, 1992). These results point to a function of the fusome in regulating the mitotic synchrony of the cells of the cyst. Accordingly, dynein trans-heterozygous mutant (*Dhc64c*<sup>3-2</sup>/*Dhc64c*<sup>6-12</sup>), which affects the structure of the fusome (fragmented and not extending through all the cells), produce egg chambers with less than 16 cells, but following the 2<sup>n</sup> rule (McGrail and Hays, 1997). This could be explained by the fact that, unlike *hts* mutants, dynein mutants maintain the ER continuity of the fusome between the cyst cells (Snapp et al., 2004), thus keeping the synchrony.



**Figure 10: The fusome.** Electron microscopy of a fusome and spectrin-rich sphere (SS, spectrosome). Thin sections of the anterior region of a germarium from a wild-type (A) or a *hts*<sup>1</sup> (B). The wild-type cysts (A) contain a large fusome (F) that extends through four ring canals (arrows) and associated with a centriole (C). The fusome can be seen to contain small membranous vesicles and to exclude most ribosomes and mitochondria. In *hts*<sup>1</sup> females, typical fusomes are not seen (B) (Lin, 1994). (C-D) Membrane and lumenal ER markers exhibit distinct diffusion properties in the fusome. FRAP of the fusome membranes revealed that Lys-GFP-KDEL (C) fluorescence recovers rapidly within the fusome, whereas Sec61α-GFP (D) recovers significantly slower (Snapp et al., 2004).

Interestingly, another skeletal protein, ankyrin, was mostly found in the stem cell spectrosome. This suggests that the molecular constitution of the spectrosome is in part different from the derived fusome, and probably developmentally regulated.

The protein TER94 (transitional endoplasmic reticulum ATPase), required for vesicles fusion during ER biogenesis, was also found localizing in the fusome (Leon and McKearin, 1999). Fusomal ER formation was suggested to be linked to cyst differentiation, as the key protein involved in this process (Bam, discussed later) directly binds TER94 (Leon and McKearin, 1999).

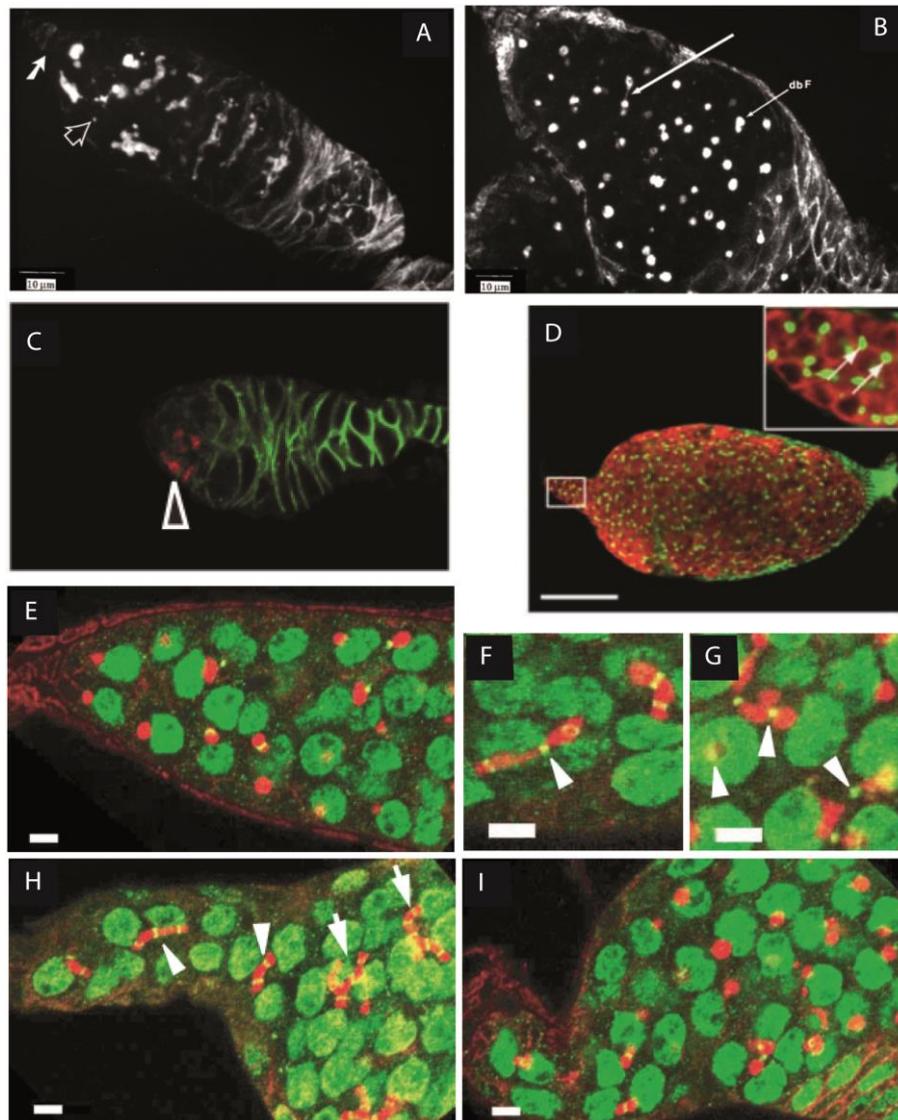
Another class of fusome components is made of microtubules-associated proteins including the plus-end binding proteins, Orbit/Mast and CLIP-190, the MT minus-end motor, dynein (referred above), and the *Drosophila* homologue of Spectraplakins, shot.

The fusome seems to be conserved between invertebrates and vertebrates. In *Xenopus laevis* an organelle with similar ultrastructure and molecular composition was observed connecting the cells of the cyst (Kloc et al., 1998). In this animal, all cells of the cyst become oocytes.



A key molecule in the process is the differentiation factor bag of marbles (Bam). Its repression in GSCs is crucial for GSC self-renewal, and its expression in cysts necessary and sufficient for differentiation (McKearin and Ohlstein, 1995). In *bam* mutant ovaries, CBs fail to differentiate and the germaria accumulates numerous single cells, resembling a tumor (Fig12B) (McKearin and Ohlstein, 1995). On the contrary, if the expression of *bam* is ectopically induced (*heat shock bam*, *hs-bam*), the GSCs differentiate and are lost from the germaria (Fig12C) (Xie and Spradling, 2000). The mechanisms that regulate *bam* expression have been extensively studied. It is well accepted that *bam* repression in the GSC is achieved by the activation of BMP signaling in these cells.

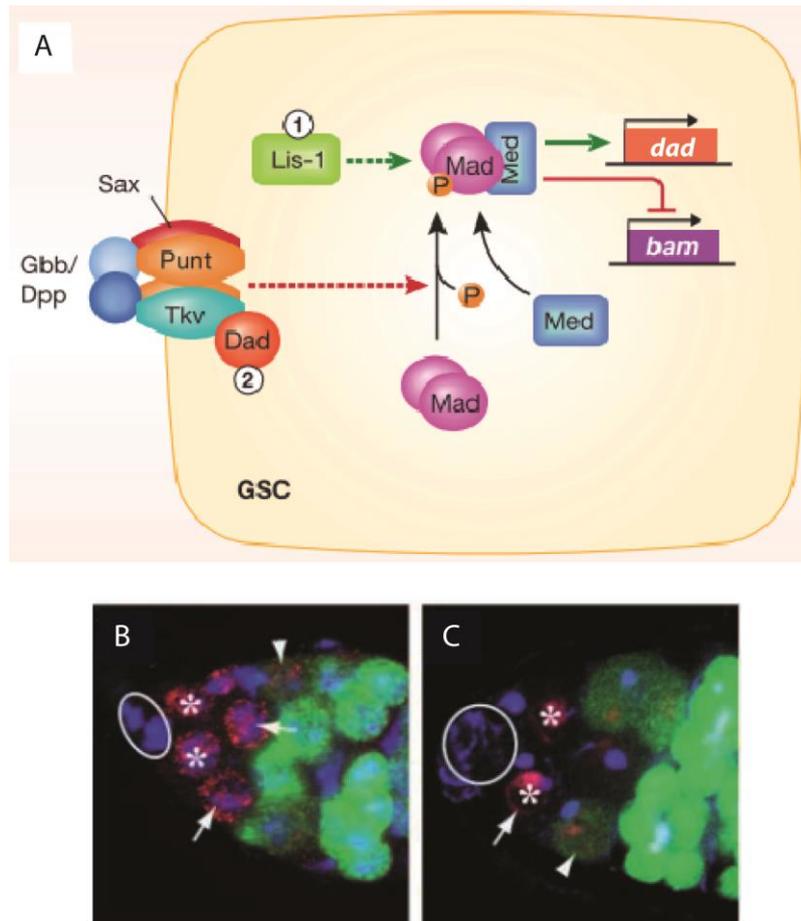
Ectopic overexpression of Dpp (Decapentaplegic), a ligand in the BMP signaling pathway, originates tumorous germaria similar to the observed in *bam* mutants (Fig12D) (Song et al., 2004). Elegant experiments showed that the *Drosophila* cysts have the capacity to fragment into single cells (cell fate reversion), similarly to what happen in pre-adult mouse germline, through the modulation of BMP signaling (Kai and Spradling, 2004). In this work, Dpp overexpression was permanently induced in flies carrying a *hs-bam*. As a consequence, tumorous germaria with mostly single cells were observed (Fig12E). When these flies were submitted to heat shock, *bam* expression was induced and the single cells started dividing as cysts with branched fusomes (Fig12F). Several hours after heat shock, Bam levels decrease, while the high levels of Dpp remain. The lack of Bam and high levels of Dpp, were sufficient to induce the fragmentation of the cysts (Fig12G-H), and 50hrs after heat shock almost all the germ cells were individualized (Fig12I). These experiments demonstrate that GSC and CB cell fate depends on the regulation of BMP signaling, which acts through Bam (Kai and Spradling, 2004).



**Figure 12: The fate of germline cells depends on the regulation of BMP signaling.** (A) Wild-type fusomes visualized by anti-Hts. Closed arrow indicates the anterior end of germarium immediately adjacent to the terminal filaments. (B) *bam86* germarium stained with anti-Hts to mark the fusome; fusomes appear as dots or dumbbell-shaped fusomes (dbF). This germarium also shows an example of a highly elongated fusome (long arrow) that can sometimes be found connecting *bam*<sup>86</sup> germ cells (McKearin, 1995). (C) Germaria from *hs-bam* females heterozygous for PZ1444 enhancer trap 72 hours after a 1-hour heat shock. Cap cells [open arrowheads] remain unchanged (Xie, 2000). (D) A *c587-gal4;UAS-dpp* germarium resulting from *dpp* overexpression in ECs is filled with single germ cells with a spectrosome. The inset shows the tip of the germarium (highlighted by a rectangle in B) at a higher magnification (4×), containing only germ cells with a spectrosome (arrows) (Song 2004). (E-I) Germaria of flies overexpressing *Dpp* (*c587-gal4;UAS-dpp*) and *hs-bam*. (E) no heat-shock, germarium full of single cells or cell pairs. (F) 20 h after heat shock showing induced 4- (arrowhead) and 8-cell (arrow) cysts with normal morphology. No single cells or cell pairs were detected (n . 100). (G) 30 h after heat shock showing a 4-cell cyst with a constricting ring canal (arrowhead). (H) 40 h after heat shock showing closed ring canals and broken fusomes (arrowheads). (I) 50 h after heat shock showing almost complete cyst breakdown (Kai and Spradling, 2004).

The BMP molecules, Dpp (Decapentaplegic) and Gbb (Glass bottom boat), are expressed by the CCs and received by the GSCs through their membrane receptors. Upon ligand binding, the BMP type II receptor Punt activates the type I receptors Sax (saxophone) and Tkv (Thickveins), which in turn phosphorylates the *Drosophila* Smad Mothers-against-Dpp (Mad; P-Mad in its phosphorylated active form). P-Mad associates with the co-Smad Medea. This complex accumulates in the nucleus and controls the expression of numerous genes (Fig13A). As P-Mad:Med has intrinsic activating properties, it is thought that, once it binds to the bam Silencer Element (SE), it recruits Shn (Schnurri) that acts as a transcriptional repressor. On the contrary, P-MAP:Med binds to Activating Elements (AE) in dad (daughters-against-dpp) regulatory region and activates its transcription. Dad is an inhibitory Smad that counteracts the BMP signaling, balancing it. Both P-Mad and Dad have been extensively used as reporters for Dpp signaling activity (Song et al., 2004). P-Mad and Dad expression are thus observed enriched in the GSC, and sporadically in the early CB (Fig13B-C).

In the opposite of what happens in the wing disc, the germline has an extremely short range of BMP signaling, harboring one single cell: the GSC (and early CB). Thus, it is thought that the restriction of the BMP signaling to the GSC is the main level of cell fate regulation. How this is accomplished is still under investigation. However, several mechanisms were already discovered.



**Figure 13: BMP signaling pathway.** (A) Intracellular regulation of the signal. (1) In GSCs (left), the Dpp signal is enhanced by stabilization of phosphorylated pMad by Lis-1, and (2) the inhibitory Smad Dad moderates the Dpp signal through competition with Mad (Harris, 2011). (B) Tip of the bam-GFP; Dad-lacZ germarium labeled for  $\beta$ -Gal (red), GFP (green) and DAPI (blue), showing that GSCs and two cystoblasts (arrows) express high Dad but no bam, and that a cystoblast (arrowhead) has low Dad and begins to express bam. (C) Tip of the bam-GFP germarium labeled for pMad (red), GFP (green), Hts (blue, fusomes), showing high pMad accumulation but no bam expression in GSCs, and low pMad but bam expression in a cystoblast (arrowhead) (Song et al., 2004).

## 1- Germline Stem Cell maintenance

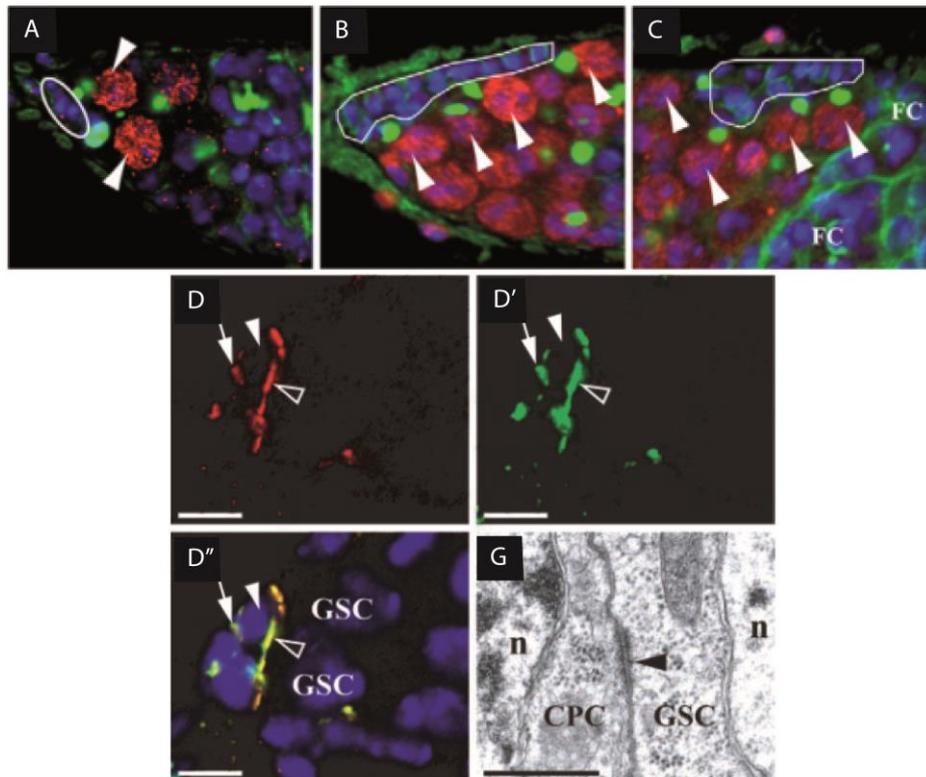
### a) Niche contribution

As referred above, the GSC niche is formed by terminal filament (TF) cells, cap cells (CCs) and anterior escort cells (aECs). Although CCs and aECs are necessary for GSC maintenance, experiments where TF cells were ablated, demonstrated that TF cells are not essential (Lin, 1993).

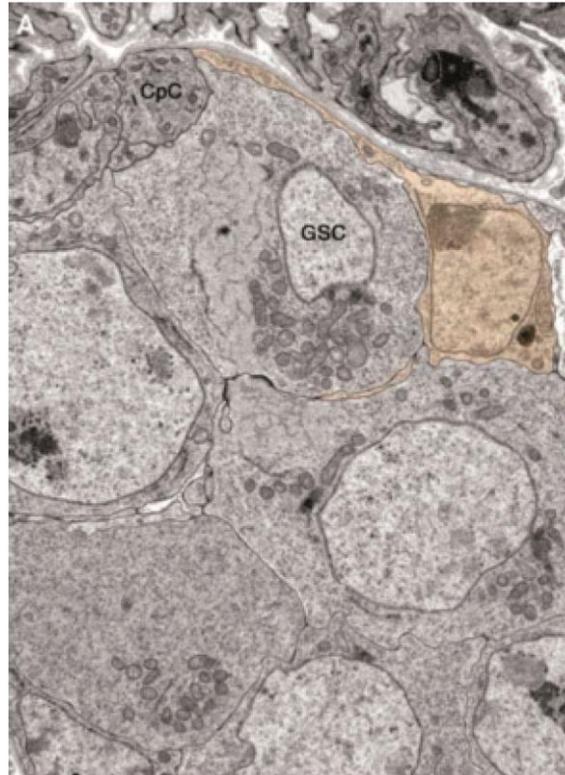
The main role of CCs is the production of the BMP signal, which will be received by the GSC and promote its self-renewal. It was shown that the glypican Dally, highly expressed in CC, is essential for GSC maintenance. Interestingly, overexpression of Dally in cells posterior to the niche (pECs) is sufficient to enhance BMP signaling and induce GSC-like expansion (Guo, 2009). Accordingly, when the CC number was increased, by expanded Notch activation (UAS- $N^{int}$ ), the number of GSC also increased. Furthermore, ectopic formation of CC was sufficient to induce high local BMP signaling and maintain GSCs (Fig14A-C) (Song et al., 2007). JAK-Stat signaling in the CC is thought to induce activation of *dpp* transcription.

CCs are also important to mediate the anchoring of the GSC to the niche. This is possible due to the accumulation of the adherent junction molecule DE-cadherin at the junctions between GSC and CC (Fig14D). DE-cadherin loss of function results in GSC loss (Song et al., 2002). Levels of DE-cadherin in the CC were shown to be regulated by insulin signaling, thought to integrate the effects of diet and age in GSC maintenance (Hsu and Drummond-Barbosa, 2009).

Anterior escort cells develop long cytoplasmic protrusions that wrap around the GSC (Fig15). These are necessary to maintain GSCs, in a mechanism dependent of JAK-Stat signaling (Decotto and Spradling, 2005).



**Figure 14: Function of Cap Cells.** (A) The tip of a *Dad-lacZ/+* germarium (control) showing that two GSCs (arrowheads) are in contact with cap cells (oval) and express high levels of *Dad-lacZ*. (B) The tip of a *c587-gal4/+; UAS-Nint/+; Dad-lacZ/+* germarium showing that an increased number of cap cells (indicated by unbroken outline) induced by the expression of an activated Notch support an increased number of GSCs (arrowheads) and cystoblasts that are positive for *Dad-lacZ*. (C) A middle portion of a *c587-gal4/+; UAS-Nint/+; Dad-lacZ/+* germarium showing that a group of ectopic cap cells (indicated by unbroken outline) close to follicle cells also support ectopic GSCs (arrowheads) that are also positive for *Dad-lacZ* (Song, 2007). (D, D', D'') The tip of a wild-type germarium labeled for DE-cadherin [(D), red], Arm [(D'), green], and nuclei [(D''), blue], respectively. The bright yellow band in (D'') indicates colocalization of DE-cadherin and Arm. (G) An electron micrograph showing the presence of adherens junctions (arrowhead) between a cap cell and a GSC (n, nuclei). CB, cystoblast; CPC, cap cell; DCs, developing cysts; FC, follicle cell; FS, fusome; GSCs, germline stem cells; IGS, inner germarial sheath cell; SSCs, somatic stem cells; TF, terminal filament cell (Song and Xie, 2002).



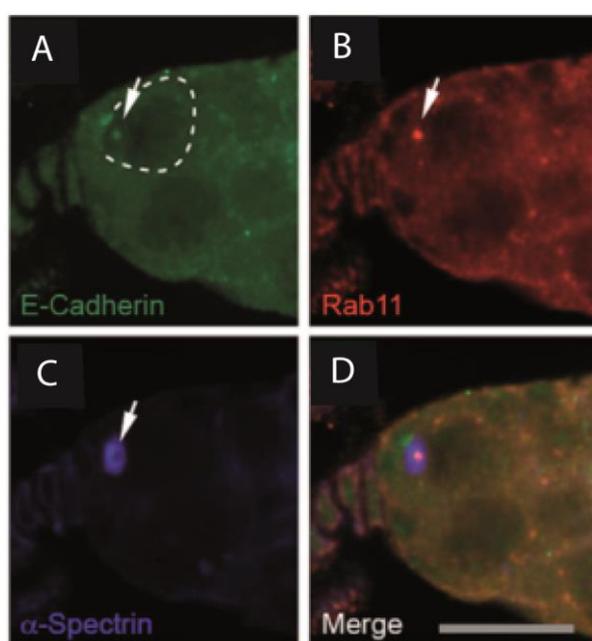
**Figure 15: Escort cell cytoplasmic protrusions.** A transmission electron micrograph showing the tip of a gerarium. An escort cell (orange pseudocolor), showing its cytoplasmic extension that wraps a GSC and contacts its cap cell (CpC) (Decotto and Spradling, 2005).

## **b) GSC cell autonomous contribution**

BMP signaling functions in the GSC to repress *bam* transcription, as referred above (Fig13A). However, low levels of Bam are observed in the GSC. Here, Bam interacts with Bgcn to repress the translation of target genes, such as DE-cadherin. Indeed, it was shown that GSCs mutant for *bam* or *bgcn* can outcompete WT GSCs due to upregulation of DE-cadherin, gaining thus more affinity to the niche (Jin et al., 2008). It is thought that GSCs have a mechanism of quality control that assures the elimination from the niche of stem cells expressing high levels of bam, and thus prone to differentiate.

To avoid the downregulation of DE-cadherin and GSC loss, eIF4A, a component of the translational initiation complex, antagonizes Bam function, by physically interacting with it (Shen et al., 2009).

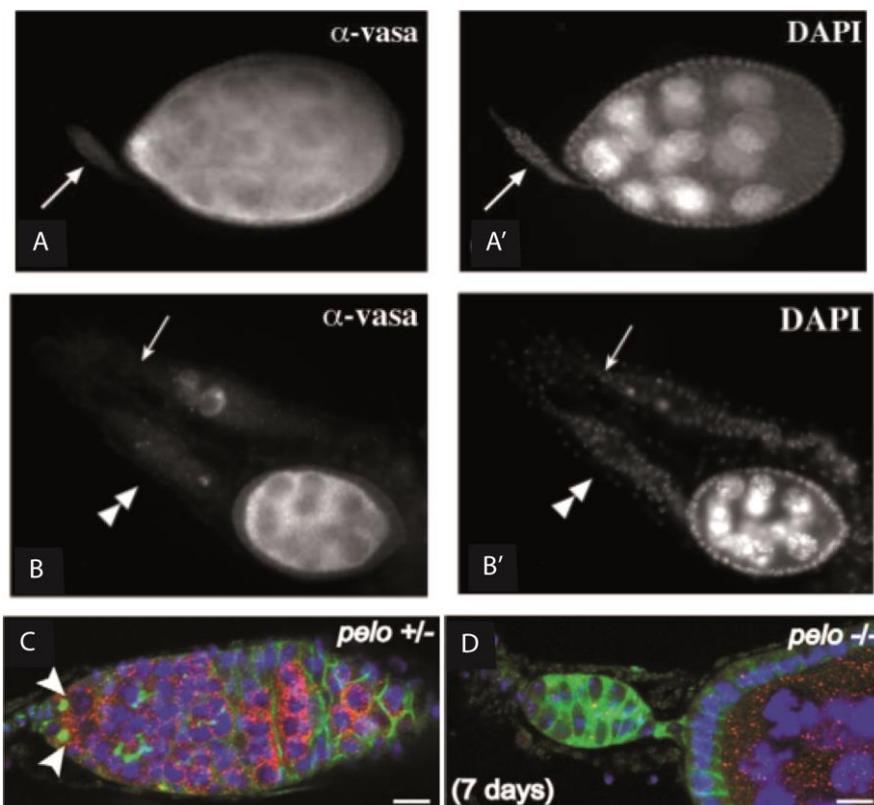
In the GSC, it was shown that DE-cadherin accumulation at the site of contact with the CCs is regulated by Rab11 coated recycling endosomes. Rab11 vesicles containing DE-cadherin are often seen associated with the spectrosome, which might promote the trafficking of these vesicles to the anterior (Bogard et al., 2007; Lighthouse et al., 2008).



**Figure 16: Rab11 is enriched on the fusome of GSCs and germline cysts.** Region 1 of a wild-type gerarium immunostained for E-cadherin (A, green), Rab11 (B, red), and the fusome marker  $\alpha$ -Spectrin (C, blue). Merged image (D). Anterior is to the left in this and all subsequent figures. A single GSC is outlined in A, with the break in the tracing (dashed line) revealing strong E-cadherin staining at the GSC-cap cell interface. The smaller dot of E-cadherin staining (arrow) superimposes with Rab11 on the fusome (arrows in B and C) (Bogard et al., 2007).

Otefin (a lamin-binding nuclear membrane protein), it was first thought to repress *bam* expression in the GSC through recruitment of the locus to the lamin repressive compartment (Jiang et al., 2008). However, it was recently shown that Otefin's function in maintaining GSC is actually independent of *bam* (Barton et al., 2013).

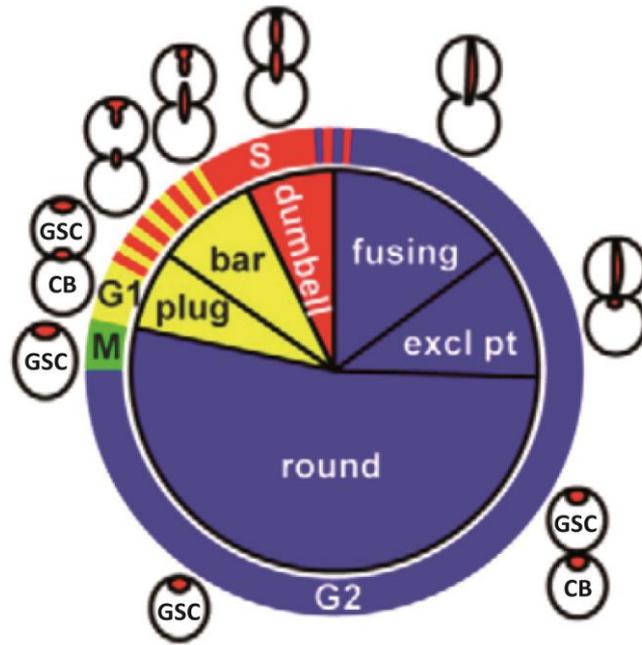
Other important players in the maintenance of GSC self-renewal, which act independently of Bam, are the complex Nanos (Nos)/Pumilio (Pum) and Pelota (Pelo) (Chen and McKearin, 2005; Szakmary et al., 2005; Xi et al., 2005). These are thought to be involved in repressing translation of gene products that are important for germ cell differentiation, and thus keep the GSC stemness. Mutants for *nos*, *pum* and *pelo* result in GSC loss (Fig17) (Forbes and Lehmann, 1998; Xi et al., 2005). A recent review by the group of Ruth Lehmann, highlights the importance of translational control in the regulation of specific GSC maintenance or differentiation-promoting factors (Slaidina and Lehmann, 2014).



**Figure 17: Mutants for *nos*, *pum* and *pelo* result in GSC loss.** (A, A') Ovariole from 5-day-old *nos* mutant female (*nos*RC/Df(3R)FX3), stained with anti-Vasa antibody to reveal the germline and DAPI to show the nuclei. A germarium depleted of germline cells (arrow) is attached to a developing stage 8 egg chamber (Forbes, 1998). (B, B') In *pum* mutant ovarioles egg chambers are frequently seen attached to empty germaria (Forbes, 1998). Ovarioles from *pelo*1 heterozygous control (C) and homozygous mutant (D). Flies labeled for Vasa (red, germ cells) and for Hts (green, spectrosomes and fusomes). (C) Germaria contain two GSCs (indicated by arrowheads) while (D) has no GSCs (Xi et al., 2005).

Cell cycle proteins were also shown to be important for GSC self-renewal. The mitotic Cyclins CycA and CycB have distinct functions in the GSC. CycB is necessary for GSC maintenance (Wang and Lin, 2005). On the contrary, degradation of CycA mediated by Ect2/APC is essential for stem cell self-renewal (Chen et al., 2009). Moreover, in this work, it was reported that the expression of a non-degradable form of CycA (but not of CycB) causes premature GSC differentiation in a Bam-independent manner (Chen et al., 2009).

GSC mitosis is followed by a short G1, an S phase and a very long G2, which is when cytokinesis between GSC and the CB is thought to be completed and the cells get physically separated (Fig18). CycE, a G1/S regulator, is atypically expressed during G2/M, and it was shown to have a role in GSC maintenance that is independent of its function in cell cycle (Ables and Drummond-Barbosa, 2013). A recent report showed that stem cells mutant for *cycE* have an impaired response to BMP signaling and are lost from the niche (Ables and Drummond-Barbosa, 2013). Diet-dependent systemic factors were shown impact on GSC proliferation and maintenance, by modulating its G2 phase. Insulin-like peptides promote GSC G2 progression via the PI3 kinase and FOXO (Hsu et al., 2008). Independently of insulin signaling, the conserved kinase TOR (Target of rapamycin) promotes GSC proliferation by modulating G2 phase (LaFever et al., 2010). This is in agreement with the observation that mutants for *TSC1* and *TSC2*, negative regulators of TOR, show loss of GSC due to precocious differentiation (Sun et al., 2010).



**Figure 18: Distribution of GSCs displaying specific fusome morphologies relative to cell cycle phases.** During M, the GSC fusome has a “round” morphology. During G1 and S phases of the next division, the GSC fusome changes its morphology to “plug”, “bar”, “dumbbell” as a new fusome structure is assembled, fused (“fusing”) to the old fusome, and partitioned between the GSC and the connected cystoblast (CB). During early G2, the GSC fusome has an “exclamation point” morphology, as the connection between the GSC and the cystoblast is severed. Later in G2, the GSC fusome becomes “round” again (Ables and Drummond-Barbosa, 2013).

## 2- Cystoblast differentiation

In the cystoblast (CB) the processes leading to differentiation repression need to be alleviated. This is mainly achieved by the blockage to of the BMP pathway and consequent *bam* expression. Different mechanisms have been described to regulate the restriction of BMP signaling to the GSC (Fig11).

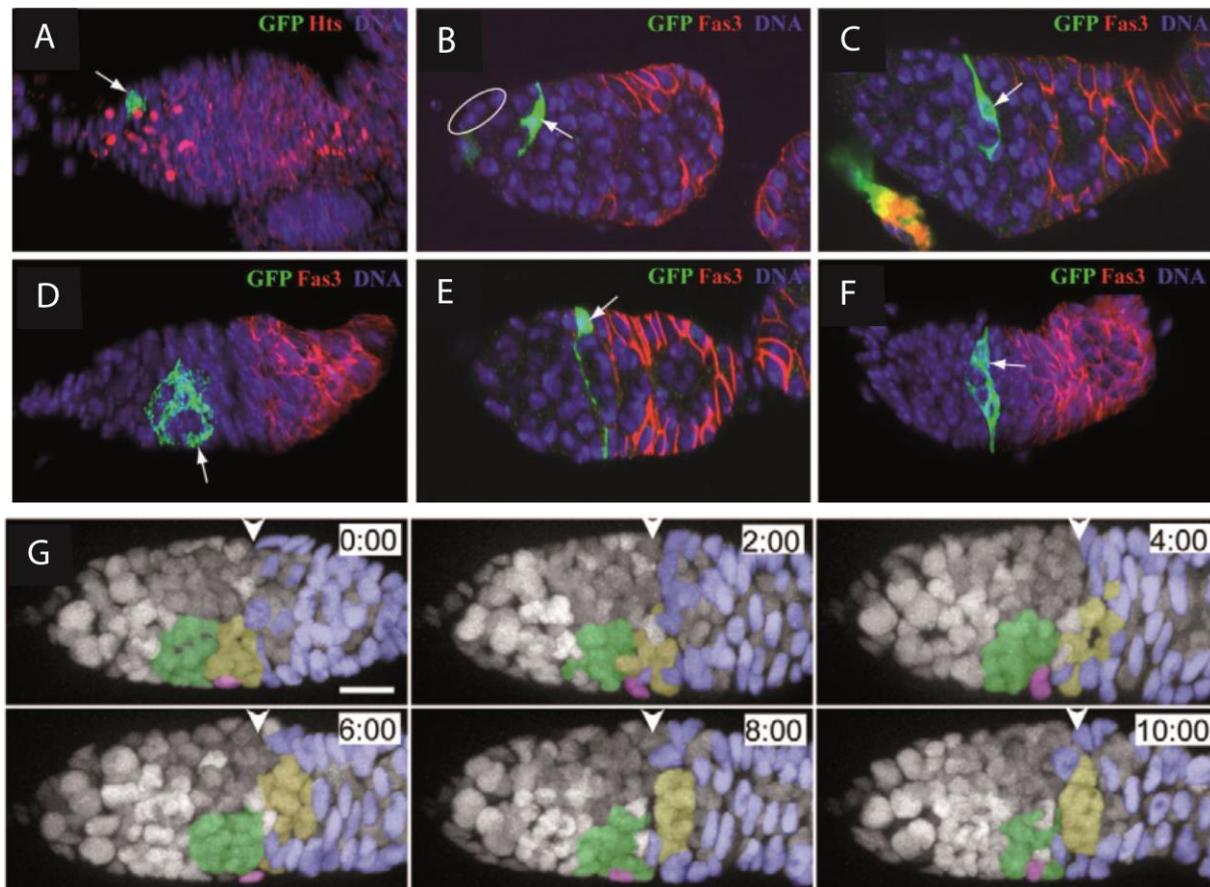
### a) Non-autonomous

Similar to aECs and GSCs, the pECs possess cytoplasmic processes wrapping the CB. These are also found in straight contact with the differentiating mitotic cysts (Fig19A-F). They divide only as needed, and remain fixed in the germarium during cyst migration (Fig19G). The cysts are transferred from one pEC to the next by their dynamic cytoplasmic processes (Kirilly et al., 2011; Morris and Spradling, 2011). Posterior escort cells (pECs) are thought to promote differentiation of the CB by restricting the BMP signal to the GSC. Indeed, it was shown that, in mutants for the small GTPase Rho, the ECs lose their processes and differentiation is impaired due to the expansion of the Dpp signal outside of the GSC niche. Rho is also thought to downregulate *dpp* expression within the pECs (Kirilly et al., 2011). The histone demethylase Lsd1 inhibits Dpp signaling in the ECs, not due to direct binding to *dpp* locus, but by regulating a diverse group of genes, including *engrailed* (Eliazer et al., 2014).

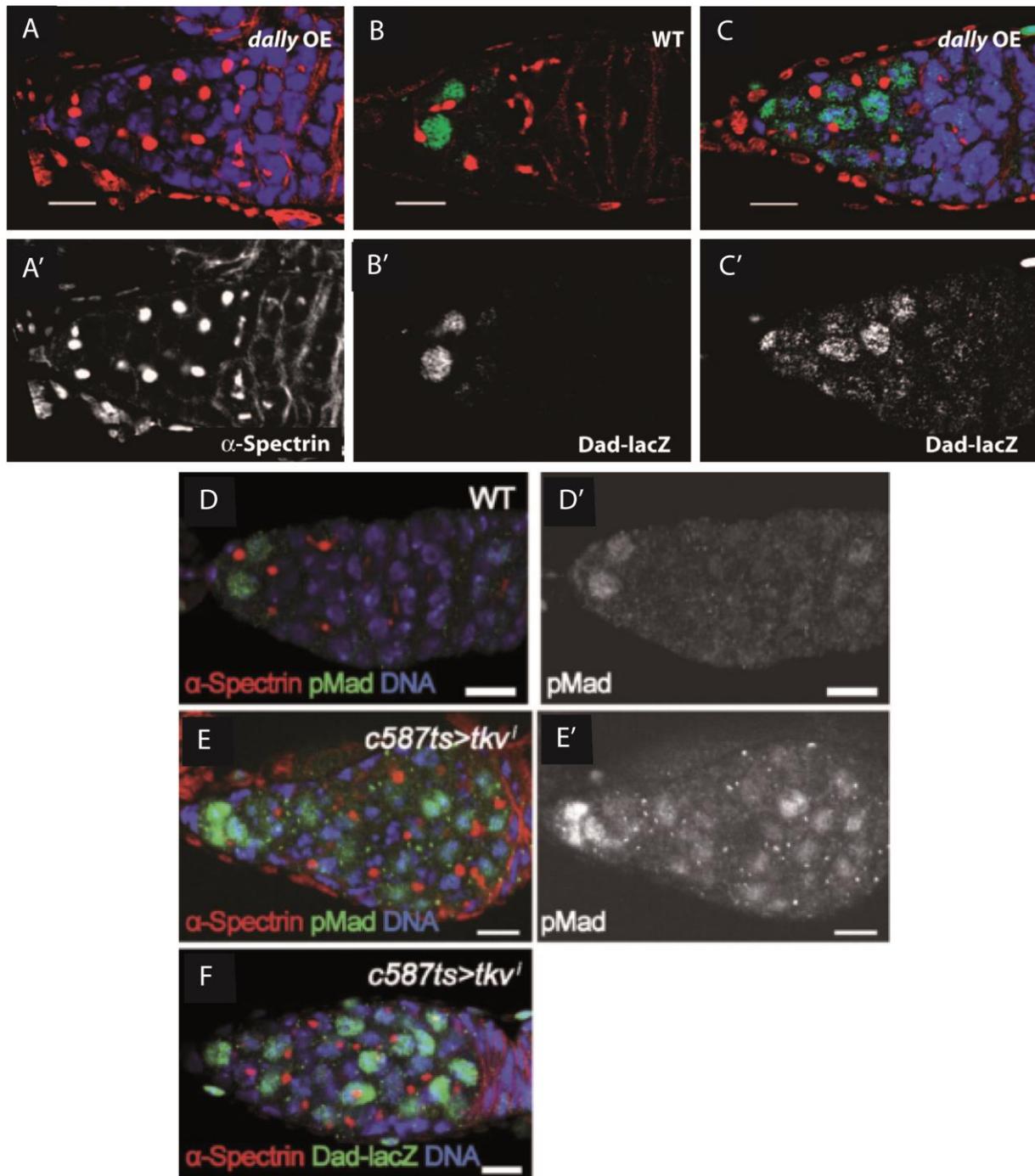
The pECs can also reduce the extra-cellular levels of the Dpp in the space adjacent to the CB. Dpp decreases due to downregulation, in the pECs, of a glypican required for DPP movement and stability, *dally* (Liu et al., 2010). Accordingly, when overexpression of Dally was induced in ECs, many germaria appeared filled in with GSC-like cells, expressing Dad (Fig20). Interestingly, downregulation of *dally* in ECs is promoted by activation of EGFR signaling coming from the germline (Liu et al., 2010). In parallel, the histone methyltransferase Egg might downregulate *dally*, independently of the EGFR pathway (Wang et al., 2011).

Recent work reported another mechanism to restrict the range of Dpp to the GSC. The authors observed that ECs express high levels of the Dpp receptor Tkv, and that this acts as a “receptor sink” to remove the excess of Dpp outside of the niche. Downregulation of Tkv in

the ECs results in differentiation defects. They further demonstrated that tkv expression was induced by multiple Wnt ligands produced by cap cells (Luo et al., 2015).



**Figure 19: Dynamics of Escort cells cytoplasmic processes (A-F)** Differentiated germ cells are wrapped by pEC cellular processes. Germaria labeled for GFP (green) and Hts (red, **A**) or Fas3 (red, differentiated follicle cells, **B-F**) containing a GFP-positive EC (arrow) at region 1 (**A-B**), region 2a (**C-D**) or the 2a/2b boundary (**E-F**). The ECs in **A** and **B** have short cellular processes encasing the cystoblast (CB) and mitotic cyst, respectively, whereas those in **C-F** have longer cellular processes wrapping around 16-cell cysts (Kirilly et al., 2011). (**G**) Germline cysts migrate normally during culture but escort cells do not accompany them. At the start of the imaging period, a region 2b germline cyst (yellow) is associated with an escort cell (magenta). During 10 hours in vitro, the cyst migrates into region 2b, acquires a characteristic lens shape and becomes surrounded by follicle cells (blue). The escort cell now associates with a younger, adjacent germline cyst (green). Arrowhead indicates 2a/2b border. Times are shown in hours:minutes (Morris and Spradling, 2011).

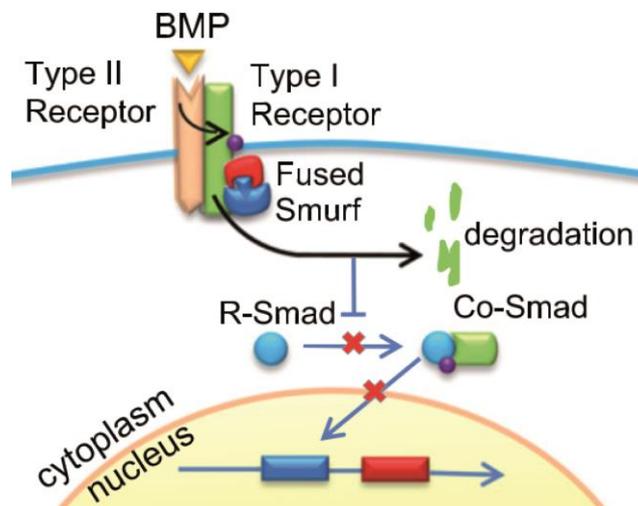


**Figure 20: Escort cells control cysts differentiation through regulation of BMP signaling. (A-C)** Red,  $\alpha$ -spectrin; green, Dad-lacZ. **(A and C)** A germarium with ectopic *dally* expression in ECs. **(A)** Ectopic spectrosome-containing cells. While Dad-lacZ was largely restricted to the anterior region in a WT germarium **(B; n = 26 germaria)**, in **(C)** Dad-lacZ was expressed in ectopic spectrosome-containing cells **(C; n = 82 germaria)** (Liu, 2010). **(D)** A WT germarium contains two pMad-positive GSCs within the niche. **(E)** A *tkv* germarium exhibits more pMad-positive spectrosome-containing cells. **(F)** A *tkv* germarium exhibits ectopic Dad-lacZ-positive spectrosome-containing cells (Luo et al., 2015).

## b) Cell autonomous

BMP signaling is also blocked cell autonomous in the CB. While the microRNA miR-184 was shown to transcriptionally repress the Dpp type I receptor *sax* (Iovino et al., 2009), Tkv was reported to be degraded. Indeed, Tkv proteolysis seems to be instructed by the serine/threonine kinase, Fused, and conducted by the ubiquitin E3 ligase, Smurf (Fig21) (Xia et al., 2010).

Brat (Brain Tumor) together with Pum, work downstream the BMP signaling cascade, to translationally repress Mad (Harris et al., 2011).

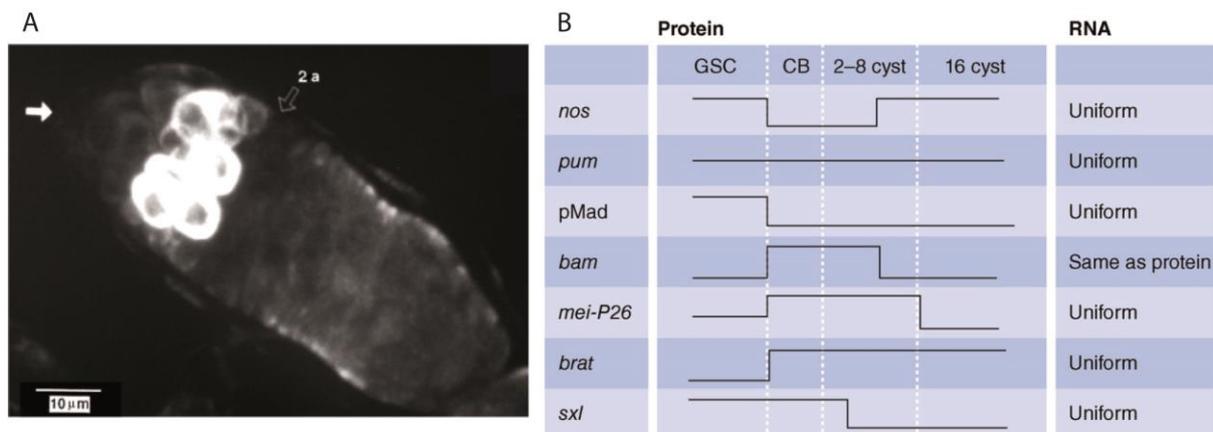


**Figure 21: Fu acts in concert with Smurf to regulate the ubiquitination of Tkv in the CB.** This generates a steep gradient of response to BMP signaling between GSCs and CBs for their fate determination. The model suggests that Fu likely acts on Tkv through targeting and phosphorylating the S238 site and subsequently leads to Tkv ubiquitination and degradation by Smurf. The downstream BMP signaling cascade would be blocked (Xia et al., 2010).

As a consequence of BMP signaling downregulation in the CB, *bam* expression is induced (Fig22). Interestingly a feedback regulation loop was observed to happen, as Bam seems to downregulate Dpp signaling. Bam overexpression is sufficient to rescue the tumorous germaria phenotype that result from the expression of a constitutively active form of Tkv (Casanueva and Ferguson, 2004).

It is thought that the main role of Bam in the CB is to prevent Nos/Pum function, thus inhibiting GSC maintenance. Epistasis analysis showed that removing Pum from *bam* mutant ovaries is sufficient to induce GSC differentiation (Chen and McKearin, 2005; Szakmary et al., 2005). In agreement, it was shown that Bam is able to physically interact with Pum, inhibiting its function (Kim et al., 2010), and to downregulate Nos transcript (Li et al., 2009).

Other proteins, such as Mei-P26, Sxl, and Vasa, are thought to repress translation of self-renewing factors in the CB, promoting its differentiation.



**Figure 22: Expression patterns of key regulators in germaria. (A)** Staining of cytoplasmic Bam in wild-type germaria. The anterior end of germarium is marked by a closed arrow. Anti-Bam.m2 antiserum was reacted against wild-type ovaries and prepared for confocal viewing using fluorescent secondary antibodies. Eight cystocytes in region 1 that are positive for BamC; the beginning of region 2a is marked by an open arrow. The micrograph presented in B was overexposed so that the outline of the germarium can be seen clearly. In all cases examined, BamC-positive cells were confined to germarium region 1. Cells in the anterior-most position, putative stem cells, were never positive (McKearin and Ohlstein, 1995). **(B)** Expression patterns in germ cells in germaria. (left) For each gene and for the phosphorylated Mad protein (pMad), changes in protein levels (high or low) are depicted during the indicated stages. (right) RNA expression patterns are summarized. Note that protein patterns are dynamic, whereas there is little change in RNA expression with the exception of *bam* (Slaidina and Lehmann, 2014).

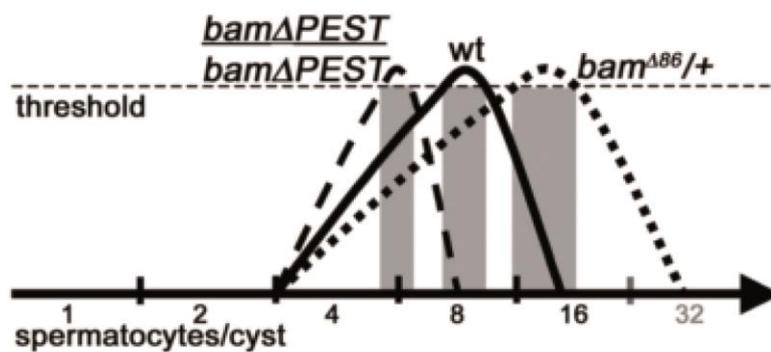
### 3- Regulation of cyst divisions

The process of *Drosophila* female cyst differentiation consists in precisely 4 rounds of cell divisions, with incomplete cytokinesis. How this number is counted, is not yet understood. However, in males the number of divisions appears to be set by the timing of Bam protein accumulation (Insko et al., 2009) (Fig23). Indeed, male germ cells heterozygous for *bam* take longer to reach the protein threshold level and have time to complete a fifth division, giving rise to cysts with 32 cells instead of 16. On the opposite, in cells expressing a stable form of *bam* (*bam* $\Delta$ PEST), high levels of the protein accumulate earlier and the cyst goes through 3 rounds of division, with only 8 cells at the end (Insko et al., 2009). Bam is not required for the initiation of the male cystoblast divisions. However in females, Bam acts earlier promoting CB differentiation and *bam* mutant germaria are mostly composed of single cells (tumors), as described above. Thus it was not yet possible to separate functions and test if it acts later in counting the number of cell divisions of the cyst. However, Bam was observed to localize on the fusome (McKearin and Ohlstein, 1995). This organelle was shown to be important to count the number of divisions, as well as to synchronize cyst mitosis. Unfortunately, the antibody that detected Bam on the fusome no longer is available, and GFP tagged Bam was absent from this structure.

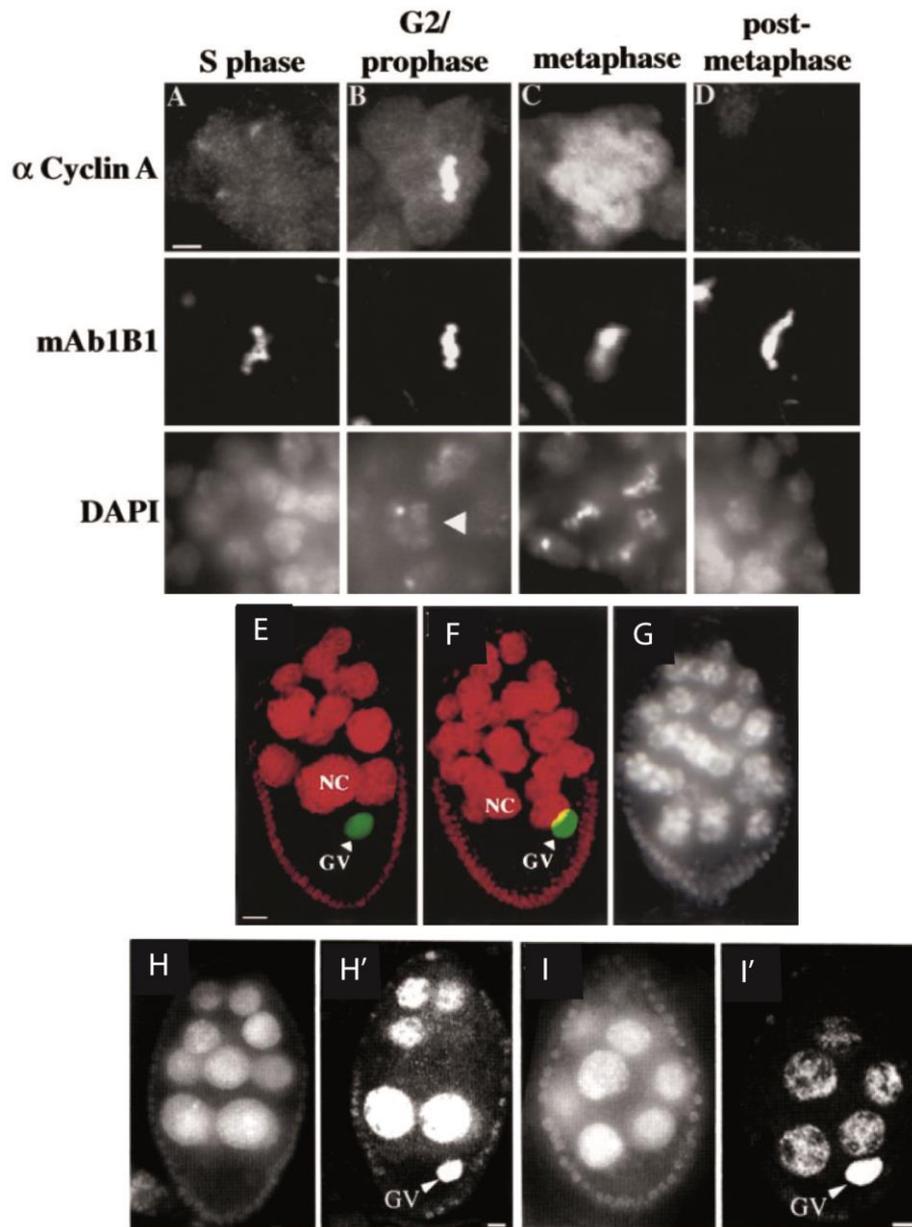
Another protein that localizes to the fusome is the mitotic Cyclin A (CycA). It accumulates in G2 at the mitosis onset, the time point when the fusome interconnects all cells in the cyst through a continuous structure (Fig24A-D). It is thought that fusome distribution could equalize the activation of CycA/Cdk1, synchronizing the entry in mitosis. Accordingly, the levels of cyclins appear to be important for the counting mechanism. Overexpression of CycA or CycB is sufficient to induce a 5<sup>th</sup> mitosis in some of the cysts, which are composed of 32 cells (Fig24E-G) (Lilly et al., 2000). Mutants for a negative regulator of these cyclins, UbcD1, give rise to 32 cell cysts, and it can be rescued by the removal of one copy of CycA or CycB. Also the G1 cyclin E is important for this process, as females carrying a hypomorphic allele of *cyclin E* frequently contain only 8 cells per cyst (Lilly and Spradling, 1996). On the opposite, mutants for *encore*, where polyubiquitinated CycE gets accumulated, originates cysts with 32 cells. This phenotype was enhanced by mutations in genes coding for components of the

SCF pathway such as *cul1*, *ubcD2* and *effete* (Hawkins et al., 1996; Ohlmeyer and Schupbach, 2003).

Even though some key players in the process were already found, how do cysts stop dividing precisely after 4 cycles is yet to be understood. *Drosophila* cysts formation is thus a powerful model to address one of the central questions for developmental biologists: how do cells count the number of mitosis.



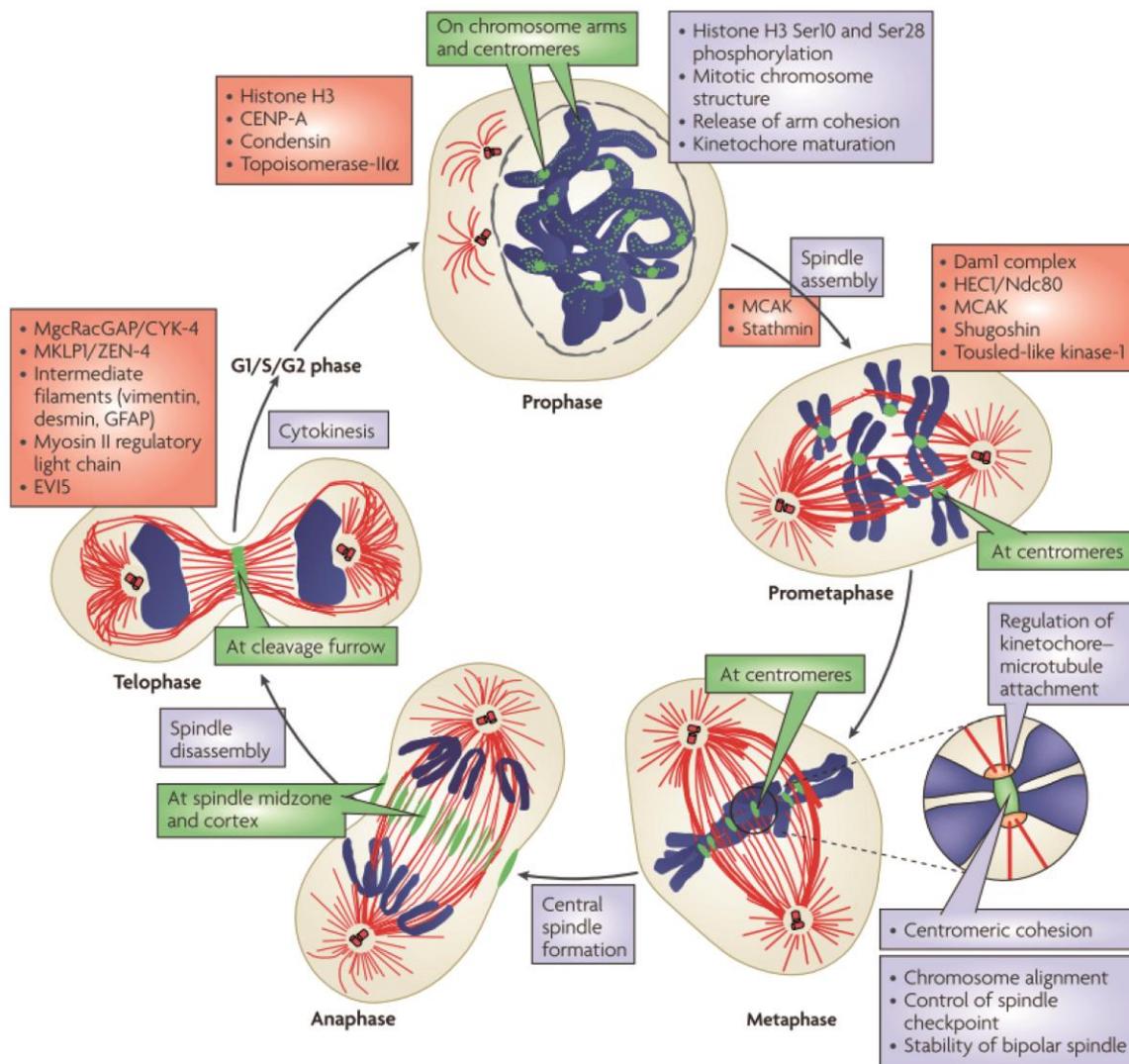
**Figure 23: Bam controls the number of cyst divisions in males.** Model: Bam protein accumulation to a critical threshold triggers the switch to spermatocyte fate following the next mitotic division, producing 16-cell spermatocyte cysts in wild-type. Bam reaches the threshold later in *bam<sup>Δ86</sup>/+*, producing many cysts with 32 or more spermatocytes. In *bamΔPEST/bamΔPEST; +/+* flies, stabilized Bam protein reaches the threshold earlier, producing many cysts with eight spermatocytes per cyst (Insko et al., 2009).



**Figure 24: The level of cyclins control number of cyst divisions in female (A–D)** Ovaries were stained with antibodies raised against *Drosophila* CycA and the monoclonal antibody mAb1B1 to highlight fusomes and follicle cell membranes. DNA is labeled with DAPI. A single cyst in the indicated cell cycle stage is shown in each part. **(A)** S phase. The distribution of CycA during S phase was determined by performing BrdU incorporation in concert with anti-CycA antibody staining (data not shown). **(B)** G2/prophase. The DNA has begun to condense (arrowhead). **(C)** Metaphase. **(D)** Postmetaphase (Lilly et al., 2000). Egg chambers from **(E)** wild-type and **(F)** Hs- $\Delta$ cyclin A (stabilized by deletion of destruction box) females were stained with propidium iodide to label nuclei and with a low concentration of anti-CycE to highlight the germinal vesicle (GV). Note that the Hs- $\Delta$ cyclin A egg chamber contains only a single GV. **(G)** A DAPI-stained egg chamber containing a 32-cell germline cyst from a Hs- $\Delta$ cyclin B female (Lilly et al., 2000).

## **CHAPTER III - AURORA B AND CYCLIN B CONTROL THE TIMING OF GSC ABSCISSION**

Recently, our lab identified a set of genes that when mutated modified the number of cells in the cysts (Mathieu et al., 2013). In this work it was shown that mutants for *aurora B* (*aurB*) and *survivin* (*svn*) frequently contained cyst with 8 cells. AurB is the kinase of the chromosomal passenger complex (CPC), which also contains Survivin, Incenp and Borealin (Ruchaud et al., 2007). The CPC is known to act during metaphase, regulating chromosome orientation and attachment to the mitotic spindle. It also plays later functions during the cell cycle, as it translocates from the centromeres to the spindle midzone. Here it promotes furrow ingression during the first steps of cytokinesis (Fig25) (Ruchaud et al., 2007), in process antagonized by CycB/Cdk-1 (Echard and O'Farrell, 2003). Aurora B was recently found to inhibit abscission, the last step of cytokinesis (discussed in detail later).



**Figure 25: Chromosomal passenger complex localization and function during mitosis.** Schematic representation of the chromosomal passenger complex (CPC) localization (green) correlated with its multiple functions (grey boxes) and principal targets (red boxes) during the different phases of mitosis relative to tubulin and chromosome dynamics. In prophase, the CPC is found on chromosome arms where it phosphorylates histone H3 on Ser10 and Ser28. It is involved in the release of arm cohesion and mitotic chromosome structure. During this phase it accumulates at centromeres where the maturation of kinetochores begins and continues through prometaphase. The CPC is required for the formation of a bipolar spindle and its stability from prophase/prometaphase to anaphase. In metaphase, it localizes at centromeres, where it has a central role in centromeric cohesion and the regulation of kinetochore-microtubule attachments. It controls the correct alignment of chromosomes on the spindle equator and the spindle checkpoint. In anaphase, the CPC translocates to the spindle midzone and appears at the cortex; it is involved in the formation of the central spindle. In telophase, the CPC concentrates at the cleavage furrow and, subsequently, at the midbody, where it is required for completion of cytokinesis. Chromosomes, blue; tubulin, red; nuclear envelope, grey. (Ruchaud et al., 2007)

Homozygous hypomorph mutant for *aurB* gave rise to 18% of 8-cell cysts, while mutated forms of *svn*, with nonphosphorylatable sites fitting with AurB consensus (SvnS125A and SvnS125A,T129A), produced around 20% of 8-cell cysts. In these egg chambers, the oocytes had 3 ring canals, suggesting that they resulted from only 3 rounds of mitosis (Figure 26B).

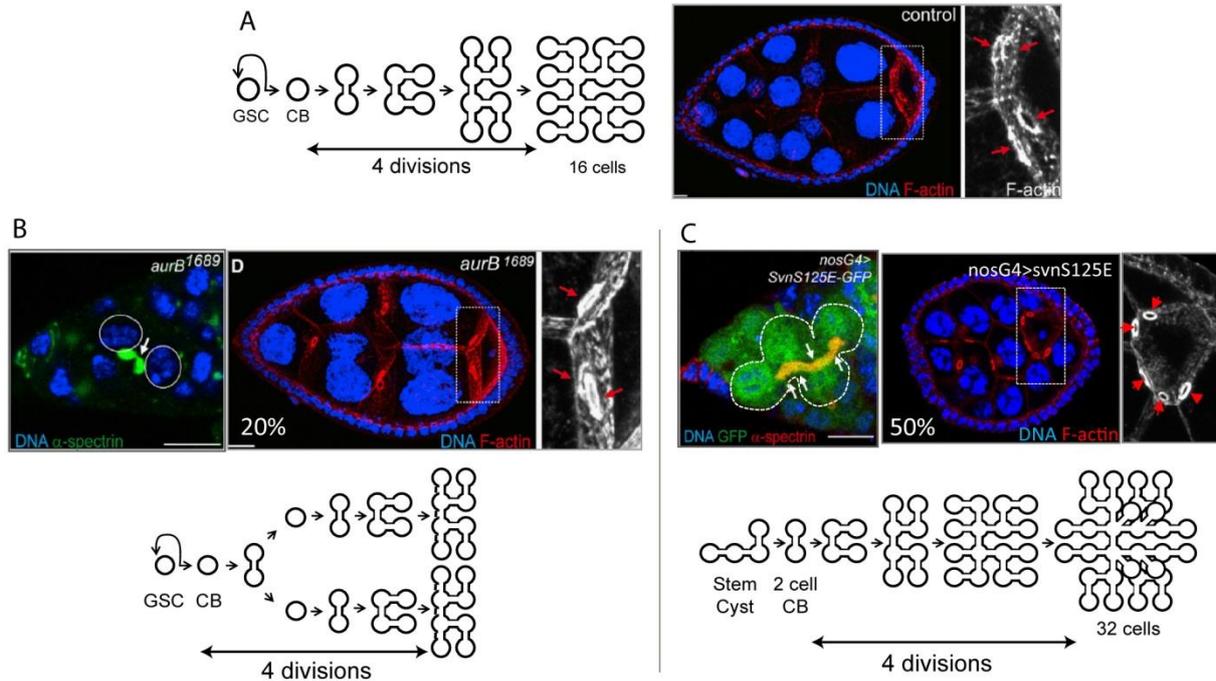
When AurB and Svn were overexpressed in the all germline cells (using *nanos>GAL4* driver), significant number of 32 cell cysts, with oocytes harboring 5 ring canals, were produced. Overexpression of a phosphomimic form of Svn (SvnS125E) resulted in almost 50% of chambers with 32 cells, or even more (Figure 26C). Very surprisingly, no phenotype was observed when the overexpression was induced specifically in the mitotic cyst cells (with *bam>GAL4* driver). It thus meant that the extra germ cells in the cyst were induced by CPC overactivation earlier in the GSC.

To explore how the stem cell could control the number of cyst divisions, live imaging of SvnS125E expressed with the *nos>GAL4* driver, was performed. GSCs were observed dividing synchronously with several neighboring cells, a phenomenon that never happens in wild type germaria. Immunofluorescence analysis in these cells showed that very often the GSC was connected through a branched fusome to up to eight other cells (Figure 26C). These cells were expressing Nanos to a similar level of wild type GSCs and not expressing Bam. Thus, due to the lack of differentiation markers, but its similarity with interconnected cysts, this structure was named stem-cyst. Interestingly, even though the cells from the stem-cyst were shown to share the cytoplasm, only the cell in contact in the niche was stained for P-Mad. Somehow the Dpp signaling seemed to be restricted to the stem cell, although the other cells were still able to repress *bam*.

The next question was then, how would this structure be formed and responsible for the development of 32 cell cysts. The overactivation of the CPC would delay the abscission between the GSC and the CB, and the next round of division would happen while the 2 cells were still connected. Eventually abscission in the stem-cyst would be induced releasing a 2-cell precursor. This group of 2 cells would behave like a cystoblast and divide 4 times. Because it started with double of the cells, it would give rise to cysts with twice the number of cells ( $2 \times 16 = 32$ ). Indeed, the breakage of the fusome separating these 2 cells from the

stem-cyst was often observed (Figure 26C). On the opposite, in the CPC loss of function, abscission between GSC and the CB seems to happen earlier, as these two cells were seen replicating non-synchronously. In the wild type, abscission occurs in G2, after S phase. It was also observed an unusual abscission in two- cell cysts that could explain the 8-cell egg chambers phenotype. These two cells, which had already been through one cycle, once separated would continue the 3 mitosis left giving rise to 2 cysts of 8 cells. Indeed, 2 cysts of 8 cells were very frequently seen one next to the other in ovarioles (Figure 26B).

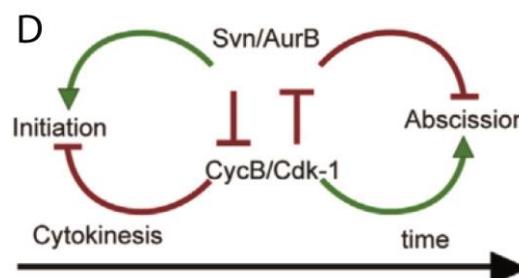
It is thought that the first division of the CB is very similar to a GSC division, as both seemed to be delayed by AurB. However, the expression of Bam in the CB triggers the differentiation program that blocks abscission in all divisions of the cyst. An interesting observation was that the 8-cell cyst phenotype of the *aurB* homozygous hypomorph was rescued when a copy of *bam* was removed. This is in agreement with the model of Bam protein level controlling the number of divisions in the cyst, discussed above (Insko et al., 2009). With one less copy of the gene, Bam protein level would take longer to reach the threshold, giving time for an extra division of the cysts. This experiment, thus points to a function of Bam controlling the number of divisions of the female cysts, in a way similar to what happens in males.



**Figure 26: The CPC and CycB/Cdk1 control the timing of germline abscission.** Deregulation of this process results in the change of cell number in the cyst. **(A)** In the WT, the CB goes through 4 divisions forming cysts of 16 cells, and oocytes with 4 ring canals. **(B)** In the homozygous hypomorphic allele of *AurB* (1689), the GSC abscission is faster and cytokinesis is complete e some of the 2-cell cysts. Each of the cells, now separated, continue the process of differentiation and divide 3 more times, giving rise to 2 sister 8-cell cysts (20%). The oocyte of these cysts harbors 3 ring canals. **(C)** In the overexpression of activated *svn* (*nos>svnS125E*), GSC abscission is slower and the next mitosis happens before cytokinesis is completed, resulting in the formation of stem-cyst. In this structure the GSC is connected to several other cells by the fusome. Once abscission happens, it releases a 2-cell cyst that is functionally equivalent to a CB, thus it divides 4 times giving rise to 32-cell cysts (50%) and oocytes with 5 ring canals. (Mathieu et al., 2013)

To further explore the role of AurB in regulating abscission, immunoprecipitation of the CPC followed by mass-spectrometry, were done to find putative targets. CycB came up as one of the interacting proteins, and 5 phosphorylation sites fitting the AurB consensus were found in its sequence. It was shown that the 32 cell cyst phenotype observed in the CPC overactivation, was rescued when one copy of *cycB* was removed. When the 5 sites were mutated into phosphomimic (*cycB5E*) and expressed using the *nos>GAL4* driver, GSC abscission was delayed and both stem-cysts and 32 cell egg chambers were formed. The control where this same mutated form was expressed specifically in the mitotic cysts (*bam>GAL4*) gave no phenotype. However, AurB consensus sites seem to be important to promote a 5<sup>th</sup> mitosis in the cyst, as the expression of a nonphosphorylatable form (*cycB5A*), or a wild type form, with the *bam>GAL4* driver gave rise to 32 cell cysts. This result is in agreement with the previous observation that CycB overexpression in the germline results in chambers of 32 cells (Lilly et al., 2000). The expression of RNAi against CyB, using the *nanos>GAL4* driver, induces the formation of stem-cyst and 32 cell cyst, but also egg chambers with 8 cells. This result points to a double function of CycB/Cdk1 in the germline, promoting abscission in the GSC and controlling the number of divisions in the cyst. With this work, a long lasting paradox may finally be solved. The overexpression of the Cdk1 activator *string/cdc25*, expected to induce extra mitosis, was seen to produce mostly egg chambers with 8 cells and some with 32 (Mata et al., 2000). The same phenotype was observed in a loss of function of Tribles, a negative regulator of *string* (Mata et al., 2000). These phenotypes are now understood to be a consequence of CycB/Cdk1 function in promoting GSC abscission.

A balance between CPC and CycB/Cdk-1 mutual inhibitions must be achieved to correctly time the processes of cytokinesis and abscission.

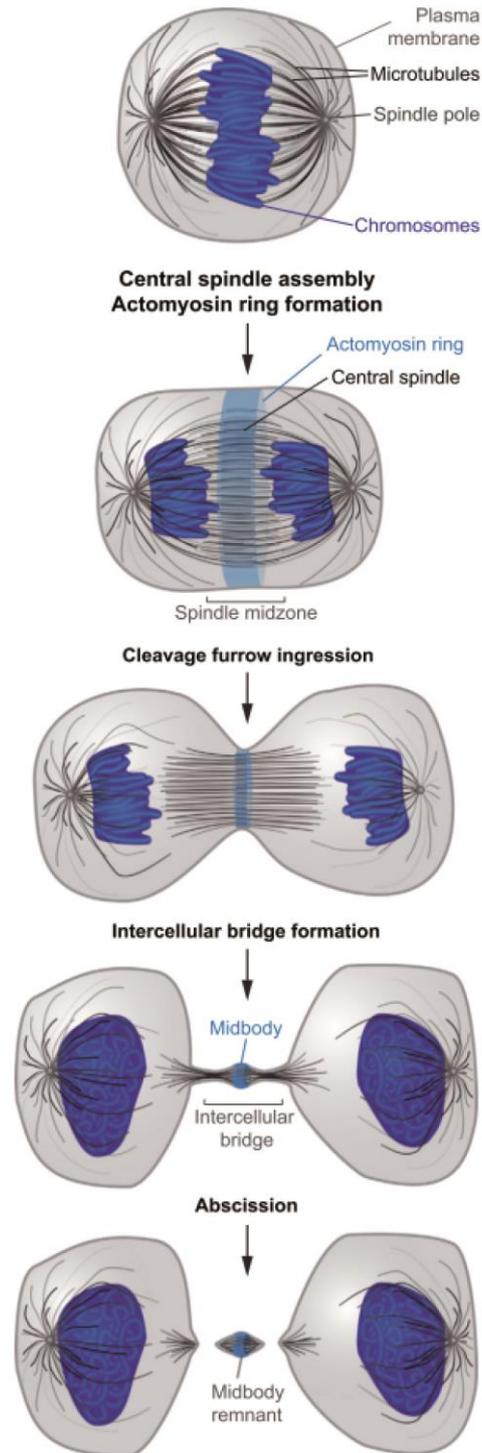


**Figure 27: Schematic model of the interactions between the CPC and CycB/Cdk1 during early and late cytokinesis.** (Mathieu et al., 2013)

## **CHAPTER IV - ABSCISSION: THE LAST STEP OF CYTOKINESIS**

Cytokinesis is the final step of cell division, when the two daughter cells get physically separated. This process can be divided into three broad stages: (1) selection of the site of plane of division by the central spindle, (2) formation of an actomyosin contractile ring, furrow ingression and formation of an intercellular bridge and (3) abscission of the midbody to separate the cells (Fig28) (Mierzwa and Gerlich, 2014).

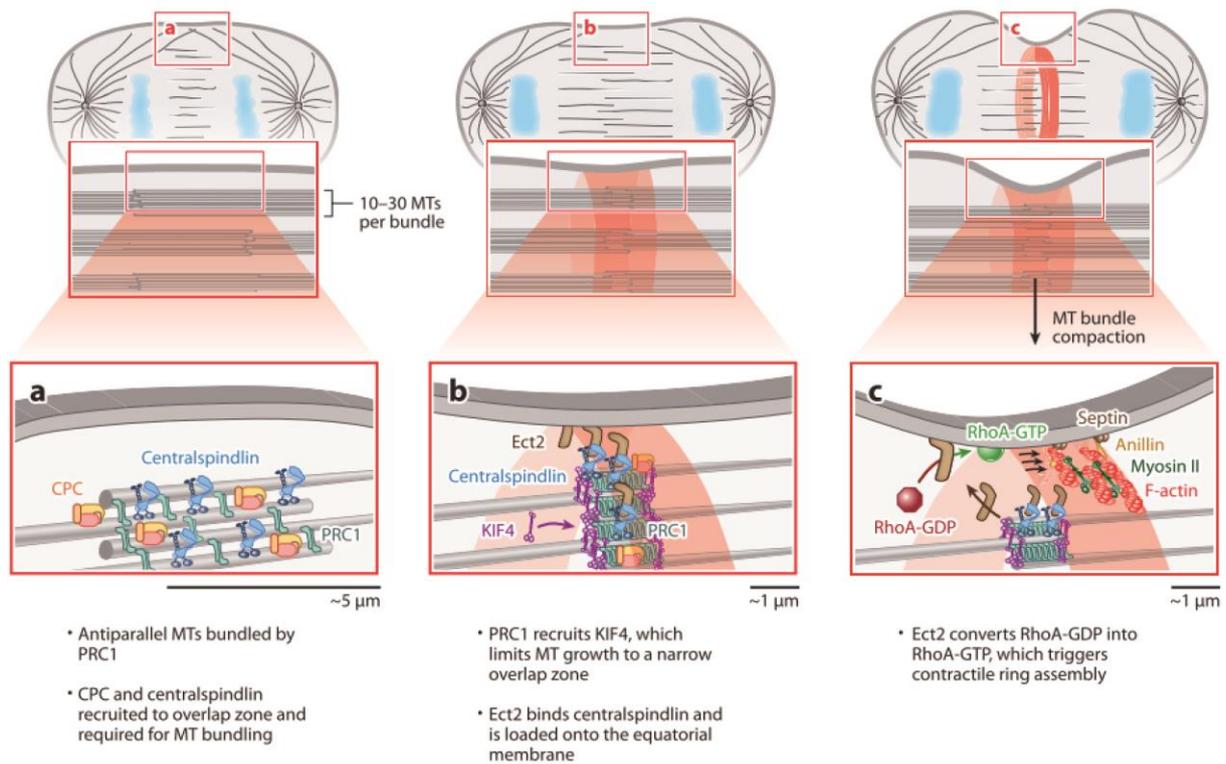
The process of cytokinesis has been vastly studied and well described in mammalian cell culture lines, thus its known molecular mechanisms come mostly from these model systems.



**Figure 28: Overview of Animal Cell Cytokinesis.** Cytokinesis initiates during anaphase, when the two sets of sister chromatids segregate toward opposing spindle poles. Microtubules of the mitotic spindle then reorganize to form the central spindle. Signaling between the anaphase spindle and the cell cortex specifies where the actomyosin ring assembles. Contraction of the actomyosin ring ingresses the attached cell cortex to form the cleavage furrow, which partitions the cytoplasm into two domains. Completion of actomyosin ring contraction results in the formation of the intercellular bridge, which contains the midbody at its center. Abscission proceeds by disassembly of the microtubules adjacent to the midbody and membrane fission to physically separate the nascent daughter cells (Mierzwa and Gerlich, 2014).

## 1- Cleavage plane specification

The cell division plane is known to be specified during anaphase, by the mitotic spindle microtubules (MTs). These consist in astral and central spindle MTs (Bringmann and Hyman, 2005; Dechant and Glotzer, 2003). It is thought that the astral MTs, which emanate from the centrosomes at the spindle poles, provide an early cue for cleavage plane specification. Their function seems to be more important in larger cells and was shown not to be required for proper cytokinesis in *Drosophila* (Basto et al., 2006). However, the function of the central spindle MTs is conserved in animal cells, as cleavage furrows formed in their absence do not progress in cytokinesis. The formation of the central spindle requires the antiparallel microtubule cross-linker PRC1 and the kinesin KIF4, a suppressor of microtubule dynamics with motor activity (Bieling et al., 2010). The centralspindlin complex has also been shown to contribute to the assembly of the central spindle and is thought to function by promoting MT bundling (White and Glotzer, 2012). This complex consists in two units of the kinesin MKLP1 (Pavarotti, Pav, in *Drosophila*) and two units of the Rho-family GAP MgcRacGAP (RacGAP50C in *Drosophila*). The centralspindlin complex is regulated by two kinases located at the mitotic spindle. The Aurora B kinase, part of the CPC complex, promotes its recruitment through MKLP1 phosphorylation. The Polo-like kinase phosphorylates MgcRacGAP, which in turn is responsible for the recruitment and activation of the Rho GEF Ect-2. This GEF protein then translocates to the cortex, at the site of the cell division plane, and is responsible for the local activation of RhoA (Burkard et al., 2009; Kim et al., 2005; Wolfe et al., 2009). This small GTPase is thought to be the master regulator of the cytokinetic ring assembly (Fig29). The centralspindlin complex was also shown to be important to anchor the ring to the plasma membrane (Lekomtsev et al., 2012).



**Figure 29: Central spindle formation and signaling.** (A) In early anaphase, PRC1, centralspindlin, and the chromosomal passenger complex (CPC) are recruited to the spindle midzone, where they promote bundling of overlapping microtubules (MTs) of opposite polarity that emanate from the two spindle poles. (B) KIF4 is recruited to the overlap zone in midanaphase by PRC1. KIF4 translocates to MT plus ends and stops their growth, limiting the length of the overlap zone. Ect2 binds centralspindlin and is loaded onto the equatorial membrane. (C) Ect2 converts RhoA-GDP to RhoA-GTP to promote contractile ring assembly (Green et al., 2012).

## 2- Assembly and constriction of the actomyosin ring

The formation of cortical contractile ring is essential for the bipartition of the cytoplasm into the two daughter cells. The main components of the cytokinetic ring are crosslinked protein filaments. These consist in bundled actin filaments, MyoII minifilaments and septin filaments, which are all crosslinked by anillin (Fig29C). Activated Rho drives the assembly of these players and the constriction of the ring. More precisely, it is responsible for the activation of formins, which promote actin polymerization, and activation of Rho-kinase, known to phosphorylate and activate MyoII.

To be able to promote contractibility, the ring must be tightly connected to the plasma membrane. This connection seems to involve the phosphoinositide PIP<sub>2</sub>, which is also concentrated at the cleavage ring (Green et al., 2012; Lekomtsev et al., 2012). In agreement, several players in the process were shown to bind or contain PIP<sub>2</sub> binding domains, such as anillin and septins. However, these two proteins do not seem to be required for the anchoring in early cytokinesis in *Drosophila* cells. They only become essential later on, after midbody formation (Kechad et al., 2012).

The contraction of the actomyosin ring induces the ingression of the cleavage furrow, resulting in the formation of the midbody. The two daughter cells remain connected by a thin intercellular bridge, which persists until abscission takes place.

### **3- Abscission**

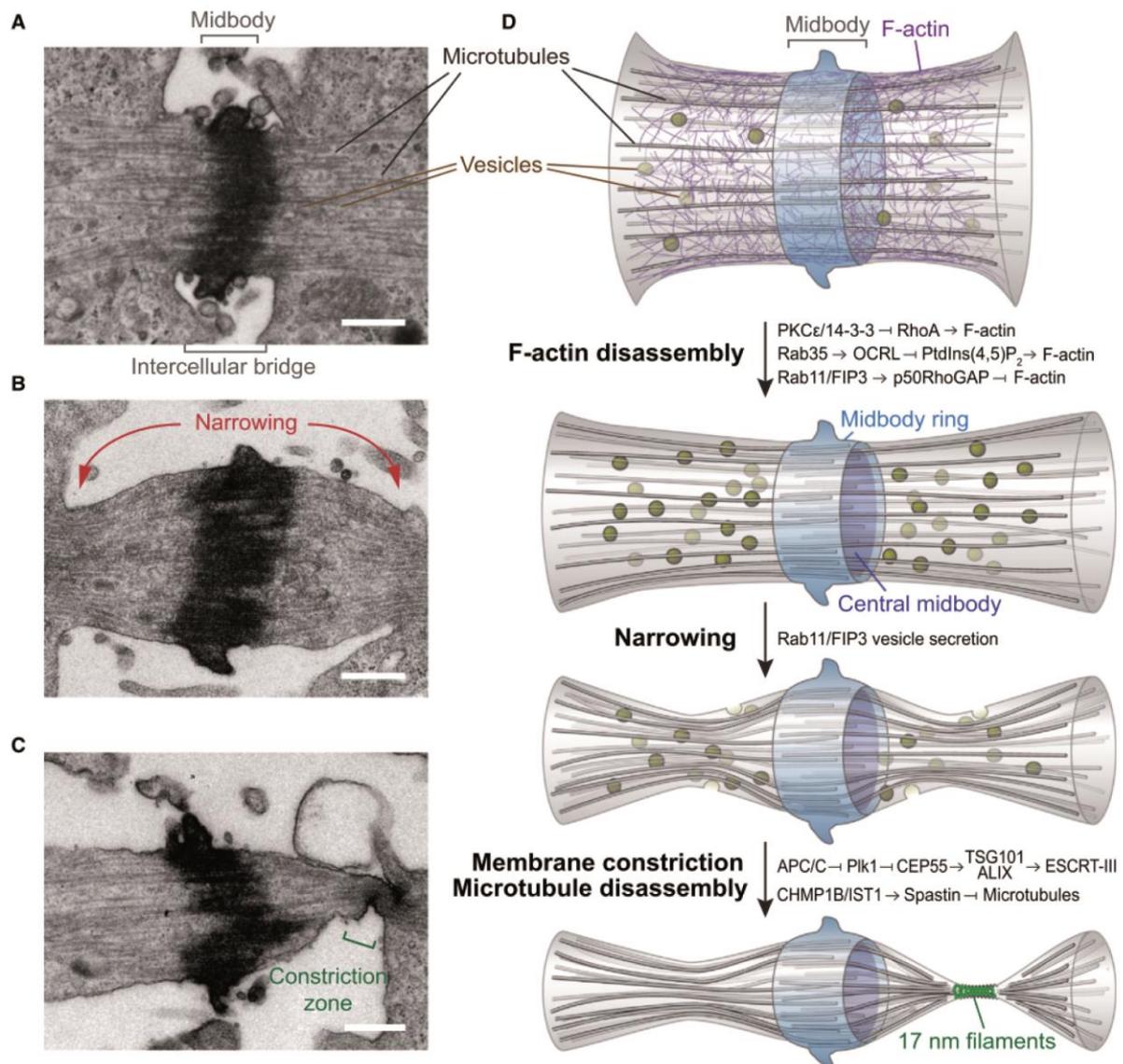
The midbody was first identified by Walther Flemming in 1891, who described it as a remnant of the spindle just before complete cleavage of the membrane. The, also called, “Flemming body” was later confirmed to be formed by a bundle of antiparallel microtubules, and observed as an electron dense structure by electron microscopy. The midbody is now known to act as a platform for nucleation of the abscission machinery (Fig30). Indeed, proteomic analysis of midbodies isolated from mammalian cells, revealed the presence of a variety of proteins essential for abscission. These include a large number of MT regulators, actin regulators and proteins involved in membrane trafficking (Skop et al., 2004).

After cleavage furrow ingression is completed, most of the actin filaments of the contractile ring disassemble (Guizetti et al., 2011), in a process dependent on the local inactivation of RhoA and recruitment of endosomal vesicles. Dambournet et al. proposed that the Rab35 GTPase controls the localization of the OCRL phosphatase at the intercellular bridge. OCRL in turn is responsible to prevent F-actin accumulation in the bridge, through hydrolysis of PtdIns(4,5)P<sub>2</sub>. Thus, mutants for OCRL present a longer abscission, which can be rescued by the addition of F-actin depolymerizing agents (Dambournet et al., 2011).

The stability of the intercellular bridge is maintained by several nonredundant factors localized at the midbody and adjacent regions, including centralspindlin and anillin. In *Drosophila* S2 cells depleted for anillin, the intercellular bridge cannot be stabilized and the midbody ring detaches from the plasma membrane (Echard et al., 2004).

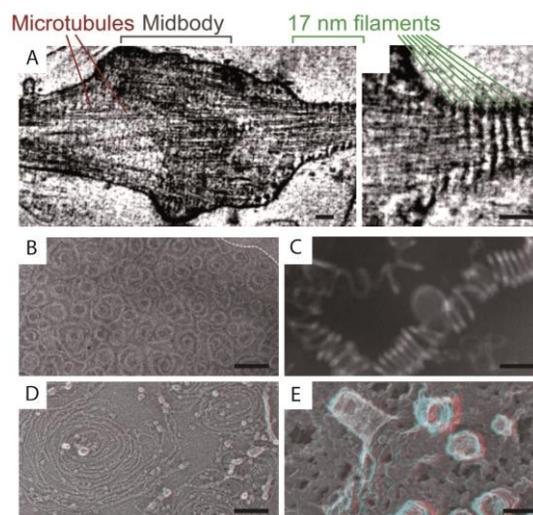
Upon midbody formation and stabilization, a secondary membrane constriction is observed on both sides of the midbody. This event depends on the recruitment of the ESCRT (Endosomal Sorting Complexes Required for Transport) machinery. ESCRT proteins were first described to be important in the formation of intraluminal vesicles, by promoting the bending and fission of the endosomal membrane (explained in detail below). Although not all the players are conserved between the process of endosomal sorting and abscission, the basic mechanism of membrane constriction appears to be similar.

Indeed, components of the ESCRT-I and ESCRT-III complexes were observed to be recruited prior to abscission. The degradation of Polo kinase allows the binding of CEP55 (Centrosomal protein 55) to the midbody-localized MKLP1 (component of the centralspindlin). This in turn promotes the recruitment of the ESCRT-I Tsg101 and the adaptor protein Alix to both sides of the midbody ring. Tsg101 is able to then recruit the ESCRT-III subunits, which interact with membranes via PtdIns3P-binding protein FYVE-CENT and TTC19. ESCRT-III are thought to directly mediate membrane constriction. At this point microtubules are severed by the AAA ATPase spastin that is targeted through direct binding to CHMP1B and IST1. (Fig30)



**Figure 30: Maturation of the Intercellular bridge.** (A–C) Transmission electron micrographs of intercellular bridges of HeLa cells at different stages of maturation. (A) Early-stage intercellular bridge appears short with bundles of straight microtubules. (B) Mid-stage, elongated intercellular bridge. Microtubule bundles appear compressed at either end, where the intercellular bridge has a reduced diameter. (C) Late-stage intercellular bridge with rippled, electron-dense cortex at a constriction zone. Microtubules at the constriction zone appear curved and highly compressed. Note that actin filaments are not visible in these images, owing to high cytoplasmic background. Scale bars, 500 nm. (D) Schematics of intercellular bridge maturation. Complete ingression of the cleavage furrow is followed by disassembly of cortical F-actin. Fusion of vesicles correlates with gradual narrowing of the intercellular bridge on both sides of the midbody. Abscission proceeds by assembly and constriction of 17 nm filaments adjacent to the midbody and simultaneous disassembly of the microtubules lateral to the midbody (Mierzwa and Gerlich, 2014).

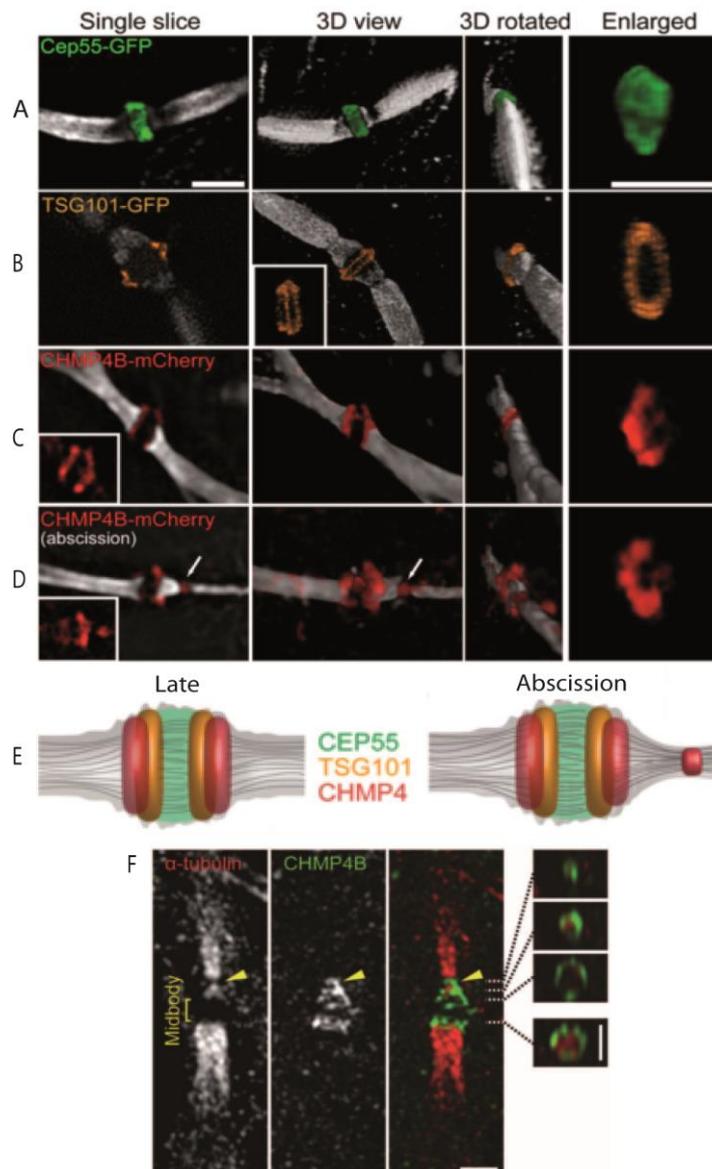
At the time of the secondary membrane constriction, adjacent to the midbody, it is observed the assembly of 17nm diameter filaments that encircle the bridge forming a spiral. The nature of these filaments, observed by electron tomography of high-pressure-frozen cells, is not yet known (Figure 31A) (Guizetti et al., 2011). However, the fact that their formation is suppressed in mutants for the ESCRT-III subunit CHMP2A, suggests that ESCRT-III may either regulate or directly polymerize into the 17 nm filaments, to exert its function. This hypothesis is support by the fact that ESCRT-III are able to form polymers in vitro, as well as on membranes, being sufficient to promote their curvature and constriction. An auto-activated mutant of the budding yeast Snf7, homolog of human CHMP4 ESCRT-III, assembled into filaments in vitro (Figure 31B). It assembled into flat spirals on lipid monolayers, and when two other ESCRT-III components were added (Vps2/CHMP2 and Vps24/CHMP3) it formed 3D coiled helices (Henne et al., 2012) (Figure 31C). Overexpression of truncated human CHMP4A in COS-7 cells resulted in the formation of flat spirals on the surface of the plasma membrane (Figure 31D). When coexpressed with full-length CHMP2A, tubular protrusions were formed (Cashikar et al., 2014) (Figure 31E).



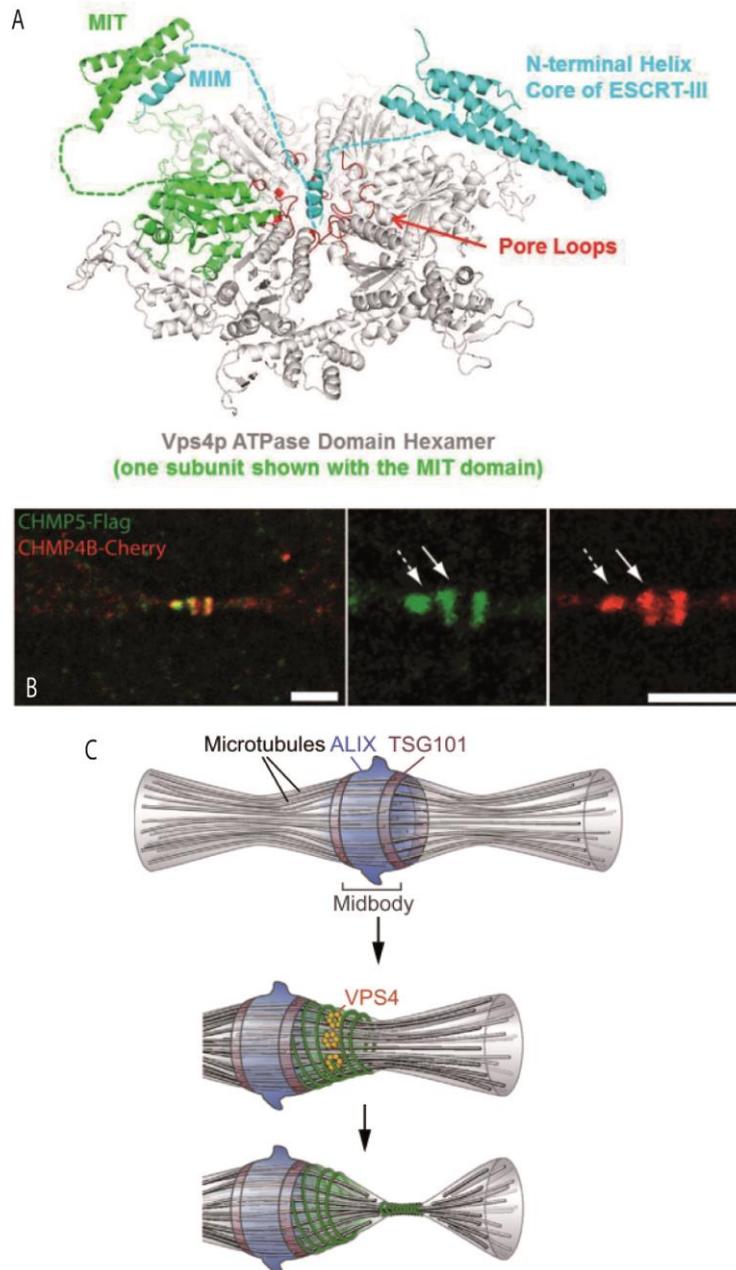
**Figure 31: Assembly of ESCRT-III filaments.** (A) Electron tomogram of a high-pressure-frozen HeLa cell with a late-stage intercellular bridge containing 17 nm filaments. On the right, enlarged section of the tomogram shown on the left reveals parallel arrangement of regularly spaced 17 nm diameter filaments at the constriction zone (Guizetti et al., 2011). (B) Auto-activated budding yeast Snf7 (Snf7R52E) assembles into flat spirals on lipid monolayers (Henne et al., 2012). (C) Addition of Vps2 and Vps24 to Snf7R52E forms 3D coiled helices (Henne et al., 2012). (D) Deep-etch electron microscopy of plasma membranes of COS-7 cells overexpressing truncated human CHMP4A results in flat spirals on the surface of the plasma membrane (Cashikar et al., 2014). (E) Coexpression of truncated CHMP4A and full-length CHMP2A leads to tubular protrusions on the plasma membrane (Cashikar et al., 2014).

The dynamics of the core and most abundant subunit of ESCRT-III CHMP4B, during abscission in HeLa cells, also support this hypothesis. CHMP4B localizes to two narrow cortical rings on both sides of the midbody before microtubule disassembly. Upon microtubules severing, it relocates away from the ring, accumulating at the secondary cortical constriction zone, where it is thought to promote abscission (Elia et al., 2011; Guizetti et al., 2011) (Fig32).

The AAA ATPase protein Vps4 was also shown to accumulate at the intercellular bridge prior to abscission (Elia et al., 2011), and be crucial for ESCRT-III-mediated membrane fission (Adell et al., 2014). In vitro experiments showed that the C-terminal MIM domains of the ESCRT-III subunits, which form the helical filaments, bind to the MIT domain at the N-terminal of Vps4. This interaction activates Vps4 central pore to engage the ESCRT-III polymers, and through pulling forces promote their depolymerization (Fig33A) (Babst et al., 2011; Han et al., 2015). Thus, it is thought that Vps4-mediated disassembly of ESCRT-III polymers may constrict membrane necks in a “purse-string” mechanism to promote scission (Fig33B-C).



**Figure 32: Localization of ESCRT proteins during abscission.** (A-D) MDCK cells expressing CEP55-GFP (A), TSG101-GFP (B), or CHMP4B-mCherry (C and D) were synchronized, fixed, stained with anti- $\alpha$ -tubulin antibodies, and imaged by SIM (Elia et al., 2011). Each panel shows (from left to right) a single slice, a 3D rendering, a 3D rendering rotated 90°, and a zoomed-in image of the structure. Microtubules are colored in white, CEP55-GFP in green, TSG101-GFP in orange, and CHMP4B-mCherry in red. (A) CEP55 form a diffusely filled structure that is  $1.4 \pm 0.15 \mu\text{m}$  in diameter and  $0.75 \pm 0.07 \mu\text{m}$  in width.  $n = 10$ . (B) TSG101 forms a tightly packed double-ring structure surrounding the microtubules at the center of the midbody dark zone (width =  $0.82 \pm 0.03 \mu\text{m}$ ). The rings are  $1.7 \pm 0.07 \mu\text{m}$  in their outer diameter and are  $0.23 \pm 0.02 \mu\text{m}$  apart. (Inset) A rotated image demonstrating the existence of two separate rings ( $n = 5$ ). (C and D) CHMP4B concentrates in two broken rings that are  $0.43 \pm 0.08 \mu\text{m}$  apart. The diameter of each broken ring is  $1.25 \pm 0.18 \mu\text{m}$ . In some cells CHMP4B also shows an additional pool that is located asymmetrically  $1.2 \mu\text{m}$  away from the center of the dark zone (arrow in D) and is perfectly colocalized to the site of microtubule constriction. (Insets) CHMP4B signal alone ( $n = 14$ ). (E) A model for ESCRT organization at the midbody integrating the SIM measurements indicated above (Elia et al., 2011). (F) Structured illumination microscopy of a late-stage intercellular bridge. HeLa cell stably expressing CHMP4B-EGFP was stained with anti- $\alpha$ -tubulin. Maximum intensity projections; dashed lines indicate cross sections. Dark zone indicates midbody position (arrowhead marks constriction zone) (Guizetti et al., 2011).



**Figure 33: Vps4 structure and localization during abscission. (A)** Two-step model for disassembly of the ESCRT-III complex by Vps4. The active Vps4 hexamer is shown with one subunit colored green. ESCRT-III substrates are recognized by binding of their MIM elements to Vps4 MIT domains, which release MIT/linker-mediated autoinhibition and allows the Vps4 pore loops (red) to engage a high affinity binding site on helix 5 of the ESCRT-III subunits and initiate translocation. **(B)** Synchronized MDCK cells expressing CHMP4B-mCherry (red) together with CHMP5-Flag (Vps4) were stained with anti-Flag antibodies (green) and imaged. Anti- $\alpha$ -tubulin staining was used to identify the intercellular bridges (not shown). Data show a high degree of overlap between the localization of CHMP4B-mCherry and CHMP5 on both the initial pool (solid arrows) and the peripheral pool (dashed arrows) (Elia et al., 2011). **(C)** Speculative model for ESCRT-III-mediated membrane constriction at the intercellular bridge. ALIX and TSG101 provide an ESCRT-III targeting platform at the midbody. ESCRT-III filaments first form cylindrical spirals, which are then split by VPS4 to release fragments. The ESCRT-III spiral fragments then constrict as they slide away from the midbody (Mierzwa and Gerlich, 2014).

#### **4- Abscission timing regulation**

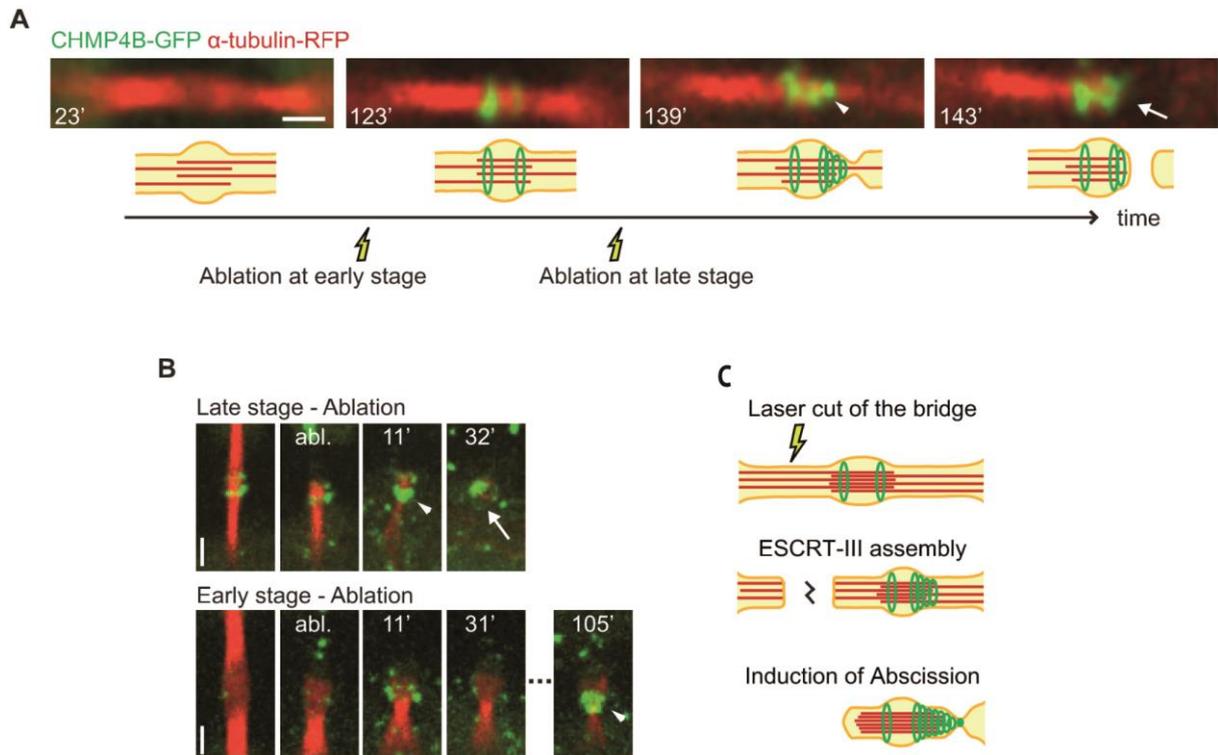
The notion that the process of abscission is subjected to timing regulation was first proposed when experiments in budding yeast showed that lagging DNA, trapped at the intercellular bridge, resulted in abscission delay (Mendoza et al., 2009; Norden et al., 2006). The so called “NoCut” checkpoint was also described to be active in human cells (Steigemann et al., 2009).

It is thus thought that abscission timing must be tightly regulated to ensure the correct separation of daughter cells. This process seems to depend on the developmental context, as the time needed to finalize abscission appears to differ among different cell types.

Different mechanisms to induce abscission have been already described.

##### **a) Tension release**

Lafaurie-Janvore et al. propose that abscission is induced when tension exerted on the intercellular bridge between the two daughter cells is released. After division, the cells, with high contractility and motility, move apart generating pulling forces and tension, which inhibit abscission. When the cells stop moving, the tension on the bridge drops, triggering the assembly of ESCRT-III filaments and abscission happens. This abscission delay would keep the cells connected until they find their correct relative and stable positions, working as a checkpoint for tissue integrity (Lafaurie-Janvore et al., 2013).



**Figure 34: ESCRT-III assembly is regulated in time by forces exerted on the bridge.** (A and B) Time-lapse sequence of control bridge cleavage (A) and ablated bridge (B). Leftmost image is before ablation (abl.). Ablations performed after CHMP4B-GFP recruitment to the midbody resulted in assembly of the conical structure on the other side 10 min later, concomitant with pinching and microtubule severing. (C) The proposed model. Cell membrane (yellow), microtubules (red), and ESCRT-III CHMP4B-GFP (green) are shown. (Lafaurie-Janvore et al., 2013).

## b) Chromosomal passenger complex

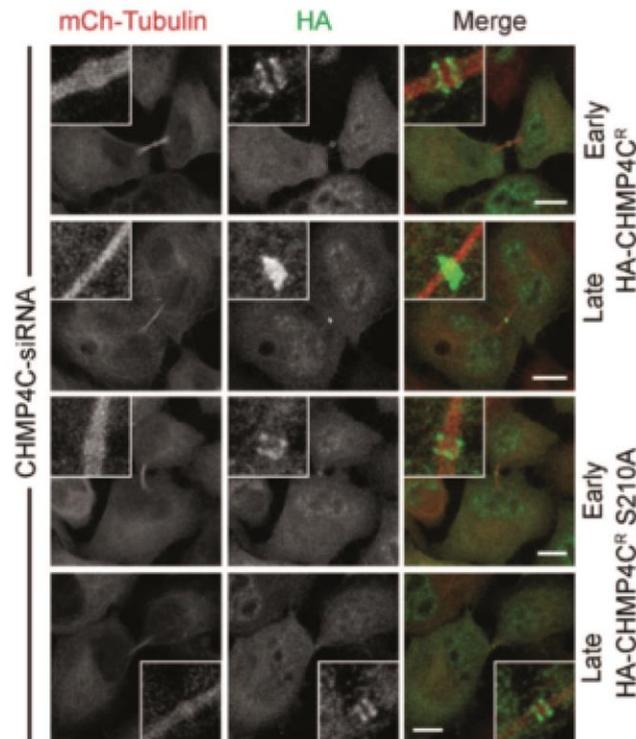
As referred above, the chromosomal passenger complex (CPC) and CycB/Cdk1 function as timers during *Drosophila* germline stem cell abscission (Mathieu, 2013). Interestingly, the CPC was shown to regulate abscission in human cells through direct interaction with ESCRT-III proteins (Capalbo et al., 2012; Carlton et al., 2012).

Yeast two-hybrid experiments in HeLa cells showed that the ESCRT-III CHMP2A, CHMP4B, CHMP4C and CHMP6 bind to the C-terminus of the CPC component Borealin (Carlton et al., 2012). In another study, Borealin purified from bacteria, was pulled down with the 3 human CHMP4 proteins (CHMP4A, CHMP4B and CHMP4C) (Capalbo et al., 2012). Interestingly,

when the authors tried to co-precipitate these proteins with Borealin extracted from HeLa cells, CHMP4C was not successful. This suggested that the interaction between Borealin and CHMP4C was regulated by pos-translational modifications in vivo (Capalbo et al., 2012). Indeed, three sites (S210, S214 and S215) were identified to be Aurora B phosphorylation sites in the C-terminal region of CHMP4C (Capalbo et al., 2012). This CHMP4 protein was also reported to localize with a different dynamic. While CHMP4A and CHMP4B were observed localizing transiently to the midbody arms at the site and time of abscission, CHMP4C initially at the arms was then redirected to the midbody central region (Fig34) (Carlton et al., 2012). To test whether AurB-dependent phosphorylation of CHMP4C could regulate its later localization at the midbody, they constructed a non-phosphorylatable form of CHMP4C (S210A). They observed that, indeed, its localization was impaired and the molecule was retained at the midbody arms (Fig34) (Carlton et al., 2012). What could be then the function of CHMP4C? Functional studies showed that depletion of CHMP4C from HeLa cells promoted a faster abscission, while over-expression resulted in abscission delay. When CHMP4C-S210A was over-expressed in CHMP4C depleted cells, abscission was no longer delayed (Carlton et al., 2012).

These results pointed thus to a model where the CPC could inhibit abscission by two different mechanisms: (1) Borealin interaction with the ESCRT-III subunits could physically block their assembly into polymers and association with the membrane; (2) AurB phosphorylation of CHAMP4C would redirect its localization from the site of secondary constriction to the central midbody, functioning as a brake in the abscission process.

The *Drosophila* only CHMP4 protein Shrub does localize at the site of abscission and physically interacts with Borealin in S2 cells. However, the AurB phosphorylation sites present in CHMP4C are not conserved in Shrub. No functional studies were developed in flies to address its function in abscission (Capalbo et al., 2012).



**Figure 35: CHMP4C localization during abscission.** H. HeLa mCh-Tub cells stably expressing HA-CHMP4CR, HA-CHMP4CR $\delta$ INS or HA-CHMP4CR S210A were treated with CHMP4C siRNA, fixed, stained with  $\alpha$ -HA and HA-CHMP4C location was scored ( $n=3\pm$ S.D.) (Carlton et al., 2012).

### c) NoCut pathway

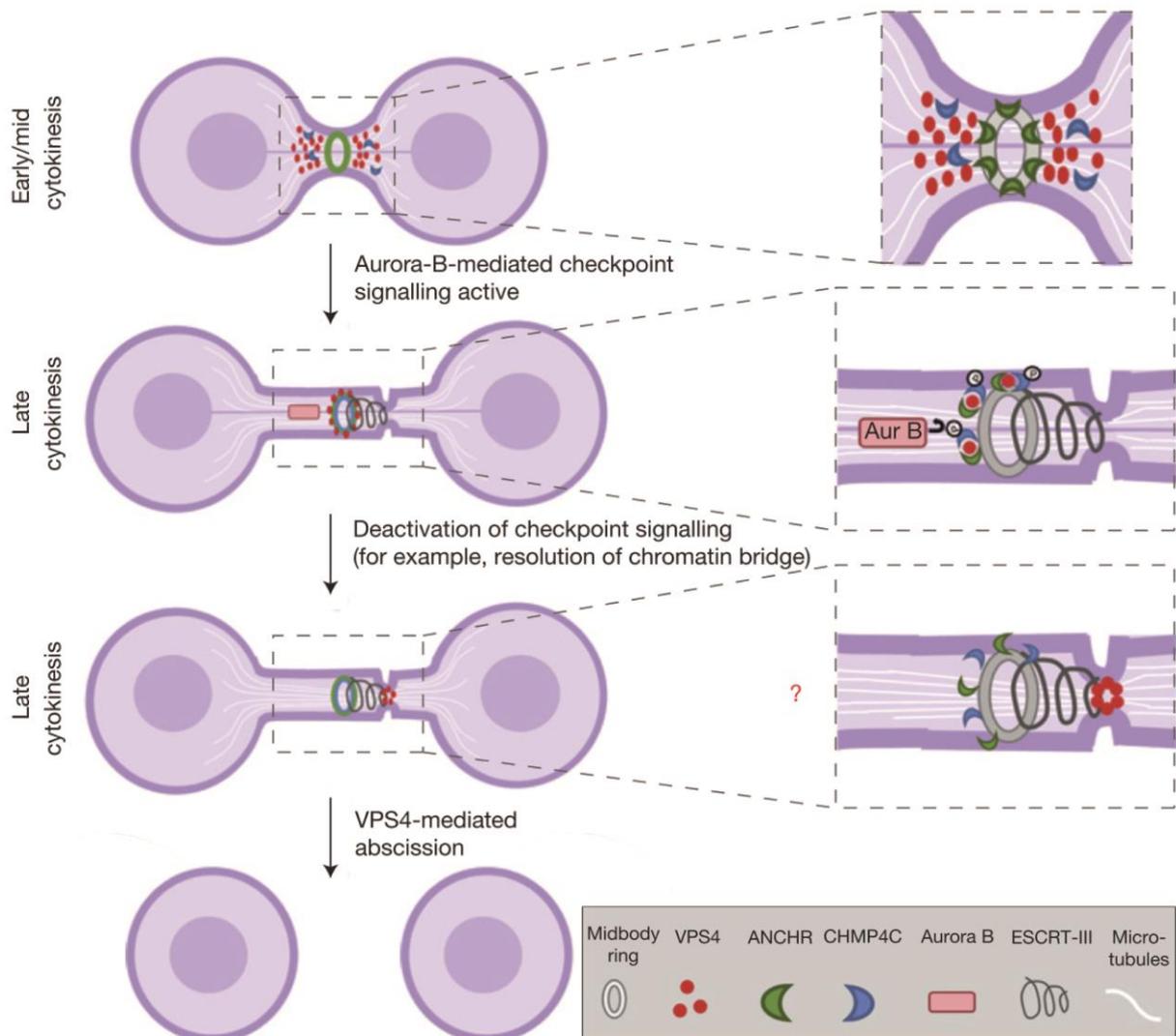
Aurora B was shown to be part of the NoCut checkpoint pathway, important to avoid premature abscission when lagging DNA is localized at the intercellular bridge.

Activated AurB, phosphorylated at T232, was reported to localize at the midbody ring when a chromosome bridge is passing through (Steigemann et al., 2009). This pool of AurB was seen to colocalize with CHMP4C, which when depleted results in the increase of Histone H2AX phosphorylation levels (Carlton et al., 2012).

The PtdIns3P-binding protein ANCHR (abscission/NoCut checkpoint regulator), was recently found to be part of this pathway, as its downregulation in HeLa cells resulted in increase of multinucleated cells. This function was demonstrated to be dependent on AurB (Thoresen et al., 2014).

Similarly to the ESCRT-III subunits, ANCHR possesses a MIM domain that is capable of binding to Vps4 MIT domain. Indeed, it was shown that, in cells with defective DNA segregation, ANCHR, Vps4, the ESCRT-III CHMP4C and AurB were all colocalizing at the central midbody. Interestingly, Vps4 was observed to be released from the midbody in cells with induced chromosome bridges and knocked down for either ANCHR or CHAMP4C.

Furthermore, Vps4 localization at the midbody was shown to be dependent on AurB activation. Thus, because AurB is known to phosphorylate CHMP4C, but not ANCHR, it was proposed that AurB promotes the formation of the ternary complex composed by ANCHR–CHMP4C–VPS4 through direct targeting of CHMP4C. Once the check-point is released and AurB inactivated, the pool of Vps4 sequestered at the midbody by ANCHR and phosphorylated CHMP4C, relocates to the midbody arms promoting abscission (Thoresen et al., 2014) (Fig36).



**Figure 36: A model for the ANCHR-mediated regulation of VPS4 on abscission checkpoint activation (NoCut).** At late cytokinesis, until abscission checkpoint signaling is terminated, ANCHR and CHMP4C retain abscission-competent VPS4 at the midbody ring. Upon inactivation of AurB, dephosphorylation of CHMP4C results in the dissociation of the ANCHR–CHMP4C–VPS4 ternary complex. This release could allow VPS4 to subsequently migrate to the abscission zone to mediate the final stages of abscission, through its effects on ESCRT-III. Image from (Thoresen et al., 2014)

## **CHAPTER V - OTHER FUNCTIONS OF THE ESCRT PROTEINS**

In the process of mammalian cells abscission, described above, only the ESCRT-I and ESCRT-III are thought to be important. Indeed, loss of function of ESCRT-0 and ESCRT-II did not result in any abscission phenotype. However, the endosomal sorting complex required for transport (ESCRT) comprises a lot more subunits and is involved in several distinct cellular functions (reviewed in (Rusten et al., 2012)).

### **1- ESCRT in multivesicular bodies biogenesis**

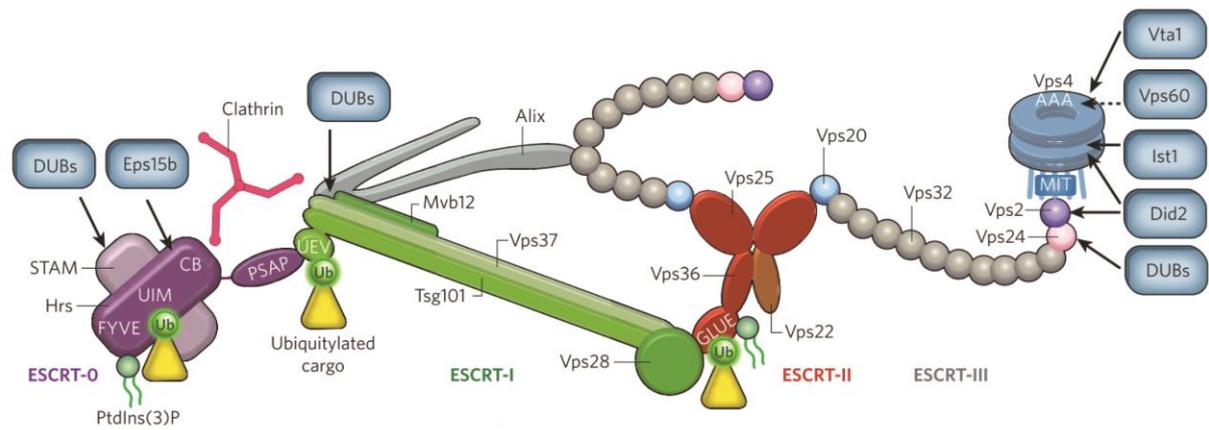
ESCRTs were first identified and well described in yeast for their function in endosomal sorting and multivesicular bodies (MVBs) biogenesis. Polyubiquitinated plasma membrane proteins, marked for lysosomal degradation, are endocytosed and translocated into early endosomes. Because these vesicles contain different types of proteins, these need to go through a sorting process. Cargoes such as nutrient receptors are recycled back to the plasma membrane. The ubiquitinated proteins are recognized by the ESCRT machinery, composed by ESCRT-0, ESCRT-I, ESCRT-II and ESCRT-III, which are recruited from the cytoplasm to the endosomal membrane in a sequential manner (reviewed in Raiborg (Henne et al., 2011; Raiborg, 2009) (Fig37). Here they promote the formation of vesicles that bud into the lumen of the maturing endosome, forming MVB. The primary lysosome then fuses with the MVB inducing the degradation of the cargoes.

The ESCRT-0 subunits Hrs and STAM, which interact via a coiled-coil domain, are the first to be recruited to the early endosome. Both proteins contain an ubiquitin-binding domain for cargo recognition, whereas Hrs also contains a FYVE domain, which specifically directs its binding to the endosomal lipid phosphatidylinositol 3-phosphate (PtdIns(3)P). The ESCRT-0 then recruits the ESCRT-I that is composed of four subunits, Tsg101, Vps28, Vps37 and Mvb12, which form an elongated heterotetramere with a long coiled-coil stalk and a globular head group. Tsg101 and Vps23, through their ubiquitin E2 variant domain (UEV), bind to the PSAP-like motifs of the ESCRT-0 Hrs. Through its headpiece, the ESCRT-I binds to

ESCRT-II. ESCRT-II complex is formed by one VPS36, one Vps22 and two Vps25, which adopt a Y-shaped structure. The Vps36 GLUE (GRAM-like ubiquitin-binding in EAP45) domain is responsible for the interaction with both the C-terminus of the ESCRT-I Vps28, and ubiquitin. It also interacts with PtdIns(3)P at the membrane and , together with the ESCRT-0 FYVE domain, provides endosomal localization specificity.

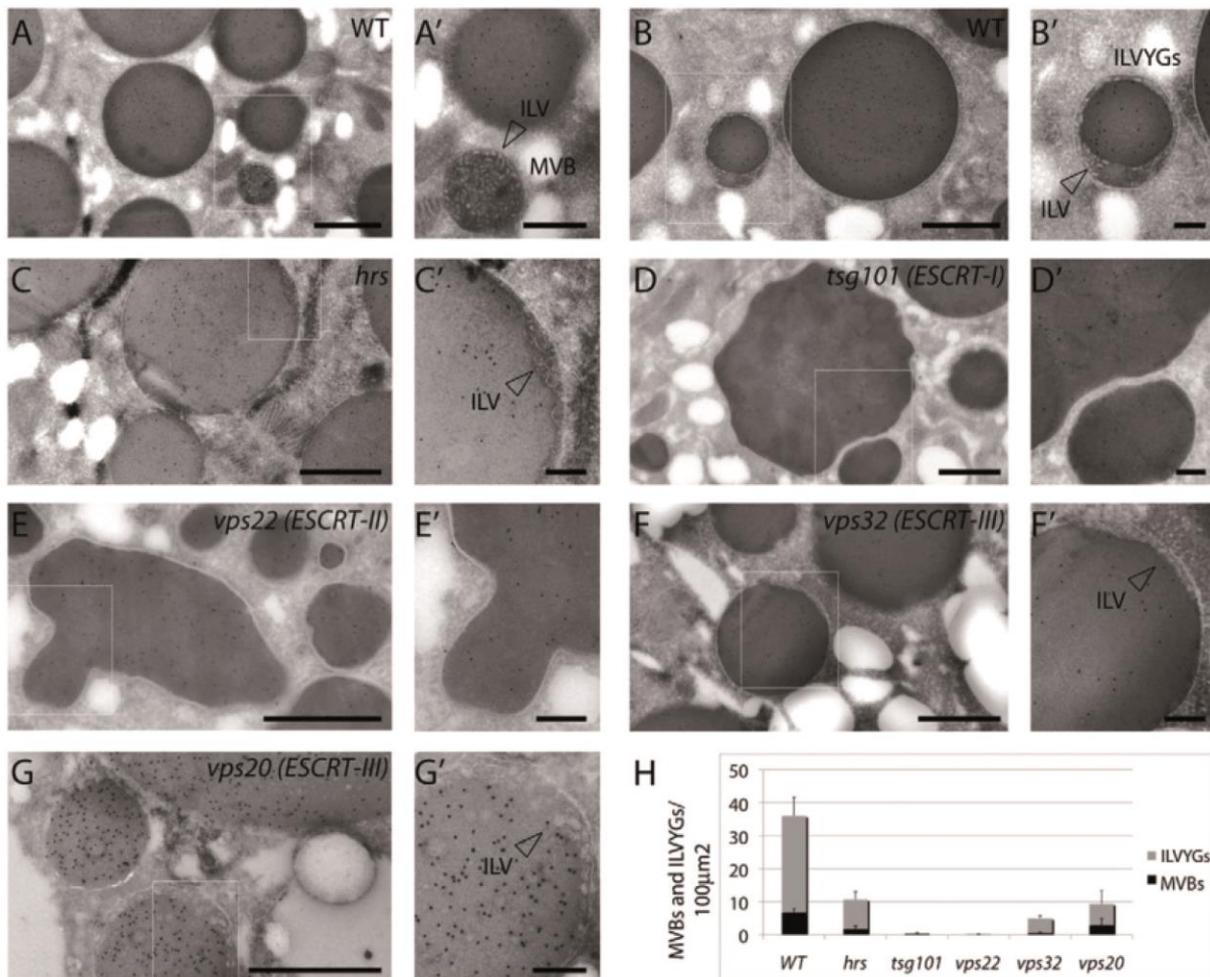
The ESCRT-III consists in 4 small subunits, Vps20, Vps32, Vps24, and Vps2 (in mammals CHMP6, CHMP4, CHMP3, CHMP2, and their isoforms). These localize as monomers in the cytoplasm, and are maintained inactive due to inhibitory interactions between their C- and N-terminus. They were described to assemble into higher-order multimers once targeted to the membrane. Indeed, Vps20, apart from being recruited and possibly activated by the ESCRT-II Vps25, it is also thought to directly interact with the endosomal membrane. Vps20 is the subunit responsible for the targeting of Vps32, the most abundant of the ESCRT-III (CHPM4 in human or Shrub in *Drosophila*). This is thought to polymerize into long filaments around the membrane, as described above. Vps32 recruits the adaptor protein Bro1/Alix (apoptosis-linked gene-2 interacting protein X), which stabilizes the filaments and promotes cargo deubiquitination, by recruiting Doa4 (degradation of alpha-4) (Luhtala and Odorizzi, 2004; Odorizzi et al., 2003). The Vps32 filaments are thought to be capped by the Vps24 subunit that then binds Vps2. This mediates the recruitment of the ATPase Vps4, which is in turn regulated by Ist1–Did2 and Vta1–Vps60. The model mechanism for the final step of endosomal membrane constriction and scission, mediated by the ESCRT-III and Vps4, would be very similar to the one already described for the cytokinetic abscission process.

Although in yeast, all the ESCRT components described above seem to work as a single functional unit to regulate MVB biogenesis, in mammalian cells and *Drosophila* that is not as clear. *Drosophila* oocytes mutant for *tsg101* (ESCRT-I) or *vps22* (ESCRT-II) have almost no endosomes containing ILVs. However, in mutants for *hrs* (ESCRT-I), *vsp32* or *vps20* (ESCRT-III) these structures, even if less abundant than in the WT, they can still be formed (fig38). It thus seems that only the ESCRT-I and ESCRT-II are absolutely required for MVB formation (Vaccari et al., 2009).



	Metazoan protein	Yeast protein	Interacting partners	Motifs/domains
ESCRT-0	Hrs	Vps27	PI3P, Ubiquitin, Tsg101, STAM, Clathrin	VHS, FYVE, DUIM, PxxP, CC, CB
	STAM 1,2	Hse1	Ubiquitin, DUBs, Hrs	VHS, UIM, SH3, CC
ESCRT-I	Tsg101	Vps23	Hrs, Alix, Ubiquitin, Cep55, Mvb12, Vps28, Vps27	UEV, PRR, CC
	Mvb12 A,B; UBAP1	Mvb12	PS, Tsg101, Vps37, Ubiquitin (yeast/UBAP1)	MABP, UMA
	Vps37 A,B,C,D	Vps37	Tsg101, Mvb12, Acidic Phospholipids	NTD, PRR
	Vps28	Vps28	Tsg101, Vps36	CTD
ESCRT-II	Vps36 (EAP45)	Vps36	PI3P, Ubiquitin, Vps28, Vps22, Vps25	GLUE, WH, NZF (yeast)
	Vps22 (EAP30)	Vps22	Acidic Phospholipids, Vps25, Vps36	HD, WH
	Vps25 (EAP20)	Vps25	Vps22, Vps36, Vps20	WH
ESCRT-III	CHMP6	Vps20	Vps25, Vps32, Vps4	MIM2
	CHMP4 A,B,C	Vps32 (Snf7)	Vps20, Vps24, Vps4, Alix, CHMP7, DUBs	MIM2
	CHMP3	Vps24	Vps32, Vps2, Vps4, Did2, PI3,5P2, DUBs	MIM1
	CHMP2 A,B	Vps2	Vps24, Vps4, Did2	MIM1
	CHMP1 A,B	Did2 (Vps46)	Ist1, Vps4, Vta1, Vps24, Vps2, DUBs, Spastin	MIM1
	Ist1	Ist1	Vps4, Did2, Vta1	MIM1, MIM2
	CHMP5	Vps60 (Mos10)	Vta1, Vps4	Unclassified MIM
	CHMP7	-	CHMP4B	MIM1, MIM2
Others	Vps4 A,B (SKD1)	Vps4	Vps20, Vps32, Vps24, Vps2, Did2, Vta1, Ist1, Vps60	MIT, AAA, $\beta$ -Domain
	LIP5	Vta1	Vps4, Vps60, Did2, Ist1, Vps20, Vps32, Vps24, Vps2	MIT, VSL
	Alix (AIP1)	Bro1 (Vps31)	Tsg101, Vps32, Cep55, DUBs	Bro1, PRR

**Figure 37: Composition and molecular interactions of the ESCRT machinery.** The figure is a composite of data obtained from studies of yeast and mammalian ESCRTs. Protein domains are labelled in white. CB, clathrin-box motif. Ub, ubiquitin. Dashed arrow indicates interaction predicted by genetic studies but not yet confirmed biochemically (Raiborg, 2009). Table listing the components of the ESCRT complexes and accessory factors, domains found in each factor and characterized binding partners. Adapted from (Schuh and Audhya, 2014).



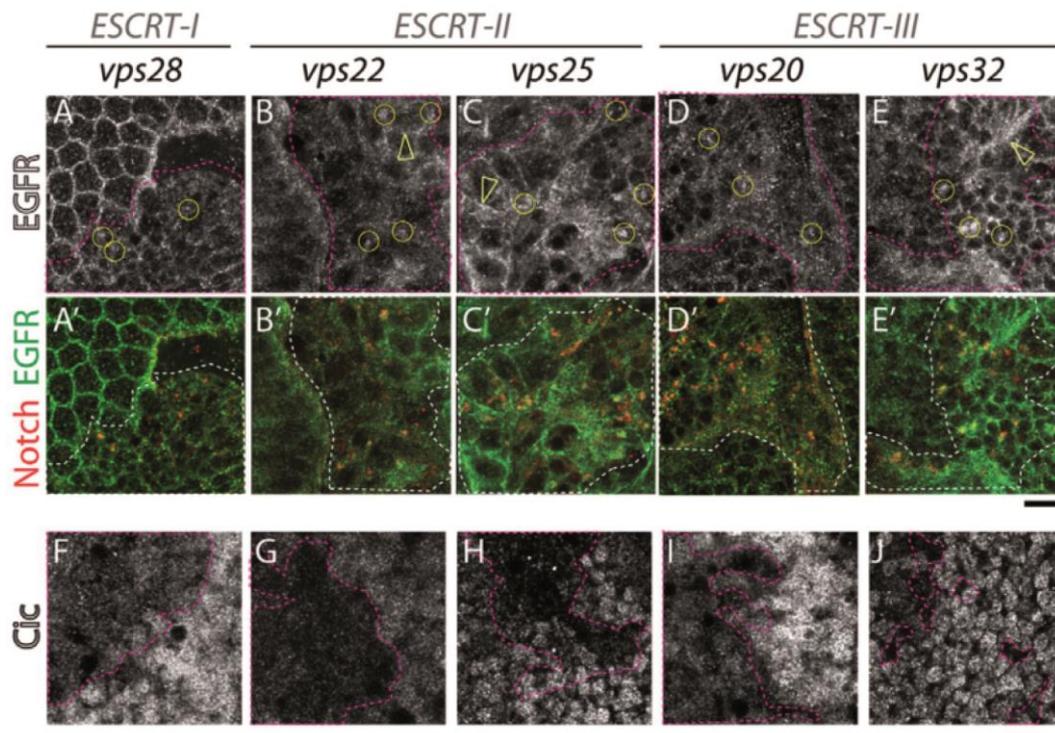
**Figure 38: Ultrastructural characterization of ESCRT-mutants.** ESCRT-I, ESCRT-II but not *hrs* or ESCRT-III are crucial for MVB biogenesis. **(A-G)** Yolk protein immunolabeling with 10 nm gold particles on cryosections of *Drosophila* oocytes. WT stage 9-10 oocytes show accumulation of yolk proteins in the yolk granules **(A)**. Isolated MVBs containing ILVs are also frequently observed in the ooplasm **(A')**. Additionally ILV-containing yolk granules (ILVYGs), derived from fusion with MVBs, are frequently observed **(B)**. Magnification **(B')** shows that ILVYGs contain ILVs. **(C)** In *hrs* (ESCRT-0) mutants, there is a similar phenotype, with presence of isolated MVBs and ILVYGs, although they are less abundant than in WT oocytes (arrowhead in **C'** indicates ILV). **(D)** In the *tsg101* (ESCRT-I) mutants, the yolk granules still accumulate with large diameters; however, ILVYGs and isolated MVBs are absent. *vps22* (ESCRT-II) mutant oocytes **(E)** also lack ILVYGs and isolated MVBs. **(D'-E')** Magnifications show lack of ILVs. Compared with WT, the *tsg101* and *vps22* yolk granules are irregularly shaped. **(F,G)** By contrast, the *vps32* and *vps20* (ESCRT-III) mutant oocytes possess regularly shaped ILVYGs as well as isolated MVBs, which are more similar to *hrs*. Scale bars: 1 µm **(A-G)**, 0.25 µm **(A'-G')**. **(H)** Quantification of the number of ILVYGs and MVBs per 100µm<sup>2</sup> in WT and mutant stage 10 ovaries. The number of ILVYGs and MVBs formed in the ESCRT-III mutants analyzed is similar to that of *hrs* mutant. By contrast, ESCRT-I and ESCRT-II mutant oocytes contains almost no ILVYGs and MVBs (Vaccari et al., 2009).

## **2- ESCRT in signaling regulation**

One of the ESCRT mutant phenotypes most studied in flies is the deregulation of signaling pathways important for development. The constant turnover of the plasma membrane receptors for signaling is dependent on the endosomal sorting pathway, where the ESCRT proteins are thought to play a crucial role. Indeed, mutations in ESCRT components result in the accumulation of receptors at the membrane of maturing endosomes. Instead of being sequestered into intraluminal vesicles (ILVs) to shut down the signal, their intracellular domain remains in contact with the cytoplasm. As a consequence, the signaling pathway is maintained over-activated and the developmental process deregulated. Even though the *Drosophila* ESCRT mutants resemble one another in several phenotypes, they impact differently on different signaling pathways: EGFR, Dpp, Wg and Notch (Vaccari et al., 2009).

### **a) EGFR signaling**

In *Drosophila* eye discs mutant for ESCRT-I (vps28), ESCRT-II (vps22 or vps25) or ESCRT-III (vps20, vps2 or vps32 (Shrb)) EGFR gets accumulated in endosomes with high levels of ubiquitinated proteins, suggesting that its degradation is reduced. This accumulation seems to be enough to promote EGFR signaling activity, as *capicua*, whose expression levels are downregulated by the pathway, is lower in the mutant cells. The oversignaling phenotype of the hypermorphic EGFR allele *ElpB1* is enhanced by the ESCRT mutations. Thus, this work showed that the ESCRT complexes -I, -II and -III are all required to downregulate EGFR signaling (Fig39) (Vaccari et al., 2009). It was previously reported that EGFR accumulates in endosomal compartments also in ESCRT-0 Hrs mutants (Jekely and Rorth, 2003).

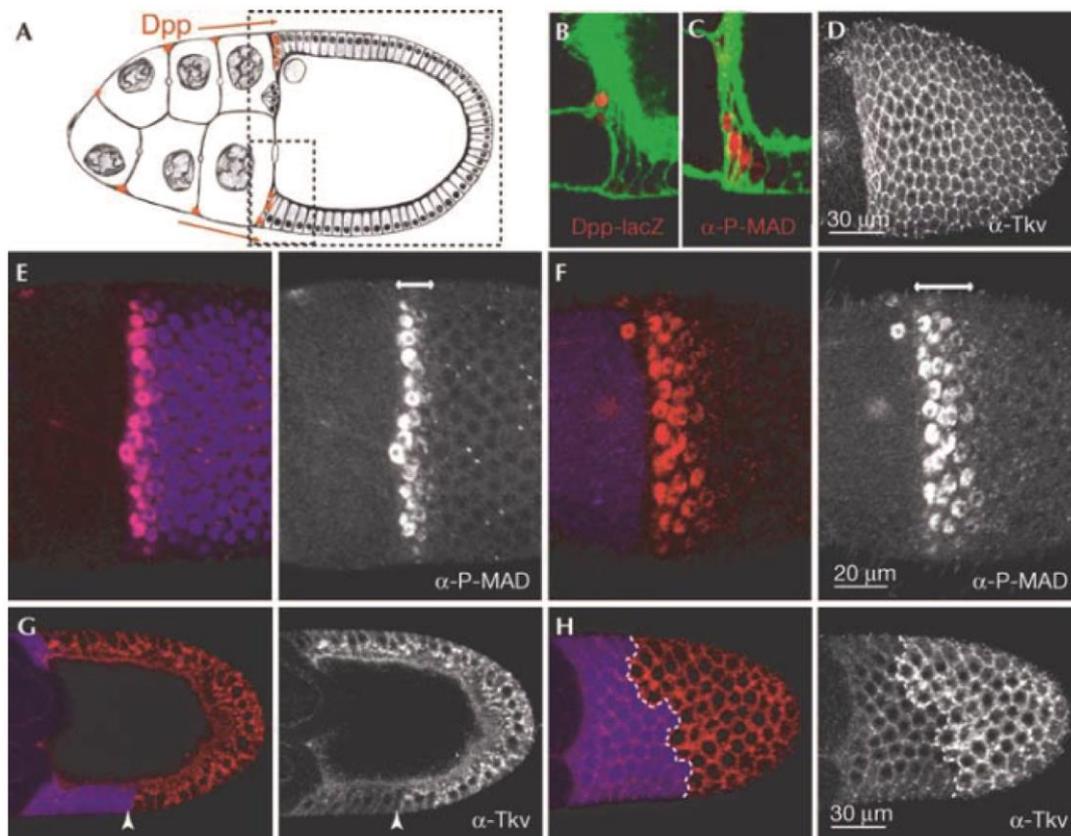


**Figure 39: EGFR accumulation and EGFR signaling is overactivated in ESCRT mutants. (A-E')** ESCRT-I, ESCRT-II and ESCRT-III mosaic eye discs stained with anti-EGFR (**A-E**) and Notch (merged in **A'-E'**); Compared with WT tissue, in mutant tissue (delimited by pink dashed lines) EGFR accumulation is seen in intracellular puncta (examples are encircled in **A-E**) that are Notch positive. Note that compared with Notch, the majority of EGFR is still detected in other cellular areas (arrowheads in **B,C,E**). (**F-J**) ESCRT-I, ESCRT-II and ESCRT-III mosaic eye discs stained with an antibody to detect expression of Capicua (Cic), a nuclear negative regulator of EGFR signaling. Compared with WT tissue, Cic expression is reduced in ESCRT mutant cells (delimited by pink dashed lines), indicating higher levels of EGFR signaling activation (Jekely and Rorth, 2003).

## b) Dpp signaling

The impact of Hrs on Dpp pathway was also analyzed (Jekely and Rorth, 2003). The Dpp receptor Tkv is expressed in all follicular epithelium of stage 10 egg chambers, while the Dpp source comes from the anterior side and induces expression of P-Mad in only 1-2 adjacent rows (Fig40). It was shown that Hrs was important to promote the degradation of Tkv, whether it was in its activated form or not, as clones in or outside the Dpp signaling range gave rise to the same levels of receptor accumulation. However, signaling overactivation was ligand dependent, as in Hrs mutants for all the follicular epithelium P-Mad expression

domain was only expanded to 3–4 rows adjacent to the Dpp source (Jekely and Rorth, 2003). Another study showed that the ESCRT-II Vps25 is also important to downregulate Tkv and shut down Dpp pathway in the developing leg (Thompson et al., 2005). The dorsal/ventral axis of the leg imaginal disc is organized by two antagonistic signals: Dpp, acting dorsally, and Wingless (Wg) that acts ventrally. Cells from the ventral side, mutant for Vps25, have the same phenotype of those expressing an activated form of Tkv, causing outgrowth from the ventral side of the leg (Thompson et al., 2005).



**Figure 40: Role of Hrs in Dpp signalling and Tkv trafficking in follicle cells.** (A) Schematic cross-section of stage 10 egg chamber. A small and a large stippled box indicate areas shown in (B, C) and (D, G, H), respectively. (D, H) Surface views of the epithelium, as are (E) and (F). Anterior follicle cells (red in (A)) express Dpp and Dpp-lacZ (see also (B)). P-MAD staining visualizes Dpp signalling activity in the cells expressing Dpp and in adjacent cells (C). The Dpp receptor Tkv is detected in all follicle cells (D). Wild-type (C, D) and hrs mosaic (E–H) stage 10 egg chambers stained with antibodies (red in double-staining) against b-galactosidase (B), P-MAD (C, E, F) or Tkv (D, G, H). (B) and (C) are also stained with phalloidin (green). Mutant cells are marked by the absence of GFP (blue) in (E–H). The boundary between hrs mutant and wild-type cells is also indicated with an arrowhead (G) or a dashed line (H). In (E) no follicle cells are mutant, and in (F) all follicle cells are mutant for hrs. The double-headed arrow (E, F) indicates the P-MAD positive domain, which is expanded when cells are mutant for hrs (Jekely and Rorth, 2003).

### **c) Wg signaling**

The processes of endocytosis and MVBs biogenesis were demonstrated to promote Wg signaling (Seto and Bellen, 2006). In mammalian cells, this was reported to be achieved indirectly by the sequestration of Glycogen synthase kinase  $3\beta$  into ILVs, inhibitor of  $\beta$ -catenin, in a process dependent on Hrs and Vps4 (Dobrowolski et al., 2012; Taelman et al., 2010).

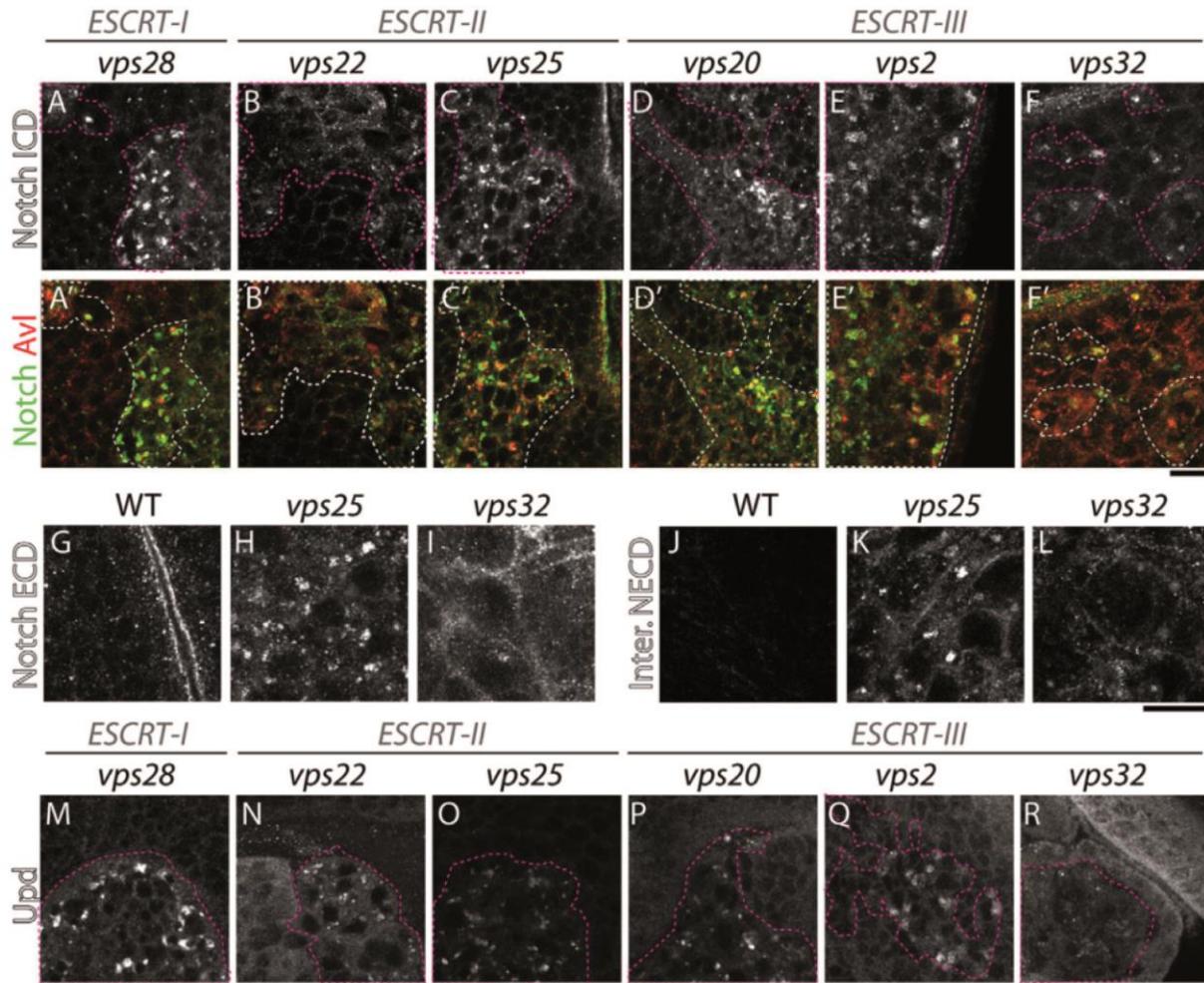
### **d) Notch signaling**

The impact of ESCRT proteins on Notch signaling pathway has also been analyzed in *Drosophila*. Cells from the imaginal disc epithelium, mutant for the ESCRT-II vps25, were reported to promote growth of WT neighboring cells. In vps25 mutant cells, Notch receptor was accumulated at the endosomal membrane resulting in its activation. This induced the ectopic expression of unpaired (Upd), which when secreted activated JAK-STAT signaling pathway in the neighboring cells and consequent growth (Herz et al., 2006; Vaccari and Bilder, 2005).

Mutants for vps28 and tsg101 (ESCRT-I), vps22 (ESCRT-II), vps20, vps32 and vps2 (ESCRT-III) also gave rise to no-autonomous growth of epithelial tissue. Similarly to vps25 mutant cells, Notch receptor was found to be accumulated in endosomes, together with an increase of ubiquitinated proteins (Moberg et al., 2005; Thompson et al., 2005; Vaccari et al., 2009). Surprisingly, Shrb (Vps32) displayed lower amounts of Notch in endosomes, which appeared to be distributed to the cell cortex. This suggests that Shrb could also function during Notch endocytosis, or that some Notch could escape endosomal trapping. Consistent with this observation, the levels of ectopic Upd expression in shrb mutant cells are lower than in the other ESCRT mutants analyzed (Fig 41) (Vaccari et al., 2009).

Even though, Notch accumulation in endosomes was also observed in mutants for the ESCRT-0 Hrs and Stam, neither Notch activation nor overgrowth resulted from it (Jekely and Rorth, 2003; Thompson et al., 2005; Tognon et al., 2014; Vaccari and Bilder, 2005). Thus, it

seems that ESCRT-I, ESCRT-II and ESCRT-III components, but not Hrs, are required to prevent ectopic Notch signaling activation in *Drosophila*.



**Figure 41: Notch accumulates in endosomes of ESCRT mutant cells leading to overactivation of signaling. (A-F)** ESCRT-I, ESCRT-II and ESCRT-III mosaic eye discs stained with an antibody recognizing the intracellular domain of Notch. Compared with WT tissue, Notch is depleted from the cellular surface and accumulates in enlarged endosomes (labeled Avl in **A'-F'**) in all ESCRT mutants except vps32 mutants, in which less Notch accumulates (**F**). (**G-L**) Notch localizes in intracellular puncta in vps25 mutants and is diffuse in vps32 mutants. Notch immunodetection (Notch ECD; **G-I**) and 5 hour Notch internalization assay (inter. NECD; **J-L**) using antibodies directed to the extracellular portion of Notch in WT (**G,J**), vps25 (**H,K**) and vps32 (**I,L**) entirely mutant eye disc tissue. In vps32 mutants, Notch accumulation in intracellular puncta is reduced, whereas cortical localization is increased compared with vps25 (especially visible on fixed tissue in **I**). Note that in **J**, very little signal is present because most Notch is degraded 5 hours after labeling. (**M-R**) ESCRT-I, ESCRT-II and ESCRT-III mosaic eye discs stained with an antibody to detect expression of the Notch target unpaired (Upd). Compared with WT tissue, Upd is ectopically expressed to varying degrees in mutant cells. Notably vps32 mutants appear to express the lowest amounts of Upd. Mutant tissue is encircled by dashed pink lines in **A-F**, **M-R** (white in **A'-F'**).

### 3- ESCRT and Lethal (2) giant discs (Lgd)

In normal cells, the Notch receptor is activated by the binding of the ligand (Delta or Serrate) to its extracellular domain (NECD). After this activation, Kuzbanian (Kuz) promotes the shedding of ectodomain of the receptor through cleavage (S2) and removal of the NECD. The intermediate NEXT (Notch extracellular truncation) is then cleaved (S3) by the  $\gamma$ -secretase, releasing the intracellular domain (NICD) into the cytosol. This goes to the nucleus and binds to the transcription factor Suppressor of Hairless (Su(H)), which induces expression of the target genes.

Recent work suggested that Notch could be activated through  $\gamma$ -secretase mediated cleavage at the endosomal membrane, in a ligand independent manner (Vaccari et al., 2008). In ESCRT-I, -II and -III mutants, which increase receptor retention in the endosome,  $\gamma$ -secretase cleavage and consequent Notch activation were enhanced (Vaccari et al., 2008).

The tumour suppressor gene lethal (2) giant discs (lgd) has been described to regulate the activity of the Notch pathway in *Drosophila*, and its loss of function results in phenotypes very similar to those of the ESCRT mutants: MVBs with less ILVs than the WT, receptor accumulation, Notch signaling activation and overgrowth. The Lgd protein is composed of four repeats of a DM14 domain, with unknown function, followed by one C2 domain in its C-terminus. C2 domains have been described to be calcium and lipid binding domains, able to mediate membrane recruitment, protein-protein interaction, protein localization and trafficking. Even though Lgd was reported to bind phospholipids in vitro (Gallagher and Knoblich, 2006), it does not associate with membranes in vivo (Childress et al., 2006; Gallagher and Knoblich, 2006; Jaekel and Klein, 2006; Troost et al., 2012). Instead, the C2 domain seems to provide protein stability and direct Lgd localization to the cytoplasm, as mutants where the C2 domain was deleted had lower amounts of the protein and localized in the nucleus. When a NES (nuclear export cassette) was added to the C2 deleted construct, it was able to rescue the null mutant. On the contrary, deletion of the DM14 domains did not have the same rescue effect, suggesting that these provide the Lgd-specific function (Troost et al., 2012). Indeed, Lgd was shown to be able to directly bind Shrb (GST-pulldown assays), through its DM14 domains (Troost et al., 2012). This cytoplasmic interaction with Shrb is

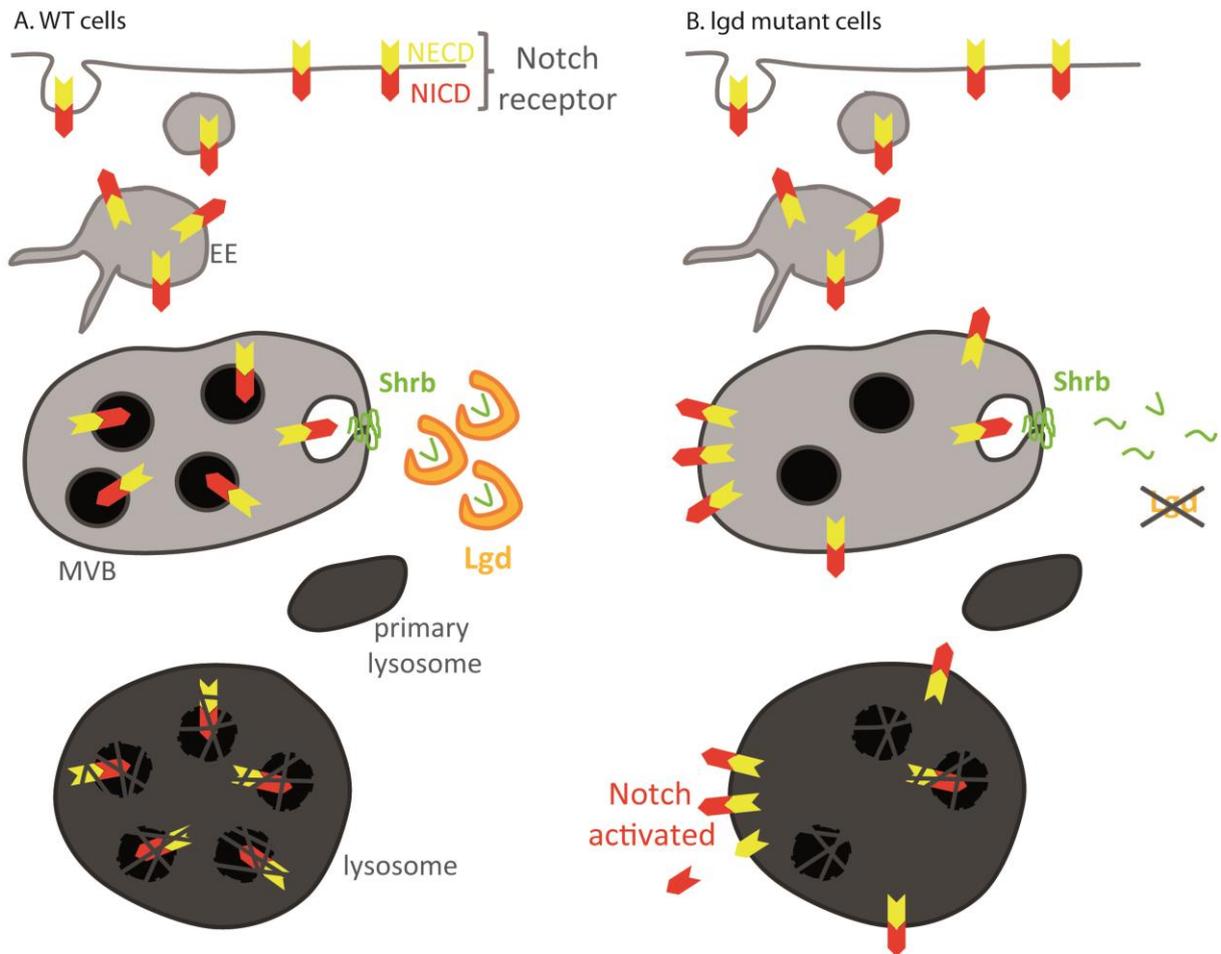
thought to stabilize Shrb monomers and facilitate its activation. However, in mammals it was reported that CC2D1A (Lgd) inhibits CHMP4B (Shrb) polymerization in vitro through the interaction of its third DM14 domain with the helical hairpin of the CHMP4B (Martinelli et al., 2012). An interesting observation was made when the removal of one copy of shrb, rescued Notch overactivation in lgd null mutant cells. The same rescue was observed with the downregulation of the ESCRT-III vps20 (Troost et al., 2012). However, the dramatic enlargement of maturing endosomes in the rescued cells, show that both genes act in concert with respect of endosome morphology. A possible explanation came with the observation that these enlarged endosomes had a great reduction in the protein Rab7, compared to the lgd single mutant cells (Schneider et al., 2013). Rab7 is known to control the fusion of the MVB with the primary lysosome, strongly suggesting that this fusion must occur to actually promote Notch activation. In agreement, downregulation of the proton pump vATPase, necessary to acidify the endosomal lumen and activate the acidic hydrolases coming from the lysosome, prevents Notch ectopic activation in lgd mutant cells (Schneider et al., 2013). Another interesting observation is that the ESCRT-0 Hrs is also able to rescue Notch activity in lgd mutant cells (Childress et al., 2006; Troost et al., 2012). This may be due to Hrs function in concentrating the receptor in the regions of ILV formation (Childress et al., 2006; Troost et al., 2012).

With these experiments, a model for how lgd mutant cells could induce Notch ectopic activation in a ligand-independent manner, was proposed (Fig42) (Schneider et al., 2013). Notch receptor would be normally endocytosed, targeted to the maturing endosomes and the ESCRT-0 Hrs would promote its clustering at the ILV formation sites. However, because the loss of Lgd induces a decrease in Shrb activity, Notch would not be efficiently trapped into the ILV. As a consequence, it would accumulate at the membrane of the MVB. Rab7 mediated fusion of the MVB with the primary lysosome would promote the activation of the hydrolases by the vATPase, which in turn induces an alternative ectodomain shedding of the receptor. This creates a NEXT-like complex that is recognized and cleaved by the  $\gamma$ -secretase present at the membrane of the lysosome. The NICD would be released into the cytosol, activating Notch target genes. The lgd mutant cells, when heterozygous for shrb, lack the protein Rab7 at their maturing endosomes. This would prevent the fusion of the MVB with the lysosome and Notch pathway would not be activated.

A surprising observation in this study was that in *shrb* single mutant cells, the activation of Notch does not require the fusion with the lysosome, as it is not affected by depletion of Rab7. It thus seems that *lgd* and *shrb* single mutant cells induce Notch activity through different mechanisms (Schneider et al., 2013).

Very interestingly, Notch ectopic activation in *lgd* mutant cells was rescued by the overexpression of other membrane proteins (UAS-*tkv*-GFP, UAS-CEN and UAS-T48-GFP). This suggests that the process of endosomal sorting required for Notch activation in *lgd* cells has a limited capacity (Schneider et al., 2013).

Recent work reported a role for Vps4 in controlling signaling, independently of its function in receptor degradation (Legent et al., 2015). Similar to other ESCRT components, *vps4* mutant cells accumulate several signaling receptors in enlarged endosomes. As expected for its function in ILV formation, mutant cells presented an increased Dpp signaling and decreased Wg signaling (described above). However, a reduction on EGFR and Notch signaling was observed in these cells. These surprising results could be explained by the fact that Vps4 is the last molecule acting in the formation of ILV. In the absence of Vps4, the membrane scission of the newly formed ILVs would be blocked. The receptors would somehow get trapped in these semi-closed vesicles, which would inhibit the access of the intracellular domain of the receptor to the cytoplasm and signaling would be blocked (Legent et al., 2015).



**Figure 42: Model of activation of Notch in lgd cells.** **A-** Notch degradation in WT cells. Lgd interacts with Shrub in the cytosol, promoting full activation of Shrub. All Notch at the endosomal membrane is incorporated in ILVs and then degraded after fusion of the MVB with the lysosome. **B-** Activation of Notch in lgd mutant cells. Loss of lgd function results in a reduction in activity of Shrub. As a consequence, a fraction of Notch receptor remains at the limiting membrane of the endosome. Upon fusion with the lysosome the activated hydrolases, possibly in combination with the acidic environment, perform alternative ectodomain shedding. This creates a NEXT-like substrate for the  $\gamma$ -secretase complex, which releases NICD into the cytosol activating Notch signaling pathway. NECD, Notch extracellular domain; NICD, Notch intracellular domain; EE, early endosome; MVB, multivesicular body. Adapted from (Schneider et al., 2013).

#### **4- ESCRT in plasma membrane fission**

A new function for the ESCRT machinery in *Drosophila* was recently reported. The ESCRT-I and -III, but not the ESCRT-0 and -II, are necessary to promote neurite scission during pruning (Loncle et al., 2015). Both the topology of the neurite severing, as well as the 'ESCRT pruning module', are very similar to those of the cytokinetic abscission (described above). However the Alix accessory protein, important for abscission, seems to be replaced by Myopic in neurite scission. Myopic, like Alix, was shown to directly bind Shrb in vivo (Loncle et al., 2015).

The mammalian process of viral budding is also very similar to the cytokinetic abscission, as it depends on ESCRT-I and -III, but also Alix (Carlton and Martin-Serrano, 2007; Morita et al., 2007) (Martin-Serrano et al., 2003).

Another function of ESCRT proteins in mediating budding of the plasma membrane was recently described. In mammalian cells, CHMP4B, CHMP2A and Vps4, were shown to be required for wound repair, promoting membrane budding and shedding (Jimenez et al., 2014).

#### **5- ESCRT in surveillance of nuclear pore complexes formation**

In budding yeast, ESCRT proteins were recently reported to have a role in the biogenesis of functional nuclear pore complexes (NPCs) (Webster et al., 2014). In this work, the yeast Vps32/Snf7 ESCRT-III protein was shown to physically interact with Heh2, member of the Lap2-emerin-MAN1 family. Heh2 localizes to the inner nuclear membrane (INM) and is thought to function in the surveillance of the NPC biogenesis, by binding to early NPC assembly intermediate, but not to mature NPCs. Bifunctional complementation studies, where N- and C-terminal domains of Venus were fused to Heh1/2 and Snf7 respectively, showed that these two proteins were able to bind in vivo. This interaction was confirmed biochemically when stabilized Snf7 was detected to bind affinity purified Heh2. Heh2 was

also shown to interact with Vps4, but not with Vps20 (ESCRT-III) or Vps23 (Tsg101, ESCRT-I). Because the known adaptor proteins responsible for the recruitment of ESCRT-III in other systems (ESCRT-II and Alix) were not shown to be important in this process, it suggests that Heh2 might take the function in the INM. Functional studies described mutants for vps4, snf7 and heh2 with similar phenotypes. Nup49, Nup protein part of the NPC, normally localizes in a punctate pattern. However in some of the mutant cells it was seen accumulating in a single cluster, forming a plaque on the nuclear envelope. EM images from vps4 mutant clusters demonstrated that they contained NUPs, with a variable relative enrichment that did not match the expected stoichiometry. This suggests that the clusters were formed by defective NPCs. The authors term these structures as SINC for storage of improperly assembled nuclear pore complexes. Interestingly, the authors showed that the SINC had a strong tendency to be retained in old mother cells. This mechanism is thought to act as a quality control system, preventing the passage of malformed NPCs to the progeny and thus ensuring that nuclear compartmentalization is maintained (Webster et al., 2014; Webster and Lusk, 2015).

Even though this seems to be an important new function in yeast, in mammalian cells disruption of ESCRT-III function do not affect markers for general NE reassembly or nuclear pore complex accumulation (Vietri et al., 2015).

## **6- ESCRT in nuclear envelope sealing**

During cell division, nuclear envelope (NE) breaks down to allow the microtubules from the mitotic spindle to capture the separating chromosomes, and reassembles once the chromosomes have separated. Vietri et al, have recently demonstrated that the ESCRT proteins have a key role in the coordination of nuclear envelope sealing and microtubules disassembly in mammalian cells (Vietri et al., 2015). The authors showed that the ESCRT-III CHMP4B and Vps4 transiently localized at chromatin discs during late anaphase (Fig43). Other ESCRT-III subunits (CHMP2A, CHMP3 and CHMP4A) and associated proteins (CHMP1A, CHMP1B and IST1) were also recruited around chromatin discs. Furthermore, correlative

light and electron microscopy (CLEM), as well as structured illumination microscopy (SIM) analysis, showed that CHMP4B was localized in regions of the NE still open, and that these seem to correspond to contact sites between MTs and chromatin.

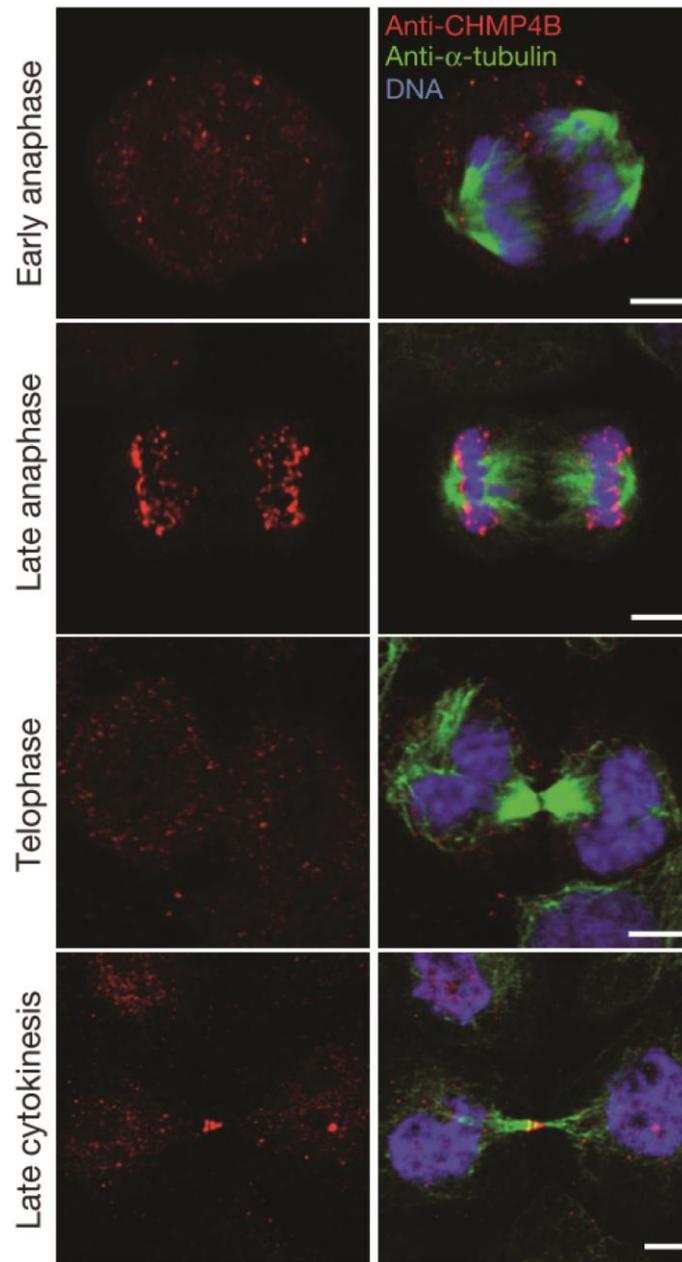
The fact that ESCRT-III were already shown to recruit the microtubule-severing enzyme, spastin, in cytokinetic abscission (Yang et al., 2008), suggested a function in MT disassembly also in this process. In agreement, mutants for CHMP2A, resulted in the depletion of spastin from the NE and consequent delay in MT disassembly. In these cells a persistence of CHMP4B enrichment was also observed.

The model presented by the authors proposes that during NE reformation, holes in the membranes are formed in sites where the MTs are still in contact with chromatin. ESCRT-III components, together with Vps4, would be then recruited to these sites. Here they would, on one hand recruit Spastin to sever the MTs, and on the other hand seal the membrane.

Interestingly, proteins already described to be important for ESCRT-III recruitment in other processes (CEP55, ESCRT-0 (Hrs), ESCRT-I (TSG101), ALIX...), do not seem to act on NE sealing. A poorly characterized ESCRT-III-like protein, CHMP7, was shown to be the one essential for the recruitment of CHMP4B (Vietri et al., 2015).

Furthermore the group of Carlton, who described the same role for the ESCRT-III in remodeling NE membrane at the sites of annular fusion, reported the function of UFD1 in their recruitment (Olmos, 2015). UFD1 is an adaptor protein of the p97 AAA-ATPase, responsible for the control of NE reformation. The authors showed that UFD1 was able to bind the ESCRT-III CHMP2A, by yeast two hybrid assay. Cells depleted for UFD1 had the recruitment of CHMP2A to the forming NE impaired, while its localization at the midbody was normal (Olmos et al., 2015).

It thus seems to be now clear that different adaptor proteins are responsible for the recruitment of ESCRT-III proteins for different cellular compartments.



**Figure 43: ESCRT-III is transiently recruited around chromatin discs during nuclear envelope reformation.** Confocal images of fixed HeLa cells in different phases of mitotic exit. Scale bars, 5  $\mu$ m. Representative confocal images of at least five captures for each stage (Vietri et al., 2015).

## INTRODUCTION TO MY PhD PROJECT

Cell division has been one of the most extensively studied processes in cell biology.

Cells in culture present many advantages to study cell division, as they can be synchronized and obtained in large quantities, allowing biochemical and cellular approaches. However, in recent years, it became clear that cell division is developmentally regulated and can vary between organisms and cell types, within the same organism. Thus, several studies and new model systems have been developed to try to dissect the different layers of developmental regulation of this process.

In our laboratory we have been interested in understanding the process of abscission in the context of *Drosophila* oogenesis. We believe that our *in vivo* model has several advantages for the study of abscission.

1) In the germarium, two types of cytokinetic abscission can be identified. The germline stem cell (GSC) divides asymmetrically with a delayed but complete abscission, and we used tools to identify the time of the final membrane scission. Later, cells forming the cyst go through 4 rounds of mitosis with incomplete cytokinesis. In these cells, abscission is completely inhibited and they stay connected by ring canals, reminiscent of the arrested contractile rings. The presence in the same developmental structure of two distinct cell types, with different abscission fates, is very useful in the search for abscission regulators. The analysis of both compartments individually may facilitate the identification of both promoting and inhibiting abscission factors.

2) The profound knowledge of this model system by our group leader, Jean-René Huynh, is a great help during the analyses of complex germline phenotypes. Indeed, years of experience in looking at different germline mutant phenotypes, recently allowed the identification of key players in the process of GSC abscission (Mathieu et al., 2013). Mutants that modify the number of germ cells in the cyst, phenotype normally associated with the regulation of the

number of mitotic divisions during cyst formation, were demonstrated to instead act earlier in the abscission of the GSC. Careful examination of the morphology and disposition of the cells in the germarium resulted in the identification of a structure never described before. This structure, named the stem-cyst, consisted in a chain of cells connected to the GSC by intercellular bridges, harboring a branched fusome similar to the mitotic cysts. The characterization of these stem-cysts allowed the identification of the origin of the phenotype and the conclusion that, in these mutants, GSC abscission was delayed.

Mathieu et al. identified thus, for the first time, GSC abscission timing regulators in the germline. The chromosome passenger complex (CPC), where Aurora B is the kinase, was shown to negatively regulate GSC abscission, while Cdk1/CycB acts to promote it (explained in detail on the Chapter III of the introduction).

3) The access to the vast number of tools developed in *Drosophila*, allows to perform functional genetic assays, not possible in other model systems. The vast collections of RNAi lines, combined with the numerous expression drivers, enable the downregulation of specific genes of interest, at different developmental timings, and in distinct compartments. Also, the generation of homozygous mutant clone cells, in a heterozygous background fly, allows the analyses of protein null phenotypes, otherwise lethal in the adult.

Taking advantage of these characteristics, our lab developed a pilot screen to better understand the process of abscission in the *Drosophila* female germline. Several transgenic flies expressing shRNA targeting kinases, phosphatases and regulators of membrane trafficking were chosen from the TRiP collection (Ni et al., 2011). These RNAi constructs, designed to be efficient in germline, were specifically expressed in germ cells using a nanos-GAL4 driver. Because previous work of the lab described the specific phenotypes expected when impairing or promoting abscission, the mutants could easily be scored. Downregulation of proteins involved in promoting abscission should result in egg chambers made of 32 germ cells and have stem-cysts present in their germaria. Impairment of proteins

inhibiting abscission would induce the formation of cysts with 8 germ cells, due to induced abscission in 2-cell cysts.

Even though other genes that seem to act in abscission were identified in this screen, my work focused on one of the proteins that resulted in the highest formation of 32-cell cysts and stem-cysts. This is the ESCRT-III protein *Shrb*, the single *Drosophila* homolog of the mammalian CHMP4. As described in the introduction, CHMP4 proteins are known to either promote, or delay abscission in culture cells. However, no functional studies, regarding abscission, were published with the fly homolog.

Thus, my work consisted in the detailed description and characterization of the abscission phenotype of *shrb*, in the *Drosophila* female germline.

The localization of *Shrb*-GFP during GSC division was also analyzed and compared with the observed in mammalian cells. To explore possible regulatory mechanisms for *Shrb* in this model system, genetic interaction studies were performed.

This work was published on the 3<sup>rd</sup> of February, 2015, in the journal PLOS Genetics. The title of this work is: Abscission is regulated by the ESCRT-III protein *Shrub* in *Drosophila* germline stem cells.

# RESULTS

## ARTICLE

**Matias, N. R., Mathieu, J., Huynh, J. R.** (2015). "Abscission is regulated by the ESCRT-III protein shrub in *Drosophila* germline stem cells." PLoS Genet 11(2): e1004653.

RESEARCH ARTICLE

# Abscission Is Regulated by the ESCRT-III Protein Shrub in *Drosophila* Germline Stem Cells

Neuza Reis Matias<sup>1,2</sup>, Juliette Mathieu<sup>1,2\*</sup>, Jean-René Huynh<sup>1,2\*</sup>

**1** Department of Genetics and Developmental Biology, Institut Curie, Paris, France, **2** CNRS UMR3215, Inserm U934, Paris, France

\* [juliette.mathieu@curie.fr](mailto:juliette.mathieu@curie.fr) (JM); [jean-rene.huynh@curie.fr](mailto:jean-rene.huynh@curie.fr) (JRH)



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**Competing Interests:** The authors have declared that no competing interests exist.

## Abstract

Abscission is the final event of cytokinesis that leads to the physical separation of the two daughter cells. Recent technical advances have allowed a better understanding of the cellular and molecular events leading to abscission in isolated yeast or mammalian cells. However, how abscission is regulated in different cell types or in a developing organism remains poorly understood. Here, we characterized the function of the ESCRT-III protein Shrub during cytokinesis in germ cells undergoing a series of complete and incomplete divisions. We found that Shrub is required for complete abscission, and that levels of Shrub are critical for proper timing of abscission. Loss or gain of Shrub delays abscission in germline stem cells (GSCs), and leads to the formation of stem-cysts, where daughter cells share the same cytoplasm as the mother stem cell and cannot differentiate. In addition, our results indicate a negative regulation of Shrub by the Aurora B kinase during GSC abscission. Finally, we found that Lethal giant discs (Igd), known to be required for Shrub function in the endosomal pathway, also regulates the duration of abscission in GSCs.

## Author Summary

Abscission is the final step of cytokinesis which allows the physical separation of sister cells through the scission of a thin cytoplasmic bridge that links them at the end of mitosis. The duration of abscission varies depending on cell types, indicating that the event is developmentally regulated. Recently, we have identified two kinases, Aurora B and CycB/Cdk-1, which regulate the timing of abscission in germ cells and in mammalian cells. However, these kinases are upstream regulators and do not perform abscission per se. Here, we show that Shrub, a potential target of Aurora B and one of the most downstream effectors of abscission, is required for complete abscission in germline stem cells. In the absence of Shrub, the mother stem cell remains linked to its daughter cells, which then share the same cytoplasm and cannot differentiate. Loss of Shrub and Aurora B have opposite effects on abscission duration suggesting that Aurora B regulates negatively Shrub. We further show that Shrub acts together with its interactor Lethal giant disc to ensure proper abscission timing.

## Introduction

Abscission is the last step of cytokinesis when sister cells linked by a thin cytoplasmic bridge become physically separated. It takes place on the side of an electron dense structure called the midbody that resides within the bridge. Unexplored for many years, this late step of cell division has begun to be characterized at the cellular and molecular level in the last decade as a result of recent advances in microscopy and genetic engineering [1]. Our understanding of abscission originates from studies carried out mainly in yeast and in mammalian cells in culture. However, features like the duration of abscission vary greatly from one cell type to another. It lasts a few hours in mammalian cells, while in sea urchin embryos, the completion of cell division only occurs during the S phase of the next cycle [2]. Abscission is completely blocked in germ cells of most species at some point during normal development [3]. How abscission timing is regulated in a developmental context remains however poorly characterized.

During abscission, membrane scission happens at a secondary ingression point in the bridge that appears just before the cut [4,5,6]. At this site, microtubules overlapping in the bridge are severed by the AAA ATPase Spastin, and actin filaments are cleared by modifications of the lipid content of the membrane mediated by the PIP2-phosphatase OCRL [7,8]. A secondary constriction is thought to be formed and then abscised, by a set of proteins belonging to the Endosomal Sorting Complex Required for Transport-III (ESCRT-III) machinery and the vacuolar protein sorting 4 (VPS4). The subunits of the ESCRT-III complex, including CHMP4B, and the most downstream component VPS4 are relocated at the exact site of the cut just before abscission occurs [5,6]. The ESCRTs have the ability to self-assemble into spiral filaments, a structure that has been described beside the midbody that would allow membrane curvature and scission [1,9]. The timing of abscission depends on the local recruitment of the ESCRT-III machinery. This can only occur after mitotic exit when PLK1 gets degraded, and thereby allows the centrosomal protein 55 (CEP55) to localize to the midbody [10]. This in turn permits the sequential recruitment of the ESCRT-I component TSG101 and ALIX, and finally the ESCRT-III machinery [11,12,13]. Although recruited by the ESCRT-I complex during Multi Vesicular Bodies (MVBs) formation, the ESCRT-II complex does not appear to be involved in abscission in mammalian cells. In mouse males spermatocytes, the binding of CEP55 to ALIX and TSG101 is inhibited, therefore abscission does not occur and a stable bridge is formed [14]. Abscission can also be blocked or delayed by the presence of lagging strands of DNA in the cytoplasmic bridge between two sister cells. Elegant work identified this checkpoint in yeast and mammalian cells, and demonstrated that it delays abscission until the lagging DNA bridges are resolved. It has thus been named the NoCut checkpoint. An important molecular player of this checkpoint is the essential mitotic kinase Aurora B [15,16]. Aurora B delays abscission by phosphorylating a member of the ESCRT-III complex, CHMP4C, a close paralog of the filament forming CHMP4B required for abscission [17,18]. Recent work suggests that this delay may be mediated by the retention at the midbody ring of the terminal effector of abscission Vps4 by CHMP4C and ANCHR proteins [19]. The temporal control of abscission is thus highly regulated by a complex molecular machinery that is still not fully understood. In addition, whether the conserved ESCRT machinery is regulating abscission in *Drosophila* has not yet been explored.

Recently, we have used the *Drosophila* female germline to study abscission in a developmental context and in a genetically amenable system [20]. *Drosophila* germ cells regulate abscission differentially according to the developmental stage. Germline stem cells (GSCs) are located at the very anterior of region 1 of the germarium, in contact with somatic cells called cap cells and escort cells, which regulate their behavior [21]. Each stem cell divides asymmetrically to generate one stem cell, which stays in contact with cap cells in the niche, and a second daughter cell

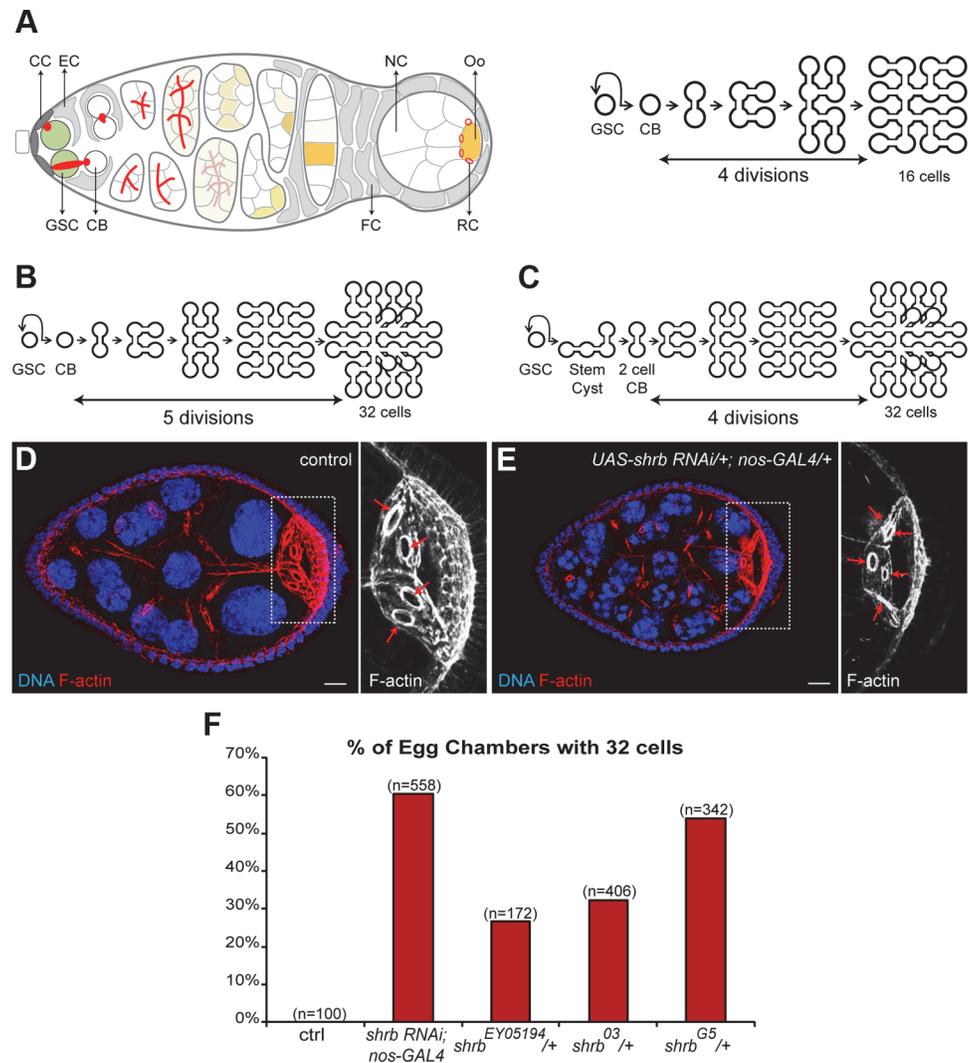
positioned outside of the niche. The daughter cell starts to transcribe the gene *bam*, which is necessary and sufficient to trigger the differentiation of the daughter cystoblast (CB). This differentiation is characterized by four rounds of synchronous and incomplete divisions, giving rise to a cyst of 16 cells made of 15 nurse cells and one oocyte. In the resulting cyst, each cytokinesis is arrested and all sister cells share the same cytoplasm through ring canals. In contrast, cytokinesis between the GSC and the CB is complete. It is, however, very slow and GSCs and CBs remain linked at least until the following S-phase. The orientation and synchrony of these divisions are controlled by a germline-specific organelle, called the fusome, which is made of ER-derived vesicles (Fig. 1A) [21]. The fusome is partly inherited from the round spectrosome of the GSCs (also made of ER-derived vesicles), and partly newly formed at the midbody during each division. Fusion between fusome precursors creates a continuum of vesicles penetrating each canal and connecting all the cells within a cyst. The fusome thus appears branched in dividing germline cysts. The fusome starts to degenerate and disappears when the germline cyst enters the meiotic zone or region 2 of the germarium [22,23].

In a genetic screen, we isolated the first mutations in *aurora B* and *survivin*, a regulatory subunit of the Chromosomal Passenger Complex (CPC) in *Drosophila* [20,24]. Using allelic series of gain- and loss-of-function of these genes, we demonstrated that the function of Aurora B as an abscission timer is conserved during the development of germline cells. Enhancing Aurora B activity delays abscission in GSCs and multiple divisions can occur before the preceding abscissions are completed. This leads to the formation of stem-cysts, a structure composed of several cells with stem cell-like properties still linked to the anterior GSC. In contrast, reducing Aurora B activity induces precocious abscission in GSCs and complete abscission in 2-cell cysts. A simple readout of these events is the number of germ cells per cyst. 32 cells or more per cyst are found when abscission is delayed in cyst, while 8 cells or less are observed in the case of a precocious abscission in the cyst [20]. In this study, we have analyzed the consequences of the loss of function of Shrub (Shrb), the single *Drosophila* homolog of CHMP4, on the development of the germline lineage. We found that Shrb was positively required for abscission, as mammalian CHMP4B; and it negatively regulated by Aurora B. In addition, we showed that Lethal giant discs (Lgd), which is known to be required for Shrb function in the endosomal pathway, was also involved in GSCs abscission regulation.

## Results

### 1. Loss of the ESCRT-III subunit Shrub (SNF7/CHMP4) in germline cells induces the formation of egg chambers with 32 cells

We performed a pilot RNAi screen for mutants affecting the number of germ cells per egg chamber. Mutations in *cyclin A*, *cyclin E* or their regulators affect the number of germ cells by modifying the number of divisions. High levels of Cyclin A or mutations in *encore* induce the formation of egg chambers with 32 cells by triggering a fifth mitosis in cysts (Fig. 1B) [25,26]. In contrast, mutations in *cyclinE* or *half-pint* give rise to egg chambers with 8 cells due to only 3 divisions [27,28]. Recently, we showed that changing the duration of abscission could alter the number of germ cells per egg chamber without modifying the number of cyst mitosis. We showed that delaying abscission in GSCs induces the formation of cystoblasts made of two cells instead of one, which results in the formation of egg chambers containing 32 cells after four divisions (Fig. 1C). In contrast, a faster abscission after the first cyst mitosis allows for the completion of cytokinesis in 2-cell cysts, and leads to the formation of two cysts of 8 cells after the remaining three divisions [20]. To find novel genes involved in these divisions, we used a collection of transgenic flies expressing shRNA designed to be efficient in germ cells (TRiP collection, [29]). We selected 230 transgenic lines targeting kinases, phosphatases and regulators of



**Figure 1. Loss of the ESCRT-III subunit Shrub in germline cells induces the formation of egg chambers with 32 cells.** (A) Schemes of a germarium (left) and germline divisions (right). At its anterior tip, the germline stem cell (GSC, green) divides asymmetrically and produces a cystoblast (CB). Cap cells (CC) maintain GSC stemness, while escort cells (EC) promote CB differentiation. The CB goes through 4 divisions forming a cyst of 16 cells, 15 nurse cells (NC) and 1 oocyte (Oo, yellow). The cyst is encapsulated by follicular cells (FC) and buds out of the germarium. The abscission of the GSC/CB is complete, while in the following 4 divisions it is incomplete. The oocyte shares the cytoplasm with the NC through 4 ring canals (RC). The spectrosome in the GSC and the fusome in its progeny (red, left scheme) are germline-specific organelles. Anterior is on the left, posterior on the right. (B and C) Schemes representing 2 possible ways of explaining the 32-cell cysts. (B) A fifth mitosis. (C) A delay in GSC abscission. (D and E) Stage 7 egg chambers from WT or *UAS-shrb RNAi/+; nos-GAL4/+* stained with DAPI (DNA, blue) and phalloidin (F-actin, red). On the right, close-up on oocytes. Red arrows indicate the four ring canals in the control oocyte and the five ring canals in the mutant background. (F) Fraction of egg chambers exhibiting 32 cells on the y axis. Genotypes are on the x axis. Scale bar: 10  $\mu$ m.

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membrane trafficking, and expressed shRNAs specifically in germ cells using a *nanos-GAL4* driver. Three females were dissected for each line and ovaries were stained with Hoechst to count the number of cells.

One line (HMS01767) induced a high percentage (61%,  $n = 558$ ) of egg chambers containing 32 cells. In these mutant egg chambers, the oocyte was linked to nurse cells by 5 ring canals

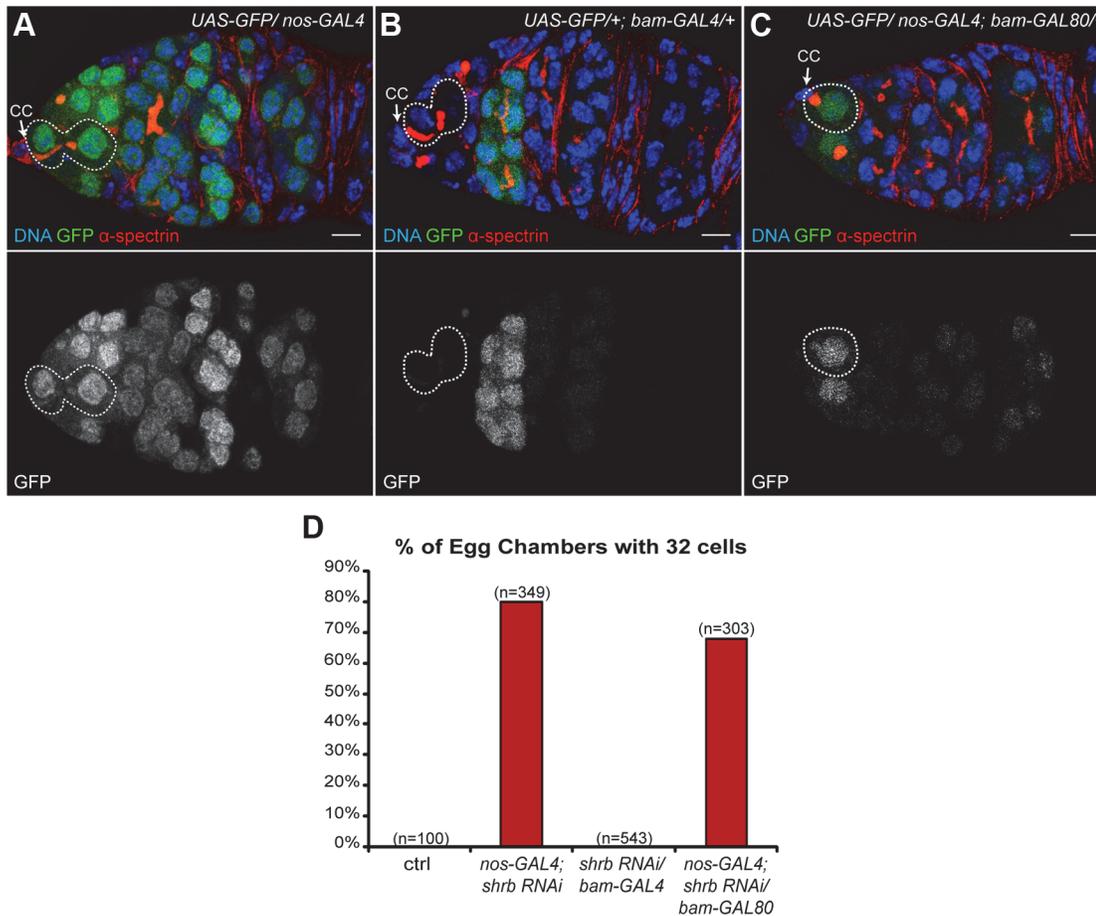
instead of 4 indicating that the extra germ cells were not the result of packaging defects (Fig. 1D-F). We also observed less penetrant phenotypes, such as binucleated cells, polyploid cells and a few egg chambers filled with tumor-like germ cells (S1 Fig.). The HMS01767 line encodes a short hairpin RNA directed against *shrub* (*shrb*). *shrb* encodes a subunit of the ESCRT-III complex and is the *Drosophila* homologue of *Snf7/CHMP4*. We tested the specificity of the RNAi line by using several *shrb* alleles: *shrb*<sup>G5</sup>, *shrb*<sup>O3</sup> and *shrb*<sup>EY05194</sup>. We could not recover any *shrb* homozygous mutant germ cells using the Flp/FRT technique. However, we noticed a high percentage of 32-cell egg chambers in flies heterozygous for these alleles: 27% (n = 172); 32% (n = 406) and 54% (n = 342) of the egg chambers has 32 cells in the *shrb*<sup>EY05194/+</sup>, *shrb*<sup>O3/+</sup> and *shrb*<sup>G5/+</sup> females, respectively. We concluded that the RNAi was specific and that the gene dosage of *shrb* was important to regulate the number of germ cells per egg chamber.

## 2. The loss of Shrub in germline stem cells induces the formation of 32-cell cysts

The cyst goes through four rounds of mitosis in a subpart of region 1 that can be identified by the expression of the gene *bam*. *bam* is weakly expressed in cystoblasts and 2-cell cysts, increases in 4-cell cysts, and peaks at 8-cell cysts before being switched-off quickly[30]. In mutants inducing one extra round of mitosis, the *bam*-expressing region is expanded posteriorly, indicating that the corresponding gene acts during the cyst divisions. In contrast, in abscission-defects mutant giving rise to 32-cell cysts, the genes are required in the GSCs before the cyst divisions. To distinguish between both possibilities, we expressed RNAi against *shrb* differentially in GSCs and dividing cysts. *nanos*-GAL4 drives expression in all germ cells of the germarium, whereas *bam*-GAL4 only in dividing cysts (Fig. 2A-B). We also expressed the same shRNA (HMS01767) against *shrb*, driven by *bam*-GAL4 to test if *shrb* was required in dividing cysts to control the number of divisions. In the later conditions, all egg chambers had 16 cells (0%, n = 543), in contrast to 80% (n = 349) of 32-cell cysts when driven by *nanos*-GAL4 (Fig. 2D). This result suggested that Shrb function is required in germ cells expressing *nanos*, but not *bam*, which are mainly the GSCs and a few pre-cystoblasts. To confirm this hypothesis, we combined *nanos*-GAL4 with a GAL80 repressor under the control of the *bam* promoter. In this background GAL80 repressed the activity of GAL4 in the *bam* expressing domain (Fig. 2C); as a consequence, *UAS-shrb-RNAi* was expressed only in GSCs and some pre-cystoblasts. In such ovaries, we found that 68% (n = 303) of egg chambers had 32 germ cells. We concluded that *shrb* was required in GSCs to regulate non-autonomously the number of germ cells per cyst.

## 3. *shrub* loss of function leads to the formation of stem-cysts

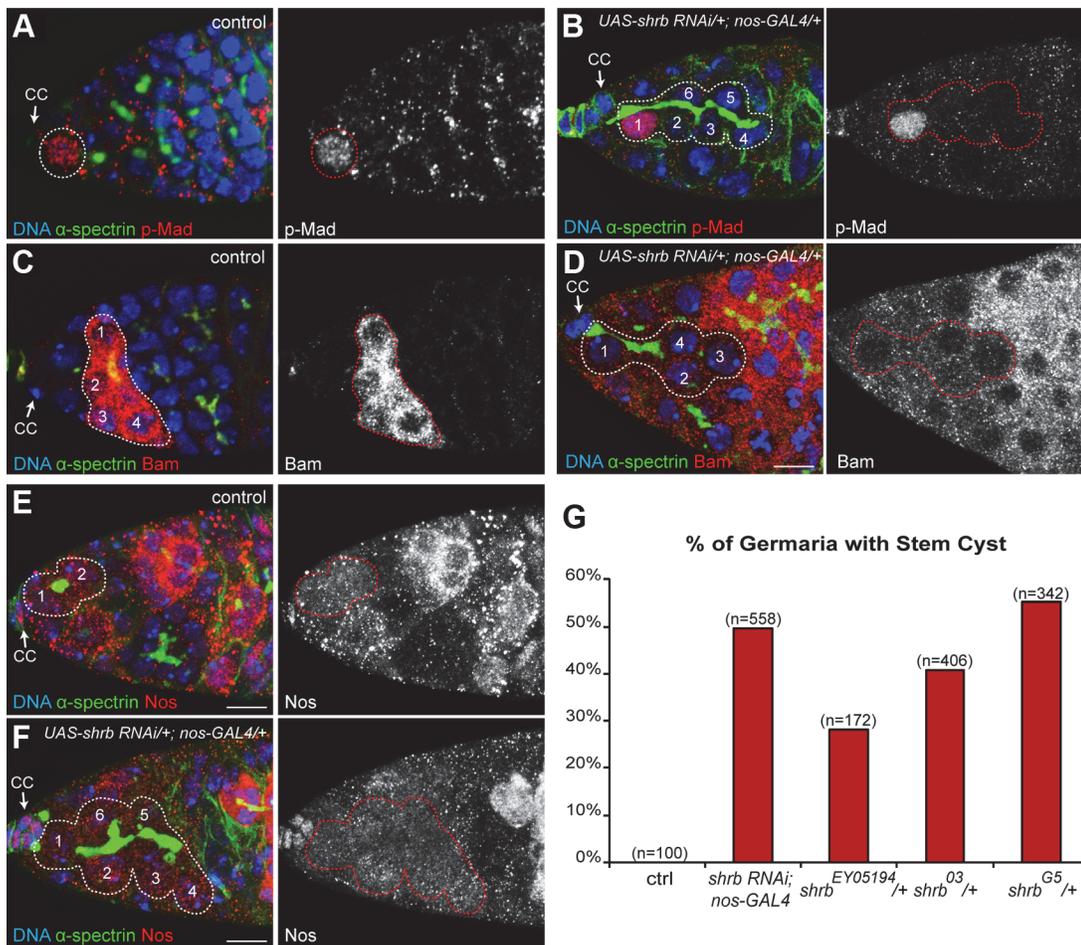
The requirement for Shrb in GSCs rather than in cysts to regulate the number of germ cells strongly indicated that Shrb regulates abscission rather than the number of cyst divisions. We had previously shown that a delayed abscission in GSCs led to the formation of group of cells that remained connected by cytoplasmic bridges. We named these clusters “stem-cysts” as they express stem-cell markers but are linked by a branched fusome as found in germline cysts. We thus looked for such stem-cysts in *nanos*-GAL4; *UAS-shrb-RNAi* ovaries. We found that the fusome of mutant GSCs did not have a round or exclamation point shape as a regular GSC, but was instead branched and apparently passing through several cells (Fig. 3A-B). Next, we examined p-Mad staining, a nuclear marker of GSC identity induced by Dpp signal secreted by the cap cells [31]. We found that the mutant GSC attached to the cap cells was positive for p-Mad, as in wild type, indicating that stem cell identity was not affected upon *shrb* loss of function (Fig. 3A-B). In wild type GSC, Dpp signaling blocks the transcription of *bam*, which



**Figure 2. The loss of Shrub in germline stem cells induces the formation of 32-cell egg chambers.** (A-C) Germaria expressing *UAS-GFP* under the control of different promoters and stained with DAPI (DNA, blue) and  $\alpha$ -spectrin (fusome, red). (A) *nos-GAL4* is specific for germline cells; (B) *bam-GAL4* is specific for early differentiated cells in the cysts; (C) *nos-GAL4; bam-GAL80* is specific for germline, excluding the early cysts. Dotted lines surround GSC/CB pairs (A and B) and GSC (C). Cap cells (CC) are indicated. (D) Fraction of egg chambers exhibiting 32 cells on the y axis. Genotypes are on the x axis. Scale bar: 10  $\mu$ m.

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is necessary and sufficient for the differentiation of the cyst and therefore, Bam protein is not present in the GSC (Fig. 3C-D) [30]. We found that upon *shrb* loss of function, all the cells that were linked to the anterior most GSC by a fusome were devoid of Bam protein. Finally, Nanos protein is weakly expressed in control GSCs, and is completely lost in early differentiating cysts (Fig. 3E) [32]. Upon *UAS-shrb-RNAi* expression, we found weak expression levels of Nanos in the anterior GSC and in cells connected to it by a branched fusome (Fig. 3F). We also observed clusters of cells with the same characteristics in ovaries heterozygous for *shrb*<sup>G5</sup> and *shrb*<sup>O3</sup> alleles. These groups of cells expressed Nanos but not Bam, like wild type GSCs. Moreover, they were also linked by a branched fusome like germline cysts (S2 Fig.) thus corresponding to our definition of stem-cysts. In addition, we found that the penetrance of stem-cysts formation was high 50% (n = 558) for *nanos-GAL4;UAS-shrb-RNAi*, and 55% (n = 342), 41% (n = 406), 28% (n = 172), for germline heterozygous for *shrb*<sup>G5</sup>; *shrb*<sup>O3</sup> and *shrb*<sup>EY05194</sup>, respectively. The high penetrance of stem-cysts was consistent with the high number of 32-cell cysts.

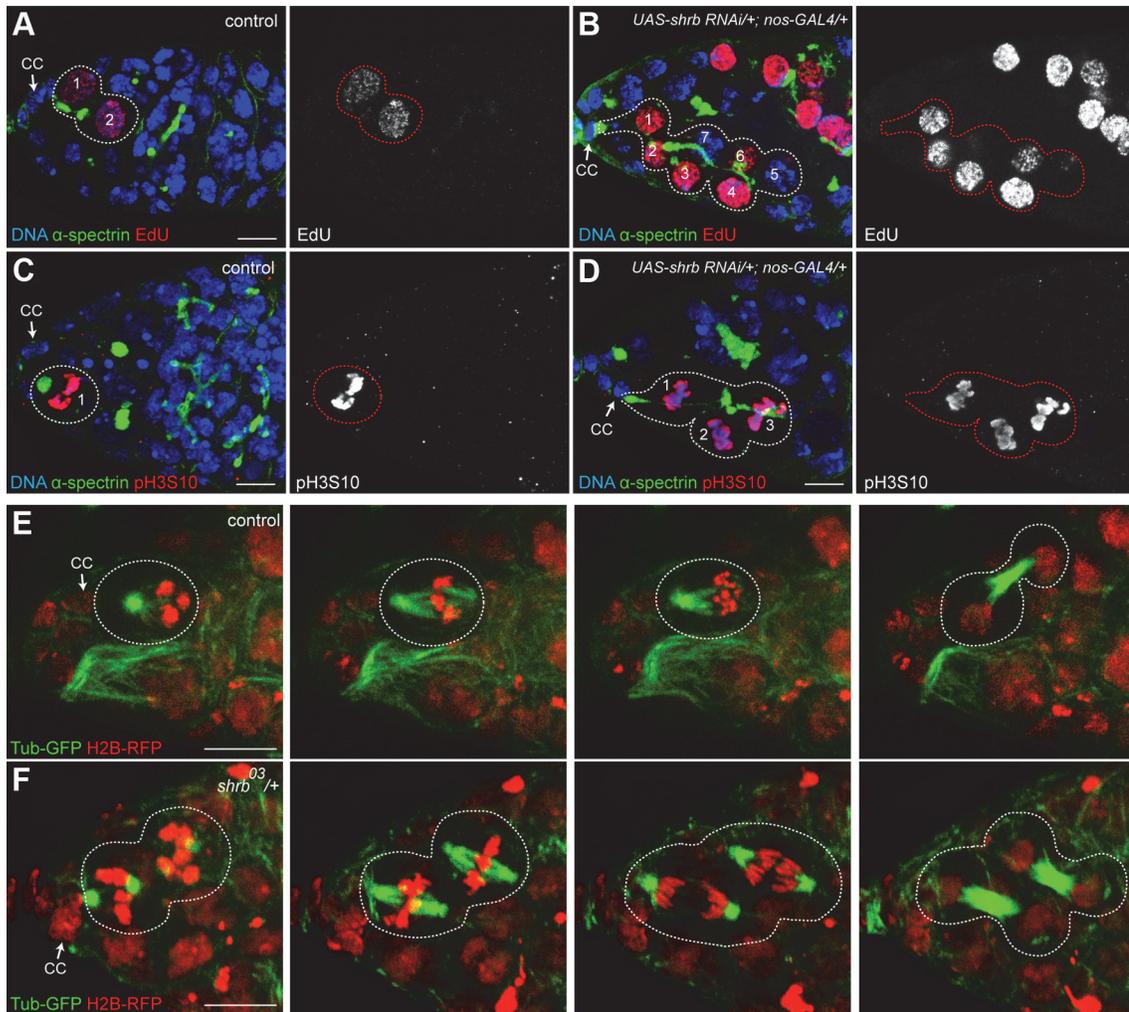


**Figure 3. *shrub* loss of function leads to the formation of stem-cysts.** Germaria of WT females (A, C, and E) or females expressing *UAS-shrb RNAi* under the control of *nos-GAL4* driver (B, D and F), stained for DAPI (DNA, blue),  $\alpha$ -spectrin (fusome, green), and either p-Mad (A and B, red), Bag of marble (Bam, C and D, red), or Nanos (Nos, E and F, red). Dotted lines surround: GSC (A), stem-cysts (B, D and F), 4-cell cyst (C) and GSC/CB pair (E). Cap cells (CC) are indicated. (G) Fraction of germaria exhibiting at least one stem-cyst on the y axis. Genotypes are on the x axis. Scale bar: 10  $\mu$ m.

doi:10.1371/journal.pgen.1004653.g003

#### 4. Stem-cysts in *shrub* mutant ovaries are caused by synchronous divisions

Stem-cysts are formed by the synchronous divisions of GSCs and connected cells, while previous abscissions have not been completed. To test the synchronicity of GSCs with other cells, we first analyzed EdU incorporation after a 15 min pulse to mark the S-phase. In control condition, we found that GSCs and their daughter cystoblasts incorporated similar amount of EdU, indicating that they were still synchronized and connected as previously described [20,22]. However, we never detected additional neighboring cells labelled by EdU (Fig. 4A, n = 45). In germarium expressing *shrb-RNAi*, we observed that in addition to the anterior GSC and its direct neighbor, additional neighboring cells had incorporated EdU, indicating that all these cells had replicated synchronously their DNA (Fig. 4B, n = 9). These cells were all linked by a common fusome. To test if the synchrony of the cell cycle extended to M-phase, we used the mitotic marker pH3. We found that in control conditions, GSCs always divided non-synchronously with their neighbors, including their daughter cystoblasts (Fig. 4C, n = 27). In contrast, in germ cells expressing *shrb-RNAi*, all cells linked to a GSC by a fusome exhibited similar levels of pH3 staining. This indicated that these cells were all performing mitosis synchronously (Fig. 4D,



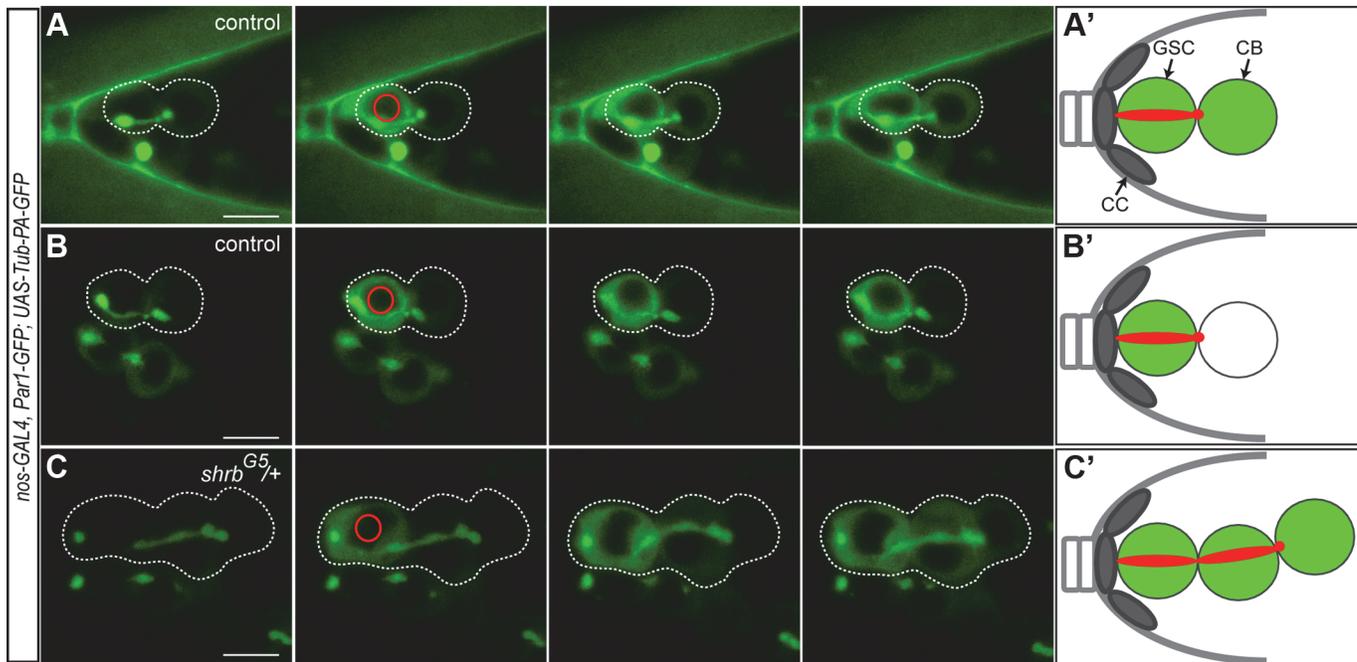
**Figure 4. Stem-cysts in *shrub* mutant ovaries are caused by synchronous divisions.** Germaria of WT females (A and C) or females expressing *UAS-shrb RNAi* under the control of *nos-GAL4* driver (B and D), stained for DAPI (DNA, blue),  $\alpha$ -spectrin (fusome, green), and either EdU (A and B, red) showing S-phase, or pH3S10 (C and D, red) to highlight mitotic cells. Dotted lines surround: GSC/CB pair (A), stem-cysts (B and D) and GSC (C). (E and F) Selected time points of live imaging experiments performed on germaria expressing H2B-RFP (chromatin, red) and G147 (tubulin, green). (E) A WT GSC (surrounded by dotted lines) undergoing mitosis alone. (F) In a female heterozygous for *shr<sup>03/+</sup>*, the GSC and its daughter CB undergo mitosis synchronously (surrounded by dotted lines). Cap cells (CC) are indicated. Scale bar: 10  $\mu$ m.

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$n = 15$ ). Finally, to directly analyze cell cycle synchrony in stem-cysts, we used live imaging of heterozygous *shr<sup>03/+</sup>* ovaries expressing an *H2B-RFP* transgene to label chromosomes, and *G147*, a protein-trap insertion marking microtubules. We were able to follow 10 GSCs undergoing mitosis, and found that 4 of them were dividing synchronously with neighboring cells (Fig. 4F). This result is consistent with the percentage of stem-cysts observed by immunostaining. Synchronous divisions were never observed in wild type control (Fig. 4E,  $n = 21$ ). Altogether, these results demonstrated that cells within stem-cysts induced by *shr<sup>03</sup>* loss-of-function were cycling synchronously.

## 5. Cells within one stem-cyst share the same cytoplasm

The cell cycle synchronicity within stem-cysts suggested that these cells shared the same cytoplasm and that *shr<sup>03</sup>* loss-of-function induced a strong delay in abscission. To determine



**Figure 5. The cells of the stem-cyst share the same cytoplasm.** Selected time points of live imaging experiments performed on germaria expressing *UAS-Par1-GFP* and *UAS-Tubulin-PA-GFP* under the *nos-GAL4* promoter. Tub-PA-GFP is photoactivated in the region defined by the red circle in the GSC, and the fluorescence diffusion to the neighboring cells is observed. (A and B) WT female GSCs in mid-G2 phase. (A) Abscission between the GSC and the CB did not yet occurred as the GFP diffuses to the CB. (B) GFP does not diffuse to the CB. Abscission has occurred and the cells no longer share their cytoplasm. (C) Stem-cyst of *shrb<sup>G5/+</sup>* female. After photoactivation in the GSC, the GFP diffuses through the 2 neighboring cells. This shows that the cells of the stem-cyst do not complete abscission and stay connected, sharing their cytoplasm. Dotted lines surround: GSC/CB pair (A and B), stem-cysts of 3 cells (C). (A', B' and C') Schematic representation of the Tubulin-PA-GFP diffusion from the GSC to the CB (A' and B') or within a stem-cyst (C'). Scale bar: 10  $\mu$ m.

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precisely when abscission took place in wild type and mutant conditions, we expressed a diffusible  $\alpha$ -tubulin tagged with a photo-activatable GFP (Tubulin-PA-GFP). Activation of the GFP in one cell allowed tracing labeled tubulin to the neighboring cell, and thus determining whether or not abscission had been completed. Indirect assays, using EdU incorporation, had already established that abscission between GSCs and cystoblast happens during or after S-phase in wild type condition [20,22]. To time more precisely abscission in GSCs, we used the shape of the fusome as a timer, as it has been shown to follow stereotypical changes during the different phases of the cell cycle [22,33]. We used germaria co-expressing Tubulin-PA-GFP with a *UAS-Par-1-GFP* transgene to label the fusome during live-imaging. We activated Tubulin-PA-GFP in one GSC with a brief pulse of a 2-photon laser, and recorded its diffusion to the attached cystoblast for each stage of the fusome cycle as defined in [33]. We observed diffusion of Tubulin-PA-GFP in the cystoblast only when the GSC and the cystoblast were visibly linked by a fusome ( $n = 61$ ). We observed diffusion in GSC/CB pairs harboring a fusome shaped as a plug ( $n = 2$ , G1 phase), a bar ( $n = 6$ , G1/S phase), dumbbell ( $n = 15$ , S phase), fusing ( $n = 16$ , G2 phase) and an exclamation point ( $n = 22$ , G2 phase, Fig. 5A, S1 Movie). However, we also observed some GSC/CB pairs linked by an exclamation point fusome, in which Tubulin-PA-GFP did not diffuse from GSC to CB ( $n = 21$ , Fig. 5B, S2 Movie). Tubulin-PA-GFP never diffused when the fusome was round (late G2 phase,  $n = 13$ ). These results indicate that Tubulin-PA-GFP can diffuse from GSC to CB until the exclamation point stage of the fusome, i.e. mid-G2 phase. We thus established that in wild type condition, abscission between GSC and CB happens during mid-G2 phase (Fig. 5). We carried out the same experiment in *shrb<sup>G5/+</sup>* mutant GSCs. We selected stem-cysts with at least three cells, including an anterior GSC, linked by a

Par1-GFP positive fusome. We activated Tubulin-PA-GFP in the anterior GSC. We observed diffusion of Tubulin-PA-GFP in all cells of a single stem-cyst in 96% stem-cysts analyzed (Fig. 5C,  $n = 27$ , S3 Movie). This result demonstrated that cells within one stem cyst shared a common cytoplasm, and that abscission was almost never completed in GSCs, although scission eventually happened at other cytoplasmic bridges of stem-cysts.

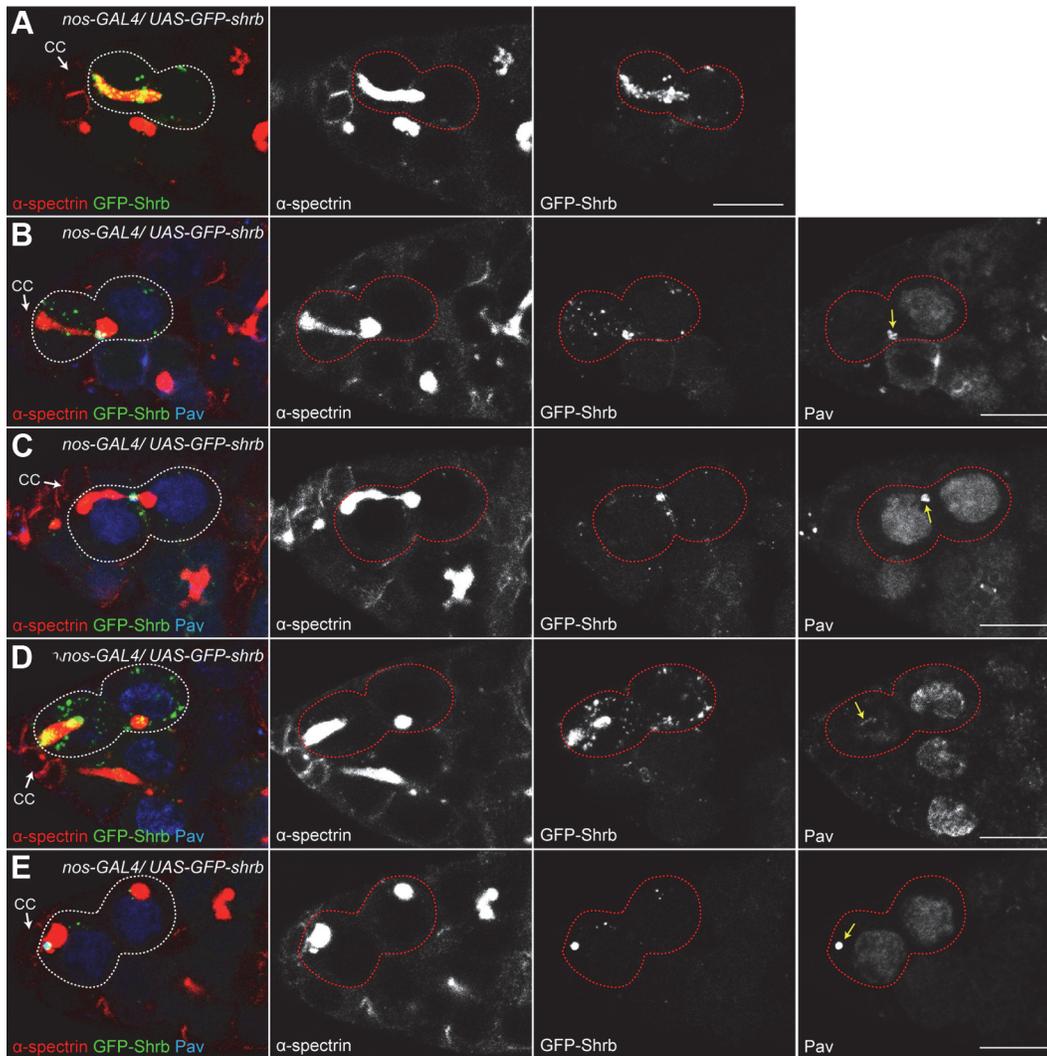
## 6. *Drosophila* Shrub localizes on the fusome and at the midbody

To analyze the dynamic localization of Shrb in germ cells, we generated an N-terminal fusion protein, GFP-Shrub (GFP-Shrb), under the control of a *UASp* promoter to express it in GSCs and germline cysts with the *nanos*-GAL4 driver. In GSCs, we found that GFP-Shrb localized as dots along the fusome and at the transient ring canal linking the GSC and CB before abscission (Fig. 6A). In mid-G2 phase, when the fusome adopts an exclamation point shape, we found that GFP-Shrb localized additionally to a strong focus at the site where the fusome splits. We hypothesized that this dot could be the midbody, and therefore co-stained ovaries for the midbody marker Pavarotti (*Pav*), which is the *Drosophila* homologue of MKLP1. We found a perfect co-localization of GFP-Shrb and *Pav* on this structure and on the surrounding ring canal (Fig. 6B). This result indicated that GFP-Shrb localized at the midbody. At a later stage, when the fusome between the GSC and the CB is about to break, both Shrb and *Pav* disappeared from the shrunk ring canal, but remained co-localized at the midbody (Fig. 6C). Similarly, after scission when the fusome is retracting to reform the typically round spectrosome in the GSC, Shrb and *Pav* co-localized at the posterior tip of the fusome (Fig. 6D). In late G2 and mitosis, we occasionally observed midbodies co-stained by *Pav* and GFP-Shrb next to the spectrosome (Fig. 6E). This dynamic behavior of GFP-Shrb is consistent with a function of Shrb in GSCs abscission, and with a recent report demonstrating that the midbody is asymmetrically inherited by the GSCs in ovaries [34]. We further observed that this asymmetric inheritance of the midbody occurs concurrently to the retraction of the fusome to reform a round spectrosome. In germline cysts of the mitotic region (region 1), we found that GFP-Shrb was almost not visible even in cytoplasmic vesicles (S3 Fig.). In contrast, in meiotic cysts (region 2 of the germarium), GFP-Shrb localized as bright dots along the fusome and at ring canals (S3 Fig.).

While studying the localization of GFP-Shrb, we noticed a weak but consistent appearance of 32-cell cysts in an otherwise wild type background. Depending on the expression levels of different insertions of *UASp-GFP-Shrb* transgenes, and using the same *nanos*-GAL4 driver, we observed between 4% and 15% of egg chambers with 32 cells. This result suggested that expression of GFP-Shrb could have a dominant-negative effect, as it gave the same phenotype as a loss-of-function of *shrub*. Alternatively, a high expression of Shrb could induce the same phenotype as a low expression. To distinguish between these alternatives, we expressed GFP-Shrb in a *shrub* heterozygous background. If GFP-Shrb acts as a dominant-negative allele, it should aggravate the 32-cell phenotype, while it should rescue it if GFP-Shrb is a functional protein. We found that the phenotype of 32-cell cysts decreased from 44% in *shrub*<sup>O3/+</sup> ovaries to 12% in *shrub*<sup>O3/+</sup> ovaries expressing GFP-Shrb (S3 Fig.). We concluded that GFP-Shrb was not a dominant-negative allele, and that abscission was very sensitive to the levels of Shrb whether increased or reduced.

## 7. Loss of Lethal giant discs (Lgd) induces the formation of stem-cysts and 32-cell egg chambers

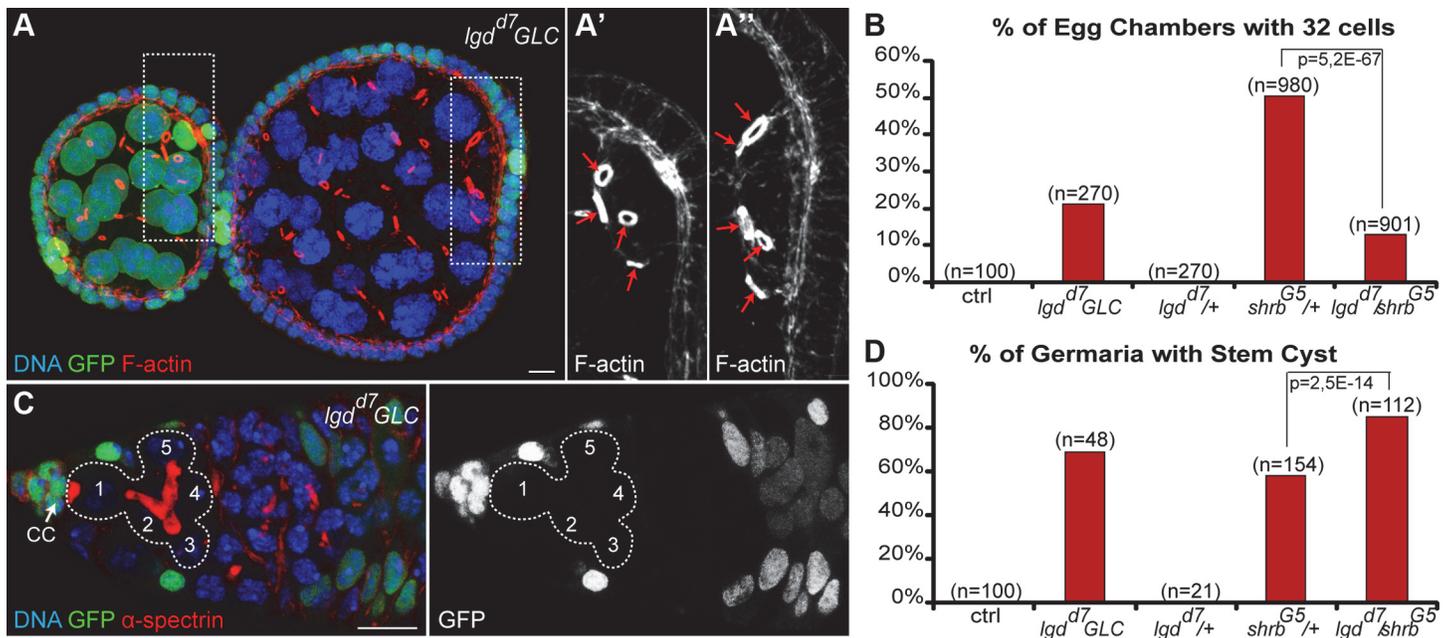
Lgd is a tumor suppressor known to regulate endosomal trafficking and is a direct interactor of Shrb. In *Drosophila* and vertebrates, Lgd was shown to interact physically with Shrb via its



**Figure 6. *Drosophila* Shrub localizes on the fusome and at the midbody.** Ovaries expressing *UAS-GFP-shrb* under the control of *nos-GAL4* and stained for  $\alpha$ -spectrin (A, red) and Pavarotti (B-E, Pav, blue) a marker of the midbody. Midbody position is indicated by a yellow arrow. (A) Before abscission, GFP-Shrb localizes to the fusome (red) and at the ring canal between the GSC and the CB (See also [S3B Fig.](#)). (B) GFP-Shrb localizes specifically at the ring canal and midbody (Pav, blue) during its constriction. (C) During abscission, GFP-Shrb is enriched at the midbody, colocalizing with Pav. The ring canal is no longer visible. (D) After abscission, the fusome retracts towards the GSC and the midbody, stained with Pav and Shrb-GFP, is segregated with it. (E) GSCs with round spectrosome (late G2, M) often show colocalization of Shrb-GFP with the midbody (Pav). Scale bar: 10  $\mu$ m.

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DM14 domain. This interaction is required in flies for Shrub endosomal function [35]. In contrast, the mammalian homologue of Lgd was suggested to be an inhibitor of CHMP4B, a Shrub orthologue [36]. To assess the function of Lgd in germ cell abscission, we induced germline clones homozygous mutant for null alleles of Lgd. We found that 21% ( $n = 270$ ) of *lgd*<sup>d7</sup> mutant egg chambers contained 32 cells ([Fig. 7A-B](#)). Furthermore, we observed that 69% of *lgd*<sup>d7</sup> mutant GSC formed stem-cysts ([Fig. 7C-D](#),  $n = 48$ ). We concluded that in the absence of Lgd, abscission is delayed in GSCs, and thus that Lgd is required positively for abscission to be completed. To investigate whether Lgd and Shrb act in the same pathway to regulate abscission, we performed genetic interactions between *lgd* and *shrb* alleles by crossing heterozygous *shrb*<sup>G5/+</sup> flies with *lgd*<sup>d7</sup> null allele. We found that the number of stem-cysts was significantly increased in *shrb*<sup>G5/lgd</sup><sup>d7</sup> (85%,  $n = 112$ ) compared to *shrb*<sup>G5/+</sup> (58%,  $n = 154$ ) ([Fig. 7D](#)). We



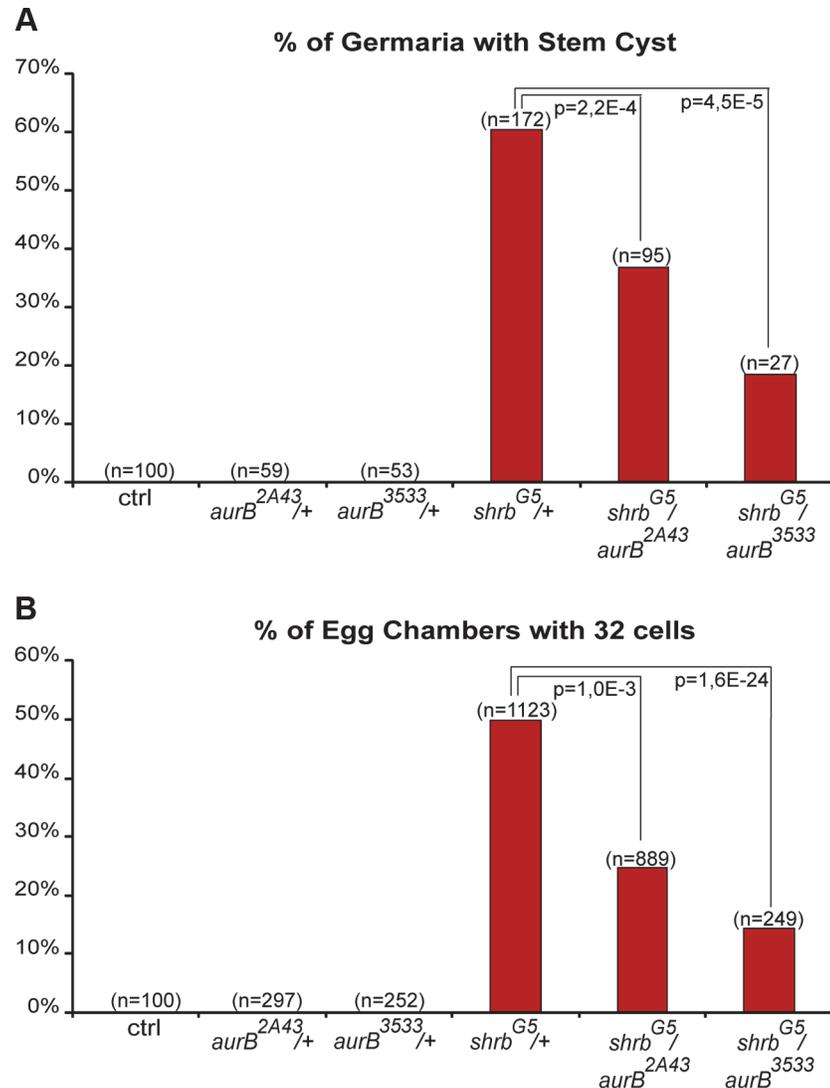
**Figure 7. Loss of Lethal giant discs induces the formation of stem-cysts and 32-cell egg chambers.** (A) Female germline clones (GLC) of *lgd<sup>d7</sup>* (*hsFlp/+; lgd<sup>d7</sup>, FRT 40A/ GFP, FRT 40A*) stained with DAPI (DNA, blue) and phalloidin (F-actin, red). Egg chamber with heterozygous germ cells (GFP, green) is made of 16 cells with a 4 ring canals oocyte (close up in A'). The neighboring egg chamber is a GLC, made of 32 cells with a 5 ring canals oocyte (close up in A''). (B) Fraction of egg chambers exhibiting 32 cells on the y axis. Genotypes are on the x axis. (C) Female germline clones (GLC) of *lgd<sup>d7</sup>* stained for DAPI (DNA, blue) and  $\alpha$ -spectrin (fusome, red). A stem-cyst composed of 5 homozygous mutant cells is shown. (D) Fraction of germaria exhibiting at least one stem-cyst on the y axis. Genotypes are on the x axis. Scale bar: 10  $\mu$ m.

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concluded that Lgd and Shrub interacted positively for GSCs abscission. Surprisingly, we found that the number of egg chambers with 32 cells was reduced in double heterozygous flies. We counted 13% (n = 901) of egg chambers with 32 cells in *shrb<sup>G5</sup>/lgd<sup>d7</sup>* flies, compared to 51% (n = 980) in *shrb<sup>G5</sup>/+* flies (Fig. 7B). This negative genetic interaction between *lgd* and *shrb* suggested that Lgd might have another function in germline cysts. We concluded that Lgd and Shrub interacted positively in GSCs and negatively in germline cysts.

## 8. Aurora-B and Shrub interact to regulate abscission in germline stem cells

In human cells, CHMP4A, B and C are three isoforms of CHMP4, the vertebrate homologue of Shrub. Both CHMP4B and C regulate abscission timing in vertebrate cells, albeit with opposite activity. CHMP4B is known to regulate positively abscission, whereas CHMP4C can delay it [17,37]. The activity of CHMP4C is regulated by Aurora B (AurB)-dependent phosphorylation [17,18]. We previously showed that in flies, AurB negatively regulates abscission, and that abscission occurs precociously in GSCs and 2-cell cysts mutant for *aurB*. In contrast, increasing the activity of AurB leads to the formation of stem-cysts as observed in *shrub* loss-of-function [20]. Shrub was further shown to interact physically with Borealin, a regulatory subunit of the AurB complex [18]. We thus tested genetic interactions between *shrb<sup>G5</sup>/+* flies and two null alleles of *aurB*, *aurB<sup>2A43</sup>* and *aurB<sup>3533</sup>*. Both single alleles *aurB<sup>2A43</sup>* and *aurB<sup>3533</sup>* have no phenotype when heterozygous (Fig. 8A-B). We found that the number of stem-cysts in double heterozygous flies *shrb<sup>G5</sup>/aurB<sup>2A43</sup>* (37%, n = 95) and *shrb<sup>G5</sup>/aurB<sup>3533</sup>* (19%, n = 27) was greatly reduced compared to *shrb<sup>G5</sup>/+* flies (60%, n = 172) (Fig. 8A). Furthermore, the number of egg chambers with 32 cells was also rescued from 50% (n = 1123) in *shrb<sup>G5</sup>/+* flies to 25% (n = 889)



**Figure 8. Aurora-B and Shrub interact to regulate abscission in germline stem cells.** (A) Fraction of egg chambers exhibiting 32 cells on the y axis. Genotypes are on the x axis. (B) Fraction of germaria exhibiting at least one stem-cyst on the y axis. Genotypes are on the x axis.

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in *shrb*<sup>G5</sup>/*aurB*<sup>2A43</sup>, and 14% (n = 249) in *shrb*<sup>G5</sup>/*aurB*<sup>3533</sup> ovaries (Fig. 8B). These results demonstrated that AurB negatively interacts with Shrb during GSCs and germline cysts divisions.

## Discussion

The duration of abscission is developmentally regulated in different cell types. We have previously shown that negative feedback loops between two kinases, AurB and CycB/Cdk-1, control the timing of abscission in *Drosophila* germline cells and in vertebrate cells in culture [20]. This mechanism is thus probably conserved in many different cellular contexts. In a genetic screen for genes regulating the number of germ cells per egg chamber, we found that loss of Shrb induced the same phenotype as the overactivation of AurB. Shrb seemed an interesting candidate as it is the *Drosophila* homologue of yeast SNF7 and mammalian CHMP4B/CHMP4C, which have been previously implicated in the regulation of abscission [17]. Furthermore, *Drosophila*

Shrub was shown to physically interact with Borealin, a regulatory subunit of the AurB complex [18]. Interestingly, CHMP4B and CHMP4C have opposite effect on abscission; CHMP4B is a positive regulator of abscission, while CHMP4C delays abscission when phosphorylated by AurB [17,37]. Our results demonstrate that Shrub is a positive regulator of abscission in GSCs, as reducing Shrub levels delayed abscission and led to the formation of stem-cysts. In addition, we found that reducing AurB levels could rescue a reduction of Shrub levels for the formation of both stem cysts and 32-cell egg chambers. This negative genetic interaction indicates that a delay in abscission caused by lower levels of Shrub can be compensated by lower levels of AurB, which accelerate abscission. We also analyzed genetic interactions between *shrub* and *lgd*, as Lgd is known to be required for Shrub function in *Drosophila* [35]. Accordingly, we found that loss of *lgd* induced the formation of stem-cysts and 32-cell egg chambers. Furthermore, decreasing Lgd levels in a *shrub* mutant background increased the delay in abscission as shown by the higher number of stem-cysts. However, we observed surprisingly that the 32-cell phenotype was rescued in transheterozygous *shrub<sup>G5</sup>/lgd<sup>d7</sup>* females. This result indicated that *lgd* and *shrub* interacted negatively in the cyst, contrasting with the positive interaction they showed in the GSC for abscission. We propose that *lgd* may have another function in the cyst that can compensate for *shrub* loss of function. We speculate that Shrub is required for the maintenance of the incomplete cytokinesis of the cyst cells. Reduced levels in *shrub<sup>G5</sup>/lgd<sup>d7</sup>* females would allow the presumptive 32-cell cysts originating from 2-cell precursors to break into two 16-cell cysts, as observed in *aurB* loss-of-function. The “rescued” 16-cell cysts could thus result from a combination of two successive phenotypes, delayed abscission in GSCs followed by a failure to maintain incomplete abscission in cysts at the oldest ring canal. Unfortunately, it is not currently possible to remove *lgd* function only in germline cysts to test this hypothesis. In addition, Lgd and Shrub are both involved in endosomal sorting, and their loss of function may therefore affect signaling pathways and other processes, complicating the interpretation of genetic interactions [38,39,40,41]. Indeed, we observed that loss of Shrub also induced additional phenotypes in ovaries, such as tumorous egg chambers.

One remarkable finding of our study is the great sensitivity of abscission to the levels of Shrub. We observed a gradation in the penetrance of phenotypes depending on the levels of Shrub in the germline. A moderate overexpression of GFP-Shrub using the *nanos*-GAL4 promoter produced less than 15% of egg chamber with 32 cells. Removing one copy of *shrub* induced up to 60% of 32-cell cysts, and the appearance of polyploid germ cells in the germarium. These polyploid cells probably resulted from a complete failure of cytokinesis, and we interpret it as a stronger phenotype than the formation of stem-cysts. Stronger phenotypes were obtained by using shRNAs targeting *shrub*, with up to 80% of egg chambers with 32 cells, many polyploid cells and in addition, the formation of egg chambers containing tumor-like germ cells. Finally, we could not even obtain homozygous *shrub* mutant cells using the Flp/FRT technique indicating that Shrub is required for cell viability in the germline. The levels of Shrub thus appear to be essential for its proper functions, including the regulation of abscission timing in GSCs. In mammalian cells, the final step of abscission is thought to be mediated by the formation of 17 nm-diameter filaments spiraling from the midbody to the constriction zone. The formation of these filaments depends on the Shrub homologue, CHMP4B, and helices of CHMP4B have been described by structured illumination microscopy [6]. However, even though CHMP4B can form filaments in cells, the filaments formed in these CHMP4B over-expressing cells had only a diameter of 5 to 6 nm [42]. Therefore, it is tempting to speculate that the helical filaments observed during abscission are hetero-polymers, comprising other components in addition to Shrub/CHMP4B subunits. Changing the stoichiometry of these components by decreasing or elevating the levels of Shrub/CHMP4B may affect the ability of the subunits to form filaments of the proper diameter to perform abscission. This could explain why overexpression of Shrub or removing one copy of *shrub* can both lead to a delay in abscission.

In mammalian cells, delay in abscission often induces the regression of the cytoplasmic furrow and the formation of bi-nucleated cells [16]. In contrast, we found that in *Drosophila* germ cells, abscission delay resulted in the formation of stem-cysts. In stem-cysts, we demonstrated that all cells shared the same cytoplasm as shown by the diffusion of Tubulin-GFP between ring canals. However, each cell remained individualized and only the most anterior cell, i.e. the cell in contact with the niche, had pMad translocated in the nucleus. This result could be explained if pMad cannot diffuse in the cytoplasm. This intriguing result could also be in agreement with the proposal that cells away from the niche are actively induced to differentiate by neighboring escort cells [43]. We speculate that posterior escort cells can promote the differentiation of distal cells in stem-cysts. Stem-cysts are formed by several rounds of mitosis of GSCs before the completion of preceding abscissions. These mitoses are synchronous and thus form stem-cysts of 2, 4 or 8 cells. However, abscission is only delayed, and ultimately takes place at the oldest ring canals, releasing cystoblasts made of 2 or 3 cells. These multicellular cystoblasts then undergo the regular four mitosis giving rise to 32-cell cyst (2×16) originating from 2-cell cystoblasts; or 48-cell cysts (3×16) originating from 3-cell cystoblasts. In this study, we have only occasionally observed 48-cell cysts, obtained with a strong over-activation of AurB. Reduction of *shrb* or *lgd* mostly generated 32-cell cysts, originating from 2-cell CBs. In agreement, we found stem-cysts mainly formed of 4 cells. We can thus speculate that abscission takes about twice as long in these mutant conditions.

In mammalian cells, the recruitment of ESCRT proteins to the midbody is tightly regulated in time to prevent premature abscission [1]. Intriguingly, we found that in *Drosophila* GSCs, GFP-Shrb localizes on the fusome long before abscission, and also later on at the midbody, which remains associated with the fusome. Since Shrub is present at the site of scission before abscission takes place, its activity must be inhibited to prevent premature abscission. Our genetic interactions between *shrb* and *aurB* suggest that AurB and the CPC could inhibit Shrb activity. There is still no evidence in *Drosophila* that AurB phosphorylates Shrb, as the residues phosphorylated by AurB in CHMP4C are not conserved in *Drosophila* Shrub. In contrast, it has been proposed that direct binding between Shrub and Borealin, a member of the AurB—CPC complex, could block Shrb activity by keeping Shrb in a closed conformation [18]. Consistent with this hypothesis, we have shown that Survivin, another member of AurB complex, is localized on the fusome in GSCs [20]. It is thus possible that precocious activity of Shrb in GSCs is prevented by its binding to the CPC on the fusome. Interestingly, we could barely detect GFP-Shrb on the fusome in dividing cysts when abscission remains incomplete. This indicates that Shrb levels are regulated developmentally, and that there is a correlation between the absence of Shrub protein on the fusome and incomplete cytokinesis in germline cysts. Furthermore, GFP-Shrub was expressed using the exogenous *nanos*-Gal4 promoter (i.e. not under the endogenous transcriptional regulation) indicating that Shrub is regulated at the protein level. This raises the exciting possibility that the absence of Shrb in dividing cysts may block abscission in differentiating germline cysts. Elegant works performed in the mouse testis have shown that TEX14 blocks abscission in spermatogonia [14]. There is no homologue of TEX14 in *Drosophila*, and what blocks abscission in the differentiating cysts remains a major question in the field. We believe that elucidating how Shrb protein levels are regulated in the fly germline cyst may help understand how incomplete cytokinesis is controlled.

## Materials and Methods

### *Drosophila* genetics

The *Drosophila* alleles or transgenes used in this study are HMS01767 (TRiP line; [29]); *shrb*<sup>G5</sup> and *shrb*<sup>O3</sup> [41]; *shrb*<sup>EY05149</sup> (Bloomington Stock Center); *H2B-RFP* [44]; *G147* [45];

*UASp-tubulin-PA-GFP* [46]; *lgd<sup>d17</sup>* [47]; *lgd<sup>EY04750</sup>* (Bloomington Stock Center); *aurB<sup>2A43</sup>* and *aurB<sup>3533</sup>* [20]; *UASp-par1-GFP* [48].

Overexpression experiments were performed using the Gal4/UASp system [49] with the *nanos-GAL4-VP16* [50] or the *bam-GAL4* [51] drivers. We generated the *bam-GAL80* construct (see later), and combined it with a *nanos-GAL4* driver devoid of VP16 activation domain (a kind gift of M. Fuller).

The germline clones were generated using the Flp/FRT technique [52,53]. Clones were induced by heat-shocking third instar larvae at 37°C for 2 hours, females were dissected 2 days after eclosion.

## Constructs

To generate the *bam-Gal80* construct, we synthesized a 3886 bp DNA fragment encoding the *bam* promoter (−898 to +133, between 5' AGATCTAACCATTGATTAAC 3' and 5'GATTTGTGTGATTTAACTTA 3'), the Gal80 coding sequence (5' ATGGACTACAACAAGA GAT 3' to 5' TCTCGCATTATAGTTTATAA 3') and terminated by the K10 terminator, between Not1 sites (euofins/MWG). The NotI fragment was then cloned into pCasper vector.

To generate the pUASp-GFP-*shrb* construct, we subcloned the *shrb* fragment from a pDONR221 vector for Nter fusion construct (a kind gift from Pier Paolo D'Avino; [18]) by LR recombination to pPGW destination vector.

Transgenic lines were generated by BestGene.

## Immunohistochemistry

Antibody staining and Hoechst staining were performed according to standard protocols. Briefly, ovaries were dissected in PBS, fixed in 4% PFA, permeabilized in PBT (PBS-0,2% Triton) for 30 min, left overnight with primary antibodies in PBT at 4°C, washed 2 h in PBT, left with secondary antibody for 2 hrs at room temperature, washed 1 h in PBT and mounted in Cityfluor.

The primary antibodies used in flies were the following: mouse-anti- $\alpha$ -spectrin (clone 3A9, DSHB) 1:500; rb anti- $\alpha$ -spectrin 1:1000 [54], rat-anti-BamC 1:1000 [30]; rb-anti-Nanos 1:200 [55]; rb-anti-pSMAD 1:100 (a kind gift of Peter ten Dijke); rb-anti-Pav 1:150 [56], rb-anti pH3S10 1:1000 (Upstate). Fluorescent secondary antibodies were from Jackson ImmunoResearch; rhodamin-phalloidin and Hoechst were from Molecular Probes.

For Edu treatment (Click-iT Edu Imaging kit, Invitrogen), ovaries were dissected in Schneider medium complemented with 10% FBS. There were then incubated at 25°C for 15 min in 20 $\mu$ M Edu in Schneider medium+ 10% FBS. Edu detection was performed according to manufacturer's instructions.

## Quantification and statistics

The number of cell per egg chamber was quantified on DAPI and Rhodamin-phalloidin stained ovaries. We counted the number of nuclei with the DAPI staining. In addition, the number of ring canals stained by phalloidin was counted for the oocyte so that chambers formed of 32 cells having 2 oocytes with 4 ring canals each (due to encapsulation defects) are not included. Quantification of the percentage of egg chambers having more or less than 16 cells were done on one day old females. Chi-square tests were used to compare the proportions of egg chambers having 16 or 32 cells.

Stem-cysts quantification was done on germaria immunostained with alpha-spectrin antibody; stem-cysts were identified as a group of 3 cells minimum, linked by a fusome, with its most anterior cell being attached to the niche. The percentage of germaria exhibiting at least

one stem-cyst was counted. Chi-square tests were used to compare the percentages observed in the different genotypes.

## Microscopy

Acquisition of Z-stacks on fixed sample was carried out on Zeiss LSM710 or LSM780 confocal microscopes. For quantification of egg chambers with 16 or 32 cells, ovaries were analyzed with a Upright Widefield Leica Microscope.

For live imaging of germarium in Fig. 4, ovaries were dissected and mounted in oil (10S, Halocarbon, Sigma) and were imaged with an inverted Confocal Spinning Disk Roper/Nikon equipped with a CCD camera CoolSnap HQ2. Time-lapse images were then treated with Fiji.

For the photo-activation experiments of Fig. 5, ovaries were dissected and mounted in oil (10S, Halocarbon, Sigma). Photo-activation was done with a 2-photon laser at 820nm (3 iterations, laser power 15%, scan speed = 6; these numbers are rough approximations for the excitation power). Imaging was done with a confocal microscope Zeiss LSM 710.

## Supporting Information

**S1 Fig. Description of the time course phenotypes of *shrub* downregulation.** Females expressing *UAS-shrb RNAi* under the control of *nos-GAL4* were dissected after 1, 2, 3 or 5 days. (A) Phenotypes in the cyst: apart from the WT looking chamber of 16 cells and those with 32 cells, chambers with encapsulation defects (B and C), as well as “tumor bags” (D) were observed. (E) Phenotypes in the germarium: apart from the WT looking cells, cells highly polyploid (F, yellow \* indicates a polyploidy cell, white \* indicates normal cell) and germaria without germ cells (G, stem cell loss) were observed. (B, C, D and G) Ovaries were stained for DAPI (DNA, blue) and phalloidin (F-actin, red). (F) Ovaries were stained for DAPI (DNA, blue) and  $\alpha$ -spectrin (fusome, red). Scale bar: 10  $\mu$ m. (TIF)

**S2 Fig. Stem cyst in *shrub* heterozygous.** Germaria of WT females (A and C) or *shrb*<sup>03/+</sup> females (B and D), stained for DAPI (DNA, blue),  $\alpha$ -spectrin (fusome, green), and either Nanos (A and B, red), or pH3S10 (C and D, red) to highlight mitotic cells. Dotted lines surround: GSC/CB pair (A), stem-cysts (B and D) and GSC (C). Scale bar: 10  $\mu$ m. (TIF)

**S3 Fig. Over-expression of GFP-Shrub.** (A) Ovaries expressing *UAS-GFP-shrb* under the control of *bam-GAL4* were stained for DAPI (DNA),  $\alpha$ -spectrin (red). GFP-Shrb (green) is expressed only in the early (mitotic) cysts in the germarium, at low levels. (B) Ovaries expressing *UAS-GFP-shrb* under the control of *nos-GAL4* were stained for DAPI (DNA),  $\alpha$ -spectrin (red). GFP-Shrb (green) is present in all germline. In the GSC and CB it is enriched in the fusome, ring canal (check also Fig. 6) and vesicles; it stays present also in the 2 cell cyst (cc), but it barely detected in 4, 8 and 16cc; in meiotic 16cc, GFP-Shrb localizes to vesicles enriched at the fusome. (C) Fraction of egg chambers exhibiting 32 cells on the y axis. Genotypes are on the x axis. (D) Females expressing *UAS-GFP-shrb* under the control of *nos-GAL4* were stained for DAPI (DNA),  $\alpha$ -spectrin (red); stem-cysts were observed. Scale bar: 10  $\mu$ m. (TIF)

**S1 Movie. Diffusion of Tubulin-PA-GFP from the GSC to its daughter CB in late G2 wild type GSC/CB pair.** Wild type GSC/CB pair expressing *UAS-PARI-GFP* (fusome) and *UAS-Tubulin-PA-GFP* under the control of *nos-GAL4* driver. The GSC and the CB are linked by an exclamation point shaped fusome. Tubulin-PA-GFP was activated in the GSC cytoplasm

between the first and second time points. Note the diffusion of the Tubulin-PA-GFP from the GSC to the CB. Time frame: 10 sec  
(AVI)

**S2 Movie. No diffusion of Tubulin-PA-GFP from the GSC to its daughter CB in late G2 wild type GSC/CB pair.** Wild type GSC/CB pair expressing *UAS-PARI-GFP* (fusome) and *UAS-Tubulin-PA-GFP* under the control of *nos-GAL4* driver. The GSC and the CB are linked by an exclamation point shaped fusome. Tubulin-PA-GFP was activated in the GSC cytoplasm between the first and second time points. Note the absence of diffusion of the Tubulin-PA-GFP from the GSC to the CB. Time frame: 10 sec  
(AVI)

**S3 Movie. Diffusion of Tubulin-PA-GFP from the anterior GSC to all cells of the stem cyst in *shrb<sup>G5</sup>/+* female.** Stem cyst of three cells expressing *UAS-PARI-GFP* (fusome) and *UAS-Tubulin-PA-GFP* under the control of *nos-GAL4* driver in *shrb<sup>G5</sup>/+* female. The GSC is linked by a linear fusome to two cells. Tubulin-PA-GFP was activated in the GSC cytoplasm between the first and second time points. Note the gradual diffusion of the Tubulin-PA-GFP from the anterior GSC to more posterior cells of the stem cyst. Time frame: 10 sec.  
(AVI)

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## Author Contributions

Conceived and designed the experiments: JM JRH. Performed the experiments: NRM JM. Analyzed the data: NRM JM JRH. Wrote the paper: JM JRH.

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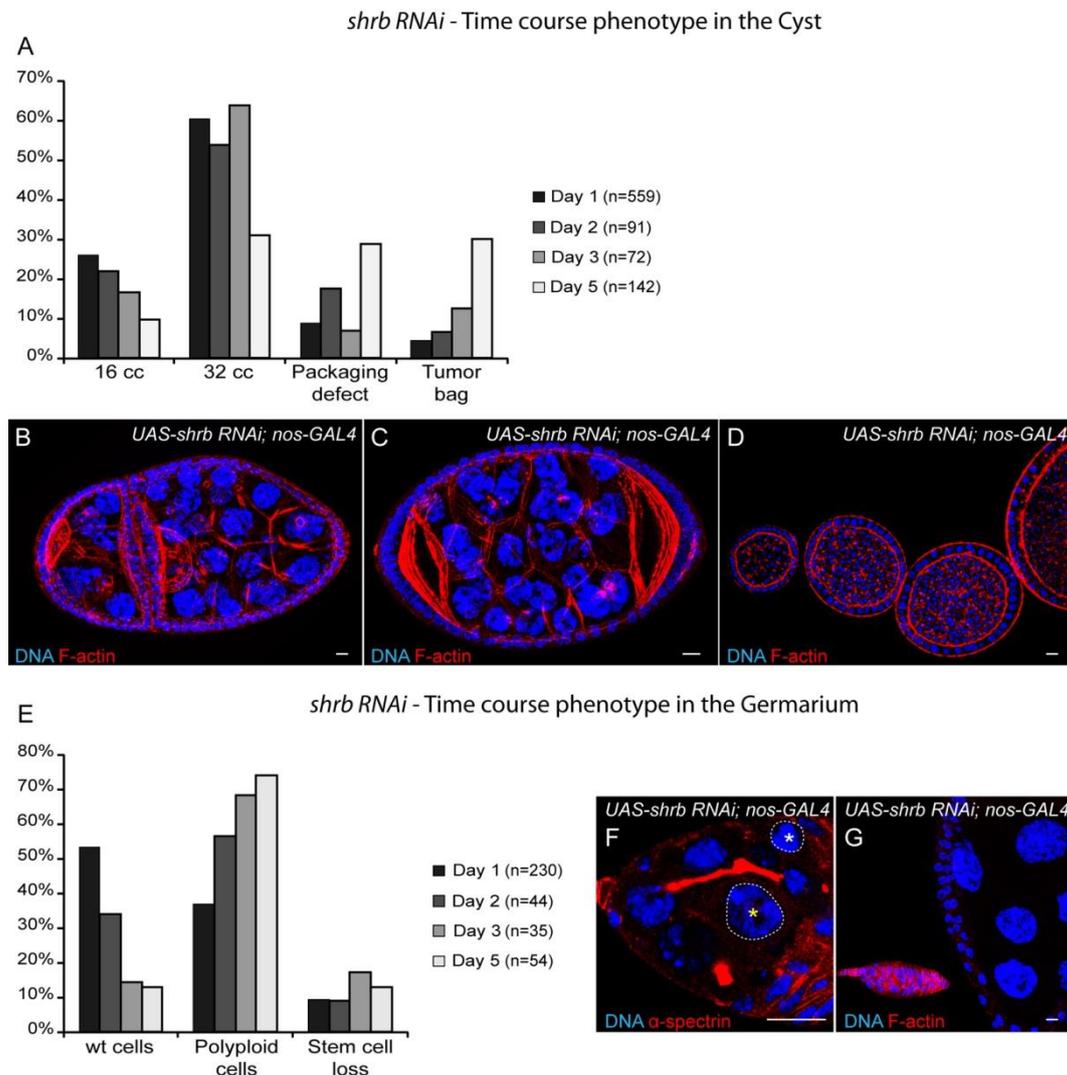
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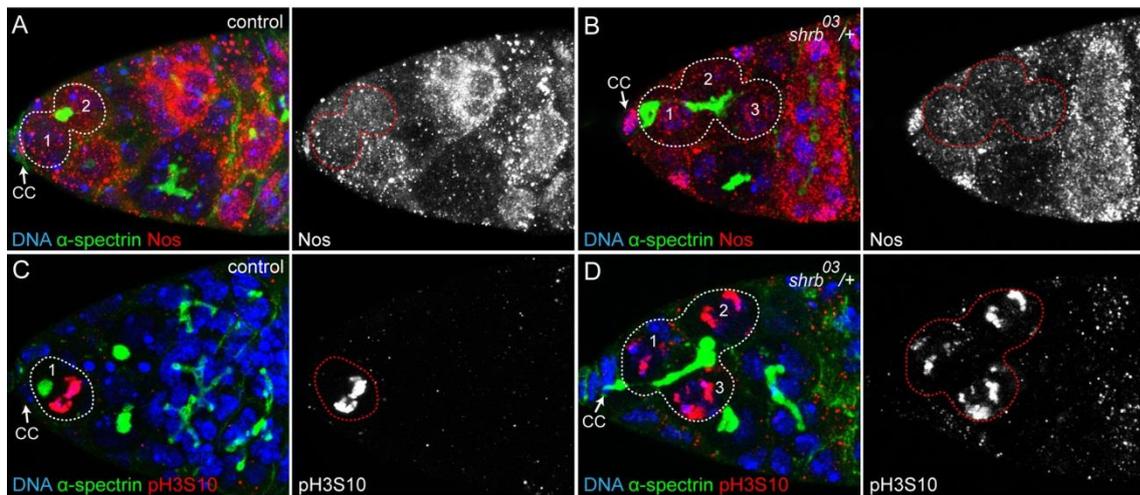
## SUPPORTING INFORMATION

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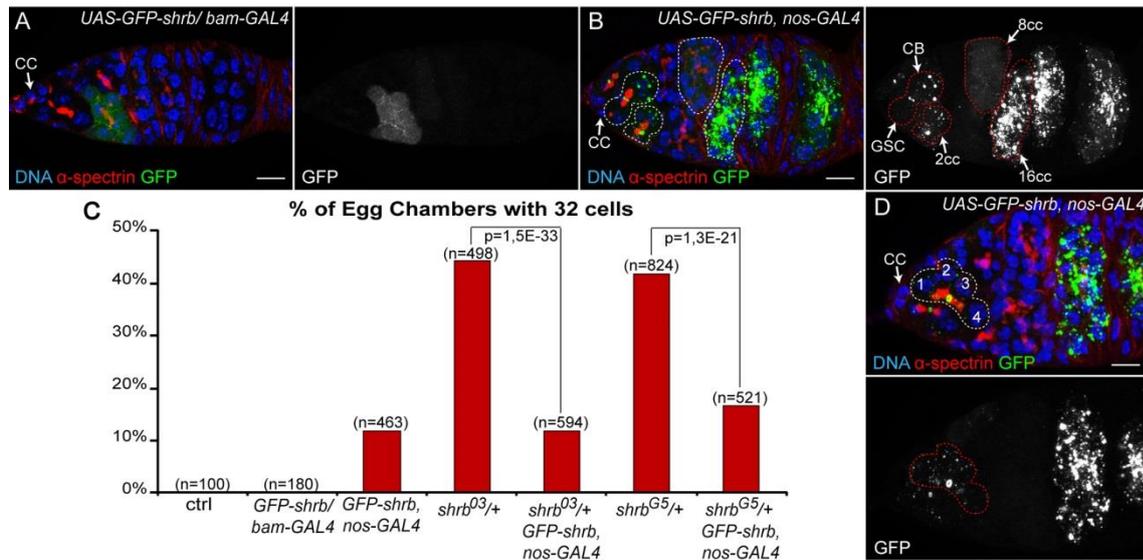
Supplementary figure 1

**Figure S1: Description of the time course phenotypes of *shrb* downregulation.** Females expressing *UAS-shrb RNAi* under the control of *nos-GAL4* were dissected after 1, 2, 3 or 5 days. **(A)** Phenotypes in the cyst: apart from the WT looking chamber of 16 cells and those with 32 cells, chambers with encapsulation defects **(B and C)**, as well as “tumor bags” **(D)** were observed. **(E)** Phenotypes in the germarium: apart from the WT looking cells, cells highly polyploid **(F, yellow \* indicates a polyploidy cell, white \* indicates normal cell)** and germaria without germ cells **(G, stem cell loss)** were observed. **(B, C, D and G)** Ovaries were stained for DAPI (DNA, blue) and phalloidin (F-actin, red). **(F)** Ovaries were stained for DAPI (DNA, blue) and  $\alpha$ -spectrin (fusome, red). Scale bar: 10  $\mu$ m.



Supplementary figure 2

**Figure S2: Stem cyst in *shrub* heterozygous.** Germaria of WT females (**A and C**) or *shrb*<sup>03/+</sup> females (**B and D**), stained for DAPI (DNA, blue),  $\alpha$ -spectrin (fusome, green), and either Nanos (**A and B**, red), or pH3S10 (**C and D**, red) to highlight mitotic cells. Dotted lines surround: GSC/CC pair (**A**), stem-cysts (**B and D**) and GSC (**C**). Scale bar: 10  $\mu$ m.



Supplementary figure 3

**Figure S3: Over-expression of GFP-Shrub.** (A) Ovaries expressing *UAS-GFP-shrb* under the control of *bam-GAL4* were stained for DAPI (DNA),  $\alpha$ -spectrin (red). GFP-Shrub (green) is expressed only in the early (mitotic) cysts in the germarium, at low levels. (B) Ovaries expressing *UAS-GFP-shrb* under the control of *nos-GAL4* were stained for DAPI (DNA),  $\alpha$ -spectrin (red). GFP-Shrub (green) is present in all germline. In the GSC and CB it is enriched in the fusome, ring canal (check also Fig.6) and vesicles; it stays present also in the 2 cell cyst (cc), but it barely detected in 4, 8 and 16cc; in meiotic 16cc, GFP-Shrub localizes to vesicles enriched at the fusome. (C) Fraction of egg chambers exhibiting 32 cells on the y axis. Genotypes are on the x axis. (D) Females expressing *UAS-GFP-shrb* under the control of *nos-GAL4* were stained for DAPI (DNA),  $\alpha$ -spectrin (red); stem-cysts were observed. Scale bar: 10  $\mu$ m.

[S1 Movie.](#) Diffusion of Tubulin-PA-GFP from the GSC to its daughter CB in late G2 wild type GSC/CB pair. Wild type GSC/CB pair expressing *UAS-PAR1-GFP* (fusome) and *UAS-Tubulin-PA-GFP* under the control of *nos-GAL4* driver. The GSC and the CB are linked by an exclamation point shaped fusome. Tubulin-PA-GFP was activated in the GSC cytoplasm between the first and second time points. Note the diffusion of the Tubulin-PA-GFP from the GSC to the CB. Time frame: 10 sec  
doi:10.1371/journal.pgen.1004653.s004

[S2 Movie.](#) No diffusion of Tubulin-PA-GFP from the GSC to its daughter CB in late G2 wild type GSC/CB pair. Wild type GSC/CB pair expressing *UAS-PAR1-GFP* (fusome) and *UAS-Tubulin-PA-GFP* under the control of *nos-GAL4* driver. The GSC and the CB are linked by an exclamation point shaped fusome. Tubulin-PA-GFP was activated in the GSC cytoplasm between the first and second time points. Note the absence of diffusion of the Tubulin-PA-GFP from the GSC to the CB. Time frame: 10 sec  
doi:10.1371/journal.pgen.1004653.s005

[S3 Movie.](#) Diffusion of Tubulin-PA-GFP from the anterior GSC to all cells of the stem cyst in *shrb<sup>G5</sup>/+* female. Stem cyst of three cells expressing *UAS-PAR1-GFP* (fusome) and *UAS-Tubulin-PA-GFP* under the control of *nos-GAL4* driver in *shrb<sup>G5</sup>/+* female. The GSC is linked by a linear fusome to two cells. Tubulin-PA-GFP was activated in the GSC cytoplasm between the first and second time points. Note the gradual diffusion of the Tubulin-PA-GFP from the anterior GSC to more posterior cells of the stem cyst. Time frame: 10 sec.  
doi:10.1371/journal.pgen.1004653.s006

## SUPPLEMENTARY RESULTS

### 1- Transheterozygous *shrb<sup>G5</sup>/lgd<sup>d7</sup>* rescued 32-cell cyst phenotype due to abscission in late cysts

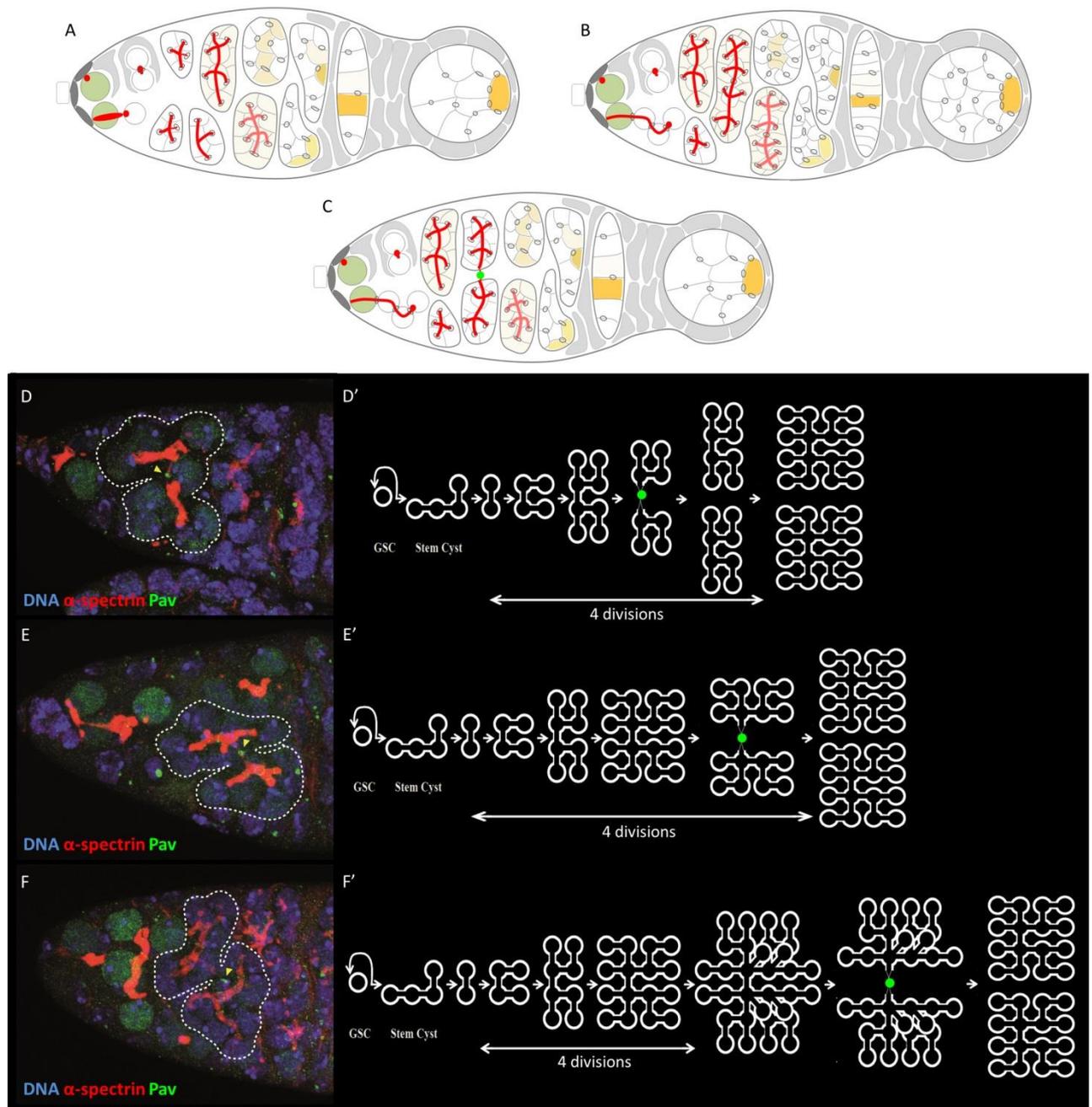
In our published article, we tested whether Lethal giant discs (Lgd) would regulate Shrb during its function in cytokinetic abscission. Indeed we observed that germline clones (GLC) of *lgd<sup>d7</sup>* (*hsFlp/+; lgd<sup>d7</sup>, FRT 40A/ GFP, FRT 40A*) gave rise to stem-cysts and egg-chambers with 32 cells, as found in *shrb* heterozygous females (*shrb<sup>G5</sup>/+*). Furthermore, when we crossed heterozygous *shrb<sup>G5</sup>* with heterozygous *lgd<sup>d7</sup>* (with no abscission phenotype) and looked at *shrb<sup>G5</sup>/lgd<sup>d7</sup>* we saw an increase in stem-cysts compared to *shrb/+* alone. These results showed that Lgd interacts positively with Shrb in promoting germline stem cell (GSC) abscission. However, very surprisingly, we observed that the number of 32-cell cysts was decreased in the transheterozygous *shrb<sup>G5</sup>/lgd<sup>d7</sup>* flies. We hypothesized that this negative genetic interaction between Lgd and Shrb could reflect another function of Lgd later in the mitotic region. It seems that Lgd function in the cyst is important to maintain the 32 cells derived from the stem-cyst.

We could make two hypotheses to explain the rescue of the 32-cell cysts in the *shrb<sup>G5</sup>/lgd<sup>d7</sup>* females: 1) decreasing the number of mitosis to three; 2) inducing abscission in the cysts and separation of two 16-cell cysts.

The first hypothesis is not likely to be true as, in both mutants separately, we did not observe any change in the number of divisions. No egg chambers with 8 cells were found.

To test the second possibility, we dissected ovaries from transheterozygous *shrb<sup>G5</sup>/lgd<sup>d7</sup>* females. The ovaries were stained with  $\alpha$ -spectrin (marker of the fusome), to identify the number of cells per cyst, and incubated with antibody against pavarotti (marker of the midbody), to observe events of complete abscission (Fig1). Midbodies were found in the mitotic region of the germaria, localized on a thin thread of fusome connecting two cells within the cyst. Very intriguingly, in all the examples observed (n=6), the cysts appear to be divided exactly in the middle, as half of the cells were on one side of the bridge and the

other half on the opposite side. The separation of the cysts in the middle, would explain the rescue of the 32 cells to two cysts made of exactly 16 cells. We thus conclude that reducing Lgd levels in *Shrb* heterozygous promotes abscission in the cysts. How abscission could be induced in these cells is not yet understood.



**Figure 1: Reduced Lgd levels in *Shrb* heterozygous promotes abscission in the cysts (A)** In the wild type gerarium, the GSC divides asymmetrically with complete abscission, giving rise to another GSC and a CB. This goes through 4 rounds of mitosis with incomplete cytokinesis, forming a cyst of 16

cells interconnected by ring canals. The fusome is represented in red, and branches into all the cells from the cyst. **(B)** In *shrb* heterozygous, stem cysts are formed due to delay in GSC abscission. Once the 2-cell precursor (CB equivalent) is separated from the stem-cyst, it divides the expected 4 times. Because it started with 2 cells instead of one, the resulting cyst contains 32 cells. **(C)** In transheterozygous *shrb<sup>G5</sup>/lgd<sup>d7</sup>* mutants stem-cysts are also formed and a 2-cell precursor released. These 2 cells go through 4 cycles of mitosis having the potential to form cysts of 32 cells. However, abscission is induced in the middle of the cyst, separating it in two equal halves and rescuing the initial phenotype. **(D-F)** Examples of breaking cysts in *shrb<sup>G5</sup>/lgd<sup>d7</sup>* mutants, staining with  $\alpha$ -spectrin (RFP, fusome) and  $\alpha$ -pavarotti (Pav, GFP, midbody). **(D)** Abscission happening in a cyst of 8 cells. **(D')** Schematic model predicting that, once the 8-cell cyst is broken, the two resulting 4-cell cysts go through two more mitosis, giving rise to two cysts of 16 cells. **(E)** 16-cell cyst going through abscission. **(E')** The model predicts that the two resulting 8-cell cysts would go through the last mitosis and form 2 cysts of 16 cells. **(F)** A cyst of 32 cells breaking into two 16-cell cysts. Yellow arrow head points the midbody in the left images, while green circle represents the site of abscission in the right schemes.

## 2- Lgd function in the mitotic cysts seems to be cell autonomous

All cells in double heterozygous *shrb<sup>G5</sup>/lgd<sup>d7</sup>* germaria have reduced *lgd* protein level. Thus, it is not known whether the cells responsible for the rescued 32-cell cyst phenotype are the germline cyst cells, or the somatic cells around them.

Unfortunately, RNAi constructs able to target Lgd in the germline are not available and thus, is not yet possible to test a cell autonomous effect, using the *bam*>GAL4 driver. We are in the process of injecting *Drosophila* embryos our own TRiP construct targeting Lgd, specific to function in the germline.

However, an RNAi line (TRiP valium 10) that works in somatic cells is available and can be used to test for a possible non-autonomous function of *lgd*. The best candidate somatic cells to exert a regulatory function in the abscission of the cyst cells are the posterior escort cells (pECs). These are in close contact with the cyst cells and are known to be important for proper cyst differentiation (see chapter II of introduction). We thus tested if the impairment of Lgd function in the pECs was sufficient to induce the breakage of the dividing cysts, as observed in the *shrb<sup>G5</sup>/lgd<sup>d7</sup>* mutants. To test this hypothesis, we expressed RNAi for *lgd* in the pECs, using the C587-GAL4 driver (activated in ECs and early follicular cells). To avoid early RNAi expression, the cross was done at 18°C and the animals were maintained at this

temperature until hatching. Then the females were moved to 25°C, to allow GAL4 activation, and dissected after 4 days. Only egg chambers with 16 cells were observed in this experiment. This suggests that Lgd does not function in escort cells to control germline abscission.

Interestingly, while in *shrb*<sup>G5</sup>/+ the percentages of 32-cell cysts and of stem-cysts are both higher and with similar values, in the *lgd*<sup>d7</sup> GLC the number of stem-cysts observed is much higher than the number of 32-cell cysts. This suggests that in the *lgd*<sup>d7</sup> GLC a rescue of the 32-cells cysts could happen. This is in agreement with a cell autonomous effect of Lgd in the cyst, as in this experiment only germline mutant clones were scored. Furthermore, this effect of Lgd loss of function seems to be independent of Shrb protein levels in the background.

### **3- Is Lgd function in GSC abscission regulated by Cdk1 phosphorylation?**

In the germarium, abscission occurs during GSC division, but does not progress during cyst divisions. One possible explanation for this observation is that the abscission machinery is differently regulated in these two compartments.

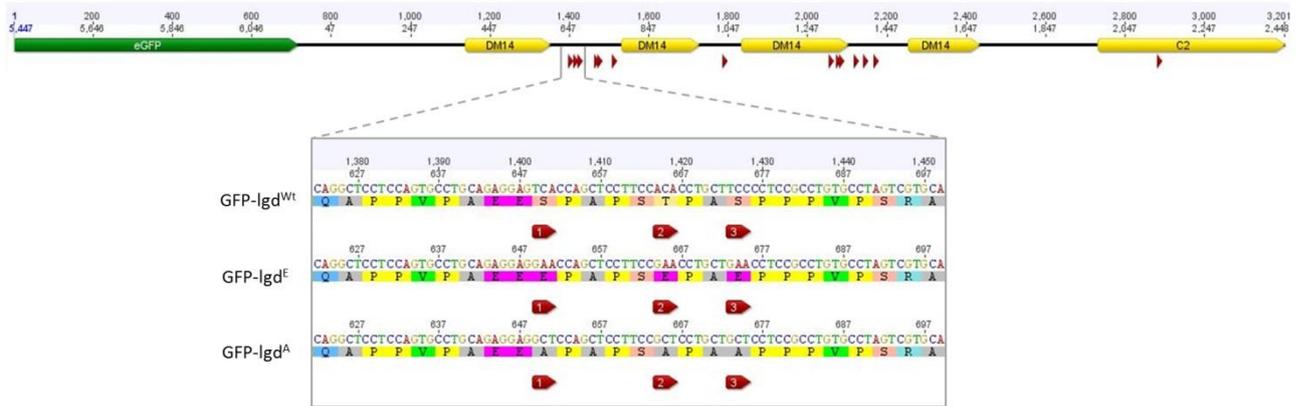
In mammalian cells, it was shown that the chromosomal passenger complex (CPC), regulates abscission timing through direct binding and phosphorylation of a mammalian homologue of Shrb (Carlton, 2012; Capablo, 2012). In *Drosophila*, even though the CPC binds to Shrb, it does not seem to phosphorylate it (Capablo, 2012). However, in our article we showed that Aurora B negatively interacts with Shrb during GSC abscission. This suggests that the CPC might inhibit Shrb polymerization through a physical blockage.

The other complex responsible for timing abscission is CycB/Cdk1. No evidences were yet found that support a regulation of Shrb by Cdk1. However, work done in mammalian cells reported that Cdk1 phosphorylates Lgd, in at least one site (Ser<sup>208</sup>) (Nakamura, 2010). This mitotic phosphorylation was shown to contribute for the ability of Lgd to bind the cohesin component Scc1.

Because Lgd seems to promote abscission in the *Drosophila* female GSC, we hypothesized that it could be induced to do so by Cdk1-dependent phosphorylation. To test this possibility, we first investigated if the *Drosophila* Lgd has any Cdk1 phosphorylation consensus sites (S/T\*-P, with the asterisk indicating the site of phosphorylation) (Holt, 2009). We found 14 of these sites in the Lgd molecule, represented in the figure 2. Many of these sites are conserved from vertebrates to flies.

To test if Lgd phosphorylation at these sites is functionally required in abscission, we constructed two different phospho-mutant forms of the protein. Both were tagged with GFP in its N-terminal and under the control of a UASp promoter. All 14 sites were transformed either into phosphomimic (*UASp-GFP-Lgd<sup>E</sup>*), or nonphosphorytable (*UASp-GFP-Lgd<sup>A</sup>*). A wild type version was also constructed to use as control (*UASp-GFP-Lgd<sup>wt</sup>*) (Fig2).

The ideal experiment to test our hypothesis would be to induce germline *lgd* mutant clones and, at the same time, express our construct in the clones. This would be possible using MARCM (RFP) flies. In this situation we would expect to have in the clones: a rescue in the formation of stem-cysts when expressing *UASp-GFP-Lgd<sup>wt</sup>*; increased percentage of stem-cysts with *UASp-GFP-Lgd<sup>A</sup>*; and maybe precocious GSC abscission with the expression of *UASp-GFP-Lgd<sup>E</sup>*.

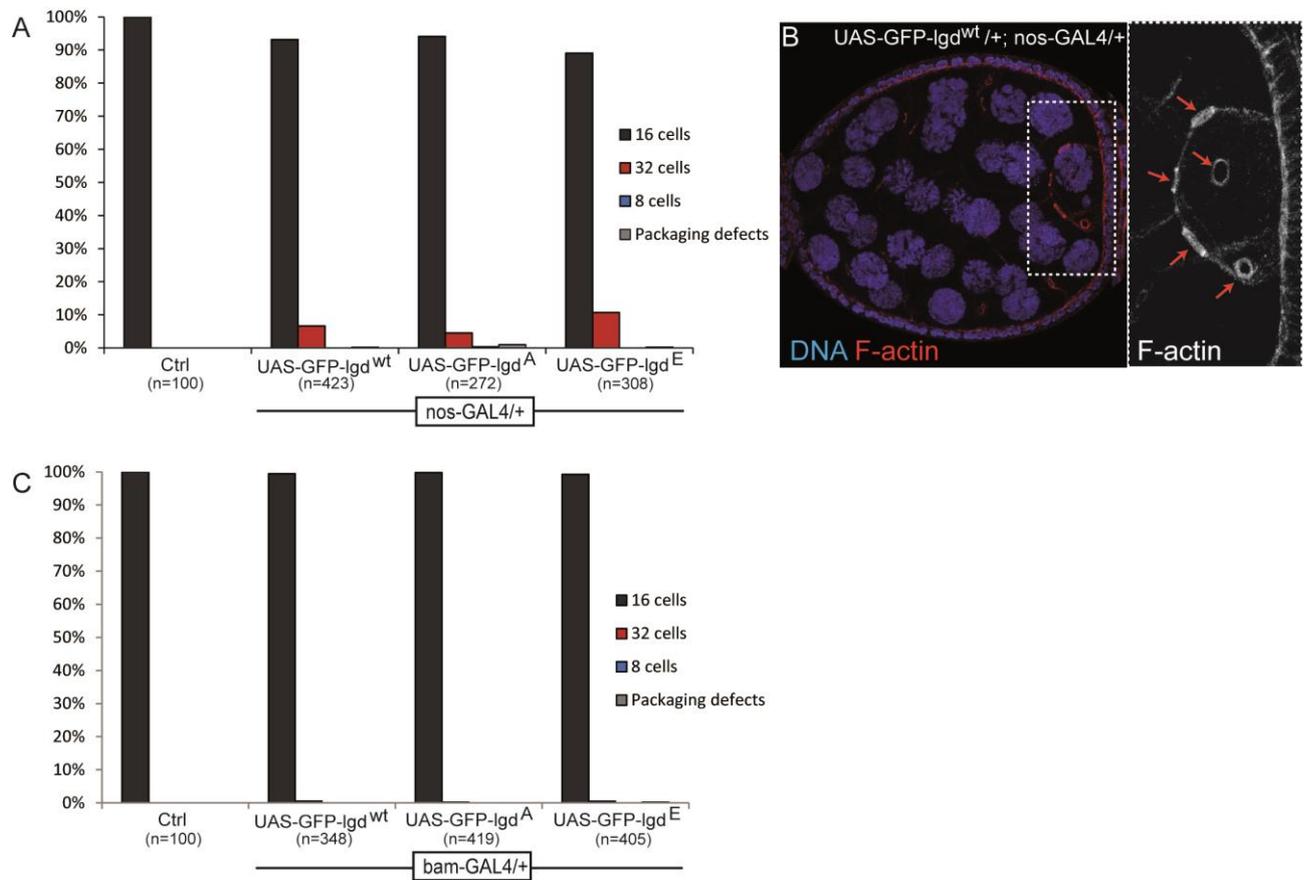


**Figure 2: Representation of the Lgd protein, tagged with GFP on the N-terminal.** Lgd has four DM14 domains, with unknown function, but shown to be important for its interaction with Shrb (Troost, 2012). On its C-terminus, Lgd has a C2 domain, shown to be important for protein stability and cytoplasmic localization (Troost, 2012). We found 14 consensus sites for Cdk1 phosphorylation in Lgd, indicated by red triangles. Holt et al. showed that Cdk1 has the tendency to phosphorylates Serine or Threonine, followed by Proline (S/T\*-P) (Holt, 2009). Zoomed out is a window of DNA and protein sequence from the different Lgd forms we constructed (*UASp-GFP-Lgd<sup>wt</sup>*, *UASp-GFP-Lgd<sup>E</sup>* and *UASp-GFP-Lgd<sup>A</sup>*), showing the different point mutations (extracted from Geneious).

### 3.1- Overexpression of GFP-Lgd in the germline results in the formation of stem-cysts and 32-cell egg chambers

We received the transgenic flies (*UASp-GFP-Lgd<sup>wt</sup>*, *UASp-GFP-Lgd<sup>A</sup>* and *UASp-GFP-Lgd<sup>E</sup>*) very recently and, as a first step, we simply expressed them using the germline driver *nos>GAL4*, in a wild type context for Lgd. With this experiment we were able to score for possible dominant negative effects of mutant Lgd forms. We observed that overexpression of any of the GFP-Lgd forms resulted in the formation of approximately 10% of 32-cell cysts (Fig3A-B). Stem-cysts were also sporadically found (Fig4E).

We then expressed these three constructs, under control of *bam>GAL4* driver, and observed that overexpression of GFP-Lgd, specifically in the mitotic cysts, had no defect (Fig3C). However, because the *bam>GAL4* is a weak driver, we will confirm this result by adding an additional copy of it (homozygous *bam>GAL4* background).



**Figure 3: *Igd* overexpression with *nos*>*GAL4*, but not with *bam*>*GAL4*, induces the formation of few 32-cell cysts. (A and C) Fraction of egg chambers exhibiting 16, 32, 8 cells or packaging defects on the y axis. Genotypes are on the x axis. (B) Stage 7 egg chamber from *UAS-GFP-Igd<sup>wt</sup>/+; nos-GAL4/+* stained with DAPI (DNA, blue) and phalloidin (F-actin, red). On the right, close-up on oocyte. Red arrows indicate the four ring canals in the control oocyte and the five ring canals in the mutant background.**

### 3.2- GFP-Lgd is enriched at the abscission site and in the fusome

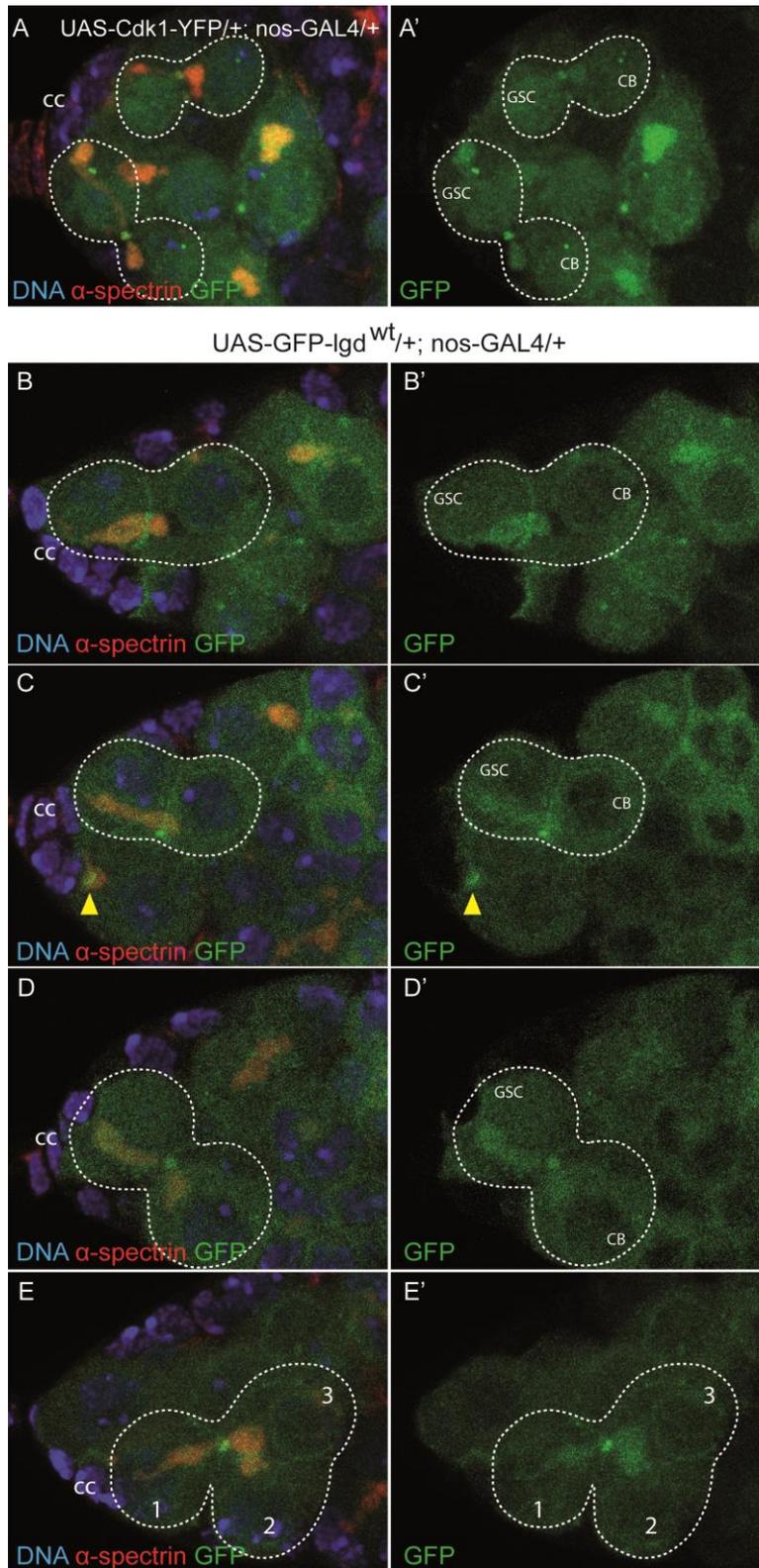
The expression of *UASp-GFP-Lgd<sup>wt</sup>*, *UASp-GFP-Lgd<sup>A</sup>* and *UASp-GFP-Lgd<sup>E</sup>*, under control *nos>GAL4* driver, allowed us to analyze protein localization following the GFP tag (Fig4B-E). We saw that the three different GFP-Lgd forms have very similar localization in the germarium. This suggests that their cellular localization does not depend on Cdk1 phosphorylation, at least when in the context of *lgd* wild type background.

Interestingly, we observed GFP-Lgd being enriched in the fusome of GSC/CB pairs, and to a less extent in some of the early mitotic cysts. We saw it localizing at the GSC ring canal, at the onset of constriction (Fig4B), and being strongly enriched at the abscission site (Fig4C-D). GFP-Lgd was also found localizing to the GSC spectrosome, probably with the midbody taken by the fusome after abscission (Fig4C). We will better characterize these lines, looking at GFP-Lgd dynamics in live imaging.

The expression of *UAS-cdk1-YFP* in the germline showed that also Cdk1 localizes to the abscission site and is enriched in the fusome (Fig4A).

The exciting fact that *Lgd* seems to colocalize with *Shrb* and Cdk1, at the site and timing of abscission, suggests that their possible regulatory interactions may occur at this location.

However, it is very surprising to observe *Lgd* enrichment at the fusome, mostly made of ER vesicles, and at the abscission site. Indeed, in S2 and KC cells (Childress, 2006), as well as in imaginal discs (Troost, 2012), *Lgd* was seen localizing at the cytoplasm, but no association with membrane structures was ever observed.



**Figure 4: Both Cdk1 and Lgd are enriched at the abscission site.** Germlaria of UAS-cdk1-YFP/+; nos>GAL4/+ (**A**) or UAS-GFP-Lgd<sup>wt</sup>/+; nos>GAL4/+ (**B-E**) females stained for DAPI (DNA, blue) and α-spectrin (fusome, green). Both YFP and GFP are in green. Dotted lines surround: GSC/CB pair (A-D) or stem-cysts (**E**). Cap cells (CC) are indicated. (**C**) Yellow arrow head points to a dot of GFP-Lgd enriched in the GSC spectrosome.

## **DISCUSSION**

The ESCRT proteins, first described in yeast to act in endosomal membrane bundling and formation of MVBs, have been recently found to act in several other cellular processes. Abscission, the last step of cell division when daughter cells get physically separated, was shown to be regulated by the ESCRT machinery. Even though this process has been well described in mammalian cultured cells, how it is regulated in different cell types of a developing organism is poorly understood. Together with our collaborators Eikenes et al., our work was the first to show in vivo ESCRT function in abscission (Eikenes et al., 2015). *Shrb*, the *Drosophila* ESCRT-III homologue of the mammalian CHMP4, appears to promote female germline stem cell (GSC) abscission. Mutations in *shrb* result in the formation of stem-cysts, a specific abscission phenotype, and consequently egg chambers with 32 cells. Interestingly, our genetic interaction experiments point to a role of the abscission timer Aurora B in regulating *Shrb*. *Shrb* appears also to interact in this process with *Lgd*, which we show to localize at the abscission site.

## **1- *Shrb* promotes GSC abscission**

After discovering that the chromosomal passenger complex (CPC) and *CycB/Cdk1* function as timers during *Drosophila* female GSC abscission, our group became interested in understanding this process in depth. Thus, a small-scale screen was performed to identify downstream regulators of GSC abscission. The ESCRT-III *Shrb* was the protein with the highest score for the abscission phenotypes. Females expressing RNAi specific for *Shrb* in the germline showed high percentage of stem-cysts and egg chambers with 32 cells.

*Shrb* downregulation in the germline results in a delay in GSC abscission. As a consequence of incomplete abscission and continue cell division cycles, a structure very similar to a cyst is formed. Several cells remain connected to the stem cell by ring canals. These so called stem-cysts were previously described by Mathieu et al. 2013 to be present both in over-activated CPC mutants, as well as in *CycB* loss of function, which induce a delayed abscission (Mathieu et al., 2013). Because stem-cysts are very similar in the different mutants, we hypothesized that they derive from the same initial defect.

Interestingly, we observed the formation of stem-cysts and egg chambers with 32 cells, in flies heterozygous for three different alleles of *shrb*. We then proceeded to the characterization of stem-cyst in *shrb* heterozygous females.

The cells from the stem-cyst are interconnected by actomyosin ring canals. To determine if they function as proper channels for cell communication we tested whether cells in the stem-cyst were sharing the same cytoplasm. We photoactivated tubulin tagged with GFP in one of the cells of the stem-cyst and checked whether the fluorescent labelling would pass to other interconnected cells. Indeed, we saw that photoactivated tub-GFP in the GSC was able to diffuse to the other cells. The same happened when tub-GFP was photoactivated in other cells from the cyst.

To functionally characterize stem-cysts, we stained mutant germaria with markers for stem cells and for differentiating cysts. Interestingly, we saw that cells of the stem-cyst were in an undifferentiated state. None of the interconnected cells expressed the differentiation protein Bam, but rather expressed Nanos in levels very similar to those of wild type GSC-CB pairs. However, phosphorylated Mad (P-Mad), which is normally detected in the GSC in response to BMP signaling, remained restricted to the nucleus of the anterior most cell of the stem-cyst in contact with cap cells (the GSC).

This result is very intriguing in two ways: 1) Why is P-Mad restricted to the GSC and does not diffuse to the other interconnected cells in the stem-cyst? 2) If Bam is thought to be inhibited in the GSC through activation of the BMP signaling, how could the BMP reporter P-Mad be restricted to the GSC and inhibit Bam be inhibited in all the other cells of the stem-cyst?

One plausible explanation on why P-Mad is restricted to the GSC could be that the fusome acts as a barrier of diffusion to certain molecules. In our photoactivation experiment, we used GFP tagged tubulin and observed its spreading from the cell where it was activated to the other cells of the stem-cyst. Tubulin is a component of microtubules, which are very much enriched in the fusome, and thus might be actively transported from cell to cell through the fusome. The same could happen for molecules that bind to endosomal membranes, or any other fusome component. However, because P-Mad does not localized at the fusome, it is not free to diffuse throughout the cells of the stem-cyst. It would be very interesting to test the capacity of diffusion of other tagged molecules throughout the stem-cyst. In agreement with this hypothesis, it is known that the transport of several molecules and organelles, from the nurse cells to the oocyte, only takes place once the fusome disappears.

Additionally, other mechanisms that promote cystoblast differentiation by counteracting the BMP signaling, could be acting at the posterior cells of the stem-cyst. They could act cell-autonomously by the active degradation of the BMP receptor and by the restriction of Dpp diffusion by the escort cells, which would then inhibit the signaling from the stem-cyst to reach the posterior cells (Kirilly et al., 2011; Morris and Spradling, 2011). However, because we know that the P-Mad antibody used in our stainings does not work very well, some molecules not detectable by microscopy might still diffuse and be able to inhibit Bam. It would be thus important to use other BMP reporters, like Dad-GFP, to test for the activity of the signaling pathway at the posterior cells of the stem-cyst.

Another interesting observation that derived from our study was that the process of GSC abscission seems to be very sensitive to the concentration of Shrb protein. Complete depletion of Shrb by induction of germline clones, leads to cell death, as no clones were ever observed. Downregulation of Shrb through expression of RNAi specific in the germline induces 60-80% of egg chambers with 32 cells and around 50% of stem-cysts. We also saw that heterozygous females, mutant for 3 different alleles of Shrb, induce between 30% (in the weakest allele) and 60% (in the strongest allele) of both 32-cell cysts and stem-cysts. Furthermore, when we overexpressed UAS-GFP-Shrb in the germline we saw the appearance of very few but persistent chambers of 32 cells and stem-cyst. Shrb overexpression could however partially rescue the strong effect of Shrb heterozygous in the formation of stem-

cysts and 32 cell egg chambers, suggesting that GFP-Shrb was functional. Defects associated with the overexpression of GFP tagged Shrb were already reported. Indeed, Sweeney et al. generated a UAS-shrub-GFP transgenic line, which they showed to act as a dominant negative in eyes and neurons (Sweeney, 2006). Even though we made our construct tagged on the N-terminal instead of C-terminal, some defects were still observed. It thus suggests that the GFP tag could be disturbing the conformation of the protein. Shrb is a very small protein, with approximately the same size as the GFP, and both N- and C- terminal might be needed for Shrb function.

However, we cannot exclude that Shrb overexpression would have by itself a phenotype, and an optimal concentration in the cell may be regulated to assure its proper function. In mammalian cells, it is thought that the helicoidal filaments, observed at abscission sites, are formed of an arrangement of different ESCRT-III proteins. These structures most probably obey to a very strict stoichiometry that allows the correct conformation and function of the filament. Once the perfect balance is broke, its function is impaired.

To understand whether the tagged version of Shrb is having an adverse effect, we could analyze flies overexpressing a non tagged form of Shrb, and an endogenous GFP tagged. Indeed, we are using the CRISPR/Cas9 genome editing technique to insert a GFP tag on the N-terminal of the endogenous locus.

## 2- Shrb localizes at the midbody

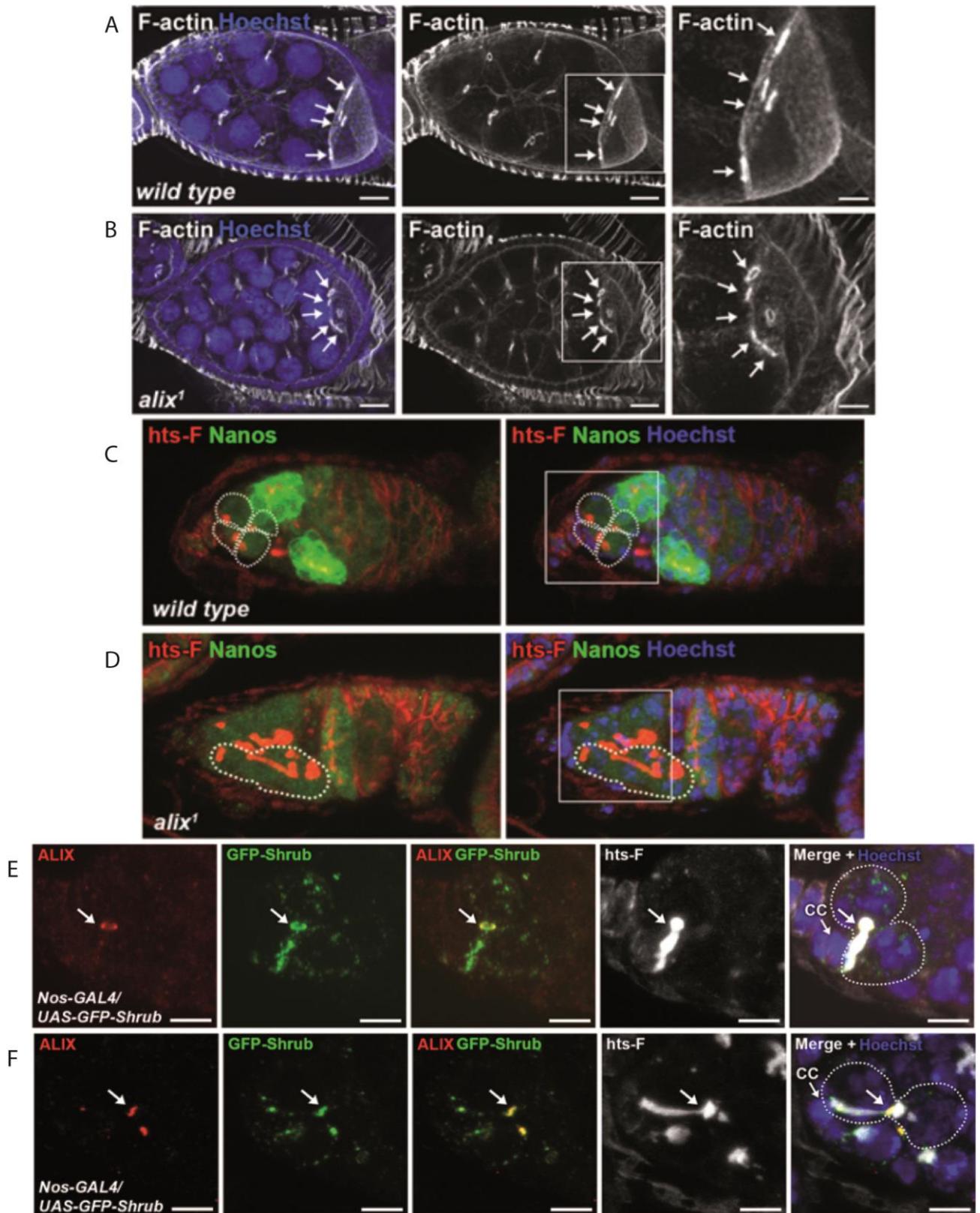
Although UAS-GFP-Shrb expression in the germline induces a slight delay in GSC abscission, it was useful to address its localization during GSC division. We used the specific shape of the fusome/spectrosome to time the GSC cycle. Before abscission in early G2 we observed GFP-Shrb strongly enriched at the ring canal between the GSC and the CB, and often present along the fusome. During ring constriction, Shrb colocalized with the midbody protein Pavarotti. Once abscission happens, the fusome retracts towards the cap cells to form the round spectrosome. During this process, we observed that in several cases the midbody, enriched with GFP-Shrb, seemed to travel with the fusome. At the end of mitosis it often localized on the spectrosome of the GSC.

While in *Drosophila* female GSC divisions Shrb is recruited very early to the contractile ring, and remains enriched at the midbody after abscission, the mammalian homologues CHMP4A, B and C, have a very different localization. CHMP4C was observed localizing initially at the midbody arms (site of abscission) and then being redirected to the midbody central region. CHMP4A and CHPM4B (the functional homologue of Shrb) were observed localizing only transiently to the midbody arms at the time of abscission (Carlton, 2012). Indeed, the dynamic localization of the mammalian ESCRT machinery during abscission is thought to be tightly regulated to define when membrane scission finally occurs (Thoresen et al., 2014). It is thought that during the NoCut pathway, AurB induces abscission delay by retaining the complex composed by ANCHR–CHMP4C–VPS4 at the midbody central region. Only when the check-point is realized, VPS4 is able to properly localize at the abscission site (Thoresen et al., 2014).

A particularity of the *Drosophila* GSC is the presence of the fusome, which seems to be always in close proximity with the ring canal and later with the midbody. It would be important to address whether these differences in Shrb dynamics are dependent on the presence of the fusome. It would also be very interesting to explore Shrb dynamic localization in other *Drosophila* cell types undergoing abscission, which do not have fusome, such as neuroblasts. The analysis of abscission sites using live high resolution microscopy would allow a better understanding of the physical relation between these different players.

In a simultaneous publication, Eikenes et al., showed that the ESCRT linker ALIX, described to promote mammalian cells abscission, also acts during GSC abscission (Eikenes et al., 2015) (Fig1). Homozygous mutants for ALIX resulted in the formation of egg chambers with 32 cells (Fig1B), as well as stem-cysts (Fig1D). They showed that ALIX colocalized with Shrb during cytokinetic abscission in ring canals (Fig1E) and later in midbodies (Fig1F). To further explore the relation between Shrb and ALIX during GSC division, they constructed ALIX forms mutated for the sites shown to mediate interaction with CHMP4 proteins. Contrary to ALIX wild type form, the mutated proteins were not able to rescue the ALIX homozygous mutants, and presented high percentage of stem-cysts and 32-cell cysts. Thus, the authors concluded that the physical interaction between ALIX and Shrb was important in promoting abscission in the female *Drosophila* GSC.

Interestingly, homozygous flies lacking ALIX protein can reach adulthood without major developmental defects. Even though, abscission does not happen properly in the follicular cells from the mutant egg chamber, resulting in bi-nucleated cells, the tissue seems to keep a wild type shape.



**Figure 1:** (A-B) *alix1* (B) mutant egg chambers frequently contain 32 germ cells. Wild type (A) with four ring canals (arrows) to the oocyte. (B) *alix1* mutant with five ring canals (arrows) to the oocyte. Ovaries were fixed and stained to visualize F-actin (white) and nuclei (blue). (C-D) Loss of ALIX causes abnormal GSC division. (A) Wild type *Nanos*-positive GSCs show normal spectrosome/fusome morphologies (GSCs and GSC-CB pairs are outlined). *Nanos*-positive GSCs in the anterior tip of *alix1*

**(D)** mutant germaria are interconnected to chains of daughter cells via abnormally long fusomes (outlined). Ovaries were fixed and stained with antibodies against hts-F (red), Nanos (green) and Vasa (white), and with Hoechst (blue). **(E-F)** ALIX and GFP-Shrub co-localize at ring canals **(E)** and midbodies **(F)** during GSC cytokinesis. GFP-Shrub was expressed under the control of Nos-GAL4. Ovaries were fixed and stained with antibodies against ALIX (red) and hts-F (white), and with GFP Booster (green) and Hoechst (blue). CC, cap cell. (Eikenes et al., 2015).

### **3- The Chromosomal Passenger Complex may inhibit Shrb during GSC abscission**

In mammalian cells, it was shown that the chromosomal passenger complex (CPC), regulates abscission timing through interaction with the Shrb homologues CHMP4 proteins. Indeed, Borealin, one of the component of the complex, was shown to directly bind CHMP4A, B and C, while the kinase Aurora B phosphorylated CHMP4C (Capalbo et al., 2012; Carlton et al., 2012). Functional studies also demonstrated that CHMP4C, when phosphorylated by Aurora B, was capable of inhibiting abscission. In agreement with the functional similarities between Shrb and CHMP4B, but not CHMP4C, it was reported that the Aurora B phosphorylation sites of CHMP4C are not conserved in *Drosophila* Shrb. However, direct binding of Shrb to the CPC via Borealin was observed, suggesting a possible regulatory function also in *Drosophila* (Capalbo et al., 2012).

Because we previously showed that the CPC functions in the GSC to inhibit abscission and control its timing (Mathieu et al., 2013), we tested whether it could possibly act through Shrub regulation. We saw that the delay in abscission seen in Shrb heterozygous mutants was less severe when we removed one copy of Aurora B. Because Aurora B heterozygous does not show any abscission phenotype, this can be considered a proper rescue and not just a sum of opposite phenotypes. Thus, it seems possible that the CPC inhibits abscission through binding and physically blocking Shrb polymerization. The generation and analysis of Shrb mutants, specifically impaired in binding to the CPC, will be very useful to directly address this question.

## 4- GSC abscission is different in females and males

Very recently, Lenhart et al. carefully analyzed the process of abscission in male *Drosophila* GSCs and uncovered interesting differences with abscission in female GSCs (Lenhart and DiNardo, 2015).

An important difference between female and male GSCs is that, while in females the GSC divides alone, in males it divides in coordination with two somatic Cysts Stem Cells (CySCs), which are also attached to the niche. For each gonialblast (Gb, equivalent to the female cystoblast) produced by the GSC, two cyst cells are generated by two CySCs. These somatic cyst cells, which are equivalent to the female escort cells, surround and encyst the Gb during the four mitotic divisions. Somatic cyst cells are known to be essential for the process of spermatogenesis.

The authors observed that the coordination in division between GSCs and CySCs was not achieved by synchronous division. Because GSC abscission happens a long time after mitosis, they suggested that GSCs and CySCs divisions could be coordinated by the timing of GSC abscission.

Indeed, they showed abscission timing to be modulated cell autonomously in two distinct phases, but also non-autonomously through the action of the somatic cyst cells (Fig2).

### a) Cell autonomous regulation

Using live cell imaging, Lenhart et al. characterized the process of male GSCs cytokinesis. They proposed that it happens in two phases, both regulated by AurB, which also functions as an abscission timer.

They saw that after mitosis, cytokinesis progressed normally with the disassembly of the central spindle and contractile ring. However, they observed the formation of a secondary F-actin ring, which they showed to act as a block for cytokinesis progression. They suggested that this block constitutes the first phase of the abscission delay. After the disassembly of

this new F-actin ring, the second phase starts with the re-initiation of cytokinesis, formation of the midbody and finally abscission.

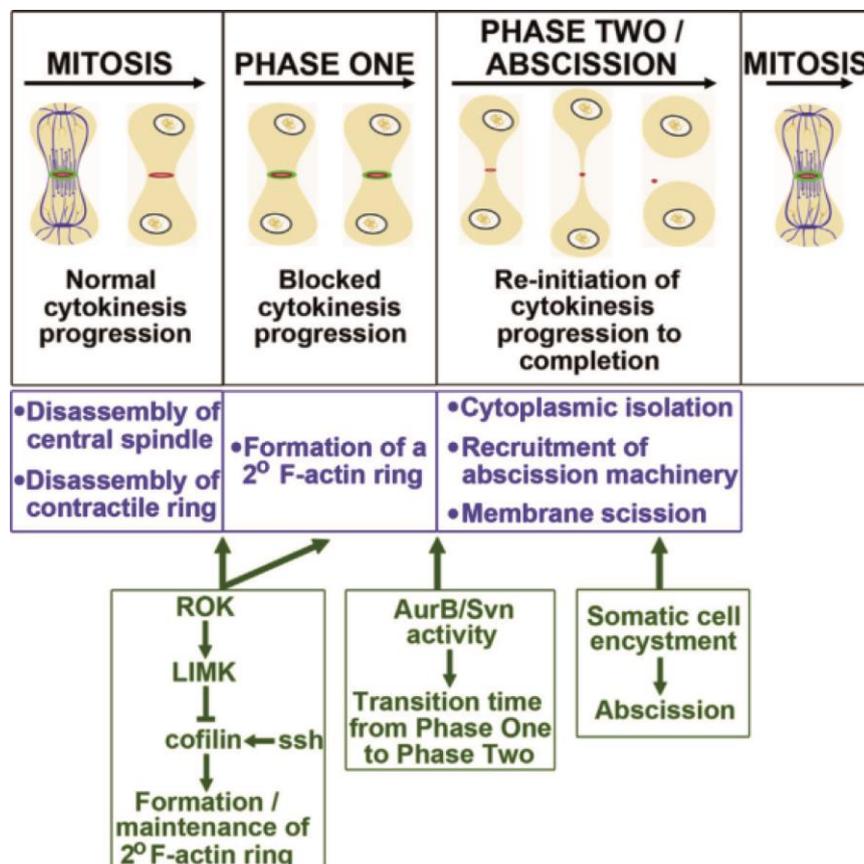
The authors then analyzed the dynamics of Shrb-GFP and observed its recruitment to the intercellular bridge only at the onset of second phase. This is in contrast with what happens in the female, where GFP-Shrb is enriched in the fusome and localizes much earlier with the contractile ring. However, the exact timing and the changes in the composition of the ring was not yet analyzed in our study. The hypothesis we had put forward to explain early Shrb recruitment was the presence of the fusome. In male GSCs the fusome is also present, but the recruitment of Shrb happens later. One explanation to reconcile these differences is that the components of the fusome could differ between the two, giving it different properties and functions. In particular, female fusome are poor in F-actin in contrast to male fusome.

The authors then went to explore the role of the CPC in the regulation of male GSC abscission. In agreement with our results, they saw that expression of an activated form of survivin (SvnS125E) prolonged phase 2. However, they did not observe the formation of stem-cysts, as we did in females (Mathieu et al., 2013; Roth, 2001). Very surprisingly, they saw that the disassembly of the new F-actin ring was anticipated, resulting in an overall timing of abscission (phase 1 plus phase 2) similar to the wild type. This novel function of the CPC was confirmed when they looked at homozygous for the hypomorphic allele of Aurora B (*AurB*<sup>1689</sup>), which in females results in precocious abscission. They saw a delay in the disassembly of the new F-actin ring and thus a later transition to phase two. This delay gave rise to the formation of chains of interconnected cells linked to the niche, similar to stem-cysts. The authors then concluded that the CPC promotes abscission of the male GSC, the opposite of what happens in the female.

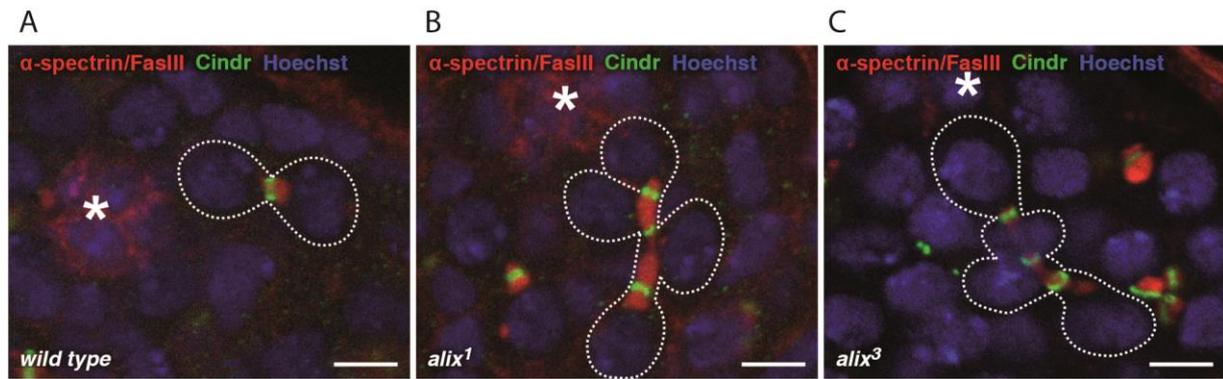
I found three ways of trying to explain the gender specific function of the CPC. 1) This new function of Aurora B in disassembling the F-actin ring is not conserved in females, and only its later function in delaying the final step of abscission in phase 2 exists. 2) There is only one phase in females, as no secondary F-actin ring is formed. Thus only the CPC function in inhibiting abscission exists. 3) In males the strong effect seen in abscission with homozygous *AurB*<sup>1689</sup> is a non-autonomous effect. As all the cells in the fly are mutant for *AurB*, the defect on GSC abscission could be due to the influence of the CySCs around it. Thus, it would be

interesting to analyze clones of germline cells, versus somatic cells to explore this possibility. A function for AuroraB in promoting abscission via actin depolymerization has never been seen in any other system.

Very interestingly, Eikenes et al. showed that loss of function of ALIX resulted in the formation of stem-cysts in males (Fig3) (Eikenes et al., 2015). Because ALIX is thought to be important in the recruitment of the ESCRT machinery, which was observed to act late in the process, this result suggests that defects in the second phase are also able to give rise to stem-cyst formation.



**Figure 2:** Schematic view of the process of male GSC cytokinesis and abscission regulation (Lenhart and DiNardo, 2015).



**Figure 3:** (A) Wild type testis tip with mGSC-gonialblast (GB) pair (outlined) in cytokinesis interconnected by an MR (Cindr, green) and fusome ( $\alpha$ -spectrin, red). (B-C) Testis tips in *alix1* and *alix3* mutants with mGSCs connected to chains of daughter cells (outlined) via MRs (green) and fusome (red). Testes in (A-C) were fixed and stained with antibodies against Cindr (green),  $\alpha$ -spectrin (red) and FasIII (red), and with Hoechst (blue). Hubs are indicated with asterisks (Eikenes et al., 2015).

## b) Non-autonomous regulation

The authors showed that, in males, the somatic cyst cells, in contact with the GSC-CB pair are important for the abscission process, in a non-autonomous way. Indeed, in somatic cyst cells expressing a dominant negative form of EGFR, which impairs encystment, the GSC abscission was strongly delayed and stem-cysts were formed. Interestingly, both phases one and two seemed to progress normally, and only the last step of membrane fission was delayed.

Moreover, EGFR signaling was shown to promote encystment through Rac1 activation and consequent actin assembly. Indeed, disruption of these downstream players resulted in a similar phenotype.

These results could explain my hypothesis that the formation of stem-cysts in homozygous mutants for *AurB*, is due to a non-autonomous effect. Indeed, *AurB* was already proposed to control cell shape by regulating Rac1, which activates a formin necessary to stabilize a selected population of microtubules, and promote the assembly of actin stress fibers at the cell periphery (Floyd et al., 2013). Thus, loss of function of *AurB* in the somatic cyst cells could result in defects on actin assembly, leading to the formation of stem-cysts.

## **5- Lethal (2) giant discs (Lgd) promotes GSC abscission and localizes at the midbody**

The tumour suppressor gene *lgd* has been described to regulate the activity of the Notch pathway in *Drosophila*, and its loss of function results in phenotypes very similar to those of *Shrb* mutants. In both *lgd* and *shrb* mutants, Notch overactivation is thought to be induced due to defects in the process of endosomal sorting. *Shrb* was shown to act in the formation of intraluminal vesicles, responsible for the sequestration of Notch in the endosomes, and proper elimination of the receptor by the lysosome. Impairment of this function, leads to the accumulation of Notch at the endosomal membrane, where it ectopically activates the signaling.

Indeed, *Lgd* was shown to directly bind *Shrb* (Troost et al., 2012). This interaction is thought to stabilize *Shrb* monomers and facilitate its activation during vesicle membrane fission.

We thus hypothesized that *Lgd* could also ensure the correct conformation of *Shrb*, during the abscission process. To test this, we first verified that the loss of function of *Lgd* had the same abscission phenotype as *Shrb* downregulation. Indeed, *lgd* germline clones resulted in the formation of stem-cysts and 32-cell cysts, characteristic of a delayed GSC abscission. Furthermore, we saw that removal of one copy of *lgd* from the heterozygous *shrb* mutant induced an increase in stem-cysts formation. This result pointed to an interaction between *Lgd* and *Shrb* during abscission. However, the number of egg chambers with 32 cells was less in the double heterozygous flies. Because we think that the 32-cell cysts are originating from stem-cysts, we were expecting to see an increase in the number of 32-cell cysts.

To try to understand this unexpected result, we explored what was happening during cyst development, and saw that abscission was taking place in the mitotic cysts. Furthermore, our analysis showed that in all cases, abscission was happening exactly in the middle of the cyst, dividing it in two equal cysts. Interestingly, because we never saw egg chambers with less than 16 cells, it suggested that the breaking cysts are those coming from the stem-cysts and destined to form 32-cell cysts. How such a rescue in the number of cells per cyst could occur is discussed below.

Even though Lgd has a phospholipid C2 binding domain in its C-terminus, it was reported to localize at the cytoplasm and to not bind to membranes in vivo. Thus the interaction with Shrb was predicted to occur in the cytoplasm (Childress et al., 2006; Gallagher and Knoblich, 2006; Jaekel and Klein, 2006; Troost et al., 2012). However, when we expressed our GFP-Lgd construct in the germline, it surprisingly localized to the fusome, as well as to ring canals and midbodies. This suggests that in the GSC, Lgd and Shrb could interact at sites of membrane constriction.

The mammalian homologue of Lgd was shown to be phosphorylated during mitosis by Cdk1, in at least one site (Ser<sup>208</sup>) (Nakamura et al., 2010). This phosphorylation seemed to contribute for the ability of Lgd to bind the cohesin component Scc1. Because we showed that Cdk1 localizes to the fusome and midbody, and more importantly function in promoting GSC abscission (Mathieu 2013), it would thus be interesting to test whether Cdk1 promotes the activation of Lgd and consequent binding to Shrb.

We mutated 14 consensus sites for Cdk1 phosphorylation in *lgd* into either phosphomimic (UASp-Lgd<sup>E</sup>-GFP), or nonphosphorytable (UASp-Lgd<sup>A</sup>-GFP) forms. To test if Cdk1-dependent Lgd phosphorylation is required for its regulatory function, we would like to express these phospho-mutated forms in a *lgd* mutant background, and check for possible abscission phenotypes. Interestingly, we noticed, in preliminary experiments, that when we expressed either a wild type form of Lgd, or the phospho-mutant forms, we obtained few chambers with 32 cells as well as stem-cysts. It thus seems that, similar to what happens in the Notch signaling pathway, overexpression of a tagged form of Lgd has a negative effect on its function.

## 6- How does *lgd* loss of function induce ectopic abscission in the cysts

We showed that when we removed one copy of *lgd* from heterozygous mutant *shrb* the percentage of stem-cysts increased, as expected for a positive regulatory interaction. It seems that Lgd acts together with Shrb to promote GSC abscission. However a very intriguing result came with the observation that the 32-cell cysts, originated from the stem-cysts, were being rescued. Even more surprisingly, we saw that the rescue was done by abscission exactly in the middle of the cysts, dividing them in two. How could this happen?

### a) Lgd has opposite functions in the GSC and in the cysts

A first hypothesis would be that Lgd has opposite functions in the GSC and in the mitotic cysts. It could undergo several different posttranslational modifications, which would change its characteristics, or have different interaction partners. An important experiment to test this hypothesis would be to downregulate Lgd specifically in the mitotic cysts. Because there is no RNAi line for Lgd that works in the germline, we are currently constructing our own TRiP valium 22 line. It will be expressed specifically in the cysts using the *bam>GAL4* driver. Another, and probably more efficient, way of removing Lgd from the cysts is by inducing Lgd protein degradation. This will be possible with the expression of a deGradFP (Caussinus et al., 2013), under the control of the *bam* promoter, in flies expressing endogenously tagged Lgd. In these flies, the deGradFP would recognize the GFP fused protein and induce its degradation by the proteasome. We are now currently testing whether our new *bam>deGradFP* works, as well as tagging the *lgd* endogenous gene in the N-terminal, using the CRISPR/cas9 genome editing technology.

If our hypothesis is correct, we would expect that, removing Lgd from the cyst, would induce abscission, and egg chambers with less than 16 cells. However, there are some observations that contradict this model. First, if Lgd downregulation in the cyst would promote abscission, we would expect to see it happening in all cysts and in all cells of the cyst. However, we only

see abscission happening in cysts that would otherwise form 32-cell cysts. Furthermore, abscission happens only between the two first cells of the cyst located in the middle. Another interesting observation is that the abscission seems to happen in later cysts. This is different from what Mathieu et al. observed in Aurora B loss of function, where the downregulation of an inhibitor of abscission induced separation earlier in 2-cell cysts.

## **b) Lgd downregulation induce abscission through cell fate modulation**

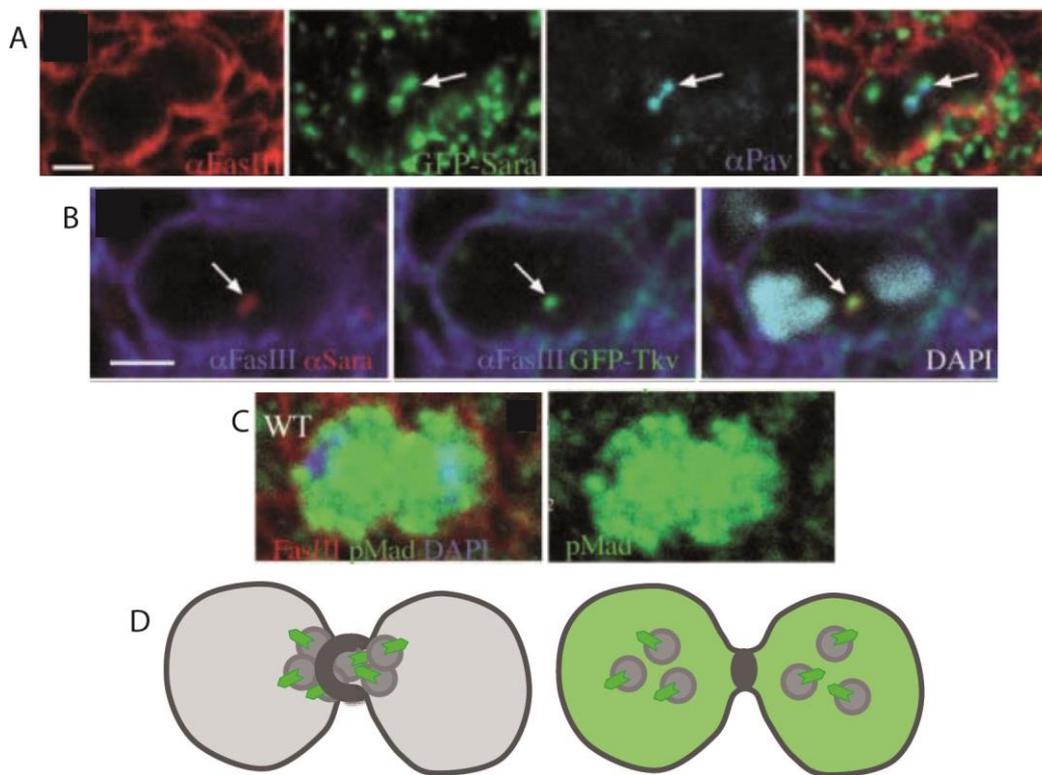
An alternative hypothesis to explain this result is to consider the context where these cells are developing. The GSC divides asymmetrically with complete cytokinesis to give rise to another stem cell and a cystoblast (CB). The CB then enters its differentiation program, which dictates the formation of cysts of 16 cells through incomplete cytokinetic cycles and no abscission. This change in cell program was shown to be tightly regulated, and several mechanisms have been described to assure proper fate of the cells in the germarium. BMP ligands (Dpp and Gbb) produced by the cap cells bind to the receptors (Tkv, Sax and Punt) on the GSC membrane and activate signaling. This signal induces the phosphorylation of Mad (P-Mad), which is responsible of *bam* transcription inhibition in the GSC. In the CB, several extrinsic and intrinsic mechanisms, such as degradation of the Dpp receptors, ensure that the BMP signaling is inactivated and *bam* is expressed.

My alternative hypothesis was based on the two following studies:

**1)** The group of Allan Spradling demonstrated that the modulation of Bam protein levels can control cell fate programming (Kai and Spradling, 2004) (explained in detail in the introduction - chapter 2, figure 12). In this work, they showed that ectopic Bam expression in all the cells from the germarium induces differentiation of the stem cells and cysts formation. More interestingly, they showed that in the differentiated germ cells, ectopic expression of Dpp from the posterior somatic cells could change cell fate. They observed that cysts, formed by interconnected cells with branched fusomes, were breaking their ring canals upon Dpp

signaling induction. It is thus possible to induce abscission in differentiating cysts, by modulating cell fate (Kai and Spradling, 2004).

2) Bokel et al. showed that, during mitosis of developing wing epithelial cells, the Dpp receptor Tkv was localized to endosomes and targeted to the spindle midzone (Fig2). Then later, during cytokinesis, the endosomes would be equally segregated, assuring equal signaling in the daughter cells. It thus seems plausible that endosomes carrying Tkv have the capacity to concentrate at the zone where the cytokinetic ring is formed (Bokel et al., 2006).



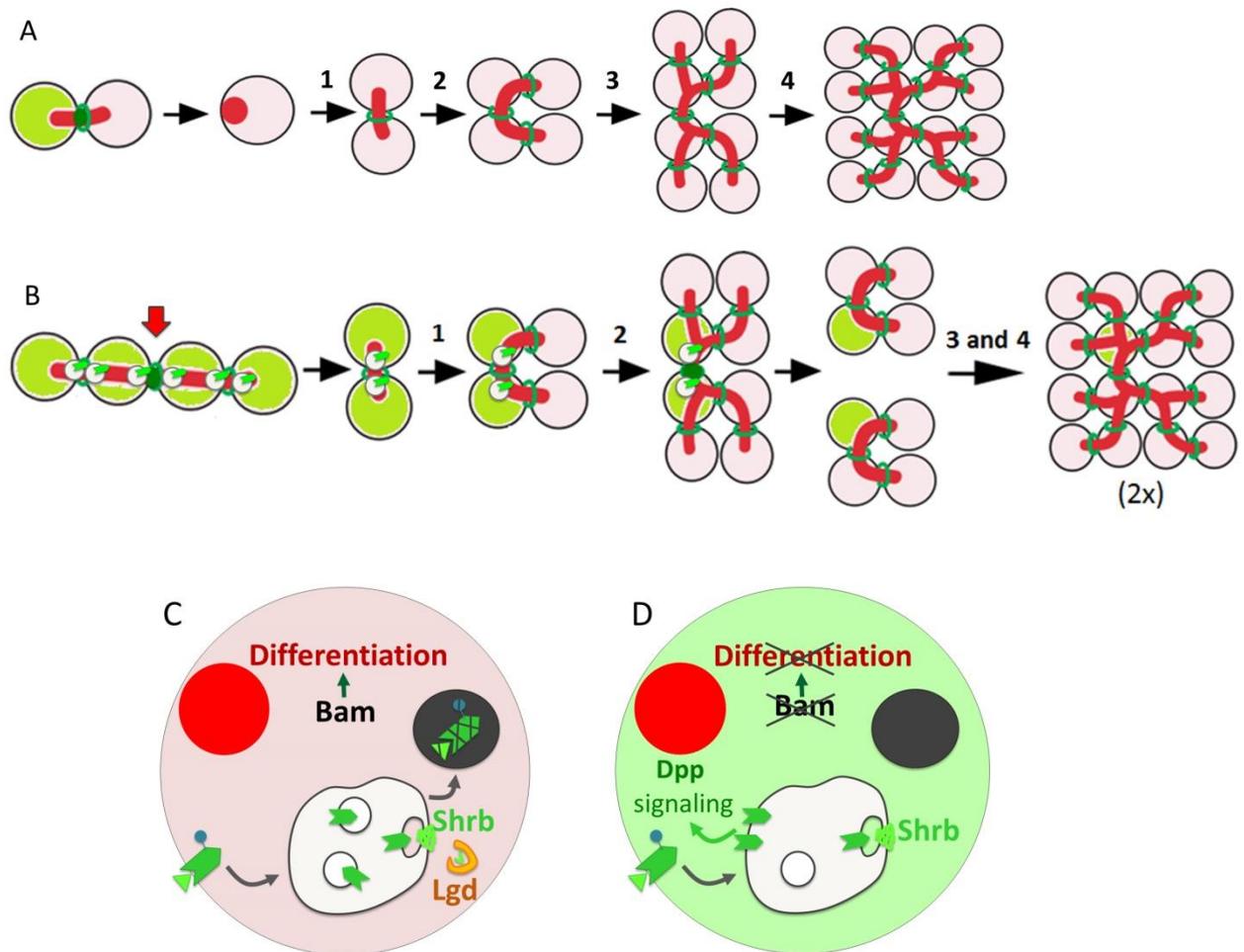
**Figure 4:** (A) GFP-Sara (green) localizes to the central spindle (arrows) marked by Pavarotti [blue] at telophase in dividing wing epithelial cells outlined with FasIII [red]. (B) A fraction of intracellular Tkv-GFP (green) colocalizes with endogenous Sara (red) at the cleavage plane (arrows). Cell was outlined by means of FasIII (blue) and DNA was marked by DAPI (light blue). (C) Equal pMad staining (green) in WT at the exit of mitosis, counter-stained with FasIII (red) and DAPI (blue) (Bokel et al., 2006). (D) Endosomes (grey circles) carrying Tkv (green icon) are localized at the spindle midzone (left). After membrane constriction the Tkv endosomes are separated equally between the two daughter cells, resulting in equal distribution of the signal.

**My hypothesis:**

In the double heterozygous *shrb* and *lgd* mutants, stem-cysts are formed due to a delay in GSC abscission. In these mutants, the other processes in which these proteins function could also be compromised. Another known function of Shrb and Lgd is the regulation of signaling pathways by the elimination of the receptors through MVBs formation. We know that one important receptor that needs to be degraded in the CB to promote its differentiation is Tkv, the Dpp receptor. Thus, one scenario is that in *shrb* and *lgd* mutants, the activated Tkv elimination is impaired, as it was previously shown in other ESCRTs mutants (Thompson et al., 2005). In this case, Tkv would accumulate in enlarged endosomes and induce Dpp signaling. Endosomes carrying Tkv were previously seen localizing to the spindle midzone of dividing cells (Bokel et al., 2006).

Thus, in the double heterozygous mutants for *Lgd* and *Shrb*, we would expect to find the endosomes with accumulated Tkv at the ring canal connecting the GSC and the CB. These, would probably be trapped within the fusome and be segregated with it in each division of the cells during stem-cyst formation. It would then be expected to have active Dpp signaling in the cells from the stem-cyst. In agreement we see that Bam is inhibited in these cells. When abscission happens in the stem-cyst, it releases a 2-cell CB precursor.

Because these two cells originate from the stem-cyst, they would have in their fusome, endosomes with activated Tkv. During cyst divisions these two cells are likely to maintain a higher level of the endosomes, as the fusome is asymmetrically segregated. The oldest cells, located in the middle of the cyst, inherit more fusome and keep the oldest fusome plugs. Thus, the two oldest cells from the cyst carrying activated Tkv, would maintain with time the original Dpp signaling, which would induce a change in their cell fate. They would behave like stem-cells, inhibit Bam and complete abscission. Abscission would then occur only between the two cells originated from the stem-cysts. In this process, the cyst would be divided in the middle, rescuing the 32-cell cyst phenotype (Fig5).



**Figure 5: (A)** Scheme of wild type dividing GSC and cyst. The GSC is the only cell with active Dpp signaling (green cell). It divides to give rise to another GSC and a CB. This goes through four mitosis and results in cysts of 16 cells. **(B)** Scheme of *shrb* and *lgd* double heterozygous. The GSC divides with incomplete cytokinesis forming stem-cysts. Due to defects in MVB formation, endosomes carrying activated Tkv are enriched at the ring canals and fusome. All cells from the stem-cyst have Dpp signaling activated, but lower in the posterior cells. A 2-cell CB precursor is released from the stem-cyst. These two cells have activated Tkv that is kept during cell division. At some point the Dpp signaling is enough to change cell fate and induce abscission between the two cells. At the end it will be formed two cysts of 16 cells. **(C)** In wild type CB, Tkv is internalized into MVB by the action of Shrb, kept in its active conformation by Lgd. Tkv is then degraded by lysosome fusion. Without Dpp signaling, Bam is expressed and promotes differentiation. **(D)** In a mutant cell for *lgd*, Shrb is not stable and do not promote efficient Tkv internalization. Active Tkv accumulates on the endosomal membrane and induces Dpp signaling. Bam is downregulated, changing the cell fate.

To test this hypothesis, we would like to check whether we see an upregulation of Dpp reporters, like P-Mad and Dad, and downregulation of Bam in the two cyst cells undergoing abscission. We will also express a non-degradable form of Bam (UAS- $\Delta$ Pest-bam) using the bam-GAL4 driver, to maintain the levels of Bam high in the cysts, thus avoiding cell fate change. We will test whether expression of a constitutively active form of Tkv, expressed in the cysts with the bam-GAL4 driver, is enough to activate Dpp signaling, downregulate Bam and promote abscission in the cyst cells.

If this hypothesis holds, we would expect to see the breaking cysts in *lgd* and *shrb* single mutants, since both act in abscission and in the degradation of signaling receptors. Indeed that seems to be the case for *Lgd* loss of function clones, as the number of 32-cell cysts is very low compared with the high number of stem-cysts. However, in *shrb* heterozygous mutants and RNAi induced downregulation, both the numbers of 32-cell cysts and stem-cysts are high. Thus no strong rescue of the 32-cell cyst phenotype seems to be happening in *shrb* loss of function. In agreement it was shown that *Shrb* is the ESCRT-III that, when mutated in the imaginal disc epithelium, induced the weaker phenotype in Notch signaling ectopic activation (Vaccari et al., 2009). Interestingly, when we dissected older ovaries mutants for *Shrb*, we noticed the appearance of tumorous germaria and egg chambers. These were full of single cells, which kept dividing. This phenotype seems to be cell fate related, and it would be interesting to characterize these cells. With these experiments, it looks clear that while *Shrb* function in abscission is very sensitive to protein dosage, its function in signaling is not.

It thus seems that different functions of *Shrb* require different levels of the protein. Thus suggesting that the stoichiometry of the ESCRT-III components forming the constriction filaments, change in the different contexts.

## **7- Lgd regulates the activity of the BMP/Dpp signalling pathway during *Drosophila* oogenesis (Morawa, 2015)**

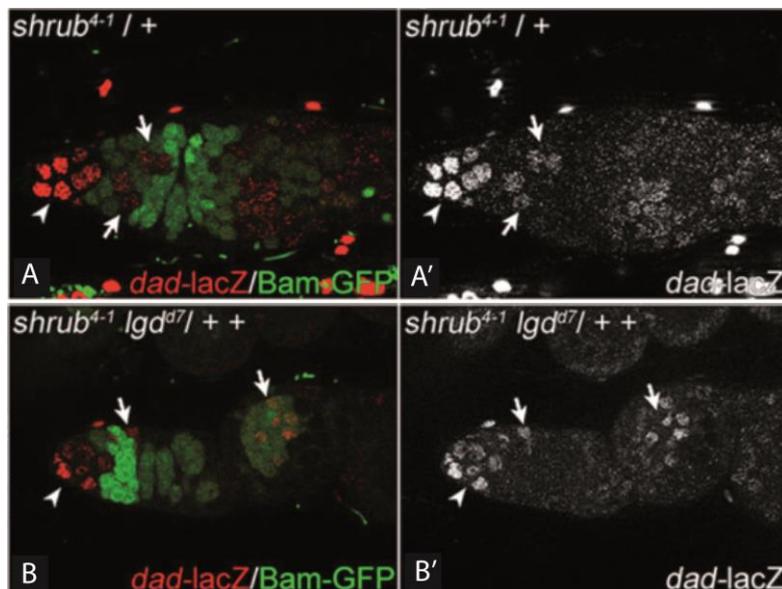
Recently, the group of Thomas Klein published their work on Lgd regulating *Drosophila* oogenesis (Morawa et al., 2015). They also showed that *lgd* mutant germline clones gave rise to egg chambers with 32 cells. However, their interpretation of this result was different from ours. They argued that the 32-cell cysts were formed due to a fifth mitosis in the cyst induced by ectopic activation of Dpp. Even though they missed the stem-cysts phenotype and thus could not conclude for a defect in abscission, interesting experiments were done. To understand whether *lgd* mutation leads to ectopic activation of the Dpp signaling pathway, they used the reporter Dad-lacZ. From their figures we can see that Dpp signaling seems to be active later in the germarium, after the mitotic region, and thus could not justify a fifth mitosis in the cyst. However, very interestingly, they observe a higher number of Dad-lacZ positive cells at the anterior tip of the germaria, while in the wild type it is only observed in the GSC. We believe that these Dpp activated cells are part of stem-cysts, and a fusome staining would clarify it. If these Dad positive cells are indeed stem-cysts, it would explain the lack of Bam expression, even though P-Mad is only observed in the GSC.

Another interesting experiment was the analyses of germline clones mutants for both *lgd* and *tkv*. They observed that the percentage of 32-cell cysts was diminished when Dpp signaling was reduced. What we think is happening is that the removal of *tkv* rescued the formation of stem-cysts, and consequently the 32-cell cysts. With no Dpp signaling the mutant GSC was probably forced to differentiate rather than divide to form stem-cysts. With time, the double mutant GSC would probably be lost due to precocious differentiation.

Then, they looked at *Shrb* heterozygous mutants and observed the presence of chambers with 32 cells, as well as ectopic Dad positive cells in the anterior of the germaria. To test for genetic interaction between Lgd and *Shrb* they looked at double heterozygous mutants. They obtained increased Dad positive cells, which are probably the stem-cysts, and a rescue of the 32-cell cyst phenotype.

To further explore the role of Dpp signaling in these phenotypes, they performed genetic interactions with fused (*Fu*), a kinase involved in the degradation of activated Tkv in the CB

(Xia et al., 2010). They saw that, when one copy of Fu was removed from both *lgd* germline clones and *shrb* heterozygous mutants, the number of Dad positive cells strongly increase. The number of 32-cell cysts was lower in *lgd* mutants and increased in *shrb* heterozygous. We suggest that the decrease on Fu would enhance the Dpp signaling and probably more cells from the stem-cysts would be turned positive for Dad-lacZ. It would be interesting to look at these mutant germaria with a marker for the fusome to properly detect the stem-cysts.



**Figure 6:** Loss of *shrub* function causes ectopic activation of the Dpp pathway. **(A)** Ectopic activation of *dad-lacZ* in *shrub/+* heterozygous germ cells. The expression of Bam-GFP is close to normal. The arrow points to cysts with cells that have ectopically activated *dad-lacZ* and have already switched off Bam-GFP in the germarium. Note, the expression of *dad-lacZ* in more than the usual 2-3 GSCs in the anterior tip of the germaria (arrowheads). **(B)** *lgd shrub* double heterozygous ovariole. Ectopic *dad-lacZ* expression is still observed in cyst in the germaria (arrows). The arrowhead points to the *dad-lacZ*-positive germ cells (Morawa et al., 2015).

## 8- How is abscission blocked in the dividing cysts?

One major question in the germline field is: How is abscission blocked in the differentiating cysts?

In mammals this process seems to be better understood. The Matzuk lab has shown that Tex14 (testis expressed gene 14) is a key player in blocking abscission of mouse spermatogonia. Elegant works have reported its localization to intercellular bridges and described how it acts to prevent abscission (Greenbaum et al., 2009; Greenbaum et al., 2011; Iwamori et al., 2010). In somatic cells, the centralspindlin component MKLP1, localized at the contractile ring, binds to CEP55. CEP55 in turn interacts with the GPPX3Y motifs of Alix and Tsg101 (ESCRT-I) and recruits them to the ring. Then the ESCRT-III components are also recruited and abscission takes place. In spermatogonia, Tex14 interacts with CEP55, through GPPX3Y motifs similar to those of Alix and Tsg101. This competition for CEP55 binding inhibits its interaction with Alix and Tsg101. Without these two proteins, the ESCRT machinery is not recruited to the intercellular bridges and abscission does not occur.

In *Drosophila*, it seems that there are no homologues of CEP55 and Tex14. Thus, how abscission is blocked in the cysts remains an open question. We speculate that the same mechanism could happen, but with different proteins, or that an alternative process to block abscission exists in *Drosophila* cysts. It would be then interesting to develop a screen to find possible abscission inhibitors in the cyst. We could express RNAi constructs from the TRiP collection, using the Bam-GAL4 driver specific for the cysts, and check for abscission events.

An interesting observation came with the analysis of GFP-Shrb localization during germline development. We saw that GFP-Shrb was barely detected in the dividing cysts. It was not localized at the ring canals and was not enriched in the fusome, as in the GSC. We could only detect low cytoplasmic levels in the cysts. This was a very surprising result as GFP-Shrub was expressed using the exogenous nanos-Gal4 promoter (i.e. not under the endogenous transcriptional regulation) and thus expected to be expressed at the same levels in all germ cells, indicating that Shrb is regulated at the protein level. This raises the exciting possibility

that *Shrb* could be differently regulated in the GSC and the differentiating cysts. *Shrb* in the GSC would be in an active and stable form to promote abscission, while in the cysts it would be posttranslationally modified and sent for degradation. Absence of *Shrb* in the cyst would then block abscission and stabilize the ring canals.

Another hypothesis to explain why abscission occurs in the GSC and not in the cyst comes from the observation that these have very different cell cycle timings. While the GSC takes 24 hours to complete one cell division, the cyst cells take 24 hours to complete the four rounds of mitosis. This suggests that, because the four divisions of the cysts occur so fast, abscission does not have time to happen and the ring canals stay blocked.

Interestingly, the first division of the CB seems to be very similar to the GSC division. Indeed, in *Aurora B* loss of function abscission is induced during CB division, and not during any of the other divisions of the cyst. We think that the intercellular bridges during the three last divisions mature and become more resistant to abscission. This could happen due to dilution of an abscission activator, or accumulation of an inhibitor. Another idea is that this process could be regulated by the differentiation protein *Bam*. Indeed we suggested that, in the double *shrb lgd* heterozygous, during the rescue of the 32-cell cyst phenotype, abscission is induced due to cell fate change by *Dpp* signal induction and *Bam* downregulation. Testing this hypothesis, would help us understand the relation between abscission and differentiation.

## **ANNEXES**

## **ANNEX 1: AURORA B IN POSITION EFFECT VARIATION**

### **INTRODUCTION**

Aurora-B is a serine/threonine protein kinase, part of the Chromosomal Passenger Complex (CPC). Survivin, Incenp and Borealin are the regulatory subunits of the complex. The CPC has been extensively studied in cell culture for its important functions in regulating mitosis. In early mitosis, the CPC is localized along chromosome arms, where it displaces HP1 from the chromatin by phosphorylating serine 10 of the histone H3 (H3S10). After, it moves toward the centromere promoting chromosome alignment. Then it concentrates at the midbody and regulates cytokinesis and abscission (Ruchaud et al., 2007).

Fillion et al. developed a study that consisted of genome-wide analyzes of binding maps of chromatin components in the *Drosophila* Kc167 cell line. This study showed Aurora-B binding to all chromatin types, and more specifically to the three heterochromatin types described (centromeric chromatin, PcG binding sites, silenced chromatin) (Filion et al., 2010). As these cells were not synchronized, the samples analyzed probably consisted in a mixture of cells in different time points of the cycle. Thus, the AurB binding maps are probably correspondent to mitotic profiles.

We then asked whether AurB loss of function could disturb chromatin regulation and, as a consequence, impact in gene expression. Because AurB is thought to be present only in dividing cells, its function on chromatin would be during mitosis. Defects in mitotic chromatin would then induce gene misregulation in interphasic cells.

To test the possible role of AurB in chromatin dynamics we chose the phenomenon of position effect variegation, where a gene presents variegated levels of expression, depending on the formation or spreading of heterochromatin. Position effect describes the variable expression of a DNA sequence, as the state of adjacent chromatin modifies it, in a quantitative and/or temporal manner. A protein that promotes heterochromatin formation (HP1, Su(var)3-9) is called a suppressor of variegation (Su(var)), while a protein that inhibits its formation (JIL-1) is called an enhancer of variegation (E(var)).

The functional assays available in the lab (given by Stéphane Ronsseray) allowed us to study CPC function in centromeric (PEV), telomeric (TPE), Polycomb dependent (DREV) and Repeat-Induced (RIGS) Heterochromatin.

## RESULTS AND DISCUSSION

### 1- Position effect variegation in centromeric heterochromatin

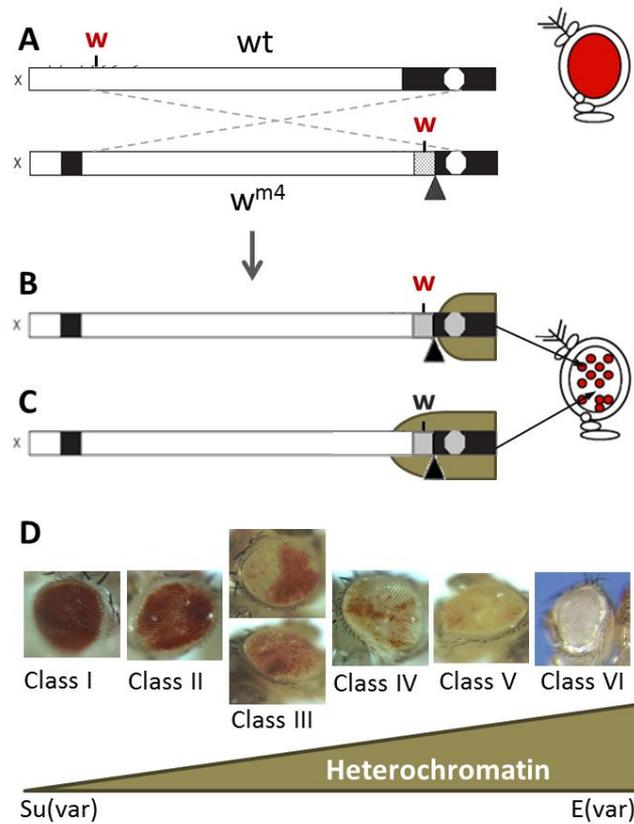
The position effect variegation in centromeric heterochromatin was studied with two different systems: Wm4 and Sbvar.

The Wm4 is an inversion in the X chromosome that juxtaposes the gene *white* and heterochromatic sequences adjacent to the centromere (Fig1). These flies present a variegated eye color that depends whether the heterochromatin spreads (white ommatidia) or do not spread (red ommatidia).

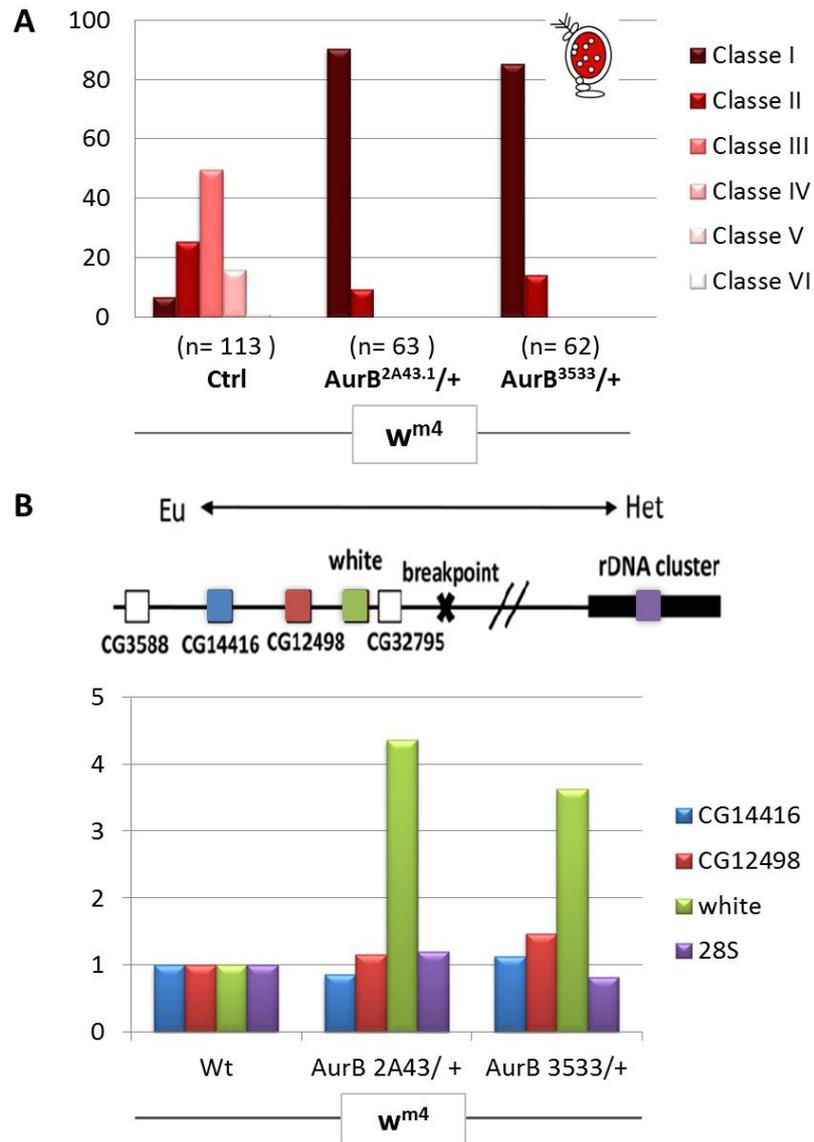
With this system we were able to test 2 different null mutant alleles for AurB.

Heterozygous mutants for AurB crossed with the W<sup>m4</sup> resulted in F1 with dark red eyes, meaning that heterochromatin did not spread through the gene *white*, which was kept active. Thus, AurB acts as a suppressor of PEV, being important for the formation of centromeric heterochromatin (Fig2A).

We then decided to take a quantitative approach using the RT-qPCR technique. Looking at the *white* gene (and neighboring genes) level of expression, in the W<sup>m4</sup> system. We saw an upregulation of *white* in the two AurB heterozygous mutant backgrounds, which validated our previous results (Fig2B). Interestingly, we observed that the neighboring genes were not equally affected. These results are in agreement with the work done by Vogel et al., which showed that the gene *white* has an intrinsic affinity for heterochromatin and is the only one detected to be repressed in the wild type W<sup>m4</sup> context (Vogel et al., 2009). Thus, it was expected to see the biggest change in gene expression in the gene *white*.



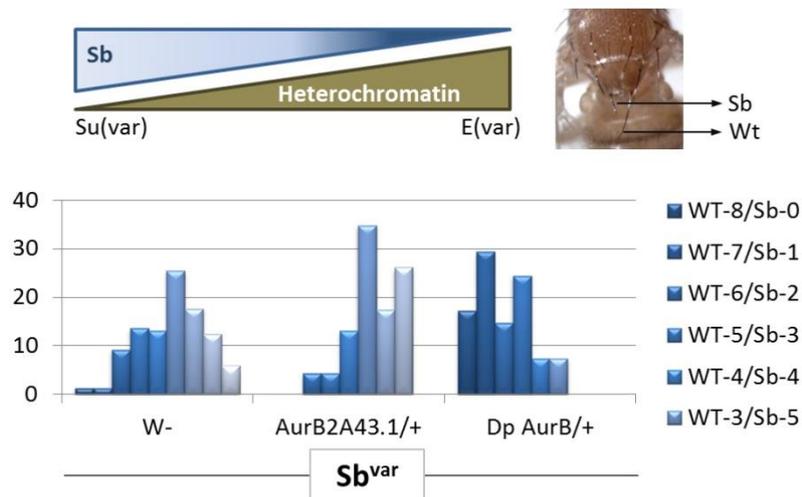
**Figure 1: (A-C)** A schematic illustration of the  $W^{m4}$  system. **(A)** The  $W^{m4}$  chromosome formed due to an X-ray-induced inversion with the break point in the pericentric heterochromatin (black arrow head). This inversion brought the gene *white* close to the centromere. **(B-C)** The gene *white* has a variegated pattern of expression in the eye. **(B)** When expressed gives rise to red ommatidia. **(C)** The spread of heterochromatin through the gene *white* induces its silencing, resulting in white ommatidia. **(D)** During the analyses of the phenotypes, the flies were scored depending on their eye color. The classes of eye color varied from I (strong red color, less heterochromatin, Su(var) phenotype) to VI (white eyes, more heterochromatin, E(var) phenotype).



**Figure 2: (A)** AurB functions as a Su(var) in the  $W^{m4}$  system. Most of the scored flies belong to the class I. Control flies are  $w^{1118}$ . **(B)** RT-qPCR done in male heads. We used the same primers published in (Di Stefano et al., 2011).

The other PEV system we tested was the  $Sb^{var}$ , which consists in a translocation of centromeric chromatin of the 2<sup>nd</sup> chromosome, close to the  $Sb$  mutation in the 3<sup>rd</sup> chromosome. These flies have a variegated expression of  $Sb$ . This has a dominant negative effect, and when expressed induces the formation of short macro-chaetes (Fig3).

We could reproduce the suppressor phenotype (short macro-chaetes) for both mutant alleles and saw that  $AurB$  duplication results in an enhancer phenotype (wild type macro-chaetes) (Fig3).  $AurB$  behaves, like most heterochromatin components already studied, as a haplo-suppressor and triplo-enhancer. We also looked at  $AurB$  over-expression and saw that, very surprisingly, it has a suppressor effect on variegation. These results show that this system is very sensitive to  $AurB$  levels.

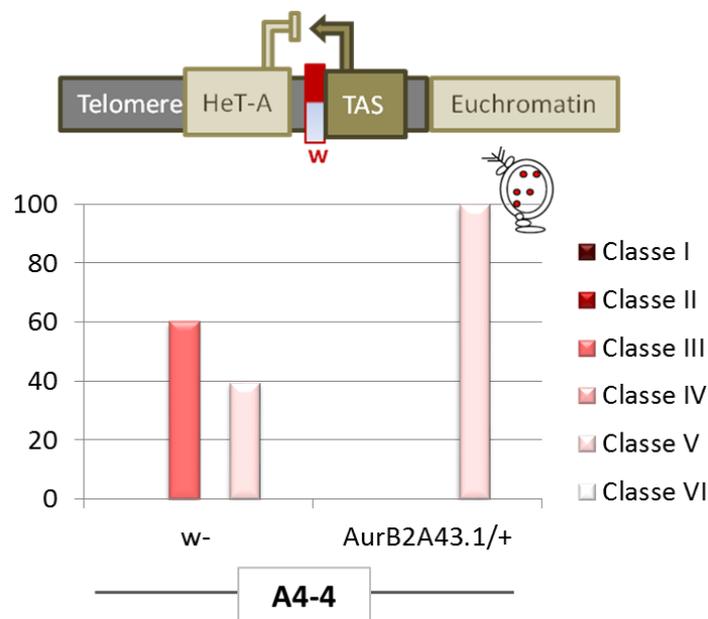


**Figure 3:** For the analysis of the  $Sb^{var}$  system the flies were scored depending on the number of wild type (Wt) and  $Sb$  macro-chaetes. We quantified always the same 8 macro-chaetes per fly. The expression of the  $Sb$  dominant negative gene, due to  $Su(var)$  effect (less heterochromatin), induces short macro-chaetes. Long Wt macro-chaetes are induced by  $E(var)$  (more heterochromatin) that silences the gene.  $AurB$  mutant heterozygous functions as a  $Su(var)$ , resulting in  $Sb$  expression and having mostly Wt macro-chaetes.

## 2- Position effect variegation in telomere-associated sequences (TAS)

We then studied the formation of telomeric heterochromatin, by looking at gene expression variegation of a white gene, inserted in the middle of telomere-associated sequences (TAS). It is thought that, while the TAS have a repressive effect on *white* expression, the terminal HeT-A transposon array has a stimulating effect (Mason et al., 2003).

Contrary to what happen on centromeres, on this system (A4-4) AurB seems to act as an E(var), counteracting the formation of heterochromatin. Flies resulting from the cross have higher number of white ommatidia than red ommatidia (Fig4).

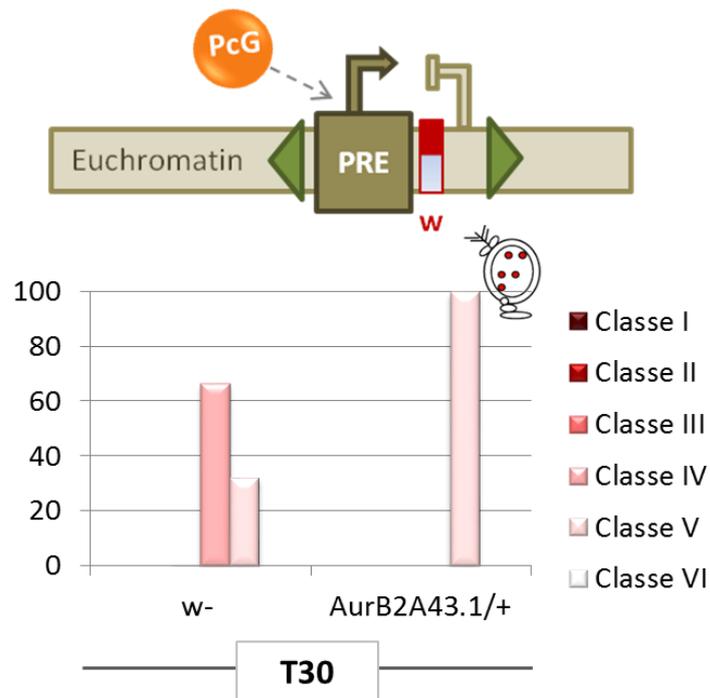


**Figure 4:** AurB functions as an E(var) in the variegation of the expression of a reported inserted in the telomeric sequences. AurB heterozygous mutant results in the inhibition of *white* expression, due to spreading of heterochromatin.

### 3- Developmental Regulated Effect of Variegation (DREV) dependent on Polycomb

We then studied the Polycomb dependent Developmental Regulated Effect of Variegation (DREV). We used a system (T30) that consists in the insertion of a P-element upstream of the *white* gene. This P-element carry polyhomeotic (ph) regulatory sequences, which function as PcG response elements (PRE) and causes a local and clonally inherited heterochromatinization (Fauvarque and Dura, 1993).

AurB, like in telomeres, functions in this system as a E(var) and the flies obtained from the cross have mostly white ommatidia (Fig5).

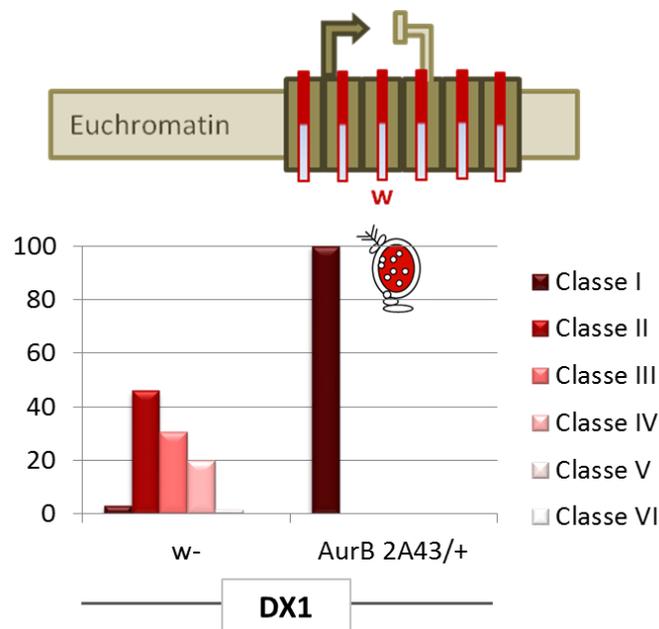


**Figure 5:** AurB functions as an E(var) in the variegation of the expression of a reported near by a PRE sequence. AurB heterozygous mutant results in the inhibition of *white* expression, due to spreading of heterochromatin.

#### 4- Repeat-Induced Gene Silencing (RIGS)

To study the Repeat-Induced Gene Silencing we used the DX1 system, which has 6 copies in tandem of a P-lacZ-white transgene, with one of them inverted. These arrays inserted on the second chromosome, create strong HP1 binding sites, generating a heterochromatic block within euchromatin (Ronsseray et al., 2001). The gene white in each of the repeats, presents a variegated expression in the eye.

We observed that mutants for AurB resulted in eyes mostly red (Fig6). Thus, AurB seems to function as a Su(var), promoting heterochromatin formation in the tandem repeats.



**Figure 6:** AurB functions as an Su(var) in the variegation of the expression of a reported inserted as tandem repeats. AurB heterozygous mutant results in the induction of white expression, due to inhibition of heterochromatin spreading.

## 5- Problem: loss of some phenotypes

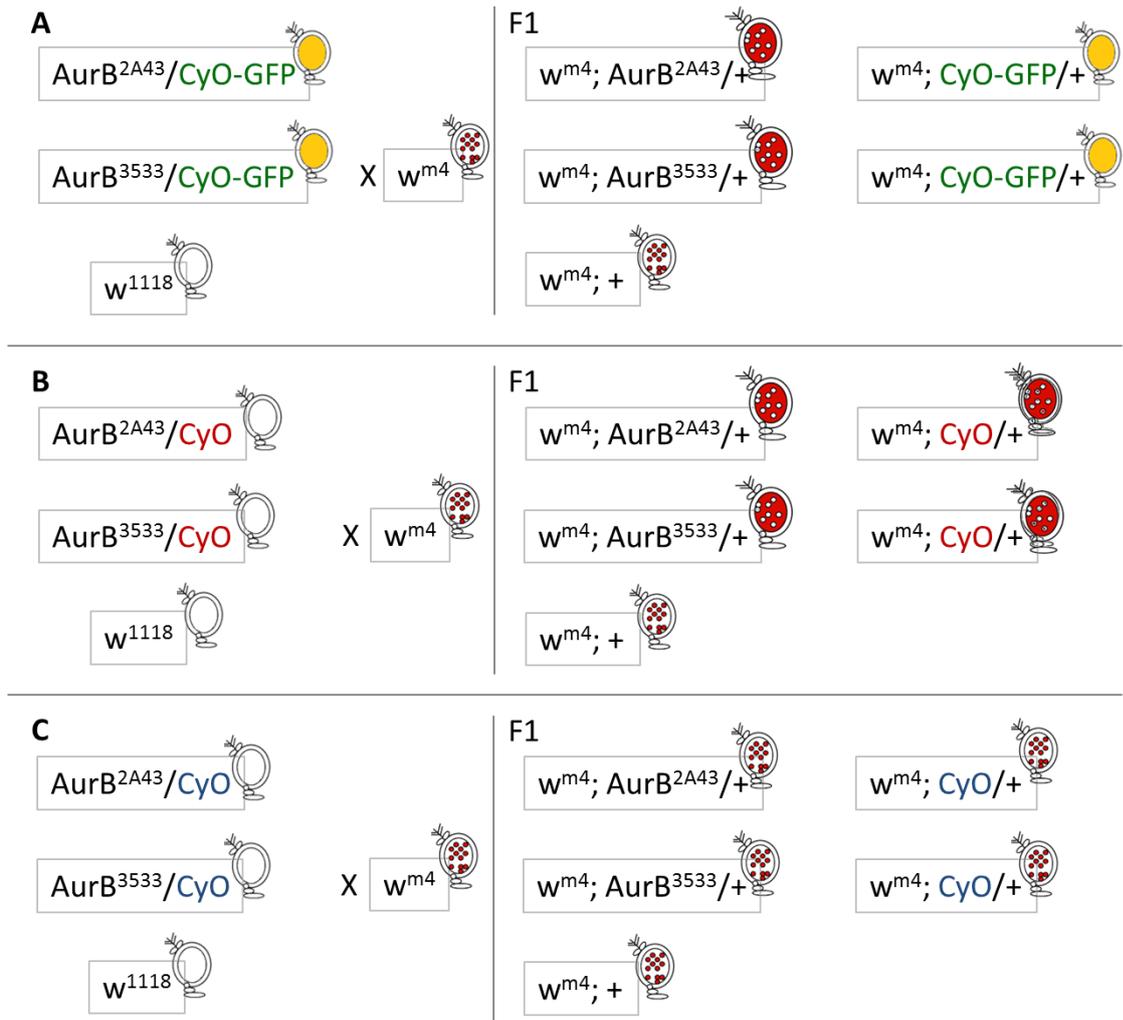
After performing the first set of experiments presented above, we were kindly advised by Stéphane Ronsseray to use the balancer chromosome CyO as an internal control for the AurB mutant flies background. Because the one we used before had inserted a white gene, these flies had colored eye and were not suitable for the analysis (Fig7A). Thus, we had to change the balancer for another CyO without a white insertion.

Very surprisingly, when we repeated the analysis with the  $W^{m4}$  system, both the flies that inherited the AurB mutant allele and those inheriting the CyO balancer, had mostly red ommatidia (Fig7B). This suggested that the new CyO balancer had accumulated a Su(var) mutation.

We thus changed once again the CyO balancer chromosome, and proceeded to new crosses with the  $W^{m4}$  flies. This time the results were even more surprising, as we lost the Su(var) phenotype of AurB mutation. Also this CyO chromosome did not present any phenotype (Fig7C).

We then used these flies to check for the AurB phenotypes in the other systems studied. Unfortunately, we realized that the effect of AurB mutation, in both the telomeric (TEV) and Polycomb dependent (DREV) heterochromatin formation, had been lost. However, the AurB Su(var) phenotype on tandem repeats (RIGS) was maintained.

We thus conclude that AurB promotes heterochromatin formation on arrays of repeated insertions of a p-element carrying a mini-white, lacZ. The effect of AurB on heterochromatin from centromeres, telomeres and Polycomb regulated sites, seems to depend on the balancer chromosome used in the experiments. We cannot conclude on these experiments.



**Figure 7: Schematic illustration of the 3 different crosses done with the  $W^{m4}$  system. (A)** Both AurB mutant alleles (2A43 and 3533) were balanced with the CyO-GFP chromosome (green). When crossed with  $W^{m4}$  flies, the resulting F1 was formed by: AurB mutants with eyes mostly red and CyO-GFP with orange eyes. **(B)** Both AurB mutant alleles (2A43 and 3533) were balanced with a new CyO chromosome (red). When crossed with  $W^{m4}$  flies, the resulting F1 was formed by: AurB mutants and CyO flies with eyes mostly red. **(C)** Both AurB mutant alleles (2A43 and 3533) were balanced with another new CyO chromosome (blue). When crossed with  $W^{m4}$  flies, the resulting F1 was formed by: AurB mutants and CyO flies with wild type variegated eyes.

## **ANNEX 2: AURORA B IN BORDER CELL MIGRATION**

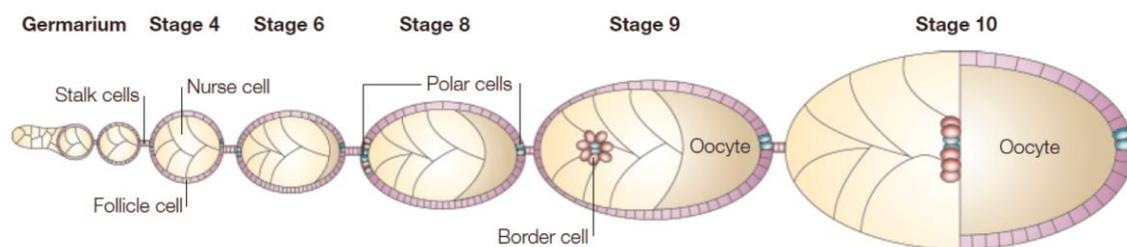
### **INTRODUCTION**

Aurora B (AurB) kinase is a well-known oncogene, described to be over-expressed in several tumor cells (Bonet et al., 2012; Hetland et al., 2013; Vischioni et al., 2006). AurB function in cell transformation has been thought to relay on its role in chromosome biorientation and in the spindle assembly checkpoint. Thus, drugs inhibiting these functions of AurB have been extensively used in cancer therapy. In the lab we discovered novel functions for AurB, using *Drosophila* as a model system. We found that this kinase is important for cell abscission at the end of mitosis (Mathieu J. 2013) and, in this project, we described unexpected function in cell migration during oogenesis.

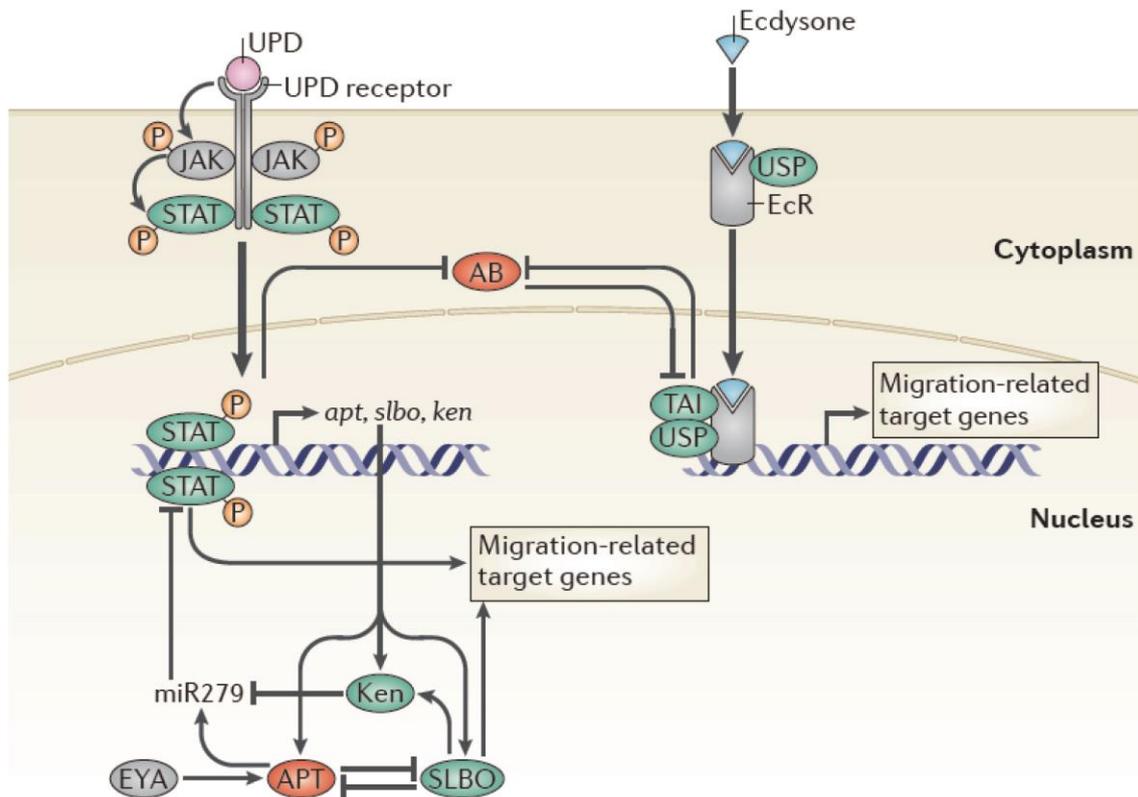
The egg chamber is the developmental unit in *Drosophila* oogenesis. One egg chamber consists of 16 germ line cells, 15 nurse cells and the oocyte, surrounded by a monolayer epithelium of somatic cells called follicle cells. Differentiation of the egg chamber requires multiple signaling events and cell interactions between and within cell layers. A very well studied process occurs at stage 9 of the egg chamber development, when a group of anterior follicular cells, called border cells, delaminate from the follicular epithelium as a tight cluster and migrate between the nurse cells to the oocyte, towards the posterior side. This process is already well characterized and is actively studied by many labs as a model for metastasis (Fig1).

The process of border cell migration is controlled by mainly two different pathways (reviewed in (Montell et al., 2012)). At stage 8, in the anterior side, two polar cells produce UPD that diffuses to the neighbor cells, where it functions as a ligand in the JAK-STAT pathway. By a cascade of phosphorylations, STAT is activated in the neighboring cells and goes to the nucleus, where it induces the transcription of Slbo (Slow border cells). Slbo is a transcription factor, responsible for the induction and over-expression of important migration factors in these cells. These cells are then specified as Border cells (BC) (Fig2).

However, they do not migrate until they receive an Ecdysone signal (steroid hormone). This hormone is expressed everywhere, but its Receptor EcR-B1 is only expressed in follicular cells, including BC. EcR-B1 forms a heterodimer with USP, and Taiman is the coactivator of the complex (Fig2). Other important factors are the guidance factors (Duchek et al., 2001). Mutants for the JAK-STAT and Ecdysone pathways impair BC migration (Beccari et al., 2002; Lehmann, 2001; Montell, 2003).



**Figure 1: Egg-chamber development in the *Drosophila* ovary.** At stage 8 the two polar cells (blue), located at the anterior side, specify 4-8 neighboring follicular cells to form the border cell cluster (red). Then at stage 9, the border cells detach from the epithelium, become invasive and carry the polar cells through the center of the egg chamber, until they reach the oocyte in the posterior by stage 10 (Montell, 2003).



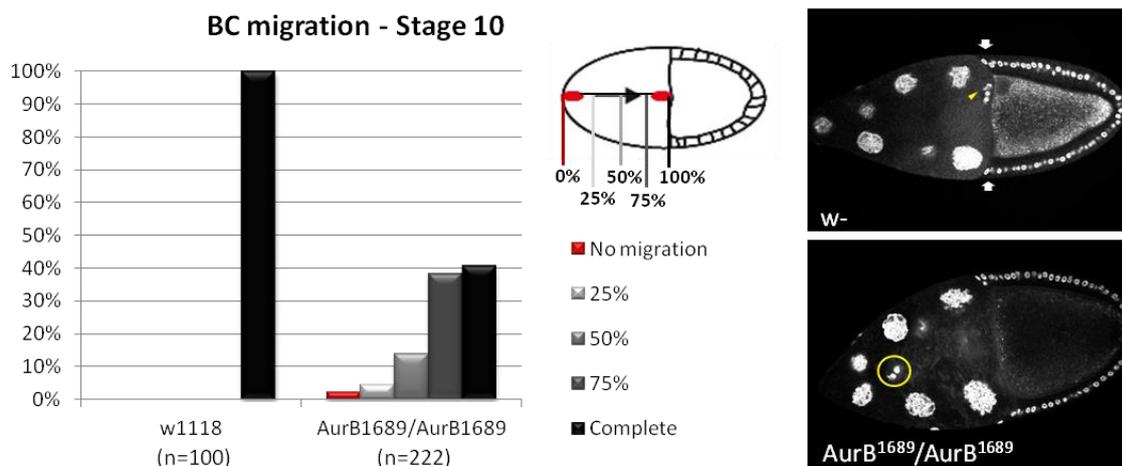
**Figure 2: Converging signaling pathways regulate border cell migration.** Local production of Unpaired (UPD) from polar cells activates the UPD receptor and JAK (Janus kinase), leading to phosphorylation and activation of STAT (signal transducer and activator of transcription). STAT signaling activates a regulatory circuit that is minimally comprised of Apontic (APT), Slow border cells (SLBO), microRNA-279 (miR-279) and Ken. APT is also activated by Eyes absent (EYA). Cells that surpass a signal threshold reinforce SLBO expression and activate downstream target genes that are needed for border cell movement. Mathematical modelling and simulation demonstrate that this set of interactions can convert a gradient of UPD into on and off states of STAT activity. The concentration of the steroid hormone ecdysone rises during stage 9, activating a nuclear hormone receptor complex comprised of Ecdysone receptor (EcR), Ultraspiracle (USP) and Taiman (TAI). Receptor activation leads to expression of downstream targets that promote border cell motility. Both the UPD and ecdysone pathways inhibit Abrupt (AB), which also feeds back to inhibit TAI (Montell et al., 2012).

## RESULTS AND DISCUSSION

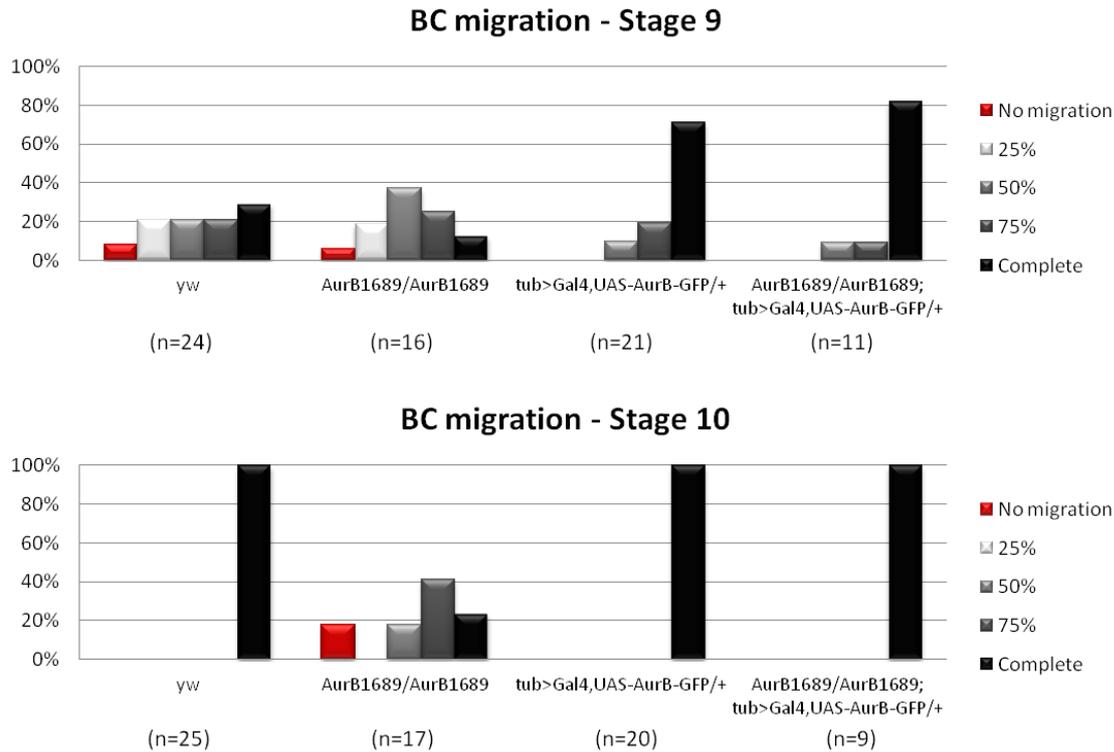
### 1- Loss of function of Aurora B results in Border cell migration delay

We saw for the first time that, in flies homozygous mutants for the hypomorph allele of AurB (*AurB*<sup>1689</sup>), the process of Border Cell migration is deregulated. These cells are not able to complete migration properly, getting delayed in 60% of the cases (Fig3). The delay was rescued when AurB was overexpressed (*UAS-AurB-GFP*) using the ubiquitous Tub>Gal4 driver (Fig4).

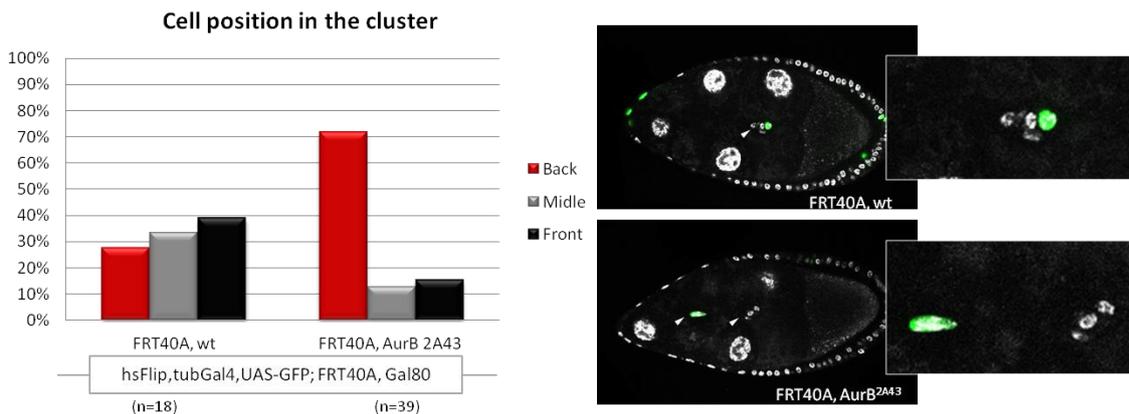
Another way of looking at the capacity of mutant cells to migrate is by checking their position in the migrating cluster. Therefore, we created single cell null mutants (*AurB*<sup>2A43</sup>) using the MARCM system. We observed that the mutant cells migrate in the back of the cluster in 70% of the cases, indicating a low capacity to migrate (Fig5).



**Figure 3: AurB homozygous for the hypomorph allele 1689 has a delay in BC migration.** We DAPI stained *w*<sup>1118</sup> and *AurB1689/AurB1689* egg chambers and quantified their stage 10 migration index. The AurB mutant egg chambers are significantly delayed in migration. Pvalue= 1,98E-23 (CHITEST in Excel).



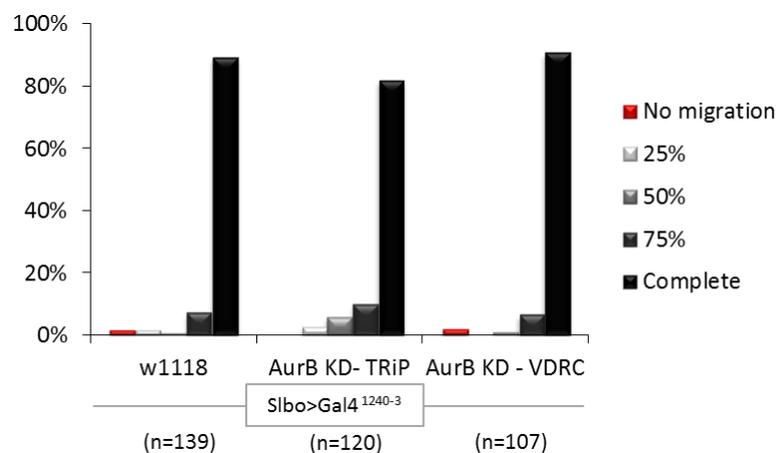
**Figure 4: Over-expression of AurB rescues the AurB mutant.** Egg chambers from the respective genotypes were stained with DAPI and their stage 9 or 10 migration indexes were quantified. At stage 9, a higher percentage of BC clusters, of both *Tub>Gal4, UAS-AurB-GFP* (unpublished) and *AurB<sup>1689</sup>/AurB<sup>1689</sup>; Tub>Gal4, UAS-AurB-GFP* have completed migration, in comparison to the control (yw) and *AurB<sup>1689</sup>/AurB<sup>1689</sup>*. At stage 10, all the analyzed genotype had complete migration, except *AurB<sup>1689</sup>/AurB<sup>1689</sup>*.



**Figure 5: AurB mutant cells tend to migrate in the back of the cluster.** We generated mutant cells using MARCM flies (*hsFlip, tubGal4, UAS-GFP; FRT40A, Gal80*). We analyzed the position, within the migrating cluster, of single mutant cells for AurB (marked with GFP). AurB homozygous mutant cells for the null allele 2A43 (*AurB<sup>2A43</sup>/AurB<sup>2A43</sup>*) were observed migrating at the back of the cluster, in 70% of the cases. (Up right) Wild type (wt) marked single cell (GFP, green), migrating in the front of the cluster. (Down right) *AurB<sup>2A43</sup>/AurB<sup>2A43</sup>* marked single cell (GFP, green), migrating behind the cluster. This is a dramatic example, where the AurB null cell detached from the migrating cluster.

We then tested the down-regulation of AurB, using 2 different RNAi lines from different collections (TRiP and VDRC collections). To express the RNAi specifically in the already specified BCs, we used the *slbo>GAL4* driver (Fig6). Surprisingly, we did not observe a phenotype in these flies.

The follicular cells stop dividing at stage 6, before the BCs are specified. Thus, our interpretation for these results was that the cells, most probably, need to go through mitosis for the phenotype of AurB loss of function to appear.



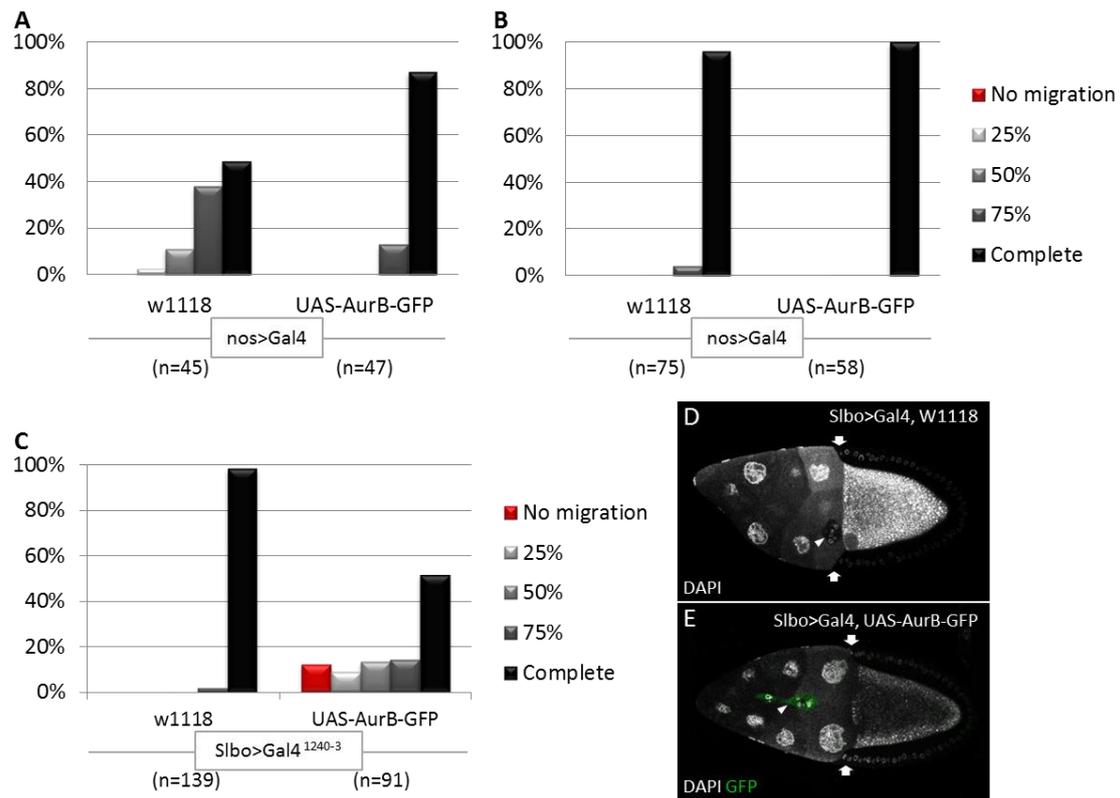
**Figure 6: AurB downregulation specifically in BC has no effect in migration.** Egg chambers from female expressing RNAi for AurB (constructs from TRiP and VDRC collections), under control of *Slbo>Gal4<sup>1240-3</sup>*, were stained with DAPI and their stage 10 migration index was quantified.

## 2- Gain of function of Aurora B impairs Border cell migration

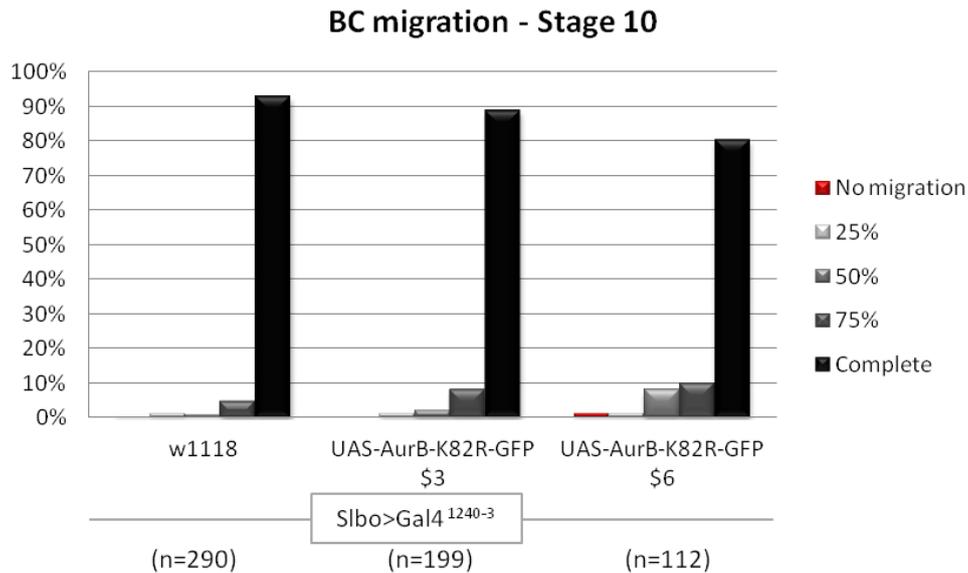
A curious observation came with the experiment described above, where we rescued the *AurB*<sup>1689</sup> homozygous with the ubiquitous overexpression of AurB. Very interestingly, in the control flies from this experiment, overexpressing AurB in a wild type context (*tub>GAL4, UAS-AurB-GFP*), we noticed that most of the clusters had already completed migration by stage 9 (Fig4). It thus seemed that overexpression of AurB induced precocious or faster BC migration.

To understand if this effect was cell autonomous, we expressed *UAS-AurB-GFP* with a driver specific for the germline (*nos>GAL4*) and with a driver specific for the BCs (*slbo>GAL4*). Very surprisingly, we observed at stage 9 a similar precocious migration with *nos>GAL4* driver. However, a delayed migration with the *slbo>GAL4* driver was observed at stage 10 (Fig7).

To check if this cell autonomous phenotype is dependent on the AurB kinase activity, we over-expressed a kinase dead form of the protein specifically in the BC (*slbo>gal4*). As we did not observe an obvious defect in migration, it thus seems that the kinase activity of AurB is important for the wild type over-expression phenotype (Fig8).



**Figure 7: AurB overexpression specifically in the germline, or BC, has opposite phenotypes in BC migration.** Egg chambers from the respective genotypes were stained with DAPI and their stage 9 or 10 migration indexes were quantified. **(A-B)** *UAS-AurB-GFP* expressed under control of the *nos>GAL4* driver, specific for germline. **(A)** Stage 9 migration index shows a higher percentage of complete migration than in the control. **(B)** At stage 10 all have completed migration. **(C-E)** *UAS-AurB-GFP* expressed under control of the *Slbo>GAL4<sup>1240-3</sup>* driver, specific for the BC. **(C)** Stage 10 migration index shows that only 50% of the clusters have completed migration ( $p = 3,34E-11$ ) (CHITEST in Excel).



**Figure 8: The delay in BC migration, seen with *UAS-AurB-GFP/Slbo>GAL4<sup>1240-3</sup>* depends on AurB kinase activity.** We DAPI stained egg chambers and quantified their stage 10 migration index. We expressed, under control of *Slbo>GAL4<sup>1240-3</sup>*, a kinase dead form of AurB (*UAS-AurB-K82R-GFP*, unpublished, \$3 and \$6 are two stocks with different insertion sites). No significant defect on BC migration.

These results suggest that AurB acts in the germline to counteract BC migration, controlling the proper speed of movement. However it does not seem to be required in the border cells for their proper migration, as downregulation of AurB in these cells has no effect. On the other hand, AurB seems to be necessary to program the cells for a correct migration, previous to their specification.

The delay in migration observed with the overexpression of AurB in the BCs, may be due to impairment of regulatory pathways where AurB normally acts. AurB is a kinase with multiple targets during mitosis. Some of them are also expressed in interphase and could be important in the regulation of BC migration.

AurB was shown to be restricted to protrusions of the cell cortex after mitosis, where it coordinates cell spreading. It is thought to act through the phosphorylation of the formin FHOD1, which is required to stabilize a selected population of microtubules and promote the assembly of F-actin fibers at the cell periphery (Floyd et al., 2013). Polarization of actin fibers

is also important during BC migration. F-actin is observed to assemble at the outer rim of the migrating cluster, and when overexpression of the actin polymerization protein Ena is induced, F-actin is seen to assemble throughout the cluster resulting in impaired migration (Lucas et al., 2013). Thus, we speculated that AurB gain of function effect in migration could be due to impairment on the assembly of F-actin. It would be interesting to look at the actin dynamics during migration in the context of AurB gain of function.

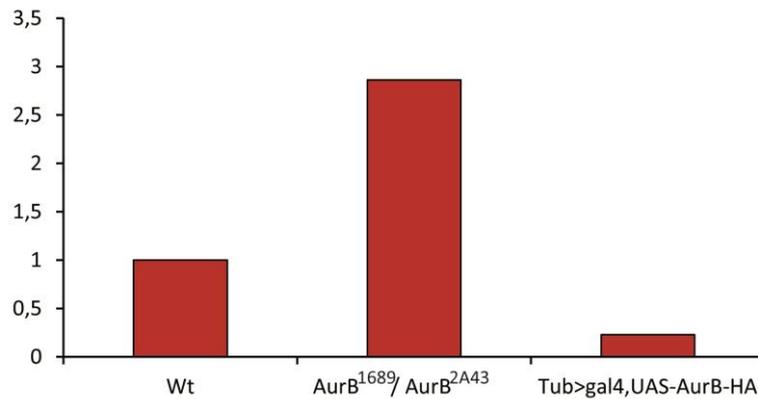
### **3- Aurora B loss of function could impact in EcR transcription regulation**

We then asked what could be the mechanism by which AurB loss of function delays abscission. One hypothesis would be that AurB loss of function would impact on chromatin during mitosis, which would result in later defects in gene expression. One of AurB best described phosphorylation targets is the Ser10 in histone H3 (H3S10).

Indeed, we saw that downregulation of AurB in BC, which are no longer cycling, have no effect in the migration. On the contrary, mutant AurB clone cells had to go through at least one round of mitosis to be formed, and thus were observed to have a delayed migration.

One of the genes that should be properly expressed to allow migration is the ecdysone receptor *ecR-B1*, expressed in follicular cells, including BC. Because we did not have access to a specific antibody for EcR-B1, took another approach to investigate its expression levels. We did an RT-qPCR to quantify EcR-B1 gene expression, using larva transheterozygous for AurB (*AurB<sup>2A43</sup>/AurB<sup>1689</sup>*), the strongest loss of function condition we can obtain. We also looked at the effect of AurB overexpression, using the ubiquitous tubulin driver.

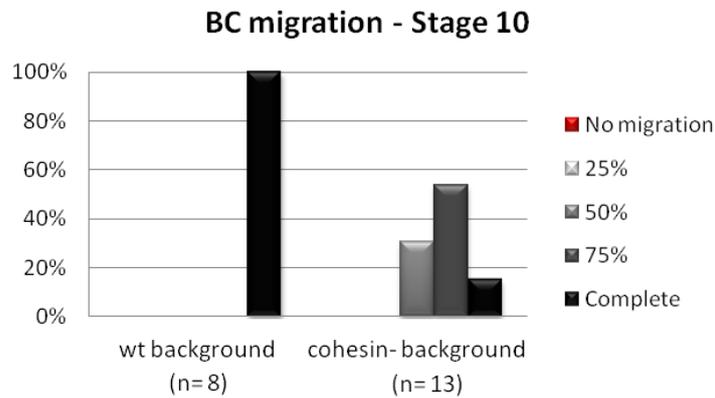
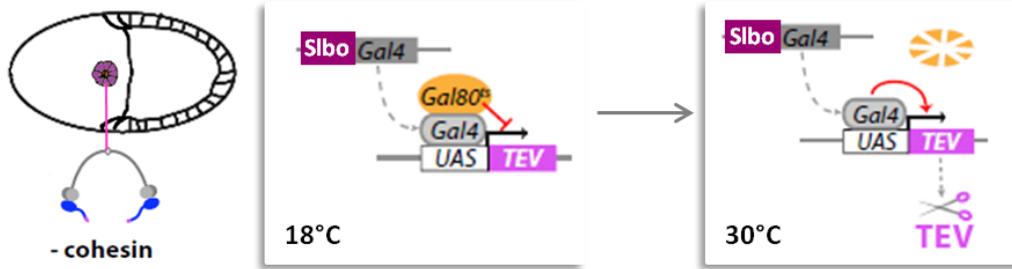
Preliminary results, suggest that AurB could act as an EcR-B1 transcriptional repressor, as the gene seems to be upregulated in the loss of function and downregulated in the overexpression conditions (Fig9). It would be important to validate these results and repeat the experiment, but using ovaries as material.



**Figure 9: AurB seems to regulate EcR transcription levels.** RNA was isolated from L3 larva from the genotypes *AurB*<sup>1689</sup>/*AurB*<sup>2A43</sup> and *tub>GAL4, UAS-AurB-HA* and use for RT-qPCR. We used primers for EcR-B1 used in (Pauli et al., 2010). EcR-B1 expression is 3 fold increased in AurB transheterozygous mutant, and decreased in AurB overexpression.

AurB is thought to facilitate release of Cohesin from the chromosomes, necessary for mitotic chromosome segregation (Nasmyth and Haering, 2009). Cohesin is also important to modulate gene transcription, by regulating chromatin topology. Indeed, it was shown to promote EcR-B1 expression in salivary glands (Pauli et al., 2010).

Over-expression of AurB in post-mitotic BC induces a delay in migration. To test if this effect could be due to impaired Cohesin regulation, and consequent EcR-B1 misexpression, we took advantage of the approach used in Pauli et al. (Pauli et al., 2010). In this work, the authors expressed the tobacco etch virus protease (TEV) specifically in the salivary glands. This protease then cleaved the cohesin component Rad21, which had a TEV recognition site, releasing the cohesin ring from the DNA. We used a similar system to express TEV protease specifically in post-mitotic BCs, in flies expressing the TEV-cleavable Rad21. We observed that when we induced TEV expression with the *slbo>GAL4* driver to promote Rad21 cleavage in BC, we observed a significant delay in migration (Fig10). It would be important to do RT-qPCR in these cells to check the expression levels of EcR-B1. It would also be interesting to do CHIP-qPCR on the EcR-B1 locus to check for the presence of cohesin, in wild type and AurB over-expressing BCs.



**Figure 10: Induced cleavage of cohesin specifically in BC results in a BC migration delay.** wt background flies:  $w^{1118}; Slbo>gal4/CyO; tubGal80(ts), UAS-NLSv5TEV, 363 (Rad21ex15, Rad21(550-3TEV)myc10)/TM6, tb$ . Cohesin- background flies:  $w^{1118}; Slbo>gal4/CyO; tubGal80(ts), UAS-NLSv5TEV, 363 (Rad21ex15, Rad21(550-3TEV)myc10)/Rad21ex3$ . All experimental flies were switched temperature for 8hrs (from 18°C to 30°C) before dissection. We DAPI stained egg chambers and quantified their stage 10 migration index.

#### **4- Problem: loss of the phenotype**

The process of Border cell migration seemed to depend on the levels of AurB and, whether the levels changed on the cluster itself or on the germline, could have opposite phenotypes. To try to understand these puzzling observations, we checked if the delay in BC migration observed in the homozygous flies for the hypomorphic allele was due to a cell autonomous or germline induced effect. We thus recombined the AurB<sup>1689</sup> allele with the FRT40A, to allow the induction of mutant cell clones. Very surprisingly, the analysis of both germline and follicular homozygous mutant clones did not show any effect on BC migration. No phenotype was found in homozygous flies for the recombined allele. We thus, unfortunately, had to conclude that the hypomorphic allele of AurB does not affect BC migration. We believe that the recombination event cleaned the chromosome from a second mutation, which when homozygous was responsible for the phenotype. Indeed, a mutation in the lethal (2) giant larvae (lgl) gene has been persistently found to accumulate in fly stocks, giving rise to BC migration defects (Denise Montell, personal communication).

Without a strong loss of function phenotype, the publication of this work could be seriously compromised. We thus felt obliged to abandon all this project and future perspectives, and start the new project on the function of Shrb in germline stem cell abscission.

We, however, believe the other presented results to be correct and meaningful: 1) Aurora B depleted cells migrate slower, at the back of the BC cluster; 2) Gain of function of Aurora B, either cell autonomous or non-autonomous, impairs BC migration; 3) Aurora B seems to impact on Ecdysone receptor transcription; 4) Cohesin cleavage, specifically in the BC, delays their migration.

### **ANNEX 3: REVIEW (Molla-Herman, 2014)**

Molla-Herman, A.; **Matias, N. R.**; Huynh, J. R. "Chromatin modifications regulate germ cell development and transgenerational information relay". Current Opinion in Insect Science, 1, 10 (2014).

# Chromatin modifications regulate germ cell development and transgenerational information relay

Anahi Molla-Herman<sup>1,2</sup>, Neuza R Matias<sup>1,2</sup> and Jean-René Huynh<sup>1,2</sup>

Germ cells transmit genetic, cytoplasmic and epigenetic information to the next generation. Recent reports describe the importance of chromatin modifiers and small RNAs for germ cells development in *Drosophila*. We also review exciting progress in our understanding of piRNAs functions, which demonstrate that this class of small RNAs is both an adaptive and inheritable epigenetic memory.

## Addresses

<sup>1</sup> Department of Genetics and Developmental Biology, Institut Curie, Paris, France

<sup>2</sup> CNRS UMR3215, Inserm U934, F-75248 Paris, France

Corresponding author: Huynh, Jean-René ([jean-rene.huynh@curie.fr](mailto:jean-rene.huynh@curie.fr))

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## Introduction

Germ cells are the only cells which are transmitted from one generation to the next, and can thus be considered immortal. Germ cells produce highly specialized cells called gametes, which have the unique capacity to convey all the information needed to build an entirely new organism. Understanding how this information is transferred from one generation to another is not only of utmost medical importance for reproductive medicine, but is also crucial to comprehend how animal shapes and forms evolve through generations. Germ cells can transmit at least three types of information: firstly, genetic information, which is the sequence of DNA corresponding to the maternal or paternal genome; secondly, cytoplasmic components, which are mainly mRNAs coding for proteins to be expressed before the zygotic genome becomes transcribed; and finally, epigenetic information, which is both nuclear and cytoplasmic, and which controls the expression of both the maternal and paternal genome. Here, we will focus on how epigenetic information is

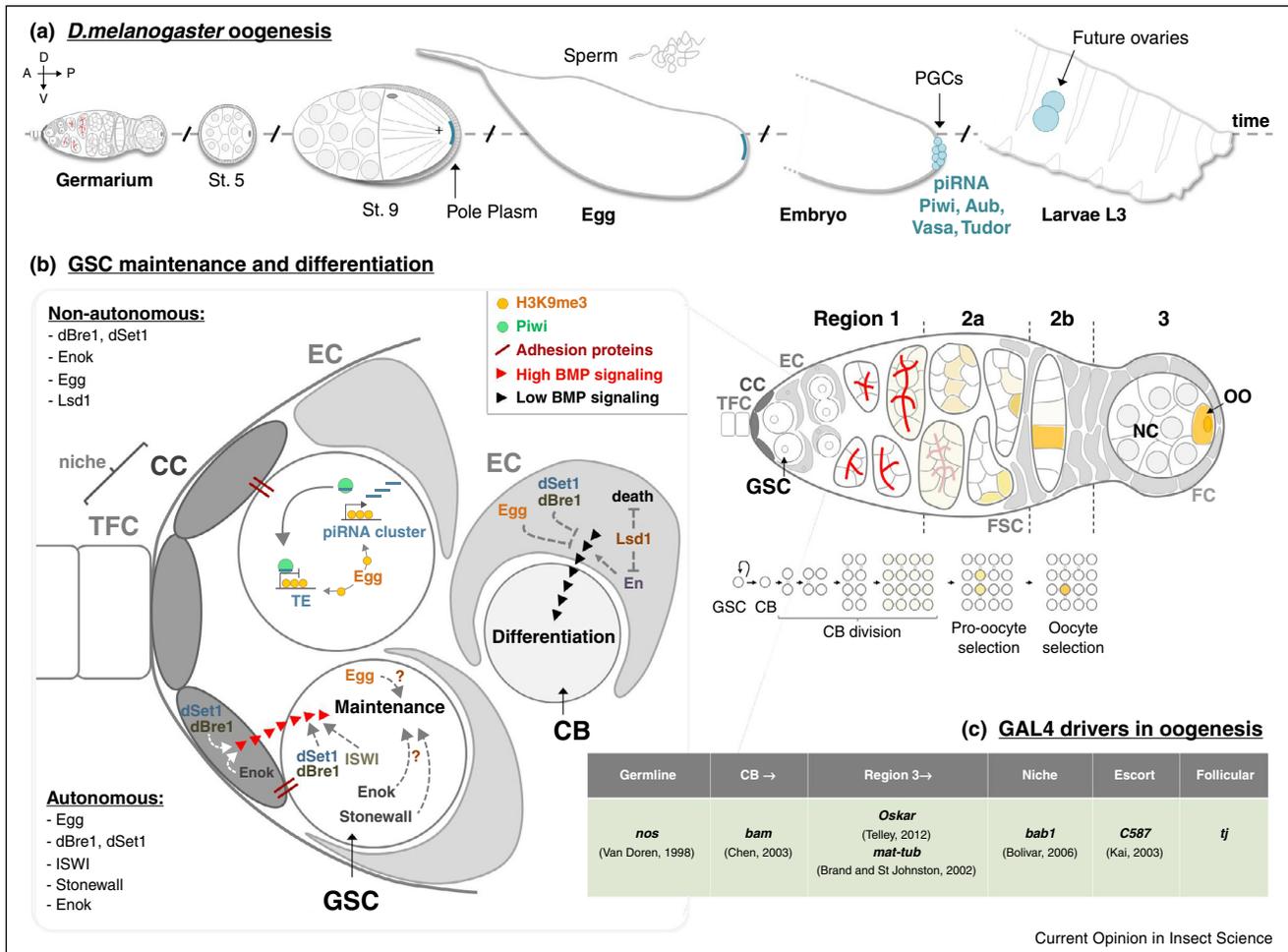
established and transmitted during the formation of the egg, the female gamete, in a process called oogenesis.

*Drosophila* ovaries are one of the best model systems to study germ cell development, and to date, there is no cell line which can recapitulate oogenesis *in vitro*. Female flies produce eggs throughout their life, and all the different stages of oogenesis are present in their gonads at any one time (Figure 1a) (reviewed in [1]). This continuous production of eggs is supplied by two types of stem cells, each generating either germ cells (GSCs), or somatic follicle cells (FSCs) surrounding germline cells (Figure 1b). Both types of stem cells are located at the tip of each ovary in a specialized structure called the germarium, where the early steps of oogenesis take place. Each GSC divides asymmetrically to produce two daughter cells, one remains a GSC, while the other differentiates as a cystoblast. This cystoblast then undergoes a series of four divisions to form a germline cyst of 16 cells. These divisions are incomplete leaving all 16 cells linked by cytoplasmic bridges. However, only one sister cell becomes the oocyte, the future egg, while the 15 other cells become nurse cells and provide the oocyte with cytoplasmic components. Nurse cells endoreplicate their DNA, become polyploid and transcribe actively their genome. In contrast, the oocyte enters meiosis, compacts its DNA into a karyosome, and is mostly silent transcriptionally. The chromatin states of nurse cells and oocytes are thus very different. Recent findings have demonstrated that chromatin marks play a very important role during the differentiation of both cell types. In addition, germ cells themselves were shown to produce small RNAs able to induce the formation of heterochromatin. These novel and exciting results made use of the exquisite genetic tools available in *Drosophila*, which allow disrupting gene function in specific cell types at precise times of development.

## Chromatin dynamics during early oogenesis

Genetic information encoded in the DNA sequence is packaged by histones into nucleosomes and chromatin (also see Mteirek *et al.*, in this issue). Access to DNA sequence is crucial for RNA transcription, DNA repair or DNA recombination, to name a few key processes. The accessibility to DNA is mainly modulated by post-translational modifications of histone tails such as methylation, acetylation, phosphorylation or ubiquitination, as there is no DNA methylation in *Drosophila* [2]. These histone

Figure 1



*Drosophila melanogaster* oogenesis: **(a)** Each ovary is formed by 15–20 ovarioles, which are strings of progressively mature egg chambers. Egg chambers bud from the germarium, the most anterior region of the ovaries. Each egg chamber has 16 germline cells (15 nurse cells and one oocyte), surrounded by follicular somatic cells. By stage 9, several factors like Piwi, Aub, Vasa, Tudor proteins, and piRNAs accumulate in the posterior of the oocyte, forming the pole plasm (blue), which will form the primordial germ cells (PGCs) in the embryo. PGCs will give rise to the adult ovaries (light blue). Antero-posterior and dorso-ventral axis are shown. The dotted gray line shows time scale and only chosen stages of the ovarioles are shown. **(b-right)** In region 1 of the germarium, germline stem cells (GSCs) divide asymmetrically giving rise to another GSC and to a cystoblast (CB). The CB divides 4 times forming a cyst of 16 cells interconnected by the fusome (red), all surrounded by a mono-layer of follicular cells (FC). Terminal filament cells (TFCs) and cap cells (CC) are somatic cells that make the GSCs niche. Escort cells (EC) are somatic cells that interact with the germline in region 1. In region 2a only two cells out of the 16 cells cyst become pro-oocytes (light yellow) and, by region 2b, only one is selected as the oocyte (yellow). The other 15 cells become nurse cells (NC). **(b-left)** Autonomous and non-autonomous factors required for GSC maintenance and differentiation. CCs are connected to the GSCs by adhesion proteins and are responsible for maintaining their stemness. Differentiation of the CB is regulated by the ECs. Both processes are controlled by a balance in BMP signaling: high BMP is important for GSC maintenance whereas low BMP is required for CB differentiation. ISWI (chromatin remodeling factor), dSet1 (H3K4 trimethyltransferase) and dBre1 (E3 ubiquitin ligase) control GSC maintenance autonomously, through BMP signaling activation [5,7]. Stonewall (chromatin remodeling factor), Enok (histone acetyltransferase) and egg (dSetDB1, H3K9 methyltransferase) act cell autonomously to maintain the stem cell [4,6,10], independently of BMP. In the niche, dSet1, dBre1 and Enok extrinsically regulate GSC maintenance, through BMP signaling activation. In Escort cells, dSet1, dBre1, Lsd1 (histone lysine demethylase) (through the transcription factor engrailed) and egg regulate CB differentiation by decreasing BMP signaling [10,11,52,53]. Egg could also regulate piRNA transcription activation and transposable elements (TEs) silencing as recently suggested [12,32]. Dotted lines represent regulation by unknown targets. **(c)** GAL4 drivers in oogenesis. *nos*-GAL4 is expressed in germline cells; *bam*-GAL4 starts being expressed in the CB; *oskar*-GAL4 is expressed from stage 1 (region 3) onwards; *mat-tub*-GAL4 is expressed from region 2 onwards. *bab1*-GAL4 is expressed in the niche in adult flies; *c587*-GAL4 is specific for EC and is weakly expressed in early FC; *tj*-GAL4 is expressed in all FC.

marks constitute an important code which regulates gene expression, chromosome structure and even nuclear architecture, and which can be transmitted through cell divisions. However, in contrast to DNA sequences, the

epigenetic landscape can be changed by environmental cues that can erase or write histone modifications [3]. In the case of germ cells, it opens the fascinating prospect that acquired epigenetic modifications could be transmitted not

only during cell division and oogenesis, but importantly to the egg cell, that is to the next generation.

Testing this hypothesis is however challenging. Genetically removing histone modifiers usually leads to oogenesis defects and sterility, impairing the analysis of the progeny. Nonetheless, it has revealed that histone modifications play a crucial role during germ cells development. Using the powerful genetic tools available in *Drosophila*, it was possible to determine when and where these modifications are required (Figure 1b). Removing chromatin remodeling factors such as ISWI or Stonewall from germline stem cells leads to their differentiation and loss, indicating that these factors are required intrinsically to maintain GSCs [4,5]. Similarly, GSCs lacking the histone acetyltransferase Enok differentiate precociously and are lost from the germarium [6]. dBre1, an E3 ubiquitin ligase, interacts with the H3K4 trimethyltransferase dSet1 in GSCs and is required for their maintenance. Histone modifiers are also necessary in cells surrounding GSCs (aka the ‘niche’), which regulate extrinsically the balance between GSCs maintenance and differentiation. For example, downregulation of dBre1 and dSet1 specifically in cap cells leads to the loss of the GSCs. In contrast, downregulation of these same genes in escort cells induces an accumulation of GSC-like cells in the germarium, indicating a failure to differentiate [7] (for more details see Figure 1b). Most of these phenotypes have been linked with defects in the Dpp (BMP-like) signaling pathway, which is the main regulator of GSCs fate in flies. The identity of the precise genes affected by these chromatin modifications remains however unknown in most cases.

Yet, some targets of *eggless* (*egg/dSetDB1*) have been identified. *Eggless* is one of the three H3K9 methyltransferases in *Drosophila*, with G9a and Su(var)3-9. It has been proposed that the same H3K9me3 mark is deposited sequentially in the germarium; first by *Eggless* in the most anterior region, then by Su(var)3-9 at the posterior of the germarium, when the fully formed egg chamber is budding off [8,9]. Intriguingly, only the loss of *Eggless* induces sterility [9], suggesting that H3K9me3 proper deposition in GSCs is crucial for germline development. Indeed, the H3K9me3 repressive mark was shown to act both in GSCs for their maintenance and in escort cells to promote GSC differentiation [9,10,11]. Importantly, several piRNA-producing clusters (see part II) have been identified as targeted by *egg* activity. In *egg* mutant, H3K9me3 marks are not deposited on these clusters, which then do not transcribe piRNAs, resulting in the activation of transposable elements and oogenesis arrest [12]. Counter-intuitively, piRNA clusters thus need this repressive mark to be transcribed. Interestingly, using weaker alleles of *eggless* it was recently shown that H3K9me3 is also required at multiple stages during oogenesis [10]. The genes or loci targeted during these stages are however unknown.

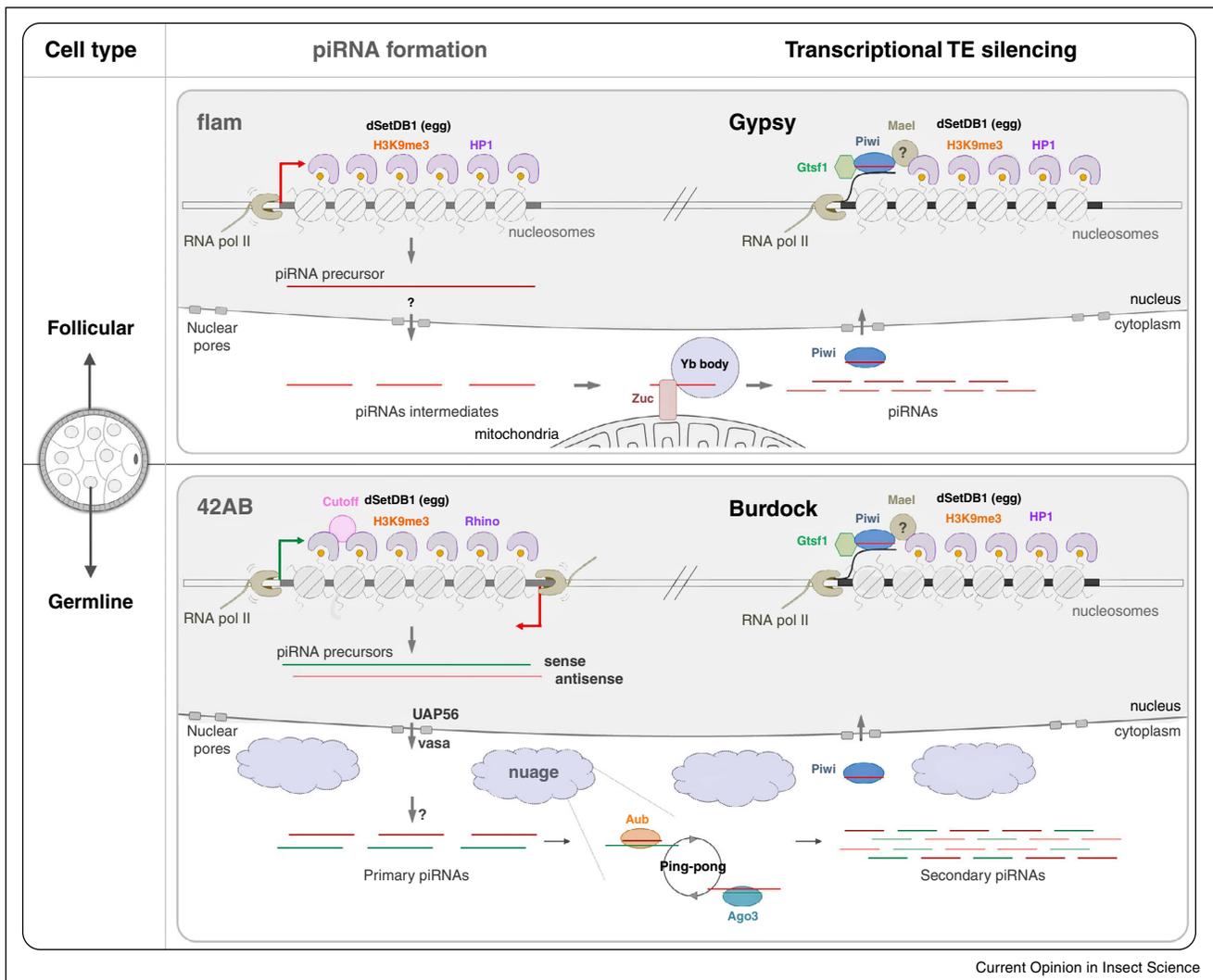
Epigenetic marks thus do not only regulate stem cells, but also many different stages of oogenesis. The determination of the oocyte was recently shown to depend on PRC2 (Polycomb repressive complex 2) [13]. This transcriptional repressor complex is known to trimethylate histone H3 on lysine 27 (H3K27me3), E(z) being the histone methyltransferase component of the complex. Genome wide analysis had identified hundreds of Polycomb targets, including the first described Hox genes, as well as cell-cycle and cell-differentiation genes [14]. In this work, Iovino *et al.* showed that in germ cells lacking E(z), the oocyte is first correctly selected, but then changes fate and develops as a nurse cell. ChIP experiments further demonstrated that PRC2 specifically targets *cyclinE* and *dacapo*, two regulators of DNA replication. The expression of the two genes is upregulated in E(z) mutants and the oocyte becomes polyploid. The authors proposed that PRC2 inhibits the transcription of these two cell-cycle genes, assuring the correct determination of the oocyte by preventing improper endocycle. Surprisingly, this new function for PRC2 is independent of PRC1 (the other Polycomb repressive complex in *Drosophila*), which is absent from female germ cells. In testis, E(z) was recently shown to be required non-autonomously in somatic cells to prevent germ cells from becoming somatic cells [15].

Histone modifiers thus play an important role during oogenesis. However, a more detailed approach is needed to identify specific targets of these modifications. Whether these histone modifications are directly transmitted to the zygote remains an open and exciting question. A tempting alternative would be that the epigenetic information could somehow be templated into the oocyte cytoplasm (by piRNAs?) and then transmitted back into the nucleus during the next generation.

### Maternal inheritance of epigenetic information

Hybrid Dysgenesis (HD) is a syndrome of genetic abnormalities resulting from the mobilization of transposable elements (TEs) in germ cells. It causes DNA damages, chromosomal rearrangements, high mutation rates and genome instability [16–19]. Transposons represent 15% of the *Drosophila* genome, and almost 50% of the human genome (reviewed in [20,21]). A better understanding of transposon silencing is thus highly relevant to human reproductive medicine. HD occurs when a male carrying several active copies of a given TE is crossed to a female, which is devoid of copies of this family. In the case of HD caused by P-elements, the female progeny (the daughters) of this cross can be completely sterile due to atrophy of their gonads (at 25°C and above). In contrast, in the reverse cross, a female carrying the TE with a naïve male, produces perfectly fertile female progeny. Females in the progeny of both crosses have the exact same genotype, however, the

Figure 2

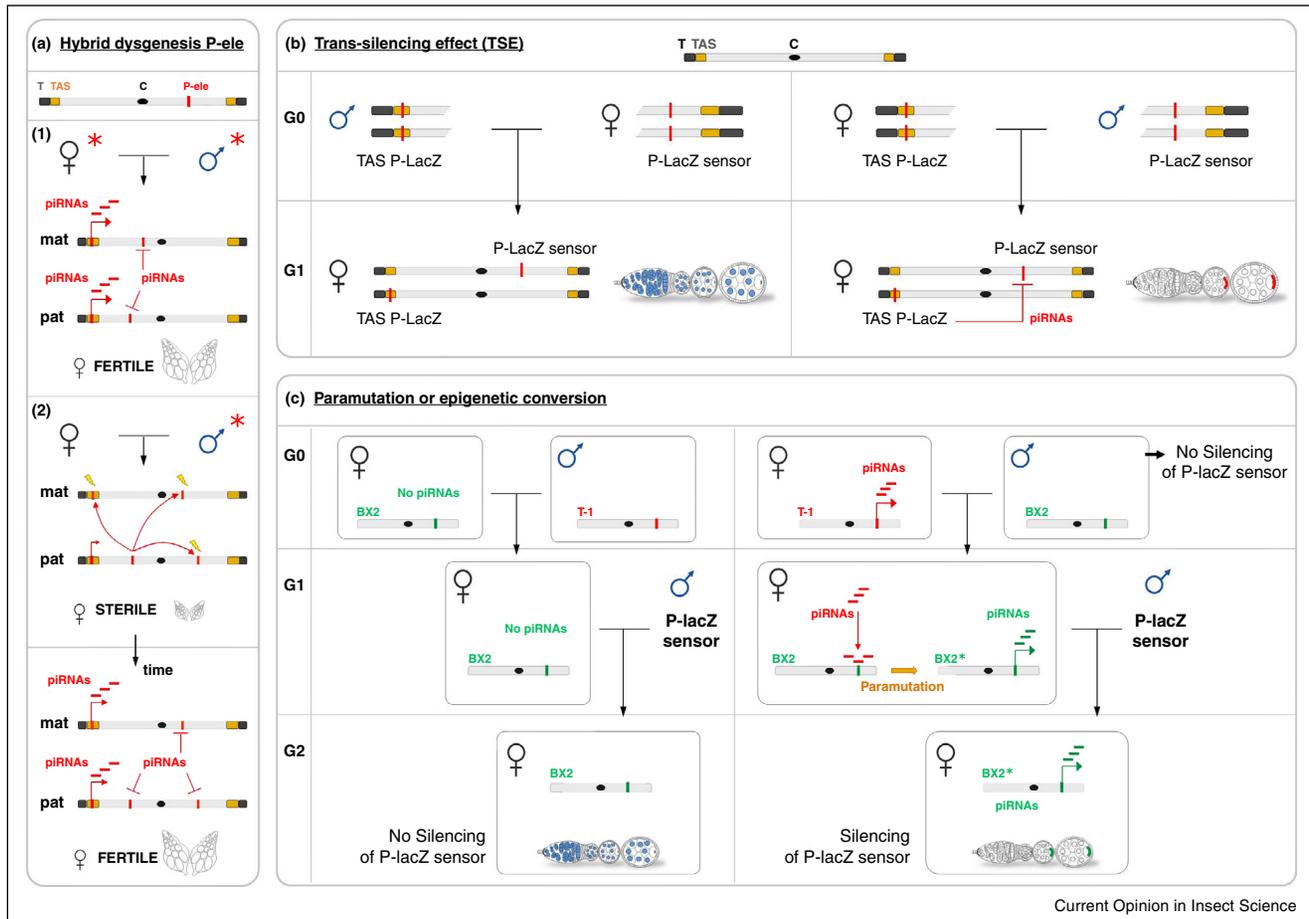


Heterochromatin marks are important for piRNA formation and for TEs silencing by piRNAs: In follicular somatic cells (top), the piRNA cluster Flamenco and a complementary TE Gypsy are two examples. The uni-strand piRNA cluster is transcribed as a long piRNA precursor (red) that exits the nucleus and is first processed by an unknown mechanism, to form piRNAs precursors. The mitochondrial nuclease zucchini (Zuc), together with the perinuclear Yb-body components, then cleave the transcript to form primary piRNAs (reviewed in [39]). Piwi is then guided by mature piRNAs to the nucleus where it can scan the genome and detect a nascent complementary transcript of Gypsy. Piwi can recruit histone methyltransferases (like egg/dSetDB1) and Heterochromatin Protein 1 (HP1), inhibiting TEs transcription by polymerase-II (Transcriptional Silencing). Piwi requires Gtsf1 (CHHC Zinc finger protein) and Maelstrom (Mael) for TE silencing, but Mael is not required for H3K9me3 deposition [32\*\*,54]. In the germline (bottom), the piRNA cluster 42AB and a complementary TE Burdock are expressed. Bistrand piRNA cluster transcription also requires heterochromatin marks and the HP1 homologue protein Rhino. Sense (green) and antisense (red) piRNA precursors exit the nucleus led by UAP56 [55,56\*]. The precursor then encounters Vasa at 'the nuage', a perinuclear cytoplasmic structure harboring several piRNA formation factors. Here the processing of primary piRNAs into secondary piRNAs through the 'ping pong' cycle takes place. In this process, Aub will preferentially bind antisense piRNAs and is guided to cleave sense primary piRNAs. Ago3, instead, will preferentially bind sense piRNAs, cleaving antisense primary piRNAs. At the same time, transcripts from active transposons can be cleaved in a post-transcriptional fashion. Piwi is eventually guided by piRNAs to induce TE transcriptional silencing similarly to follicular cells [20,33\*\*,34\*\*].

progeny of the second cross inherits a cytoplasmic factor transmitted by the oocyte cytoplasm that is absent in the first cross and that represses P element activity (Figure 3a) [22]. Although HD was first described in the 1970s, the molecular understanding of this phenomenon led to one of the most exciting discoveries in recent years. With the advent of Next Generation Sequencing (NGS) technologies, it was discovered that this

epigenetic information is carried by a large population of small RNAs (23–28 nt long) called piRNAs (Piwi-associated RNAs), which are produced by several loci interspersed throughout the genome. The sequences of these piRNAs are complementary to transposons sequences, allowing them to guide Argonaute family proteins such as Aubergine and Ago3 to transposons transcripts. Once recognized, these transcripts are then eliminated by

Figure 3



Current Opinion in Insect Science

**(a) P-element linked hybrid dysgenesis.** A *Drosophila* chromosome with: Telomeres (T, dark gray), Telomeric Associated Sequences (TAS, yellow), Centromere (C, black) and P-element insertions (red). (1) mat, maternal; pat, paternal. A female and a male having P-elements (red stars) are crossed and the resulting female can be fertile, since maternally inherited piRNAs homologous to the P element, silence in the progeny germline, both the maternally and paternally inherited P copies. (2) When a female devoid of P-elements and a male carrying P-elements are crossed, the resulting female can be sterile (rudimentary ovaries after development at 25°C and above). This happens because there is no maternal transmission of piRNAs homologous to the P-element to silence the paternally inherited P copies, and P-element mobilization creates DNA damages. However, with time, piRNAs homologous to P-elements will be produced by P copies inserted in paternally inherited piRNA clusters. In addition, mobilized P-elements can insert into piRNA producing loci (such as TAS) and can contribute to piRNA production, reinforcing P-element repression. Oogenesis and fertility can thus be restored with time. **(b) Trans-Silencing-Effect (TSE).** A *Drosophila* chromosome with Telomeres (T), Telomeric Associated Sequences (TAS) and Centromere (C). TAS-P-lacZ corresponds to a P-element containing lacZ sequences. This can silence in the germline the expression of homologous sequences located in euchromatic regions of the genome through piRNAs. LacZ repression can be followed by the absence of β-gal staining in ovaries. (Left) The cross of a male harboring the TAS P-lacZ with a female carrying a euchromatic P-lacZ gives rise to a G1 female unable to silence the euchromatic P-lacZ, and β-gal is observed. (Right) Crossing a female harboring the TAS P-LacZ transgene with a male containing a euchromatic P-lacZ gives rise to a G1 female, in which maternally transmitted piRNAs (red) can repress paternally transmitted lacZ expression [57,58]. **(c) Paramutation or epigenetic conversion.** Two arrays of P-lacZ insertions called BX2 (green) and T-1 (red) have the same DNA sequence, but only T-1 produces piRNA able to silence a homologous P-lacZ sensor. This silencing can be followed by the lack of β-gal expression. (Left) A BX2 female is crossed with a T-1 male. The resulting BX2 female (G1), which does not contain the T-1 allele, is then crossed with a male carrying a P-lacZ sensor. This sensor is not silenced, as shown by the presence of β-gal staining (data not shown in [43]). (Right) In the reverse cross, a T-1 female is crossed with a BX2 male. The resulting BX2 female (G1), which does not contain the T-1 allele, is now crossed with a male carrying a P-lacZ sensor. The absence of β-gal staining demonstrates that the sensor is silenced, and that BX2 is now able to silence the sensor. In this cross, the G1 BX2 female has inherited piRNAs from the T-1 mother, and the BX2 locus is now able to produce piRNAs in the absence of the T-1 allele. The BX2 locus is now transformed (paramutated) into a BX2\*, capable of piRNA production. BX2\* female can also transmit piRNAs to a naïve BX2 and paramutates it into a BX2\*\* allele; BX2\*\* becomes paramutagenic (reviewed in [42,43]).

Post-Transcriptional Gene Silencing (PTGS) (Figure 2) [23–25,26\*]. Furthermore, this genome defense system is adaptive. It was recently shown that flies can adapt to novel TE invasion within their lifetime [27]. If an active

transposon gets inserted into one of the piRNAs loci, novel complementary piRNAs will be generated, and this transposon will then be silenced and oogenesis rescued (Figure 3a).

How is this maternal cytoplasmic transmission achieved? It was known for a long time that the piRNA-pathway proteins, Aubergine, Vasa, Piwi or Tudor localized in the germ plasm at the posterior of the oocyte (Figure 1a) (reviewed in [28,29]). It raises the possibility that piRNAs are associated with these proteins in the germ plasm and are inherited by the PGCs when they bud off from the posterior of the embryo. This population of piRNAs inherited from the mother could help to kick-start TE silencing in the next generation [19,22,30]. How exactly piRNAs are able to establish this acquired epigenetic information is being intensively studied.

### Heterochromatin marks activate piRNA cluster transcription, but inhibits transposon expression

In contrast to Aubergine and Ago3, Piwi localizes mainly in the nucleus, leading to the hypothesis that Piwi-piRNAs complexes could also silence TEs at the transcriptional level (TGS) (reviewed in [31]). This hypothesis has been demonstrated thoroughly by four different groups [32<sup>••</sup>,33<sup>••</sup>,34<sup>••</sup>,35<sup>••</sup>]. They have shown that piRNAs can guide Piwi to nascent TE transcripts, where Piwi induces the formation of heterochromatin with the deposition of H3K9 methylation marks (Figure 2). These marks recruit the heterochromatin protein HP1, which locally reduces elongation by Pol-II and thus silences transcripts expression. These transcripts derive mainly from transposons, but the repression can spread to nearby endogenous genes, which become collateral victims (see also Mteirek *et al.*, in this issue). Remarkably, ectopic insertions of piRNA-complementary sequences are sufficient to recruit Piwi, HP1 and Su(var)3-9 and to induce the formation of heterochromatin at these ectopic euchromatic sites [35<sup>••</sup>,36<sup>•</sup>,37<sup>•</sup>]. piRNAs are thus necessary and sufficient to induce *de novo* heterochromatin formation. Haifan Lin's group study proposes a model where Piwi first recruits HP1, which in turn recruits Su(var)3-9 to add H3K9 methylation marks, resulting in further recruitment of HP1 molecules to stabilize the repressive state of the chromatin [35<sup>••</sup>]. However, the putative HP1-binding sites on Piwi protein have been recently shown to be dispensable for Piwi-mediated transposon silencing [38]. Further studies are thus needed to understand how Piwi recruits HP1. piRNAs are mainly (but not only) produced by 140 loci or clusters in the *Drosophila* genome [23]. These clusters are thought to be transcribed into long transcripts made of intermingled TE sequences, and processed into small piRNAs by complex mechanisms (Figure 2) (reviewed in [39] and see Chambeyron and Seitz, in this issue). Since these transcripts contain TE sequences, piRNAs could also recognize these long transcripts and induce the deposition of H3K9me3 at these loci, which is required for piRNA cluster transcription as shown in *eggless* mutants (Figure 2). This loop could thus create a positive feedback for piRNA cluster transcription. It was recently shown that individual TE insertions can also be a source of bidirectional piRNAs in the germline [40<sup>•</sup>]. Again, these piRNAs

could lead to the deposition of H3K9me3 marks at these sites, but this time transposons would be silenced, through a negative feedback loop. The recent discovery of the first paramutation in *Drosophila* showed that piRNAs can also initiate a feedforward loop [41<sup>••</sup>]. A paramutation is the transfer of an acquired epigenetic state to a homologous locus, resulting in a meiotically heritable alteration in gene expression (Figure 3c) (reviewed in [42,43]). de Vanssay *et al.* showed that a locus not producing piRNAs, called BX2, can be converted into a piRNA producing locus simply by homologous piRNAs inherited maternally. After its conversion, BX2\* can now produce piRNAs for the following generations, and can even convert a naïve BX2 locus into producing piRNAs [41<sup>••</sup>]. Collectively, these works thus demonstrate that piRNAs themselves could serve as an epigenetic memory, deposited in the egg, able to point out in the next generation what are the TEs to be silenced and what are the piRNA clusters to be expressed. piRNAs are thus a mean to transfer the information, but how this information is then used to define a locus as a source of piRNAs still remains unclear (reviewed in [44]). The H3K9me3 mark is part of the answer (see above). It is also known that the HP1 homologue Rhino binds specifically piRNA clusters expressed in germ cells, and promote their transcription in both sense and anti-sense directions (Figure 2). Recently, genomewide genetic screens have uncovered most of the genes required to make piRNAs. Interestingly, several of these genes such as dSet1, Lsd1, egg, Caf1, His2av, Asf1, etc. are histone modifiers or chromatin dynamic regulators [45<sup>••</sup>,46<sup>••</sup>]. It will now be a challenge to understand how each of these genes helps to create a chromatin environment which defines a locus as a heritable source of piRNAs.

### Conclusions and perspectives

A combination of advanced fly genetics and next generation sequencing has thus placed *Drosophila* oogenesis at the forefront in our understanding of epigenetic inheritance. The recent works reviewed here show that histone modifications and chromatin dynamics play a crucial role in germ cells differentiation. The next challenge is now to uncover the target genes, which are deregulated when these epigenetic marks are disrupted. These analyzes are done routinely with pure populations of cells cultured *in vitro*. *Drosophila* ovaries, however, contain several types of cells at different stages of development. It thus remains a challenge to isolate enough cells at a similar stage to perform omics analysis. It will also be important to test the functions of these histone modifications not only by disrupting the enzymes that write these marks, but also by mutating histones directly. A major step toward this goal has been achieved recently using elegant genomic engineering [47].

The discovery of piRNAs in flies, together with elegant work performed with *C. elegans* (*Caenorhabditis elegans*), bring us closer to understand how epigenetic memory is

transmitted from one generation to the next [48,49]. Works done in other insect species demonstrate that these mechanisms are widely conserved in this class (see Chambeyron and Seitz, in this issue). The situation is also similar to piRNA-driven silencing of TEs in mammals, where piRNAs guide DNA *de novo* methylation of TE insertions at each generation [50]. The making of piRNAs in any species is however not fully understood. Major efforts have been made to catalog all the genes required to generate piRNAs. It now remains a challenge to decipher the precise function(s) of each of these genes [45,46]. These studies have also revealed that only 60% of piRNAs target transposon sequences. What is the function of the remaining 40% of piRNAs? Do piRNAs also regulate the expression of endogenous genes [32,40,51]? And is this regulation transmitted to the next generation? These are exciting lines of research for the future.

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