Cellular architecture & rab protein distribution in Drosophila melanogaster male accessory glands

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Cellular Architecture & Rab Protein Distribution in *Drosophila melanogaster* Male Accessory Glands

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N.B. - La thèse doit porter la déclaration précédente et remplir les conditions énumérées dans les "Informations relatives aux thèses de doctorat à l'Université de Genève".

Synopsis

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"The best thing in life is to go ahead with all your plans and your dreams, to embrace life and to live everyday with passion, to lose and still keep the faith and to win while been grateful. All of this because the world belongs to those who dare to go after what they want. And because life is really too short to be insignificant."
Charlie Chaplin

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Résumé:

Contrairement aux idées reçues, le fluide séminal n'est pas un simple liquide permettant le transport des spermatozoïdes; ce plasma représente 95% de l'éjaculat et affecte la fertilité des mâles de nombreuses espèces. Par exemple chez les Mammifères, la présence du fluide séminal dans l'éjaculat améliore la santé de la descendance et augmenterait le taux de grossesse lors de fécondations *in vitro* ou inséminations artificielles. Ainsi, pour des raisons éthologiques et économiques, comprendre comment le fluide séminal et ses composants contribuent à la fertilité et la fécondité des mâles, se révéla comme un sujet de grand intérêt. Un des organismes les plus étudiés dans ce domaine est la Drosophile, *Drosophila melanogaster* ; le fluide séminal du mâle affecte profondément le comportement et la physiologie de sa conquête après accouplement ; une paire de glandes annexes, les glandes accessoires (homologues fonctionnels de la prostate), participent majoritairement à la composition du plasma séminal et la nature des composés séminaux sont majoritairement conservés chez les Mammifères. Mon projet de doctorat s'inscrit dans

ce domaine en ce focalisant sur les glandes accessoires elles-mêmes *ie* quelle est leur architecture cellulaire et comment sécrètent-elles les molécules dans le fluide séminal?

Les glandes accessoires de la Drosophile mâle sont faites d'un épithélium encerclant un lumen central, le tout enroulé dans des fibres musculaires. La couche cellulaire est composée de deux types cellulaires sécréteurs et bi-nucléés, les cellules principales et les cellules secondaires. Chacune de ces cellules joue a rôle important dans la fertilité du mâle en sécrétant, notamment, des molécules affectant la réponse post-copulatoire chez la femelle. La fonction des cellules principales est plutôt bien comprise, contrairement à celle des cellules secondaires ; la fonction des cellules secondaires semble, cependant, reposer sur les larges vacuoles présentes dans leur cytoplasme. Pour approfondir notre connaissance sur le rôle des cellules secondaires, nous décidâmes d'étudier la nature de ces compartiments proéminents. De ce fait, nous partîmes du principe que ces larges vacuoles ressemblaient à de larges compartiments intracellulaires, et pour cela nous criblâmes une collection de Drosophile permettant d'observer l'expression de régulateurs du transport vésiculaire, les protéines Rab.

Cette collection comporte des lignées où les 27 gènes *rab* de la Drosophile ont été étiquetés de manière endogène par une protéine fluorescente, YFP; nous permettant ainsi d'observer ces protéines à leurs niveaux d'expression endogènes et dans les tissues qui leurs sont propres. En criblant cette librairie par microscopie confocale, nous fîmes plusieurs observations. Premièrement, la distribution des protéines Rab associée à celle des marqueurs de polarité apico-basale dans l'épithélium des glandes accessoires confirma la polarité de ce tissu et montra que l'architecture cellulaire suggère des échanges entre les cellules principales et secondaires. Deuxièmement, quatre protéines Rab (Rab6, Rab7, Rab11 & Rab19) associés à des fonctions intracellulaires différentes, sont ancrées à la

membrane de différentes sous-populations parmi les larges vacuoles. Troisièmement, bien que la plupart de ces compartiments sont caractérisés par la présence d'une seule protéine Rab, des expériences de co-expression révélèrent néanmoins l'existence de certaines vacuoles avec deux « identités » Rab. Enfin, ces larges vacuoles pourraient être des compartiments matures naissant de la fusion de compartiments plus petits et leur formation serait basée sur la présence de certaines protéines Rab.

Mes données de doctorat fournissent des indices sur le fonctionnement des glandes accessoire. Cet épithélium sécréteur est particulier et son étude permettrait, par exemple, d'obtenir plus d'information sur la fonction de Rabs plutôt méconnues, telles Rab14 et Rab19, exprimées spécifiquement dans les cellules secondaires. La fertilité de la gente masculine affichant une baisse depuis plusieurs années, comprendre comment les glandes accessoires produisent les protéines du fluide séminal permettrait de déceler le problème et comment le contrer.

Abstract:

Contrary to popular belief, the seminal fluid is not a simple transport medium for the spermatozoa. It represents 95% of the ejaculate and has been shown to play an important role in male fertility in many species. For example, in mammals, it has been shown that sperm separated from the seminal fluid produce poorer quality offspring than sperm together with seminal fluid. How the components of the seminal fluid affect male fertility is now a subject of great interest. One of the most studied organisms with regard to the effect of seminal fluid on male fertility is *Drosophila melanogaster*. Its seminal fluid has drastic effects on female behaviour and physiology that increases male reproductive success. A pair of male accessory glands makes the *Drosophila* seminal fluid components; functionally, they

are the homolog of the mammalian prostate gland. My PhD project centres on the cells that make up the *Drosophila* male accessory glands and determining how they are organized and how they produce chemicals that will become part of the seminal fluid.

The *Drosophila* male accessory glands are made of an epithelium surrounding a central lumen, all wrapped in muscle fibres. The epithelial cell layer is made of two secretory and bi-nucleated cell types: the main cells and the secondary cells. Both of these cells play an important role in male fertility by secreting the seminal fluid products that produce the female post-mating response. The role of the mains cells is quite well understood. They make up 96% of the gland and have been shown to produce most of the known proteins that trigger post-mating responses. However, the function of the secondary cells is less unclear. Mutations that affect their development seem to result in the modification of main cell products such that they lose some of their efficacy. As their cell morphology is dominated by prominent large vacuoles filling their cytoplasm, we decided to investigate the large vacuoles in order to better understand their function in inducing the post-mating response. We started this investigation with the hypothesis that these features are similar to intra-cellular trafficking compartments and thus, examined the localization of the master regulators of vesicular transport, the Rab proteins.

Using a library in which each of the 27 *Drosophila rab* genes were fused to YFP at their endogenous genomic location, we were able to observe these proteins at their endogenous expression levels and in their endogenous tissues. By screening this library using confocal microscopy, we shed light on two main things. First, the distribution of the Rab proteins in the accessory gland epithelium; second, by combining these Rab fusion proteins with apical-basal membrane markers, we showed that this tissue is polarized and its cellular architecture suggests exchanges between the secondary cells and the mains cells.

Third, we found that four Rab proteins (Rab6, Rab7, Rab11 and Rab19) localize to different subsets of large vacuoles in the secondary cells, suggesting different sub-populations of vacuoles with different functions. Fourth, although most of the large vacuoles carry one Rab protein, co-expression and selective RNAi experiments showed that there are some vacuoles with two Rab identities, suggesting that these vacuoles may mature or modify their activity over time.

My PhD provides clues into the functioning of the accessory glands and provides a solid foundation from which new experiments can be performed. As this secretory epithelium is particular and expresses a few rarely expressed Rabs of unknown function, its study could give insights on the function these poorly characterized Rab proteins (for example, Rab14 and Rab19). Since male fertility has seen a marked decrease over the past few decades, a better knowledge about the making of the seminal fluid and how male accessory glands contribute to this process is of great immediate interest.

Introduction

Reproduction is an essential property of living organisms. It can generally be described as the sum of the biological processes that permit a species to sustain its existence through the creation of new individuals. There are many types of reproduction in the living world, each with their advantages and disadvantages. Some of these methods will be discussed below.

The most fundamental division in reproductive mechanisms is that of asexual *vs* sexual reproduction. In asexual reproduction, progeny arise from a single organism by a mitosis-based process. Thus, the resulting offspring are identical to their parent. Asexual reproduction is fast and relatively energy efficient. However, it can be disadvantageous if deleterious mutations are present in the parents, as these mutations will be passed along to their offspring. Thus, this form of reproduction allows a species to quickly reproduce in a favourable environment but does not allow for a lot of variation in the progeny to cope with changing environmental conditions. Asexually reproducing organisms have therefore created different ways to provide genetic variation in their progeny such as error prone replication and fast reproduction times. Asexual reproduction is used by many species including bacteria and many plants and fungi.

Sexual reproduction is a form of reproduction based on the fusion of specialized cells called gametes. Each gamete generally arises from a meiosis event, where the genetic material of an individual parent is halved. Thus, each gamete contains only half of the genetic material of an individual (haploid). Offspring arise when gametes, generally from two different individuals, fuse to create a new individual (diploid). In most cases, the gametes

stem from two different sexes. The male gametes are called the sperm, while the female gametes are called the oocyte (ovum). Although sexual reproduction has been estimated to be twice as costly energetically, it has the advantage of making individuals different from their parents. As such, it produces variation in a population that can dilute the effect of deleterious mutations as well as create new phenotypes that can be advantageous under certain conditions. In spite of its high energetic costs, the advantages of sexual reproduction have made it the most common form of reproduction in the living world (Crow & Kimura, 1965).

Sexual reproduction can be further divided into two categories based on where the gamete fusion event takes place. If the gamete fusion event takes place in the environment, it is called external fertilization, whereas, if the gamete fusion event occurs within the body of one of the parents, it is called internal fertilization. External fertilization has the advantage of not requiring physical contact between the parents and is thus often used by sessile organisms that simply eject their gametes into the environment. Internal fertilization, however, often requires physical contact between the parents. In most species that use internal fertilization, the male parent ejects its sperm into the female genital tract where the eggs are fertilized. In this way, the gametes are protected from the environment and environmental predators until fertilization can be achieved. This method also allows the parents to select their mates and thus, theoretically helps to increase the evolutionary fitness of their progeny (Boundless. "External and Internal Fertilization". *Boundless Biology* Boundless, 26 May. 2016. Retrieved 22 Jan. 2017)

As the issue of mate selection implies, the critical point of reproduction is to produce offspring that will live to reproduce themselves. Thus, to favour one's reproductive success, one must try to insure that one's own progeny are favoured in the next generation over

those of others. Some organisms simply give birth to many progeny in hopes that some of them will survive to adulthood. For example, sea turtles often lay up to 200 eggs in a single nest. Due to their numerous predators, however, only about 10% of these offspring will ever make to adulthood. Other organisms, like humans, take the opposite path and try to maximize the viability of a more limited number of offspring (Lack, 1954).

Another distinction between mating strategies lies around monogamy *vs* polygamy. Monogamy is when one male and one female cohabitate and copulate only exclusively with each other. Polygamy, on the other hand, allows both males and females to have multiple mates. Monogamy is somewhat rare compared to polygamy, though it is thought to be advantageous for the care of offspring. Polygamy allows individuals to combine their genetic material in more ways to increase genetic variation and thus buffer against a fluctuating environment (Fletcher et al., 2015). In organisms with internal fertilization and development, it also allows for males in particular, to have a larger number of progeny. Since males and females are often produced in similar numbers, this means that males will generally be competing for a limited number of females. Thus, polygamous males have developed ploys to insure their reproductive success over those of competing males. For example, in species where few males monopolize reproduction over a limited number of females such as in some primates, squirrels and polar bears, males use infanticide to eliminate the progeny of rival males and make the female available again for mating (Lukas & Huchard, 2014). Besides this “barbarism”, other species use more “elegant” methods such as “mate-guarding” their conquests. Examples of this sort of progeny protection can be seen in insects like fruit flies and crickets (Wolfner, 1997; Simmons et al., 2014), as well as in some rodents (Bromfield, 2014). This “mate-guarding/mate remote-control” is mostly achieved through the use of seminal fluid components transferred to the female during

mating that affect her physiology and behaviour. These changes occurring in the female after mating are grouped under the name of the Post-Mating Response (PMR).

In mammals, although sperm is the most important constituent of the seminal fluid, it actually only makes up about 2-5% of the seminal fluid volume (http://www.news-medical.net/health/What-is-Semen.aspx). The rest of the seminal fluid is made up of proteins, sugars, lipids, ions and other molecules made by specialized secretory glands associated with the male reproductive tract. In *Drosophila*, these glands are called the male accessory glands (AGs), whereas in mammals, the same role is fulfilled by the combination of the seminal vesicles, prostate and bulbo-urethral glands. The testis, for the most part, produces only the sperm.

For a long time, the seminal fluid was thought of as a simple transport medium for the sperm. Although, the seminal fluid represents 95-98% of the male ejaculate, the fertilizing capacity of the male ejaculate was attributed solely to the spermatozoa. In fact, except for the nutrients present in the seminal fluid to help nourish the sperm throughout their journey through the female reproductive tract, the seminal fluid was even thought to have a negative impact on fertilization (Chang, 1957). Thus, for *in vitro* fertilizations (IVFs), sperm was generally washed and used without seminal fluid. Recent studies, however, have now started to change our view on the importance of the seminal fluid. Several mammalian studies now show that the seminal fluid not only interacts with the female reproductive tract to influence whether a pregnancy will occur (Tremellen et al., 2000), but also affects the post-natal health of the resulting progeny (Bromfield et al., 2014). As male infertility is the cause of 20-70% of unfertile couples (Tournaye et al., 2016), understanding the roles played by seminal fluid components can have an important impact on fertility treatments.

Although a number of mammalian studies have now been performed in rodents and humans, much of the work on how the seminal fluid influences male reproductive success has been performed in insects. Given the importance of insects in agriculture and disease propagation, male fertility/infertility has been of incredible economic interest. Because of the genetics available in the model insect, *Drosophila melanogaster*, studies of *Drosophila* seminal fluid have yielded important results.

I- The accessory glands

In vertebrates and invertebrates, male fertility requires the testes and the AGs. In mammals, the epididymis, the seminal vesicles, the prostate and the bulbo-urethral gland consecutively add their secretions to the passing spermatozoa coming from the testes (Fig.1A-B). The known functions of these secretions include: sperm capacitation, increasing sperm motility, protecting sperm from the female immune system and regulating the ejaculatory processes (Verze et al., 2016). In *Drosophila* a single pair of AGs (or paragonia) secretes seminal fluid proteins (Sfps) into the seminal fluid that will join the sperm in the ejaculatory duct before being transferred to the female (Fig.1C). Although based on their structure (a pair of sac-like organs) and their tissue origin (mesoderm), the *Drosophila* AGs are more similar to the mammalian seminal vesicles (Riva, 1967; Bairati, 1968). However than the prostate, which is of endodermal origin, the secretions made by the glands and some developmental similarities, cause scientists to feel like the *Drosophila* AGs are the functional-homologs of the prostate gland (reviewed by Wilson et al., 2017). Because of this, I will now compare the mammalian prostate gland with the *Drosophila* AGs.

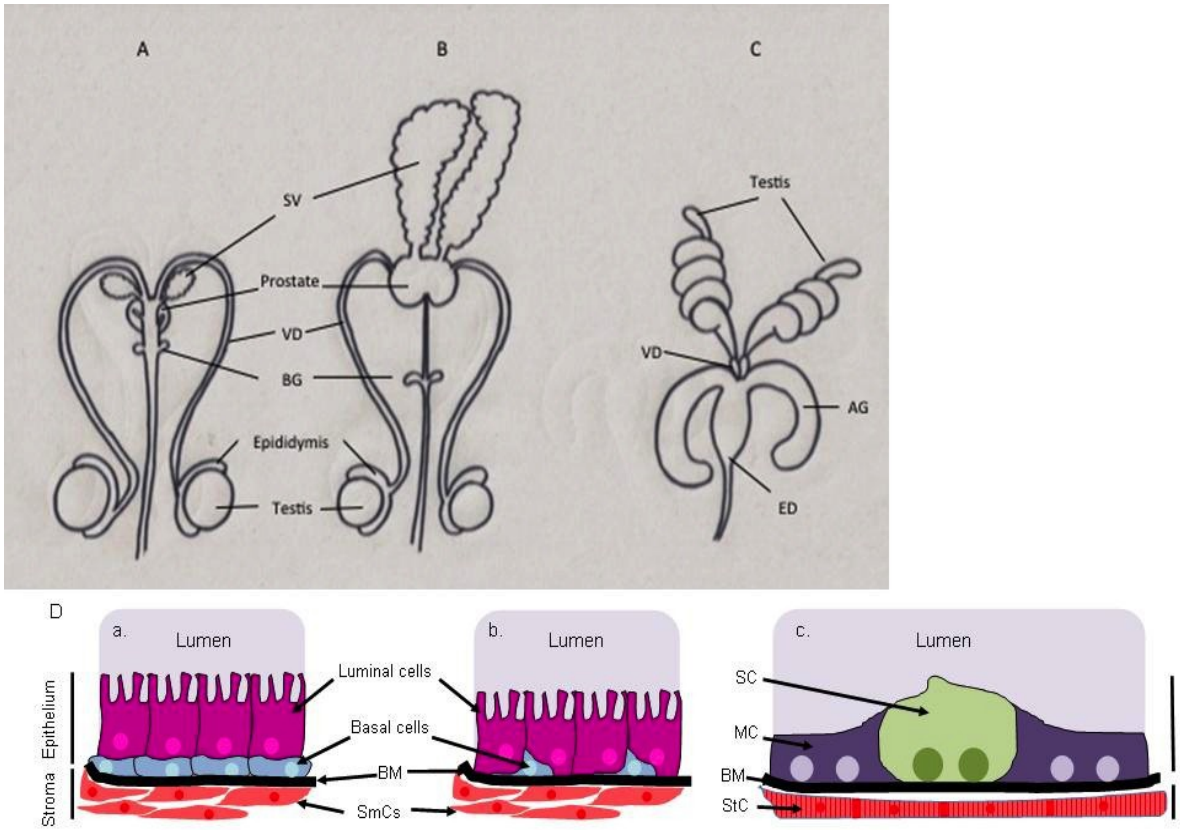


Figure 1. The genital tract of different model species
On the top, drawing (not to scale) of the human (A), mice (B) and *Drosophila* (C) genital tracts. Abbreviations: SV, seminal vesicle; VD, *vas deferens*; BG, bulbo-urethral gland; AG, accessory gland; ED, ejaculatory duct.
On the bottom, schematic representations of the cellular organization of the human (Da.) and mice (Db.) prostates; the cellular architecture of the tip of a Drosophila accessory gland is also schematized (Dc.). In human and rodents the prostatic epithelial compartment is composed of the luminal secretory cells and the basal cells, whereas the stromal/mesenchymal compartment is made of smooth muscle cells (SmCs) (Da. &Db.). Drosophila accessory glands are made of a single epithelial layer made of two cell types at the distal tip, the secondary cells (SC, lavander) and the main cells (MC, khaki); the glands are surrounded by striated muscle cells (StCs). All these epithelia rest on a basal membrane (BM) and face the lumen on their apical side. These schemes are adapted from descriptions done by Marker et al., 2003 and Bairati, 1968.

1) The mammalian prostate

The prostate is an endodermal tissue whose growth begins during fetal life and ends at sexual maturity (Marker et al., 2003; Prins & Putz, 2008; Huang et al., 2007; Javed & Langley, 2014; Powers & Marker, 2013). In the early embryo, the urogenital sinus (UGS) grows in a way indistinguishable between males and females. It is not until late embryogenesis, when the fetal testes start to secrete androgens, such as testosterone, that

the prostate gland starts to differentiate. Although the initial steps of prostate gland development happen in the embryo, a vast majority of its growth and differentiation (outgrowth of the epithelial buds, branching morphogenesis and lumen formation) does not occur until after birth and continues until sexual maturity (Timms, 2008; Marker et al., 2003; Powers & Marker, 2013). Once mice and rats are sexually mature, they have what is called a tri-lobar prostate (ventral, dorso-lateral and anterior prostates for mice; ventral, lateral and dorsal prostates for the rats). Although human prostates do not share the same lobed structure, they do have three zones (transition, central and peripheral zones) defined by specific sets of secreted proteins and secretory activity (Marker et al., 2003).

The prostate lobes/zones are made of three compartments; a central lumen, an epithelium that surrounds the lumen, and a stromal/mesenchymal compartment that surrounds the epithelium (Marker et al., 2003). The epithelium is made of two cell types: the columnar secretory luminal cells that secrete proteins from their apical surface toward the lumen, the basal cells that form a continuous layer between the luminal cell layer and the basement membrane in the human prostate (Fig. 1Da) or interspaced between basal corners of luminal cells and the basement membrane in rodent prostates (Fig. 1Db) and the rare neuro-endocrine cells (Marker et al., 2003; Kwon et al., 2014). The stroma layer includes a smooth muscle layer that maintains the epithelium in an appropriate microenvironment, and can regulate the gland homeostasis by sensing the extracellular area (Verze et al., 2016). The muscle layer is also used to expel the secretions of the prostate (Marker et al., 2003; Timms & Hofkamp, 2011; Powers & Marker, 2013) (Fig. 1Da-b).

As I mentioned earlier, prostate development is initiated by secretion of testosterone from the fetal testes. This circulating hormone, after being transformed into 5-α-dihydrotesterone (DHT) and, DHT induces prostate specific developmental genes via its

binding to UGS nuclear androgen receptors (nARs) in the mesenchyme and epithelium. The role of the androgens is not only developmental as they are also required to maintain gland homeostasis in the adult and to regulate gene expression in a lobe-specific manner (Marker et al., 2003; Huang et al., 2007; Timms, 2008; Verze et al., 2016).

Besides the androgens, the *Hox* genes are also very important in prostate development and tissue maintenance (Javed & Langley, 2014). The *Hox* genes were first identified in *Drosophila melanogaster* for their role in the determination of segmental identity along the anterior-posterior (A-P) axis (reviewed in Maeda & Karch, 2011; McGinnis & Krumlauf, 1992; Lewis, 1978). Subsequently, orthologous genes with similar functions in segmental identity were found in other species. In rodents and humans, the posterior *Hox* genes of the *Abd-B* type (*Hoxa9-a11*, *Hoxa13*, *b13* and *d13*) are expressed in the developing and adult prostate (Prins & Putz, 2008). Many of the *Hox* genes are lobe/tissue specific. For example, *Hoxa13* and *Hoxd13* are postnatally up regulated in the ventral prostate (VP) and current data suggests that *Hoxb13* is necessary in the maintenance of the prostatic epithelium (Prins & Putz, 2008; Huang et al., 2007; Javed & Langley, 2014).

Many other factors have been implicated in the development and function of the prostate in mammals, including: the Wnt, the Hedgehog (Hh), and the Bone-Morphogenetic-Proteins (BMPs) pathways (involved in prostate growth and development) (Prins & Putz, 2008; Timms & Hofkamp, 2011; Wilson et al., 2017; Montano & Bushman, 2016). Recently a peptide-hormone, relaxin, has also been shown to be involved in human prostate development and function (Ivell et al., 2016).

2) The *Drosophila* accessory glands

The functional homolog of the prostate in *Drosophila* is thought to be the pair of male AGs (Wilson et al., 2017). The AGs are sac-like organs having an epithelial monolayer of cells surrounding a central lumen, all wrapped by a layer of striated muscle fibres (Susic-Jung et al., 2012). The epithelium is made of two bi-nucleated and secretory cells types: the main and the secondary cells (Fig.1 Dc & Fig. 2). The polygonally shaped main cells (MCs) cover ninety-six per cent of the AG. The remaining four percent are the large, spherically-shaped secondary cells (SCs) that are interspaced between MCs at the distal tip of the glands and contain large vacuoles filling their cytoplasm (Bertram et al., 1992) .

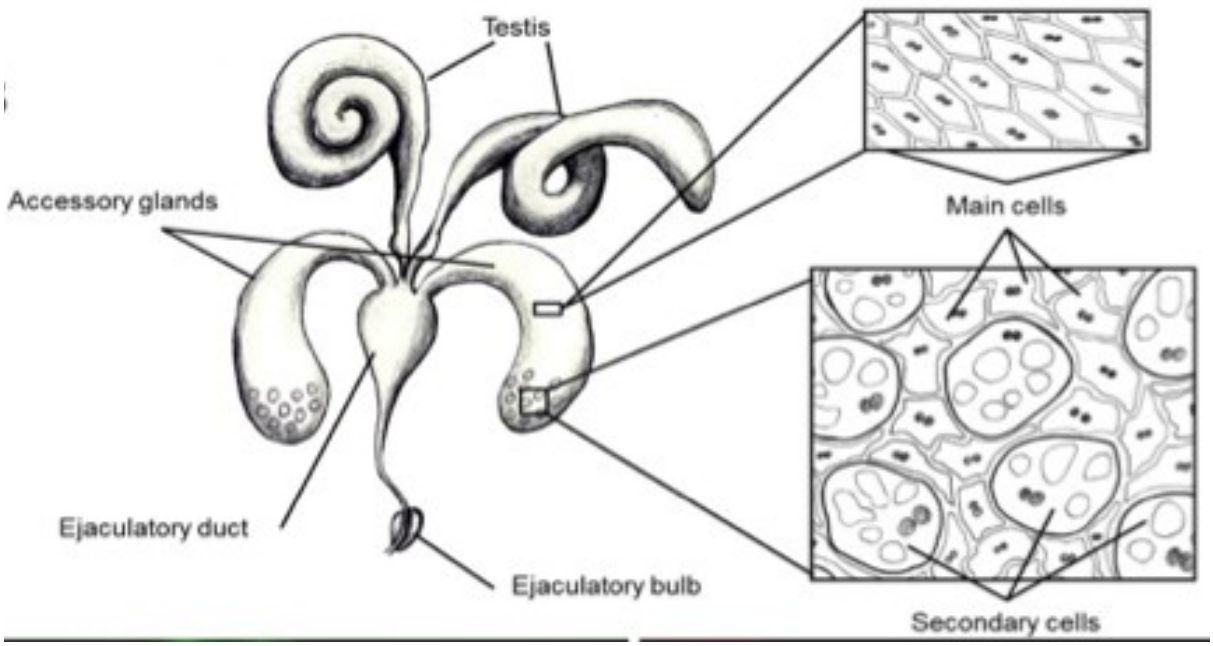


Figure 2. The accessory glands are made of two cell types (Gligorov et al., 2013)
J. Sitnik's drawing from Gligorov, Sitnik et al., 2013 that depicts the male reproductive tract (the pairs of testis and accessory glands, the ejaculatory duct and the ejaculatory bulb. Each accessory gland is made of two secretory cell types, the main cells, which cover 96% of the surface of the accessory glands (top insert) and the spherically-shaped secondary cells, only located at the distal tip of the gland, interspersed among the main cells (bottom insert). Both cell types are bi-nucleated (top and bottom inserts, black dots) (Gligorov et al., 2013).

There are two types of large vacuoles in the SCs: ones simply called “vacuoles”, and others called “filamentous bodies” (Bairati, 1968) or “dense core granules” (DCGs) (Corrigan et al., 2014). The filamentous bodies are so called, due to the odd filamentous structures that they contain. The identity of these filaments is still unknown, though some have suggested that they may be composed, at least in part, by RNA (Tandler et al., 1968) anb may be secretory vesicles since their content can be found into the lumen (Bairati, 1968; Redhai et al., 2016).

The development of the AGs, like many sex-specific tissues is determined by the sex-determination genes that act downstream of *Sex-lethal* (*Sxl*) (Chapman & Wolfner, 1988). Male mesodermal cells expressing the fibroblast growth factor (FGF) homologue, Breathless (Btl), are recruited into a part of the male genital disc primordium where they differentiate into epithelial cells and proliferate under the control of the *Pax* gene, *paired* (*prd*) (Xue & Noll, 2002; Minami et al., 2012). Ten hours after puparium formation (APF), small epithelial buds start to grow. Between twenty-four and forty hours APF, the lumen is created by an epithelial closure that is then covered by mesodermal cells (Susic-Jung et al., 2012). The AG growth and maturation continue to some degree until six days after eclosion (Ruhmann et al., 2016), though male flies are largely capable of productive matings after only one day. In insects, there is no evidence of androgen hormonal pathways. Still, AG maturation is under hormonal control, where juvenile hormone (JH) has been shown to be important for its development (Simmons et al., 2014; Schemshedini et al., 1990; Wilson et al., 2003; Kubli, 2003).

How the two secretory cell types of the gland are specified is still unknown however, a number of different transcription factors are differentially expressed in the two cell types (Bertram et al., 1992). For example, the Pax gene *prd* and the transcriptional repressor Defective proventriculus (Dve) are two genes whose cell-type specific expression changes in

the two cell types. *prd* is a *pair-rule* segmentation gene required for a proper segmentation, post-embryonic viability and male fertility (Xue & Noll, 2002). *Prd* also is required for the development of the AGs and for the expression of some AG products (Xue & Noll, 2002). In adults, its expression is dynamic depending on the age and sexual activity of the male. Prd is present in the nuclei of both MCs and SCs in newly eclosed, virgin male AGs. If males are not allowed to mate, then *prd* expression dramatically decreases until it only remains detectable in SC nuclei by ten days. Mating of the males induces *prd* expression in both MCs and SCs (Xue & Noll, 2002).

Dve is another transcription factor that is found in both the main and secondary cell types. *dve* expression starts twenty-four hours APF in both cell types. After forty-eight hours, however, its expression becomes strongly restricted to the SCs. The temporal regulation of *dve* expression in the MCs is known to require Prd (Minami et al., 2012), linking the two transcription factors in MC development. Loss of *dve* has confirmed its importance in the development of both of these cells types as mutants in *dve* show morphological changes in the MCs and the loss of the secondary cell lineage. Both *prd* and *dve* mutants also show defects in male fertility, highlighting the importance of these factors and the AGs in male fertility (Xue & Noll, 2002; Minami et al., 2012).

The role of the MCs in male fecundity and fertility is well established. These cells are the production sites of most of the Acps that have been shown to play a role in the female PMR, such as the Sex-peptide (SP) (Wolfner 1997; Kalb et al., 1993). Recent data from our laboratory and H. Nakagoshi's group have shown that the SCs are also required for male fertility and fecundity. Besides the work on *dve* (Minami et al., 2012), we have found that the *Hox* gene *Abdominal-B* (*AbdB*) (Gligorov et al., 2013) and a male-specific, long-non-

coding RNA (*msa*) are required for SC development and function (Maeda et al. (in preparation)).

3) Seminal fluid components and accessory gland products

The seminal plasma regulates the fertilizing capacity of the sperm via a number of different actions in both mammals and insects. Among the different effects are: providing energy for sperm motility, protecting against the hostile environment of the female genital tract (Thomson & Marker, 2006; Simmons et al., 2014), stimulating a female immune response (that in mammals seems to be important for increasing pregnancy rates) (Bromfield et al., 2014; Bromfield, 2016), modifying ovulation rates (Koene, 2016; Herndon & Wolfner, 1995; Chapman et al., 2001) and changing the female genital tract morphology to increase sperm performance (England et al., 2013; Wong et al., 2008; Adams & Wolfner, 2006). Although many seminal fluid proteins (Sfps) display a high rate of evolution (thought to play a role in speciation in insects) (Almeida & DeSalle, 2016; Meslin et al., 2015), some classes of Sfps are quite well conserved from insects to humans (proteases, anti-microbial, proteases inhibitors, sperm-binding proteins, anti-oxidants) (Dean et al., 2011; Findlay et al., 2008; Rubinstein & Wolfner, 2014; Simmons et al., 2014).

a. The different classes of accessory glands products

One class of seminal fluid proteins that is highly implicated in fertility in many species is that of the proteases. For example, the human prostatic epithelium secretes serine proteases like the kallikreins (KLKs). The kallikreins are a group of 15 serine proteases that includes KLK3 (prostate-specific antigen (PSA)), whose presence in serum is used as an early

indicator of prostate cancer. Proteolytic activation of some of the 10 prostate produced family members has been shown to occur within the female genital tract (Pampalakis & Sotiropoulou, 2007; Kryza et al., 2016; Verze et al., 2016) where it is thought to help liquify the seminal fluid to increase sperm motility. In *Caenorabditis elegans* and some insects, serine proteases have been shown to be important for sperm activation (Smith & Stanfield, 2012; Simmons et al., 2014). Meanwhile in *Drosophila*, it has been shown that 25% of the Sfps are proteases or proteolytic regulators (LaFlamme & Wolfner, 2013; Findlay et al., 2008). Though the function of most of these proteolytic reactions is still unknown, some have been shown to be involved in regulating different processes in reproduction. For example, CG11864, an Sfp predicted to be an astacin-type metalloprotease has been shown to be involved in the cleavage of Ovulin, a hormone that stimulates ovulation, and Acp36DE, a molecule implicated in initiating sperm storage (LaFlamme & Wolfner, 2013; Ravi Ram et al., 2006). Interestingly, although this protein is made in the male, it does not seem to be active until it is placed within the female reproductive tract, where is cleaves its targets. It turns out that forty per cent of the *Drosophila* accessory gland proteins (Acps) are secreted as pro-hormones, whose processing/cleavage occurs within the female genital tract (Ravi-Ram et al., 2006; LaFlamme & Wolfner, 2013; Rubinstein & Wolfner, 2014). The large number of pro-hormones hints that proteases should play a very prominent role in regulating male fertility.

Other seminal fluid components have anti-microbial functions or somehow interact with the female immune system. In mammals, it has been shown that the cytokine inducing protein, TGF-β, is a component of the seminal fluid (Bromflield, 2016; Gutsche et al., 2003, Sharkey et al., 2012) and that activation of the inflammation response aids in embryo implantation probably by deadening the immune response to paternal antigens (Bromfield

et al., 2016). Interestingly, a similar decrease in female immune reactivity is seen in many other animals, including *Drosophila* (Fedorka et al., 2007; Short & Lazzaro, 2010, Short et al., 2013) and bumble-bees (Korner & Schmid-Hempel, 2003). In *Drosophila*, much of this immune-suppression seems to be due to the Sfp, Sex-peptide (SP), though other seminal fluid components also seem to be involved in the process as well (Short et al., 2013). Although the *Drosophila* TGF-β homologue, *dpp*, is expressed in the fly AGs, so far, no evidence has linked this molecule to immuno-suppression in the fly (Redhai et al., 2016).

Ions too make up an important part of the seminal fluid. It turns out that prostate cells take up more Zn^{2+} than any other tissue in the human body. It is therefore no surprise that Zn^{2+}concentration is elevated in seminal plasma and that lower levels of Zn^{2+} have been correlated with lower fertility (Zhao et al., 2016). The Zn^{2+} ion is imported by transporters, ZIP 1-4, into the cytoplasm of the prostatic epithelial cells. It is then sequestered in an androgen-dependant manner into intra-cellular vesicles and then redistributed by Zn^{2+} exporters, ZnT1-10. The prostatic epithelium then regulates its release toward the seminal fluid where it is known to aid in sperm release, motility and survival (Khelleher et al., 2011; Verze et al., 2016). Each lobe/zone of the prostate varies in Zn^{2+} content; the dorsal lobe (DL) and peripheral zone (PZ) of the rat and human prostates, respectively, are the most enriched in Zn^{2+} and are involved in prostatic fluid secretion (Khelleher et al., 2011). Within the epithelium, the distribution and the expression of the Zn^{2+} transporters change depending on the cell polarity, on the lobe/zone identity and on sexual maturity. ZIP1 is present on the baso-lateral membranes to uptake Zn^{2+} from the circulatory system, while ZIP2-4 are present on the apical surface to uptake Zn^{2+} from the seminal fluid. Although ZnT1 decreases with age, ZnT2 increases in the anterior lobe of the rat prostate and remains constant in the other lobes after sexual maturity. As the organism ages, however, the

prostatic and seminal fluids levels of Zn^{2+} tend to decrease, which correlates to a decrease in male fertility (Khelleher et al., 2011). Recently, transcriptome data from *Drosophila* AGs suggest that SCs express high levels of a Zn^{2+} permease, *fear-of-intimacy* (*foi* also called ZIP6), as well as other transporters like the Zn^{2+} exporter, ZnT1 (also called ZnT63C), and the Zn^{2+} importer, ZIP3 (also called Zip89B) (Gligorov et al., in preparation). The role for Zn^{2+} in *Drosophila* is still largely unknown, though many proteases, like CG11864 are thought to be Zn^{2+}-dependent metalloproteases (Mueller et al., 2004).

Other categories of proteins known to be secreted by the prostate and *Drosophila* AGs are the sperm binding proteins, CRISPs (Koppers et al., 2010, Findlay et al., 2008; Sitnik et al., 2016) and mating plug proteins (Bretman et al., 2010; Avila et al., 2015[a,b]; Findlay et al., 2008).

b. The post-mating response

Although it is now clear that the seminal fluid plays in important role in male fertility in most internally fertilizing organisms, this was not the case earlier. Thus, many of the early studies on the importance of seminal fluid in fertility concentrated on insect model organisms. In *Drosophila*, it has been known for some time that mating influences a wide range of responses in the female, both physiology and behavioural (reviewed by Wolfner, 1997). Among these changes are an increase of ovulation and ovipositioning (Chapman et al., 2001), an increase of proteinaceous food intake (change of quantity and quality of food) (Hussain et al., 2016), an ability to store and nourish sperm, a decrease in female innate immunity, a decrease in female lifespan , and a decrease in receptivity to additional matings (reviewed by Avila et al., 2011). The summation of these responses is generally known as the

post-mating response (PMR). Here, I will concentrate on the *Drosophila* PMR and the molecules that mediate these processes.

The *Drosophila* female transcriptome is modified after mating; the expression of 1700 genes changes in the female during the first three hours post-mating. These genes play a role in immunity, metabolism and protein modification. However, a subsequent matings mostly change only the immunity-related genes. Although many molecules contribute to the changes seen during the PMR, it turns out a large number of changes seem to be due to the presence of a single molecule from the seminal fluid, called the Sex-peptide (SP) (Avila et al., 2011).

Sex-peptide

Sex-peptide (SP; Acp70A) is a 36 amino-acids protein that induces a vast number changes in the female, post-mating. For this reason, and the fact that it was discovered and largely characterized in Switzerland, it has often been compared to a Swiss army knife for the *Drosophila* male (Kubli & Bopp, 2012). Like a Swiss army knife, different regions of SP polypeptide chain mediate different responses.

The C-terminal part of SP is probably the most active region of the peptide. It has been shown to be involved in the decreases in female receptivity and siesta sleep, the increases in food intake, ovulation, and ovipositioning and probably mediates much of the SP-induced reduction of female lifespan (for review, Kubli & Bopp, 2012). It has also been shown to be required for the release of stored sperm from the female sperm storage organ (Avila et al., 2010).

The C-terminus of SP is thought to function through a seven-transmembrane domain, G-protein coupled receptor, called the Sex-Peptide Receptor (SPR) (Yapici et al., 2008). It is expressed in the central nervous system (CNS), as well as in the oviduct and the

sperm storage organs. There are still some uncertainties surrounding the SPR and whether or not it is the only receptor for SP (Haussman et al., 2013).

Although SPR is expressed in the oviduct and the sperm storage organs, it now seems that the primary area of SP signalling is in the *fruitless* expressing neurons of the CNS (Kubli & Bopp, 2012). It has been shown that SP binds to the areas where SPR is present in both the female reproductive tract and the CNS (Yapici et al., 2008; Tsuda & Aigaki, 2016). However, there are mutant forms of SP that can bind to the oviduct and the sperm storage organs (locations of SPR localization) but cannot bind to the SPR in the CNS. These mutant forms show no induction of the PMR, suggesting that the CNS is the location of SP action. Furthermore, SP injected directly into the haemolymph of the fly, will induce a PMR in much less time than a regular mating (Ding et al., 2003). If one adds in mutations that increase the permeability of the blood brain boundary of the fly, some PMR responses occur almost instantly (Haussman et al., 2013). Given that, after mating, SP can be found in the haemolymph at concentrations that can induce the PMR, it has been proposed that the CNS is the primary location of SP signalling.

Some of the uncertainties about the SPR come from experiments performed with SPR mutants. It turn out that introducing SP directly into the brain (via a leaky blood brain barrier and SP injection into the haemolymph, or direct expression of SP in the CNS) is able bypass the requirement for SPR in inducing the PMR, though higher concentrations of SP and longer times are required to see the response (Yapici et al., 2008). Thus, it has been proposed that SPR may play a role in the SP response to increase SP efficacy or transport, but that it is not the true receptor for SP (Haussman et al., 2013). In fact, recent work has shown that SPR can also bind a second set of molecules called the myo-inhibitory peptides (MIPs) (Poels et al., 2010). The MIPs have been shown to be involved in sleep control and

odorant/gustatory responses in *Drosophila* (Oh et al., 2014; Hussain et al., 2016). These responses have been shown to require the SPR as well as the MIPs. Interestingly, MIPs do not seem to be able to trigger most the SP dependent responses. Injection of the MIPS cannot mimic a PMR (Poels et al., 2010). The one exception to this is that SP triggers a change in female eating behaviour that is also a response triggered by the MIPs (Hussain et al., 2016). Based on these data, it may be that the SPR does mediate some phenotypes of the PMR, but that it is not the main receptor of SP.

The N-terminal region of SP has other functions. First, this region seems to be important for SP localization to the sperm (Avila et al., 2011). During copulation, SP is transferred to the female in two forms. The first is free floating SP in the seminal fluid and the second is SP that is attached to the sperm tail. The vast majority of SP transferred to the female is in the free floating form. It is thought to trigger the initial large increase of the PMRs during the first few days after mating. But much of this free-floating form disappears during the first 24 hours, post-mating. The female often ejects a lot of the seminal fluid during the first hours after mating and eject more with the laying of the first eggs. Yet the PMR lasts for up to 10 days. This turns out to be due to a second source of SP, one that is attached to the sperm themselves. Female *Drosophila melanogaster*, like many organisms, store sperm after mating and can use these sperm to fertilize eggs for days after their sexual encounter. As the sperm is stored, the female also stores SP. This SP is then slowly cleaved off of the sperm to perpetuate the PMR for several days. These two sources of SP correlate to the phases of the PMR, called the short and long-term PMRs. Mutations that prevent the attachment of SP to the sperm result in females displaying the PMR for only 2-3 days after mating (Ravi Ram et al., 2007; Minami et al., 2012). Proteins known to be involved in this process are the lectins CG1652 and CG1652, the protease inhibitor CG17575 and the

protease CG9997. It is interesting to note that three of these products (all but CG9997) seem to be made by the SCs of the AG and not the MCs (Gligorov et al., 2013; Ravi-Ram & Wolfner, 2009).

The N-terminal part of SP also seems to be important for the induction of the *Drosophila* hormone, juvenile hormone (JH) (Moshitsky et al, 1996). The consequences of this induction of JH can be many and are now just starting to be investigated. For example, it has been shown that females switch their diet after mating to increase protein consumption at the expense of carbohydrates (Hussain et al., 2016). This switch in diet is accompanied by a remodelling of the gut to better digest this new source of nutrients. Recently, this has been shown to be due to a burst of JH synthesis in the female that is caused by SP (Reiff et al., 2015). Also caused by this JH increase is the reduction of female pheromone synthesis, which could cause females to become less attractive to potential mates (Bontonou et al., 2015). Finally, it is known that JH release also causes the post-mating reduction in the female immune capacity (Short et al., 2012). Given the number of roles that JH plays during the life of *Drosophila*, these few examples could simply be the tip of the iceberg and more phenotypes may be attributed to JH and the N-terminus of SP. Note that since the N-terminus of SP stays attached to the sperm during the long term-storage, these phenotypes are short-term in nature.

Ovulin

A second molecule derived from the AGs with known biological activity in the PMR is Ovulin (Acp26Aa or mst355a). Ovulin is a 264 amino-acids glycoprotein that increases ovulation rates for 24 hours after mating (Chapman et al., 2001). Ovulin is made as a pro-peptide that is activated as the result of a protease cascade with the serine protease CG10586 cleaving and activating the metallo-protease CG11864, which in turn activates

Ovulin (Ravi Ram et al., 2006; LaFlamme et al., 2012; LaFlamme & Wolfner, 2013). Both of these proteases are constituents of the male seminal fluid but the activation cascade is only activated once these proteins are placed within the female reproductive tract (LaFlamme et al., 2014).

The molecular mechanisms, by which Ovulin induces ovulation is still unclear, as no Ovulin receptor has yet been found. However, the result of Ovulin stimulation is the contraction of the ovarian muscles and the relaxation of the oviduct muscles that allows eggs to be pushed out of the egg chamber. These processes are known to be controlled by octopaminergic (OA) neurons. Indeed, it has been shown that Ovulin signalling requires OA neurons and that Ovulin stimulates the increases in synaptic button formation of OA neurons with the female reproductive tract (Rubinstein & Wolfner, 2013). It is still unclear if these functions are direct, through interaction of Ovulin with the OA neurons present in the female reproductive tract or if Ovulin must signal first through the CNS. Given that some effects of Ovulin happen within 1.5 hours post mating (muscle relaxation), and other require up to 6 hours to manifest (button formation), it is likely that direct, local stimulation and more systemic effects are both important for mediating Ovulin's effect on the PMR (Heifetz & Wolfner, 2004).

II- The role of the secondary cells in the post-mating response

As mentioned earlier, the seminal fluid is made by the male AGs. The level of production of the constituents of the seminal plasma depends on AG age, size, the number of MCs and on mating activity (Santosh & Krishna, 2013). Most of the Sfps are produced by

the MCs (Acp95EF, Ovulin and SP; (Kalb et al., 1993)). Given that the MCs make up 96% of the gland, much is known about how the MC components of the seminal fluid, like SP, affect the PMR (reviewed in Wolfner, 1997). But there is another secretory cell type that makes up the remaining 4% of the gland, called the SCs. Recently, others and we have shown that these cells too play a role in the PMR (Minami et al. 2012; Gligorov et al., 2013; Sitnik et al., 2016). Indeed, we now know that there are Sfps that are SCs-specific (CG1656, CG1652, CG17575; (Gligorov et al., 2013)) or present in both cell-types (Ovulin; (Chapman et al., 2003 & Fig. 7)). Here I will review what we know about the role of the SCs in the PMR.

1) The contribution of both accessory glands secretory cell types

Outside of the early cytological studies (DiBennedeto et al., 1990; Bairati, 1968), very little was known of the SCs until quite recently. This was because very few, SC-specific genes were known. A few years ago, we found that the *Hox* gene, *AbdB*, was expressed in the SCs of the AG and was excluded from the MCs. Because of our lab's long-term interest in *AbdB*, we were able to use our collection of genetic tools to isolate a mutation in the *AbdB cis*-regulatory region that specifically knocked out *AbdB* expression in the SCs, without affecting its expression during early segmental development. The absence of *Abd-B* expression in the SCs led to a major cytological phenotype (loss of the large vacuoles, characteristic of the cell type), as well as a phenotype affecting the PMR. This mutation, $iab6^{cocu}$, when mated to *wild-type* females, caused an initial PMR response, but not an extended PMR. As mentioned above, the PMR can be divided into two phases, the short-term response (ShTR) and the long-term response (LTR). The ShTR is caused by the free floating SP in the seminal fluid, while the LTR is caused by the SP that is bound to the sperm and slowly released from the

female sperm storage organ. The LTR is what allows for the PMR to last for longer than 2-3 days. Thus, mates of *iab6*^{cocu} flies caused a PMR that lasted only 3 days (Gligorov et al., 2013).

The PMRs assayed in these studies were the stimulation of egg laying and loss of receptivity to additional mates. These PMRs are both SP-dependent. But SP is a MC product. How does a mutation affecting SCs have a phenotype on a MC product? The answer to this question is still not perfectly clear. What is known is that there are 4 known Sfps that are required for SP binding to sperm. Two of these products are made in the SCs, the two lectins CG1652 and CG1656. It turns out that both of these products are improperly glycosylated in the *iab6*^{cocu} mutant seminal fluid (Gligorov et al., 2013). What effect this improper glycosylation has on the PMR is still unknown though it suggests that glycosylation of these products, or others, is required to properly attach SP to the sperm.

A third protein that was also found to be improperly glycosylated was Ovulin (Gligorov et al., 2013). Although, this Sfp is present in both cell types at the level of protein, its production site is still unclear (Monsma et al., 1990; Chapman et al., 2003 & Fig. 7). Early antibody staining experiments showed that in one-day post eclosion, virgin males, Ovulin could be seen in both cell types, but that after 5 days it is limited to the large vacuoles of the secondary cells. If the males are mated, then the MC expression restarts. Thus, the protein seems to be made in both cell types, but accumulates in the SC large vacuoles (Monsma et al., 1990). However, RNA-seq studies from our lab show that Ovulin expression in the SCs is actually quite low, but that it is, in fact, one of the highest expressing genes in the AG as a whole (Gligorov unpublished and Sitnik et al., 2016). This is supported by results where ablation of MCs causes loss of ovulin expression in the gland (Kalb et al., 1993). Thus, Ovulin protein is seen in the SCs, but most of its production seems to come from the MCs. This is

interesting because our glycosylation results show that all of the Ovulin protein in the gland is differentially glycosylated in *iab-6*^{cocu} mutants (Gligorov et al., 2013; Sitnik et al., 2016). So, somehow, the effect in the SCs in *iab-6*^{cocu} mutants causes changes in a product primarily made in the MCs. We still do not know how this is accomplished. However, the Ovulin antibody results could also suggest a hypothesis where Ovulin protein may be transported into the SCs during its maturation process.

Besides the PMR defects and the effect on glycosylation, one of the main defects in *iab6*^{cocu} AGs is their loss of the large vacuoles characteristic of SCs. The role of these vacuoles is still known. So far we know of some products that can be seen in these vacuoles including angiotensin-I converting enzyme (ANCE) (Rylett et al., 2007), Ovulin (Monsma et al., 1990; Fig. 7) and the transforming growth factor beta homologue, Dpp (Redhai et al., 2016). The correlation between the loss of vacuoles, the PMR phenotype and the changes in glycosylation suggest that the large vacuoles of the SCs are essential to properly glycosylate Sfps and that these defects cause the PMR phenotypes (Gligorov et al., 2013). As the large vacuoles of the SCs are vesicles, reminiscent of intra-cellular compartments/organelles, we hypothesize that these vacuoles are primarily involved in the transport of molecules. To characterize the SCs in more detail, my project centred on finding a function for these large vacuoles. To do this, we examined the Rab proteins that bind to these intracellular compartments. Interestingly, many Rab proteins have been already linked to the prostate/AGs (Steffan et al., 2014; Corrigan et al., 2014; Redhai et al., 2016) and male fertility (Tiwari & Roy, 2008; Kwon et al., 2014; Redhai et al., 2016). However, before discussing the Rabs directly, I will first introduce the intracellular trafficking pathways. At least three intra-cellular trafficking pathways exist; endocytosis, exocytosis and transcytosis.

2) Intracellular trafficking

a. Endocytosis

For endocytosis, material from the cell surface is internalized and invaginated to join the endocytic compartment; the first endocytic compartment is called the early-endosome (EE). From the EE, the endocytosed cargo is sorted and is forwarded along a number of different routes. It can return to the PM via the recycling pathways. It can be sent to the lysosome for degradation. Or it can join the *trans*-Golgi network (TGN) for resorting and transportation through the secretory pathway (Segev, 2011). To correctly dispatch the different internalized materials, EE forms sub-domains with tubular structures budding of the EE. While molecules localized on the newly synthesized tubular membranes are directed to the PM or the TGN; the molecules remaining in the main body of the EE are targeted for degradation. To get membrane molecules fated to be degraded to the centre of the EE small vesicles are often internalized from the EE membrane and placed into the centre of the EE. This leads to the EEs having some vesicular structure in their core. Besides the difference in membrane morphology, the two sub-domains also differ in pH (Jovic et al., 2010).

To compensate for the membrane components removed by internalization during endocytosis, recycling pathways exist within the cells to return endocytosed material to the PM. It turns out that most of the molecules endocytosed are recycled and only a small fraction joins the degradative pathway. Some components are quickly recycled *ie* directly from EEs to the PM, while others are slowly recycled. The slowly recycled products travel through an EE tubular membrane extensions from which recycling endosomes (REs) sort and are directed toward the PM (Grant & Donaldson, 2009).

Endosomes fated for the degradative pathway will first mature from EEs into late-endosomes (LEs). Unlike the EE, which often displays tubular protrusions, the LE is mostly round and often contains internal vesicle, like the centre of the EE. From the LE, some components can still be recycled to the PM. Those components not recycled can then follow the LE as it fused to the lysosome where the acidic environment and the digestive enzymes can degrade the internal components (Huotari & Helenius, 2011).

b. Exocytosis

Newly synthesized proteins intended for secretion are co-translationally made in the rough endoplasmic reticulum (ER). From the ER, the proteins are placed into vesicles that fuse with the Golgi apparatus. The Golgi apparatus is a membranous organelle present in all Eukaryotic cells. This compartment is made of several cisternae (flattened disc-like structures) divided into three zones: *cis*-, medial- and *trans*-Golgi. Each Golgi cisternae region contains characteristic sets of enzymes that allow it to post-translationally modify proteins contained within it (glycosylation, sulfation, phosphorylation…). The modifications that the protein receives often determine its fate and final destination. From the *cis*-Golgi, the molecules are generally moved towards the *trans* face of the Golgi (via the medial cisterna) through multiple vesicle budding and fusion reactions. In the *trans*-Golgi, the products can be further modified and are then sorted for delivery to other intracellular compartments or for secretion at the PM (Segev, 2011; Papanikou & Glick, 2014). As mentioned above, the TGN also can receive material from the endocytic pathway to be rerouted to the PM or toward other organelles/compartments such as the lysosomes (from British Society for Cell Biology).

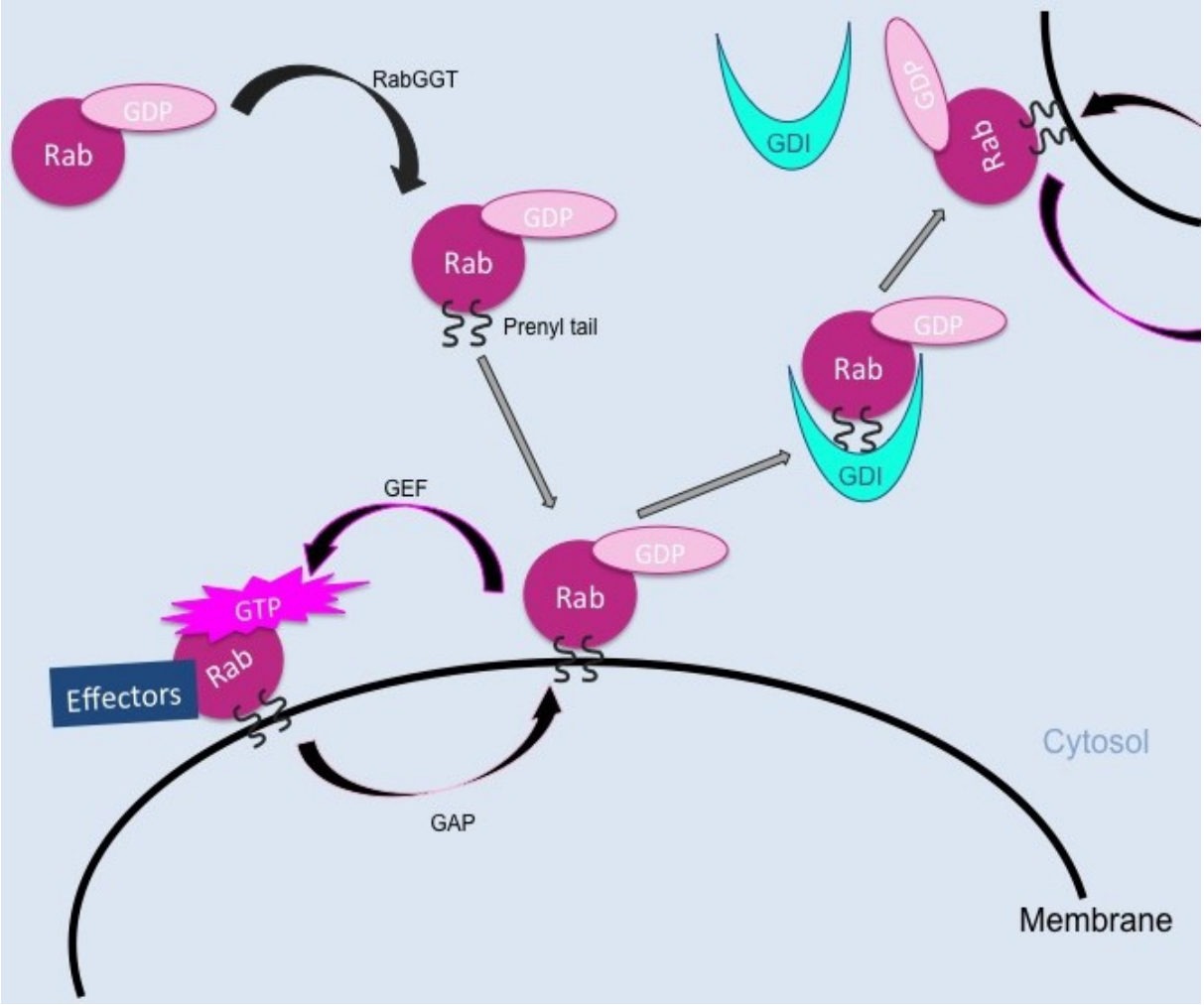
c. Transcytosis

Transcytosis is an intra-cellular pathway allowing transporting products from one side of the cell to the other side. This pathway is mostly used in cells with an apico-basal polarity (Bhuin & Roy, 2014). In many instances, transcytosis resembles endocytosis with recycling to the PM except that the PM to which the products are recycled is on different sides of the cell. In these instances, products are taken up from one side of the cell as endosomes, fuse to the EE, move to the LE or recycling endosomal compartment, and then are secreted to a different cell surface. In other cases, such as transport across the blood brain barrier, vesicles simply take products from one side of the cell and move across the cell to fuse with the PM at a different location (De Bock et al., 2016).

d. The Rabs

The Rab proteins are monomeric, lipid-modified GTPases, largely conserved from yeast to human. They cycle between an inactive GDP-bound form and an active GTP-bound form. The newly synthesized Rab proteins are in a stable GDP-bound complex in the cytosol. They soon become membrane associated through the action of a Rab geranylgeranyl transferase (RagGGT), that attaches two prenyl tails that allows it to associate with its target membrane. Once stimulated by donor compartments, the Rab exchanges its GDP molecule for a GTP molecule through the action of a GTP exchange factor (GEF). In the GTP bound form, the Rab can bind to different effector molecules to perform its function (vesicle targeting, fusion, budding, coat association…). When the GTP is hydrolysed though the GTPase activity of the Rab and its GTPase activating protein cofactors (GAPs), the GDP-Rab is released into the cytosol by the GDP dissociation inhibitor (GDI). This “steady complex” GDP-Rab-GDI can then be reactivated by targeting the Rab once again to its appropriate

membrane (reviewed in Seabra et al., 2002; Stein et al., 2003; Mitra et al., 2011; Stenmark, 2009; Hutagalung & Novik, 2013; Bhuin & Roy, 2014). The Figure 3, just below, depicts the cycle of activation of the Rabs as described above.



<u>Figure 3.</u> The Rab proteins cycle between a cytosolic inactive GDP-bound form and a membrane-anchored active GTP-bound form.
Schematic representation of the cycle of activation of the Rab proteins as described above. The newly synthesized cytosoplic Rab protein (dark-pink circle, on the top left) is GDP-bound (light-pink circle) and dually prenylated in the cytosol by a Rab geranylgeranyl transferase (RabGGT). This form will join and anchor to the membrane of the targeted compartment. Through the action of the GTP Exchange Factor (GEF), the GDP is changed in GTP (magenta star); this active form is able to interact with different effector proteins (blue rectangle) to achieve its function. Then the Rab interacts with its associated GTPase activating protein (GAP) leading to the hydrolysis of the GTP to GDP. The GDP- Rab is then taken in charge by a guanine dissociation inhibitor (GDI) and this complex (GDI-Rab-GDP) can be targeted to another donor compartment (on the top right).

Many Rab proteins are associated with specific transport pathways. For example, Rabs 1, 2, 6 and 8 are primarily associated with exocytosis while Rabs 4, 5, 7 and 11 are characteristic of the endocytic and recycling pathways (Fig. 4). Within these pathways the different Rabs bind to specific types of vesicles or compartments. Rab5 is an EE-marker, Rab7 is present on the LEs and lysosomes, Rab11 mostly marks the REs and/or exocytic compartments; Rab6 generally marks the Golgi. Figure 4 summarizes the compartments marked by the different Rab proteins (Fig. 4).

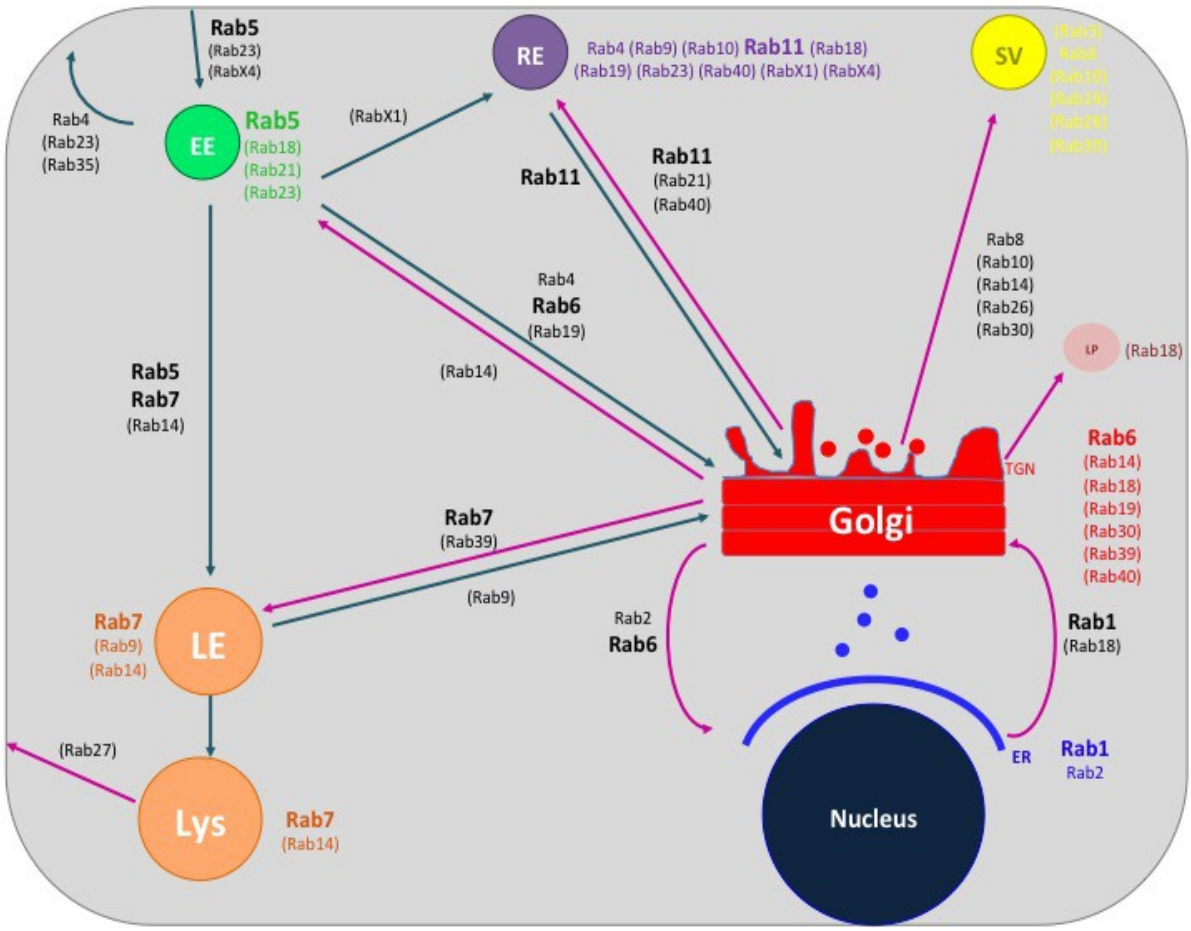


Figure 4. The Rab GTPases mark specific intra-cellular trafficking steps.
Schematic representation of a non-polarized cell (in grey) where the different core compartments for intra-cellular trafficking are depicted; an early-endosome (EE, light-green), a recycling-endosome (RE, purple), a secretory vesicle (SV, yellow), a late-endosome (LE, orange), the Golgi apparatus in red composed of three cisternae and the *trans*-Golgi network (TGN), a lipid droplet (LP, light-pink), a nucleus and the endoplasmic reticulum (ER) in blue. The Rab proteins written in bold are the core Rabs (Rab5, Rab6, Rab7 and Rab11). The Rabs can be present on different compartments (colored Rabs) and mediate different transport steps (black Rabs). The arrows represent the exocytic (magenta) and the endocytic/recycling (dark-turquoise) pathways. The non-canonical Rabs are between parentheses

Because the Rabs often mark similar types of compartments in different tissues, knowing the Rab that marks a compartment can give scientists an idea as to their function. In *Drosophila* AGs, some large vacuoles of the SCs have already been identified as lysosomal acidic compartments (Rab7-positive) and secretory vesicles (Rab11-positive) (Corrigan et al., 2014) but to better understand how the AGs function and how the different parts (lumen, SCs and MCs) communicate between each other we decided to go further by screening a large fly library of endogenously YFP-tagged Rab proteins (Dunst et al., 2015).

Based on genomic sequences, there are 33 Drosophila *rab* genes present in one single copy, but only 27 are apparently expressed (Chan et al., 2011; Zhang et al., 2007; Dunst et al., 2015). The Rab proteins are evolutionary conserved from yeast to human, but not all Rabs exist in every species. For example, there are six Rabs that exist in all sequenced *Drosophila* species that are absent from the genomes of mice and humans (Rabs X1-6). These six Rabs share the genomic characteristic of being composed of only one protein-coding exon whereas the other Rabs have multiple coding exons (Pesgraves, 2005).

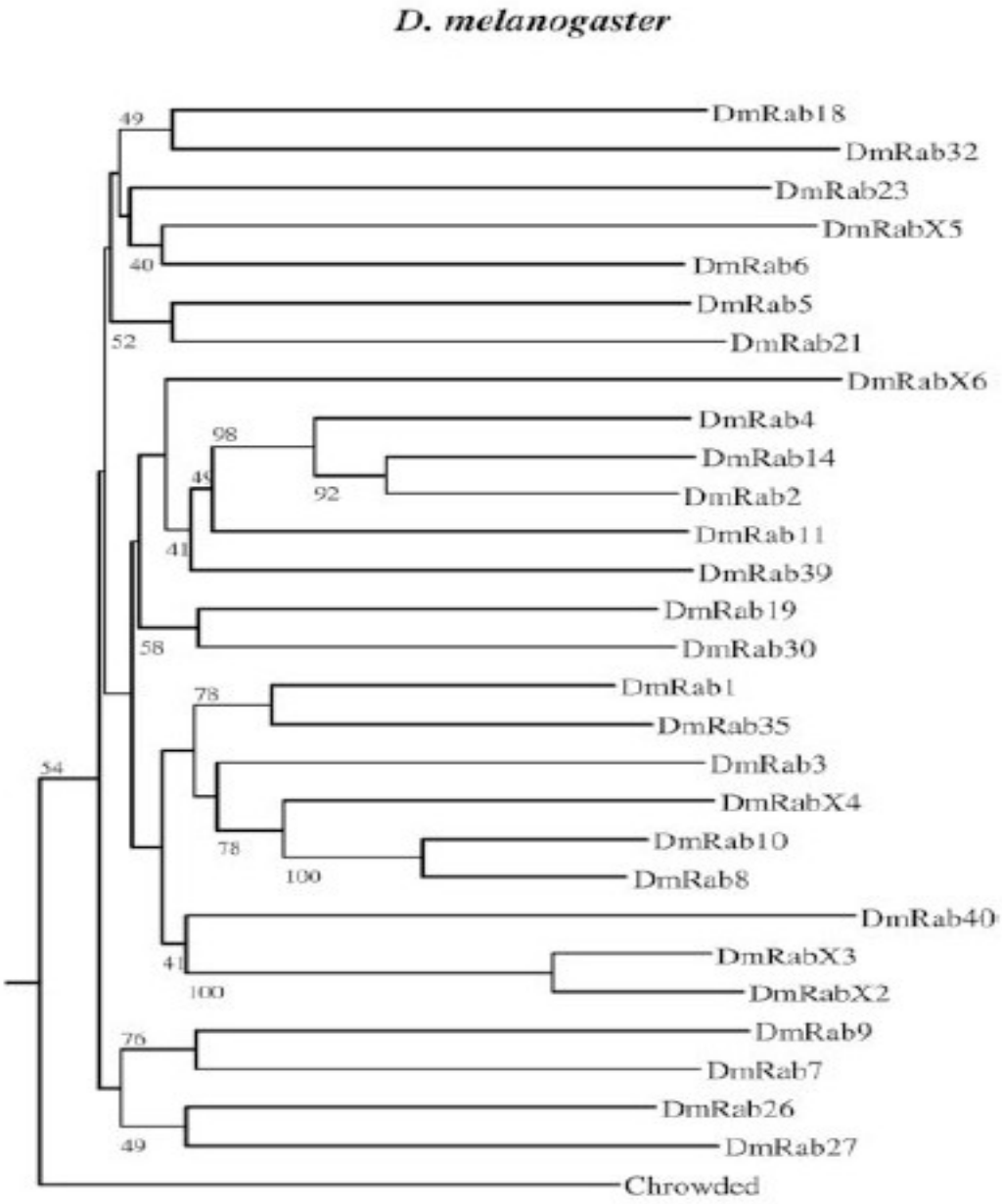


Figure 5. Evolution of the Rab proteins (Pereira Leal & Seabra, 2001)
Phylogenetic tree of the 27 *Drosophila* Rab proteins studied (from Pereira Leal & Seabra, 2001). The numbers on the branches represent the the divergence between proteins .

Among the Rab GTPase family, 5 Rabs are thought to be core Rabs, meaning that they are present in almost all eukaryotic cells and regulate essential intra-cellular transport routes (Rab1, Rab5, Rab6, Rab7 and Rab11). Rab1 regulates the transport of newly synthesized proteins from the ER to the Golgi apparatus, Rab5 is the main regulator of early endocytosis, Rab6 mediates most of the routes from, within and to the Golgi apparatus,

Rab7 is a late-endocytic Rab protein and Rab11 is involved in the transport of products toward the PM via REs. Three other Rab proteins can be lost in some cell types but still have a canonical function. Rab2 regulates retrograde transport of cargos from the Golgi to the ER, Rab4 is involved in early-endocytic functions and Rab8 is associated to secretion of molecules toward the PM (Fig. 4). The last nineteen GTPases are called "tissue-specific" Rabs that seem to enhance the Rab repertoire (Fig. 5). They mostly appear in the Metazoans where membrane trafficking became more complex. The specific function for many of these Rabs is still unknown in large part due to their rarity (Lütcke et al., 1995; Dunst et al., 2015; Zhang et al., 2007; Fig. 4). For example, some Rabs are specifically expressed in *Drosophila* central nervous system (CNS) and other neurons like Rab3, Rab26, Rab27 and RabX4 (Harris & Littleton, 2011; Chan et al., 2011; Dunst et al., 2015). Thus, characterization of these tissue-specific Metazoan Rabs, may lead to canonical functions for these less used Rab proteins. Unfortunately, finding functions for these uncharacterized Rabs is difficult as over-expressing tagged Rab proteins has shown artifactual phenotypes, such as aggregate formation, morphological changes in organelles and disturbances in trafficking pathways (Chan et al., 2011; Leiblich et al., 2012). Therefore, to study the Rabs, one needs to examine them in their normal, cellular environment.

3) Review of Rab-YFP^{Myc} expression patterns in *Drosophila* tissues

To study the Rabs in their native environment, the laboratory of Susan Eaton generated a Drosophila library where the 27 *Drosophila* rab genes have been knocked-in as N-terminal YFP^{Myc} tagged Rabs (YRabs); thus the Rab proteins can be observed at their endogenous levels of expression in their endogenous tissues (Dunst et al., 2015). Using

these lines, they generated a Rab database FLYtRab, where they list the distribution and the sub-cellular location of the Rabs within different types of *Drosophila* tissues. They also aimed at establishing a link between cell polarity and the Rab distribution by comparing YRab expression in polarized and unpolarized cells. As I made use of their library and their work provides an excellent reference with which to compare the analysis of my work, I will review the findings of the S. Eaton's laboratory in their investigation of the Rabs in the multiple tissues of the developing fly.

In their work, S. Dunst and collaborators wanted to examine intracellular trafficking in multiple polarized tissues as well as some unpolarized tissues to determine if the secretion apparatuses change. They examined multiple polarized epithelial cells like the neurons from the CNS, the salivary gland cells (SG), the ovary follicular epithelia cells (OFE) and the wing disc cells (WD). Finally, they also examined the unpolarized cells of the *Drosophila* fat body (FB). For OFE and WD, different developmental stages were observed: early and late. At early-stages, OFE cells are cuboidal and interact with germ cells via their apical surface. Meanwhile at later stages, these epithelial cells become columnar, squamous and contact the oocyte. The WD cells contact the lumen via their apical side and the haemolymph on the basal side. From the third larval instar however, the cuboidal cells of the WD become pseudo-stratified on one side and squamous on the other one.

Canonical exocytic Rabs

Rab1 and Rab2 are ubiquitous Rabs that regulate trafficking between the ER and the Golgi apparatus (Fig. 4). Both of them are present in the cell body and the neuropil of the neurons, with YRab1 being more enriched in the cell body. Their pattern of expression is stable in the neurons through differentiation, but varies with age in the other tissues. In the

other tissues examined, YRab1 and YRab2 start off as uniformly distributed, but as the cells age, these Rabs start to localize to one side of the cell. For example, in the OFE YRab1 and YRab2 become basally localized after stage 9 of oogenesis. This happens at the time when the OFE cells start to rearrange their cytoskeleton, move towards the posterior of the egg chamber and increase their contact with the oocyte. A similar basal or basal-medial localization is seen in the SG, though YRab1 is often present on larger features than YRab2 in the later stages and is more highly expressed. The opposite localization seems to happen in the WD, where both Rabs seem to localize to junctions on the apical side. This change in localization, much likes those found in the other tissues correlates with physical changes occurring in the cell. In the case of the WD cells, these changes coincide with the WD cells of the disc proper (the cells becoming the actual wing) becoming pseudostratified. Interestingly, as part of the system that transports to and from the ER and Golgi, one might expect these Rabs to be present near the nuclei. In the case of the WD cells, this does not seem to be the case as the nuclei are often found more centrally in these cells.

Another core Rab canonically associated with the secretion machinery, Rab6, has been shown to associate with the Golgi in number of tissues (Bhuin & Roy, 2014; Liu & Storrie, 2015). Perhaps as expected, YRab6 shows a similar pattern of localization to YRab1 and YRab2 in most of the tissues tested. The one exception being in the WD where, although it is enriched one apical surface, it is also present at lower levels throughout the entire cell body within the cells of the pseudostratified layer.

Rab8 is normally associated with secretory vesicle transport of molecules sorting from the TGN to the PM or REs. It is uniformly distributed in the neurons and its pattern is not affected by neuronal differentiation. In the other tissues, YRab8 follows a pattern similar

to the other canonical secretory Rabs though it is localized basally in the WD cells prior to pseudostratification, upon which it reverses its polarization.

The placement of these Rabs should indicate the direction of the basic secretory machinery in these cells. It would therefore follow that in the OFE and SG, much of the secretory pathway is focused towards the baso-lateral side of the cells. This, however, seems at odds with the known function of these cells. The SG secretes large amounts of glue protein to aid in puparation. These proteins are stored in the lumen of the gland, which is formed by the apical surfaces of the cells. In the WD as well, there may be some oddities as the secretory machinery seems to be focused towards the apical side of the cell, but it is known that a number of important signalling factors, like Wg and Hh, are secreted towards the basal side of the cell (Strigini & Cohen, 2000; Callejo et al., 2011). Thus, it seems likely that in these cells, other Rabs might take up the role of secretory Rabs to complement or enhance there ability of these cells to increase the polarized secretion of molecules.

Rab canonically associated to endocytosis and recycling

Rab5 is a core endocytic protein mainly known for marking EEs (Bhuin & Roy, 2014; Harris & Littleton, 2011). Within the cell types examined, YRab5 localization varies remarkably. For example, YRab5 is uniformly expressed in the neurons and SG suggested that endocytic internalization occur all along the neurons, but is apical in the WD. While Rab5 pattern does not seem to change overtime in the WD, it goes from apically localized in the early OFE to uniform in the late OFE. Thus, YRab5 localization is dynamic, probably reflecting the need for endocytosis at different places at different times.

YRab4 expression differs somewhat from YRab5. Rab4 is generally associated with the transport of proteins away from the EE to the RE or towards to the PM for fast recycling

in *Drosophila* and mammals (Bhuin & Roy, 2014; Mohrmann et al., 2002; Gillingham, Sinka et al., 2014). Because of the linked functions, Rab4 often localizes with Rab5. This can be seen in some tissues examined by Dunst et al., (2015); for example, in the neurons both Rabs are uniformly distributed. Likewise in the OFE, Rab4 and Rab5 show similar dynamics, where they both start out apically localized but later lose this polarized localization at oogenesis stage 9. However in the SG, YRab4 is basally located, while YRab5 is not. This is in contrast to the early WD where YRab5 is localized to the apical side of the cell and YRab4 is not. In later pseudostratified, WD cells, however, both Rabs are localized to the apical surface facing the disc lumen. Thus, although the functions of Rabs 4 and 5 are often linked, they do seem to perform more specialized functions that require them to be at least partially separated sometimes.

Rab7 is a core Rab that is known to mark the late-endosome (Bhuin & Roy, 2014; Harris & Littleton, 2011) accepting vesicles from the EE marked by Rab5. Its distribution highly resembles that of YRab4 with the exception of being localized to the baso-medial portion of the cell in the late OFE, while YRab4 is expressed more uniformly. The location of Rab5 and Rab7 are thought of as a reflection of high endocytic activity (Marois et al., 2006). YRab5 and YRab7 are enriched at the contact zone between the follicular epithelium and the germ cells and at the apical face of the pseudostratified WD cells, suggesting that these are locations of high endocytic activity.

Where Rab7 marks the late endosomes that are often the precursors to degradation in the lysosomes, Rab11 is a core Rab that marks the RE and vesicles trafficking to and from the RE (Bhuin and Roy, 2011; Bhuin & Roy, 2014; Kessler et al., 2012 & Fig. 4). In the CNS and OFE, YRab11 localization looks much like that of YRab4 and YRab5. However in the SG and the WD the patterns are quite different. In the SG, YRab11 expression is polarized

towards the apical surface with lower levels of expressed more basally. This is very different from YRab4, which is stickily basal and the YRab5, which is uniformly expressed. In the WD, YRab11 shows a strange bipolar localization in the pseudostratified cells. This observation supports the idea that Rab11 has pleiotropic function during wing development as recycling, exocytosis and transcytosis of products, is required to maintain the cell shape and the cytoskeleton (Bhuin & Roy, 2011; Yamazaki et al., 2016). Examining the non-polarized cells of the FB cells also highlights some peculiarities in endocytic Rabs. Although Rab4 is a Rab that is almost always associated with export from the EE, it is actually not a core Rab. In instances where Rab4 is absent, it is often thought that Rab11 might take over its role (Woichansky et al., 2016). YRab11 is uniformly distributed within the cytoplasm of FB cells but there are some cortical punctate. It may be that the recycling of products from these un-polarized cells uses the core Rab, Rab11 rather than Rab4 for the recycling of molecules (Bhuin & Roy, 2011). Perhaps in un-polarized cells, the core Rab are used to fullfill the "basic" intra-cellular transport steps.

The Metazoan and/or "tissue-specific" Rab proteins

By examining the localization of the Rabs with highly characterized roles in intracellular trafficking, Dunst et al. could localize the likely places in cells where the different trafficking events occur. However, this led to some strange findings. For example, as mentioned earlier, the SG is thought to secrete the majority of its secretory output into the lumen surrounded by the apical faces of the SG cells. Yet, according to the localization of key secretory Rabs, like Rab8, most of the secreted molecules should be sent to the baso-lateral sides of the cells. As there are many Rabs that are expressed in a tissue specific manner and many Rabs seem to be able to perform more than one function, it seems likely

that one of the tissue specific Rabs may be used in highly polarized secretory cells for polarized secretion.

Based on published work, Rab10 would be one candidate. Based on sequence comparisons, Rab10 is closely related to the secretory Rab8 (Zhang et al., 2007 & Fig. 5) and has previously been shown to be involved in a number of processes in different cell types, including exocytosis (Huntagalung & Novick, 2011; Mitra et al., 2011). In particular, Rab10 has been shown to be involved in secretion to the basal surface of cells (Lerner et al., 2013). The results of Dunst et al. (2015) indicate that a role for Rab10 in basal secretion is possible, as YRab10 is localized to the baso-lateral side of the cell in both the SG and late stage OFE cells. As Rab8 is also present in these same places, it seems likely that Rab10 would be at best used redundantly with Rab8 in the secretion pathway. As Rab10 has also been shown to play a redundant function with Rab11 in recycling in the *Drosophila* CNS and trachea, it may not be playing a role in secretion at all (Chan et al., 2011; Jones et al., 2014).

In fact, when we look over the localization of Rabs known to be involved in secretion (for example, Rab3, 26, 27), none of them seem to have the proper pattern of localization to explain the apical secretion seen in the SGs or the basal secretion in the WD. Thus, we must look at less characterized Rabs to potentially fullfill the role. Two potential candidates to accomplish the role of an apical secretion Rab in the SG are Rabs 19 and 30. Rab19 and its closest Rab family relative, Rab30, have largely unknown function (Zhang et al., 2007; Fig. 5). In the literature, some have indicated that Rab19 is associated to Golgi apparatus and has a tissue-specific expression in mice (Sinka et al., 2008; Seabra et al., 2002; Lütcke et al., 1995). Previous studies in the *Drosophila* brain suggest that the over-expressed Rab19 affects recycling like Rab11 (Harris & Littleton, 2011; White et al., 2015). However, Dunst et al. showed that both YRab19 and YRab30 expression in the CNS is enriched in the synapses,

where secretion occurs. Both of these proteins are also enriched on the apical side of the SG. As these are the only two Rabs to be localized to the apical side, it is tempting to hypothesize that these two Rabs could fullfill the role for apical secretion in the SG.

Regarding what Rabs might be involved in basal secretion in WD cells even more unclear. Looking over the basally localized Rabs, we find that only YRab11 is localized to the basal surface, though it is localized bipolarity to both the apical and basal surfaces. Although Rab11 is normally associated with RE, it is possible that it may play a role in basal secretion as Rab11 vesicles do move to the PM and many processes feed into the RE pathway. It is therefore interesting to note that HH molecules made in the WD cells are secreted to the basal side of the membrane. However, this basal secretion is accomplished in a roundabout way. According to Callejo et al., HH protein is actually secreted both apically and basally, but the apical secretion is reinternalized to then be rerouted to the basal surface. This could mean that the initial secretion can be directed to the apical surface (or simply not directed) and that only through the recycling pathway does HH reach its correct location of action. A similar rerouting mechanism may be playing a role in Wg signalling, as Wg too has been shown to be internalized in endosomes on both the apical and baso-lateral surface to control its diffusion (Marois et al., 2006).

Although Dunst et al. (2015) examined all of the *Drosophila* Rab proteins, most of the other Rab proteins showed little or no specific localization with respects to cell polarity. The exceptions to this being the neuronal-specific Rabs and YRabX1. Four YRabs are CNS-specific (YRab3, YRab26, YRab27 and YRabX4). The distribution and the expression levels of these CNS-specific YRabs are increasing during neuronal differentiation. These Rabs are enriched in neuronal projections supporting a previously hypothesized function for these Rabs in vesicle release at synapses in both *Drosophila* and mammals (Harris & Littleton, 2011; Chan

et al., 2011; Zhang et al., 2007; Bhuin & Roy, 2014). Rab3 and Rab27 are secretory-vesicles markers while RabX4 and Rab26 are present on REs in *Drosophila* (Harris & Littleton, 2011; Chan et al., 2011). Rab27 has also been shown to play a role in lytic vesicles release in mammals (Bhuin & Roy, 2014). Given the similar expression profiles of these Rabs, it is perhaps not too surprising to find that these four Rabs are closely related on the evolutionary tree (Fig. 5).

YRabX1 seems to localize like Rab5. Consistent with this, previous data has indicated that RabX1 may be the link between EEs and REs (Chan et al., 2011; Harris & Littleton, 2011; Woichansky et al., 2016). The predicted roles of the Rabs are summarized in the Table 1 (below).

Rabs	CNS	OFE	WD	SG
1	**ER-Golgi Neuronal cell-body**	**ER-Golgi Basal side Polarization with oogenesis**	**ER-Golgi Apical side Polarization with pseudostratification**	**ER-Golgi Basal side**
2	Golgi-ER Uniform	Golgi-ER Basal side Polarization with oogenesis	Golgi-ER Apical side Polarization with pseudostratification	Golgi-ER Basal side
3	Neuronal synaptic release Neurons-enriched	ø	ø	ø
4	Recycling and/or EE-TGN Neuronal cell-body Neuroblast-enriched	Fast recycling to the germ cells Apical side Depolarization with oogenesis	Fast recycling Apical side Polarization with pseudostratification	Fast recycling Basal side
5	**Neuronal endocytosis Uniform Neurons-enriched**	**Endocytosis of germ cells products Apical side Depolarization with oogenesis**	**Endocytosis of luminal products Apical side**	**Endocytosis in a subset of cells**
6	**Golgi-related transport Neuronal cell body**	**Golgi-related transport Basal side Polarization with oogenesis**	**Golgi-related transport Uniform**	**Golgi-related transport Basal side**

7	**Late-endocytic pathway Neuronal cell-body**	**Late-endocytic pathway Apical-to-basal switch during oogenesis**	**Late-endocytic pathway Apical side Polarization with pseudostratification**	**Late-endocytic pathway Basal side**
8	Golgi-PM Uniform	Golgi-PM Basal side Polarization with oogenesis	Golgi-PM Basal-to-apical switch with pseudostratification	Golgi-PM Basal side
9	? Glia-specific	?	?	?
10	?	Golgi-PM Basal side Polarization with oogenesis	?	Golgi-PM Basal side
11	Recycling Uniform	Recycling toward germ cells Apical side Depolarization with oogenesis	Apical & basal recycling Bi-polarization with pseudostratification	Apical & basal recycling
14	ø	?	ø	ø
18	? Neuroblast-enriched	ER-Golgi Basal side Polarization with oogenesis	Golgi-related transport Uniform	ER-Golgi and/or recycling Basal side
19	Synaptic release	Secretion toward the germ cells Apical side Depolarization with oogenesis	?	Apical secretion
21	Neuroblast-enriched	?	Endocytosis Uniform	Endocytosis Uniform
23	Synaptic endocytic fast recycling	Fast recycling to the germ cells Depolarization with oogenesis	Fast recycling before pseudostratification	ø
26	Neuronal synaptic recycling Neurons-enriched	ø	ø	ø
27	Neuronal synaptic release Neurons-enriched	ø	ø	ø
30	Synaptic release	Secretion toward the germ cells Depolarization with oogenesis	?	Apical secretion
32	ø	ø	ø	ø
35	?	Fast endocytic recycling and actin assembly Polarization with oogenesis	Fast recycling Uniform	Apical & basal fast recycling and actin assembly

39	Golgi-LE Neuronal cell-body	Golgi-LE Apical side Depolarization with oogenesis	Golgi-LE Uniform	Golgi-LE Basal side
40	Neuroblast-enriched	?	?	?
X1	Recycling Neuronal cell-body	Recycling to the germ cells Apical side Depolarization with oogenesis	Apical recycling to the lumen	Recycling Uniform
X4	Neuronal synaptic recycling Neurons-enriched	ø	ø	ø
X5	ø	ø	ø	ø
X6	ø	?	?	ø

Table 1. Summary of the predicted functions fulfilled by the Rab proteins in the different *Drosophila* tissues observed by Dunst et al. (2015)

This table depicts what could be their function in view of their distribution, expression levels and known function. The core Rabs are written in bold, the canonical exocytic and endocytic/recycling Rabs are written in magenta and dark-turquoise, respectively; and in brown there are the CNS-specific proteins. Rab32 and RabX5 were undetected in all the tissues observed (ø). Polarization/depolarization means that the Rab proteins acquire/loose their apical- or basal-enriched distribution and uniform is for Rabs uniformly distributed in the cytoplasm of the epithelial cells. The "?" can signify that I do not know if there are used in the cells observed in view of their expression pattern as Rab9 or Rab40 or I do not have any idea of their function as fo RabX6. Abbreviations: CNS, central nervous system; OFE, ovarian follicular epithelium; WD, wing disc; SG, salivary gland; ER, endoplasmic reticulum; EE, early endosome; LE, late endosome; TGN, *trans*-Golgi network; PM, plasma membrane.

III- My PhD project

The *Drosophila* accessory glands are thought to be the functional homolog of the mammalian prostate. The two tissues share a number of similarities, not only in terms of the function they perform, but also in the molecules they express. For example, both tissues secrete Angiotensin-I-converting enzyme, ANCE and TGF-β molecules. Although we understand that AGs are not prostate glands, given the number of similarities, we feel that a better understanding of *Drosophila melanogaster* male accessory glands could shed light on how the prostate and the seminal fluid composition can contribute to male fertility. But even

without the similarity in organs, the processes and strategies used by flies and humans are turning out to be quite similar. As more data becomes available, it seems that processes that were once thought of as insect specific are actually present in many organisms. Indeed, it is now quite clear that non-sperm-related seminal fluid components influencing male reproductive success in most species examined.

During my PhD I have investigated the cell biology of the *Drosophila* AGs. I have shown that the basic shape of the *Drosophila* accessory glands suggests exchanges between the two types of polarized secretory cells. Due to our lab interest in the role of the *Abd-B* transcription factor, I have mostly focused on the secondary cells, which express *Abd-B*. The function of these cells is still poorly understood at a molecular level, but at a physiological level, is known to be important for inducing a sustained PMR. This function in the PMR seems to relate to vesicular transport via the large vacuoles filling their cytoplasm. The screening of the fly YRab library revealed that different sub-populations of vacuoles exist within the secondary cells; these sub-groups can cooperate in or regulate the different trafficking pathways such as exocytosis and endocytosis. Furthermore, using rab-specific RNAi, I have been able to show that many of these vacuoles are interrelated, requiring one another to form in the secondary cells. Thus, the Rab identity of the vacuoles could represent different maturing steps in the life of a compartment whose changes reflect the maturation steps of in the cargo proteins they transport.

Results

I-The accessory glands are the sites of important trafficking networks

1) The cellular organization of the accessory gland epithelium suggests intercellular exchanges between the main and the secondary cells.

The *Drososphila* accessory glands (AGs) or paragonia are part of the adult male genital tract. The glands composed of two sac-like lobes, each made up of a monolayer of secretory cells surrounded by a layer of muscle cells eclosing a central lumen (Fig. 6A-C). Forming during the pupariation, the AGs differentiate from mesodermal cells associated with the male genital disc (Nöthiger et al., 1977; Ahmad & Baker, 2002; Chapman & Wolfner, 1988; Susic-Jung et al., 2012). Among genes required for its specification are the male forms of the sex determination genes and the *pair-rule* gene, *paired* (*prd*), a transcription factor required for AGs development and maturation (Xue & Noll, 2002; Li et al., 2015).

The AGs are the production site of the seminal fluid proteins (Sfps) often called the accessory gland proteins (Acps). In *Drosophila*, it has been shown that some of these proteins promote a Post-Mating-Response (PMR) in mated female, which refers to the changes behaviour and physiology a female undergoes after mating (Wolfner, 1997). In the male, the Sfps are stored in the lumen of the AGs and upon mating, it is expelled from the glands through the action of the muscle layer wrapping and squeezing the AGs. The Sfps joins the sperm (produced by the testes) in the anterior part of the ejaculatory duct and this

semen is transferred into the female genital tract. The AGs and their secretions are required for male fertility (Xue & Noll, 2002).

Mono-nucleated and striated muscle fibres surround the AGs (Susic-Lung et al., 2012). By staining the paragonia with an F-actin probe, I could see the muscle layer wrapping the gland in a mummy-like manner that allows a kind of undulatory squeezing of the glands to mix and/or expel the seminal fluid (Fig.6A-B; Annexe 10A-B).

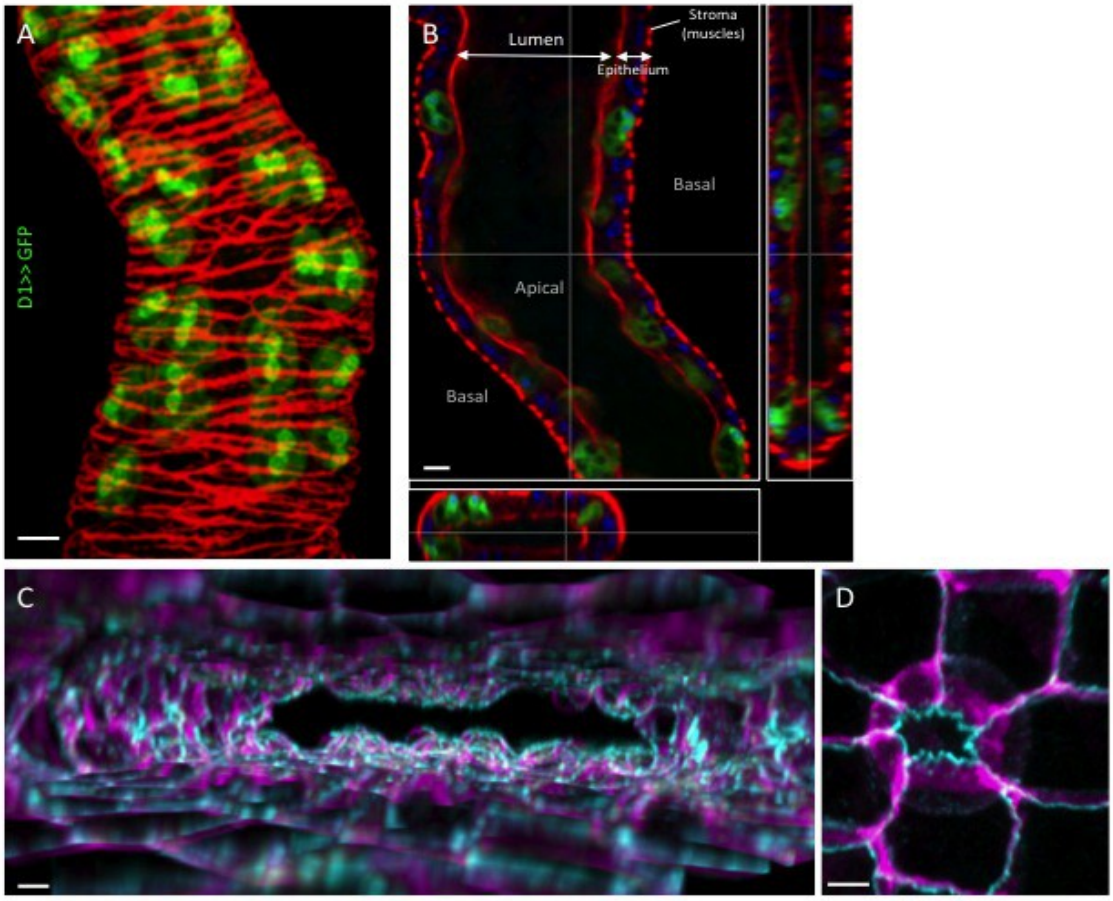


Figure 6. The basic shape of the accessory glands suggests intra- and inter-cellular transport routes.
3D-projection (A) and sagittal view (B) of accessory glands expressing GFP (green) under the control of the secondary-cell-specific *AbdB* enhancer (D1). The glands are stained by an actin probe (Phalloidin, red) and a nuclei marker (DAPI, blue). Apical and basal entitle the lumen and the extra-glandular sides of the glands, respectively.
3D view of the tip (C) and of a secondary cell (D) from accessory glands stained by a baso-lateral membrane marker (DLG; magenta) and an apical junctions marker (DCAD; cyan). Scale bars A & B, 15µm; C, 10µm and D, 5µm.

Structural studies of the AGs by electron microscopy showed that the cells of the glands, in addition to being bi-nucleated and secretory, are also polarized. The are two morphologically different secretory cell types making up the AGs: the main cells (MCs) making up 96% of the tissue and the secondary cells (SCs), located at the tip of the gland interspersed between MCs, making up the remaining 4% of the gland (around forty cells per lobe). The MCs are flatter and hexagonal whereas the SCs are round and filled by large vacuoles (Bairati, 1968; Bertram et al., 1992) (Fig. 6B & 6D).

Very few studies have been performed to understand AGs development and function. Because of this, many questions remain about their cellular organization, their developmental determination, and their tissue organization (the peculiar location in the SCs at the distal part of the gland). To get a better idea about the cellular organization of the cells of the AGs, as well as to better understand their cellular polarity, I used confocal microscopy to examine two cell polarity markers: DE-Cadherin (DCAD) as an apical surface marker and Disc-Large (DLG) as a baso-lateral junction marker (Knust & Leptin, 1996; Woods et al., 1996). Using these markers, I found that the AG epithelium is indeed polarized (Bairati, 1968) with the apical side of the cells facing the lumen and the baso-lateral side facing the muscle layer. The SCs, which are embedded among the MCs at the tip of the gland, actually seem to extrude out the cell layer and into the lumen (Fig. 6C). Interestingly, however, this does not lead to a large lumen exposed surface, as the MCs seem to climb up over the SCs. Thus, the contact surface of the SCs with the luminal fluid is very small while the contact surface with the MCs is large (Fig.6C-D). The large surface of contact between the secondary and the main cells could suggest that the SCs favour exchanges with the MCs rather than secreting materials toward the lumen.

2) The study of the Rab proteins highlights the putative trafficking routes within the accessory glands

Previous studies proposed that the two cell types of the AGs display different mechanisms of secretion. While MCs have been suggested to use merocrinic secretion methods with secretory vesicles fusing to the membrane, it has been suggested that SCs might use a kind of holocrine secretion where the whole cell would release its contents into the lumen (Bairati, 1968; Leiblich et al., 2012). It must be pointed out, however that while the proteins produced by this secretory tissue are relatively well characterized, little is truly known about the cell biology of the two cell types forming the AGs and about their methods of secretion. Thus, we were interested in the large contact surface between the MCs and SCs and if this contact surface was used to exchange material between the two cell types (Fig. 6C-D).

The modification of the Acp, Ovulin may support this hypothesis. Ovulin (also known as Acp26Aa or msP 355a) is an Sfp that is thought to be primarily made in the MCs and is responsible for the peak of ovulation observed shortly after mating (Monsma & Wolfner, 1988; Herndon & Wolfner, 1995). Ovulin protein ejaculated into the female is glycosylated in a specific way (Gligorov, Sitnik et al., 2013). However, this post-translational modification is affected in the mutant, *iab-6*^{cocu} that affects only the SCs (Gligorov, Sitnik et al., 2013). Thus, Ovulin provides evidence of that the two cell types cooperate in the making of some Acps. As glycosylation is generally thought to be an intracellular protein modification event, taking place in the Golgi and ER, it suggests that Ovulin is transported into the SCs to complete its maturation. Supporting this hypothesis, we see Ovulin protein concentrated in some

vacuoles of the SCs; the lumen and basal extracellular space in the MCs using the Ovulin antibody (Fig.7A-B).

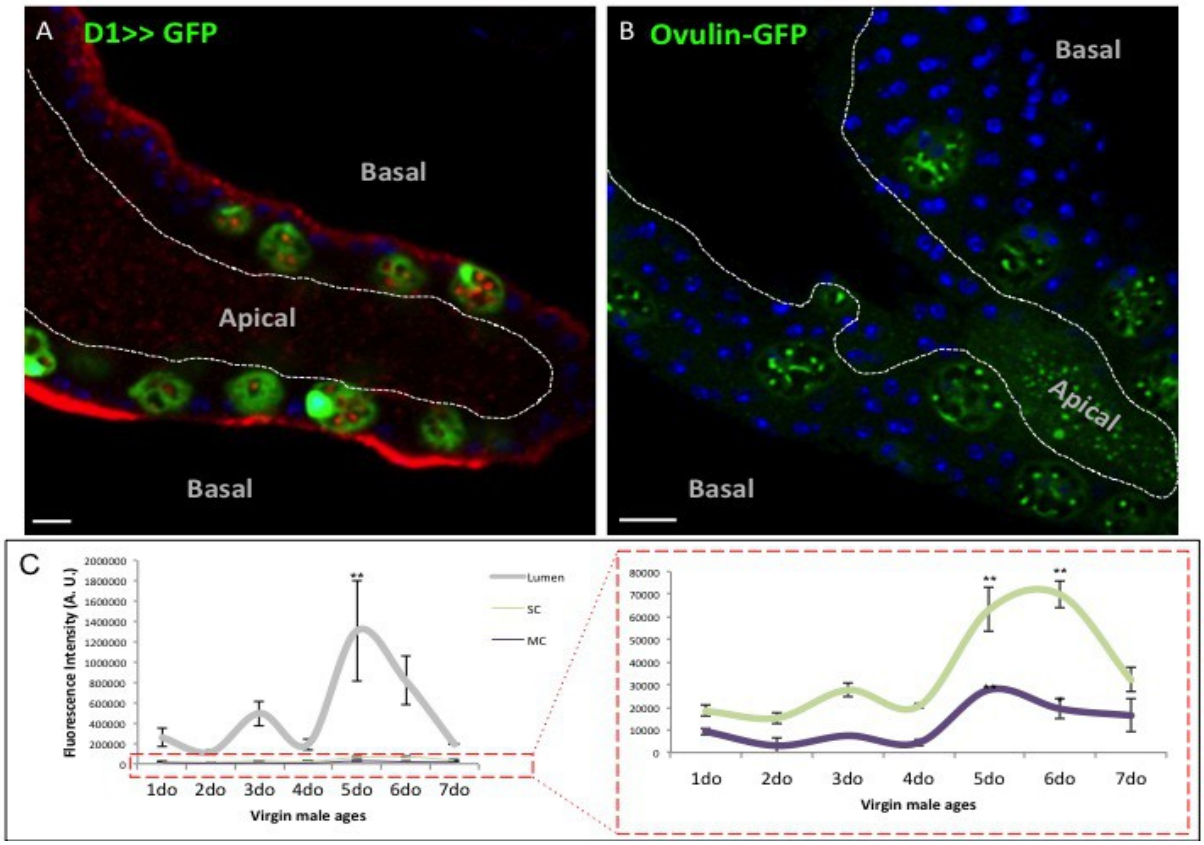


Figure 7. Ovulin is present in the large vacuoles of the secondary cells.
On the top, Z-section of the tip of an accessory gland expressing GFP in a secondary-cell specific manner (green), stained with Ovulin antibody (red) and DAPI (blue) (A). Z-section of an accessory glands expressing GFP under the control of Ovulin promoter (green) and stained with DAPI (blue) (B). The dashed line separates the cell layer from the lumen (apical). The extra-glandular side and the lumen are entitled basal and apical, respectively. Scale bar, 15µm.
On the bottom, expression levels of Ovulin in the main cells (MC, khaki), the secondary cells (SC, lavander) and the lumen (light grey) of accessory glands from virgin males at different ages (1 to 7 days-old). These glands have been fixed and stained with an Ovulin antibody (C). Statistics, p<.05 (*); p<.01 (**); non-significant (ns).

In order to examine the trafficking routes existing within the AGs, we decided to investigate the Rab proteins. Rab proteins are small GTPases controlling membrane trafficking pathways and they generally localize to specific sub-cellular compartments. Thus, they can be useful in identifying specific compartments or fusion events (Fig. 4). Unfortunately, antibodies specific for most of the Rabs do not exist and over-expression of

tagged Rabs has been shown to lead to artifactual phenotypes (Corrigan et al., 2014; Chan et al., 2011). Because of these limitations, we used a collection of strains in which each of the 2 7 *Drosophila* rab genes have been fused at their endogenous locus with *YFP* coding sequence. This rab knock-in library was created by Dunst et al. (2015) and made available to us prior to their publication thanks to the kindness of Drs. Marko Brankatsch and Susan Eaton at the Max Planck Institute of Dresden. Using this *rab* library, we performed a study of the different sub-cellular localization of the YFP-knocked-in Rabs (YRabs) by confocal microscopy. Since the YRab proteins are observed at their endogenous expression levels and have been previously demonstrated as functional by Dunst et al. (2015), I will simply refer these proteins as Rabs. I observed that most of the Rabs, including the five core Rabs (Rab1, Rab5, Rab6, Rab7 and Rab11), are present in both cell types. Two Rabs (Rab14 and Rab19) appear specific for the SCs and a few Rabs are undetectable in the secretory epithelium (Rab3, Rab9, RabX4) or in the whole tissue (Rab23, Rab26, Rab27, Rab30, Rab32, Rab40, RabX5, RabX6) (Table 2; Annexes 1-3 & 12).

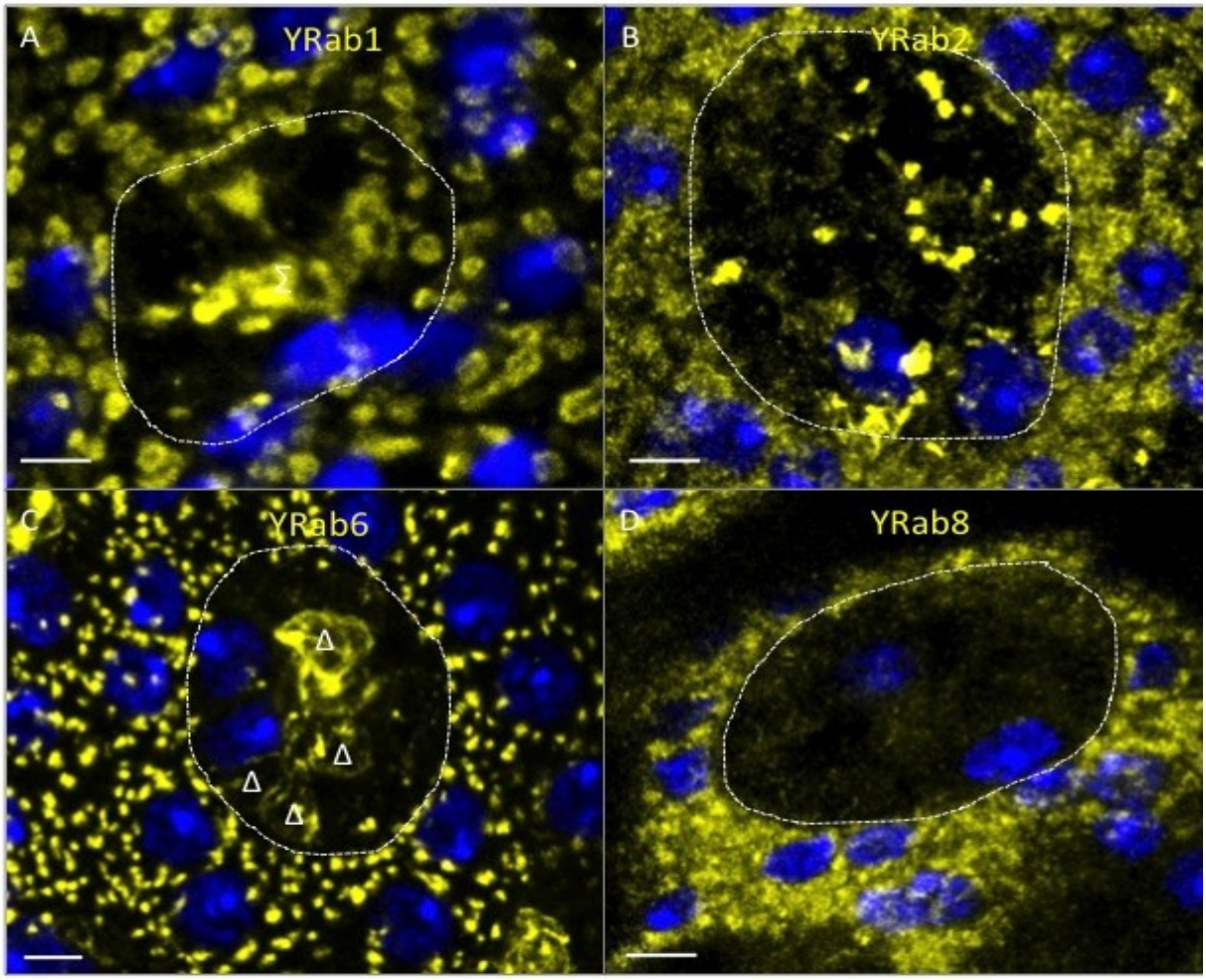
In the following sections I will describe the expression patterns of these Rabs in the AGs (summarized in Table. 2) and catalogue them on the basis of their known function, their associated-compartments and/or their patterns or levels of expression. Different terms are used in this thesis to describe the distribution of the Rabs in the AGs; I use “large vacuoles” to refer to membrane-delimited, large empty-appearing compartments, “small compartments” for features whose intra-vesicular space is not visible; “puncta” for compartments smaller than 1µm of diameter, and “diffuse” for spread and indistinguishable features. When I refer to compartments as being uniformly distributed, I am indicating that they are present homogeneously in the whole cytoplasm and when I say that compartments are cortical or peripheral, I mean at very closed to the cellular membrane. Lastly, I would like

to restate that the basal side of the cells faces the muscle layer while the apical side faces the luminal space of the AGs (Fig. 4B; Annexes 10 & 11).

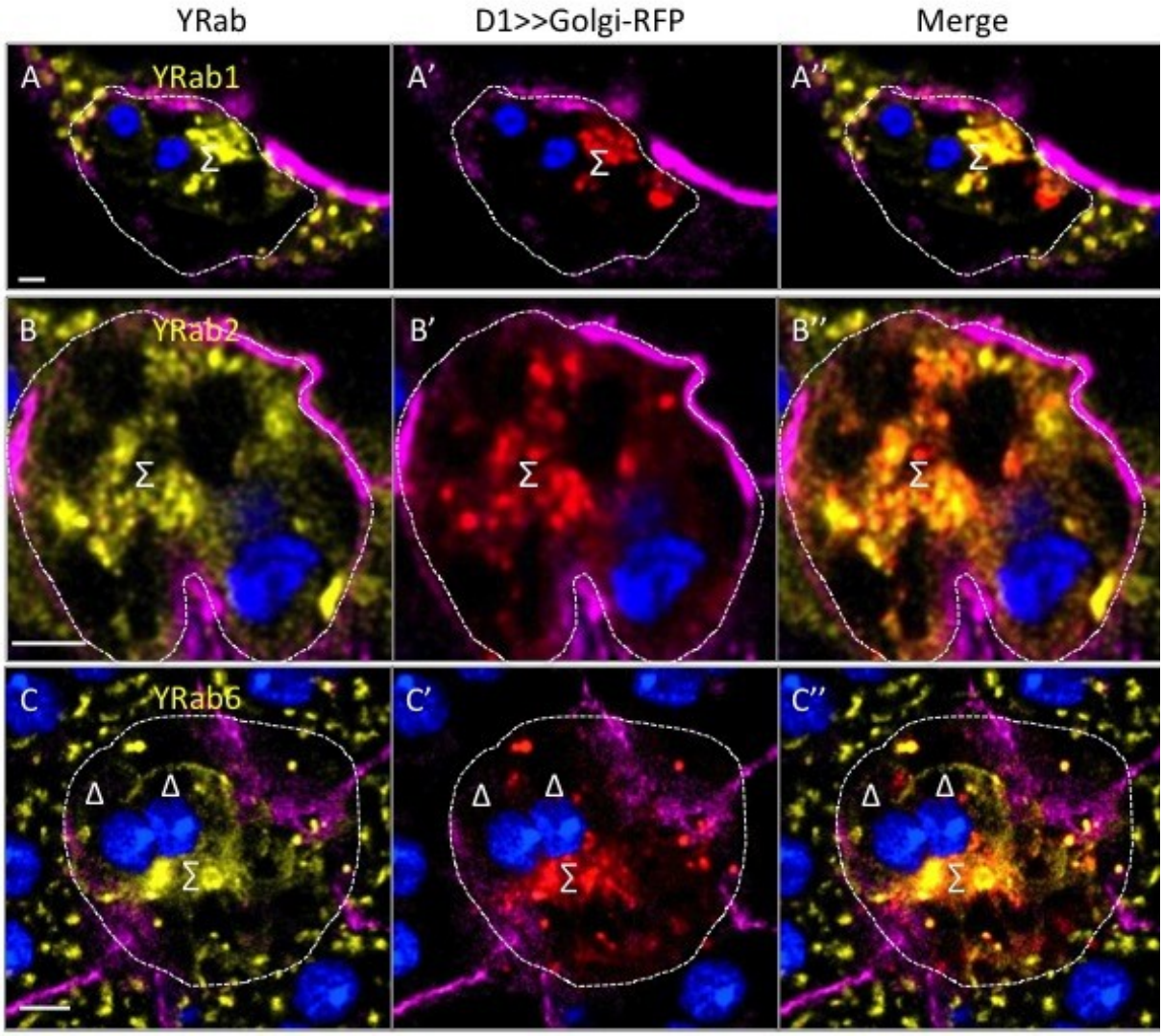
a. Rabs canonically associated with secretion: Rab1, Rab2, Rab6 & Rab8

Rab1 is a core Rab, canonically involved in endoplasmic reticulum (ER)-Golgi transport and the secretion pathway (Bhuin & Roy, 2014 and Fig. 4). In the MCs, Rab1 localizes on small, basal-medial compartments such as described in the post-mitotic salivary glands epithelial cells (Dunst et al., 2015). The particularity comes from Rab1 pattern in the SCs where I primarily detect it on a very large, central, filamentous-like structure, starting from the basal side of the cell and extending into the medial portion of the cell (Fig. 8A, Σ). Surrounding this central area of Rab1 localization are Rab1 positive smaller vesicles (Fig. 8A; Annex 1A). Based on its canonical function, the Rab1 distribution could indicate the localization of the Golgi apparatus in the SCs where it would form a large and central channel. In order to confirm this, I used our SC-specific Gal4 driver to express a Golgi-specific fluorophore (Golgi-RFP, Rickhy & Lippincott-Schwartz, 2010) (Fig.9A-A''). Examining the Golgi-RFP marker confirms a perfect co-localization of Rab1 and Golgi-RFP in the SCs (Fig. 9A-A''). Therefore it seems like Rab1 marks the Golgi apparatus in the SCs. If Rab1 also marks the Golgi in the MCs,then it would indicate that the Golgi is present in the MCs as small, basally localized structures. This is consistent with structures identified as Golgi by electron-microscopy analysis in the MCs (Bairati, 1968). It is important to note that at this level of resolution, it is difficult to see if this central large structure observed in the SCs represents one compartment or reflect the existence of numerous vesicles that are brought together by exclusion from the large vacuoles present in the cytoplasm of the SCs.

Rab2 is known to mediate Golgi-ER retrograde transport (Bhuin & Roy, 2014 and Fig. 2) and its sub-cellular localization in the epithelium of the AGs is reminiscent of Rab1. In the SCs however Rab1 and Rab2 distribution are not perfectly similar (Fig. 6A & 6B, respectively). While Rab1 is uniformly present on the large central channel and filamentous-like structures throughout the basal/medial cytoplasm (see above and Fig. 8A, Σ), Rab2 strongly marks vesicles at the peripheral tips of the filaments and only weakly labels the central channel (Fig.8B & Annex 1B). Nevertheless, the Golgi identity of the Rab2-positive features in the SCs have been confirmed by Golgi-RFP staining (Fig. 9B-B''). The differences between Rab1 and Rab2 could indicate that Rab2 marks only a portion of the Golgi apparatus or that Rab2 might also mark the ER. To investigate this further, I used an ER marker, KDEL-RFP (Rikhy & Lippincott-Schwartz, 2010). Using this marker, I saw a diffuse red-fluorescent signal throughout the cytoplasm of the SCs along with some strong punctae surrounding the central channel and apically located (Annexes 5A-A'' & B-B''). Although, I was unable to observe a co-localization of KDEL-RFP with Rab1, I did notice some Rab2 punctae that do. Thus the differences between Rab1 and Rab2 staining can be attributed somewhat to Rab2 marking the ER as well as the Golgi.



<u>Figure 8.</u> Distribution and expression pattern of the canonical secretory Rab proteins in the accessory glands. Z-projection enlargements of secondary cells (surrounded by dashed lines) from YRab1(A), YRab2(B), YRab6 (C) and YRab8 (D) accessory glands. The glands are stained by a nuclei marker, DAPI (blue). Rab1 is enriched on the central channel (A, Σ) and Rab6 is present on 4 large vacuoles (C, Δ). To see more cf. Annex 1A-D. Scale bars, 5µm.



<u>Figure 9.</u> Rab1, Rab2 and Rab6 are Golgi Rab proteins.
Confocal Z-sections enlargement of YRab1 (A-A''), YRab2 (B-B'') and YRab6 (C-C'') accessory glands expressing Golgi-RFP, in red, specifically in the secondary cells (surrounded by a dashed line). The glands are stained by a baso-lateral marker (DLG, magenta) and a nuclei marker (DAPI, blue).
YRab1 (A, yellow) and YRab2 (B, yellow) co-localize with Golgi marker (A'–A'' and B'-B''). Although Rab6 basal and centered structure (C-C'',∑) co-localizes with Golgi, the large vacuoles do not (C-C'', Δ). Scale bars, 5µm. To see co-expression experiments between other Rabs (Rab14, Rab18, Rab21) and Golgi marker, cf. Annex 4A-C.

Rab6 is also typically associated with Golgi-ER retrograde transport, but also intra-Golgi trafficking particularly towards the *trans*-Golgi network (TGN) membranes (Mitra et al., 2011; Huntalgalung & Novick, 2011; Seabra et al., 2002 and Fig. 4). In the MCs, Rab6 shows a similar distribution and pattern as Rab1 and Rab2 *ie* basal/medial small compartments. In the SCs, however, Rab6 localization is slightly different than Rab1. While

Rab6 labels some parts of the central channel, it is also seen on a few, medial/apical large vacuoles (see below and Fig. 6C,Δ), giving its visualization a “champagne cup-like” appearance (Fig. 8C; Annexes 1C & 13). Interestingly, most of the basal and medial Rab6-positive compartments are also Golgi-RFP-positive whereas the large vacuoles are not (Fig. 9C-C’’).

Finally, Rab8 is known as a secretory vesicle (SV) marker and to mediate protein transport from the TGN towards the plasma membrane (PM) (Seabra et al., 2002; Lee Ang et al., 2003; Huntalgalung & Novick, 2011; Bhuin & Roy, 2014 and Fig. 2). Rab8 expression patterns are clearly different between the two cell-types of the AGs while the expression levels are not (Fig. 8D & Annex 12). While it is notably present in the MCs on highly packed and large compartments uniformly distributed in the cytoplasm, I only observe a very weak diffuse signal in the SCs (Fig. 8D; Annex 1D). This difference could suggest that the MC secretion machinery utilizes Rab8 but the SCs use another regulator.

b. Rabs canonically associated with endocytosis and recycling: Rab4, Rab5, Rab7 & Rab11

When materials are internalized from the plasma membrane (PM), they are sorted and sunken in vesicles, called early-endosomes (EEs). From the EEs, numerous options are available. For instance they can 1) be delivered back to the PM by recycling routes, 2) join the Golgi apparatus, or 3) be degraded in the late-endocytic pathway (Jovic et al., 2010 and Fig. 4). Different Rab proteins mediate each of these pathways.

Rab5 is known as an EE marker involved in endocytic internalization and EE fusion (Harris & Littleton, 2011; Bhuin & Roy, 2014). Rab5 primarily labels the basal-medial punctate in the MCs. While basal/medial punctuate features are also observed in the

cytoplasm of the SCs, there is also a concentration of Rab5 positive punctae in the centre and the periphery of the cell (Fig. 10B; Annex 1F). While this could be due to the spatial constraint obliged by the large vacuoles filling the cytoplasm of the SCs, it may also indicate a function for EEs in this region of the SCs. Corrigan et al. (2014) previously showed that overexpressed Rab5 was present on some apical, centrally-clustered punctate structures. The localization that I see could be similar to the structures they reported (Annex 9B-B''). According to their work, they see these vesicles near late endosomes and they believe they are involved in the switching of EE to late endosome (LE) (Deretic, 2005; Rink et al., 2005).

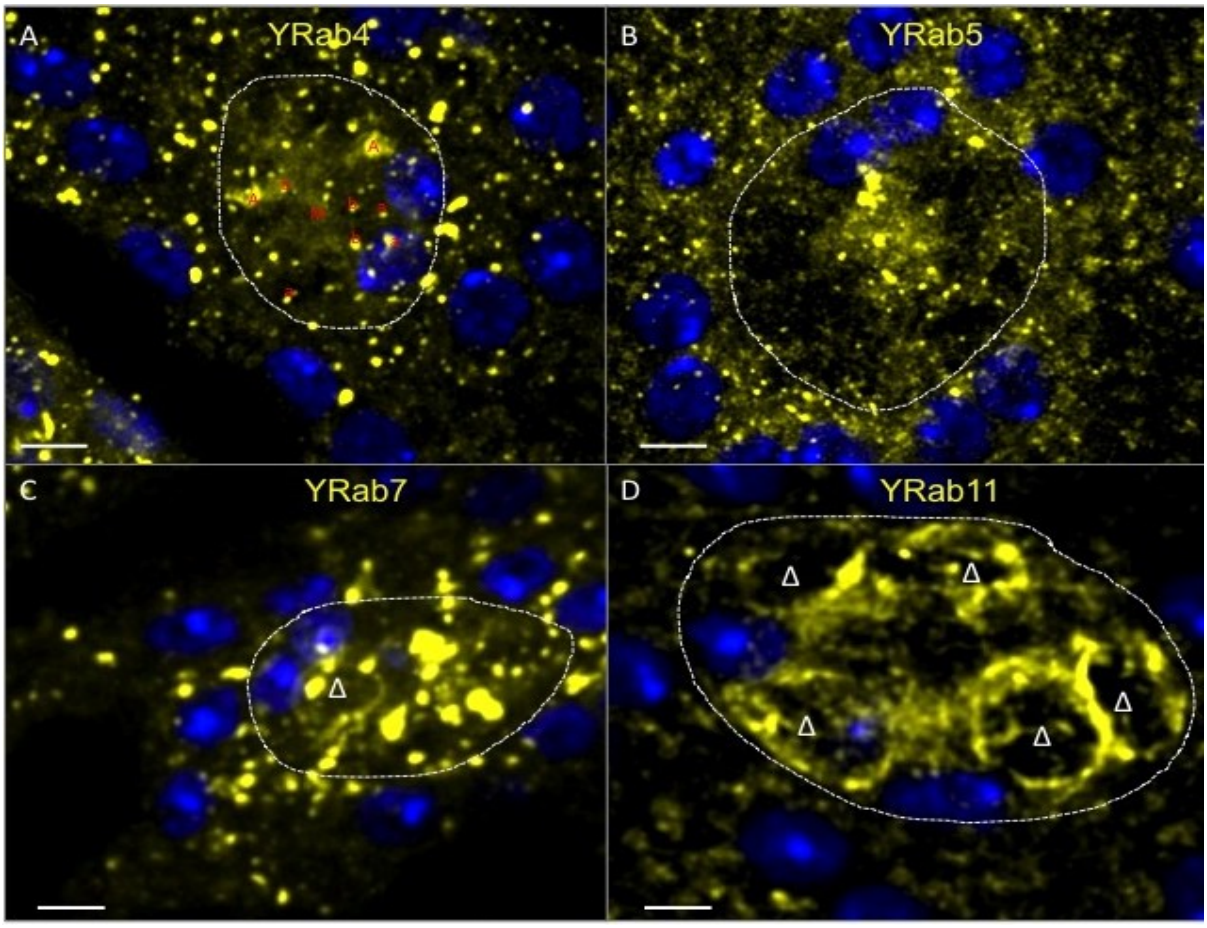


Figure 10. Distribution and expression pattern of the canonical endocytic and recycling Rab proteins in the accessory glands.
Z-projection enlargements of secondary cells (surrounded by dashed lines) from YRab4(A), YRab5(B), YRab7(C) and YRab11(D) accessory glands. The glands are stained by a nuclei marker, DAPI (blue). YRab7 and YRab11 mark some large vacuoles (C-D, Δ). To see more cf. Annex 1E-H.
YRab4 (A) is present on different features; basal and apical punctae (b & a, respectively), apical small compartments (A) and a medial centered diffuse feature (M) (see also Annex 1E). Scale bars, 5µm.

Rab7 is known as a LE and lysosomes marker. These are compartments of the degradation pathway. In MCs, this core Rab is uniformly distributed as small compartments throughout the cytoplasm. In the SCs, however, its localization is more complex. First, it labels punctae and small compartments centrally-clustered and scattered throughout the cytoplasm. This could be similar to its pattern in the MCs and could be intermediate late endosomes (iLEs) and mature LEs, respectively (as suggested by Corrigan et al., 2014 and Annex 9D-D''). Intriguingly Rab7 also labels one or two large vacuoles that are more apically located. Using a lysotracker marker, Corrigan et al. previously showed that overexpressed Rab7 could label lysotracker marked compartments that they called the acidic compartments (Fig. 10C, Δ; Annexes 1G & 9D-D'').

Rab4 can be found on EEs and on recycling endosomes (REs); this GTPase is involved in the recycling of proteins from EEs or REs directly back to the PM (Bhuin & Roy, 2014 and Fig. 4) and may also play a role in EE to Golgi transport (Gillingham, Sinka et al., 2014 and Fig. 4). In the MCs, Rab4 is strongly present on basal/medial punctae, much like Rab5. Meanwhile in the SCs, I observed different structures including peripheral puncta both apical and basal (Fig. 10A & Annex 1E, "a" & b, respectively), medial centred diffuse structures (Fig. 10A & Annex 1E, "M") and a few cortical small compartments on the apical side (Fig. 10A & Annex 1E, "A").

Where Rab4 mediates fast recycling, Rab11 regulates recycling through the recycling endosomes (REs) (Bhuin & Roy, 2014 and Fig. 4) as well as exocytosis (Bhuin & Roy, 2011;

Woichanski et al., 2016). In the MCs, this core Rab shows the same distribution as Rab7 (small compartments throughout the cytoplasm). In the SCs, Rab11 is primarily present on large vacuoles located at the PM around the basal/medial part of the cell (Fig. 10D, Δ; Annex 1H). We also see some small punctate features present in the centre of the cell on the apical side. These different concentrations of the Rab11 could suggest that recycling occurs through different compartments depending their destination. Perhaps recycling towards the MCs uses the vacuoles while recycling towards the lumen uses the apical punctate (Fig. 10D; Annex 1H).

Our observations of the Rabs canonical generally involved in secretion, endocytosis and recycling in the AGs show some interesting aspects. First, we observed that most of the Rabs involved in exocytosis and endocytosis seem to mainly localize to the basal and/or latero-basal side of MCs. Second, in the SCs, some of the vacuoles are marked by Rabs canonically associated with specific compartments (Golgi, RE, lysosome). Third, secretion in the SCs probably uses a non-canonical Rab, given that Rab8 is only weakly expressed in these cells.

c. Rabs marking large vacuoles of the secondary cells: Rab6, Rab7, Rab11 & Rab19

One of the main aims of my thesis work was to learn more about how the SCs function. As the SCs are filled by unusually large vacuoles, they were one of my main subjects of interest. Of the collection of Rabs, I found that four that associated with the large vacuoles (Rab6, Rab7, Rab11 and Rab19). Each of these four Rabs labels only a subset of the large vacuoles, suggesting that they harbour different identities and functions (Fig. 8C; Fig. 10C-D; Fig. 11D; Annexes 1C, G-H & 2C). While Rab6, Rab7 and Rab11 are core Rabs, which

were already discussed (present in all Eukaryotic cell types), the 4th one, Rab19 is secondary-cell specific and of unknown function. In general, Rab19 marks one to four large vacuoles (Fig. 11C, Δ; Annex 2C) that are apically located. In fact, of the vacuoles, the Rab19 vacuoles are usually the most apically localized. Besides the large vacuoles, Rab19 also labels medial/apical features surrounding the central channel as well as basal punctae and small compartments (Fig. 11C; Annex 2C). This apically enriched location correlates with Rab19 distribution in another secretory tissue, the salivary glands, where it was proposed to mediate apical secretion (Dunst et al., 2015).

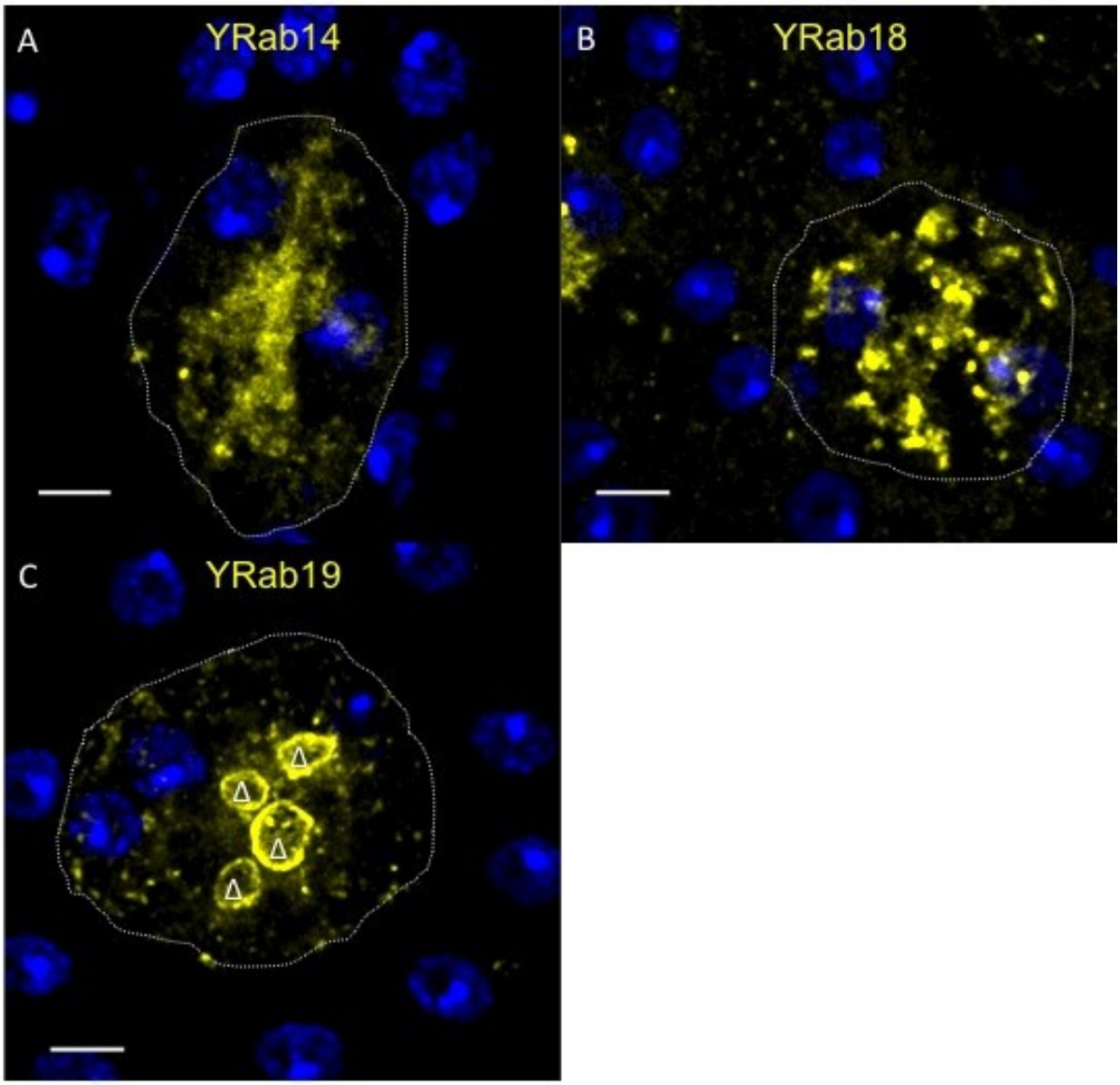


Figure 9. Distribution and expression pattern of the non-canonical Rab proteins mostly present in the secondary cells of the accessory glands.
Z-projection enlargements of secondary cells (surrounded by dashed lines) from YRab14(A), YRab18(B), YRab19(C) accessory glands. The glands are stained by a nuclei marker, DAPI (blue). Rab19 is present on 4 apical large vacuoles (C, Δ). To see more, cf. Annex 2A-C. Scale bars, 5µm.

d. Non-canonical Rab proteins present in the accessory glands epithelium: Rab10, Rab14, Rab18, Rab19, Rab21, Rab35, Rab39, RabX1

I will briefly speak about the non-canonical Rabs that are expressed in the AGs, but whose expression is less well defined. I will first describe Rab14 and Rab18 (Fig. 11A-B; Annex 2A-B), which are more strongly expressed in the SCs than in the MCs (Annex 12). Then, I will say few words about, Rab21, Rab39, Rab10, Rab35 and RabX1 (Annex 2D-F).

Like Rab19 (see above and Fig. 9C), Rab14 appears secondary-cell-specific (Fig. 9A; Annex 2A). This GTPase is thought to be involved in both endocytic and exocytic pathways (Bhuin & Roy, 2014; Kitt et al., 2008; Junutula et al., 2004 and Fig. 4). Rab14 signal is uniformly distributed throughout the cytoplasm and is strongly enriched in smaller vesicles in the centre of the SCs (Fig. 11A; Annex 2A). Among these Rab14-positive compartments, some co-localize with the Golgi-RFP marker in the punctae and central structure (Annex 4A-A''). Furthermore, I observed that Rab14 features juxtapose the signal from a secretory vesicle marker Tomato-myristoylation (Annex 6C-C''). These observations could support an exocytic function of Rab14 in the SCs.

Rab18 has been proposed to be an intermediate in ER-Golgi trafficking and to mediate lipid droplets formation (Bhuin & Roy, 2014; Gillinghan, Sinka et al., 2014 and Fig. 4). Rab18 is basally distributed on small punctae within the MCs cytoplasm; in the SCs, it is primarily located in the centre of the cell as punctate features and larger compartments (Fig. 11B & Annex 2B). In agreement with a role of Rab18 in ER-Golgi trafficking, some Rab18 punctae co-localizes with the Golgi-RFP marker and the ER-marker, KDEL-RFP (Annexes 4B-

B'' & 5C-C'', respectively), though there are also some of the Rab18-positive compartments that do not co-localizes with Golgi-RFP but rather follows the contours of the Golgi (Annex 4B-B'').

I have also determined the distribution of additional Rab proteins that I did not investigate further during my PhD, although they are present in AGs epithelium. These include Rab10, Rab21, Rab35, Rab39 and RabX1. I will only mention briefly their expression patterns here and I have placed their localization patterns in the annexes to this thesis (Annexes 2E-F & 12).

Rab10 is evolutionary closely related to Rab8 (Lerner, McCoy, et al., 2013; Bhuin & Roy, 2014; Zhang, 2007 and Fig. 5) and therefore is thought to be part of the secretion machinery. Rab10 appears uniform and diffuse in both cell types. Occasionally, Rab10 can be more seen more-strongly expressed and perhaps apically-enriched in the SCs relative to the MCs (Annexes 12 & 2Fa-a''). However this difference is not consistently seen and does not seem to correlate with any of the factors tested.

Rab35 and RabX1 are also found in the SCs. In most cases, the distribution of Rab35, which is thought to be involved in fast recycling and actin assembly (Bhuin & Roy, 2014) is similar to the distribution of Rab10 in main and secondary cells (*ie* uniform and diffuse). Occasionally centrally located, basal/medial compartments are also labelled by Rab35 in the SCs (Annex 2Fb-b''), but as with Rab10, this is not always seen.

RabX1 function is unknown but some data suggest a role in recycling (Harris & Littleton, 2011; Woichansky et al., 2016). In the MCs, RabX1 is uniformly distributed in the cytoplasm, while in the SCs there is a diffuse signal throughout the cytoplasm as well as an enriched signal in the centre of the apical side cell (Annex 2Fc-c'').

Rab39 has been shown to be involved in both secretion (from the Golgi (Gillingham, Sinka et al., 2014 and Fig. 4)) and endocytosis (Stein et al., 2003). Rab39 is present in both cell types defining small compartments in the basal/medial side in the MCs and centrally located compartments in the medial/apical part of the SCs (Annex 2E).

Rab21 has been proposed to do a number of diverse functions in the cell. It has been seen on EEs, like Rab5 (Pellinen et al., 2006; Bhuin & Roy, 2014 and Fig. 4), but also on vesicles involved in TGN to PM apical secretion (Seabra et al., 2002) and REs (Harris & Littleton, 2011; Chan et al., 2011). Rab21 signal is uniform and weak in the MCs but is enriched in the centre of the SCs, primarily as apical punctate compartments (Annex 2D). Furthermore, the distribution of the punctae seems to following the contours of the central Golgi channel with some co-localization (Annex 4C-C''); these observations could support a role in export from or import to Golgi in these cells.

e. Undetectable Rabs in the epithelium of the accessory glands: Rab3, Rab9, Rab23, Rab26, Rab27, Rab30, Rab32, Rab40, RabX4, RabX5 & RabX6.

Among the Rabs that are absent from the epithelium but present somewhere in the AG are Rabs 3,9 and X4. These are present at the synaptic buttons of the neurons innervating the muscle layer of the AG. This observation is in agreement with the report that the evolutionary related Rab3 and RabX4 are neuronal-specific (Dunst et al., 2015; Chan et al., 2011). Rab3 is mostly known of as a secretory vesicles marker (Chan et al., 2011; Harris & Littleton, 2011; Bhuin & Roy, 2014), whereas RabX4 may belong to the group of Rabs involved in endocytosis and recycling (Chan et al., 2011; Oberegelsbacher et al., 2011). Rab9 is mainly known as a glial marker (Chan et al., 2011; Dunst et al., 2015). The presence of these 3 neuronal Rabs on the muscle layer suggests that these neurons dictate the

contraction of the AGs (Tayler et al., 2012 & Annex 3). The remaining 8 Rabs (Rab23, Rab26, Rab27, Rab30, Rab32, Rab40, RabX5 and RabX6) are undetectable in the whole AGs (Annex 12).

In order to get knowledge about the function of the SCs that likely rests on the presence of the large vacuoles (Gligorov, Sitnik et al., 2013), I focused on the Rabs that mark these prominent compartments.

3) The secondary cells contain different subpopulations of large vacuoles with shared and distinct functions

The large vacuoles are the landmarks of the SCs of the AG and their integrity is required to elicit a proper PMR (Gligorov, Sitnik et al., 2013). My PhD project aims at identifying the function of these large organelles. Are they all identical, being storage organs or are there different kinds of vacuoles involved in different processes? If there are different types of vacuoles, how do they relate to each other? Are they part of a linear maturation process, or do various vacuoles originate from different compartments? Electron microscopy studies already performed in the late 60ties (Bairati, 1968) revealed the presence of a subclass of vacuoles that are electron dense, harbouring "filamentous bodies" suggesting the existence of at least 2 types of vacuoles. More recently, a live-imaging study in which overexpressed tagged version of Rabs are expressed in a tissue specific fashion from a transgene, also revealed the existence of at least two types of vacuoles in the SCs. While the 1st type of vacuoles contains "dense-core granules" (DCGs) and are marked by Rab11, the 2nd type could be lysosomes or "multi-vesicular bodies" (MVBs) and are marked by Rab7 (Corrigan et al., 2014; Sedhai et al., 2016).

During my screening of the collection of knocked-in Rabs, I identified 4 Rabs that mark the large vacuoles (Rab6, Rab7, Rab11 and Rab19) (Fig. 8C; Fig. 10C-D; Fig. 11C). I also observed that Rab6- and Rab19-positive vacuoles could also contain a dense core. While the SCs contain a certain number of large vacuoles, these 4 Rabs appear to label only a fraction of them and with different patterns, suggesting that the large vacuoles can be specific sub-cellular membrane compartments with different functions.

Among these vacuole-labelling Rabs, 3 are core Rabs (Rab6, Rab7 and Rab11). Being core Rabs, these Rab labelling may tell us something about their function in the SCs (discussed in Discussion part). Nonetheless, the 4th Rab labelling the large vacuoles is Rab19 whose function remains unknown. The apical location of Rab19-positive vacuoles in both the SCs of the AG as well as in the cells of the salivary glands, another specialized secreting gland, suggest that they may be regulating apical secretion within the lumen.

As mentioned above, Rab6 has a “champagne cup”-like distribution in the SCs (Annexes 1C & 13A), where the basal and medial parts co-localize with Golgi-RFP marker while the medial/apical large vacuoles do not (Fig. 9C-C’’). Because Rab6 can also be present on *trans*-Golgi apparatus membranes (Bhuin & Roy, 2014), I looked at the expression of Arf79F-GFP (Armbruster & Luschnig, 2012) in the SCs. Arf79F is the fly Arf1-GTPase ortholog, which is known as a TGN marker (Robinson et al., 2011). The Arf79F-GFP pattern is very similar to Rab6 distribution including it presence on large vacuoles (Annex 13B). Although, co-expression experiments have not been performed, these parallel observations are in line with a role of Rab6 in transport from and across the Golgi toward the apical side of the SCs. It also indicates that the Rab6 vacuole may have TGN-like function.

Rab7 is known as a mediator of the intracellular degradation pathway where endocytosed proteins are hydrolysed. The similarity between the patterns of Rab7 and a

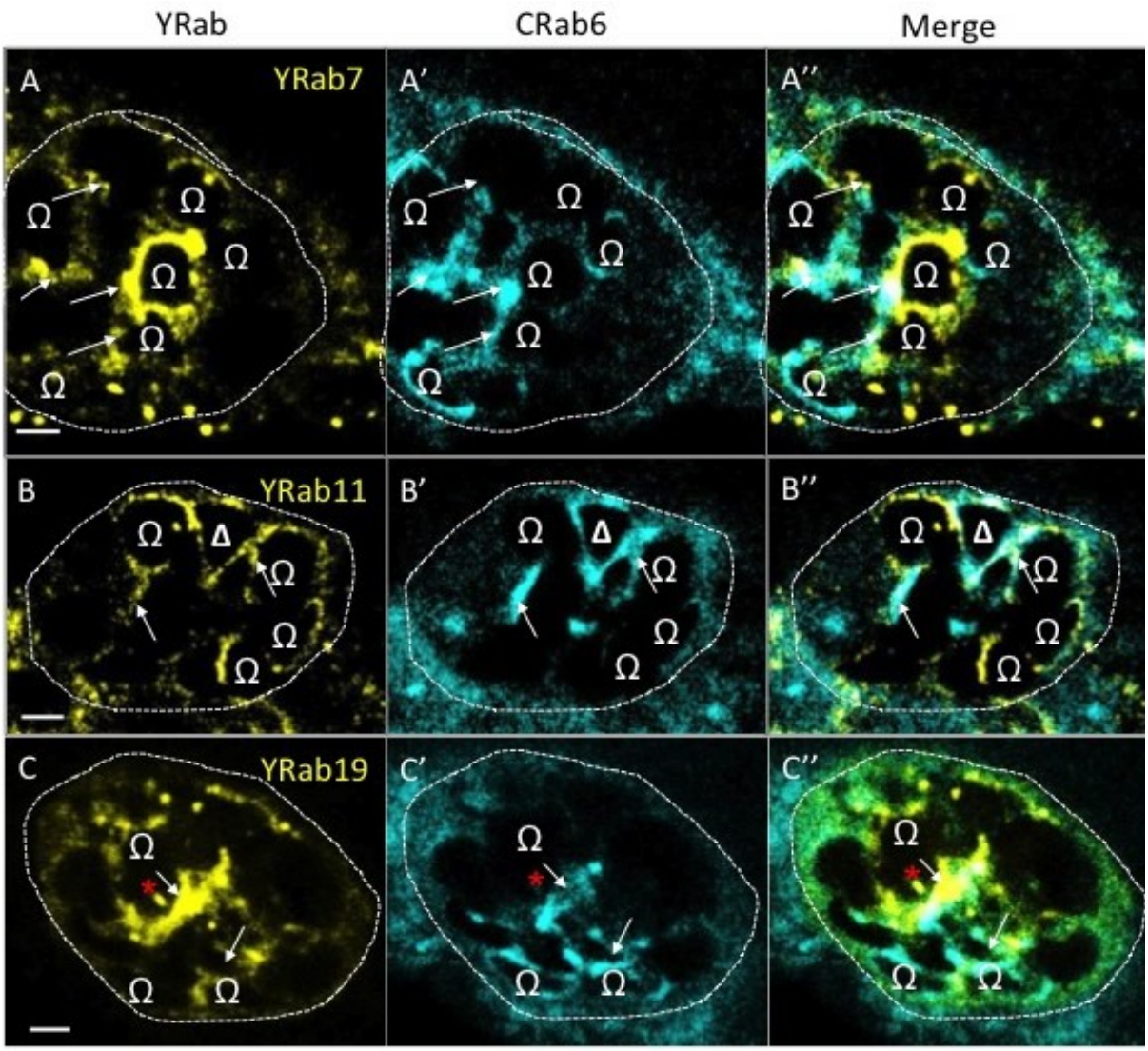
lysosomal marker, Lamp1-GFP (Chang & Neufeld, 2009) in the SCs supports this late-endocytic function of Rab7 in the SCs (Annexes 13C and 13D, respectively). As mentioned above, Rab7 marks one or two large vacuoles (Fig. 10C). Clive Wilson's laboratory has shown that some of these Rab7-positive large vacuoles are also acidic compartments containing exosomes, suggesting that these vacuoles are lysosomes or MVBs (Corrigan et al., 2014). My results are in agreement with those of the Wilson lab (Annex 9D-D'').

Rab11 is involved in the recycling pathway *ie* the pathway by which internalized PM components can be delivered back to the cell surface (Bhuin and Roy, 2014). The large vacuoles labelled by Rab11 are cortical and form a ring around medial part of the SCs near the PM (Fig. 10D; Annex 1H). This is similar to what Corrigan and collaborators observed about the distribution of the Rab11-positive vacuoles in the SCs, although they called them secretory vesicles. Second, the location of Rab11-positive vacuoles suggests that recycling and/or exocytic events can occur at the border of the SCs with the MCs, suggesting intercellular exchanges in AGs.

As already mentioned Rab19 function is unknown but may be involved in apical secretion (Dunst et al., 2015). In order to gain further insights into Rab19 function in the SCs, I have investigated if mating can induce any change in its distribution and/or levels. It turned out that the number of Rab19-positive large vacuoles does not seem to vary depending on the number of mating. Also its expression level in the SCs based on YFP fluorescence intensity does not seem to vary in a consistent manner with increased mating (Annex 14A). This led us to hypothesize that Rab19 compartments in the cell may be stable organelles and not secretory vesicles. However, during my observations of Rab19 vacuoles, I noticed that Rab19-positive bulbs or shrunken vacuoles were present in the lumen of 3 day-old or older virgin males (Annex 11). This lead to the possibility that Rab19 vacuoles might be secreted

slowly by a non-merocrine pathway but that the secretion/renewal process was not dependent on sexual activity.

So far, I have shown that there are 4 Rabs that label the vacuoles, but do these Rabs label different or overlapping compartments? Do these 4 Rabs indicate 4 compartments or more (or less)? To investigate if this further, I performed co-localization experiments using two differently marked Rab knock-in libraries (also kindly provided by Marco Brankatschk in Susan Eaton's laboratory). One interesting result came from lines containing YFP-tagged Rab11 (Rab11) and CFP-tagged Rab6 (CRab6). While there are distinct CRab6- and YRab11-positive vacuoles, I was also able to observe large vacuoles marked with both CRab6 and YRab11 (Fig. 12B-B'', Δ; Annexe 8B, Δ).



<u>Figure 12.</u> Two Rab proteins can co-exist on same large vacuoles.
Confocal Z-sections of secondary cells enlargement (surrounded by dashed lines) of accessory glands co-expressing YRab7 (A), YRab11 (B) or YRab19 (C) in yellow and CRab6 in cyan (A'-C').
Rab7 and Rab6 can be both present in punctate features (A-A'', *) but not on large vacuoles (A-A'', Ω). Rab7 and Rab6 can also co-localize on some portions of large vacuoles shown by an arrow as well as Rab19 and Rab6 (C-C''; Ω).
However, some large vacuoles can be compartments with two identities, they can be both CRab6-and YRab11 positives (B-B'', Δ). To see more, cf. Annex 8A-C. Scale bars, 5µm.

To gain further insights into the function of these vacuoles marked by the two Rabs, I expressed a secretion-pathway marker, Tomato-myristoylation (Tom-myr; Pfeiffer, 2010.9.22) in the SCs of males producing both the YRab11 and theCRab6 proteins (Fig. 13B-B'''). Myristoylation is a post-translational modification occurring between the Golgi

apparatus and the PM allowing proteins to anchor them to the intracellular membrane (Simon & Aderem, 1992; Aicart-Ramos et al., 2011; Heinrich et al., 2014). Interestingly, the CRab6-YRab11-positive large vacuoles are Tom-myr-positive supporting a role for these tri-labelled vacuoles in secretion. Further support for the role of these vacuoles in secretion come from recent studies in *Drosophila* photoreceptors, where it was shown that Rab6 positive vesicles are redistributed from the TGN to join with Rab11-positive vesicles to mediate apical secretion (Iwanami et al., 2016). In the case of the SCs, these tri-labelled vacuoles are primarily located at the border of the SCs with MCs (Fig. 13B-B''', Δ). Taken together these data suggest that the Rab6-Rab11-positive vacuoles could play a role in secretion of proteins from the SCs toward the MCs, thus giving further support to the intercellular exchanges hypothesis within AGs.

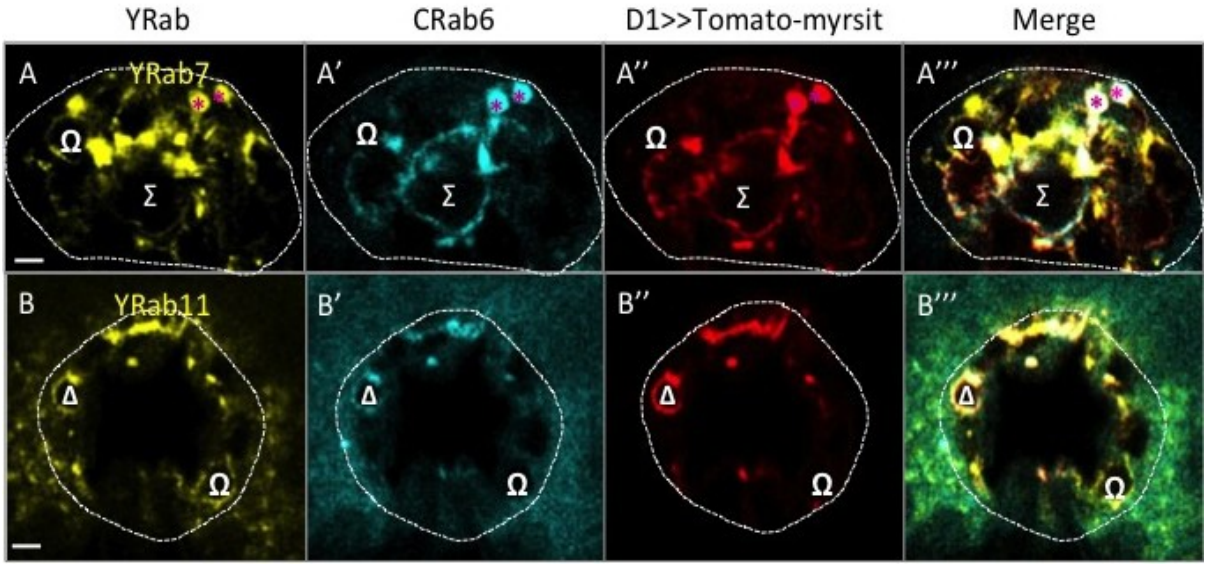


Figure 13. Rab6 and Rab11 co-mark some large vacuoles involved in secretion
Confocal Z-sections of secondary cells enlargement from accessory glands co-expressing YRab7 (A) or YRab11 (B) with CRab6 in cyan (A' and B', respectively) and the secretory marker, Tomato-myristoylation, in red (Tom-myr, A'' & B'', respectively). The secondary cells are surrounded by a dashed line.
Some small YRab7 compartments are clustered around the central channel (A-A'', Σ) while others, more peripheral, can co-localize with both CRab6 and Tom-myr (A-A''', asterix) but the large vacuole do not or partially (A-A''', Ω). Cortical basal/medial large vacuoles involved in secretion (Tom-myr-positive) can possess both Rab6 and Rab11 (B-B''', Δ). Some vacuoles only bound by Rab11 do not seem involved in secretion (B-B'', Ω). Scale bars, 5µm.

I performed similar experiments in which I combined CRab6 with YRab7 or YRab19 but failed to detect large vacuoles totally marked by both CFP and YFP signals. However, in some small compartments and at portions of vacuoles some co-localize can be observed (Fig. 12A-A'' & C-C''; Annex 8A & C). These observations agree with the idea that these vacuoles might exchange cargo or that there is a maturation sequence involving a change of membrane identity (Poteryaev et al., 2010; Del Conte-Zerial et al., 2008; Rink et al., 2005). For completeness I also attempted reciprocal co-expressions studies in which I combined CRab11 and YRab6, YRab7 or YRab19. Unfortunately, the weakness of the CRab11 signal did not give clear answer about possible co-localization; thus I was not able to confirm that Rab7 and Rab11 can be present on the same vacuoles (Corrigan et al., 2014) and it remains unclear if any Rab11 positive vacuoles can also carry Rab19, since it is has been shown that some Rab11-postive DCGs can be secreted apically toward the lumen (Redhai et al., 2016).

As mentioned above, the presence of Rab19 vacuoles at the apical surface in the salivary glands and the AGs suggest a role into secretion towards the lumen. However most of the usual secretory features that I have detected in the AG are associated with Rab6 and Rab11. Given the observation that I was not able to detect co-localization of Rab19 with Rab6, the pathway through which Rab19 is associated with secretion in the AG, if it exists, remains uncharacterized. In fact, based on the lack of co-localization of the Tom-myr reporter with Rab19 (Tom-myr labels more peripheral large vacuoles whereas Rab19 is primarily on more centred and apical vacuoles (Annex 6D-D'')) may indicate that Rab19 is not involved in the secretion of SC made proteins. However, it remains possible that recycled elements might be trafficked via Rab19 vacuoles.

Taken together, these observations suggest the existence of several trafficking pathways mediated by the large vacuoles (see in Discussion part; Fig. 18 & Fig. 19), some

directed toward the MCs and others turned to the lumen. The co-localization experiments also point to the possibility that proteins may be sequentially transported through the different vacuoles before reaching their final destination. This could be done through intermediate vesicular transport vesicles, by vacuole fusion events or by the vacuoles themselves changing their identity. In order to shed light on the relationships between the vacuoles, we decided to first observe the expression patterns of the Rab labelled vacuoles at different developmental time points (Fig. 14) and then to perform epistasis experiments by knocking down individual Rabs and examining the resulting phenotypes on the other vacuoles (Fig. 15).

4) Rabs & vacuoles in the secondary cells: a story of relationships and maturation

The presence of vacuoles with two Rab associated with their membranes could suggest of a maturation process in which vacuoles progressively change compartment identity. Alternatively it may reflect a specific stage in which 2 Rabs are required for proper trafficking regulation. In order to distinguish between these 2 possibilities, I first performed a developmental time point analysis of the 4 vacuolar Rabs (Rab6, Rab7, Rab11 and Rab19) in virgin males during the first 10 days after eclosion (Fig. 14; Annex 8).

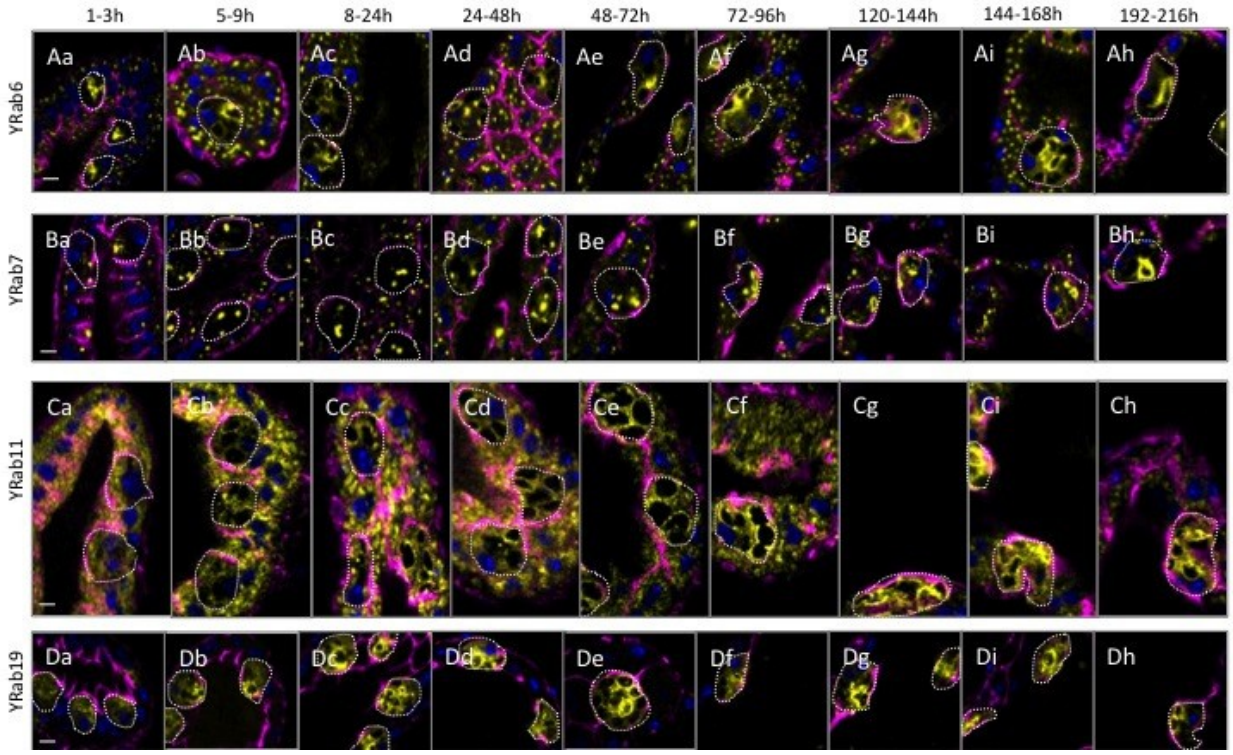


Figure 14. The large vacuoles form at different time points of male development
Confocal sections enlargement of secondary cells from YRab6 (A), YRab7 (B), YRab11 (C) and YRab19 (D) accessory glands dissected and fixed at different time point of virgin males development. YRab6 vacuoles are present since the first hours of male life (Aa), YRab11 and YRab19 ones appear between five and nine hours after (Cb and Db, respectively) birth while YRab7 large vacuoles are visible since males are three days-old. The YRab are in yellow, the accessory glands are stained by a membrane marker, DLG, in magenta and by a nuclei marker, DAPI, in blue. To see the expression levels of the different Rabs at different virgin male ages, cf. Annex 16. Scale bars, 5µm.

The vacuoles labelled by Rab6, Rab7, Rab11 and Rab19 all require at least 5 days to reach a stable pattern. In general, the pattern of these 4 Rabs starts by punctate features and then progressively, Rab-positive large vacuoles appear. Only Rab6-positive vacuoles seem to be present in SCs during the first hour post-eclosion (Fig. 14Aa). Rab19 (Fig. 14Db) and Rab11 (Fig. 14Cb) vacuoles become visible after 5 hours and Rab7 large vacuoles only start to be present after three days (Fig. 14Bf). Based on these results, we can say that if these vacuoles form from a maturation process, then Rab6 could be a precursor to all of the vacuoles and Rab19 vacuoles must not be required for the formation of the other ones. Examining the expression of the Rabs over the later days yielded a fairly stable pattern

(Annex 17). This data provides some cell biological support to recent studies showing that AGs require about six days to reach maximum functionality (Ruhmann et al., 2016).

To further test the vacuole maturation hypothesis, I performed an epistasis analysis. Using SC-specific RNAi-mediated knockdown, I systematically inactivated each of the four large vacuoles-specific Rabs and monitored the presence of the 3 remaining Rabs (Fig. 15).

As mentioned above, Rab6 is the first vacuole to form in the maturing SCs and hence could be the precursor all of the other vacuole types. Consistent with this possibility, I found that Rab6 knockdown results in a loss of all the large vacuoles. In their place are numerous smaller vesicles labelled with the other Rab proteins (Fig. 15A'-D'). Also of note is that the Rab7 and Rab11 signals seem to decrease in Rab6 knockdown SCs (Fig. 15E) and that the Rab7, non-vacuole compartment location appears more central than in *wild-type* conditions (Fig. 15B-B'), though this later observation could be simply be due to the absence of physical constraints placed on the cell by the large vacuoles. In any case, Rab6 appears to be required for the formation of the other large vacuoles. It is worthwhile noticing that the loss of large vacuoles and the resulting circular-to-pentagonal shape of the SCs observed upon Rab6 knock-down is reminiscent of the phenotype observed in *iab-6*^{cocu} males in which *AbdB* expression in the SCs is lost (Gligorov, Sitnik et al., 2013 & Fig. 16C-C'').

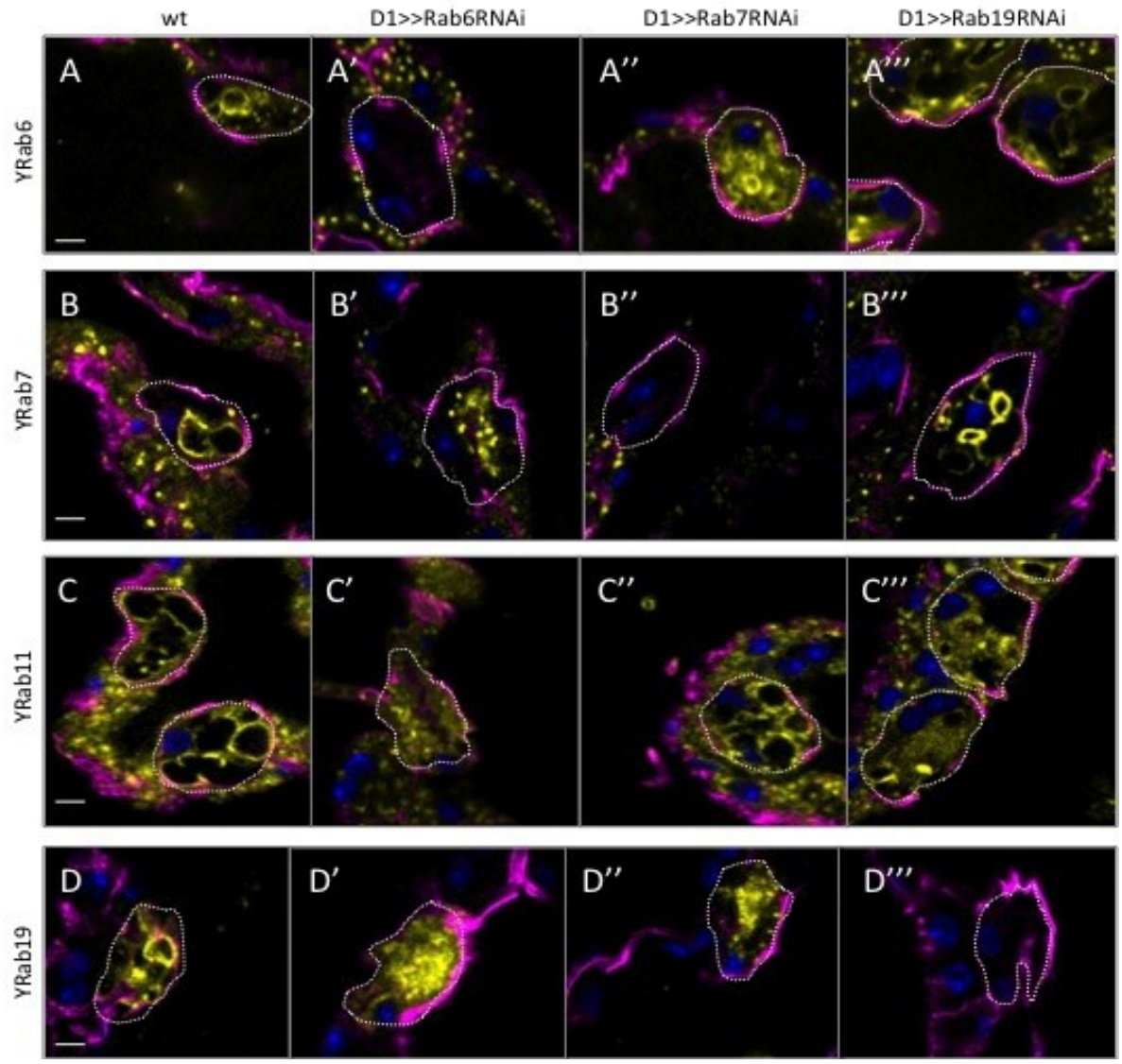
The depletion of Rab7 in the SCs leads to the loss of the Rab19-positive large vacuoles and affect the number and the size of the Rab6 vacuoles (Rab6 vacuoles become more numerous but smaller) (Fig. 15A''-D''). These observations suggest that Rab7-positive vacuoles may constitute an intermediate step in the pathway leading to the maturation of the Rab19 matured compartments. However, this cannot be true because we know that the Rab19 vacuoles appear long before the formation of the Rab7 vacuoles. Thus, it must be that Rab19 vacuoles require elements from the other Rab7 compartments to form. As Rab7

labels LE, it could indicate that Rab19 vacuoles are downstream in the trafficking pathway from the LE.

Rab11 vacuoles are still present in the absence of Rab7 or Rab19, indicating that the formation of Rab11 vacuoles does not require Rab7 and Rab19 marked elements (Fig. 15C-C’’’). This result does not mean, however, that these vacuoles function completely independent from one another. In fact, Rab11 signal increases in SCs depleted of Rab7 (Annex 15) and may indicate that by blocking the Rab7-associated vacuole pathway, we favour the creation of Rab11 vacuoles.

The knockdown of Rab19 seems to have no effect on the other large vacuoles (Fig. 15A’’’-D’’’). This suggests that Rab19 positive vacuoles are a final step in a Rab maturation process.

Unfortunately, I was not able to see the effects of the loss of Rab11. RNAi against Rab11 driven by our DI driver is lethal at the first larval instar. Although this driver is not expressed anywhere in the male reproductive tract outside of the secondary cells, there is probably some leakiness elsewhere in the fly that is causing this lethality.



<u>Figure 15.</u> Epistasis relationships exist between the Rabs labeling the large vacuoles of the secondary cells. Confocal sections enlargement of secondary cells from YRab6 (A), YRab7 (B), YRab11 (C) and YRab19 (D) accessory glands stained with DLG, in magenta and DAPI, in blue. The secondary cells are specifically depleted of specific Rab *ie* Rab6 (A'-D'), Rab7 (A''-D'') or Rab19 (A'''-D'''). In absence of Rab6 in the secondary cells, all the YRab-positive large vacuoles disappear compared to *wild-type* (A'-D' and A-D, respectively; Fig. 14A and A'', respectively). The loss of Rab7 affects Rab6 and Rab19 large vacuoles presence (A'' and D'') while the loss of Rab19 do not affect any of the other YRab proteins (A'''-D'''). To see the effects of the Rabs knockdowns on YRabs expression, cf. Annex 15). Scale bars, 5µm.

5) Relationship between Rab-specific vacuoles and the female post-mating response

My work has now established the existence of numerous compartments regulating trafficking within the SCs of the AG (Fig.18 & Fig. 19). With the possibility of inactivating the Rabs conditionally through RNAi we can now address the function of these compartments in regulating the PMR in females.

As mentioned earlier, all of the large vacuoles disappear when Rab6 is knocked-down. This is reminiscent of the phenotype observed in the *iab-6*^{cocu} mutant (Fig. 16A-A''). I therefore wondered how similar the *iab6*^{cocu} and Rab6 knockdown phenotypes were at a more detailed level. I started this investigation by comparing the Golgi morphology and distribution in the SCs of the two lines. As Rab6 labels part of the TGN, we might expect the Golgi to be disturbed in the Rab6 knockdown but not in the *iab6*^{cocu} SCs. In fact, however, with both conditions, I observed similar phenotypes. Where in *wild-type* conditions, the Golgi apparatus is centred and has a filamentous-like distribution (Fig. 16B; Annex 13E) while in both loss-of-function conditions, there are several large Golgi-RFP-positive punctate features spread through the cytoplasm of the SCs (Fig. 14B'-B''). This could mean that *iab6*^{cocu} and Rab6 knockdown result in the same defect with respect to the Golgi. Alternatively, it could simply be that the Golgi is unaffected in both conditions and that the *wild-type* distribution pattern is imposed on the Golgi by the presence of the large vacuoles in the cells.

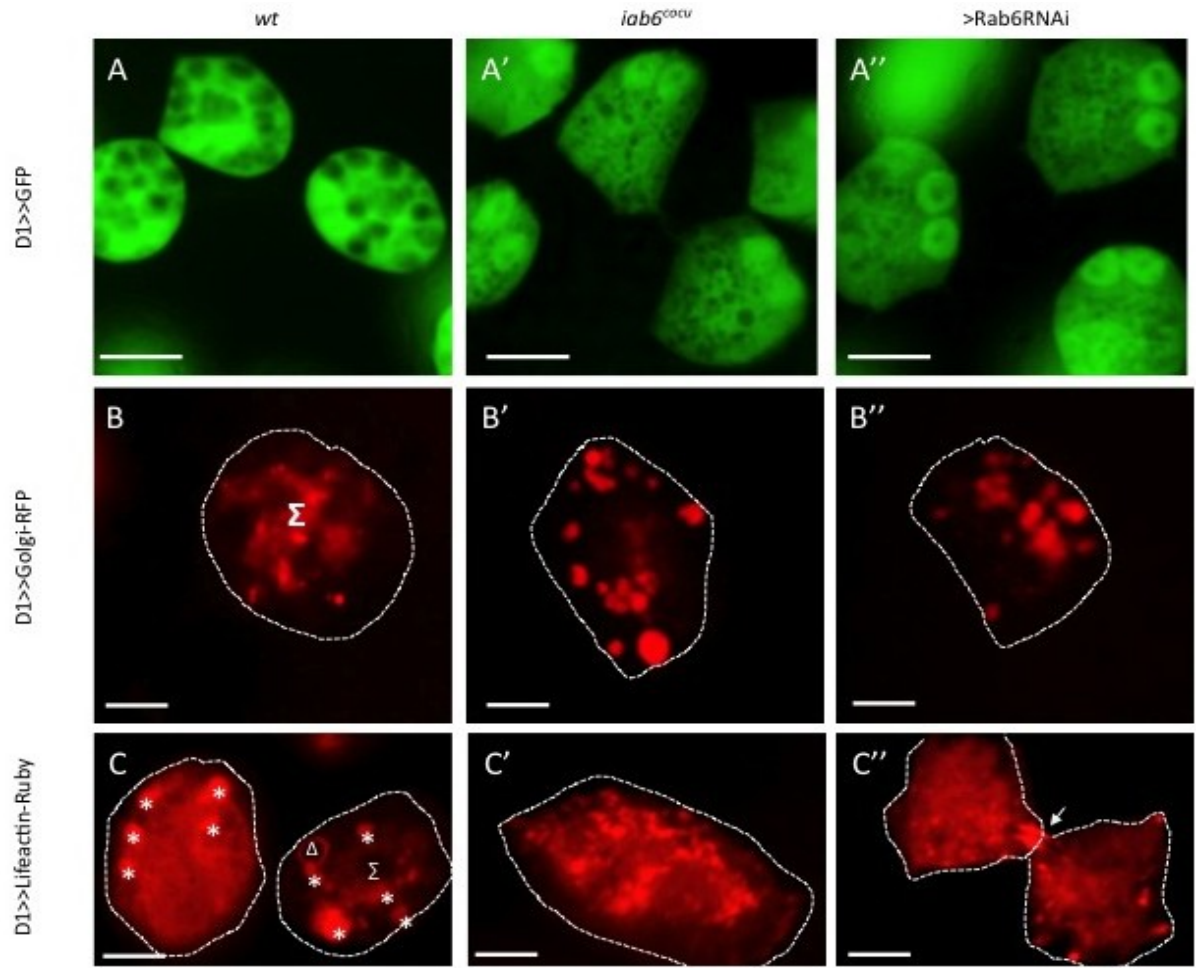


Figure 16. The loss the large vacuoles in the secondary cells disrupts the organelles morphology and distribution.

Fluorescent microscope picture enlargement of secondary cells from live accessory glands expressing GFP (A-A''), Golgi-RFP (B-B'') or Lifeactin-Ruby (C-C'') markers in a secondary-cell specific manner. Rab6 depletion seems to phenocopy *iab6*^{cocu} mutant (A''-C'' and A'-C', respectively). In both conditions, the mutant conditions result in a round-to- pentagonal shape modification of the secondary cells (A'-C' and A''-C'') and the disappearance of the large vacuoles to leave the room for smaller vesicles (A-A''). In *wild-type* conditions, Golgi apparatus is mainly present as a centered filamentous-like structure (Σ), which expands between the secondary cells as well as punctate features (B); however in secondary cells from *iab6*^{cocu} mutant accessory glands (B') as well as in secondary cells depleted of Rab6 (B''), the Golgi apparatus is spread through the cytoplasm as large round-shaped compartments. Actin primarily shows a network-like organization in *wild-type* secondary cells, it seems also to be present into large granules (C, *), on punctate features, on some vacuoles membrane (C, Δ), and to surround a central channel (C,Σ).In secondary cells without large vacuoles, Lifeactin-Ruby signal looks more diffuse and "cloudy" with punctate features (C'-C''). In some cases, these defective secondary cells tend to contact each other by actin-postive bridges shown by an arrow (C''). Scale bars, 5µm.

I also examined the actin cytoskeletal network in both loss-of-function conditions. Like the Golgi distribution, the actin cytoskeleton seems to be disrupted in both mutant lines

when compared to *wild-type*. In *wild-type* conditions, the actin cytoskeleton is organized into numerous F-actin filaments, many defining a central cylindrical channel (Fig. 16C; Annex 10C). In the absence of vacuoles, this organization seems lost, the signal seems more diffuse as if the number of polymerization sites and/or actin-containing vesicles may increases while their size decreases (Fig. 16C'-C'').

Thus, the loss of vacuoles is associated with organelle and cytoskeletal abnormalities. To relate the intra-cellular organization of the SCs with the PMR, I began testing the receptivity of females mated by *wild-type versus* Rab knockdown males. Preliminary results showed that Rab6 and Rab7 depletion in the SCs leads to premature re-mating as observed with *iab6*^{cocu} mutants (females mated to experimental males frequently re-mate after only four days instead of the ten days seen with females mated to *wild-type* males). We observe no changes in receptivity of females mated to Rab19 depleted males. This does not mean that Rab19 vacuoles show no effect on the PMR, as receptivity is only one of many PMR phenotypes that can be monitored. However, these results do support our hypothesis that the large vacuoles and notably the Rab6- and Rab7-positive ones play an important role in female receptivity.

II-Tracking Ovulin to understand the dynamic & the directions of the trafficking routes in the accessory glands

As mentioned throughout this text, our data suggests that seminal fluid proteins (Sfps) such as Ovulin are produced by the MCs but require the vacuoles of the SCs for full post-translational modification (Gligorov, Sitnik et al., 2013). We also know that some

vacuoles contain enzymes (Rylett et al., 2007) and some contain Acps such as Ovulin (Fig. 7 and Monsma et al., 1990). We therefore, wondered if the vacuoles of the SCs are the location for post-translational modifications of main cell products or if they simply transport secondary cell products to the lumen to mediate the protein modifications. To answer to these questions we decided to mark a MC produced Acp and follow its path to the lumen in real time. The obvious choice for this Acp was Ovulin.

Ovulin time points experiments with virgin male AGs stained with an anti-Ovulin antibody (Monsma et al., 1990) and fixed at different ages showed that Ovulin reaches its highest level of expression in both the lumen and the cell layer after five to six days post-eclosion. This developmental time point corresponds to the age of full maturity of the AGs (Fig. 7C). As expected, right after one mating, Ovulin levels decrease both in the epithelium and the lumen of mature males (Annex 18 B).

While Ovulin seems to be primarily made by the MCs, some evidence suggests that Ovulin is transferred between both cell types of AGs (Kalb et al., 1993; Gligorov, Sitnik et al., 2013). Several experiments showed that this Acp could be found in both the main and secondary cells (Monsma et al., 1990; Chapman et al., 2003). I confirmed that Ovulin is present in both cell types, as well as in the lumen by staining virgin *wild-type* male AGs with Ovulin antibody and by examining Ovulin-GFP expressing AGs (Lung & Wolfner, 1999). As observed by Monsma and co-workers, I saw that Ovulin could be found within the large vacuoles of the SCs (Monsma et al., 1990 & Fig. 5A-B). To gain further insight, I decided to look at Ovulin protein levels in the different parts of the AGs, whose SCs miss specific Rab proteins (Annex 16). In preliminary experiments where I knocked-down YRab7, YRab11 or YRab19 by expressing a RNAi directed against the YFP tag of the YRabs in a SC- specific manner, I found that the loss of Rab7 and Rab19 in the SCs seems to affect Ovulin levels in

the SCs while the absence of Rab11 does not. Interestingly, only the absence of Rab7 in the SCs affects the presence of Ovulin in the other compartments that are the MCs and the lumen of the AG (Annex 16). Taken together, these data could suggest that Ovulin might mainly traffic via Rab7-bound compartments within the AGs.

At this point, Ovulin is a molecule known to traffic across the 2 cell types of the AG to then end up in the lumen before being transferred to females upon mating. To gain insights into the direction of Ovulin trafficking I have created a strain expressing an Ovulin-GFP that can be photo converted using the mEos fluorochrome (detailed in Material & Methods). Although we already had an Ovulin-GFP protein under the control of the Ovulin promoter, the high levels of Ovulin expression made it difficult to use this construct for live imaging experiments. The levels of Ovulin present in the lumen and cells made it hard to track small amounts of Ovulin moving in the gland. To bypass this problem while keeping the specificity of the transgene, we decided to use a photoconvertible form of Ovulin where Ovulin made from a single cell or intracellular compartment can be tracked in real time. To do this, the Acp26Aa genomic sequence, containing the coding regions, was amplified by polymerase chain reaction (PCR) and fused at its C-terminus to the photoactivable mEos3.2 protein (Zhang et al., 2012) (Fig. 17A). Then, to create the transgenic flies, the plasmid containing the fusion protein sequence, Ovulin-mEos, was placed on a PhiC31 attB containing plasmid and injected into flies containing an integration platform on the the second chromosome (Bischof et al., 2007). The mEos3.2 protein is advisable for trafficking experiments (Zhang et al., 2012); in its non-converted state, mEos emits in green fluorescence, then after photo-activation by using a ultra-violet laser, mEos switches irreversibly to emit in the red spectra (Fig. 17C). This technique should allow us to distinguish the trafficking of photo-activated Ovulin (red) from the newly synthesized Ovulin

(green). Preliminary controls showed that Ovulin-mEos is present in AGs, mainly in the MCs but also in the vesicles of some SCs; this fusion protein is transferred to the female during mating and the green-to-red photoconversion works (Fig. 17B-D).

The first experiment we would like to perform with this tool is simply to photoactivate Ovulin in a large group of main cells near the secondary cells and watch where this protein goes. Although we believe Ovulin is trafficking from main cells to the secondary cells, this experiment will prove this. We can also see which compartments this Ovulin traffics through by combining Ovulin-mEos with the Rab-YPF lines. If we can show that Ovulin traffics through the secondary cells from the main cells then we will have goes we will have discovered a new mechanism by which proteins can be modified and show how the two cell types collaborate to make and mature a biologically important molecule. We can then begin to ask why this sort of compartmentalization is necessary. First we could examine things like glycosylization of Ovulin protein in the different RNAi knockdown lines. If Ovulin is modified sequentially, then we might be able to isolate numerous intermediate products in its maturation and test if these forms of Ovulin have modified biological activity.

Thus, if these experiments work, we would gain a better understanding of how trafficking events occur within the different part of the AG, what the SCs do and how they contribute to male fertility and reproductive success.

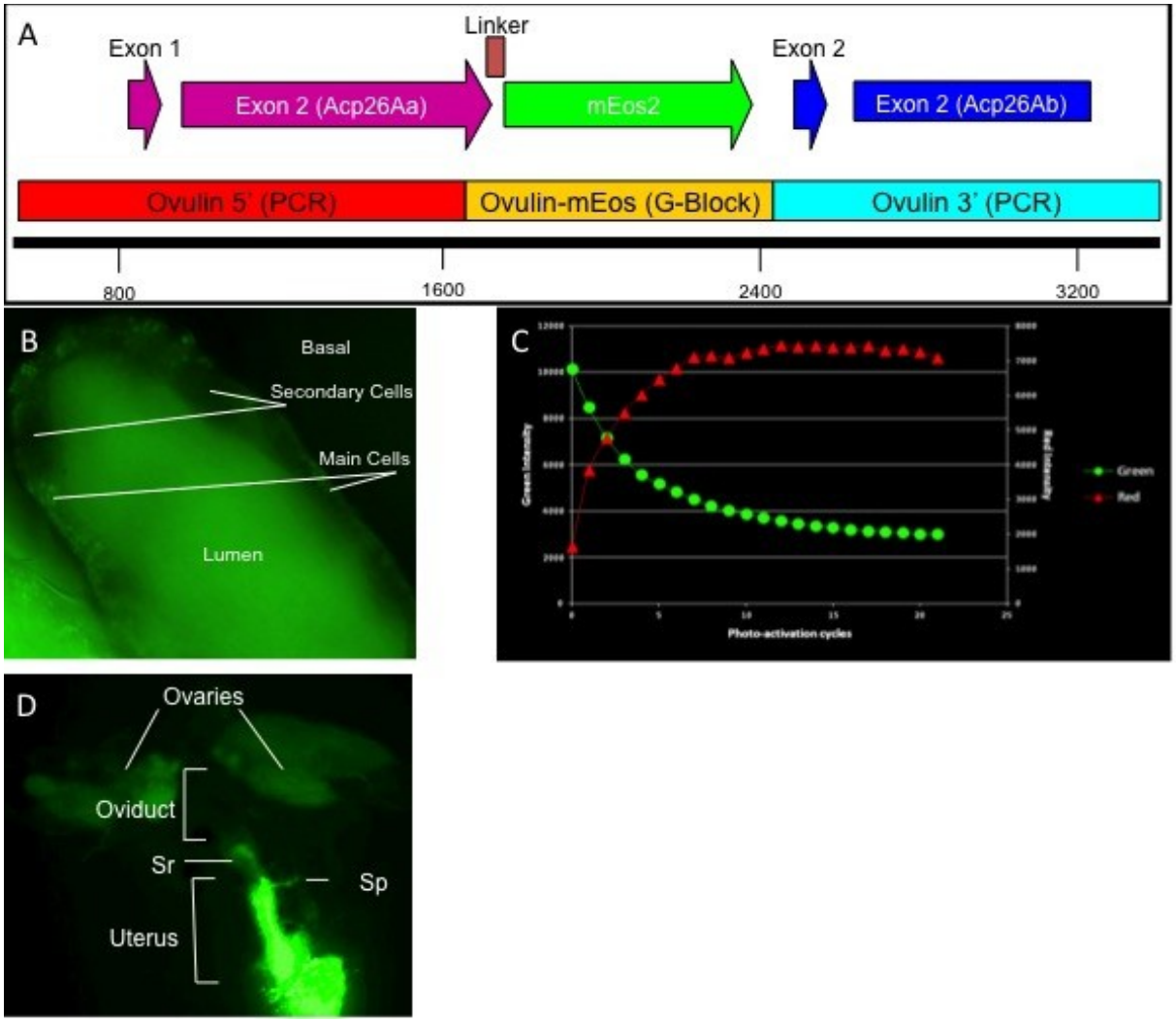


Figure 17. A photo-activable Ovulin to shed light on the direction of trafficking in the accessory glands
Representation of the construction Ovulin-mEos injected and integrated in the chromosome II (A). *On the top*, the large arrows represent coding sequences while the rectangles are not. *On the bottom*, the techniques used to make the construction; the 5' and 3' regions of Acp26A genomic sequence have been amplified by PCR while G-block have been made containing mEos3.2 sequence as well as adapted primers sequences allowing to ligate and then clone these three pieces together.
Fluorescent picture of the tip of a live accessory gland from an Ovulin-mEos male (B). The non-converted green fluorescent protein is visibly present into the lumen as a diffuse signal and in the main cells as large punctae. The secondary cells appear as black wholes in the accessory glands epithelium.
Quantification of green and red fluorescent intensities depending on the number of photo-activation cycles (C). As the number of photo-activation cycles increases, the green signal decreases while the red one increases.
Fluorescent picture of a live genital tract taken from a *wild-type* female mated by an Ovulin-mEos male (D). This picture has bee taken five minutes after mating and the green-fluorescent Ovulin-mEos protein is visible in the female uterus.

Materials & Methods

Fly stocks

Male collections were performed at 25°C. *D1-Gal4* was generated in the lab, YFP-tagged Rabs (YRabs), *UAS-Tomato-myristoylation*, *UAS-KDEL-RFP*, *UAS-LampI-GFP*, *UAS-Arf79F-GFP*, *UAS-Lifeactin-Ruby*, *UAS-Golgi-RFP* and *UAS-gfpRNAi* lines were provided by S. Eaton's lab. *UAS-Rab6RNAi* (ID100774), *UAS-Rab19RNAi* (ID103653) were available from Vienna Drosophila Resource Center, *UAS-Rab7RNAi* line was from M. Gonzalez-Gaitan's laboratory and *Ovulin-GFP* (Fig. 4B) was from M. F. Wolfner's laboratory.

Immunochemistery

Male accessory glands were dissected in ice-cold Grace's Insect Medium (GIM, BioConcept), fixed for 20 minutes with 4% Formaldehyde (Sigma) at room temperature and stained with one or more of the following antibodies over-night at 4°C: anti-Disc Large (Developmental Studies Hybridoma Bank (DHSB)), anti-DE-cadherin (DHSB), Phalloidin-546 (Life Technologies) and anti-GFP (Invitrogen, Annex 12). The detection (except Phalloidin-546) could require incubation of the glands with a secondary fluorescent antibody for 90 minutes at room temperature. All the samples were mounted in Vectashield mounting medium with or without DAPI (Vector Labs) on a cover-slip bridge. The pictures were mostly taken with a Zeiss LSM700 confocal microscope (Bioimaging Platform University of Geneva) but some samples were imaged with a Zeiss LSM510 UV (MPI-CPG, Dresden, Annex 12), a Zeiss LSM510 Axioplan 2 (C. Wilson's laboratory, Oxford university, Annex 9) or a OMX V3 Optical

Microscope (Micron, Oxford University, Annexes 8 & 9). Those images were evaluated using the FIJI and IMARIS softwares.

Quantification of Ovulin levels in the cells

Accessory glands were prepared as explained before, incubated with an anti-Ovulin antibody, which was immunodetected via a secondary fluorescent antibody, Alexa Fluor 555 (Thermofisher). The glands were imaged with a Zeiss LSM700 and the fluorescence intensity of the red channel was determined using FIJI software. The measurements were done Z-stacks of glands. The area of each cell was determined and the background was subtracted in function of it giving the Corrected Total Cell Fluorescence (CTCF) value for each cell. All the negative values were automatically changed to zero on Excel Office software. Slides background was used for background subtraction. Error bars, Standard Error of Mean (SEM). For the statistics, a One-Way ANOVA analysis and a *Tukey* HSD Test were performed (http://vassarstats.net); number of cells, N= 2-18 (*ie* 1-10 accessory glands) (Fig. 5C) or an un-paired *t*-test has been performed to compare the average of values obtained for mated samples to the virgin ones (http://www.graphpad.com); number of cells, N= 24-423 (*ie* 2-13 accessory glands) (Annex 18)

For the Annex 16, for each slide of an image (each 0.8µm), from the apical to the basal side, the cell area was determined and the fluorescence intensity in this area was measured by FIJI. The background was subtracted for each slide in function of it giving the Corrected Total Cell Fluorescence (CTCF) value for each section. All the negative values were automatically changed to zero on Excel Office software. To obtain the total fluorescence per cell, the CTCF for each slide of a cell were added. Slides background was used for

background subtraction. For the statistics, an ANOVA test was performed using PRISM software; number of accessory glands, N= 6-17.

Quantification of the expression levels of the 27 YRabs screened in the cells

During the screening of the library, an anti-GFP antibody has been use to ensure detection in case of weak signal. The glands were prepared as explained previously and the GFP was revealed via a secondary antibody, Alexa Fluor 488 (Thermofisher). After acquisition, the images were evaluated and the signal intensity measured by FIJI software. The area of each cell Z-stack was determined and the background was subtracted in function of it giving the Corrected Total Cell Fluorescence (CTCF) value for each cell. All the negative values were automatically changed to zero on Excel Office software. Oregon R accessory glands were used for background subtraction. Error bars, SEM. For the statistics an unpaired *t*-test was performed between the average of fluorescence intensities obtained for secondary cells and main cells (http://www.graphpad.com). Number of cells, N= 3-32 (*ie* 2-10 accessory glands).

Quantification of YRab signal in the secondary cells

Endogenous YFP fluorescence levels in the secondary cells were determined using FIJI software. For each slide of an image (each 0.8µm), from the apical to the basal side, the cell area was determined and the fluorescence intensity in this area was measured by FIJI. The background was subtracted for each slide in function of it giving the Corrected Total Cell Fluorescence (CTCF) value for each section. All the negative values were automatically changed to zero on Excel Office software. To obtain the total fluorescence per cell, the CTCF for each slide of a cell were added. Canton S or *UAS-RabRNAi* accessory glands were used for background substraction. Error bars, SEM. For Annex 14, an unpaired *t*-test was

performedto compare values of mated samples with the virgin ones (http://www.graphpad.com); number of cells, N= 68-241 (*ie* 4-17 accessory glands). For Annexe 15, an unpaired *t*-test was performed to compare values of Rab knockdown glands with *wild-type* ones; number of cells, N= 36-286 (*ie* 18-70 accessory glands). For Annex 17, an unpaired *t*-test was performed between the fluorescence intensity value obtained at a time-point and at the previous one; number of accessory glands, N= 1-10.

Generation of the Ovulin-mEos transgenic line

1- Construct: The aim is to fuse the photo-activable GFP, mEos 3.2, to the C-terminal of the coding region of Ovulin (Acp26Aa).

First, Ovulin genomic region sequence has been identified on FlyBase and the stop codon Acp26Aa coding sequence have been found (MacVector software); this will be the fusion site with mEos. Then, primers located around 1kb up- and down-stream of the fusion site were identified (Ovulin5' S & Ovulin 3' AS) as well as primers surrounding the fusion site that could be used for assembling and defining the 5' and 3' regions (Ovulin5' AS & Ovulin3' S).

Second, the gBlock Gene Fragment Ovulin-mEos 3.2 (OvmEos2, 796bp) has been deisgned on MacVector, generated and delivered by Integrated DNA Technologies (IDT); a linker was inserted instead of the stop codon of the exon 2 of Acp26Aa and right after this linker, the open reading frame of mEos 3.2 was positioned in order to be in frame.

Third, the GIBSON Assembly was designed; overlapping oligos were designed by NEBuilder in order to insert the three fragments (Ovulin5' (1165bp), OvmEos2 (796bp) and Ovulin3' (1001bp)) in the pAttB plasmid (7406bp). Oligos were provided by Eurofins and the sequences are found below;

Ovulin5'S: CTAGCGGATCCGGAATTGGGGCCTCGACTAAAGCACCCGATGC;

Ovulin5' AS: GATTTCCCTGACTAGGATTTGTCTCCGTGGGTACATCGG;

Ovulin3' S: AGAACAGAAACAATAGTATTGAGTCATTGCAAGACCTTCTAATTGCGC;

Ovulin3' AS: CGGCCGCAGATCTGTTACGCACCACGGGGACCACAGGTAGC.

Four, the four fragments were assembled following GIBSON Assembly protocol (HiFi DNA Assembly Protocol). The clone obtained was confirmed by sequencing (Fasteris).

2- Generation of the transgenic line:

The pAttB-Ovulin-mEos3.2 DNA sample has been injected by Bestgene service in the germ-line of PhiC31-51C flies allowing a site-specific integration of the transgene in the chromosome II (Bishoff et al., 2007).

Four individual transgenic lines have been obtained (Ov-mEos M1-5) and checked by fluorescence microscopy (Zeiss Axioplan).

3-Live imaging & Photo-activation tests

Ov-mEos males were dissected in ice-cold GIM and the accessory glands were kept in GIM at 4°C. A gland was then embedded in a 37°C live-imaging mounting medium made of GIM and Fibrinogen. Thrombine was then added making Fibrinogen polymerize, which created filaments allowing us to form a cage around the gland. Once polymerization, the encaged gland was covered by GIM. The sample was kept at 4°C until imaging.

Photo-activation tests were carried out with an Olympus Fluo View 1000 microscope (M. Gonzalez-Gaitan's facility) with the help of E. Derivery. Photo-activations cycles were performed via pulses of laser 405 during which the live tissue is imaged simultaneously in green and red channels. The green- and red-fluorescence intensities were measured by FIJI software for each photo-activation cycle.

Discussion

During my PhD, I analysed the cellular architecture of the *Drosophila* accessory glands (AGs) and the possible interactions between its two epithelial-cell types: the main cells (MCs) and the secondary cells (SCs). The main aim of my project was to shed light on how the SCs function through a better understanding of the large vacuoles filling their cytoplasm. To do this, I examined the expression patterns of the Rab proteins that act as master regulators of intracellular vesicular transport using Rab knock-in fluorescent protein fusions. The use of this knock-in library was key to my studies because it allowed me to examine the *wild-type* expression and localization of these proteins. By documenting their distribution and expression pattern in the AGs and comparing them to these expression patterns to other secretory cell types previously studied, we have been able to hypothesize possible transport routes within the AGs. These studies provide a solid foundation from which we can test operation and function of the different cells of the AG.

I- The *Drosophila* accessory glands are polarized secretory tissues

At a structural level, an epithelium is mostly defined as a sheet of cells bound together through cell junctions. In *Drosophila* there are two primary types of cell junctions. First, there are the adherens junctions (reviewed in R-Boulan & Macara, 2014). These junctions delineate the apical side of the cells and are marked by epithelial cadherin (E-cad). Then, on the baso-lateral side of insect cells, there are the septate junctions. Unlike the

adherens junctions that form a relatively tight ring around the apical side of the cell, the septate junctions are often present along much of the lateral surface of the cell, making ladder-like structures along the apical-basal axis in cross section images. Disc-Large (Dlg) is a membrane-associated protein that is present in septate junctions and is required for the basal-lateral identity of polarized tissues (Knust & Leptin, 1996; Woods et al., 1996). Using the cell polarity markers *Drosophila* E-cad (DCAD) and Dlg, we were able to show that the cells of the AG are highly polarized in nature with the apical side facing the lumen and the basal side facing the exterior of the gland (Fig. 6C-D). These results are largely in support of previous studies (Bairati, 1968; Leiblich et al., 2012).

We also were able to determine the basic shape of the cells of the gland. Once again, these results are similar to that previously described, though our images provide a better view of the cells in three dimensions. Perhaps the best structural study of the cells came from Bairati. (1968), using high resolution EM on sectioned AGs; A. Bairati describes the MCs as being “prism-shaped” and the SCs as being “pear-shaped”. Our use of cell polarity markers allowed us to examine the surfaces of the cells to extend this description. My results show that the MCs look to be a fairly uniform sheet of cells, but with an interesting feature on the apical side. Phalloidin staining showed that the apical surface of the MCs seems to have actin-filled, cilia-like structures emerging from the surface of the cells and into the lumen (Annex 10A-B). These structures are probably what Bairati describes as “irregular cytoplasmic expansions” on the apical side of the MCs (Bairati, 1968). As MCs are known to produce most of the Sfps secreted by the gland, these filaments could represent roadways by which cargo molecules are transported towards the lumen. As these structures are also reminiscent of gut microvilli, the expanded surface area exposed to the lumen could also serve as an ideal surface through which elements in the lumen can be endocytosed.

As mentioned above, the SCs have a “pear-like” shape. In 3D reconstructions of the glands, we see that these cells protrude into the lumen of the gland. But this protrusion does not result in more SC surface exposed to the lumen. In fact, DCAD staining shows that the SC surface facing the lumen is quite small, being restricted by the MCs that seem to crawl up over the SCs (Fig.6D). Furthermore, as seen for the MCs, Phalloidin staining revealed cilia-like actin filaments emerging from the apical surface of the SCs (Annex 10A-B).

This seemed odd to us, as the SCs, like the MCs, are thought to be secretory in nature and would be expected to secrete their products towards the lumen, where the Sfps are stored. We had thus predicted that their surface facing the lumen would be larger, reflecting the importance of the surface for the targeting of the secretory machinery or from the increased membrane deposition from the secretion of the large, SC vacuoles.

Secretory Rabs in the main cells

Consistent with the secretions of the gland being sent to the lumen, we would also expect that the secretory systems of these cells would be oriented towards the apical/luminal side of the cells. To look at this, we examined the Rab proteins distribution and localization in the AGs. In the MCs, I found that many Rabs are localized predominantly in the basal/medial parts of the cell. This is true for Rabs involved in early- (Rab4, Rab5, Rab21) and late-endocytosis (Rab7, Rab39), recycling (Rab11) and secretion (Rab1, Rab2, Rab6, Rab18). This could suggest that a substantial portion of the trafficking routes in the MCs is dedicated to the transport of material towards and from the basement membrane rather than towards the lumen (Fig.8; Fig.10; Fig.11B; Annexes 1, 2B & 2E). These observations seem at odds with the apical secretion model. Interestingly, this distribution is reminiscent of another secretory tissue, the salivary gland (SG), where secretion should also be oriented towards an apical lumen (Dunst et al., 2015). It has been suggested that these

cells use non-canonical Rabs to help in directed secretion towards the apical side. In the case of the SGs, this was proposed to be Rab19 and Rab30 due to their localization on the apical side of the SG cells. Although these Rabs have never directly been shown to be involved in the transportation of cargo towards the PM, the fact that both Rabs are present in neuronal projections where directed secretion is required, suggests that these Rabs might be used in the secretory pathway (Dunst et al., 2015). Unfortunately, neither Rab19 nor Rab30 is present in the MCs to fulfil this function. Thus, if there is apically directed secretion in the MCs, another Rab must be present for secretion destined for the apical surface. Because we have not found this apically localized secretory Rab, it may be that secretion in the MCs is not directional, using, for example, the non-localized Rab8 protein to secrete everywhere, but using basal endocytosis to retrieve improperly directed cargo (Fig. 8D; Annex 1D). A similar mechanism has been shown to take place in the wing disc in their secretion of the Hedgehog protein (Callejo et al., 2011). Alternatively, it is also possible that MC products are not secreted apically and reach the lumen via an alternative pathway.

Rab protein localization suggests non-canonical transport pathways within the secondary cells

The pattern of the Rabs within the SCs is very different from that seen in the MCs and suggests different trafficking routes. Being larger cells, the SCs show a striking polarity with regards to the Rabs. We can find Rabs that are specifically on the medial/apical side (Rab39, Golgi-LE transport & RabX1, recycling; Annexes 2E & 2Fc-c'', respectively), on the basal/medial side (Rab1, Rab2, Rab18) (Fig. 8A-B; Fig. 11B; Annexes 1A-B & 2B) and on both sides of the cell but with different patterns across the apical-basal axis of the cell (Rab6, Rab7, Rab11, Rab19) (Fig. 8C; Fig. 10C-D; Fig. 11C; Annexes 1C, 1G-H & 2C). For example,

Rab6, Rab7 and Rab19 mark uniformly distributed punctate features basally and large vacuolar compartments on the apical side of the cell (Fig. 8C; Fig. 10C; Fig. 11C; Annexes 1C, 1G & 2C). Meanwhile, Rab11 marks basal/medial located large, cortical vacuoles and apical punctate structures (Fig.10D; Annex 1H).

One of the main interests of my studies was trying to understand the polarity of secretion in the AGs and the SCs in particular. However, Rab8, a Rab that is canonically associated with secretory vesicles, is very weakly expressed in the SCs (Fig. 4; Fig. 8D; Annexes 1D & 12). Given these results, the SCs probably secrete apically using non-typical pathways. Here, I will summarize what I have discovered about the SCs and hypothesize how apical secretion might be accomplished in these cells.

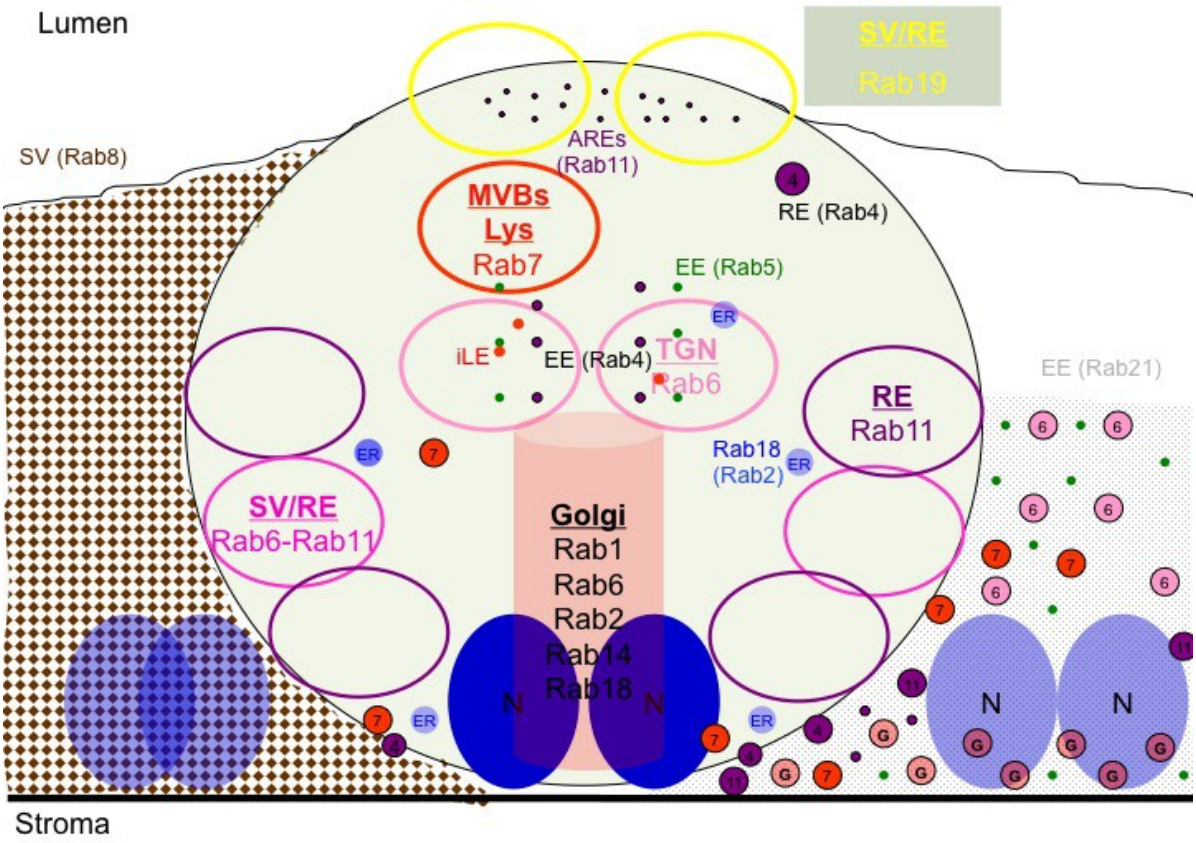


Figure 18. The epithelium of the accessory glands is polarized and secretory
Schematic representation of the different compartments labelled by the Rab GTPases within both cell types. In the drawing, a central, round-shaped, secondary cell is surrounded is by two main cells. The nuclei are present basally (N) and the Golgi apparatus is represented by the central cylinder in the SCs or as small salmon circles (G) in the MCs. The Endoplasmic reticulum location (ER) is based on the expression of the KDEL-RFP reporter expressed specifically in the SCs and is labelled in blue. The empty large circles are the large vacuoles and the colour-filled small circles are the large/small compartments. In one MC, I have shown the Rab8 punctate features as brown squares. The location of the Rab proteins on this different features and organelles are indicated. The location of the Golgi apparati (G) in the MCs is based on the Rab1, Rab2 and Rab6 expression pattern. The differences in the two MCs are purely aesthetic for clarity. Abbreviations: G, Golgi; SV, secretory vesicles; RE, recycling endosome; iLE, immature late endosome; EE, early endosome; TGN, *trans*-Golgi network; ER, endoplasmic reticulum; ARE, apical recycling endosome.

Using the Golgi-RFP marker, we have shown that the Golgi apparatus is located primarily on the basal-medial portion of the SCs cells at the beginning of a large central channel (Annex 13E). Consistent with this result, within this channel, we found a number of elements marked by Rab proteins known to mark the Golgi apparatus (Fig. 9 & Annex 4). Interestingly, however, the different Rabs show slightly different localization patterns, perhaps reflecting specialized compartments within the early secretory pathway. For example, Rab1 and Rab2 are known to regulate the transport between the ER and Golgi. In other cells, both of these Rabs have been shown to bind to both the ER and the Golgi (particularly the *cis*-Golgi) (Bhuin & Roy, 2014; Dunst et al., 2015). In the SCs, Rab1 is enriched in the large basal/medial central channel and perfectly co-localized with the Golgi-RFP marker (Fig. 8A; Fig. 9A-A''; Annex 1A), while Rab2 is present on basal/medial peripheral punctae (Annex 1B). Given the co-localization of the Golgi-RFP marker with Rab1 and other Golgi-related Rabs, like Rab6, we believe the central channel staining represents Rab1's role in Golgi trafficking (Fig. 9). KDEL-RFP, a marker for the ER can be seen as a diffuse cloud together with punctuate features surrounding the central channel throughout the cytoplasm of the cell (Annex 13F). Some of the Rab2 punctae co-localize with basal-medial parts of this cloud (Annex 5B-B''). Based on these results, we believe that the ER is located in the basal-medial portion of the cell and that Rab2 may be localizing to a subset of

ER. Another Rab that should be mentioned here is Rab18. Rab18 is a protein thought to be involved in the transport of products from the Golgi and can localize with the Golgi and ER (Bhuin & Roy, 2014; Gillingham, Sinka et al., 2014). Indeed, in examining the localization of Rab18, I found that it co-localized strongly with most of the KDEL-RFP and Golgi-RFP signal (Annexes 4B-B'' & 5C-C''). The differences between Rab18 localization and that of Rab2, may indicate that Rab18 marks all of the ER and Golgi (and the general transport of newly synthesized molecules in the Golgi and ER), but that Rab2 marks a more specialized part of ER. Given the localization of the Golgi to the central channel, these results also suggest that the central channel might be the main thoroughfare for protein trafficking in the SCs towards the apical surface.

S. Dunst et al. have suggested that Rab19 and Rab30 might mediate apical secretion in the SG. As in the SG, Rab19 is found on the apical side of the SCs, as a large vacuole structure. As Rab19 vacuoles are the most apical vacuole structure in the SCS, it is possible that these cells use Rab19 for apically directed secretion, like the model proposed for the SGs (Fig. 11C: Annex 2C). One caveat to this model is the fact that very little Tomato-myristoylated punctae can be found in the Rab19 vacuoles. This suggests that Rab19 vacuoles are probably not the destination for products made by the SCs and directed toward the PM. This, however, does not mean that Rab19 vacuoles do not process-recycled products for apical secretion, simply that SC cell made products do not traffic through these vacuoles.

If Rab19 vacuoles represent part of an apical pathway directed towards the PM, then how does it release its products into the lumen? A. Bairati claims to have captured vacuoles in the process of fusing to the luminal apical membrane in his EM studies (Bairati, 1968). However, I have never seen this in all of the glands that I have examined, nor have I seen the

Rabs that mark the vacuoles located on the PM. I have tried to increase my chances of witnessing these events by mating the males multiple times to empty the contents of the lumen and looking at the glands after different amounts of time post-mating. During this process, I never managed to capture a vacuole-PM fusion event. Also, if I compare the SCs from mated and virgin males, the number of vacuoles never seems to change. Thus, if Rab19 vacuoles are large secretory/recycling vesicles, they either do not frequently fuse to the PM or they fuse and are replaced at a rate too fast to capture. Alternatively, Rab19 vacuoles could simply represent a compartment from which molecules are secreted via smaller secretory vesicle intermediaries since I can sometimes observe myristoylated punctae features around the Rab19 vacuoles (Annex 6D-D''). In my examination of Rab11 localization, I noticed many Rab11 positive punctae in the apical region of the cells (Annex 1H). Perhaps Rab11 could be transporting molecules from Rab19 vacuoles to the cell surface. As Rab11 is a Rab associated with recycling endosomes that traffic back towards the plasma membrane, I believe this may be a possibility.

II- The Rab proteins can reflect different transport pathways within the secondary cells

Most of the Rab proteins show an odd pattern within the SCs relative to other cell types. This could be due to the spatial constraint imposed by the presence of the large vacuoles in the cytoplasm. However, it must also be noted that the SCs contain a very specific Rab expression profile, some highly expressed (Rab4 & Rab18), some less common Rabs being specifically expressed (Rab14 & Rab19) and some common Rabs poorly or not

expressed at all (Rab8) (Annex 12). Here, I will discuss the oddly expressed Rabs in hopes of gaining further insight into the function of the SCs.

The Rabs highly expressed in the secondary cells

In SCs, Rab4 is present on features with different sizes and distribution: peripheral basal and apical punctae and centrally clustered punctae (Annex 1E; Fig. 10A). Rab4 is thought of as an early-endocytic Rab that can mediate fast recycling or transport of EEs to the TGN (Bhuin &Roy, 2014; Mohrmann et al., 2012; Ghillingham, Sinka et al., 2014). But Rab4 has also been shown to sometimes function redundantly with the core recycling GTPase, Rab11 (Woichanski et al., 2016). As Rab11 is primarily present on vacuolar structures located in the cortical-medial zone and on centred punctate features apically (Fig. 10D; Annexe 1H), this could suggest that the basal Rab4-labelled punctae may be basal recycling endosomes. Interestingly, the Rab4-positive apical and basal punctae are mostly cortical, suggesting that Rab4 could mediate fast recycling of internalized material. Whether this recycling is toward the stromal compartment (basal side) or toward the covering MCs (apical) or both is still unclear (Annex 1E). Meanwhile, the centrally clustered punctate features surround the central channel, which we have shown, contains the Golgi apparatus (Annex 13E). Based on this, the central Rab4 compartments may represent Rab4's role in the transport of EE content to the Golgi apparatus.

The secondary-cell specific Rab proteins

Two Rab proteins are SC-specific *ie* Rab14 and Rab19 (Fig.11A & C; Annex 2A & C). At the sequence level, Rab14 is most closely related to the exocytic Rab2 and the endocytic Rab4 and has been implicated in both exocytic and endocytic pathways (Fig. 4 & Fig. 5). Rab14 can be an intermediate in the EE-to-LE maturation (Fig. 4; Garg & Wu, 2013) and/or play a role in the transport of cargo from the TGN to the endosomal compartments (Fig. 4;

Bhuin & Roy, 2014; Kitt et al., 2008; Junutula et al., 2004). In the SCs, Rab14-bound features primarily co-localize with the Golgi apparatus and some punctae co-localize with our myristoylation marker. This suggests that SCs use Rab14 in the biosynthetic pathways, trafficking from the Golgi towards the PM (Annexes 4A-A'' & 6C-C''). As Rab14 is not well expressed in *Drosophila* (Dunst et al., 2015), the SCs may be the ideal location to study the function of this fairly rare Rab in *Drosophila* (Fig. 11A).

Rab19's function is mostly unknown, though it has been shown to be localized to the Golgi in both *Drosophila* S2 cells and in Mammalian cells (Sinka et al., 2008; Huntagalung & Novick, 2013; Seabra et al., 2002) and to the recycling endosomes in *Drosophila* neurons (Chan et al., 2011; Harris & Littleton, 2011; White II et al., 2015). In light of its apical distribution within different secretory epithelium like the SGs, and its enrichment in axonal termini, Rab19 has been suggested to play a role in apical secretion (Dunst et al., 2015). This could fit with the pattern of Rab19 within the SCs where its major area of localization are at large, very apically located, vacuoles, facing the lumen (see above, Fig. 11C; Annex 2C).

Interestingly, we also found some Rab19-bound "blobs" within the lumen of >3 days-old virgin males (Annex 11). Based on their size and the fact that the SCs seem to be the exclusive source of Rab19 in the AG, we believe these luminal "blobs" are the actual SC Rab19 vacuoles being released into the lumen. If this is true, then this could be a vital clue into how SCs secrete products into the lumen. As we have never seen a SC secrete things into the lumen, Rab19 vacuoles in the lumen could tell us if the cells use an apocrine, holocrine or merocrine form of secretion. To distinguish between these options, I would like to seen if a second membrane surrounds the Rab19 vacuoles in the lumen or if they are floating "naked" in the lumen. In apocrine secretion, cells essentially bud off parts of themselves with cellular components contained within these buds. Thus, if SCs use apocrine

secretion, the Rab19 vacuoles should exist in the lumen as vesicles within vesicles. In holocrine secretion, the cells burst open to release their contents. If the Rab19 vacuoles are present within the lumen, exposed to the luminal fluid, then holocrine secretion might be used (as suggested by Bairati, 1968). However, if this were the case, then we might expect to find large vacuoles marked with other Rabs to be present in the lumen. The fact that we do not find these other vacuoles sheds some doubt on this mechanism of secretion, but cannot exclude it. In merocrine secretion, vesicles fuse with the membrane to release their contents. Thus, if Rab19 vacuoles would fuse with the PM, then we would not expect to see large Rab19 vacuoles in the lumen. However, it is possible that the Rab19 vacuole is first sequestered within a second membrane before fusion. As we have seen that none of the Rabs co-localize with Rab19, we believe this to be unlikely. Still, it has been shown that SCs secrete exosomes into the lumen that are contained within large vacuoles (MVBs and DCGs) (Corrigan et al., 2014; Redhai et al., 2016). Thus, there is a precedent for vesicles being present within other vesicles in this cell type.

Other vacuoles-labelling Rab proteins and vacuole maturation

Vacuoles that contain the exosomes have been shown to be marked by Rab7, sometimes Rab7 and Rab11. Rab7 marks vacuoles with a low pH and thus seem to be mature late endosmes (LEs) or lysosomes. However, it has been shown that multi-vesicular bodies (MVBs) are also acidic and thus may represent related compartments (Corrigan et al., 2014). A subset of Rab11-labeled large vacuoles have been called dense core granules (DCGs) because they contain light and electron-dense elements that have been suggested to be secretory vesicles (SVs) (Corrigan et al., 2014; Redhai et al., 2016). These DCGs have been shown to contain the BMP homolgue, Dpp, that is required for MVB formation or secretion (Rylett et al., 2007; Redhai et al., 2016). Corrigan et al. have suggested that the doubly

marked Rab7/11 arise from fusion events between Rab11-positive vacuoles and immature Rab7-positive LEs (iLEs) and that Rab11-positives vacuoles and MVBs can interact to exchange exosomes (Corrigan et al., 2014). It is interesting to note that I was unable to observe co-localization of Rab7 and Rab11 on vacuoles. One possibility for this discrepancy is that I used Rabs that were endogenously expressed, fused to different fluorophores, while Corrigan et al. used overexpression constructs. As the CFP marker that I used fused to Rab11 was quite weak (though, due to it being a knock-in construct, probably reflective of its wild-type expression levels), it may have prevented the detection of Rab11/7 vacuoles. However, I must also point out that overexpression of Rabs is known to occasionally lead to artifactual results (Chan et al., 2011; Corrigan et al., 2014). In fact, in their work, they show that overexpression of Rab11 causes morphological changes in other cellular compartments in the SCs. Therefore, it is possible that some affinity exists between Rab7 and Rab11 that allows for co-localization of these Rabs under some circumstances, when an excess of Rab11 is present.

My work, for the most part, does not contradict the work done by Corrigan et al. I find that Rab7 is present on one or two large vacuoles that may be lysosomes or MVBs. I find that Ovulin might be present in these vacuoles, which could be an indication of lysosomal degradation or sequestration into MVBs. For Rab11, I find that it is present on a number of large vacuoles that form a ring around the medial portion of the SCs, near the PM. Portions of these large vacuoles seem to co-localize with our Tom-myr marker for secretion. Thus, Rab11 vacuoles may be involved in the secretion of SC products (like Dpp), as well as being involved in recycling. Its location, however seems to indicate that any secretion coming from the Rab11 vacuoles would be oriented towards the lateral part of the cells, facing the MCs rather than the lumen.

The 4th vacuolar Rab is Rab6. From what we know of Rab6 from the literature, it seems to label the TGN. As we have previously shown in the lab in collaboration with the lab of M. F. Wolfner, a number of Acps are improperly glycosylated in *iab-6*^{cocu} mutants, which lack vacuoles (Gligorov, Sitnik et al., 2013). Given that much glycosylation takes place in the TGN, it seems likely that the Rab6 compartment plays a large role in SC function. In my work, I found that Rab6 marks a number of large vacuoles located apically, suggesting a role in the pathway towards apical secretion. Consistent with this possibility, within the *Drosophila* photoreceptors, Rab6 is located on the TGN as well as co-localizing with Rab11 on some vesicles directed to the apical membrane (Iwanami et al., 2016). I, too, have found that Rab6 vacuoles often co-localize with Rab11 at one or two large vacuoles. These double Rab-labelled vacuoles are also marked by the myristoylation marker and thus, may similarly perform a function in secretion (Fig.13B-B'''). However, unlike in the photoreceptor cells, these doubly labelled vacuoles are generally found at the medial PM next to an adjacent MC. This suggests that these vacuoles might be involved in a biosynthetic and/or recycling pathway from the Golgi apparatus of the SCs towards the MCs (Fig.12B-B''; Fig.13B-B'''; Annex 8B). Alternatively, it is possible that Rab6/11 vacuoles later move towards the apical Rab6 vacuoles for secretion, perhaps losing the Rab11 marker along the way.

The doubly marked Rab6/11 vacuoles suggest the possibility of a maturation process in vacuole development. Given the function of the SCs, it may be possible that all of the vacuoles are part of pathway to transport cargo to the lumen. Although we do not believe this to be exclusively the case, as the Rab7 and some Rab11 vacuoles seem to have very different appearances, a subset of vacuoles could form a transport pathway. Perhaps Rab6/11 vacuoles represent the capture of an intermediate species along this pathway. This is not so different from what happens in endocytosis where the Rab proteins have to be

coordinated to allow the sequential transport and/or maturation steps of cargos. For example, EEs have to mature or be converted into LEs and this can be seen via a switch of the activated membrane-bound Rabs (Rink et al., 2005; Del Conte-Zerial et al., 2008; Poteryaev et al., 2010; Huotari & Helenius, 2011). Alternatively, the doubly labelled vacuoles could be the result of a fusion of Rab11 and Rab16 vacuoles to bring recycled components to a TGN-like compartment for modification.

My developmental time points show that SCs change during the first few days after mating. We show that Rab6-labelled large vacuoles are the first visible, appearing during the three first hours after birth (Fig.14Aa). Rab11 and Rab19-positive vacuoles don't appear until around five-to-nine hours after eclosion (and continue to mature for much longer) (Fig.14Cb & Db, respectively). Thus it is possible that Rab6 vacuoles are required for Rab11 vacuole to appear. Although ideally, I would have liked to perform live imaging to watch the development of the glands, I had difficulty keeping glands alive long enough to continuously image the very slow process of vacuole maturation on live glands. Because of this, we attempted to understand the relationship between vacuoles by performing epistasis-type experiments using RNAi. These experiments showed that all of the large vacuoles require the presence of Rab6 to be formed, consistent with the idea that the large vacuoles may require "maturation" from a Rab6 compartment (Fig. 15B-B', C-C' & D-D'; Fig. 16A-A''). However, since the depletion of Rab6 also affects the morphology of the Golgi apparatus itself, as well as the actin network (Fig.16B & B", C & C''), it is hard to know if the effects we see are due to Golgi Rab6 defects or vacuole Rab6 defects. One point in favour of the vacuole argument is that the RFP-Golgi marker seems to be expressed as well in Rab6 knockdown as in *wild type*, though its localization is modified, probably due to the lack of the large vacuoles in these cells.

These knockdown experiments also showed that knockdown of Rab7 affected Rab6 and Rab19 vacuoles. As Rab7 compartments are downstream of the Golgi, these experiments may indicate a strong relationship between Rab7 and Rab6 and Rab19, consistent with the idea of compartment maturation.

Possible intercellular trafficking between the main cells and the secondary cells

Previously, we showed that Ovulin, a protein thought to be made primarily in the MCs, was not correctly glycosylated in *iab-6*^{cocu} mutant males (Gligorov, Sitnik et al., 2013). As *iab-6*^{cocu} affects the expression of BX-C gene products in the SCs, we wondered how SCs could affect the post-translational modification of a mostly MC product. We hypothesized three main possible mechanisms by which this could happen. First, glycosylation of MC-produced Ovulin could be modified in the lumen by the secretion of SCs enzymes. Second, MCs could be signalled by SC products to change the glycosylation pattern of Ovulin. Or third, Ovulin could be modified in the SCs after an uptake from a MC source. As glycosylation is generally an intracellular modification made in the Golgi apparatus, we disfavoured the first hypothesis. Also, when looking at Ovulin protein, we noticed that all of the Ovulin protein ejaculated was improperly glycosylated (Gligorov, Sitnik et al., 2013). If this process was occurring in the lumen, we might expect to find a mixture of Ovulin glycosylation products, as newly secreted Ovulin protein should be entering the lumen as others are being modified.

The second hypothesis also seemed strange to us as we wondered why the MCs would need a SC product to make Ovulin properly? One reason to depend on the two cell types to make a protein properly might be to compartmentalize certain substances from mixing with another in an improper environment (as in a protease cascade). But this would not be the case if MCs simply switched from improper to proper glycosylation of Ovulin.

Ovulin would be made in its proper form in the same cell. Thus, this second possibility seemed unlikely, though not completely ruled out.

For the reasons above, we initially favoured the third model, where MC products might be trafficked into the SCs. If MC products were secreted to a place outside of the lumen, then this would allow all products to be correctly modified using an intracellular enzymatic reaction before being placed into the lumen.

A number of the findings from my work support this idea. First, there is the shape of the cell. As seen from my initial characterization of the cell polarity of the cells of the gland, we know that the SCs have a small apical face, but a large contact surface with neighbouring MCs. In fact, looking at the cells, it seems like the MCs are actually climbing up, out of their plane of cells to cover as much of the SCs as possible. This is suggestive of intercellular exchanges between the cells. Next there is the distribution of the Rab proteins in both the MCs and the SCs. For example, Rab8, the traditional secretory Rab is present all over the MCs instead of apically oriented and no traditional secretory Rabs seems to be highly concentrated on the apical side of the cells. As Ovulin is one of the most highly expressed proteins in the AG, one would suspect a high concentration of secretory Rabs facing the apical lumen. However, Rab8 is not concentrated on the apical side of the cell. In fact, based on its localization, one might expect to see most of the secretions being sent to the basal and lateral sides of the cells. This is supported by our Ovulin antibody staining experiments where we see the Ovulin antibody staining the basal side of MCs.

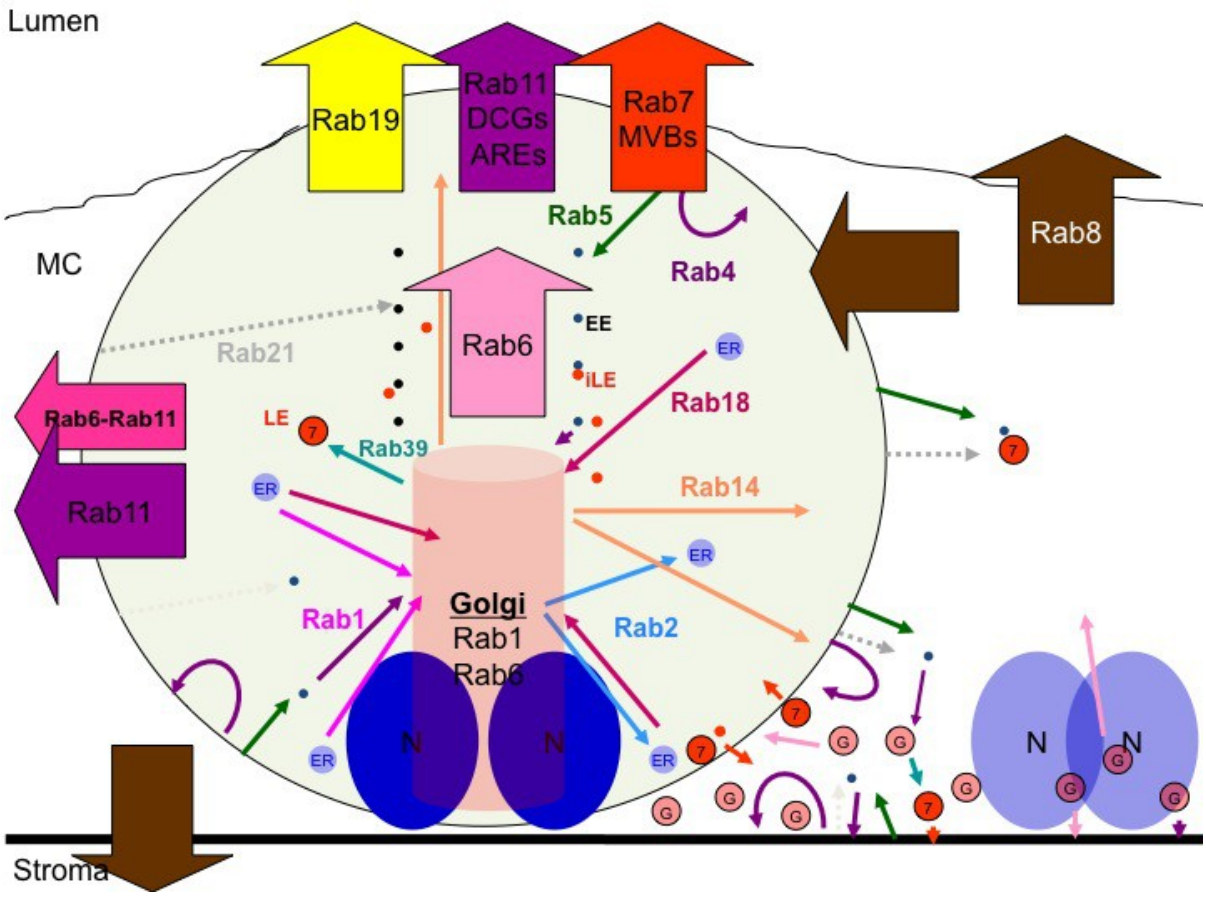


Figure 19. Overview of the putative Rab-mediated trafficking pathways within the epithelium of the accessory glands.

This schematic representation of a round-shaped secondary cell and its neighbouring two main cells depicts the putative trafficking routes that could take place within both cell types based on my data and that of other.

The main cells (MCs) are binucleated (N) have numerous plenty Golgi apparatuses (G). They may use Rab8 as a master regulator of secretion toward the SCs, the lumen and the stromal compartment (brown); most of the other exocytic pathways are only present basally (Rab6, light-pink; Rab39, turquoise; Rab18, Rab1 and Rab2 are represented via Golgi apparatuses distribution (G). SCs can internalize material from the MCs directly or via the stroma using Rab5 (dark-green) or Rab21 (dashed-light-grey) vesicles. The late endocytic (orange, Rab7) pathways seem located at the same places as the early endocytic ones indicating that the recycling routes are likely located on the basal side (Rab4, light-green; Rab11, purple) as well as the Rab4-regulated transport of EE content to the Golgi.

The secondary cells (SCs) are bi-nucleated (N) and have a channel-like Golgi apparatus (salmon-pink). The SCs secrete products toward the lumen via the Rab7-positive multi-vesicular bodies (MVBs, orange, large arrow) (Corrigan et al., 2014), the Rab11-positive dense-core-granules (DCGs, purple, large arrow) (Redhai et al., 2016), the apical recycling endosomes (AREs), and probably by Rab19-labelled large vacuoles (yellow, large arrow). Rab6 seem to mediate the intra-Golgi transport and the TGN-sorting component directed towards the apical plasma membrane (light pink, large arrow). Rab2 may mediate retrograde transport from Golgi to the endoplasmic reticulum (ER) while Rab1 and Rab18 may convey newly synthesized molecules to the Golgi apparatus. The products endocytosed (EE, black dots) from the lumen or the MCs by Rab5 (dark-green arrows) or Rab21 (dashed light-grey arrows) are likely directed to the centre of the SCs where they can be transferred to the Golgi via Rab4 (light-green arrow) or fast recycled by Rab4 (curved light-green arrows). On the basal side, the materials internalized by Rab5 are mainly cortical and could be fast recycled or convey to Golgi via Rab4. Rab7 is present on centrally clustered punctuate that may be immature late endosomes (iLEs, orange dots) (Corrigan et al., 2014) and larger compartments uniformly distributed that may be mature late endocytic compartments. The content of the late endosomes (LEs) can come from early endosomes (EEs, black dots) or from the Golgi. The Golgi-to-late endosome trafficking may be regulated by Rab39 while EE-to-LE transfer may

be due to a Rab5-to-Rab7 switch (Del Conte-Zerial et al., 2008; Poteryaev et al., 2010). Finally, the SCs may convey chemicals toward the MCs via Rab11-positive or Rab6-Rab11-positive large vacuoles (purple and fushia large arrows, respectively).

In the SCs, we see Ovulin concentrated in the vacuoles (Fig 7A). Although this could be due to SC expression of Ovulin, our latest RNA-seq experiments show that Ovulin is only weakly expressed in the SCs. Given that Ovulin is one of the most highly expressed proteins in the AG (Sitnik et al., 2016), most of the Ovulin protein must be made in the MCs. The Rab localization in the SCs would also be consistent with an intercellular exchange model where SCs would take up Ovulin made by the MCs, modify it and re-secrete it into the lumen. For example, Rab4, an endocytic Rab, is enriched on the baso-medial side of the cells. If MCs secrete towards their basal or medial sides, then this Rab would be present to take help take up these secretions (perhaps along with Rab5). Also, Rab6, a TGN marker makes up many large vacuoles in the SCs. If these vacuoles retain a function similar to the TGN, then this compartment could be used to modify proteins taken up from external sources. Live imaging of AGs from transgenic flies expressing our photo-activable Ovulin will be useful in shedding light on the direction of the trafficking in this reproductive tissue (Fig.17). Using this tool, we should be able to know if the MCs secrete Ovulin toward the extra-cellular space existing between the epithelium and the stromal muscle layer (Fig.1Dc; Bairati, 1968) and if the SCs internalize Ovulin from this source. We could also follow Ovulin through the SCs to identify the compartments by which it travels to get to the lumen. If I had to speculate on what I think may be happening in the AGs regarding Ovulin, I would suggest that Ovulin is made by the main cells and secreted towards the basal extracellular space. From there, the SCs uptake this Ovulin using the endocytic proteins, Rab5 and Rab21 and then transfer it for post-translational modification to a Rab6 compartment via Rab4 vesicles (Fig. 19). Then, to be transported to the lumen, different pathways could be used: Ovulin could be transferred

via the late endocytic pathways involving Rab39 and Rab7, via the DCGs using Rab11 or via an apical secretion pathway regulated by Rab19 (realizing that the formation of the Rab19-positive large vacuoles require the presence of Rab7) (Fig.19; Fig. 15D'').

Ovulin could then be released into the lumen through fusion of the large vacuoles to the PM or perhaps through small vesicle transport from the large vacuoles. If the later situation is the case, then I would suspect Rab14 vesicles or Rab11 vesicles might play the role conveying Ovulin to the apical PM (Fig.11A; Fig. 10D; Annexes 2A & 1H). Figure 19, above, summarizes this possible trafficking network within the AGs (Fig. 19).

III- Connecting the Rab-mediated transport, the secretion of the seminal fluid proteins and male fertility

The PMR is affected in the absence of the large vacuoles of the SCs (Gligorov, Sitnik et al., 2013), but Ovulin is not involved in most of the PMRs monitored in the traditional PMR assays (Herndon & Wolfner, 1995). Most of the PMR response phenotypes discovered in our original *iab-6*^{cocu} mutant allele can be attributed to problems with the SP pathway. As mentioned in the introduction, SP is able to elicit a long term PMR (LT-PMR) because it gets tethered to the sperm for storage. *iab-6*^{cocu} mutants seem to be deficient in this tethering process and thus, show no LT-PMR. Two secondary cell-specific Sfps seem to play a role in allowing SP to bind to sperm. These proteins are the lectins, CG1652 and CG1656 (Gligorov, Sitnik et al., 2013; Ravi-Ram & Wolfner, 2009). In *iab-6*^{cocu} mutants, both of these proteins are also improperly glycosylated (Gligorov et al., 2013). Although we do not know how glycosylation affects the activity of these proteins, it is tempting to hypothesize that

improper glycosylation of these proteins may be the cause of the *iab-6*^{cocu} mutant phenotype. Preliminary receptivity assays suggest that the knock-down of Rab6 and Rab7 decrease the time-frame before females re-mate after an initial mating, much like *iab-6*^{cocu}. This suggests that the Rab7 and Rab6 vacuoles are required for the proper PMR. Thus, if glycosylation is the key contribution of the SCs in male fertility Rab6 and Rab7 compartments (directly or indirectly) may play an important role in this process. It would be extremely interesting to test this hypothesis, but it would be extremely difficult. First, we would have to know if the glycosylation state of these proteins affects the PMR. To do this, we could start by mapping the modification sites on CG1652/1656 and see if the elimination of the glycosylation sites affects the PMR. Ideally, we would be able to produce the exact improper glycosylation patterns in these proteins and test for SP sperm binding without affecting other processes. This would require the creation of an in vitro assay, probably involving the purification of many proteins. As this seems impractical at this time, we could simply verify that SP binding to sperm is affected in glycosylation mutant flies. To test if the different Rab vacuoles are involved in the process of glycosylation, we could reexamine the glycosylation patterns after knocking down the different Rab proteins.

Conclusions:

When I started this project very little was known about the cell biology of the AGs and I realize that even now, after all of the work I did on these glands, we still know very little. Many of the questions we had when I started my PhD are still unanswered. The reason

for this, of course, is that when I started so little was known about the gland that I had to learn the basic details of the system before I could actually do the experiments. Every detail I discovered led to more questions about the cells that we needed to know in order to interpret our results. For example, we thought we would be able to see fusion of the vacuoles to the PM. I literally looked at thousands of cells and never found a single vacuole in the process of fusing to the membrane (or vacuole marking Rabs localizing to the PM as evidence of a recent fusion event). This led to the question of whether the vacuoles do not fuse to the PM or if they just require the right stimulus? This led to experiments with mating males and re-examining the vacuoles, from which we found no vacuoles fusing. Since a negative result is only suggestive, I cannot truly conclude on this issue. And every step was like this. Are we looking at the right stage? We have to do time courses. Is there a problem using fixed samples? Let's try live imaging. Can we do live imaging? Etc. This is not a complaint or an excuse; it is just a fact of working on a new system.

Still, I think we have learned a lot about the cells of this gland during my thesis and we are now in a good position to delve into the problems we initially set out to answer. Technically, we know the conditions for the best imaging of fixed and live samples. We know how to keep glands alive for longer imaging experiments. We know, or have a good idea, about the stages and mating conditions to use in the study. And we have the tools to follow a real AG protein as it traffics through the gland.

On the cell biology level, we learned a lot about the structure of the cells and where the different Rab compartments are in the cell. Although my work has been duplicated a bit by the lab of Clive Wilson, one must understand that there are great differences between his work and mine. First and foremost is the fact that my work uses knocked-in Rab lines, while his uses overexpression constructs. When we started my work, we had to make a choice. We

too could have had access to the Rab overexpression lines. But consulting with people in the field of secretion, we learned that these lines sometimes yield artifactual results due to over/miss expression. Adding too many Rabs can lead to Rabs labelling compartments that they are not meant to or change trafficking pathways in the cells (Chan et al., 2011; Corrigan et al., 2014). The work of the Wilson's laboratory even mentions that their Rab11 expression results in substantial changes in the morphology of the vacuoles (Corrigan et al. 2014) Thus, we decided to focus on the Rab knock-in lines that we were fortunate enough to gain access to ahead of publication. These lines allowed us to perform experiments similar to and beyond those of the Wilson Lab with physiological amounts of proteins. Also, as we were looking at the endogenously expressed Rabs, we could see what is actually present in the cells instead of what happens when we express a marker in the cells. This is important because, although we initially thought Rabs would tells us a lot about the nature of the compartments, it turns out that Rabs can be used for different things in different places. Although, in general, Rabs can be used to know something about a process, but it is not absolute identification. In fact, from my work, I think we learned more about the kinds of things the individual Rabs label, than information on the compartments labeled based on the Rabs.

Besides the cell biology of the AG cells, I think understanding how a prostate-related organ operates could lead to general insights into male fertility (Xue & Noll, 2002; Verze et al., 2016). Developmentally, the two tissues are quite similar. The AGs and prostate development require the Pax genes (Montano & Bushman, 2016; Xue & Noll, 2002) and posterior Hox genes to form (Prins & Putz, 2008; Huang et al., 2007). Interestingly, *Hoxb13* and *AbdB* are highly expressed at the distal tip of the ducts and are required for Sfp secretion in their respective species (Montano & Bushman, 2016; Gligorov, Sitnik et al.,

2013). As we learn more about human seminal fluid, we are seeing that some of the same processes and/or concepts are used in human and *Drosophila* seminal fluid (McGraw et al., 2015; Wilson et al., 2017). As a quick examples, both *Drosophila* AGs and Mammalian prostates secrete TGF-≅ homologues to increase male reproductive success (Wilson et al., 2017; Redhai et al., 2016; Leiblich et al., 2012; Montano & Bushman, 2016; Prins & Putz, 2008) and both prostatic epithelial cells and *Drosophila* glandular SCs have apical Rab11-postive features (Goldenring et al., 1996). I hope that my work will somehow help scientists understand more about how the seminal fluid is made, and perhaps, understand how male fertility is influenced by the seminal fluid (Atikuzzaman et al., 2016; Bromfield, 2016). As male infertility has been seen increasing over the last few decades, this could be important for many couples in the future.

Abbreviations

AbdB:	Abdominal-B
Acp:	Accessory gland protein
AG:	Accessory Gland
ANCE:	Angiotensin-I Converting Enzyme
A-P:	Antero-Posterior
APF:	After Puparium Formation
BMP:	Bone Morphogenetic Proteins
Btl:	Breathless
CNS:	Central Nervous System
CRab:	CFP^{HA}-tagged Rab
CRISP:	Cystein-Rich Secretory Protein
DCAD:	*Drosophila* epithelial cadherin
DCG:	Dense Core Granules
DHT:	5-α-dihydrotestosterone
DL:	Dorsal Lobe
DLG:	Disc-Large
Dpp:	Decapentaplegic
Dve:	Defective proventriculus
EE:	Early Endosome
ER:	Endoplasmic Reticulum
FB:	Fat Body

FGF:	Fibroblast Growth Factor
GAP:	GTPase Activating Protein
GDI:	GDP Dissociation Inhibitor
GDP:	Guanosine Di-Phosphate
GEF:	GTP Exchange Factor
GFP:	Green Fluorescent Protein
GTP:	Guanosine Tri-Phosphate
Hh:	Hedgehog
Hox:	Homeobox
iab:	infra-abdominal
IVF:	*in vitro* fertilization
JH:	Juvenile Hormone
KLK:	Kallikrein
LE:	Late Endosome
MC:	Main cell
MIP:	Myo-Inhibitory Peptide
msa:	male-specific abdominal
mst:	male-specific transcript
MVB:	Multi-Vesicular Body
Tom-myr:	Tomato-Myrsitoylation
nAR:	Nuclear androgen receptor
OA:	Octopaminergic
OFE:	Ovarian Follicular Epithelium
PM:	Plasma Membrane

PMR:	Post-mating response
Prd:	Paired
PZ:	Peripheral Zone
RagGGT:	Rab geranylgeranyl transferase
RE:	Recycling Endosome
RFP:	Red Fluorescent Protein
SC:	Secondary cell
Sfp:	Seminal fluid protein
SG:	Salivary Gland
SP:	Sex-peptide
SPR:	Sex-Peptide Receptor
Sxl:	Sex lethal
TGF:	Transforming Growth Factor
TGN:	*trans*-Golgi Network
UGS:	Urogenital Sinus
VP:	Ventral Prostate
WD:	Wing Disc
Wg:	Wingless
Wnt:	Wingless-related integration site
YFP:	Yellow Fluorescent Protein
YRab:	YFP^{Myc}-tagged Rab
ZIP:	Zipper
Zn:	Zinc
ZnT:	Zinc Transporter

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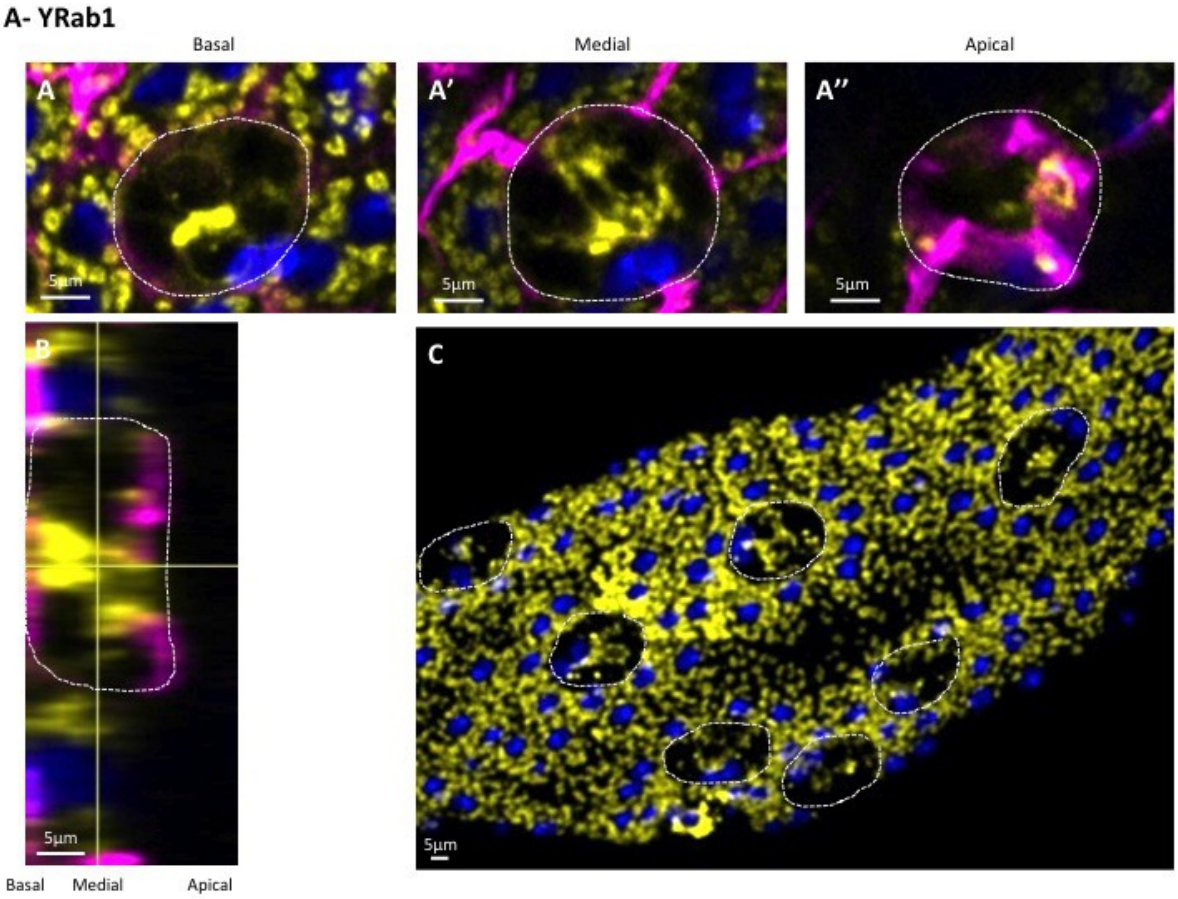
	Main Cells	Secondary Cells
Rab1	Basal/medial small compartments	Basal-to-medial/apical large centred filamentous-like structure
Rab2	Basal/medial small compartments	Basal/Medial punctae at the peripheral tips of large centred filamentous-like structure
Rab3	Synaptic buttons of the muscle layer	
Rab4	Basal/medial puncta	Basal cortical puncta, centrally-clustered medial puncta & apical cortical small compartments
Rab5	Basal/medial puncta	Basal puncta & centrally-clustered medial/apical puncta
Rab6	Basal/medial small compartments	Basal/medial large centred filamentous-like structure & 2-5 large medial/apical large vacuoles
Rab7	Basal/medial small compartments	Basal-to-apical centrally-clustered puncta and small compartments & 1-2 apical large vacuole
Rab8	Basal-to-apical compacted large compartments	Very weak diffuse signal
Rab9	Synaptic buttons of the muscle layer	
Rab10	Basal-to-apical weak diffuse signal +/- small compartments	Basal-to-apical weak diffuse signal +/- intense medial/apical central diffuse signal
Rab11	Basal/medial puncta	Basal/medial cortical large vacuoles & apical centrally-clustered puncta
Rab14	Undetected	Basal-to-apical centrally clustered intense diffuse signal & puncta
Rab18	Basal puncta	Basal small compartments, medial aggregate of puncta & small compartments
Rab19	Undetected	Basal puncta, central medial/apical channel-like structure & 1-4 apical large vacuoles
Rab21	Basal/medial diffuse signal	Medial centrally clustered puncta
Rab23	Undetected	
Rab26		
Rab27		
Rab30		
Rab32		
Rab35	Basal-to-apical diffuse signal	Basal-to-apical diffuse signal +/- intense centred basal/medial diffuse signal
Rab39	Basal/medial small compartments	Medial/apical centred small compartments
Rab40	Undetected	
RabX 1	Basal-to-apical weak diffuse signal	Basal-to-apical weak diffuse signal +/- apical centrally-clustered puncta
RabX 4	Synaptic buttons of the muscle layer	
RabX 5	Undetected	
RabX 6		

Table 2. Recapitulative table of the Rab proteins distribution in Drosophila accessory glands epithelium.
In accessory glands, there are two epithelial cell types, the main cells and the secondary cells. Here there are the expression patterns of the Rab-YFP^{Myc} observed during the library screening.
The basal side is the basement-membrane one while the apical side is turned to the lumen. The "vacuoles" are compartments delimited by a membrane and filled by fluid (www.vocabulary.com) and what I call "small compartments" are the features where the containing space is not visible. In term of size, large vacuoles ≈ 1.5-10µm; small compartments ≈ 1-1.5µm; puncta ≤ 1µm.
In light-grey are the Rab proteins that are not expressed in the epithelium but in the muscles layer while "undetected" Rab are expressed neither in the epithelium nor in the muscle layers.
The centred filamentous-like structure is likely Golgi apparatus (Annexe 13).

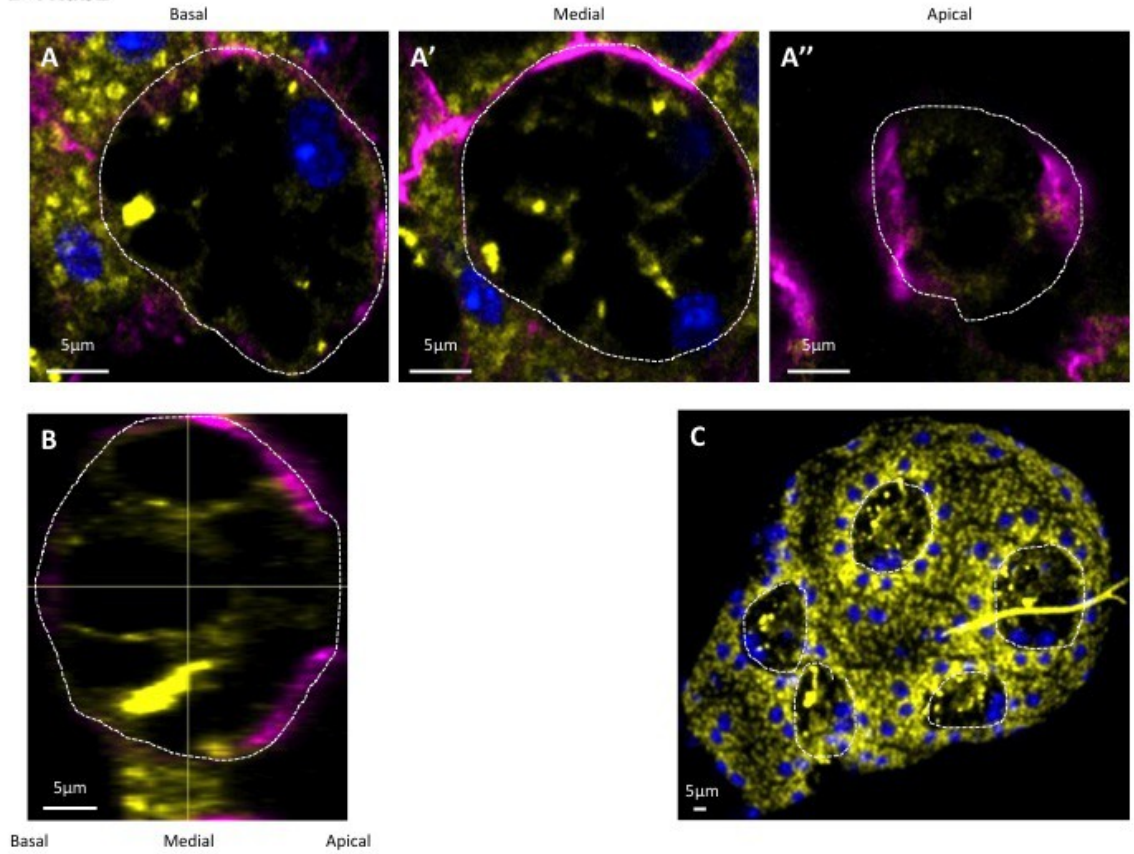
Annexes

<u>Annex 1.</u> Catalog of the "canonical" Rab proteins in the accessory glands

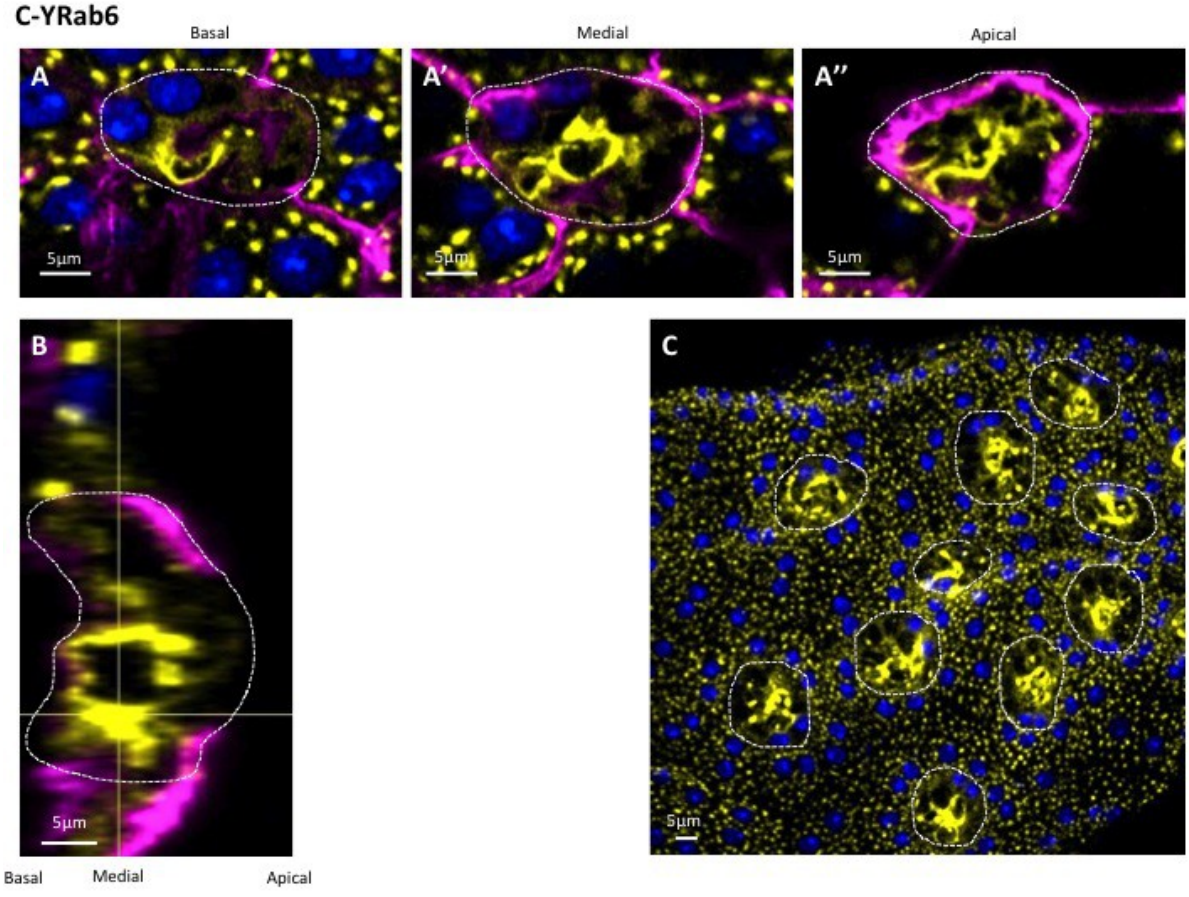
Confocal sections (**A-A''**) and sagittal view (**B**) of secondary cells enlargements (surrounded by dashed lines) from YRab accessory glands stained by a baso-lateral marker, DLG (magenta) and a nuclei marker, DAPI (blue). Z-projections of epithelium from YRab accessory glands (**C**) stained by DAPI (blue) and where the secondary cells are surrounded dashed lines. The YRab are yellow-coloured (A-C). The exocytic-related Rab (Rab1 (A-YRab1), Rab2 (B-YRab2), Rab6 (C-YRab6), Rab8 (D-YRab8)) and the endocytic/recycling Rab (Rab4 (E-YRab4), Rab5 (F-YRab5), Rab7 (G-Rab7), Rab11 (H-YRab11)) are present in this first annex. Scale bars, 5µm.

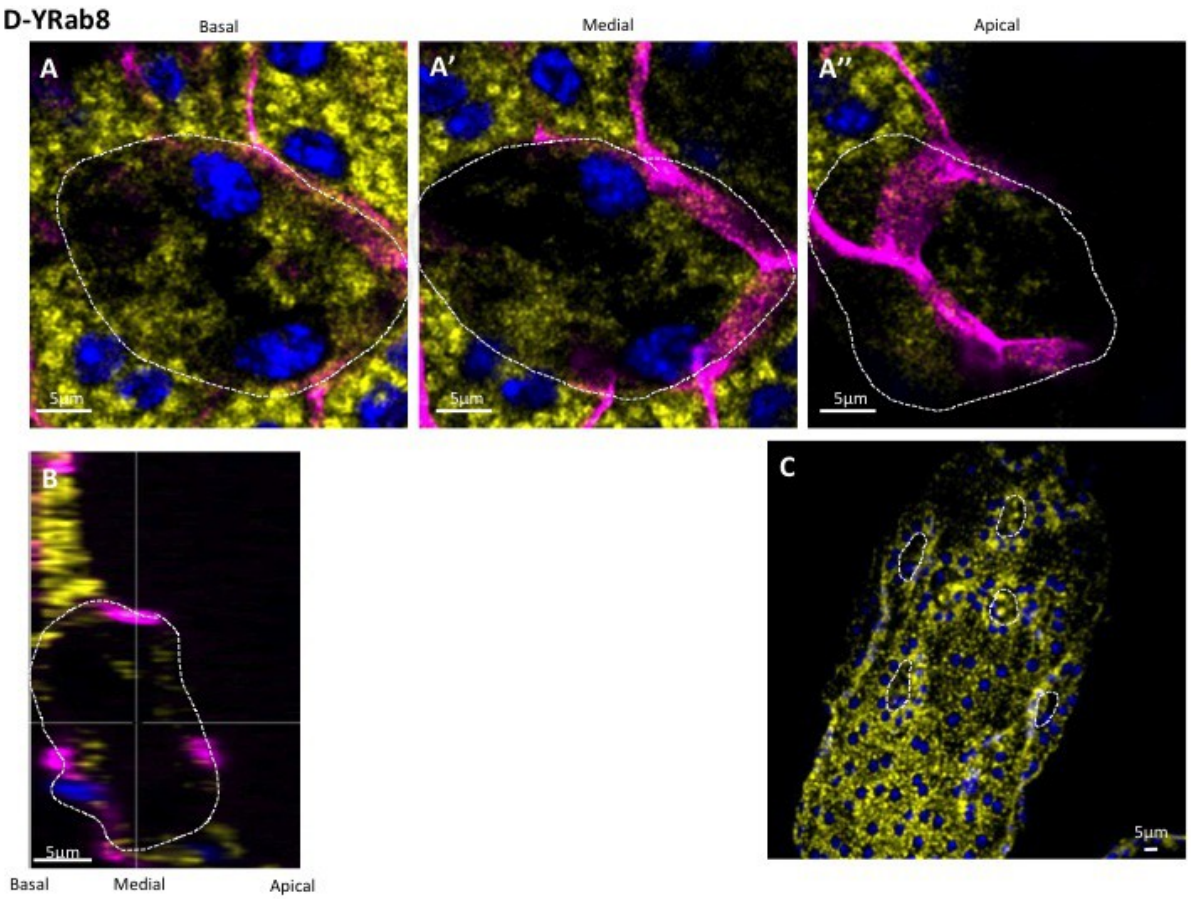


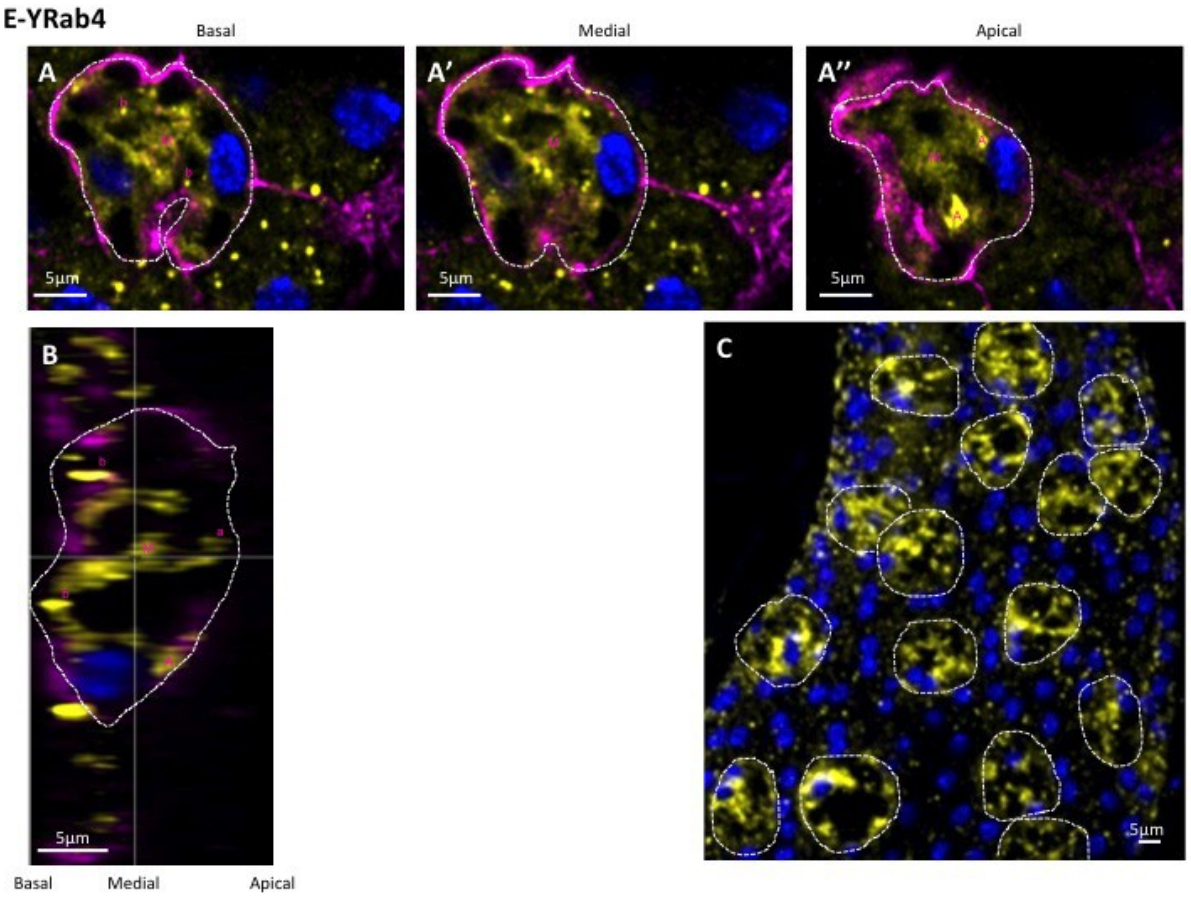
B-YRab2



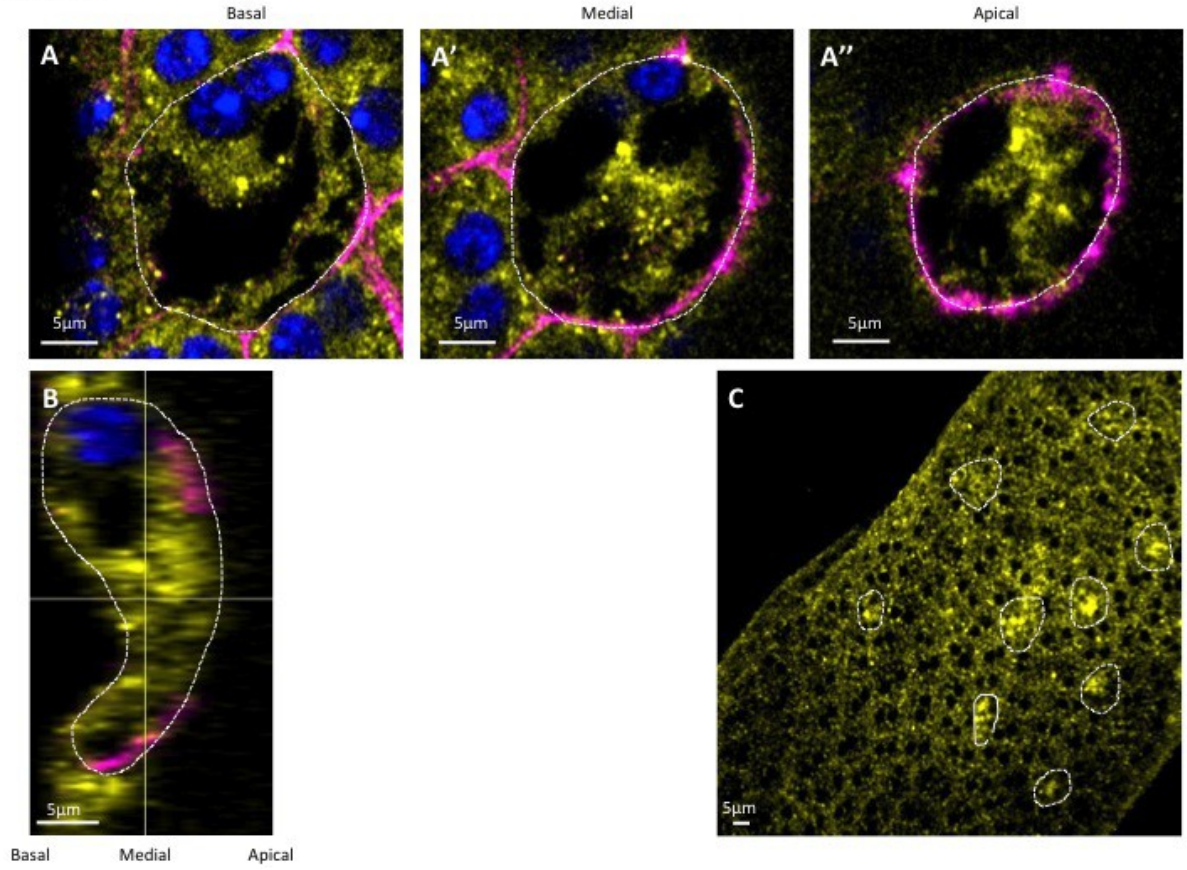
C-YRab6



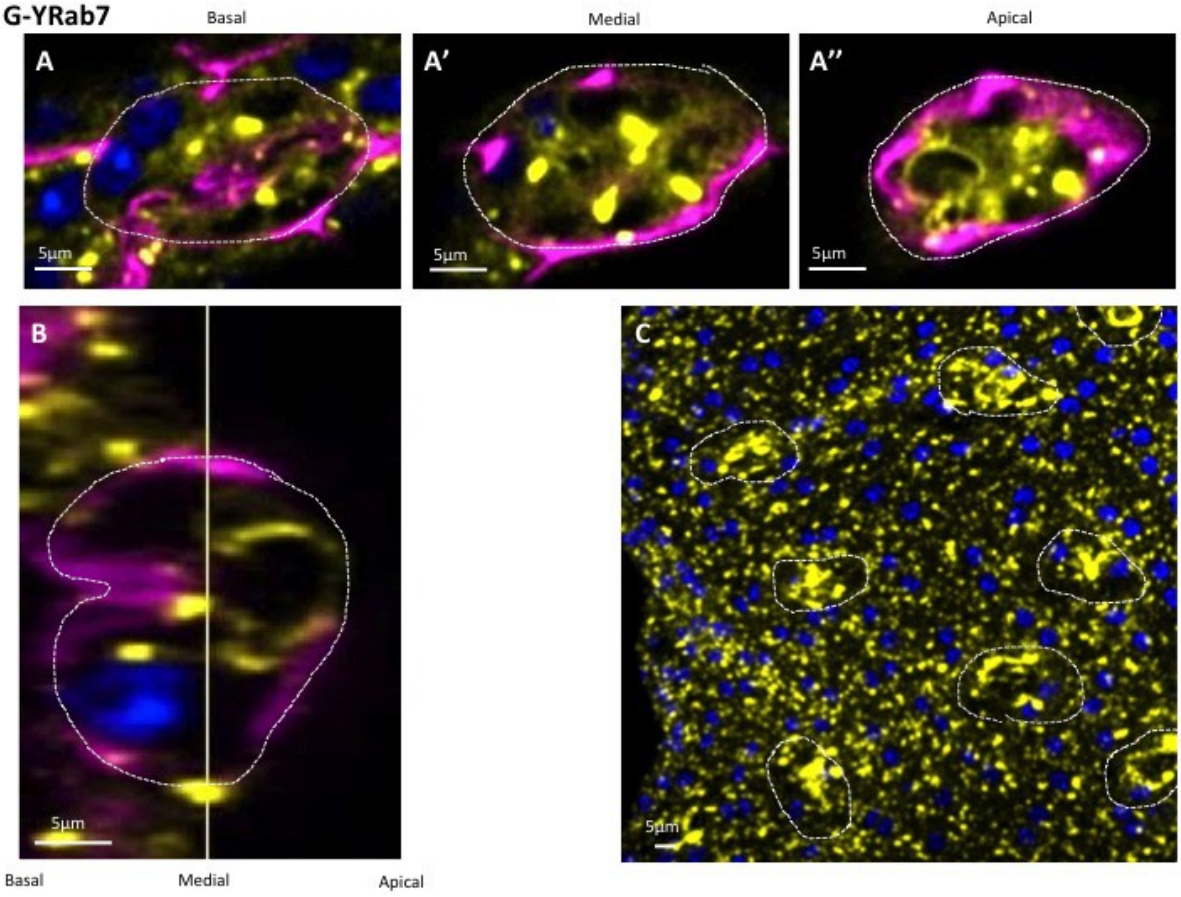

D-YRab8
Basal
Medial
Apical
A
A'
A''
5µm
B
C
5µm
Basal
Medial
Apical

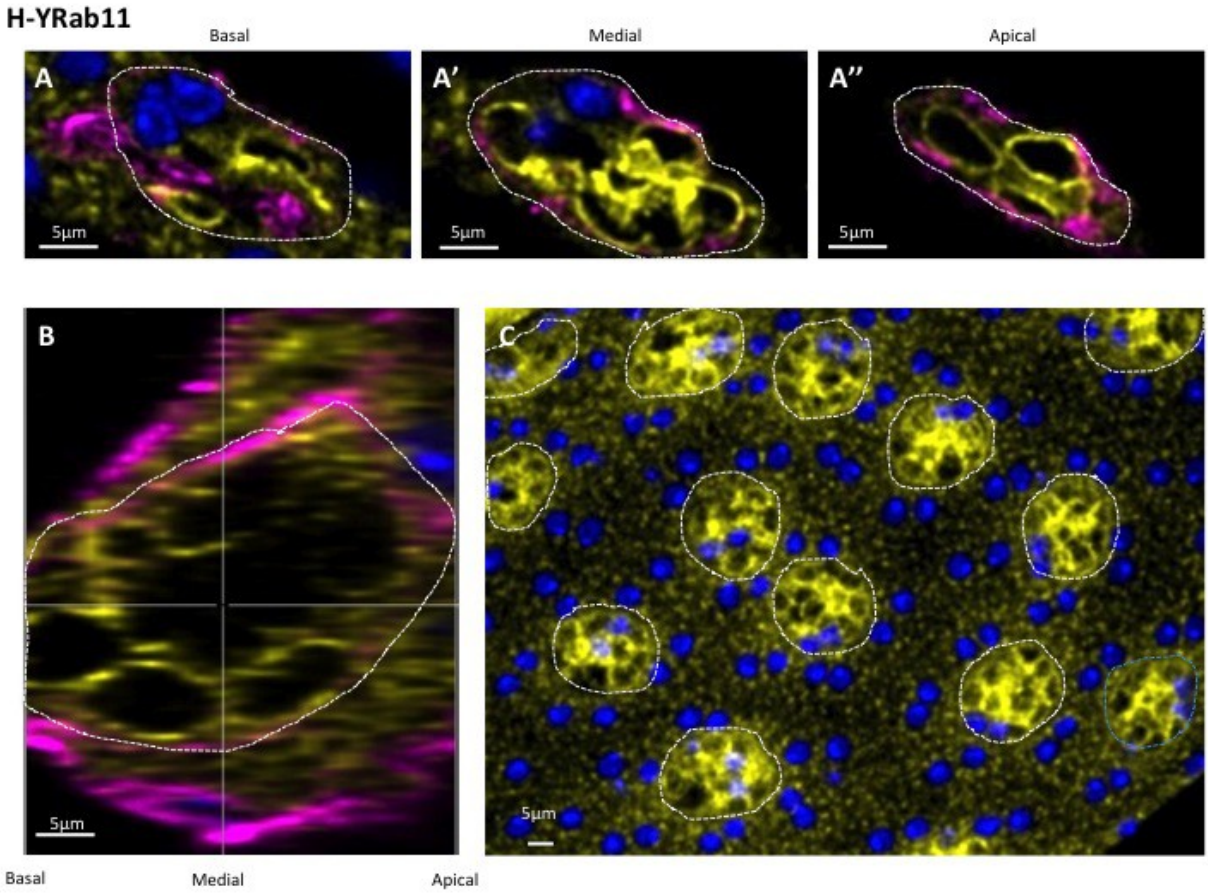

E-YRab4
Basal
Medial
Apical
A
A'
A''
5µm
B
C
5µm
Basal
Medial
Apical

F-YRab5



G-YRab7

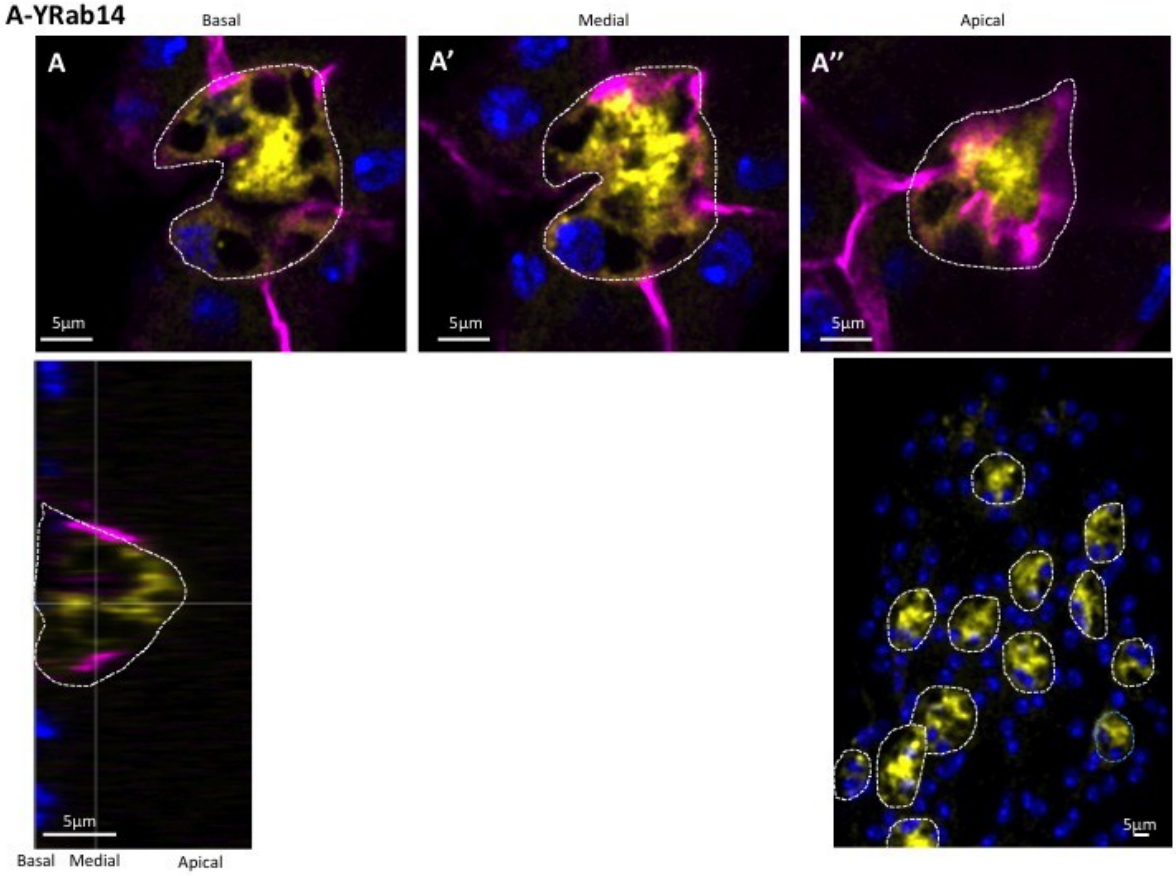


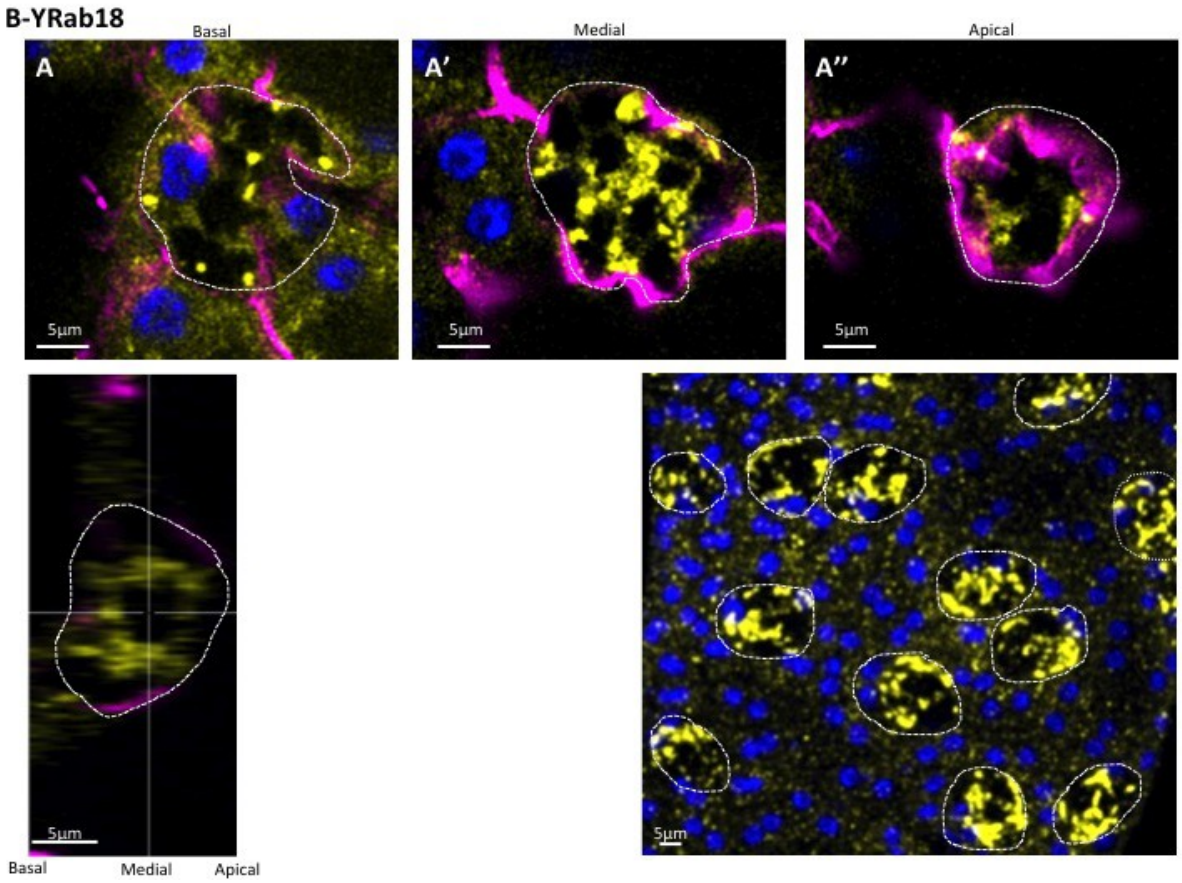

H-YRab11
Basal
Medial
Apical
A
A'
A''
5µm
5µm
5µm
B
C
5µm
5µm
Basal
Medial
Apical

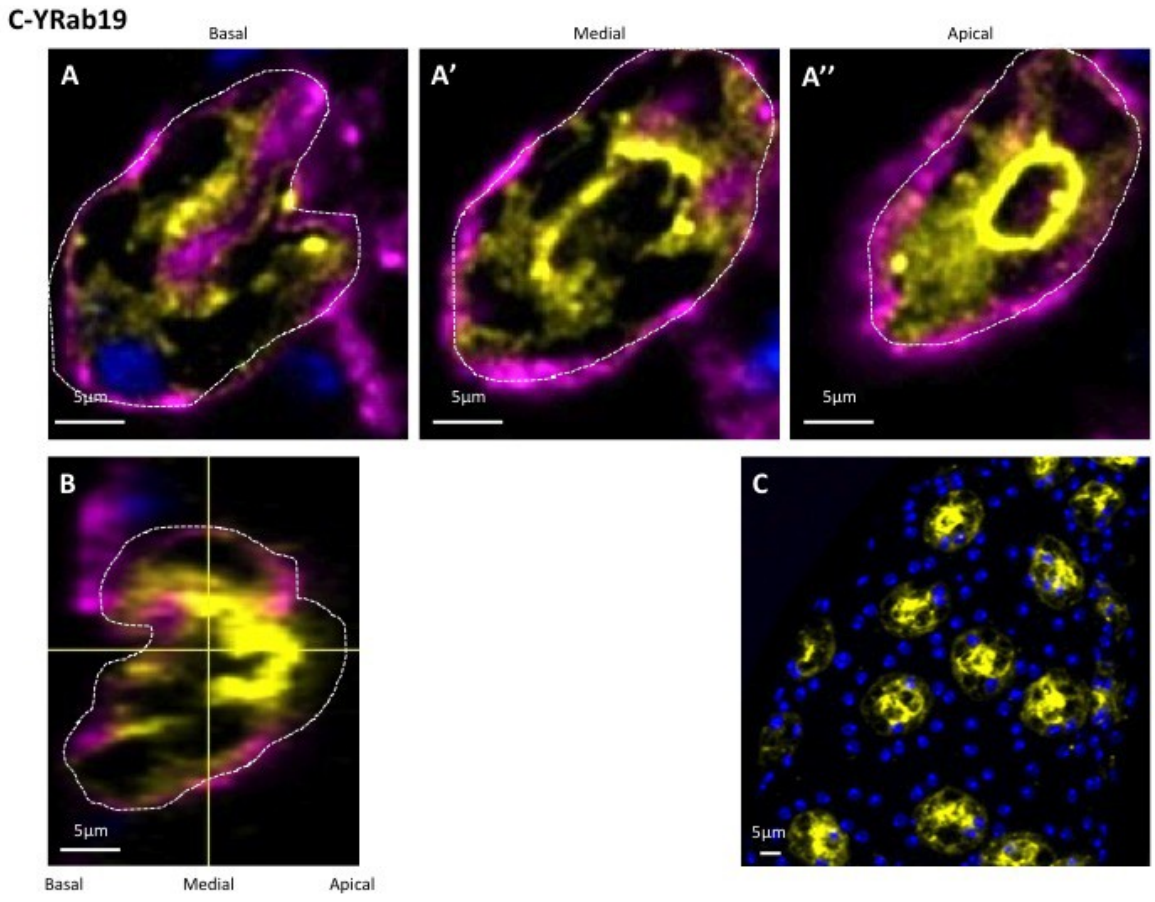
<u>Annex 2.</u> Catalog of the "non-canonical" Rab proteins in the accessory glands

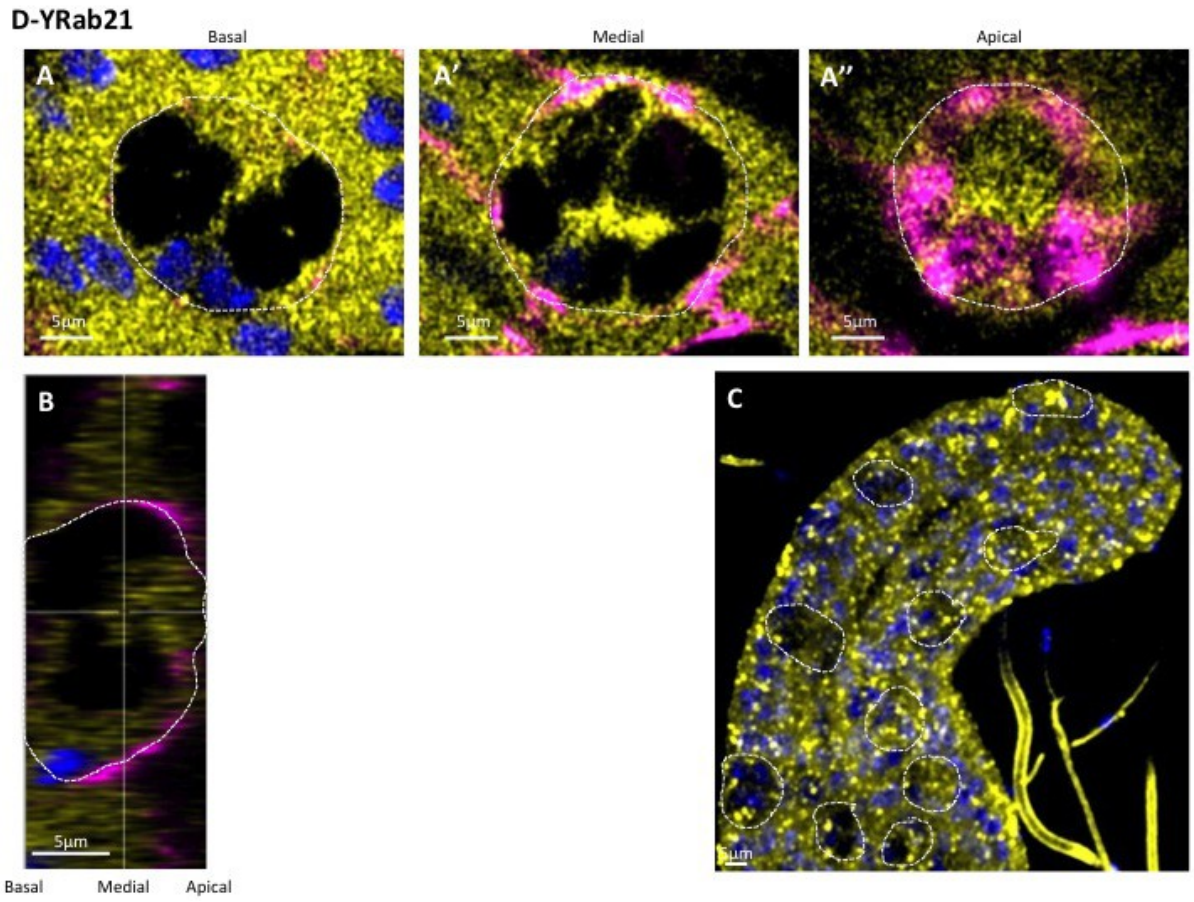
For YRab14 (A-YRab14), YRab18 (B-YRab18), YRab19 (C-YRab19), YRab21 (D-YRab21) and YRab39 (E-YRab39) : Confocal sections (A-A'') and sagittal view (B) of secondary cells enlargements (surrounded by dashed lines) from YRab accessory glands stained by DLG (magenta) and DAPI (blue). Z-projections of YRab accessory glands (C) stained by DAPI (blue) and where the secondary cells are surrounded dashed lines. The YRab are yellow-coloured (A-C).Scale bars: A-D, 5µm; Ea-b, 5µm; Ec, 10µm.

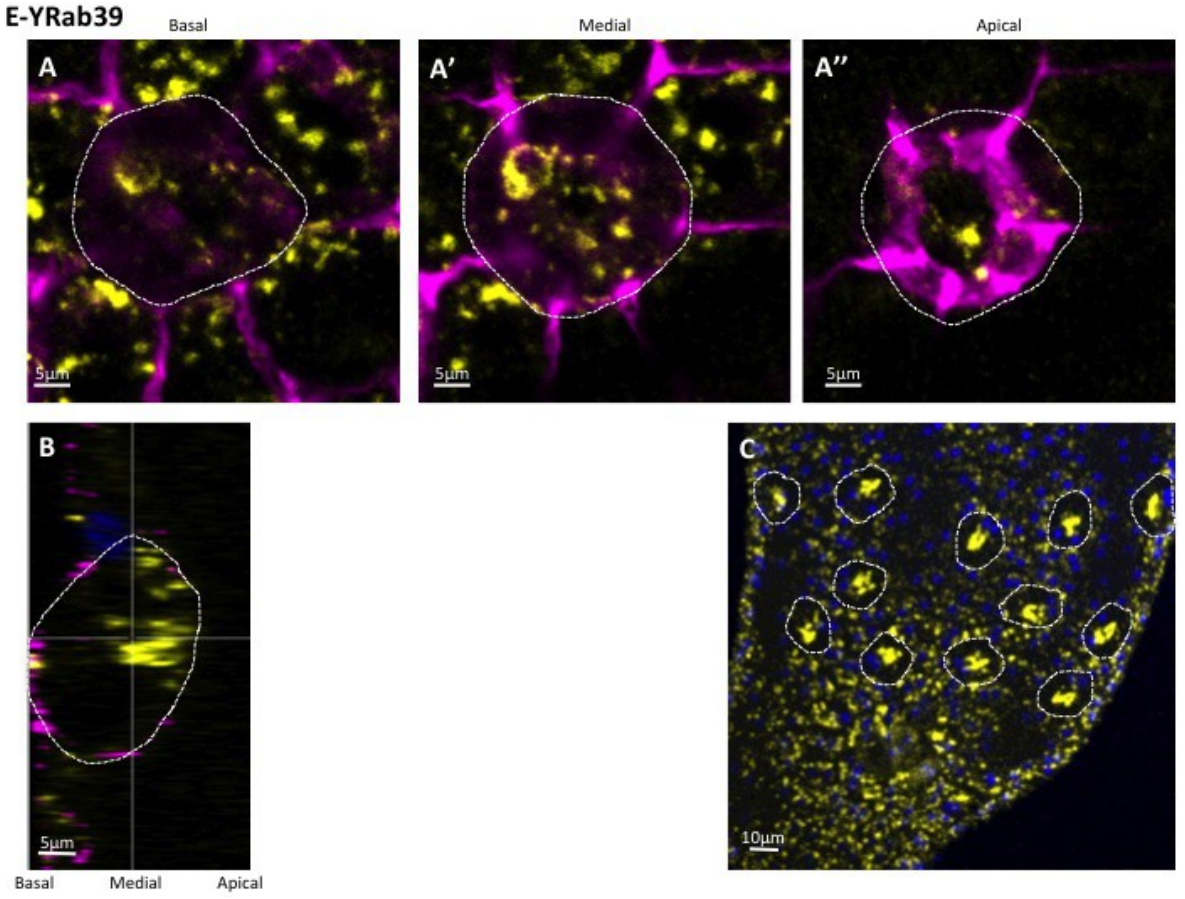
For YRab10 (Fa-a''), YRab18 (Fb-b'') and YRabX1 (Fc-c''): Sagittal views of secondary cells enlargement from YRab accessory glands stained by DLG (magenta) and DAPI (blue) (a-c). Z-projections of epithelium from YRab accessory glands stained with DAPI (blue) depicted the different patterns that we can observe for these YRab depending of the gland (a'-a''; b'-b''; c'-c''). Scale bars: Fa-c & Fb', 5µm; Fa' & c' and Fa''-c'', 10µm)

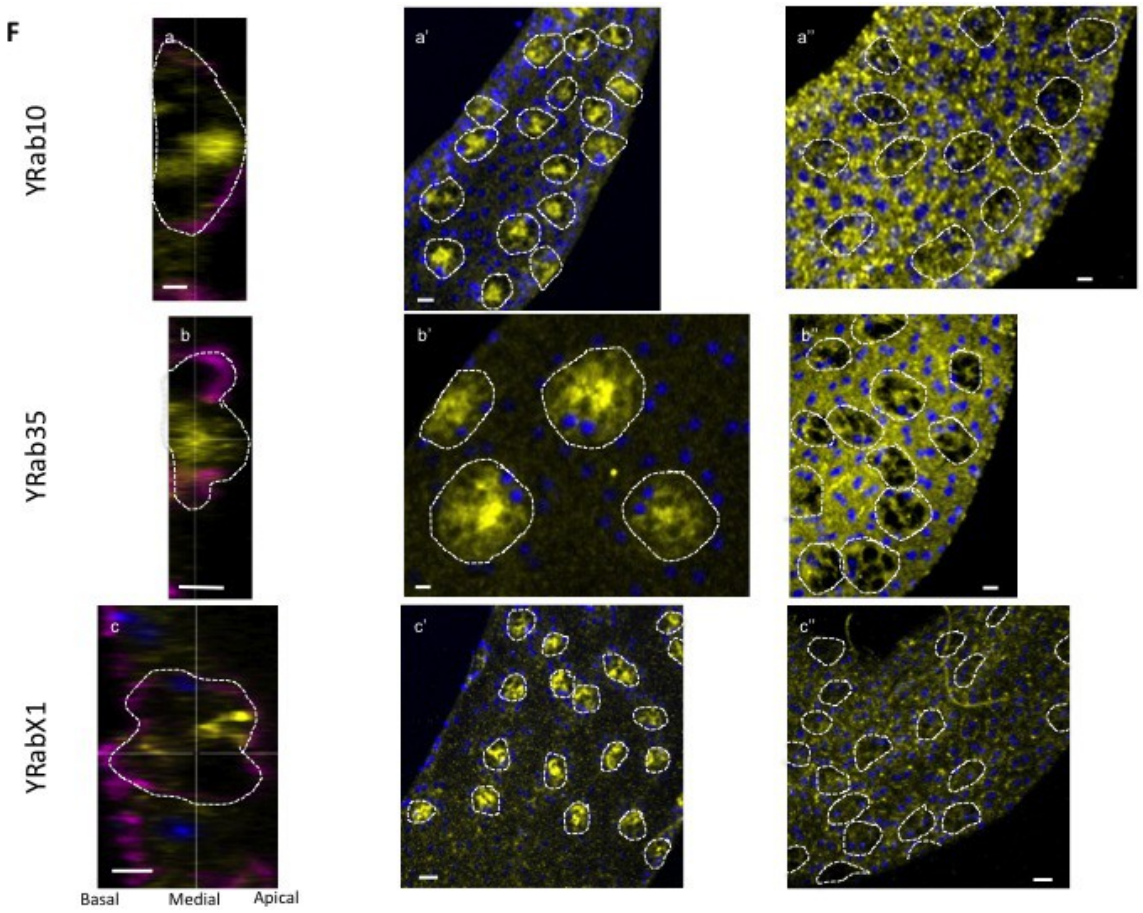



B-YRab18
Basal
Medial
Apical
A
A'
A''
5µm
5µm
5µm
5µm
5µm
Basal
Medial
Apical

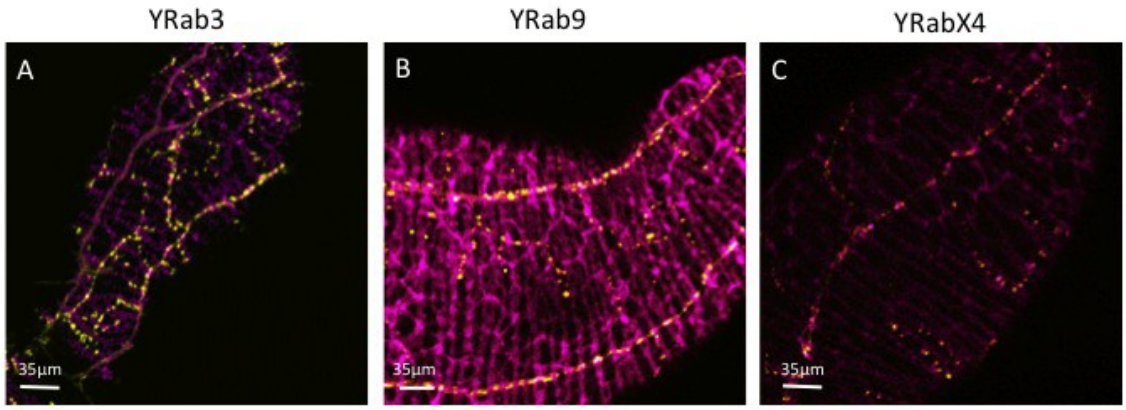

C-YRab19
Basal
Medial
Apical
A
A'
A''
5µm
5µm
5µm
B
C
5µm
5µm
Basal
Medial
Apical


D-YRab21
Basal
Medial
Apical
A
A'
A''
5µm
B
C
Basal
Medial
Apical


E-YRab39
Basal
Medial
Apical
A
A'
A''
5µm
B
C
10µm
Basal
Medial
Apical



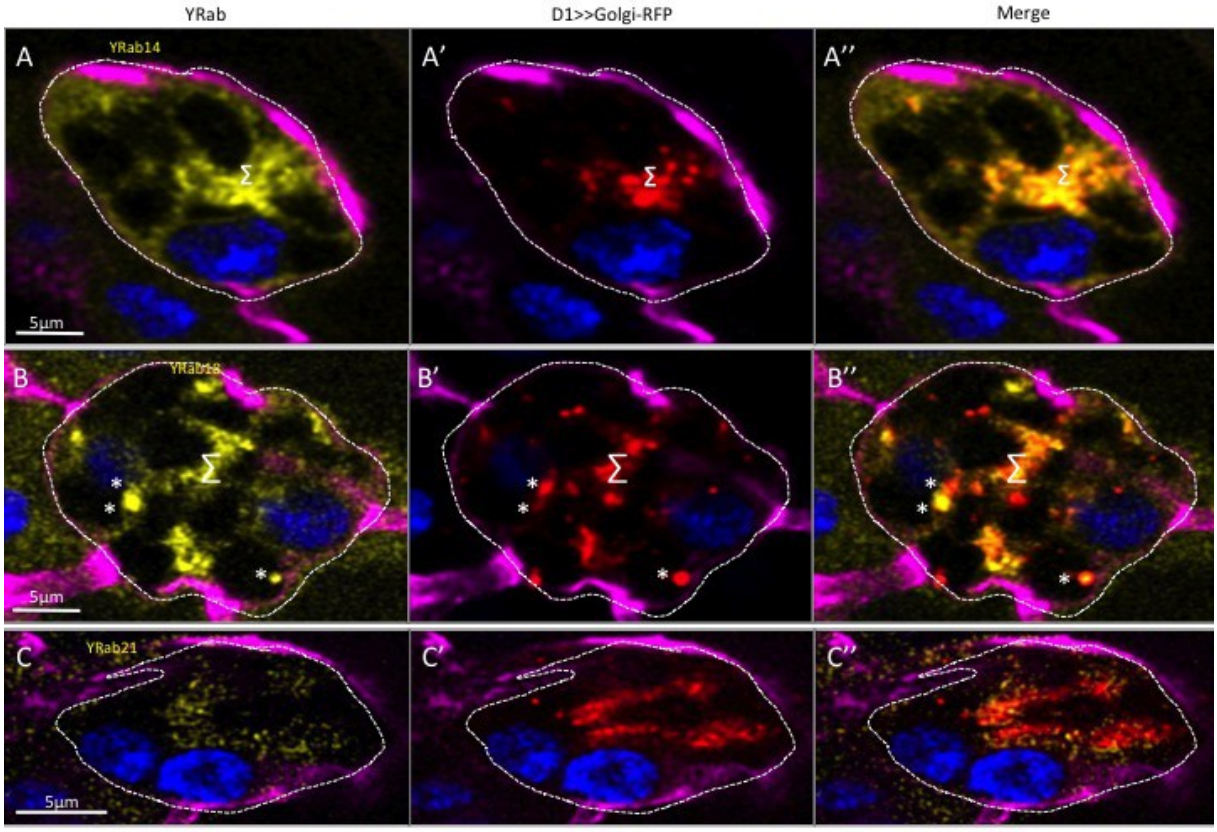
<u>Annex 3.</u> Rab3, Rab9 and RabX4 are present on neurons innervating the muscle layer of the accessory glands
Confocal sections of YRab3 (**A**), YRab9 (**B**) and YRabX4 (**C**) accessory glands stained with GFP antibody (yellow) and with the baso-lateral membrane marker, Disc-Large (magenta). Scale bars, 35µm.



<u>Annex 4.</u> Rab14, Rab18 and Rab21 could be intermediate in Golgi transport in the secondary cells

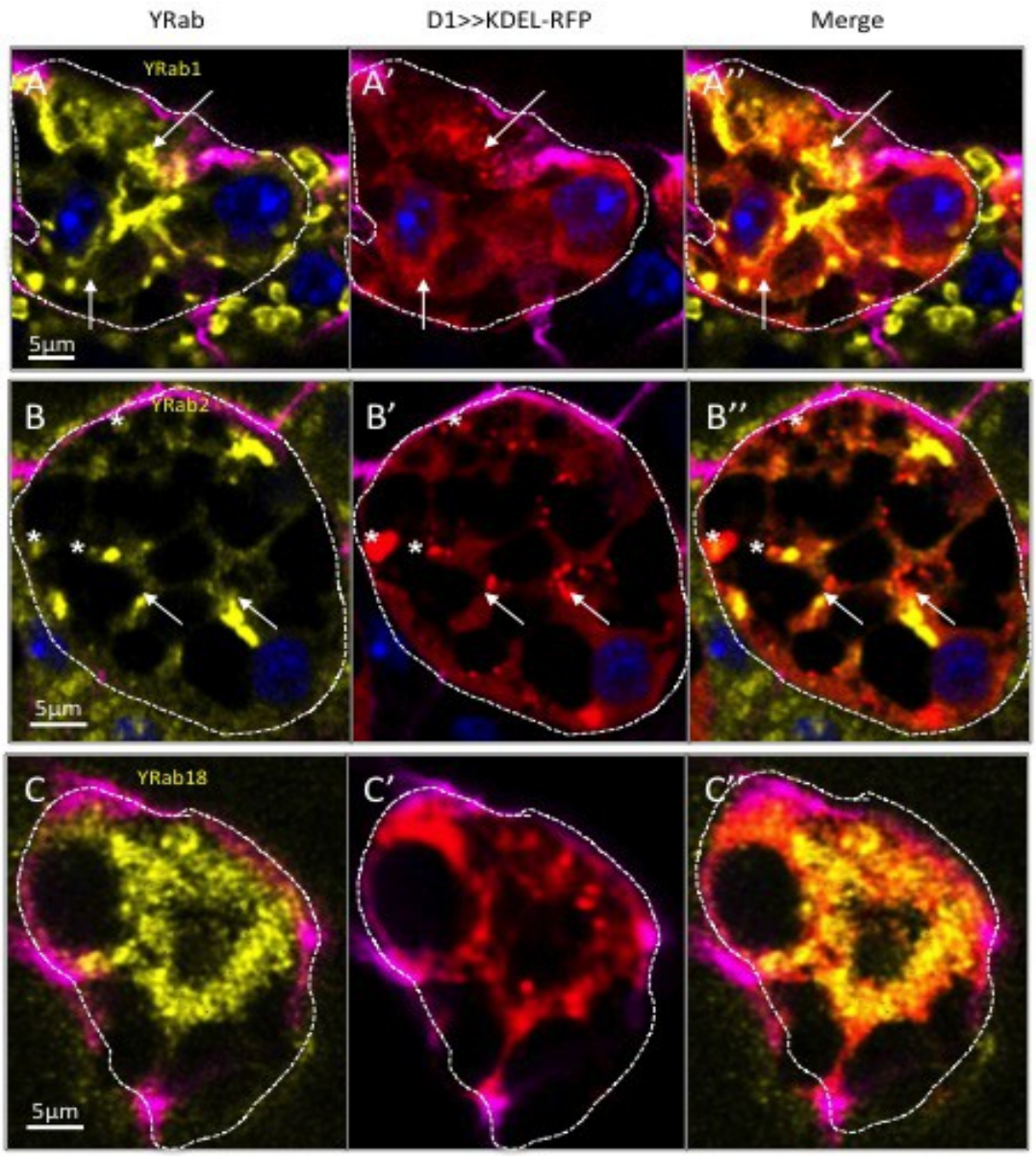
Confocal Z-sections enlargement of YRab14 (**A-A''**), YRab18 (**B-B''**) and YRab21 (**C-C''**) accessory glands expressing Golgi-RFP, in red, specifically in the secondary cells (surrounded by a dashed line). The glands are stained by a baso-lateral marker (DLG, magenta) and a nuclei marker (DAPI, blue).

The central structure marked by YRab14 and YRab18 (A-B, ∑) co-localizes with Golgi-marker (A'-A'' ; B-B''), YRab18 some small compartments and punctuate following colocalize, too (B-B'', *). YRab21-positive diffuse features surround the central channel, it seems to follow the Golgi outline without co-localizing (C'-C''). Scale bars, 5µm.



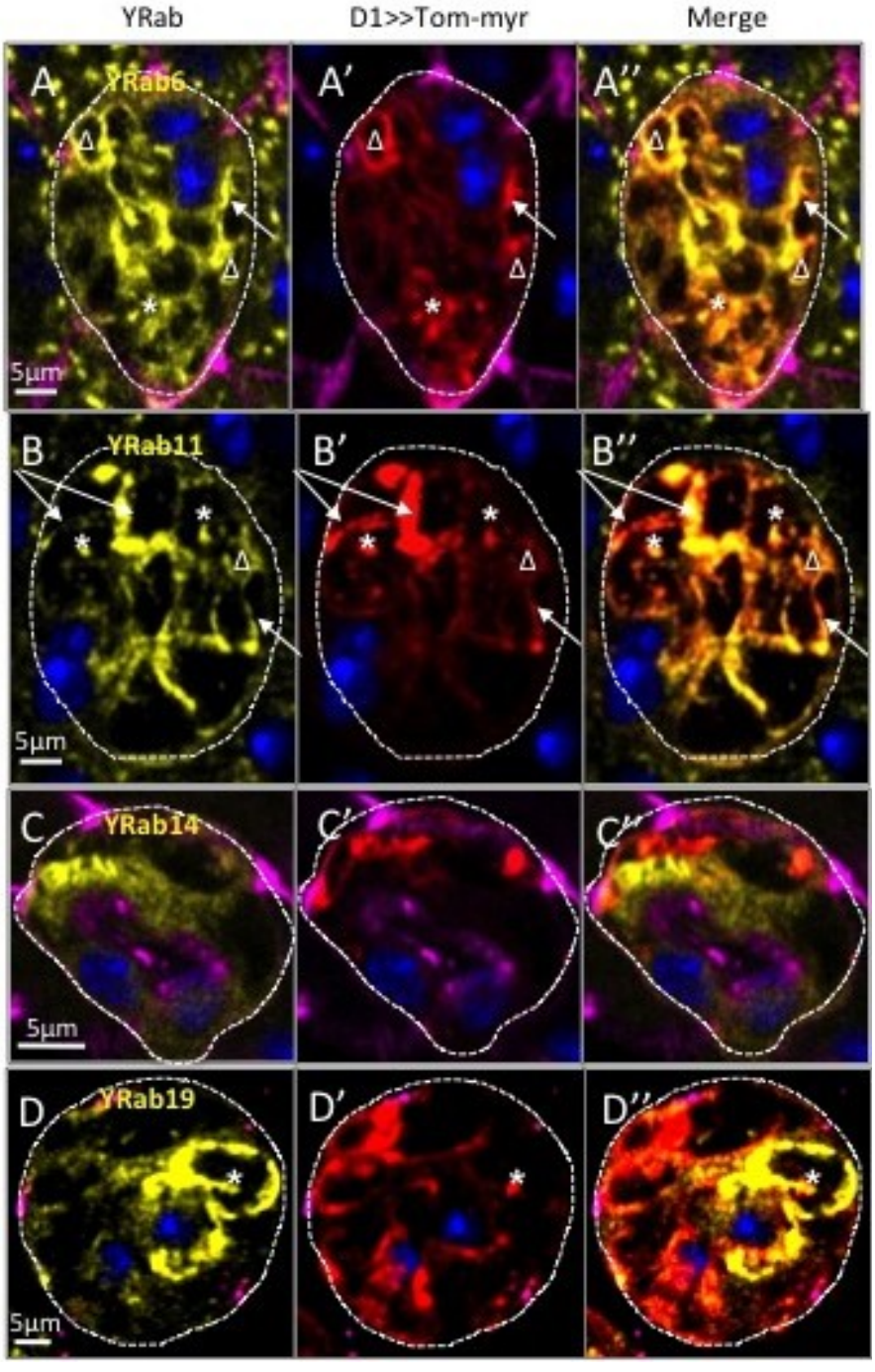
<u>Annex 5.</u> Rab1, Rab2 and Rab18 are likely mediating ER-Golgi transport

Confocal Z-sections enlargement of YRab1 (**A-A''**), YRab2 (**B-B''**) and YRab18 (**C-C''**) accessory glands expressing KDEL-RFP, in red, specifically in the secondary cells (surrounded by a dashed line). The glands are stained by a baso-lateral marker (DLG, magenta) and a nuclei marker (DAPI, blue). Although, few YRab2 punctuate colocalize with KDEl (B-B'', *), most of the YRab2 features are nearby KDEL ones without co-localizing as seen also for YRab1 (B-B''; A-A'', arrows). However, Yrab18 seem to mostly co-localize with KDEL (C-C''). Scale bars, 5µm.

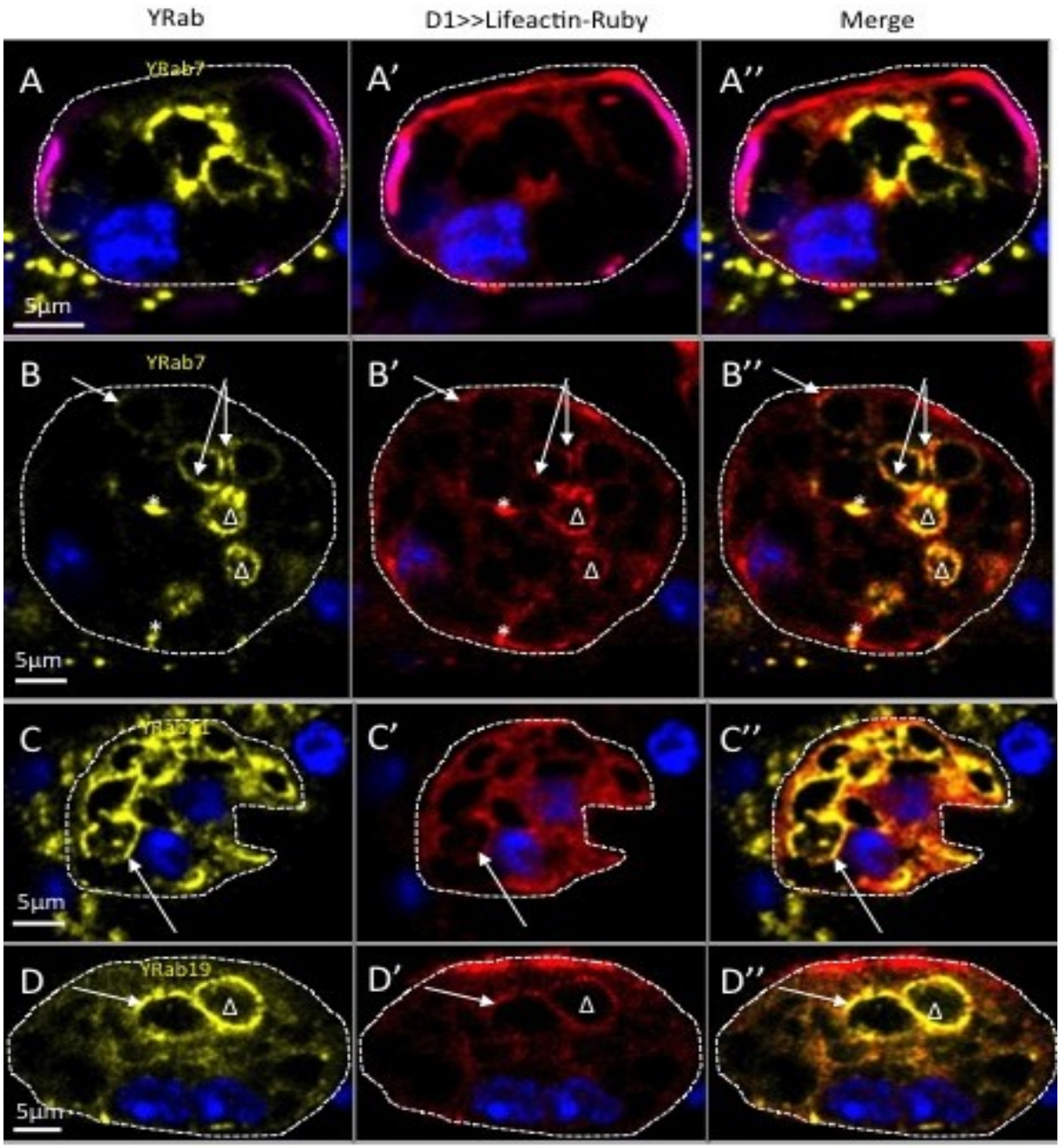


<u>Annex 6.</u> Rab6 and Rab11 large vacuoles can mediate protein delivery to the plasma membrane

Confocal Z-sections enlargement of YRab6 (A-A''), YRab11 (B-B''), YRab14 (C-C'') and YRab19 (D-D'') accessory glands expressing in a secondary-cell-specific manner, the secretory vesicles marker, Tommato-myristoylation in red. The secondary cells are surrounded by a dashed line. Some large vacuoles (Δ) (A-A''; B-B''), portion of large vacuoles (arrows) (A-A''; B-B'') and punctuate (*) (A-A''; B-B''; D-D'') can be YFP-Tomato-positive. Only 1-2 large vacuoles bound by Rab6 or Rab11 could be involved in plasma-membrane-directed vesicular transport and these vacuoles are primarily at the limiting membrane between the secondary- and the main-cells (Δ) (A-A''; B-B''). Rab19 large vacuoles are differently distributed than the myristoylated ones *ie* more apical and centered; however they can contain punctuate features directed to the plasma membrane (*) (D-D''). For Rab14, YFP signal does not co-localize with Tomato-myristoylation signal, but it seems to be following its contour (C-C''). Scale bars, 5µm.



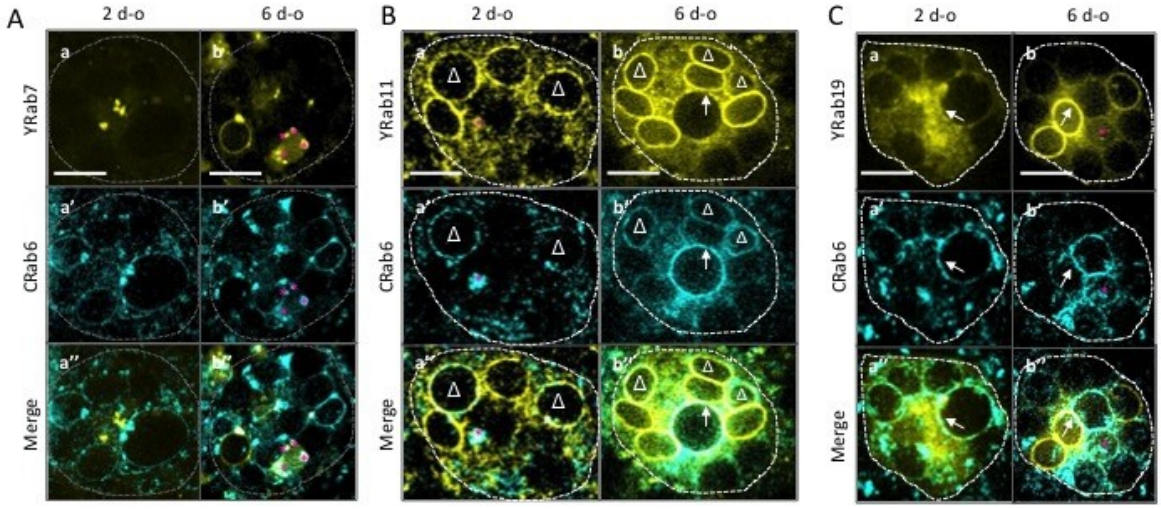
<u>Annex 7.</u> Rab7 and Rab19 large vacuoles could regulate actin-bound vesicles transport.
Confocal Z-sections enlargement of YRab6 (**A-A''**), YRab7 (**B-B''**), YRab11 (**C-C''**) and YRab19 (**D-CD'**)accessory glands expressing a F-actin marker, in red, specifically in the secondary cells (surrounded by a dashed line). The glands are stained by a baso-lateral marker (DLG, magenta) (**A-A''**) and a nuclei marker (DAPI, blue) (**A-D''**).
Rab6 compartments do not seem actin-bound (A-A'') while Rab7 and Rab19 do (B-B''; D-D''); some large vacuoles (Δ), portion of large vacuoles (arrows) and punctuate features (*).Scale bars, 5µm.



<u>Annex 8.</u> Co-expression experiments at different time point of male development

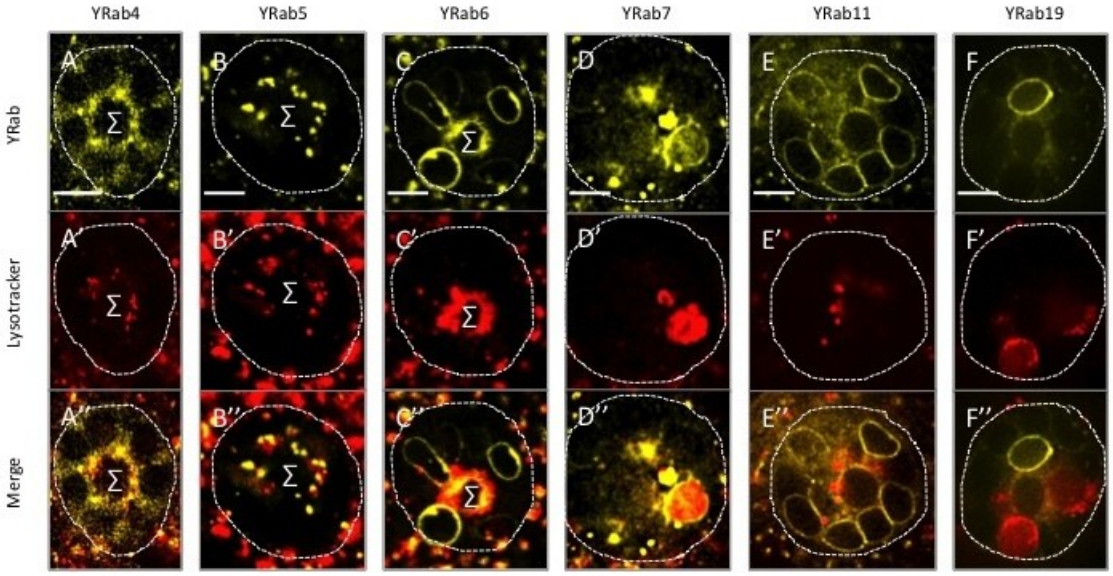
OMX microscopy picture of secondary cells enlargements from live accessory glands expressing YRab7 (**A**), YRab11 (**B**) or YRab19 (**C**) together with Rab6CFP (**A, B and C**).

The number of large vacuoles in the secondary cells seem to increase between accessory glands from males at 2 and 6 day-old (**a-a' & b-b'**) while their size apparently decreases. YRab7 and YRab19 do not co-label the same large vacuoles than CRab6 (**Aa''-b'' and Ca''-b''**), whereas YRab11 can (**B, Δ**). However, some portions of the large vacuoles (arrow), small compartments or punctate features (magenta asterix) can carry two different membrane-bound Rab GTPases. Scale bars, 5µm.



<u>Annex 9.</u> Co-expression experiments of YRab proteins and a lysosomal marker

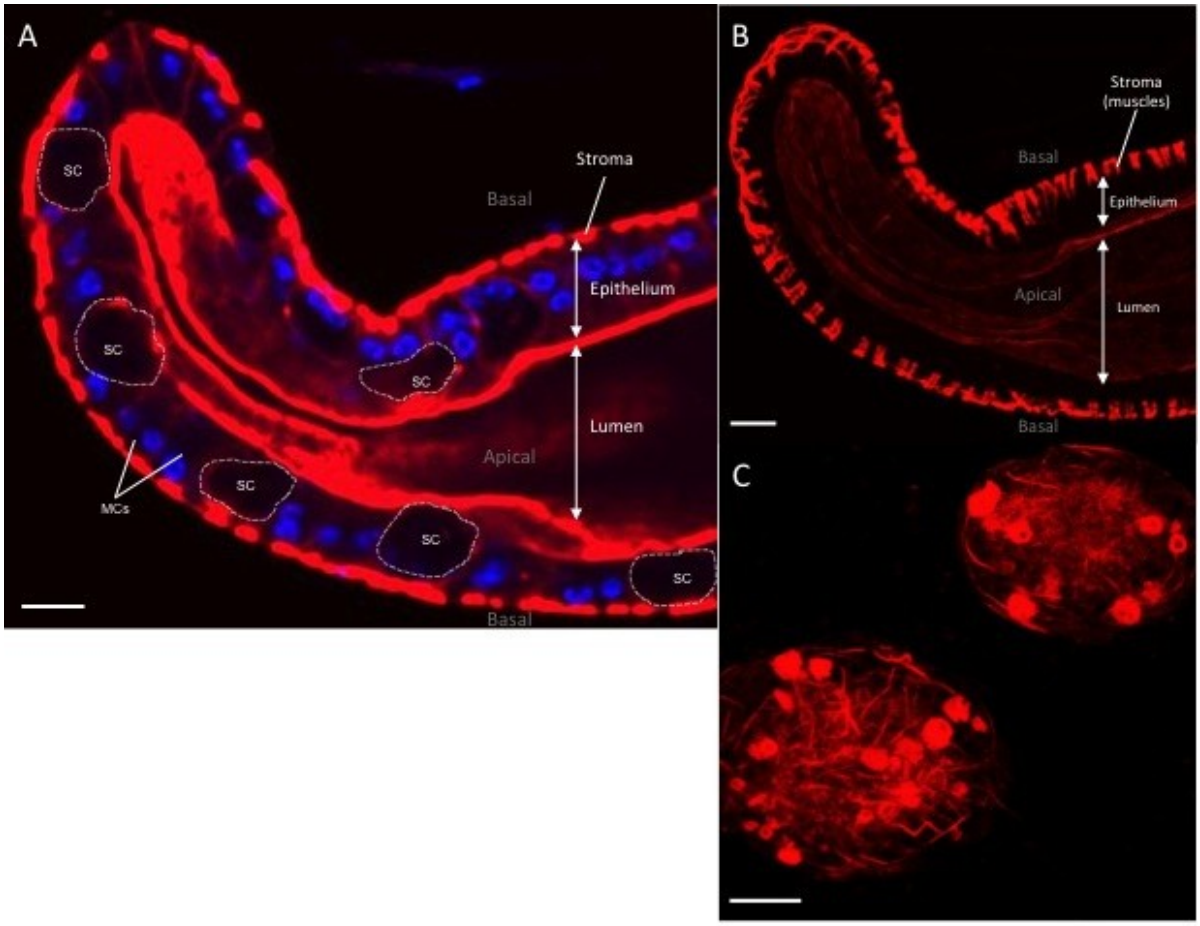
OMX microscopy section of secondary cells enlargements from live accessory glands expressing YRab4 (**A,A''**), YRab7 (**D, D''**), YRab11 (**E, E''**) or YRab19 (**F, F''**) and confocal section of secondary cells enlargement from live accessory glands expressing YRab5 (B-B'') or YRab6 (C-C''). All these accessory glands are stained by a lysosomal marker (A'-A'' to D'-D''). All YRab are in yellow and the secondary cells are surrounded by a dashed line. The accessory glands are from virgin 6days-old males. When it was possible, the central channel is depicted by Σ. Scale bars, 5µm.



<u>Annex 10.</u> The accessory glands are polarized tissues with a dense actin network

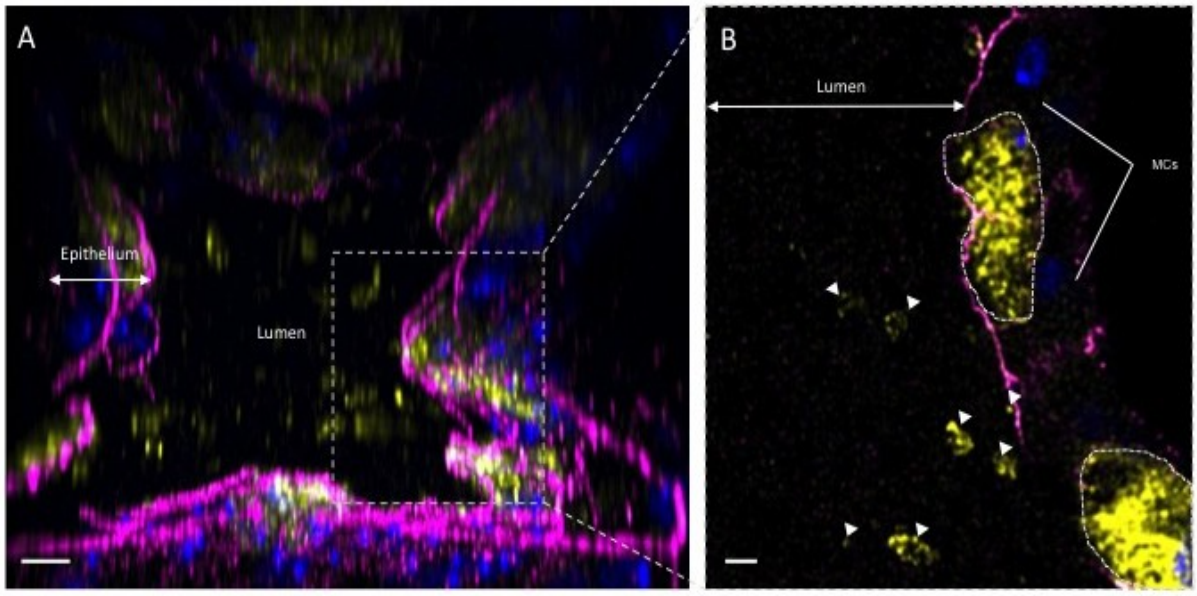
Confocal Z-section (**A**) and Z-projection(**B**) of 3 days-old male accessory glands stained by a fluorescent actin probe (red). Most of the epithelial cells have their nuclei present on the basal side (**A**; DAPI, blue). The epithelium rest on a basal membrane closed to the muscle layer while on the apical side, actin-filaments seem to emerge from the cells toward the luminal space (A & B). Scale bars, 15µm

Confocal Z-projection of secondary cells expressing a F-actin reporter gene (Lifeactin-Ruby) in a cell-type-specific manner (C); the actin network is enriched apically and centered. Also, some Actin-bound vesicles and/or polymerizing actin (Yu et al., 2011). Scale bar, 5µm.



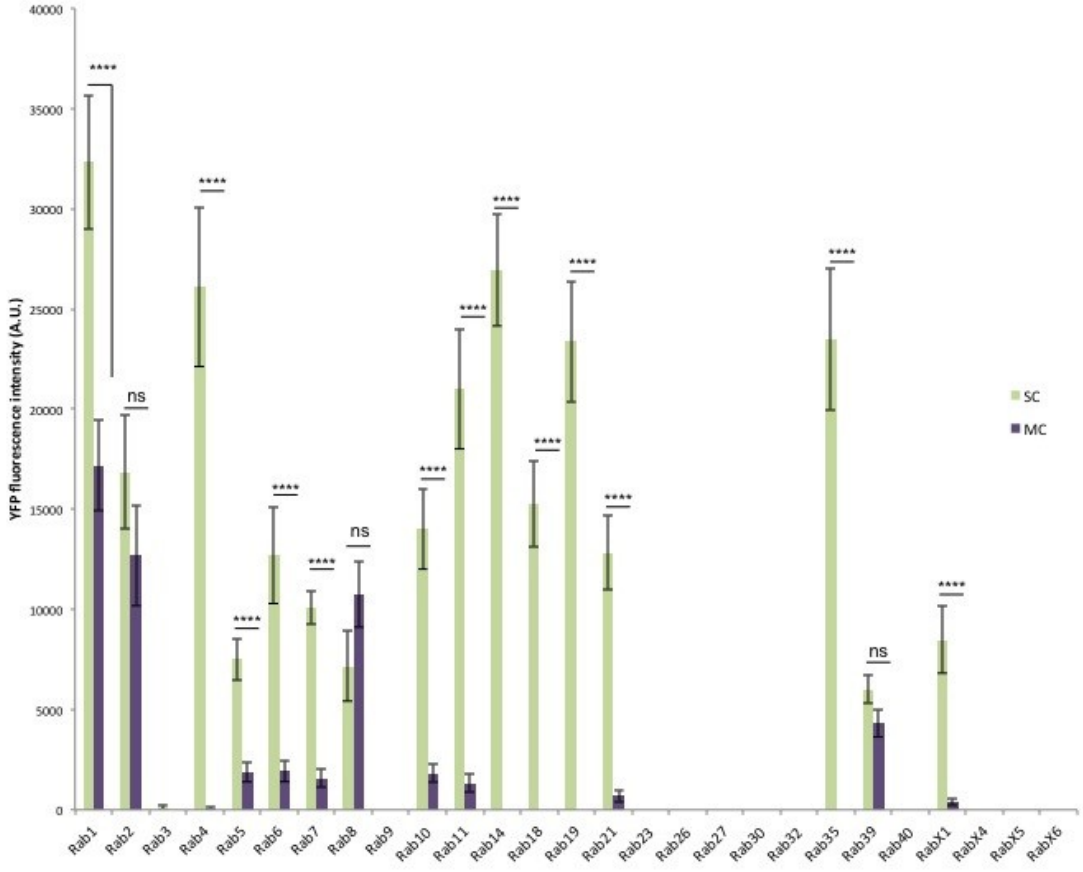
<u>Annex 11.</u> The secondary cells secreted Rab19-bound vesicles into the lumen

3D-view snapshot (**A**) and confocal section (**B**) of a YRab19 (yellow) accessory gland from a 3 days-old male. The gland is stained by an apical marker, DE-Cadherin (magenta) and the nuclei probe (DAPI, blue). YRab19 positive "bulbs" are visible in glandular lumen since virgin males are 3 days-old (**A**&**B**) Some are shown with arrow-heads (**B**); their size vary from puncta to ≈ 2-4µm of diameter. Scale bars, 5µm



<u>Annex 12.</u> The expression levels of the Rab proteins in both accessory gland cell types

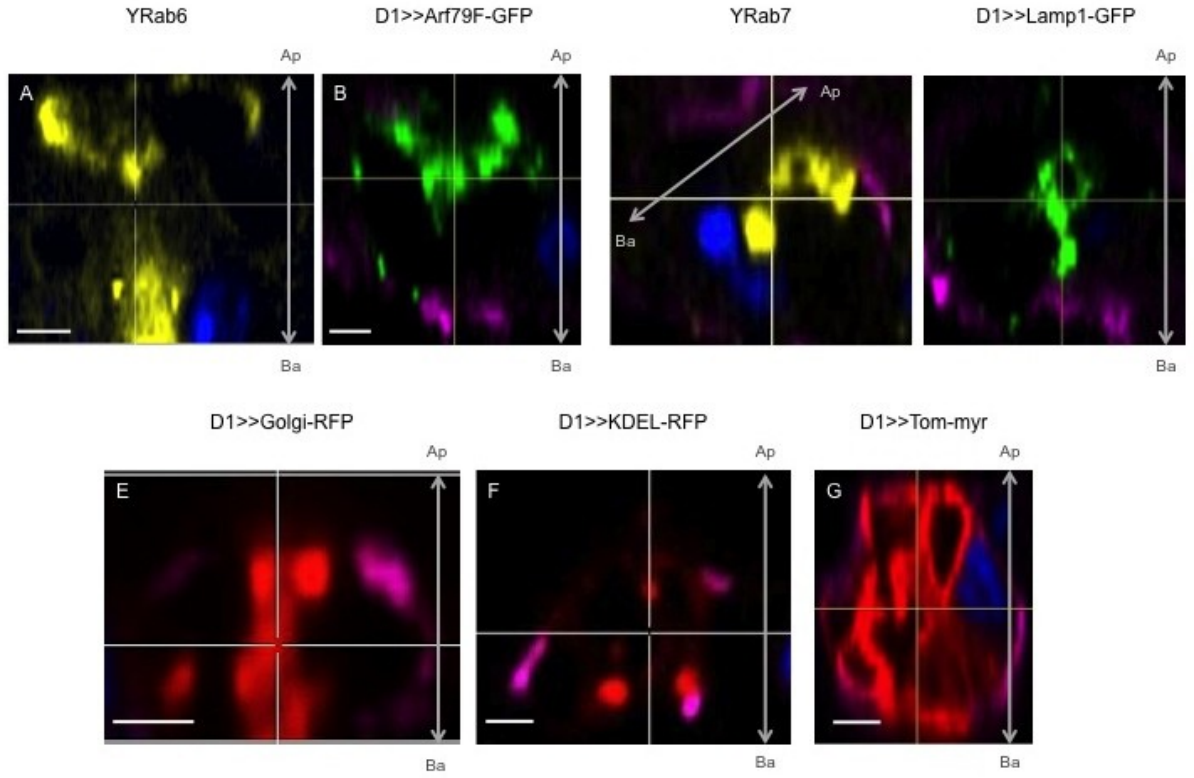
Histogram representing the expression levels of the 27 *Drosophila* YFP-tagged Rab in the secondary cells (SC, lavender) and the main cells (MC, khaki). These accessory glands were fixed and stained with an anti-GFP antibody. This quantification has been done on accessory glands from males whose age and mating status were not taken in account. Statistics, $p<0.0007$ (****); $p>0.08$ (non-significant, ns).



<u>Annex 13.</u> The distribution of intra-cellular markers in the secondary cells

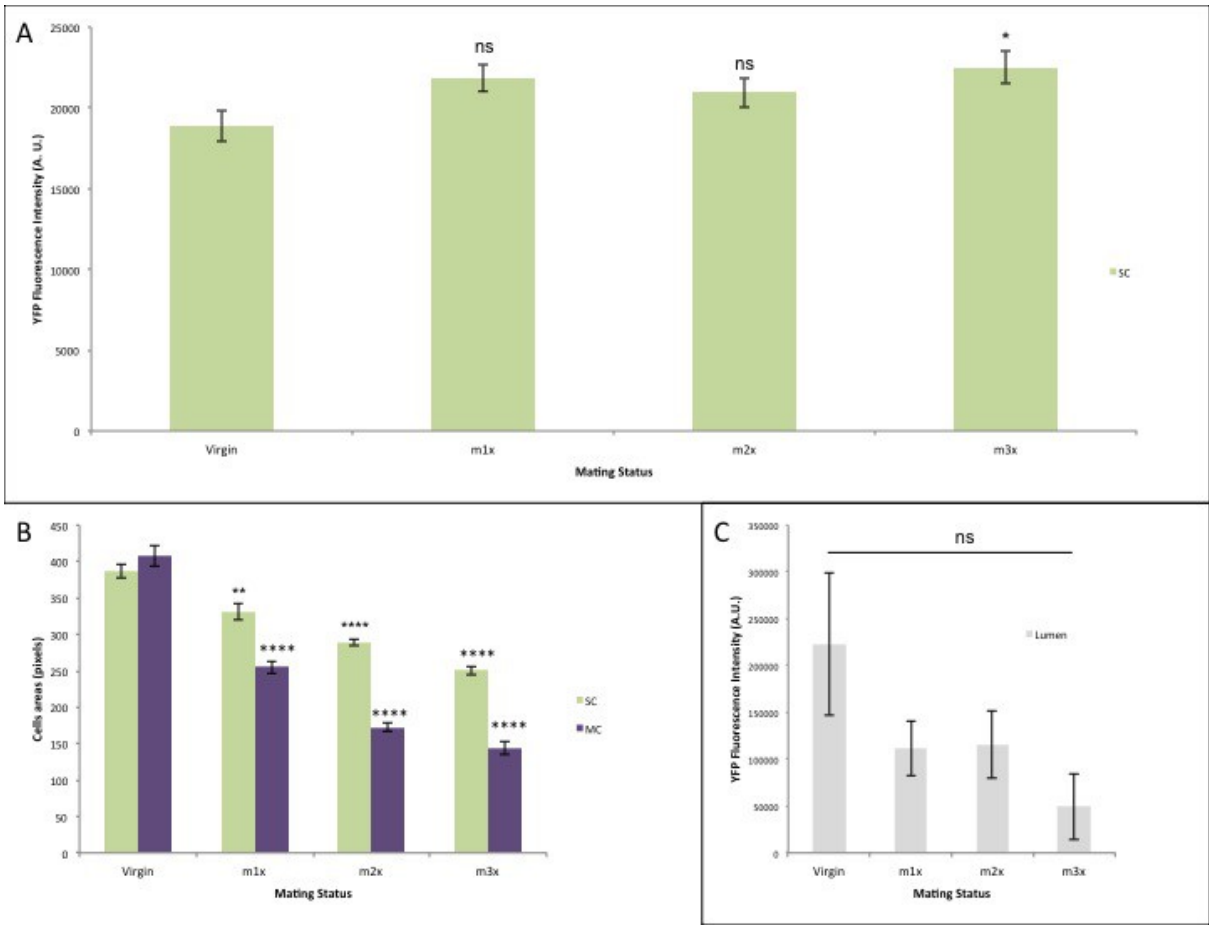
Sagittal view enlargements of secondary cells from accessory glands expressing YRab6 (A), YRab7 (C) and the intra-cellular markers in a secondary cell-specific manner: Arf79F-GFP (B), Lamp1-GFP (D), Golgi-RFP (E), KDEL-RFP (F) and Tomato-myristoylation (Tom-myr, G). The apical-basal (Ap-Ba) axis is represented as double-ends arrows. Scale bars, 5µm.

The apical distribution of Rab6 (a large vacuole) (A) is reminiscent of the *trans*-Golgi network marker, Arf79F-GFP (B); while the expression pattern of Rab7 looks like the lysosomal marker, Lamp1-GFP (C and D, repectively). The Golgi apparatus is distributed from the basal to the apical face of the secondary cells as a large central channel (E); there are several round-shaped endoplasmic reticulum, which seem to surround the contour of the central channel (F). The compartments containing myristoylated proteins are mainly cortical but they seem to follow the central channel outlines (G).

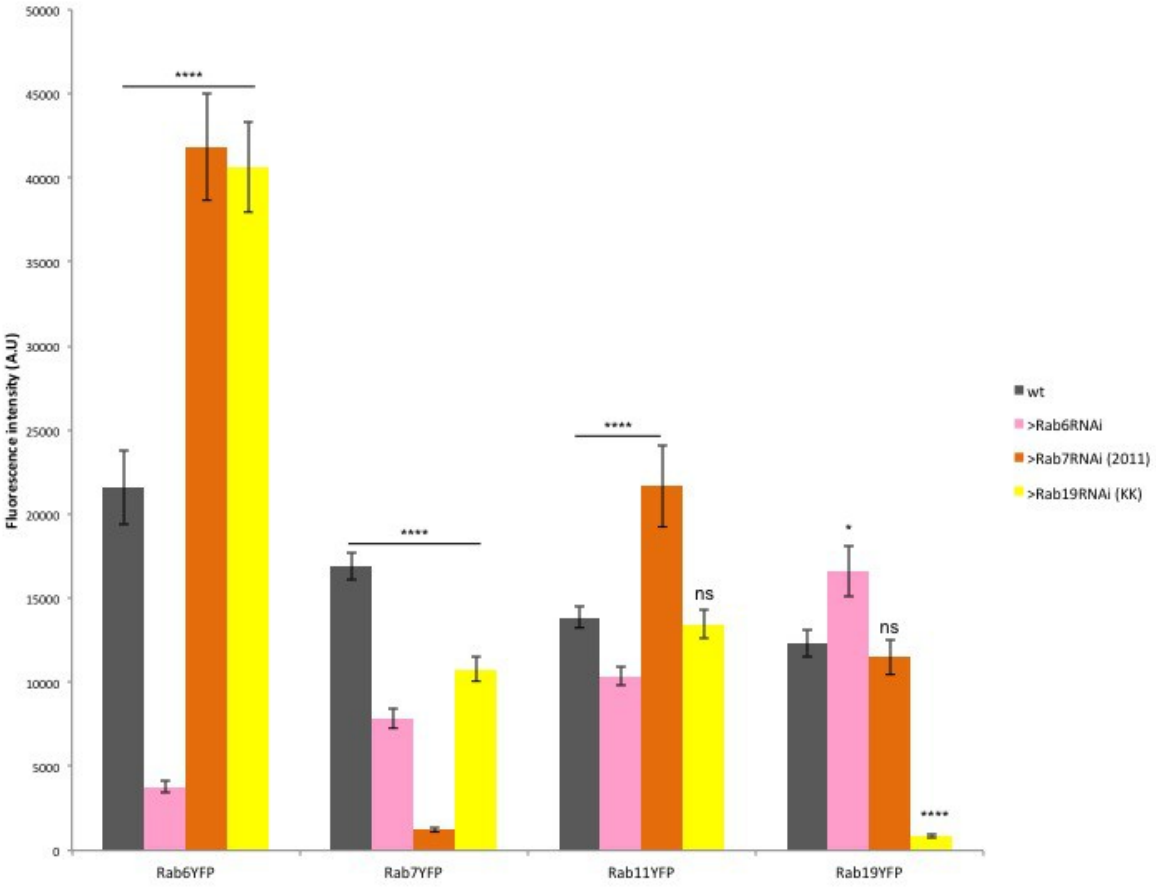


<u>Annex 14.</u> The effects of mating on the expression of Rab19 and the secondary cell area

Histograms depicted the expression levels of Rab19 in the secondary cells (A) and the lumen (B) of accessory glands from virgin males and males that mate once to three times. Mating does not seem to affect significantly the expression of Rab19 in the secondary cells and the lumen, even if in the lumen the tendency seem to be a decrease. The fact that Rab19 levels do not vary in the secondary cells support that previous studies showing that the expression of secondary-cell specific transcript are not affected by mating (Bertram et al., 1992). Regarding the cell size (B), mating seem to decrease the area of both gland cell types, main cells (MC, lavender) and the secondary cells (SC, khaki). However, the observation that the size of the secondary cells tends to decrease by mating do not correlate with previous data showing that mating stimulates the growth of the secondary cells (Leiblich et al., 2012); this could be due to the fact that I measured the area of the secondary cells while they measured the size of their nuclei. Statistics, $p<0.0001$ (****); $p=0.0020$ (**), $p=0.0315$ (*); $p>0.050$ (non-significant, ns).

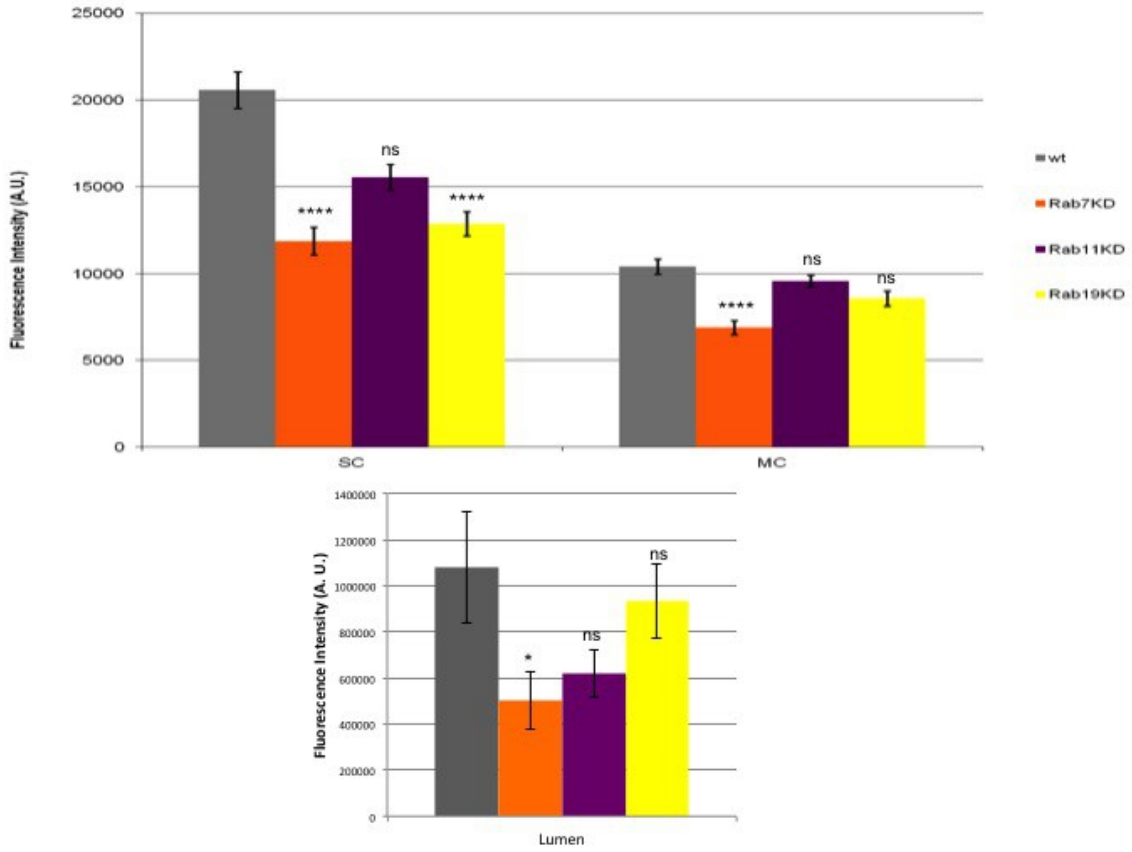


<u>Annex 15.</u> The depletion of a specific Rab protein can affect the expression of the other ones
Histograms representing the expression of Rab6, Rab7, Rab11 and Rab19 in *wild-type* condition (grey), in absence of Rab6 (pink), Rab7 (orange) and Rab19 (yellow). Statistics, p<0.0001 (****); p= 0.0263 (*); p>0.5 (non-significant, ns).

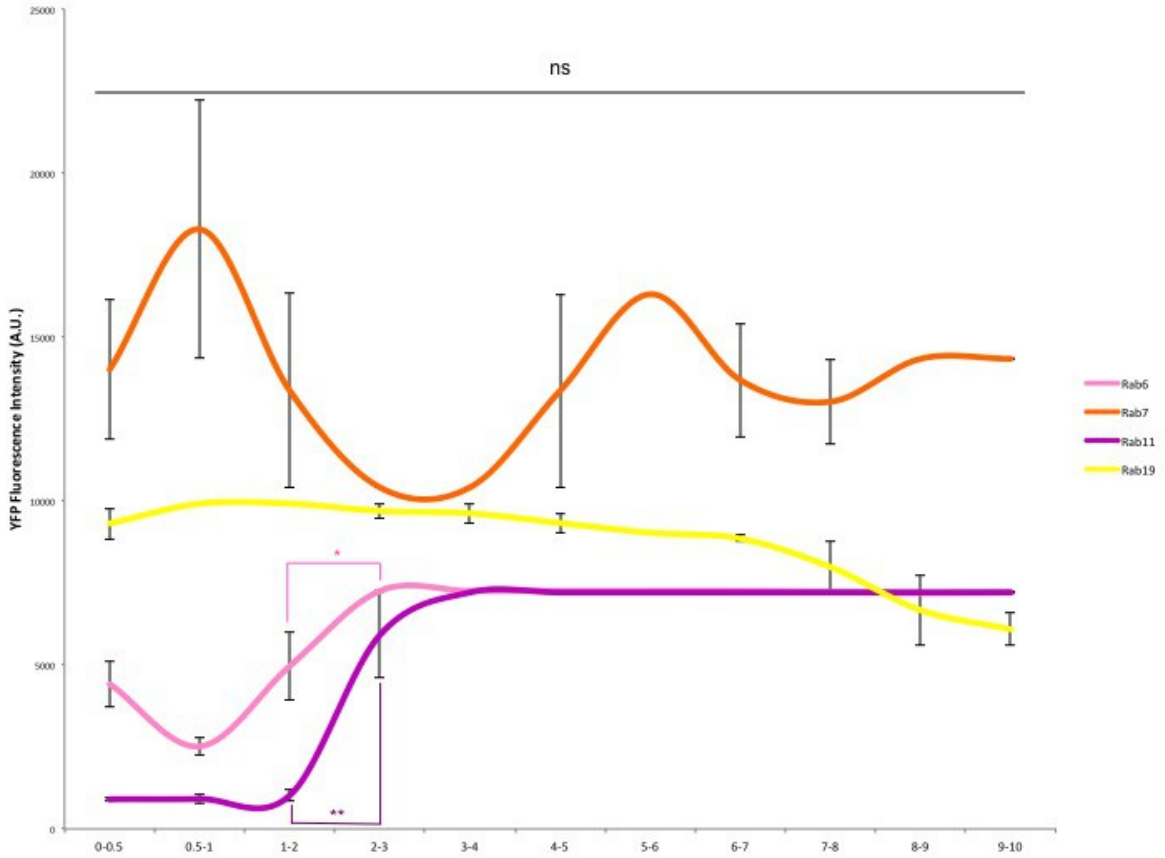


<u>Annex 16.</u> The depletion of specific Rab in the secondary cells can affect the presence of Ovulin in the different compartments of the accessory glands

Histograms depicted the levels of Ovulin in the secondary cells (SC), main cells (SC) (on the top) and in the lumen (on the bottom) from accessory glands where a GFP- RNAi have been expressed in the secondary cells of YRab7 (orange), YRab11 (purple) and YRab19 (yellow) accessory glands from 3 days-old virgin males. The glands have been stained with an Ovulin antibody. These are preliminary results done on few accessory glands.



<u>Annex 17.</u> The expression levels of the Rab proteins in the secondary cells depending on male age
Curves representing the expression levels of Rab6 (pink), Rab7 (orange), Rab11 (purple) and Rab19 (yellow) in secondary cells of accessory glands from virgin males at different ages. There is a significant burst of Rab6 and Rab11 expression since two day post-eclosion and these levels are maintained with maturation. Although Rab7 expression seems cycling, these variations are statistically insignificant. Rab19 expression does not vary along ages. Statistics, p= 0.0059 (**); p=0.0233 (*); p>0.1 (non-significant, ns).



<u>Annex 18.</u> The effects of mating on the presence of Ovulin in the accessory glands

On the top, histogram depicted the presence of Ovulin in three days-old virgin males (A, 3do), mated once and dissected right after mating (A, 3dom1x) or dissected one day after mating (A, 3dom1x1d).

On the bottom, histograms depicted, the presence of Ovulin in accessory glands dissected from four-to-seven days-old males unmated (B, 0x) or mated one, two or three-times (B, 1x, 2x or 3x, respectively).

In 3 days-old males, mating does not seem to affect the levels of Ovulin (A), while in older males it does (B). Expectidly, mating reduces the presence of Ovulin in the seminal fluid container *ie* the lumen (light-grey) as well as in the main cells (MC, lavender) and the secondary cells (SC, khaki). Statistics, p<0.0003 (****); p=0.0236 (*); p>0.3 (non-significant, ns).