Multiple Distinct Roles for the Rab11 GTPase in the Somatic Cells of the *Drosophila* Egg Chamber

By

Jiang Xu

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Chairperson – Robert S. Cohen

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Victoria L. Corbin

_____

Robert E. Ward IV

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Erik A. Lundquist

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Jennifer M. Gleason

Date Defended: 01-20-2011

The Dissertation Committee for Jiang Xu

certifies that this is the approved version of the following dissertation:

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Chairperson - Robert S. Cohen

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Abstract

The *Drosophila* egg chamber provides an excellent system in which to study the specification and differentiation of epithelial cell fates because all of the steps, starting with the division of the corresponding stem cells (called follicle stem cells or FSCs), have been well described and occur many times over in a single ovary. Here I investigate the role of the small *Rab11* GTPase in follicle stem cells (FSCs) and in their differentiating daughters, which include main body epithelial cells, stalk cells and polar cells. I show that *rab11*-null FSCs maintain their ability to self renew, even though previous studies have shown that FSC self renewal is dependent on maintenance of E-cadherin-based intercellular junctions, which in many cell types, including *Drosophila* germline stem cells, requires Rab11. I also show that *rab11*-null FSCs give rise to cells that enter polar, stalk, and epithelial cell differentiation pathways, but that none of the cells are able to complete their differentiation programs and that the epithelial cells undergo premature programmed cell death. Finally I show, through the induction of *rab11-null* clones at later points in the differentiation program, that Rab11 suppresses tumor-like epithelial cell growth. Thus, *rab11*-null epithelial cells arrest differentiation early, assume an aberrant cell morphology, delaminate from the epithelium, and invade the neighboring germline cyst. These phenotypes are associated with defects in E-cadherin localization and a general loss of cell polarity. While previous studies have revealed tumor suppressor or tumor suppressor-like activity for regulators of endocytosis, this study is the first to identify such activity for regulators of endocytic recycling. This study also supports the recently emerging view that distinct mechanisms regulate junctional stability and plasticity in different tissues.

Acknowledgements

I would like to thank all the people who have contributed to the work presented in this dissertation: my mentor Bob Cohen, for his wise guidance and superb training over my entire graduate career; previous and current members of the Cohen lab including, but not limited to, Dr. Lan Lan, Nicholas Bogard, Sui Zhang, and Cristin Gustafson, for their vital contributions to my research; Dr. David Moore and Heather Shinogle, for their excellent technical support in confocal microscopy.

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Last, but not least, I would like to thank my family members whom have backed me firmly in my graduate career, especially my wife Yan Zhang, my son PeiYuan Xu, my father XingMing Xu, and my mother Nan Jiang.

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Chapter I

Introduction

This thesis describes multiple distinct roles of a membrane trafficking organizer, the Rab11 GTPase, in the germline and somatic cells that comprise the *Drosophila* egg chamber. Important events during *Drosophila* oogenesis that involve Rab11 are introduced below.

1.1 Stem Cell Maintenance and Differentiation in the *Drosophila* Germarium

Drosophila females have a pair of ovaries each composed of 16 to 20 ovarioles. Each ovariole contains a string of egg chambers at consecutive stages of development with the most mature egg chambers located at the posterior, and a germarium located at the anterior, which contains the stem cells that give rise to the egg chambers.

At the anterior end of the germarium are 8-10 disk-like terminal filament cells (TFCs) with the most posterior TFC joined immediately by 5-7 cap cells (CCs) (Kirilly and Xie 2007) (Figure 1.1). Two to three germline stem cells (GSCs) are anchored to cap cells by adherens junctions (AJs) (KIRILLY and XIE 2007; SONG *et al.* 2002), and ensheathed by 4-6 escort stem cells (ESCs). GSCs are maintained in a microenvironment or a "niche" formed by the local somatic cells, especially the cap cells (KIRILLY and XIE 2007; SONG *et al.* 2002; XIE and SPRADLING 2000). The niche cells repress *bag-of-marbles (bam)* transcription in the GSCs by secreting Decapentaplegic (Dpp) and Glass-bottom-boat (Gbb) proteins (SONG *et al.* 2004; XIE and SPRADLING 1998; XIE and SPRADLING 2000), both members of the Bone morphogenic protein (BMP) family of secreted signaling molecules. GSCs self-renew and generate differentiated progeny through asymmetric cell division along the anterior-posterior axis. Each GSC continuously divides, giving rise to another GSC and a cystoblast. The cystoblast subsequently divides four times with incomplete cytokinesis to form a 16-cell cyst

with a shared cytoplasm. As the germline cyst moves away from the new GSC, it is first covered by escort cells produced by ESCs, then surrounded by pre-follicle cells (pre-FCs) derived from two somatic follicle stem cells (FSCs) located in the middle of the germarium (Margolis and Spradling 1995). Finally, the 16 cell cyst buds from the germarium to form an individual egg chamber.

1.2 Cell Fate Specification in the Germline Lineage during Early Oogenesis

A germline specific organelle called the fusome plays an important role in the division of the GSC and in specifying its daughter cells' fates (Huynh and St Johnston 2004). The fusome originates as a vesicle- and Spectrin- rich structure called the spectrosome in the GSC (DE CUEVAS *et al.* 1996; LIN *et al.* 1994), and helps the GSC's asymmetric division by anchoring one pole of the mitotic spindle to the anterior cortex of the GSC (DENG and LIN 1997; SONG *et al.* 2004; XIE and SPRADLING 1998; XIE and SPRADLING 2000). The anterior daughter cell of the GSC inherits the AJ and about two thirds of the fusome, and remains in the niche and maintains its GSC identity; the posterior daughter cell receives the rest of the fusome and becomes the cystoblast (DE CUEVAS *et al.* 1996; DENG and LIN 1997; MCGRAIL and HAYS 1997). The asymmetric behavior of the fusome continues throughout the subsequent divisions of the cystoblast such that one cell ultimately has more fusome material than the other 15 daughter cells and it is always this cell that becomes the oocyte (Huynh and St Johnston 2004).

1.3 Cell Fate Specification in the Somatic Lineage during Early Oogenesis

FSCs give rise to three groups of follicle cells: follicle epithelial cells (FECs; gray in

Figures 1.1 and 1.2), which form a single layer epithelium around the germline cyst; stalk cells (blue in Figures 1.1 and 1.2), which separate neighboring cysts into individual egg chambers; and polar cells (red in Figures 1.1 and 1.2), which are located at both ends of the egg chamber and are involved in multiple important signaling events (ROTH and LYNCH 2009; ST JOHNSTON and AHRINGER 2010). Cell fate specification of the FSC lineage depends on cell-cell interaction (ST JOHNSTON and AHRINGER 2010; WU *et al.* 2008) (Figure 1.2), as the two FSCs give rise to pre-FCs with no cell fate preference (MARGOLIS and SPRADLING 1995; NYSTUL and SPRADLING 2010). As the 16-cell cysts are pushed toward the posterior of the germarium, pre-FCs derived from the two FSC migrate over and cover the cyst (NYSTUL and SPRADLING 2010). Some of these pre-FCs stop dividing to become polar or stalk cells, while the other pre-FCs continue to divide and surround the cyst to form a single layer of epithelium (NYSTUL and SPRADLING 2010).

Anterior polar cells of an egg chamber are specified starting as early as when pre-FCs contact the germline cyst and continuing through stage 2 (NYSTUL and SPRADLING 2010; ROTH and LYNCH 2009). The Delta/Notch pathway plays an important role throughout this process (ASSA-KUNIK *et al.* 2007; GRAMMONT and IRVINE 2001; LOPEZ-SCHIER and ST JOHNSTON 2001; NYSTUL and SPRADLING 2010; TORRES *et al.* 2003; VACHIAS *et al.* 2010). About 4-6 pre-FCs at the anterior pole of an egg chamber receive a strong Delta signal from the germline cyst, upregulate Notch, and are specified as pre- polar cells, but only the two cells expressing the highest level of Notch survive apoptosis and eventually become the anterior polar cells of that egg chamber (VACHIAS *et al.* 2010). Specification of the

posterior polar cells of an egg chamber starts after the specification of the anterior polar cells of the adjacent older egg chamber, but the posterior polar cells are not fully committed until much later during oogenesis and will not be discussed here (NYSTUL and SPRADLING 2010; ROTH and LYNCH 2009; TORRES *et al.* 2003; VACHIAS *et al.* 2010).

After being specified by the Delta signal from the cyst, the anterior pre-polar cells also produce Delta themselves to induce another 6-8 pre-FCs that are not in direct contact with the older cyst to become stalk cells (ASSA-KUNIK *et al.* 2007; GRAMMONT and IRVINE 2001; TORRES *et al.* 2003). Stalk cell specification requires sustained Delta signaling from the pre-polar cells of the older egg chamber, and the correct number of stalk cells relies on normal amount of Delta signals (ASSA-KUNIK *et al.* 2007; GRAMMONT and IRVINE 2001; LOPEZ-SCHIER and ST JOHNSTON 2001; ROTH and LYNCH 2009; TORRES *et al.* 2003; VACHIAS *et al.* 2010). Starting from stage 2, the initially clustered stalk cells intercalate to form a single file stalk and this process requires induction of the JAK/STAT pathway by Unpaired signals from the anterior polar cells of the adjacent older egg chamber (BAKSA *et al.* 2002; MCGREGOR *et al.* 2002; ROTH and LYNCH 2009).

1.4 Maintenance of the Follicle Epithelium (FE)

The *Drosophila* follicle epithelial cells (FECs) derive from FSCs and are polarized with apical membranes facing the germline cyst and basal membranes facing an extracellular matrix (Figure 1.3). The neighboring FECs also contact each other through lateral cell junctions to act as a paracellular barrier and to form a continuous sheet of epithelium (St Johnston and Ahringer 2010). The proper development of the FECs requires their contact to

the basement membrane, the germline cyst, and their neighboring FECs (ST JOHNSTON and AHRINGER 2010; TANENTZAPF *et al.* 2000). Besides these spatial cues, the Crumbs complex apical to the AJ, and the Bazooka (PAR-3) complex in the AJ play important roles in the development and maintenance of the follicle epithelium (FE) (ABDELILAH-SEYFRIED *et al.* 2003; TANENTZAPF *et al.* 2000). The Cadherin-Catenin complex in the AJ is essential for maintaining the FE, but not for its early development (DOBENS and RAFTERY 2000; TANENTZAPF *et al.* 2000). Mutation of any of these polarity regulation genes causes mature FE to lose polarity and form multi-layers (ABDELILAH-SEYFRIED *et al.* 2003; BROWN 2000; DOBENS and RAFTERY 2000; ST JOHNSTON and AHRINGER 2010; TANENTZAPF *et al.* 2000).

The Scribble complex genes *scribble (scrib)*, *discs-large (dlg)*, and *lethal giant larvae (lgl)* have been identified as *Drosophila* nTSGs (neoplastic Tumor Suppressor Genes). They are organizers of the Septate Junction (SJ), which is the invertebrate counterpart of the vertebrate Tight Junction, but is positioned basal to the AJ. Mutation in any of the Scribble complex genes allows FECs to over-proliferate and invade in between the germline cells as a multi-layered sheet (Bilder 2004).

1.5 Membrane Trafficking and the Rab GTPases

Vesicles bud from donor membranes, move along elements of the cytoskeleton, and dock on and fuse to target membranes, in a process known as membrane trafficking (Figure 1.4). A group of small GTPases called Rab (Ras-related protein in brain) proteins were identified more than twenty years ago as essential regulators of membrane trafficking (SALMINEN and NOVICK 1987; SCHMITT *et al.* 1986; TOUCHOT *et al.* 1987). Over the years,

additional Rabs have been identified in all studied eukaryotes, and now Rab GTPases form the largest branch of the Ras superfamily; there are 11 Rab proteins in yeast (Zerial and McBride 2001), 31 in *Drosophila* (ZHANG *et al.* 2007), and more than 70 in mammals (Colicelli 2004).

The general Rab structure is highly conserved in all Rabs and across species (BRIGHOUSE *et al.* 2010; PEREIRA-LEAL and SEABRA 2001; VALENCIA *et al.* 1991). Intracellular Rabs are attached to membranes through lipid modification of C-terminal cysteine residues. These modifications are carried out by a class of enzymes known as Rab geranylgeranyl transferases (CASEY and SEABRA 1996; SEABRA *et al.* 1992). Just as Ras GTPases work as molecular switches, Rab proteins also cycle between active GTP-bound and inactive GDP-bound states in regulating membrane trafficking and this cycle involves Rabs' Mg^{2+} and guanine binding motifs (PM/G motifs) (BARBACID 1987; BOURNE *et al.* 1990; BOURNE *et al.* 1991; VALENCIA *et al.* 1991). Guanosine nucleotide exchange factors (GEFs), which exchange GDP for GTP in Rabs, and GTPase-activating protein (GAPs), which facilitate the Rabs' GTP hydrolysis ability, play important roles in the active-inactive cycle of Rabs (SCHWARTZ *et al.* 2007; STENMARK 2009; ZERIAL and MCBRIDE 2001). Rab family domains (RabFs) have been shown to be conserved in all Rabs and across species, and are thought to determine interactions with the general Rab regulators such as Rab escort proteins (REPs), which are involved in Rab prenylation, and GDP dissociation inhibitors (GDIs), which are involved in Rab membrane release (Alory and Balch 2000; Pereira-Leal and Seabra 2000; Pereira-Leal and Seabra 2001).

Each Rab uniquely mediates trafficking from a specific donor membrane to a specific target membrane (Stenmark 2009). For example, Rab11 controls the trafficking of vesicles from recycling endosomes to the plasma membrane, while Rab2 mediates retrograde trafficking from Golgi to ER (Endoplasmic Reticulum) (SCHWARTZ *et al.* 2007; STENMARK 2009). The emerging view is that each Rab recruits a unique set of four different effectors or effector complexes (EI-EIV), with each effector mediating a distinct trafficking step. Thus a membrane trafficking event is mediated by only one Rab. A Rab protein first binds to its EI or EI complex on the donor membrane, which facilitates cargo selection and vesicle budding. One good example is Rab9 and its EI, TIP47 (CARROLL *et al.* 2001). To move the cargo along the cytoskeleton to the target membrane, the Rab protein then binds to its EII, which is a motor protein or member of a motor complex. For example, Rab11 and its two EIIs, myosin motor MyoV and dRip11, form a complex to facilitate vesicle transport (LI *et al.* 2007). The EIII docking effectors in some cases are shared by different Rabs, like the exocyst on the plasma membrane (TERBUSH *et al.* 1996), which serves both Rab11 in the endocytic recycling pathway (JAFAR-NEJAD *et al.* 2005; WU *et al.* 2005; ZHANG *et al.* 2004), and Rab8 in the secretory pathway (MAZELOVA *et al.* 2009). EIV proteins facilitate membrane fusion by apparently mediating binding between v-SNARE on the vesicle and t-SNARE on the target membrane (LEHMAN *et al.* 1999).

Since all Rab proteins' prenylation domains, PM/G motifs, and RabFs are highly conserved, these structures are less likely to contribute to each Rab's unique role in membrane trafficking and its specific effector binding preference. Switch regions I and II

of Rabs undergo dramatic conformational changes between GTP-bound and GDP-bound forms, and have been shown to be involved in effector binding of GTP-bound active Rabs (OSTERMEIER and BRUNGER 1999; SHIBA *et al.* 2006; STENMARK 2009). Four Rab sub-family specific regions (RabSF1-4) have been identified to be highly conserved (58.4%-92.3% identical) within each of the Rab sub-families, but quite diversified (14.4%-34.2% identical) in all other Rabs, suggesting they are important for Rab-specific effector and regulator binding (Pereira-Leal and Seabra 2000). Rabs form two putative effector binding surfaces with RabSF1, RabSF3, RabSF4 and part of switch II on one side, and RabSF2, switch I and II on the opposite side (OSTERMEIER and BRUNGER 1999; PEREIRA-LEAL and SEABRA 2000; SHIBA *et al.* 2006).

1.6 Functions of Rab11

Drosophila Rab11 is the only *Drosophila* homologue of the human Rab11 subfamily, which consists of Rab11a, Rab11b, and Rab25 (Rab11c), and is about 82% identical in amino acid sequence to them (Figure 1.5). *Drosophila* Rab11 directs trafficking from the recycling endosome to the plasma membrane. Some of its effector proteins are know: its EII and EIII are MyoV-dRip11 complex and Sec15, respectively. Rab11's possible EI effector is Nuclear-fallout (Nuf), a homologue of Arfophilin-2 (RIGGS *et al.* 2003) and its candidate EIV is a *Drosophila* nTSG, Lethal-giant-larvae (Lgl), which is the homolog to the yeast Sro7p and Sro77p, two SNARE regulators with redundancy to each other (LEHMAN *et al.* 1999) (Figure 1.4).

Rab11 and its effectors are involved in a wide variety of cellular processes and

morphogenesis events. *Drosophila* Rab11 is essential for: *oskar* mRNA localization and cell polarization in the oocyte (DOLLAR *et al.* 2002; JANKOVICS *et al.* 2001); stem cell maintenance in the GSC (BOGARD *et al.* 2007); proper cytokinesis in spermatocytes (GIANSANTI *et al.* 2007; ROBINETT *et al.* 2009); syncytial nuclear division and cellularization in the embryo (PELISSIER *et al.* 2003; RIGGS *et al.* 2003); asymmetric cell division and cell-cell signaling in the sensory neuron lineage (EMERY *et al.* 2005; JAFAR-NEJAD *et al.* 2005); cell intercalation in the trachea (SHAYE *et al.* 2008); epithelial polarization in the embryonic ectoderm (ROETH *et al.* 2009), and polarized secretion in the photoreceptors (BERONJA *et al.* 2005; LI *et al.* 2007; SATOH *et al.* 2005). This wide variety of Rab11 functions reflects the different cargoes being transported through Rab11 mediated membrane trafficking in different cell types.

There is growing evidence to support the proposal that endocytic recycling, and especially the Rab-dependent pathway, is an important component of cell migration (JONES *et al.* 2006), and that this pathway is altered during tumor metastasis (MOSESSON *et al.* 2008). The Rab11 pathway has been implicated in epithelial-mesenchymal transition, cell migration, and tumor cell invasion by cell culture studies using dominant-negative Rab11 GTPase mutations (POWELKA *et al.* 2004; PRIGOZHINA and WATERMAN-STORER 2006; YOON *et al.* 2005). Moreover, Rab11 sub-family gene levels are altered in invasive tumor cells and malignant tumor samples (CHENG *et al.* 2010; CHENG *et al.* 2004; GEBHARDT *et al.* 2005; GOLDENRING *et al.* 1999; NAM *et al.* 2010; WANG *et al.* 2004; YOON *et al.* 2005).

Two obstacles stand in the way to our understanding Rab11-mediated membrane

trafficking, and its normal and abnormal functions. First, how do the cell culture studies relate to cell processes in living multi-cellular organisms? For example, studies in tumor cell lines and tumor samples from patients have somewhat contradicting results as Rab25 (Rab11c) is shown upregulated in some cases (CHENG *et al.* 2004; WANG *et al.* 2004), but downregulated in others (CHENG *et al.* 2010) during metastasis. Second, observation of *rab11* phenotypes in cell culture studies relies on the transfection of dominant-negative Rab11, which successfully blocks Rab11 function but doesn't allow fine analysis of the different steps in Rab11-mediated membrane trafficking.

In *Drosophila*, it is possible to make a null allele of a specific gene by homologous recombination (PARKS *et al.* 2004) and generate mosaic animals to study the gene function even if the null allele is lethal (PARKS *et al.* 2004; PERRIMON 1998). It is then possible to introduce back into the mosaic animal a wild-type allele to rescue the phenotype, or to study the gene's functional domains with chimera transgenes (Rubin and Spradling 1982; Spradling and Rubin 1982).

This dissertation shows multiple distinct roles of Rab11 during *Drosophila* oogenesis: in chapter 2, I describe the phenotypes of a *rab11-null* mutation in the *Drosophila* somatic follicle cell lineage; in appendix chapter 1, I show that a Rab11/Rab2 chimera protein rescues some but not all phenotypes described in chapter 2; appendix chapter 2 describes the functions of Rab11 in germline stem cell maintenance and differentiation.

Figure 1.1

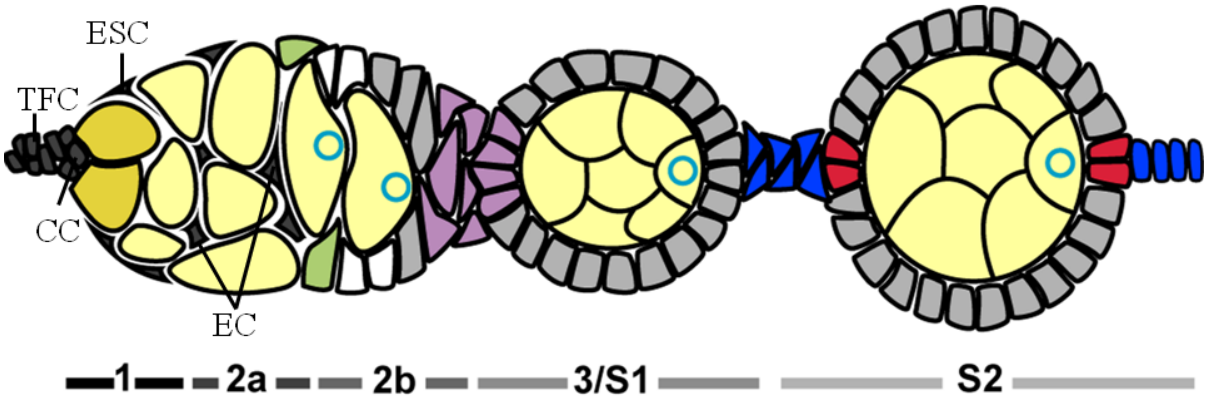

Figure 1.1 Diagram of the *Drosophila* germarium and a budded stage 2 (s2) egg chamber. Anterior is to the left in this and all subsequent images, unless otherwise noted. Germarial regions 1-3 are indicated below the diagram, where region 3, corresponds to a stage 1 egg chamber (3/S1). Terminal filament cells (TFCs) and cap cells (CCs) reside at the most anterior end of the germarium. Germline stem cells (GSCs; dark yellow) are anchored to the CCs and ensheathed by escort stem cells (ESCs). GSCs give rise to cystoblasts, which in turn give rise to the germline cysts (light yellow). Oocyte nuclei are depicted by blue circles. Germline cysts are first covered by escort cells (ECs) derived from ESCs, and then by pre-follicle cells (pre-FCs; white) derived from the follicle stem cells (FSCs; light green), which reside at the 2a/2b junction. Some pre-FCs stop dividing (purple) and eventually become polar (red) and stalk (blue) cells. The rest of the pre-FCs keep dividing and become follicle epithelial cells (FECs; grey).

Figure 1.2

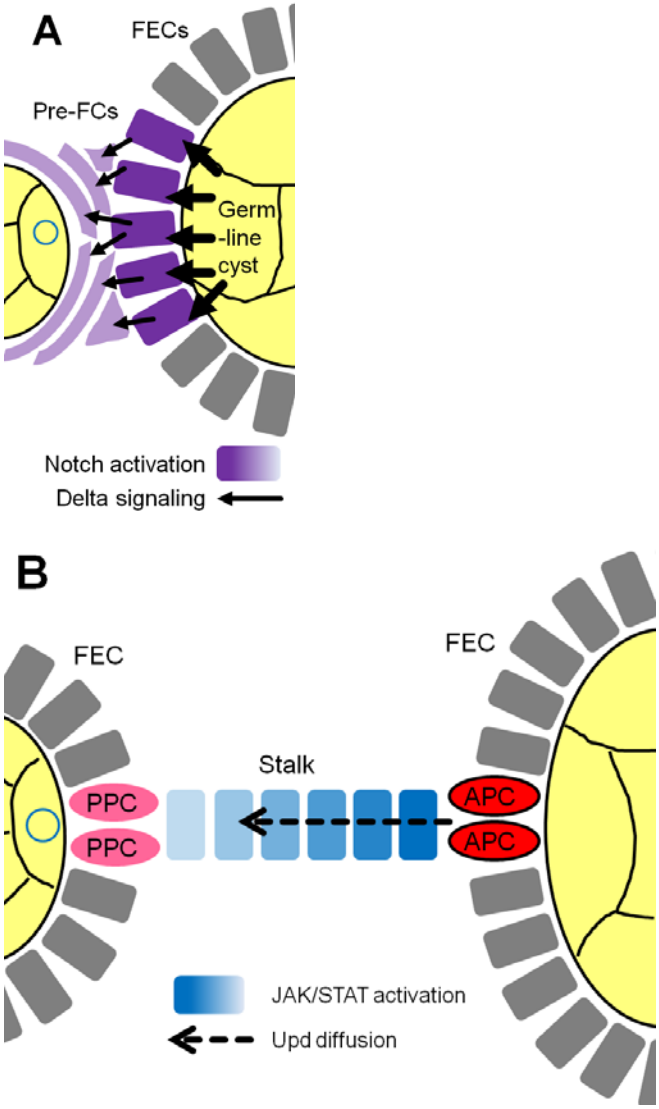
A
FECs
Pre-FCs
Germ
-line
cyst
Notch activation
Delta signaling
B
FEC
FEC
Stalk
PPC
PPC
APC
APC
JAK/STAT activation
Upd diffusion

Figure 1.2 Diagram for the specification of follicle cell fates and intercalation of stalk cells. Germline cells are light yellow. Oocyte nucleus is depicted by a blue circle. (A) At stage 1 of oogenesis, 4-6 pre-follicle cells (pre-FCs) at the anterior pole of an egg chamber have encountered high levels of Delta from the germline cyst (light yellow), upregulate Notch, and are specified as pre-polar cells (dark purple). These pre-polar cells also produce Delta themselves and induce another 6-8 pre-FCs that are not in direct contact with the older cyst to become stalk cells (light purple). The follicle epithelium cells (FECs) are shown in gray. (B) At stages 2-4, two pre-polar cells of the older egg chamber that express the highest level of Notch become anterior polar cells (APCs; red), while other pre-polar cells undergo apoptosis. The APCs of the older egg chamber express Unpaired (Upd), which activates the JAK/STAT pathway in the stalk cells (blue) and induces them to intercalate. Posterior polar cells (PPCs; pink) are specified later by mechanisms that are not yet well understood. FECs are shown in gray. (A) and (B) are based on ASSA-KUNIK *et al.* 2007, ROTH and LYNCH 2009, and VACHIAS *et al.* 2010.

Figure 1.3

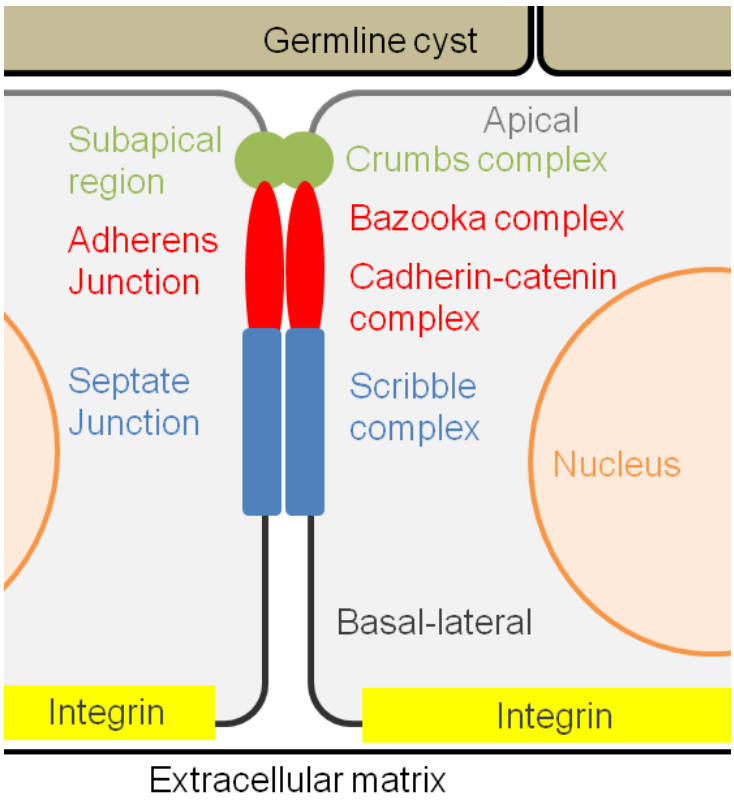
Germline cyst
Subapical region
Apical
Crumbs complex
Bazooka complex
Adherens Junction
Cadherin-catenin complex
Septate Junction
Scribble complex
Nucleus
Basal-lateral
Integrin
Integrin
Extracellular matrix

Figure 1.3 Diagram for the intercellular junctions and polarity of the FECs. Follicle epithelium cells (FECs) are connected to each other through intercellular junctions and they face the germline cyst with their apical membranes. Crumbs complex defines the subapical region. Bazooka (PAR-3) complex and Cadherin-catenin complex are in the AJ (Adherens Junction). Scribble complex proteins are organizers of the SJ (Septate Junction). FECs interact with the extracellular matrix through Integrins. Figure is base on KNUST and BOSSINGER 2002 and ST JOHNSTON and AHRINGER 2010.

Figure 1.4

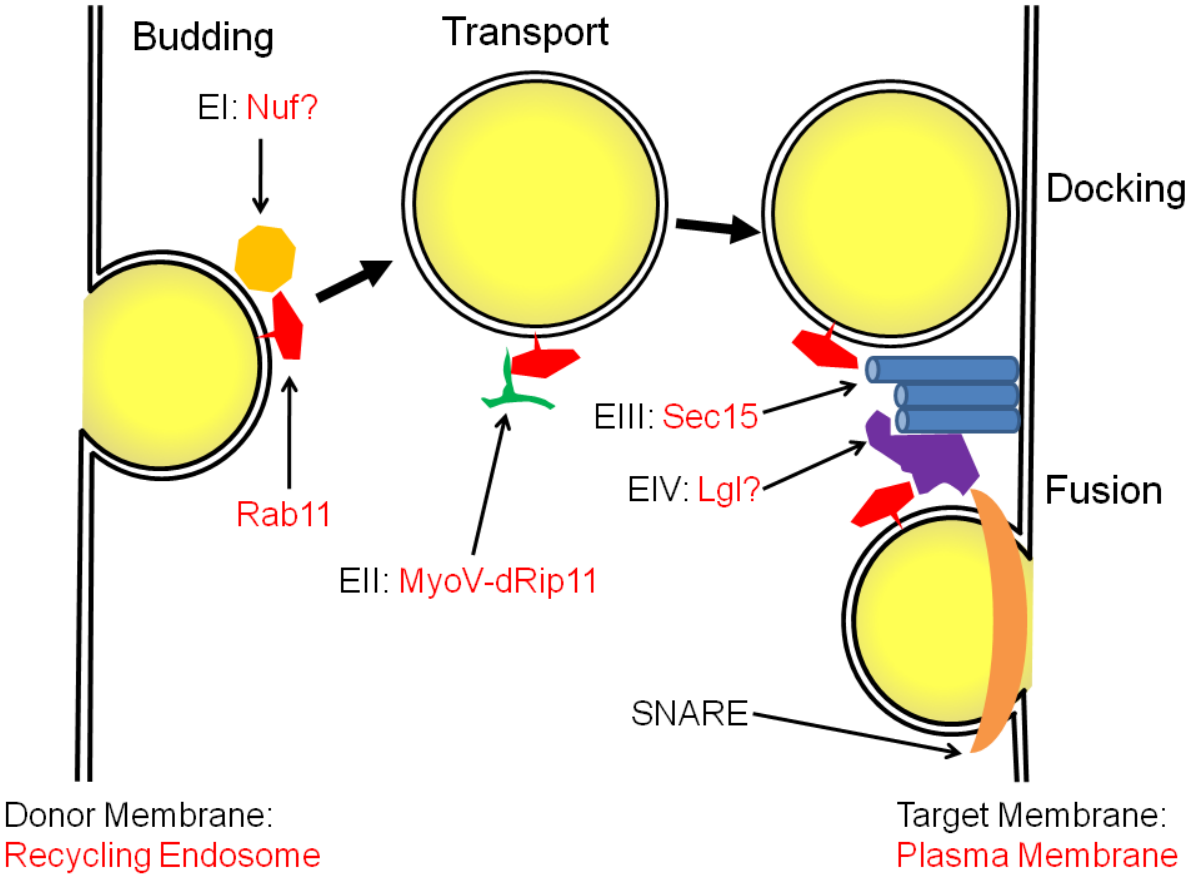
Budding
Transport
EI: Nuf?
Docking
EIII: Sec15
EIV: Lgl?
Fusion
Rab11
EII: MyoV-dRip11
SNARE
Donor Membrane:
Recycling Endosome
Target Membrane:
Plasma Membrane

Figure 1.4 A unique set of effectors assist Rab11 in membrane trafficking.

Black text shows the general theme of Rab-mediated membrane trafficking: vesicles bud from donor membrane, transport along elements of the cytoskeleton, dock on and fuse with target membrane. Four putative classes of effectors (EI-EIV) assist each Rab in every step of membrane trafficking. Red text shows Rab11-mediated trafficking from the recycling endosome to the plasma membrane, assisted by a unique set of effectors. *Drosophila* Rab11's EII and EIII are MyoV-dRip11 complex and Sec15 respectively, and its possible EI effector is Nuclear-fallout (Nuf), a homologue of Arfophilin-2. Rab11's candidate EIV is a *Drosophila* nTSG, Lethal-giant-larvae (Lgl), which is the homolog to the yeast Sro7p and Sro77p, two SNARE regulators with redundancy to each other.

Figure 1.5

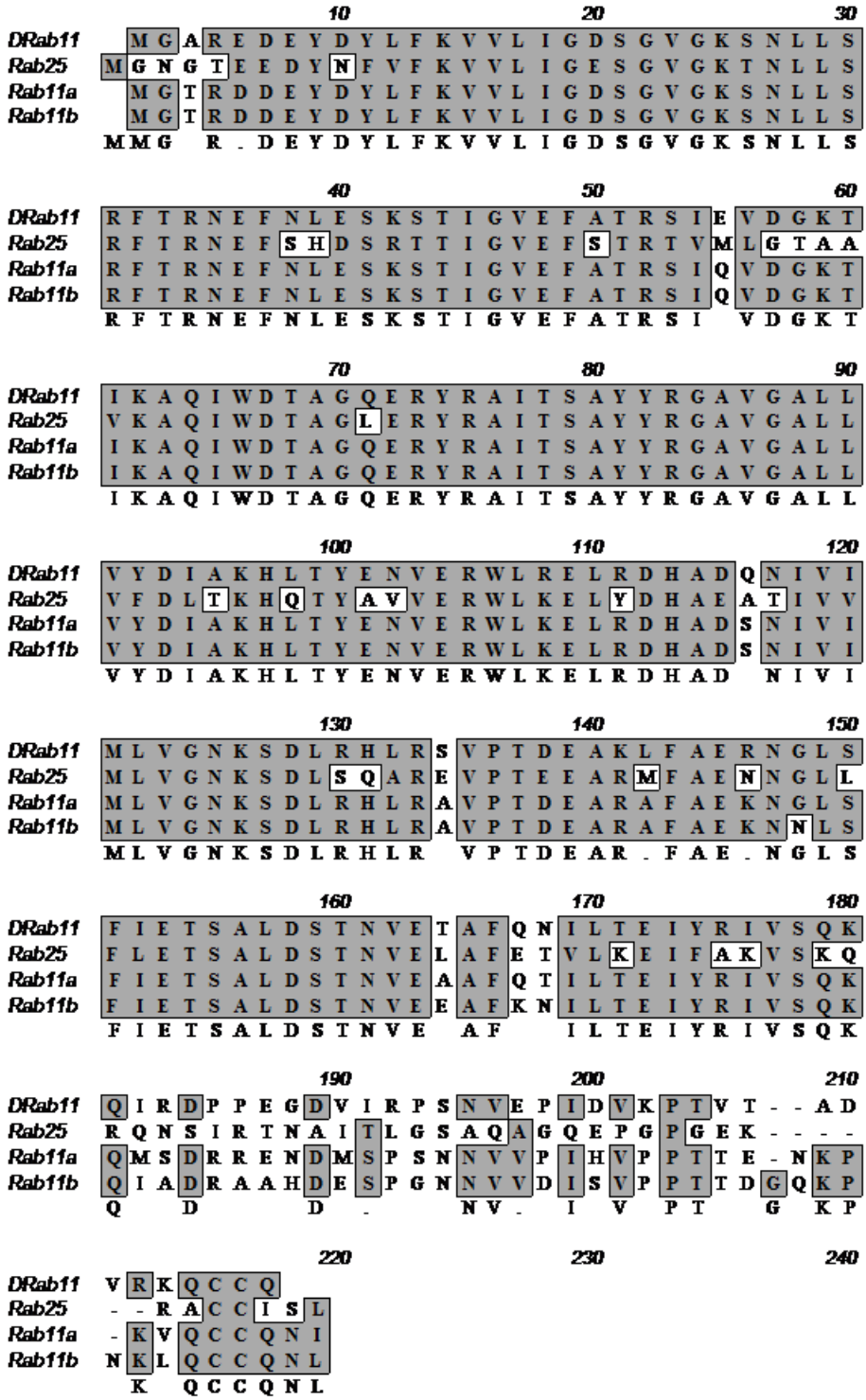
10 20 30
DRab11 MGAREDEYDYLFKVVLIGDSGVGKSNLLS
Rab25 MGNGTEEDYNFVFKVVLIGESGVGKTNLLS
Rab11a MGTRDDEYDYLFKVVLIGDSGVGKSNLLS
Rab11b MGTRDDEYDYLFKVVLIGDSGVGKSNLLS
MMG R.DEYDYLFKVVLIGDSGVGKSNLLS
40 50 60
DRab11 RFTRNEFNLESKSTIGVEFATRSIEVDGKT
Rab25 RFTRNEFSHDSRTTIGVEFSTRTVMLGTAA
Rab11a RFTRNEFNLESKSTIGVEFATRSIQVDGKT
Rab11b RFTRNEFNLESKSTIGVEFATRSIQVDGKT
RFTRNEFNLESKSTIGVEFATRSIVDGKT
70 80 90
DRab11 IKAQIWDTAGQERYRAITSAYYRGAVGALL
Rab25 VKAQIWDTAGLERYRAITSAYYRGAVGALL
Rab11a IKAQIWDTAGQERYRAITSAYYRGAVGALL
Rab11b IKAQIWDTAGQERYRAITSAYYRGAVGALL
IKAQIWDTAGQERYRAITSAYYRGAVGALL
100 110 120
DRab11 VYDIAKHLTYENVERWLRELRDHADQNIVI
Rab25 VFDLTKHQTYAVVERWLKELYDHAEATIVV
Rab11a VYDIAKHLTYENVERWLKELRDHADSNIVI
Rab11b VYDIAKHLTYENVERWLKELRDHADSNIVI
VYDIAKHLTYENVERWLKELRDHADNIVI
130 140 150
DRab11 MLVGNKSDLRHLRSVPTDEAKLFAERNGLS
Rab25 MLVGNKSDLSQARETPTEEARMFAENNGLL
Rab11a MLVGNKSDLRHLRAVPTDEARAFAEKNGLS
Rab11b MLVGNKSDLRHLRAVPTDEARAFAEKNNLS
MLVGNKSDLRHLRVPTDEAR.FAE.NGLS
160 170 180
DRab11 FIETSALDSTNVETAFQNILTEIYRIVSQK
Rab25 FLETSALDSTNVELAFETVLKEIFAKVSKQ
Rab11a FIETSALDSTNVEAAFQTILTEIYRIVSQK
Rab11b FIETSALDSTNVEEAFKNILTEIYRIVSQK
FIETSALDSTNVEAFILTEIYRIVSQK
190 200 210
DRab11 QIRDPPEGDVIRPSNVEPIDVKPTVT--AD
Rab25 RQNSIRTNAITLGSAQAGQEPGPGEK----
Rab11a QMSDRRENDMSPSNNVVPIHVPPTTE-NKP
Rab11b QIADRAAHDESPGNNVVDISVPPTTDGQKP
QD D.NV.IVPPTGKP
220 230 240
DRab11 VRKQCCQ
Rab25 --RACCISL
Rab11a -KVQCCQNI
Rab11b NKLQCCQNL
KQCCQNL

Figure 1.5 Amino acid sequence is highly conserved between *Drosophila* Rab11 and the human Rab11 family proteins. DRab11 is the *Drosophila* Rab11. Rab11a, Rab11b, and Rab25 (Rab11c) belong to the human Rab11 family.

Chapter II

The *Drosophila* Rab11 GTPase is required for cell viability and possesses tumor suppressor protein-like activities in the egg chamber epithelium

2.1 INTRODUCTION

The *Drosophila* oocyte develops within a highly organized group of cells called the egg chamber. Each egg chamber consists of a cyst of germ cells and a surrounding monolayer epithelium comprised of somatic follicle cells (HORNE-BADOVINAC and BILDER 2005). The cyst originates from a single cell, the cystoblast, which undergoes four asymmetric rounds of division, each with incomplete cytokinesis, to produce a 16-cell cyst, with only one cell destined to differentiate as the oocyte. Each of the remaining cells adopts a nurse cell fate and is responsible for the synthesis of the vast majority of RNAs and proteins that nurture and pattern the future egg and embryo. The follicle epithelial cells are derived from ovarian mesoderm and function critically in a number of germ-soma signaling events that polarize the oocyte and are additionally responsible for the secretion of the eggshell and other egg coverings. After secretion of theses coverings, the epithelial and nurse cells are targeted for programmed cell death (PCD), leaving the mature egg behind, which is passed through the oviduct and fertilized.

Egg chambers are formed and mature in assembly-line fashion along the anteroposterior axis of tube-like structures called ovarioles. Each of the ~15 ovarioles that comprise the Drosophila ovary contains an anterior compartment, called the germarium, where egg chambers are assembled from the differentiating progeny of germline and somatic follicle stem cells (GSCs and FSCs, respectively), and a posterior compartment, called the vitellarium where egg chambers mature through 13 morphologically distinct stages (s2-14). The germarium is further subdivided into 4 regions denoted from anterior to posterior as

regions 1, 2a, 2b, and 3 (Figure 2.1A). The GSCs are located at the anterior tip of germarial region 1, while the FSCs are located at the junction of germarial regions 2a and 2b. Each stem cell population is anchored in place by adherens junctions (Ajs) to neighboring niche cells (KIRILLY and XIE 2007). Egg chamber formation begins when a GSC divides to produce an anterior cell, which retains its Ajs and GSC identity, and a posterior cell, called a cystoblast, which differentiates. As new cystoblasts are formed, older ones are pushed posteriorly while dividing to produce 2-, 4-, 8- and finally 16-cell cysts. As a 16-cell cyst reaches the region 2a/2b junction it is pushed up against a pool of about 32 pre-follicle (undifferentiated) cells, which causes it to flatten across the full diameter of the germarium. Approximately half of the pre-follicle cells in this pool are derived from one FSC, while the remaining pre-follicle cells are derived from the other FSC (NYSTUL and SPRADLING 2010). Although mixing between the two pre-follicle cell populations is sometimes observed, it is generally the case that one population migrates over and covers one half (anterior or posterior) of the germline cyst, while the other population migrates over and covers the other half of the cyst (NYSTUL and SPRADLING 2010). The cyst and associated pre-follicle cells round up as they move into region 3, where they are known as a stage 1 (s1) egg chamber. Specialized "stalk" cells (see below) at the anterior end of the egg chamber subsequently adopt a wedge-like shape and intercalate to form a single-cell wide bridge that causes the egg chamber to bud into the vitellarium while remaining connected to the next (younger) egg chamber (Figure 2.1A).

The faithful assembly and polarization of the egg chamber requires the sequential

specification of three distinct follicle cell types through multiple cell-to-cell signaling events. The three follicle cell fates are: anterior and posterior polar cells, which function as signaling centers; stalk cells, which form the above mentioned bridges between adjacent egg chambers and are also responsible for orienting the oocyte within the follicle cell epithelium; and main-body epithelial cells, which secrete the egg shell and regulatory factors that polarize the mature egg and future embryo (HORNE-BADOVINAC and BILDER 2005). Anterior polar cells are specified first, when the Notch signal, Delta, is released from the germline cyst of the region 3 (s1) egg chamber (Figure 2.1A). This signal induces 4-6 pre-follicle cells at the junction of germarial regions 2b and 3 to adopt a polar cell fate (Figure 2.1A, red cells) (GRAMMONT and IRVINE 2001; LOPEZ-SCHIER and ST JOHNSTON 2001). The newly induced polar cells then themselves release Notch and JAK/STAT signals, which act together to induce ~6 neighboring pre-follicle cells to adopt the stalk cell fate (Figure 2.1A, blue cells) (LOPEZ-SCHIER and ST JOHNSTON 2001; MCGREGOR *et al.* 2002). The newly induced stalk cells up-regulate E-cadherin (E-cad), as does the presumptive oocyte of the adjacent, region 2b germline cyst, which eventually results in stable positioning of the oocyte at the posterior end of the cyst (Figure 2.1A) (GODT and TEPASS 1998; GONZALEZ-REYES and ST JOHNSTON 1998). Non-induced pre-follicle cells (that cover more lateral regions of the germline cyst) differentiate as epithelial cells and surround and accompany the cyst as it moves into region 3 as a stage 1 egg chamber (Figure 2.1A, grey cells). The region 3 germline cyst then releases Delta, and the whole process of follicle cell specification and oocyte positioning is repeated in the adjacent younger cyst.

As the s1 egg chamber buds into the vitellarium, all but two of the anterior polar cells are targeted for PCD (BESSE and PRET 2003) (and see Figre 2.1A). Posterior polar cells are also specified at this time, presumably via the conversion of posterior stalk cells to the polar cell fate through continued exposure to the Delta (TORRES *et al.* 2003). While polar and stalk cells do not divide after their specification, epithelial cells continue to divide through ~s7, increasing their cell number from about 30 to over 1000, which is necessary to maintain coverage of the germline cyst, which grows continuously over the course of oogenesis (HORNE-BADOVINAC and BILDER 2005).

Although much has been learned about the signaling events that control egg chamber formation and maturation, little is known about the membrane trafficking pathways that underlie these events. We have previously shown that Rab11, best known for its role in trafficking vesicles from recycling endosomes to the plasma membrane, is required in the germline to maintain GSC identity and to correctly orient the cyst within the surrounding epithelium (BOGARD *et al.* 2007). Both of these requirements are met, at least in part, through Rab11's ability to traffic E-cad from the fusome, a germline specific organelle, to the plasma membrane, and thus, to fortify important contacts between germline and neighboring somatic cells (BOGARD *et al.* 2007). Given the strong expression of Rab11 in the somatic components of the ovary and the known role of E-cad in maintaining FSC identity and in polarizing epithelial cells (SONG and XIE 2002; ST JOHNSTON and AHRINGER 2010), we decided to investigate the role of Rab11 in the follicle cell lineage. Unexpectedly, we find that Rab11 is not required for the maintenance of FSC identity. Antibody stains for specific

follicle cell fates indicate that *rab11-null* cells are able to respond to inductive signals and initiate specific differentiation programs, but are unable to complete them and are targeted for cell death or are otherwise unable to carry out their intended functions. Finally, we show that the loss of Rab11 from cells that have already committed to the epithelial pathway causes an early arrest of differentiation, loss of cell polarity, and invasive cell behavior.

2.2 METHODS

Drosophila *genetics*

Fly culture and crosses were carried out according to standard procedures (ASHBURNER *et al.* 2005). The wildtype stock was *w*, *w His2AV::GFP* , or *w Hrb98DE::GFP* (BOGARD *et al.* 2007; MORIN *et al.* 2001). The *rab11-null, rab11*$^{\Delta FRT}$, has been previously described (BOGARD *et al.* 2007). To generate homozygous *rab11*-null clones, we crossed *w; rab11-null/TM3, Sb* females to *y w hsp::FLP; FRT5377, His2AV::GFP /Tm3, Sb* or *y w hsp::FLP; FRT5377, Hrb98DE::GFP* males, where *FRT5337* corresponds to the centromere-proximal FRT insertion element that was used to make the *rab11-null* allele (BOGARD *et al.* 2007). F1 3rd instar larvae or 2- to 3-day adults were heat shocked for 1 hour at 37^{o}C on 2 consecutive days. Cells (in Sb+ adults) homozygous for the *rab11-null* allele were identified by the absence of GFP fluorescence. Two *sec15*-null mutants were used, *sec15*^{1} and *sec15*^{2} (MEHTA *et al.* 2005), and identical results were obtained with each. To generate *sec15*-null clones, we crossed *w; FRT82B sec15-null/TM3, Sb* females to *y w hsp::FLP; FRT82B, His2AV::GFP/ TM3, Sb* males. Two- to three-day, Sb+ F1 adults were

then heat shocked as described for the *rab11*-null clones.

Immunocytochemistry and confocal microscopy

Ovaries were fixed and immunostained as described (BOGARD *et al.* 2007; DOLLAR *et al.* 2002). Primary antibodies were used at the following concentrations: Rat anti-Rab11 (1:500) (DOLLAR *et al.* 2002); Sec15 (1:2000; a gift from H. Bellen), Nuf (1:200) (RIGGS *et al.* 2003), phospho-histone H3 (1:250; Upstate Biotech.), and GFP (1:250; Invitrogen). All other primary antibodies were obtained from the Hybridoma bank and used at the following concentrations: E-cad (1:40), Eya (1:250), Fas3 (1:50); Orb (6H4) (1:20); Fas2 (1:50); Discs Large (1:250), beta-integrin (1:2), and LamC (1:50). Secondary antibodies were purchased from Jackson labs and used at the manufacturer's recommended concentrations. Apoptotic cells were identified by incubating fixed cells with PhiPhiLux G2D2 (Cal Biochem), which stains activated caspase 3, according to manufacturer's recommended conditions. Stained ovaries were mounted in 4% n-propyl gallate (Sigma) in 90% glycerol, 10% phosphate buffered saline. Images were collected on Olympus 3L Spinning disc or Zeiss Meta 510 laser scanning confocal microscopes.

2.3 RESULTS AND DISCUSSION

Rab11 is not required to maintain FSC identity

Previous studies using partial loss-of-function alleles revealed roles for Rab11 in the germline, but failed to reveal any requirement for the protein in somatic follicle cells (DOLLAR *et al.* 2002; JANKOVICS *et al.* 2001). The strong expression of Rab11 and its

effectors in somatic follicle cells [(DOLLAR *et al.* 2002); and see Figures 2.3F, G and 2.4A-C] led us to re-examine Rab11's role in follicle cells using the recently described *rab11*-null allele, $rab11^{\Delta FRT}$ (BOGARD *et al.* 2007). We first set out to determine if Rab11 is required in FSCs to maintain stem cell identity. We thought this was a likely possibility given previous findings that FSCs are attached to niche intergermarial cells (IGCs) via E-cad-mediated Ajs (KIRILLY and XIE 2007; SONG and XIE 2002) and that the delivery of E-cad to the plasma membrane requires Rab11 in a number of different cell types (DESCLOZEAUX *et al.* 2008), including *Drosophila* GSCs (BOGARD *et al.* 2007). We used the FRT-FLP system, to generate *rab11-null* clones that were marked by the loss of nuclear GFP (nGFP) (Methods). As a control, we generated similarly marked wildtype (*rab11*+) clones. All clones were induced at a low frequency (see Methods) to ensure that the vast majority of recovered clones (i.e., GFP-negative cells) were derived from a single parent cell. Unless otherwise noted, all clones were examined 10 or more days after clone induction (ACI) to ensure that they were derived from FSCs; clones derived from FSC daughter cells, or other cells in the follicle cell lineage would be transient in existence, and expected to exit the germarium by day 3 and the entire ovariole by day 9 (KIRILLY and XIE 2007). As anticipated from these conditions, less than 20% of the examined ovarioles contained marked (GFP-negative) clones and such clones were large, accounting for approximately half of the total number of follicle cells in the germarium and/or in young (s1-3) egg chambers. The average size of the *rab11-null* clones was virtually identical to that of the *rab11*+ control clones. These findings indicate that *rab11-null* FSCs and their immediate descendants are viable and divide at similar rates to

their wildtype counterparts.

We calculated the half-life of the *rab11-null* and wildtype FSCs by plotting the percentage of germaria that contained marked follicle cell clones as a function of time (i.e., 11, 16 and 24 days) after clone induction (ACI). To our surprise, we found that the half-life of *rab11-null* FSCs was nearly identical to that of wildtype FSCs (17.2-18.7 days versus 19.8-21.2 days; see Table 2.1). By comparison, depletion of Rab11 or E-cad from GSCs results in an ~4-fold reduction in GSC half-life (BOGARD *et al.* 2007; SONG *et al.* 2002). We conclude from these findings that Rab11 is not required for the maintenance of FSC identity, or for FSC viability. These findings suggest that Rab11 is not required for E-cad trafficking in FSCs. However, we could not test this idea directly, since E-cad levels are generally low in FSCs and not reliably detectable by immunostaining. The strong requirement for Rab11 in E-cad trafficking in GSCs and in the maintenance of GSC identity, versus our results in FSCs, nevertheless indicates that FSCs and GSCs rely on different pathways to maintain needed levels of surface E-cad. Further support for this idea comes from the observation that strongest concentrations of intracellular Rab11 and E-cad in GSCs occur in the fusome (BOGARD *et al.* 2007), an organelle that is absent from FSCs (DE CUEVAS and SPRADLING 1998; DENG and LIN 1997).

Rab11 is not required to initiate stalk, polar, and epithelial follicle cell differentiation pathways

To determine whether the daughters of *rab11-null* FSCs could respond to external signals and faithfully initiate stalk, polar, and epithelial differentiation programs, we

immunostained ovarioles with antibodies directed against Lamin C (lamC), which specifically stains stalk cells, or Eyes absent (Eya), which specifically stains epithelial cells (Figure 2.1A'). We also included antibodies against Traffic jam (Tj), which stains epithelial and polar cells, but not stalk cells, and/or E-cad, which stains all follicle cells (Figure 2.1A'). As before, we carried out our analyses 10 or more days ACI to ensure that all of the recovered *rab11*-null (GFP-negative) cells were derived from *rab11-null* FSCs. We also generated control *rab11-null* clones that contained two copies of a wildtype *rab11* transgene. These clones, designated *rab11-null; P[rab11+]*, were also marked by the loss of nGFP.

The immunostain experiments identified two distinct populations of *rab11-null* follicle cells, one that stained positively for lamC (and negatively for Tj) as expected for stalk cells (Figures 2.1B, C), and another that stained positively for Eya (and Tj) as expected for epithelial cells (Figures 2.1D-D'''). Consistent with their putative stalk cell identity, the *rab11-null*, lamC-positive cells were clustered in small groups of about 6 cells/egg chamber and located at or near the junctions of adjacent egg chambers (see arrowheads in Figures 2.1C and 2.3A, A'). Also, in two rare cases present in the same egg chamber, the two groups of cells aggregated together. The *rab11-null,* Eya-positive cells formed large clones that included lateral regions of the egg chamber (Figures 2.1D-D'''). Mosaic egg chambers became severely disorganized at later stages of oogenesis, primarily due to cell death (see below). Unfortunately, this early cell death precluded confirmation of *rab11-null* cell identities by immunostaining for proteins that are expressed in terminally differentiated follicle cells. Nevertheless, the LamC and Eya staining patterns clearly identified two

distinct populations of *rab11-null* cells. For simplicity, we will henceforth refer to the LamC-positive cells as stalk cells and the Eya-positive cells as epithelial cells, with the understanding that the immunostain experiments show the *rab11-null* cells are able to *initiate,* but not necessarily, complete stalk and epithelial cell differentiation programs.

Two observations indicated that *rab11-null* FSCs also gave rise to follicle cells that initiated polar cell differentiation. First, we observed two populations of LamC-negative (i.e., non-stalk) *rab11-null* cells: one that underwent PCD during s1-3 (see below), and another that survived at least through s7 (arrows in Figures 2.3A, A'). These longer living cells were generally present in pairs and located at or near the anterior or posterior pole of the egg chamber, consistent with a polar cell or polar cell-like fate, but they never took on the tear-drop shape appearance of fully differentiated polar cells. Second, nearly 100% of the mosaic egg chambers contained clusters of *rab11-null* stalk cells at either their anterior and/or posterior ends. Since the specification of the stalk cell fate requires signals from polar cells, we argue that polar cells must have existed (at least) at the time these stalk cell clusters were specified. While we cannot rule out the possibility that all of the recovered *rab11-null* stalk cells were induced by wildtype polar cells, this possibility is difficult to reconcile with the high frequency at which the *rab11-null* stalk cell clusters were recovered; lineage tracing experiments show that stalk and neighboring polar cells generally originate from the same FSC. We interpret these findings to mean that *rab11-null* FSCs give rise to cells that *initiate,* but do not complete, polar cell differentiation. Taken together, we conclude that Rab11 is not required for the initiation of polar cell, stalk cell, or epithelial cell

differentiation.

Epithelial cells derived from rab11-null *FSCs are targeted for program cell death during stages 1-3 of oogenesis*

The epithelial cells derived from *rab11-null* FSCs did not persist beyond s4 or s5 (note the absence of GFP-negative follicle cells in Figures 2.2A, B) and many of the recovered s3-5 egg chambers contained gaps in their epithelial layers (Figure 2.2C, yellow dashes). These results suggest that *rab11-null* epithelial die during s1-3. The size and frequency of the gaps decreased with egg chamber age and were typically gone by stage 6 (Figures 2.2A, B), presumably due to compensatory divisions and replacement by neighboring wildtype cells. To visualize cell death directly, we incubated ovaries 10-12 days ACI with a fluorescently-tagged substrate for activated caspase-3 protein (Methods). Such stains revealed increased cell death of *rab11-null* cells compared to neighboring wildtype cells in s1-3 egg chambers (Figure 2.2D). The majority of such deaths occurred in s3 egg chambers, where 50% or more of the *rab11-null* cells stained positively for the death marker (Figure 2.2D). By comparison, less than 1% of the wildtype cells ever stained positive for the death marker (Figure 2.2D, and data not shown). The premature death of *rab11-null* epithelial cells was rescued by a wildtype *rab11* transgene (Figure 2.2E; and see Figures 2.1F, F'), and thus, is directly attributable to the loss of *rab11* gene activity.

We speculate that the different survival times of *rab11-null* cells depends on the number of cell divisions they undergo. The *rab11-null* epithelial cells die prematurely because they cannot deliver new membrane material to the cell surface in response to

epithelial cell growth and division. In support of this idea, Rab11 is required to deliver new membrane material to the cell surface during the cellularization of the *Drosophila* syncytial blastoderm (PELISSIER *et al.* 2003). By extension, the relatively long survival times (at least through s7) of putative *rab11-null* stalk and polar cells, might be due to the fact that these cells stop dividing at s1 and undergo fewer rounds of cell division overall (HORNE-BADOVINAC and BILDER 2005). Presumably, there is a secondary mechanism to add new membrane to FSCs as these cells successfully divided many times in the absence of Rab11 (see above).

Stalk cells derived from rab11-null *FSCs are viable, but fail to elaborate functional stalks*

While *rab11-null* FSCs gave rise to cells that initiated stalk cell differentiation (e.g., over-express LamC) none of these cells completed their differentiation program. Specifically, none of the *rab11-null* stalk cells upregulated surface E-cad expression (data not shown) or organized themselves into a recognizable stalk. Consistent with these findings, we recovered many compound egg chambers that contained two or more germline cysts encased in a single continuous epithelium (Figures 2.3A, A'). In some cases, a single massive compound egg chamber filled the entire ovariole (Figure 2.3D). The compound nature of these egg chambers was confirmed by immunostaining for Orb (TAN *et al.* 2001), which revealed at least 2 oocytes in each case (Figures 2.3B, B'). Fused egg chambers, i.e., egg chambers that contained a single, stalk-less layer of follicle cells between adjacent germline cysts, were also commonly recovered (Figure 2.3C, and see Figure 2.2B). All of the examined compound and fused egg chambers contained *rab11-null* stalk (lamC-positive)

cells, but they were located next to, rather than between, adjacent egg chambers (see arrowheads in Figures 2.3A, A' and Figure 2B). A similar combination of compound and fused egg chambers are produced by mutants for the Notch and JAK/STAT pathways, which are defective in the specification of polar and stalk cell fates (GRAMMONT and IRVINE 2001; LARKIN *et al.* 1996; LOPEZ-SCHIER and ST JOHNSTON 2001; VACHIAS *et al.* 2010).

We wondered whether the lack of fully differentiated *rab11-null s*talk cells reflected a defect in stalk cell differentiation per se or a defect in stalk cell induction, e.g., due to improper specification or differentiation of *rab11-null* polar cells. To distinguish between these possibilities we examined mixed clones that contained both *rab11-null* and wildtype stalk cells. (To this end, we moved the time of our analyses up to 5-8 days ACI to favor recovery of small clones that were induced in pre-follicle cells rather than FSCs.) We recovered two such mixed clones. In each case, the wildtype stalk cells were organized into a recognizable stalk (Figures 2.3E-E'''), while the neighboring *rab11-null* stalk cells were excluded from the stalk proper (dashed cells in Figures 2.3E-E'''). The fact that the two stalk cell populations responded differently to the same polar cell signal (environment) provides strong evidence that Rab11 is required in stalk cells to complete the stalk cell differentiation program and form a stalk. This data further indicates that Rab11 is required cell autonomously in stalk cells for their differentiation. Consistent with this interpretation, we detected very strong expression of both Rab11 and its effector Nuf1 (RIGGS *et al.* 2003) in presumptive stalk and polar cells at the junction of germarial regions 2b and 3 (Figures 2.3F, G). The basis for the block in stalk cell differentiation is not clear from our data, but could

reflect poor reception or processing of Notch and/or JAK/STAT signals from the neighboring polar cells, and/or inefficient trafficking of E-cadherin, which normally accumulates at the leading edge of stalk cells as they intercalate to form a single-cell wide stalk (TEPASS *et al.* 2001).

Rab11 and its effector, Sec15, are required for the terminal differentiation of epithelial cells

Because the epithelial cells derived from *rab11-null* FSCs died shortly after they were specified, the experiments described above could not test whether Rab11 is needed to maintain epithelial cell polarity and/or other aspects of epithelial cell behavior. Such roles seemed likely given that Rab11 and two of its best characterized effectors, Sec15 and Nuf, are expressed in follicle epithelial cells throughout oogenesis (Figures 2.4A-C; and data not shown). To investigate a possible role for Rab11 in more mature epithelial cells, we shifted our analysis point up to 2-6 days ACI to favor the recovery of clones induced in pre-follicle and/or young s1-3 epithelial cells. We reasoned that such cells would survive into late stages of oogenesis, provided that Rab11' role in epithelial cell survival is transient in nature. This approach proved useful as nearly half of the recovered s4-8 egg chambers contained clones of *rab11-null* cells (Figure 2.4). The vast majority of these cells were located in lateral regions of the egg chamber (Figures 2.4D-F) and/or over-expressed Eya (Figure 2.5B; and data not shown) consistent with the idea that they had adopted an epithelial cell fate. This was the expected result, as clone induction requires cell division and stalk and polar cells stop dividing by s1. Unlike the epithelial cells recovered from *rab11-null* FSCs, the ones examined 2-6 ACI days remained viable through 8 (Figures 2.4, 2.5). (We could not

look later than s8, without delaying our analyses to 6-8 days ACI, which had the complication of recovering egg chambers derived from *rab11-null* cells FSCs). The examination of clones 2-6 days ACI also allowed us to recover of s4-8 epithelial cells homozygous for a null allele of *sec15* (Figure 2.4G), which encodes a component of the exocyst and a Rab11 effector required for the docking of vesicles to the plasma membrane (HSU *et al.* 2004; LANGEVIN *et al.* 2005; ZHANG *et al.* 2004). The vast majority of recovered egg chambers contained a completely wildtype germline. Rare egg chambers with a mutant germline cyst were not analyzed to eliminate complications in data interpretation.

All of the recovered s4-8 *rab11-null* epithelial cells arrested differentiation early as evident by their strong expression of Fas3 (Figure 2.4D), a protein that is normally strongly expressed only in s3 and younger epithelial cells (RUOHOLA *et al.* 1991). Three observations together rule out the alternative possibility that the Fas3-positive *rab11-null* cells trans-differentiated from an epithelial to a polar cell fate, which is also characterized by strong expression of Fas3. First, as mentioned above, all of the *rab11-null* cells strongly expressed Eya (Figure 2.5B'), which is characteristic of epithelial cells, but not of polar cells (BAI and MONTELL 2002) (Figure 2.1B'). Second, the *rab11*-null cells, like normal epithelial cells, divided until s 7, as evident by phospho-histone 3 expression (Figures 2.4E-F''), whereas, polar cells do not divide beyond s1. Third, the *rab11*-null cells delaminated, but they did so prior to s9 and they did not recruit other (i.e., neighboring wildtype) cells into the cluster. Also, the delaminated *rab11*-null cells migrated in random

directions, rather than toward the nurse cell-oocyte border (see below). The *sec15*-null cells also arrested differentiation early as evident by their strong expression of Fas3 (Figure 2.4G). However, in contrast to the *rab11*-null cells, which survived for up to 6 days ACI, nearly half of the *sec15*-null cells were targeted for PCD by 2 days ACI (Figure 2.4H). While these data are consistent with the idea that Sec15 is an effector of Rab11 in follicle cell differentiation, they further indicate that Sec15 has a Rab11-independent role in cell viability, which is not unexpected given Sec15's well-described role as a Rab8 effector in the docking of Golgi-derived vesicles to the plasma membrane (LANGEVIN *et al.* 2005; MEHTA *et al.* 2005).

Rab11 behaves as a neoplastic tumor suppressor-like protein in follicle epithelial cells

The *rab11-null* epithelial cells exhibited a variety of neoplastic-like behaviors, including the above mentioned block in differentiation, loss of cell polarity, and the ability to invade neighboring cell masses. The loss of cell polarity was initially apparent in the gross morphology of the mutant cells, which became progressively more rounded over time. By 5 days ACI, a majority of the *rab11-null* cells were completely rounded up and displaced from the epithelium (Figure 2.4D' and Figures 2.5B-G). The loss of cell polarity inferred by this cell morphology was confirmed by immunostaining for protein markers of cell polarity (Figure 2.5A). Most telling, E-cad and Discs large (Dlg), which establish apical-basal membrane polarity through their organization of adherens and septate junctions (BILDER 2004; BILDER *et al.* 2000; BILDER and PERRIMON 2000; TEPASS *et al.* 2001), respectively, were absent from the plasma membrane and concentrated in intracellular compartments, or,

in the case of Dlg, dispersed throughout the cytoplasm (Figures 2.5C, D). A complete loss of cell polarity was also indicated by immunostaining for Fasciclin 2 (Fas2) (Figure 2.5E), a putative membrane anchor for Dlg. The basal membrane marker beta-integrin also showed aberrant expression (Figures 2.5F, G) and was uniformly distributed on the cell surface. Previous studies with *Drosophila* embryos revealed a role for Rab11 in maintaining Ajs (ROETH *et al.* 2009), but did not uncover a requirement for Rab11 in maintaining the localization of Dlg, Fas2 and/or other known or putative components of septate junctions. One possible explanation for this difference is that the embryonic studies used dominant negative and hypomorphic alleles of *rab11*, which may not have completely eliminated Rab11 function.

The invasive behavior of the *rab11-null* cells is distinct from that described for mutations in characterized *Drosophila* tumor suppressor genes (tsgs), which include the septate junction organizers, *discs large, scribble,* and *lethal giant larvae,* and two regulators of endocytosis, *avalanche*, and *rab5* (BILDER 2004; LU and BILDER 2005). The *rab11-null* cells invaded surrounding tissues in clusters that were fully detached from the epithelium and that ranged in size from as few as 2 cells (e.g., Figures 2.4D and 2.5E) to well over 50 (Figure 2.5G). In contrast, previously characterized tsg mutant cells invade surrounding tissues as large multi-layered sheets that remain attached to the epithelium (HARIHARAN and BILDER 2006). In essence, the invasive behavior of previously characterized tsg mutant cells is akin to tissue over-growth, while that of the *rab11*-null cells more closely parallels the behavior of metastatic tumor cells of higher animals (CHAMBERS *et al.* 2002). However,

the *rab11*-null epithelial cells did not undergo extra rounds of cell division, a defining phenotype of all "true" tumor suppressor genes; immunostains for phospho-histone 3 show that *rab11-null* cells cease dividing by stage 7 of oogenesis, like their wildtype counterparts. *Drosophila's* previously characterized tsgs also have no or only subtle roles in suppressing follicle cell over-proliferation (BILDER 2004). Indeed, the evidence that these genes suppress over-proliferation stem entirely from analyses of larval tissues, most notably imaginal discs. Whether suppression of over-proliferation in larval tissues is fundamentally different, or simply easier to demonstrate, than suppression of over-proliferation in adult follicle epithelial cells is unclear. To date, we have been unable to recover *rab11-null* clones in imaginal discs and other larval tissues, which may reflect a unique role for Rab11 in the survival of such cells. In light of these data, we propose that Rab11 protein be considered as tumor suppressor-*like* protein.

Consistent with our findings that loss of Rab11 promotes migratory behavior in non-migratory cells, recent studies have shown that reduction of Rab11 increases the motility of mammalian cells induced to migrate by wounding (JONES *et al.* 2006; PRIGOZHINA and WATERMAN-STORER 2006). Paradoxically, Rab11 expression is up-regulated in human skin and breast carcinomas and certain other metastatic cell populations (CHENG *et al.* 2004; FAN *et al.* 2004; GEBHARDT *et al.* 2005; GOLDENRING *et al.* 1999; JONES *et al.* 2006; WANG *et al.* 2004; YOON *et al.* 2005). It will be of interest to determine if Rab11 loss and up-regulation interfere with the same or different trafficking pathways and how these pathways affect normal versus cancerous cell migrations. One current difficulty in sorting out Rab11's role

in the migration of normal and cancerous cells is the lack of information regarding the identity of Rab11's cargoes and the fate of such cargoes when Rab11 is absent or over-expressed. More information in this area is needed, as is a more extensive survey of the involvement of Rab11 in the migratory behaviors of normal and cancerous cells.

Table 2.1 *rab11-null* FSCs have a wildtype or near wildtype half-life

	% marked germaria at different days ACI (total number of germaria counted)			
genotype of marked clones	11	16	24	Half-life
FRT5377, rab11-null	18.1 (204)	14.8 (203)	11.2 (155)	17.2-19.0 days*
FRT5377, rab11+	18.7 (315)	15.7 (191)	12.1 (298)	19.8-21.2 days*

* half-lives were calculated for each of the two intervals 11-16 days and 16-24 days using the equation half-life = elapsed time (days) X log 2/log [initial percentage/end percentage], which assumes that FSC loss occurs randomly, and thus linearly over time.

Figure 2.1

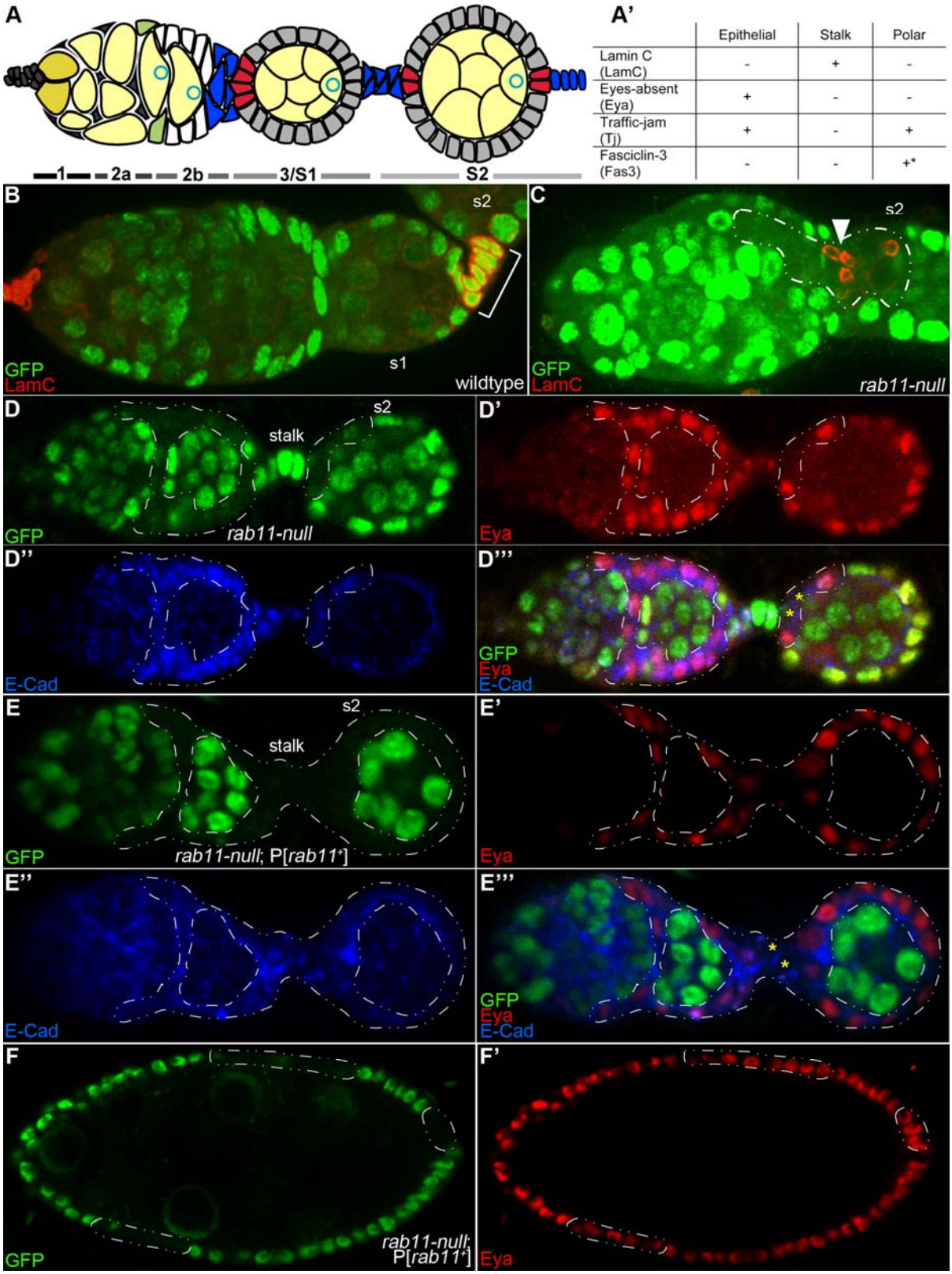
A
1
2a
2b
3/S1
S2
A'
Epithelial
Stalk
Polar
Lamin C (LamC)
- + -
Eyes-absent (Eya)
+ - -
Traffic-jam (Tj)
+ - +
Fasciclin-3 (Fas3)
- - +*
B
s2
s1
GFP
LamC
wildtype
C
s2
GFP
LamC
rab11-null
D
s2
stalk
GFP
rab11-null
D'
Eya
D''
E-Cad
D'''
GFP
Eya
E-Cad
E
s2
stalk
GFP
rab11-null; P[rab11+]
E'
Eya
E''
E-Cad
E'''
GFP
Eya
E-Cad
F
GFP
rab11-null; P[rab11+]
F'
Eya

Figure 2.1 *rab11-null* FSCs give rise to at least two types of cells, one resembling stalk cells and another resembling epithelial cells. (A) Diagram of the *Drosophila* germarium and a budded stage 2 (s2) egg chamber. Anterior is to the left in this and all subsequent images, unless otherwise noted. Germarial regions 1-3 are indicated below the diagram, where region 3 corresponds to an s1 egg chamber. GSCs (dark yellow) reside at the extreme anterior end of region 1 and give rise to cystoblasts as well as to 2-, 4-, 8- and 16-cell germline cysts (light yellow). Oocyte nucleus, blue circle. Two FSCs (green) reside at the germarial region 2a/2b junction and give rise to undifferentiated pre-follicle cells (white) and three types of differentiated follicle cells: epithelial (grey), polar (red) and stalk (blue). (A') Expression summary of cell fate markers used in this study, where '+' indicates that the marker is expressed and '-' indicates that the marker is not expressed. The asterisk indicates that Fas3 expression is specific for polar cells, only after stage 3. (B-F') Confocal images of immunostained germaria and/or egg chambers 10-12 days ACI. (B) Wildtype germarium and s2 egg chamber immunostained for nGFP (green) and LamC (red). Stalk cells are denoted by the bracket. The non-bracketed LamC-positive cells at the left of the figure are niche cap cells, which also over-express LamC (HSU and DRUMMOND-BARBOSA 2009). (C) Mosaic germarium, and s2 egg chamber immunostained with nGFP (green) and LamC (red). A *rab11-null* clone is outlined with the dashed white line. The arrowhead points to a cluster of stalk (LamC-plus) cells within the clone. (D-D''') Mosaic germaria with adjacent s2 egg chambers immunostained for nGFP (green), Eya (red), and E-cad (blue). *rab11-null* clones are outlined with the dashed line. Most of the cells within the clone have

adopted an epithelial cell fate as evident by their expression of Eya. The yellow asterisks in the merged image (D’’’) highlight a cluster of two, or possibly three, Eya-negative cells, which have presumably adopted a stalk or polar cell fate. (E-E’’’) Control *rab11-null;* P[*rab11*+] clones in a germarium and s2 egg chamber immunostained and labeled as in (D-D’’’). It may be noted that the germarium shown contains only marked (GFP-negative) follicle cells, indicative of a rare event in which both FSCs were targeted for FLP-induced recombination. (F, F’) Control *rab11-null;* P[*rab11*+] clones in an s8 egg chamber immunostained for nGFP (green) and Eya (red). Marked clones are outlined with the dashed line.

Figure 2.2

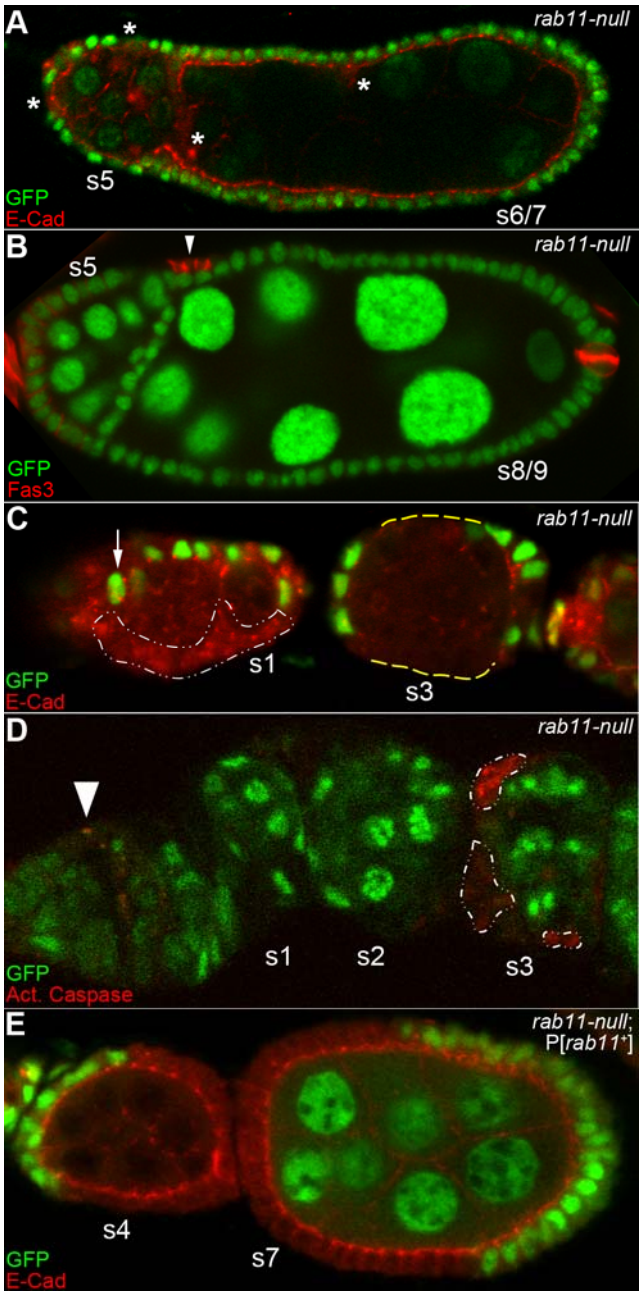
A
rab11-null
s5
s6/7
GFP
E-Cad
B
rab11-null
s5
s8/9
GFP
Fas3
C
rab11-null
s1
s3
GFP
E-Cad
D
rab11-null
s1
s2
s3
GFP
Act. Caspase
E
rab11-null;
P[rab11+]
s4
s7
GFP
E-Cad

Figure 2.2 Rab11 is required for the survival of follicle epithelial cells. (A-E) Confocal images of immunostained germaria and/or egg chambers 10-12 days ACI, with *rab11-null* cells marked by the absence of nGFP. All egg chambers are derived from mosaic germaria that contained one *rab11*-null FSC and one wildtype FSC as evidenced by direct examination of the adjacent germarium and/or the presence of *rab11-null* cells in the egg chambers themselves. The stages of the compound and fused egg chambers are estimates based on the size of the nurse cell nuclei, the distance of the fused/compound egg chamber from the germarium, and/or its position relative to other more easily staged egg chambers within the same ovariole. (A) An s5 egg chamber fused to a compound egg chamber containing two ~s6/7 germline cysts encased in a single continuous epithelium and immunostained for nGFP (green) and E-cad (red). *rab11-null* cells, which are marked with asterisks, are more abundant in the s5 egg chamber than in the adjacent older ones. The positions of the *rab11-null* cells in the compound egg chamber are consistent with a stalk or stalk-like identity (discussed more fully in Text and see Figure 2.3). (B) An s5 egg chamber fused to an s8 or s9 egg chamber immunostained for nGFP (Green) and Fas3 (red). The arrowhead points to a cluster of 2-3 *rab11-null* cells that over express Fas-3. While such expression is indicative of a polar cell fate, we cannot rule out a stalk cell fate for these cells as stalk cells that fail to incorporate themselves into a functional stalk are also known to over-express Fas3 (ASSA-KUNIK *et al.* 2007). No *rab11-null* epithelial cells are seen in this plane of focus. Other focal planes (not shown) contained no or only a few *rab11-null* epithelial cells (not shown). The two strongly expressing Fas3 cells at the posterior end of

the s8/9 egg chamber are wildtype polar cells, although the GFP signal is weak at the focal plane shown. (C) Germarium and s3 egg chamber immunostained for nGFP (Green) and E-cad (red). The arrow points to a putative wildtype FSC. A large *rab11-null* clone in the germarium is outlined in white. The dashed yellow line in the adjacent s3 egg chamber highlights two large gaps in the epithelium as evident by the absence of E-cad expression and also Nomarski imaging (not shown). (D) Germarium and s2 and s3 egg chambers immunostained for nGFP (green) and counterstained for activated caspase 3, a marker for PCD. Approximately half of the *rab11-null* cells (outlined with the dashed line) in the s3 egg chamber stain positively for activated caspase 3. The apparent weaker staining of some of the *rab11*-null cells is not evident at other focal planes (not shown). The activated caspase 3 staining activity at the junction between germarial regions 1 and 2a (arrowhead) is likely to correspond to escort cells, which are known to be targeted for PCD at this stage (DECOTTO and SPRADLING 2005). (E) Control *rab11-null* ; P[*rab11*+] clones in s4 and s7 egg chambers immunostained for nGFP (green), E-cad (red).

Figure 2.3

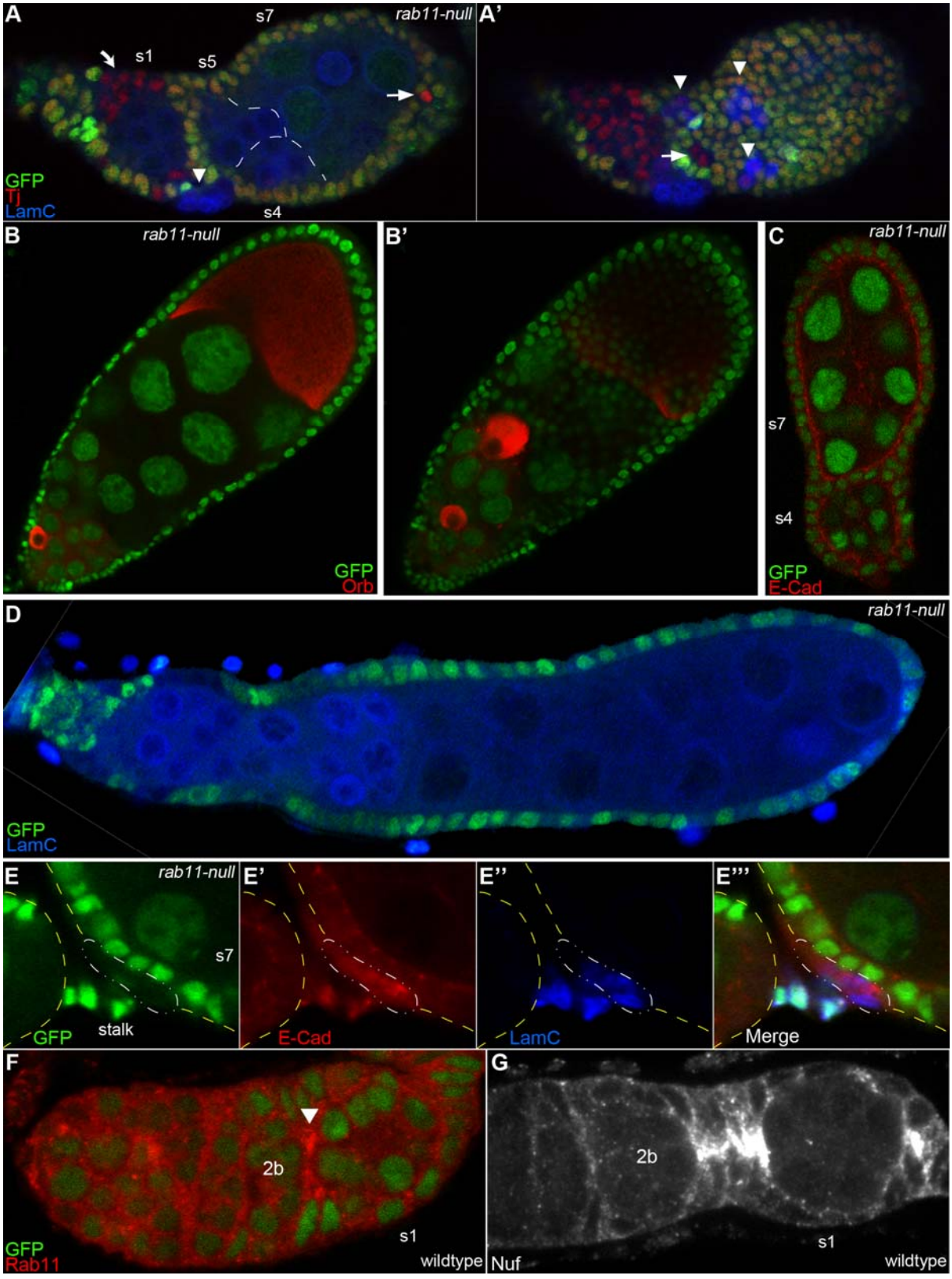
A
s7
rab11-null
s1
s5
GFP
Tj
LamC
s4
A'
B
rab11-null
GFP
Orb
B'
C
rab11-null
s7
s4
GFP
E-Cad
D
rab11-null
GFP
LamC
E
rab11-null
s7
GFP
stalk
E'
E-Cad
E''
LamC
E'''
Merge
F
2b
s1
GFP
Rab11
wildtype
G
2b
s1
Nuf
wildtype

Figure 2.3 *rab11-null* stalk cells fail to organize themselves into a functional stalk and are associated with fused and compound egg chambers. (A-D) Confocal images of immunostained germaria and/or egg chambers 10-12 days ACI, with *rab11-null* cells marked by the absence of nGFP. (A, A') Two different focal planes of an s1 (germarial region 3) egg chamber fused to a compound egg chamber containing 3 germline cysts (approximate germline cyst borders outlined with white dashes) immunostained for nGFP (green), LamC (blue), and Traffic jam (Tj) (red). The arrowheads point to putative *rab11-null* stalk (LamC-positive) cell clusters. As described in the text, such clusters contain ~ 6 cells each and are located at or near the junctions of fused and compound egg chambers. The arrows point to candidate *rab11-null* polar cells (see Text), while the curved arrow points to a clone of *rab11-null* pre-follicle cells, which also stain positively for Tj. (B, B') Two different focal planes of a compound egg chamber immunostained for GFP (green) and the oocyte marker, Orb (red). (C) Fused egg chamber immunostained for nGFP (green) and E-cad (red). Anterior at bottom. (D) Massive compound egg chamber immunostained for nGFP (green) and lamC (blue). The LamC-positive nuclei correspond to germ cells and ovariole sheath cells, which are distinguishable from stalk cells by their sizes and position. (E-E''') Enlarged confocal image of a mosaic stalk cell cluster immunostained for nGFP (green), LamC (blue), and E-cad (red) 5-6 days ACI. The borders of the flanking egg chambers are indicated with the dashed yellow line. The *rab11-null* stalk cells (enclosed in the dashed white line) are excluded from the stalk proper. (F, G) Wildtype germaria immunostained for (F) nGFP (green) and Rab11 (red), or (G) Nuf (white), a Rab11 effector protein (RIGGS *et al.*

2003). The arrow in (F) points to enriched expression of Rab11 in presumptive stalk and polar cells at the junction of germarial regions 2B and 3 (s1). The region 2b/3 (s1) junction is expanded in (G) as stalk cell formation is more advanced in this particular germarium.

Figure 2.4

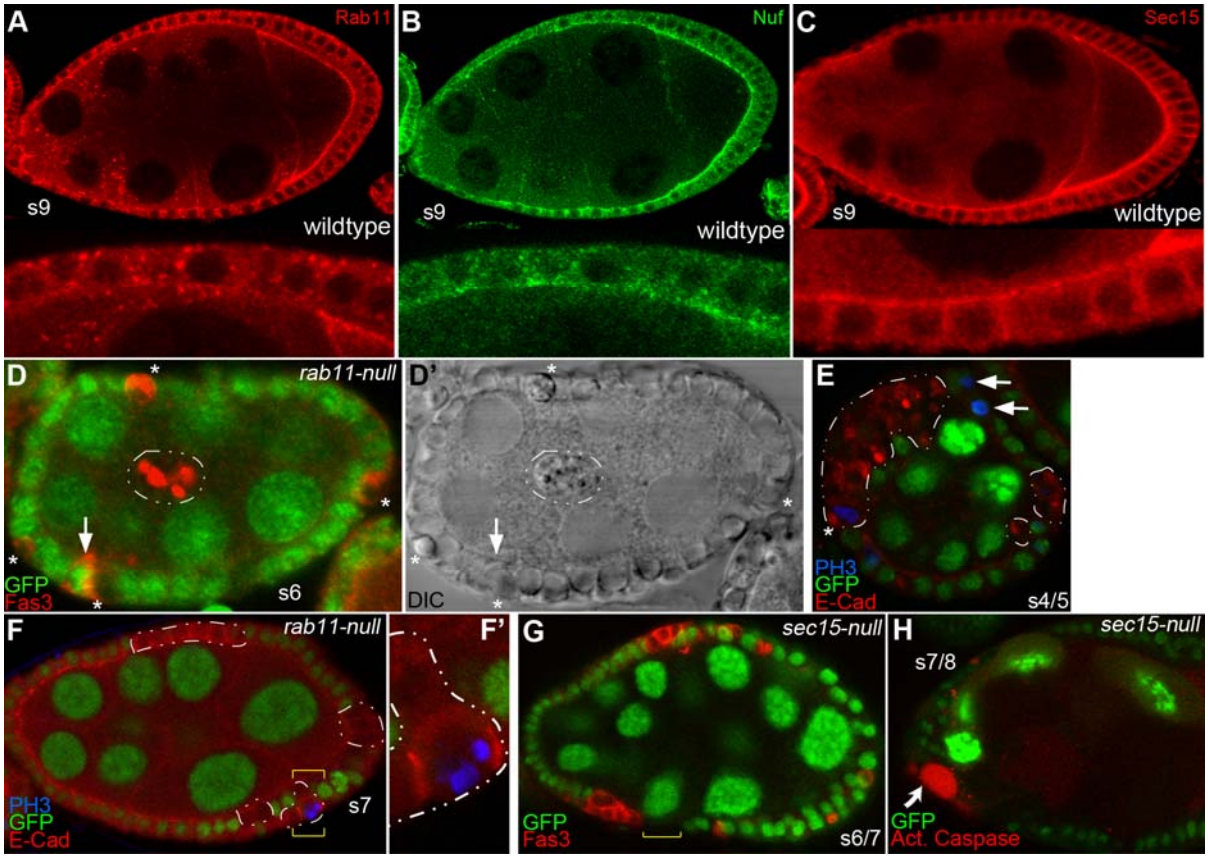
A
Rab11
s9
wildtype
B
Nuf
s9
wildtype
C
Sec15
s9
wildtype
D
rab11-null
GFP
Fas3
s6
D'
DIC
E
PH3
GFP
E-Cad
s4/5
F
rab11-null
PH3
GFP
E-Cad
s7
F'
G
sec15-null
GFP
Fas3
s6/7
H
sec15-null
s7/8
GFP
Act. Caspase

Figure 2.4 *rab11-null* epithelial cells arrest differentiation early, but continue to divide through s6 or 7, while *sec-15 null* cells arrest differentiation early and are targeted for programmed cell death. (A-C) Confocal images of wildtype s9 egg chambers immunostained for (A) Rab11 (red), (B) Nuf (green), or (C) Sec15 (red). Magnified views are shown at the bottom of each panel. (D) Mosaic s6 egg chamber immunostained for nGFP (green) and Fas3 (red) 4-6 days ACI. The asterisks point to *rab11-null* cells still embedded in the epithelium. A cluster of 3 or 4 *rab11-null* cells that have delaminated from the epithelium and invaded the germline cyst is enclosed in the dashed white line. All of the *rab11-null* cells over-express Fas3 consistent with an early arrest of epithelial cell differentiation (see Text). The arrow points to a wildtype polar cell, which also over-expresses Fas3. (D') Light micrograph of egg chamber shown in (D). (E) Mosaic s4/5 egg chamber immunostained for nGFP (green), E-cad (red) and phospho-histone 3 (PH3) (blue) 3 days ACI, with *rab11-null* cells enclosed by the dashed white lines. The asterisk marks a dividing (PH3-positive) *rab11-null* cell, while the arrows point to two dividing wildtype cells. (F) Mosaic s7 egg chamber immunostained as in (E), with *rab11-null* cells enclosed by the dashed white lines. (F') Magnified view of the region bracketed in (F), where a pair of dividing *rab11-null* (PH3-positive) cells are clearly evident. (G) Mosaic *sec15-null* s6/7 egg chamber immunostained for nGFP (green) and Fas3 (red) 2 days ACI. All of the *sec15-null* (GFP-negative) cells over-express Fas3 again consistent with an early arrest of epithelial cell differentiation. The yellow dashed line highlights a gap in the follicle cell epithelium, presumably due to PCD of *sec15-null* cells. (H) Mosaic *sec15-null*

s7 egg chamber immunostained for nGFP (green) and counterstained for activated caspase-3 (red) two days ACI. The arrow points to an apoptotic *sec15-null* cell.

Figure 2.5

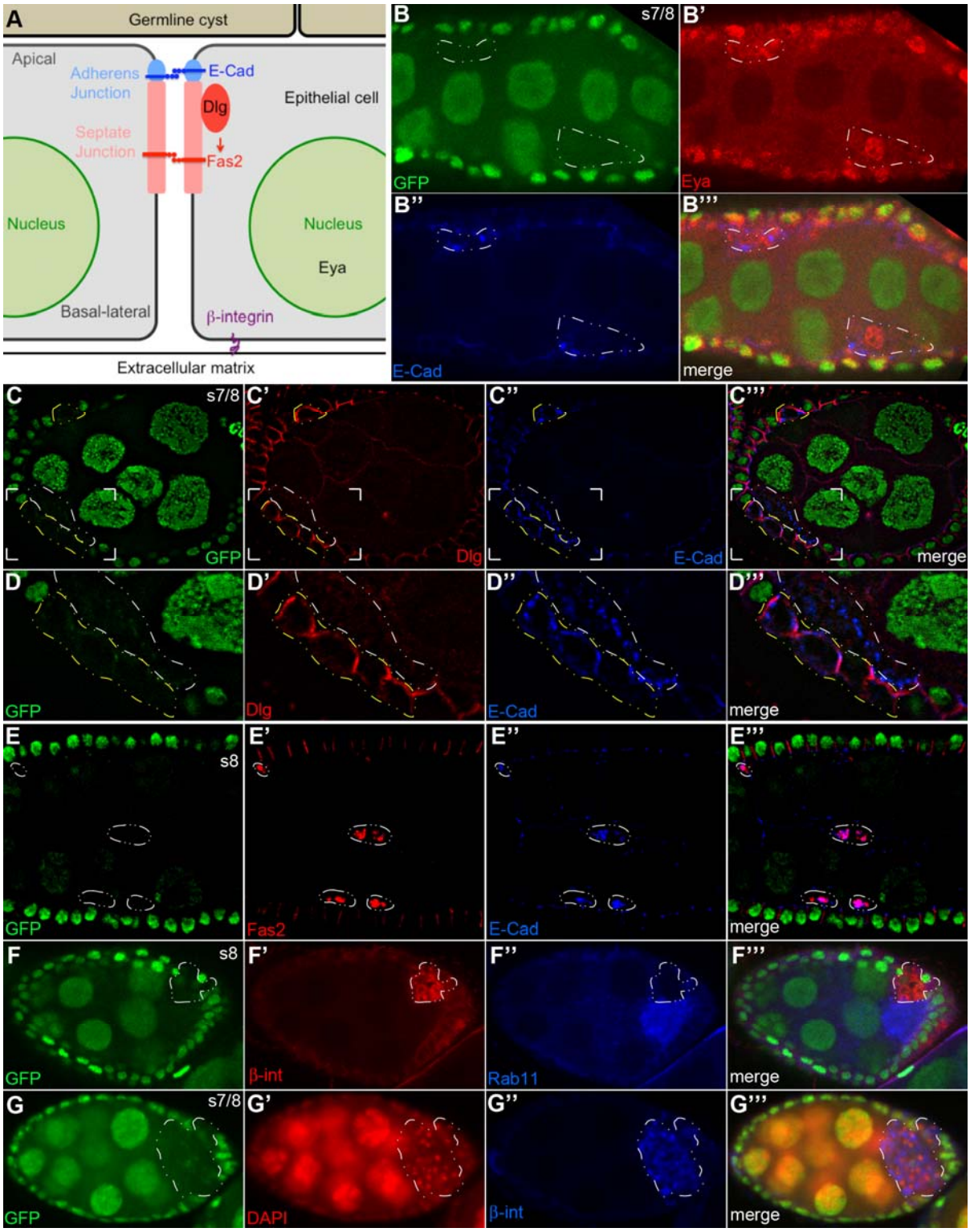
A
Germline cyst
Apical
Adherens Junction
E-Cad
Dlg
Epithelial cell
Septate Junction
Fas2
Nucleus
Nucleus
Eya
Basal-lateral
β-integrin
Extracellular matrix
B
s7/8
GFP
B'
Eya
B''
E-Cad
B'''
merge
C
s7/8
GFP
C'
Dlg
C''
E-Cad
C'''
merge
D
GFP
D'
Dlg
D''
E-Cad
D'''
merge
E
s8
GFP
E'
Fas2
E''
E-Cad
E'''
merge
F
s8
GFP
F'
β-int
F''
Rab11
F'''
merge
G
s7/8
GFP
G'
DAPI
G''
β-int
G'''
merge

Figure 2.5 *rab11-null* follicle cells lose their polarity, delaminate from the epithelium and invade the neighboring germline cyst. (A) Schematic diagram of follicle epithelial cell polarity. Markers used in this study are highlighted [adapted from (ST JOHNSTON and AHRINGER 2010)]. (B-G'') Confocal images of mosaic stages 7/8 egg chambers 4-6 days ACI. The *rab11-null* clones are marked by their absence of GFP expression and are outlined with dashed lines. (B-B''') nGFP (green), Eya (red), E-cad (blue). All of the *rab11-null* cells stain positive for Eya, consistent with an epithelial cell fate. In contrast to the strict apical expression pattern of E-cad in neighboring wildtype cells, the protein is highly enriched in intracellular compartments in the *rab11-null* cells (also see D'' and E''). (C-C''') nGFP (green), Discs large (Dlg) (red), E-cad (blue). (D-D''') Enlarged views of the bracketed regions shown in (C-C'''). Note that *rab11-null* cells that are still embedded in the epithelium (outlined in yellow) exhibit wildtype (basolateral) expression patterns for Dlg and near normal (mostly apical) expression pattern for E-cad. In contrast, the *rab11-null* cells that have delaminated from the epithelium (outlined in white) exhibit a vesicular staining pattern for E-cad, while Dlg is dispersed throughout the cell and thus barely detectable. (E-E''') nGFP (green), Fas2 (red), E-cad (blue). Three clusters of delaminated *rab11-null* cells are outlined. Each cluster contains two cells. None of the cells exhibit apical-basal polarity as evident by the vesicular-like staining pattern of both Fas2 and E-cad. (F-F''') nGFP (green), ß-integrin (ß-int) (red), Rab11 (blue). Note, the donut-shape distribution pattern of ß-int, which suggest that some of the protein is still on the cell surface.

All of the other examined cell surface markers exhibit a strictly intracellular staining pattern in delaminated cells. The circled *rab11-null* clone, along with the one shown in (G) is situated in a bubble between the epithelium proper and the germline cyst, which partially accounts for the weak ß-int signal in the flanking wildtype epithelial cells. Nevertheless, the ß-int signal was reproducibly more intense in the *rab11-null* epithelial cells than in wildtype epithelial cells. (G-G"') nGFP (green), DAPI (red), ß-int (blue). A large (> 50 cells) *rab11-null* clone in the posterior portion of the egg chamber is circled. Note that the ß –int staining pattern in this clone is more vesicular in nature than that in the previous panels. Most other similarly large clones were also located in the posterior portion of the egg chamber and like the one shown wedged between the follicle cell epithelium and the oocyte.

Chapter III

Discussion

The work presented in this dissertation reveals that the Rab11 GTPase, a membrane trafficking organizer, has multiple roles in *Drosophila* oogenesis.

First, I showed that Rab11 is required for the maintenance of germline stem cell (GSC) identity (appendix 2) but not follicle stem cell (FSC) identity (chapter 2). Because E-Cadherin (E-Cad) trafficking is known to be required in the FSC for maintaining FSC identity (Song and Xie 2002), it shows that Rab11 is not required for E-Cad trafficking in FSCs. However, this cannot be tested directly, because E-Cad levels are low in FSCs and not reliably detectable by immunostaining. Previous studies on Rab11 showed its requirement in E-Cad trafficking in the *Drosophila* embryo ectoderm for maintaining the Adherens Junctions (AJs) (ROETH *et al.* 2009). I also showed that Rab11 is required in trafficking E-Cad in the follicle epithelial cells (FECs) for maintaining their polarity (chapter 2) and in the germline stem cells (GSCs) for maintaining stem cell identity (appendix 2). These results together indicate that different types of cells may rely on different pathways to maintain needed levels of surface E-Cad. Supporting this idea, the strongest concentrations of intracellular Rab11 and E-Cad in GSCs occur in the fusome (BOGARD *et al.* 2007), an organelle that is unique in the germline cells (DE CUEVAS and SPRADLING 1998; DENG and LIN 1997), and newly synthesized E-Cad proteins are found to localize to Rab11-possitive recycling endosomes after exiting the Golgi complex and before targeting for the plasma membrane in epithelial cells (Lock and Stow 2005).

Second, I showed that Rab11 is not required for the specification of polar, stalk and epithelial cell fates in the follicle cell lineage, but is required for the terminal differentiation

and/or intercalation of stalk cells and the survival of FECs and polar cells.

As shown in chapter 2, stalk, polar and epithelial cells are specified from *rab11*-null FSCs, but no sub-lineage fully elaborates its cell fate. Stalk cells that are mutant for *rab11* accumulate E-Cad in the cytoplasm rather than on the plasma membrane, and they do not intercalate, resulting in fused egg chambers. Furthermore, within a single mosaic stalk, *rab11*-null cells do not organize into a single file line while wild-type cells do. Since the *rab11*-null stalk cells are presumably exposed to the same extracellular signals as their wild-type neighbors (ASSA-KUNIK *et al.* 2007; BAKSA *et al.* 2002; MCGREGOR *et al.* 2002; TORRES *et al.* 2003), this result shows that Rab11 is required autonomously in the stalk cells for terminal differentiation and/or intercalation. This result is also consistent with the enrichment of Rab11 and its effector Nuclear-fallout (Nuf) in the polar and stalk cells between germarium region 2b and 3.

FECs and polar cells are also specified from *rab11*-null FSCs, but most of the FECs undergo apoptosis before stage 3 and polar cells before stage 7, resulting in almost no *rab11*-null clones recovered in stage 3 or later egg chambers (chapter 2). One explanation is that the *rab11*-null cells die prematurely due to their inability to deliver new membrane material to the cell surface in response to cell growth and division. In support of this idea, Rab11 is required to deliver new membrane material to the cell surface during the cellularization of the *Drosophila* syncytial blastoderm (PELISSIER *et al.* 2003). By extension, the relatively long survival times (at least through s7) of putative *rab11*-null stalk and polar cells, might be due to the fact that these cells stop dividing at s1 and undergo fewer rounds of

cell division overall (Horne-Badovinac and Bilder 2005).

Third, I showed that when *rab11* is mutated in the mature FECs, these cells lose their polarity, delaminate from the follicle epithelium, and invade in between the germline cells, much like the *Drosophila* neoplastic tumor suppressor gene (nTSG) mutants. However, there are distinct differences between epithelial cells that are mutant for *rab11* and nTSGs. *rab11*-null FECs usually invade the surrounded tissues as groups that are fully detached from the epithelium and that consist of as few as 2 cells, while previously characterized nTSG mutant cells only invade the germline cyst as large multi-layered sheets that remain attached to the epithelium (Hariharan and Bilder 2006). In essence, the invasive behavior of previously characterized nTSG mutant cells is akin to tissue over-growth, while that of the *rab11*-null cells more closely parallels the behavior of metastatic tumor cells of higher animals (CHAMBERS *et al.* 2002). However, nTSG mutant cells over-proliferate in imaginal disks (Bilder 2004) while no *rab11*-null cells can be recovered in these larval tissues, possibly reflecting unique roles for Rab11 in the survival of such cells.

Consistent with the above findings that loss of Rab11 promotes migratory behavior in non-migratory cells, recent studies have shown that reduction of Rab11 increases the motility of mammalian cells induced to migrate by wounding (JONES *et al.* 2006; PRIGOZHINA and WATERMAN-STORER 2006). Paradoxically, Rab11 expression is up-regulated in human skin and breast carcinomas and certain other metastatic cell populations (CHENG *et al.* 2004; FAN *et al.* 2004; GEBHARDT *et al.* 2005; GOLDENRING *et al.* 1999; JONES *et al.* 2006; WANG *et al.* 2004; YOON *et al.* 2005). It will be of interest to determine if Rab11 loss and up-regulation

interfere with the same or different trafficking pathways and how these pathways affect normal versus cancerous cell migrations. The current difficulty in sorting out Rab11's role in the migration of normal and cancerous cells is the lack of information regarding the identity of Rab11's cargoes and the fate of such cargoes when Rab11 is absent or over-expressed. More information in this area is needed, as is a more extensive survey of the involvement of Rab11 in the migratory behaviors of normal and cancerous cells.

In addition to the above findings of Rab11's roles in *Drosophila* oogenesis, I also have constructed a transgene with chimeric Rab11 that contains a five amino acid substitution at residues 38-42 (P[$rab11^{38/42}$]) and introduced this transgene into *rab11*-null follicle cells (appendix 1). When P[$rab11^{38/42}$] is expressed in the *rab11*-null stalk cells, these cells express E-Cad on the plasma membrane like wildtype stalk cells and intercalate to form a functional stalk. When P[$rab11^{38/42}$] is tested for its ability to rescue lethality in polar cells and FECs, *rab11*-null polar cells with P[$rab11^{38/42}$] are not recovered in mid- and late-stages (>stage 6), while FECs derived from *rab11*-null FSCs with P[$rab11^{38/42}$] are viable, but exhibit a series of tumor-cell-like behaviors such as loss of polarity, delamination from epithelium and invasion into neighboring tissues.

It is interesting that the P[$rab11^{38/42}$] transgene rescues stalk cell intercalation but not most other Rab11 functions, e.g., polar cell differentiation/survival and epithelial cell maintenance. It is also not clear why P[$rab11^{38/42}$] restores the correct localization of polarity organizers such as E-Cad in the stalk cells but not in the FECs. It may be that the P[$rab11^{38/42}$] transgene produces reduced level of protein and stalk cell differentiation only

requires small amounts of Rab11. Alternatively, the Rab11$^{38/42}$ protein may lack specific Rab11 activity. That is, although the Rab11$^{38/42}$ protein cannot bind to transport effectors such as dRip11, it can bind to the other effectors to perform budding, docking and membrane fusion functions. It is possible that random diffusion of Rab11-tethered vesicles can bring enough cargo to close proximity to the plasma membrane to be docked and fused in the stalk cells but fail to provide enough cargo in the FECs. While we cannot fully distinguish between the above possibilities, it is noteworthy that the 38/42 substitution interferes with the *in vitro* binding of Rab11 to dRip11 but not to Sec15 (Lan and Cohen, unpublished data). A future study of Rab11's effectors binding to the 38/42 domain, such as dRip11 in *Drosophila* ovary might help answer this question.

One caveat of this chimeric Rab11 study is that the wildtype transgene P[*rab11*^{wt}] and the chimeric transgene P[*rab11*$^{38/42}$] are inserted randomly into the *Drosophila* genome and they can land in chromosome regions that are not transcribed at the same level, a phenomenon known as position effects (LEVIS *et al.* 1985), even though these transgenes are constructed in the same way using endogenous *rab11* promoter. Thus differences in these transgenes' abilities to rescue *rab11* phenotypes may reflect a difference in gene product level, rather than different Rab11 activity. In future studies, precise transformation like the ϕC31 integrase system that allows transgenes to be inserted into the same position should be used to eliminate position effects (GROTH *et al.* 2004).

Taken together, findings in this dissertation show that Rab11 is involved in multiple important biological events such as stalk cell terminal differentiation and/or intercalation, cell

survival, suppression of nTSG-like behavior in epithelial cells, and stem cell maintenance. These functions of Rab11 are tightly related to its role in trafficking of polarity organizers such as E-Cad in different cell types. In addition, the rescue study with Rab11 transgenes demonstrates a method to use chimeric proteins to analyze the functional domains of a Rab GTPase in different cell types.

Appendix I

Functional Studies of a Rab11 Effector Binding Domain

4.1 Introduction

To fully understand Rab11-mediated membrane trafficking and its functions, it is important to analyze how Rab11 interacts with its effectors in carrying out vesicle budding, transport, docking and fusion with membranes in different cell types.

Rab11-FIPs (Rab11 family interacting proteins; also known as FIPs) are Rab11 effectors that share a highly conserved 20 amino acid RBD (Rab11-binding domain) at their C-termini. With five members [RCP (Rab coupling protein; also known as FIP1), FIP2, FIP3, FIP4, and Rip11 (FIP5)], FIPs form the biggest group of Rab11 effectors (HORGAN and MCCAFFREY 2009). FIPs are categorized into two sub-families. Class I FIPs all have a C2 domain (a putative calcium –binding domain) at their N-termini (HORGAN and MCCAFFREY 2009). The only class I FIP in *Drosophila* is dRip11 [*Drosophila* Rip11; (LI *et al.* 2007)].

Drosophila Rab11 and dRip11 are involved in polarized secretion in *Drosophila* photoreceptors (BERONJA *et al.* 2005; LI *et al.* 2007; SATOH *et al.* 2005). It has been shown in our lab that binding to dRip11 involves Rab11's amino acid residues 38-42 (Lan and Cohen, unpublished data), the region where Rab11's switch I region overlaps with the Rab sub-family specific region 2 (RabSF2) (Figure 4.1). Structural studies show that human Rab11binds to the RBD of FIP3 via switch region I (SHIBA *et al.* 2006). The Rab switch regions undergo dramatic changes when Rab changes from the inactive GDP-bound state to the active GTP-bound state, and are involved in effector binding (BOURNE *et al.* 1991; SHIBA *et al.* 2006; STENMARK 2009; STENMARK and OLKKONEN 2001). In humans, RabSF2 is highly conserved (92.3% identical on average) within each sub-family but quite diversified

among all other Rabs (34.2% identical on average) (Pereira-Leal and Seabra 2000). *Drosophila* Rab11's switch I and RabSF2 sequences are identical to those of human Rab11a and Rab11b (Figure 4.1).

One of Rab11's closest paralog is Rab2, and Rab2 is involved in retrograde trafficking from Golgi to ER (PEREIRA-LEAL and SEABRA 2001; STENMARK 2009). Rab2 and Rab11 share similar GTPase structure, but some surface residues are different, including the whole RabSF2 domain (Figure 4.2). The subtle structural difference between these two Rabs are believed to result in different effector preference and ultimately their different functions in membrane trafficking.

Chimeric proteins have proven very useful in studying membrane trafficking organizers among other applications (BRENNWALD and NOVICK 1993; DUNN *et al.* 1993). In this chapter, I describe how I used site-directed mutagenesis to generate a transgene with chimeric Rab11 (P[$rab11^{38/42}$]) that contains a five amino acid substitution at residues 38-42 with Rab2 sequence and tested this transgene's ability to rescue *rab11* phenotypes I described in chapter 2. This transgene rescues some, but not all, *rab11* functions in *Drosophila* follicle cells. E.g., it rescues *rab11*-null stalk cell defects and follicle epithelial cell (FEC) lethality, but not polar cell lethality or FEC polarity defects. In addition, P[$rab11^{38/42}$] also rescues polarity defects in oocytes mutant for $rab11^{2148}$, a hypomorphic *rab11* allele. These findings suggest that not all Rab11 effectors are required in different cell types to carry out Rab11's functions. P[$rab11^{38/42}$] rescues *Drosophila* E-Cadherin (DE-Cad) mis-localization in *rab11*-null stalk cells but not in FECs, suggesting different levels of DE-Cad or different

DE-Cad trafficking pathways are required in these cells.

4.2 MATERIALS AND METHODS

Chimeric Transgene P[*rab11*$^{38/42}$]

CaSpeR4X6.3kRab11 (DOLLAR *et al.* 2002), a CaSpeR4 vector with the 6.3 kb genomic *rab11* gene fragment (*rab11*^{wt}) was used to generate a Rab11/Rab2 chimeric gene, *rab11*$^{38/42}$. A PCR fragment of 2.8 kb was amplified from CaSpeR4X6.3kRab11 with a forward primer (5'-TTTATTTTCTAGACCTAATGTGTT-3', upstream of a Nhe I site) and a reverse primer (5'-CAGGTCGTGCACGGGCTGGAATTCATTGCGCGTGAAACG-3', containing an Apal I site and the 5' was mutated to encode for Rab2 sequence). The 2.8 kb PCR fragment was then subjected to Nhe-Apal I double digestion to yield a 1.3 kb fragment with Rab11's amino acids 38/40 mutated to those of the Rab2's. Another PCR fragment of 1.5 kb was amplified from CaSpeR4X6.3kRab11 with a forward primer (5'-CAGCCCGTGCACGACCTGACGATTGGCGTTGAGTTTGC-3', containing an Apal I site and the 5' was mutated to encode for Rab2 sequence) and a reverse primer (5'-CAATTTATGCGCACATGACAA-3', downstream of a Bgl II site). This 1.5 kb PCR fragment was then subjected to Apal I-Bgl II double digestion to yield a 1.2 kb fragment with Rab11's amino acids 40/42 mutated to those of the Rab2's. The 1.3 kb fragment, the 1.2 kb fragment, and a 11.5 kb Nhe–Bgl II double digest fragment from CaSpeR4X6.3kRab11 were ligated together to generate the *rab11*$^{38/42}$ construct CaSpeR4X6.3kRab11-38/42.

The transformation vectors CaSpeR4X6.3kRab11 and CaSpeR4X6.3kRab11-38/42

were then microinjected into w^{1118} flies according to standard protocols (RUBIN and SPRADLING 1982; SPRADLING and RUBIN 1982). Two independently transformed lines with P[$rab11^{wt}$] or P[$rab11^{38/42}$] on the second chromosome were established.

***Drosophila* Genetics**

Fly culture and crosses were carried out according to standard procedures (ASHBURNER *et al.* 2005). The wildtype stock was *y w hsp::FLP, Hrb98DE::GFP* (BOGARD *et al.* 2007; MORIN *et al.* 2001). The *rab11-null,* $rab11^{\Delta FRT}$, and a *rab11* hypomorphic mutation, $rab11^{P2148}$ have been previously described (BOGARD *et al.* 2007; DOLLAR *et al.* 2002). To generate homozygous *rab11*-null clones, we mated *y w hsp::FLP; rab11-null/TM3, Sb* flies to *y w hsp::FLP; FRT5377, Hrb98DE::GFP* flies, where *FRT5337* corresponds to the centromere-proximal FRT insertion element that was used to make the *rab11-null* allele (BOGARD *et al.* 2007). F1 3rd instar larvae or 2- to 3-day adults were heat shocked for 1 hour at 37^{o}C on 2 consecutive days. Cells (in Sb^{+} adults) homozygous for the *rab11-null* allele were identified by the absence of GFP fluorescence.

For the rescue analysis, flies harboring a Rab11 transgene (P[$rab11^{wt}$] or P[$rab11^{38/42}$]) on the second chromosome were crossed to either $rab11^{\Delta FRT}$ flies to make a *w; rab11-transgene; rab11-null/TM6b, Tb* stock, or to $rab11^{P2148}$ flies to make a *w; Rab11-transgene;* FRT^{82B}, $rab11^{P2148}$*/TM6b, Tb* stock. The *w; rab11-transgene; rab11-null/TM6b, Tb* females were then crossed to *y w hsp::FLP/Y; FRT5377, Hrb98DE::GFP* males, and heat-shock of the Tb^{+} progeny was carried out as described above. The *w; rab11-transgene;* FRT^{82B}, $rab11^{P2148}$*/TM6b, Tb* females were mated to *y w*

hsp::FLP/Y; FRT^{82B}*, P[ovo*^{D1}*] /TM3, Sb* males to test rescue of the *rab11* transgenes in the germline clones, and heat-shock of the Sb^+, Tb^+ progeny was carried out as previously described (DOLLAR *et al.* 2002).

Immunocytochemistry and Confocal Microscopy

Ovaries were fixed and immunostained as described (BOGARD *et al.* 2007; DOLLAR *et al.* 2002). Primary antibodies were used at the following concentrations: Oskar (1:1000; a gift from Dr. Paul Macdonald), GFP (1:250; Invitrogen), DE-cadherin (1:40; DSHB), Eyes-absent (1:250; DSHB), and LamC (1:50; DSHB). Secondary antibodies were purchased from Jackson labs and used at the manufacturer's recommended concentrations. Stained ovaries were mounted in 4% n-propyl gallate (Sigma) in 90% glycerol, 10% phosphate buffered saline. Images were collected on Olympus 3L Spinning disc or Zeiss Meta 510 laser scanning confocal microscopes.

4.3 RESULTS AND DISCUSSION

P[*rab11*$^{38/42}$] Rescues *rab11*-null Stalk Cell Defects

To test the function of the Rab11 38/42 domain, a chimeric transgene, P[*rab11*$^{38/42}$] was made and introduced into *rab11*-null mutants. The chimeric transgene has the overall Rab11 structure but the 38/42 domain mutated to that of Rab2's. A wildtype transgene, P[*rab11*^{wt}], was used as a positive control (DOLLAR *et al.* 2002). Two fly lines with P[*rab11*^{wt}] on the second chromosome were tested. One line completely rescued *rab11*-null flies as *rab11*$^{-/-}$ flies with one copy of P[*rab11*^{wt}] were viable and fertile (data not shown).

Two fly lines with P[*rab11*$^{38/42}$] on the second chromosome were also tested and one line rescued some but not all *rab11* phenotypes and was used for this study, while the other line did not rescue any *rab11* phenotype. Due to position effects (LEVIS *et al.* 1985), some transgenes might have landed in chromosome regions that were not actively transcribed, even though these transgenes were constructed in the same way using endogenous *rab11* promoter. The P[*rab11*$^{38/42}$] line used in this study did not show different rescue patterns with one or two copies. Since P[*rab11*$^{38/42}$]; *rab11*$^{-/-}$ flies were not viable (data not shown), all studies were carried out using mosaic analyses (BOGARD *et al.* 2007).

Follicle cell clones were analyzed 14 days after clone induction (ACI) to ensure that they were derived from *rab11*-null FSCs (BASTOCK and ST JOHNSTON 2008; KIRILLY and XIE 2007). As described in chapter 2, Rab11 is required autonomously in the stalk cells for their terminal differentiation and/or intercalation. When P[*rab11*$^{38/42}$] is expressed in *rab11*-null stalk cells, these cells (Figures 4.3B-B’’’, white dashed circle; GFP-negative, LamC positive) expressed E-Cad normally (arrows in Figures 4.3A’ and 4.3B’) and formed a functional stalk. This result shows that P[*rab11*$^{38/42}$] rescues *rab11*-null stalk cell defects.

Since mosaic ovariole (n>100) that had intercalated wildtype stalk cells and older *rab11*-null anterior polar cells (APCs) in the same egg chamber junction has never been recovered, it suggests that *rab11* is also required in the APCs for stalk cell terminal differentiation and/or intercalation as these events require Delta and Unpaired signals from the APCs (ASSA-KUNIK *et al.* 2007; TORRES *et al.* 2003). However, this idea is difficult to examine with *rab11*-null clones alone because most stalk cell and APC clones are derived

from the same follicle stem cell (FSC) and *rab11* is required autonomously in the stalk cells for terminal differentiation and/or intercalation. In egg chambers that had one copy of P[$rab11^{38/42}$] and that were entire *rab11*-null follicle cell clones (Figures 4.3C-D'''), the stalk cells failed to intercalate and clustered together between egg chambers. Since P[$rab11^{38/42}$] rescues *rab11*-null stalk cell defects, the failure of stalk cell intercalation in the P[$rab11^{38/42}$]; *rab11*-null follicle cell clones is attributed to a lack or reduction of external cell signals. This result is consistent with the idea that *rab11* is required in the APCs for stalk cell terminal differentiation and/or intercalation, and P[$rab11^{38/42}$] does not rescue the APC defects.

Taken together, the above results show that P[$rab11^{38/42}$] restores the function of *rab11*-null stalk cells. They also suggest that a Rab11-dependent signal from the older egg chamber, presumably Delta or Unpaired from the APCs (BAKSA *et al.* 2002; MCGREGOR *et al.* 2002; ROTH and LYNCH 2009) is required for stalk cell terminal differentiation and/or intercalation, and P[$rab11^{38/42}$] does not rescue the APC defects. One simple explanation is that while effectors that bind to the 38/42 domain of Rab11 are not essential in the stalk cells, these effectors are indispensable in the APCs of the older egg chamber to signal the stalk cells to fully differentiate and/or intercalate.

P[$rab11^{38/42}$] Rescues *rab11*-null FECs Lethality but Not Their nTSG-like Behavior

The P[$rab11^{38/42}$] transgene was also tested in follicle epithelial cells (FECs), because *rab11* is required both for them to survive and maintain their polarity (Chapter 2). When mosaic flies were observed 15 days ACI, almost no *rab11*-null FEC clones could be

recovered in stage 3 or older egg chambers (Chapter 2). In contrast, large FEC clones could be recovered in *rab11*-null mosaic egg chambers that also harbor one copy of P[$rab11^{wt}$] (Figurs 4.4A-A'') or P[$rab11^{38/42}$] (Figures 4.4B-B''), suggesting that both transgenes could rescue FEC lethality. However, FEC clones with the P[$rab11^{38/42}$] transgene lost their polarity as indicated by their increased accumulation of DE-Cad all over the plasma membrane and sometimes intracellularly (Figurs 4.4B'), and they formed multi-layers, similar to *rab11*-null FECs described in chapter 2. When mosaic flies were observed earlier, at 8 days ACI to favor clones in developed FECs, clone cells with the P[$rab11^{38/42}$] transgene delaminated from the epithelium (Figure 4.4D''' arrow head) and invaded into the germline cyst (Figure 4.4D''' arrow) just like *rab11*-null FECs (chapter 2), while clones with the P[$rab11^{wt}$] transgene maintained their polarity and behaved like wildtype cells (Figure 4.4C-C''').

These results show that *rab11*-null FEC clones with one copy of the P[$rab11^{38/42}$] transgene can survive beyond stage 3, but instead of forming a single layer epithelium, they lose polarity, delaminate from the epithelium, and invade the germline cyst. These results demonstrate that the 38/42 domain of Rab11 is required for FEC maintenance. In extension, Rab11's effectors that bind to the 38/42 domain are likely involved in FEC maintenance too. As *rab11* is required only transiently for FEC survival (chapter 2), it is possible that not all its effectors are required for this event.

P[*rab11*$^{38/42}$] Rescues Polarity Defects in *rab11*^{2148} Oocytes

Since previous studies have shown a requirement for Rab11 in the oocytes for

maintain their polarity in late stage egg chambers (DOLLAR *et al.* 2002; JANKOVICS *et al.* 2001), it would be interesting to test whether the P[$rab11^{38/42}$] transgene could rescue the polarity defects in *rab11* mutant oocytes. Because *rab11*-null follicle clone phenotypes could complicate observations of germline clones and data interpretation, a hypomorphic *rab11* allele (DOLLAR *et al.* 2002), $rab11^{2148}$ that showed no obvious defect in follicle cells was used. Germline clones of $rab11^{2148}$ were generated, and mis-localized Oskar (Osk) protein in oocytes (arrow in Figure 4.5A) could be rescued by one copy of P[$rab11^{wt}$] (arrow in Figure 4.5B), or one copy of P[$rab11^{38/42}$] (arrow in Figure 4.5C). Consistent with this result, $rab11^{2148}$ homozygous flies with one copy of P[$rab11^{wt}$] or one copy of P[$rab11^{38/42}$] were both viable and fertile (data not shown).

Since $rab11^{2148}$ is a hypomorphic mutation that produces small amount, but otherwise normal Rab11 (DOLLAR *et al.* 2002), it is not clear if P[$rab11^{38/42}$] works alone or in synergy with the Rab11 protein from $rab11^{2148}$ to rescue the polarity defects in oocyts.

Figure 4.1

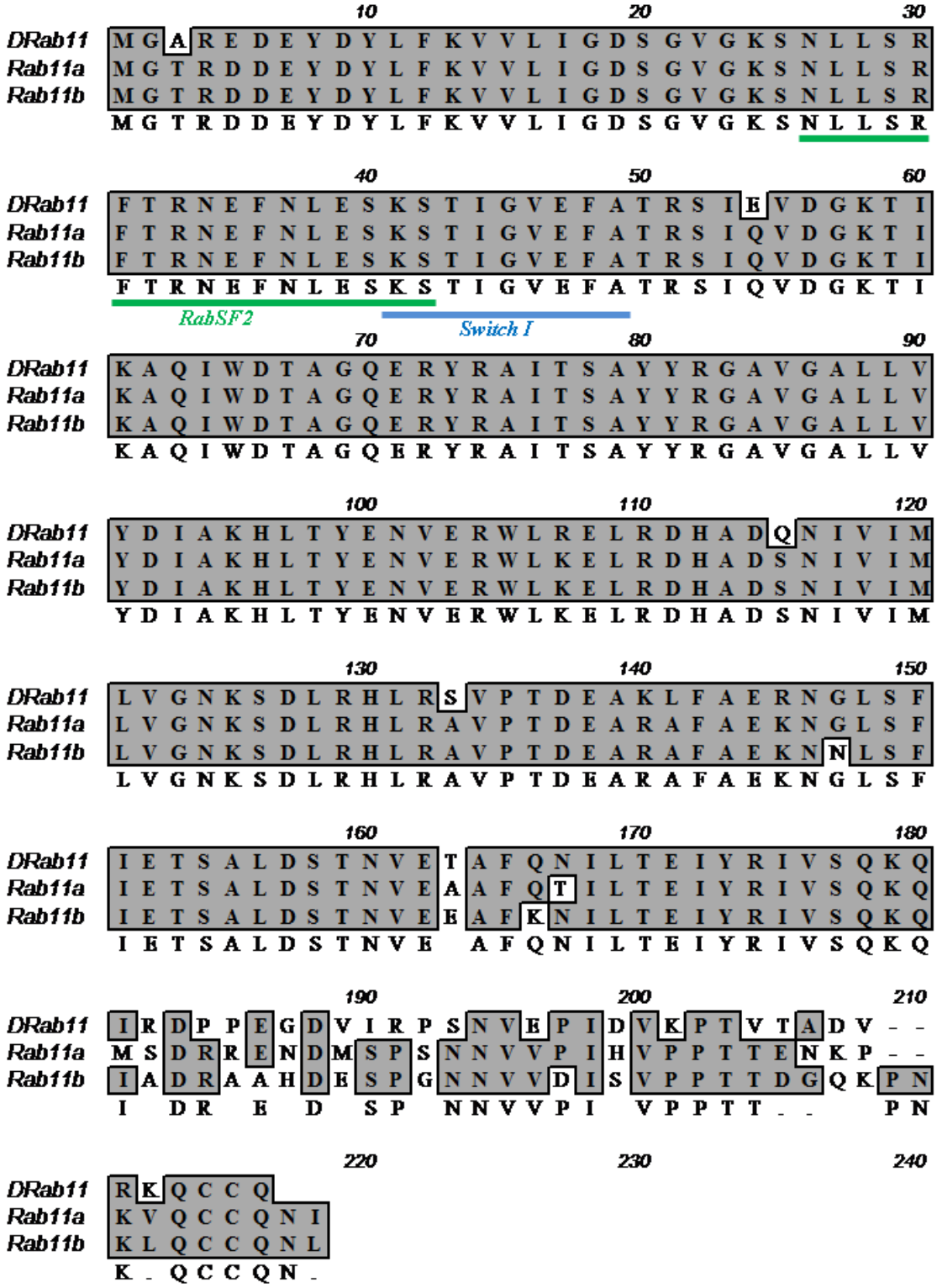
10 20 30
DRab11 M G A R E D E Y D Y L F K V V L I G D S G V G K S N L L S R
Rab11a M G T R D D E Y D Y L F K V V L I G D S G V G K S N L L S R
Rab11b M G T R D D E Y D Y L F K V V L I G D S G V G K S N L L S R
M G T R D D E Y D Y L F K V V L I G D S G V G K S N L L S R
40 50 60
DRab11 F T R N E F N L E S K S T I G V E F A T R S I E V D G K T I
Rab11a F T R N E F N L E S K S T I G V E F A T R S I Q V D G K T I
Rab11b F T R N E F N L E S K S T I G V E F A T R S I Q V D G K T I
F T R N E F N L E S K S T I G V E F A T R S I Q V D G K T I
RabSF2
Switch I
70 80 90
DRab11 K A Q I W D T A G Q E R Y R A I T S A Y Y R G A V G A L L V
Rab11a K A Q I W D T A G Q E R Y R A I T S A Y Y R G A V G A L L V
Rab11b K A Q I W D T A G Q E R Y R A I T S A Y Y R G A V G A L L V
K A Q I W D T A G Q E R Y R A I T S A Y Y R G A V G A L L V
100 110 120
DRab11 Y D I A K H L T Y E N V E R W L R E L R D H A D Q N I V I M
Rab11a Y D I A K H L T Y E N V E R W L K E L R D H A D S N I V I M
Rab11b Y D I A K H L T Y E N V E R W L K E L R D H A D S N I V I M
Y D I A K H L T Y E N V E R W L K E L R D H A D S N I V I M
130 140 150
DRab11 L V G N K S D L R H L R S V P T D E A K L F A E R N G L S F
Rab11a L V G N K S D L R H L R A V P T D E A R A F A E K N G L S F
Rab11b L V G N K S D L R H L R A V P T D E A R A F A E K N N L S F
L V G N K S D L R H L R A V P T D E A R A F A E K N G L S F
160 170 180
DRab11 I E T S A L D S T N V E T A F Q N I L T E I Y R I V S Q K Q
Rab11a I E T S A L D S T N V E A A F Q T I L T E I Y R I V S Q K Q
Rab11b I E T S A L D S T N V E E A F K N I L T E I Y R I V S Q K Q
I E T S A L D S T N V E A F Q N I L T E I Y R I V S Q K Q
190 200 210
DRab11 I R D P P E G D V I R P S N V E P I D V K P T V T A D V - -
Rab11a M S D R R E N D M S P S N N V V P I H V P P T T E N K P - -
Rab11b I A D R A A H D E S P G N N V V D I S V P P T T D G Q K P N
I D R E D S P N N V V P I V P P T T _ _ P N
220 230 240
DRab11 R K Q C C Q
Rab11a K V Q C C Q N I
Rab11b K L Q C C Q N L
K _ Q C C Q N _

Figure 4.1 Amino acid sequence is highly conserved between *Drosophila* Rab11 and the human Rab11 family proteins. DRab11 is the *Drosophila* Rab11. Rab11a and Rab11b belong to the human Rab11 family. Green line shows the RabSF2 motif (aa 26-42) and blue line shows the Switch I region (aa 41-49). Notice these two domains are identical between *Drosophila* and human Rab11s.

Figure 4.2

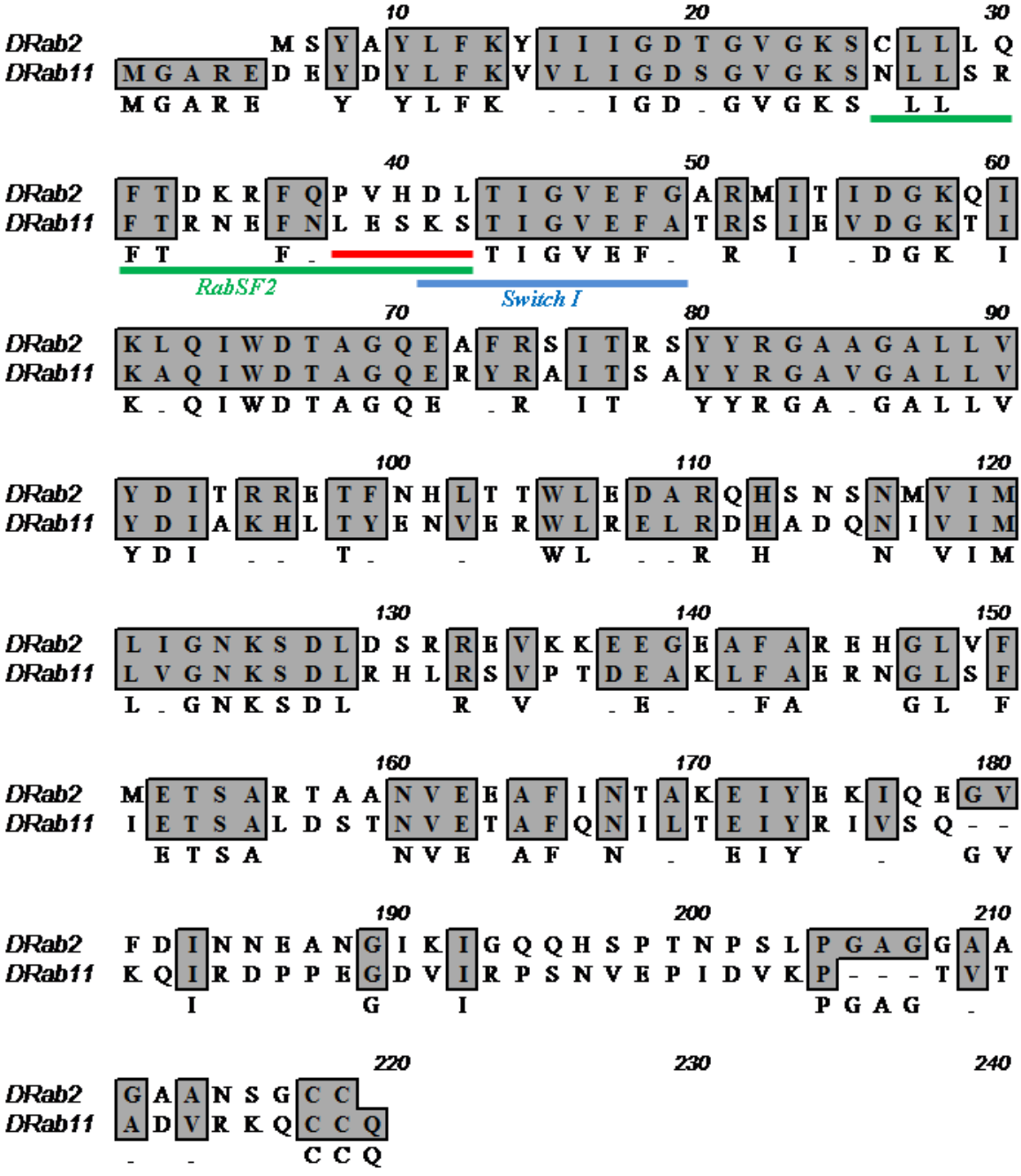

Figure 4.2 Rab11 and Rab2 share similar structure but have different 38/42 domains. Green line shows the RabSF2 motif (aa 26-42) and blue line shows the Switch I region (aa 41-49). Red line shows the 38/42 domain, which has been shown to be involved in binding between Rab11 and dRip11. In the P[*rab11*$^{38/42}$] construct, the Rab11's 38/42 domain has been changed to that of Rab2's.

Figure 4.3

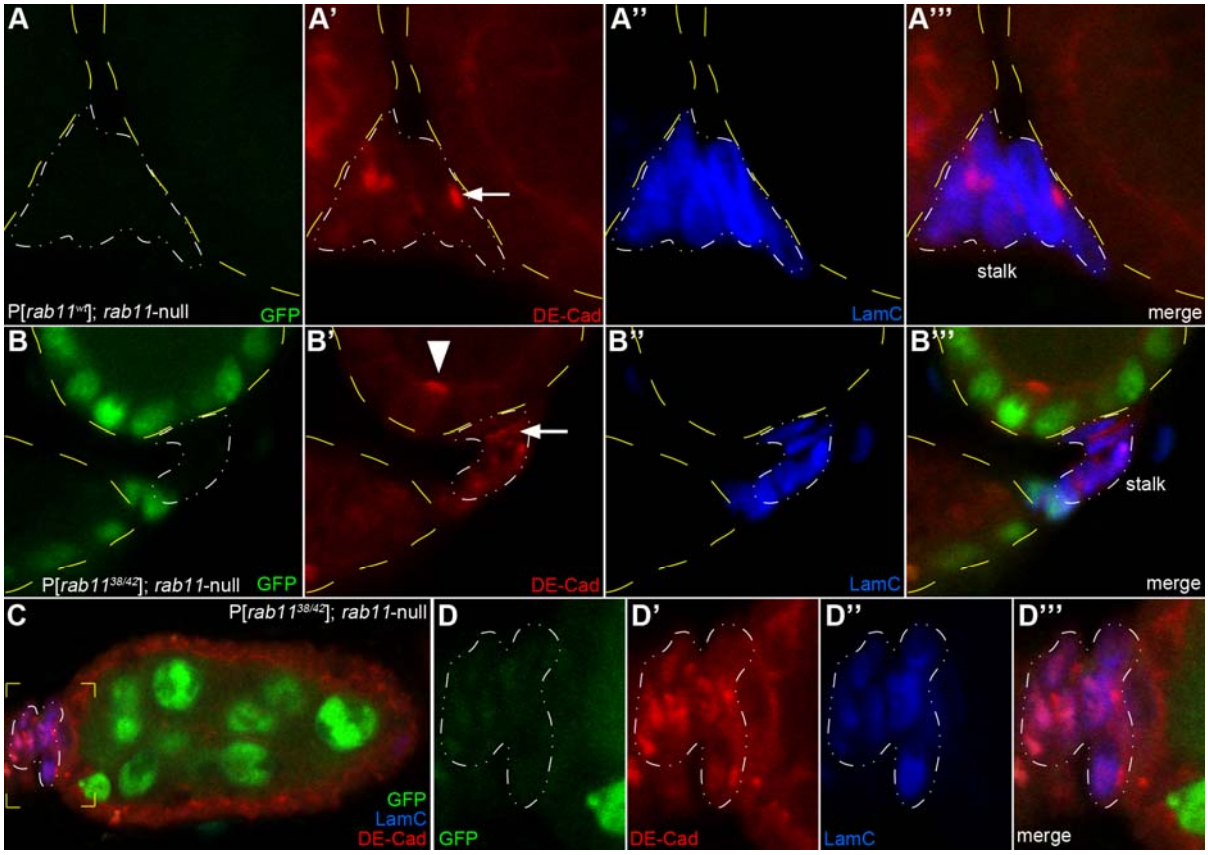
A
A'
A''
A'''
P[rab11^w1]; rab11-null
GFP
DE-Cad
LamC
stalk
merge
B
B'
B''
B'''
P[rab11^38/42]; rab11-null
GFP
DE-Cad
LamC
stalk
merge
C
P[rab11^38/42]; rab11-null
GFP
LamC
DE-Cad
D
D'
D''
D'''
GFP
DE-Cad
LamC
merge

Figure 4.3 P[*rab11*$^{38/42}$] rescues *rab11*-null stalk cells. (A-D'') Mosaic *rab11*-null egg chambers stained for nuclear GFP (green), Lamin-C (LamC; blue), and *Drosophila* E-Cadherin (DE-Cad; red) 14 days after clone induction, with one copy of the wildtype transgene P[*rab11*^{wt}] (A-A'''), or one copy of the chimeric transgene P[*rab11*$^{38/42}$] (B-D'''). Anterior is at left. The loss of endogenous *rab11* is marked by the absence of nuclear GFP in (A-D'''). (A-A''') Enlarged confocal images of an egg chamber junction. The borders of the flanking egg chambers are indicated with the dashed yellow lines. The P[*rab11*^{wt}]; *rab11-null* stalk cells (GFP-negative, LamC-positive) are enclosed in the dashed white line. Note these stalk cells express DE-Cad normally (arrow in A') and have intercalated. (B-B''') Enlarged confocal images of an egg chamber junction. The borders of the flanking egg chambers are indicated with the dashed yellow lines. The P[*rab11*$^{38/42}$]; *rab11-null* stalk cells (GFP-negative, LamC-positive; enclosed in the dashed white line) express DE-Cad normally (arrow in A') and have intercalated. Note the anterior polar cells of the older egg chamber (arrowhead in A') are *rab11* wildtype (P[*rab11*$^{38/42}$]; *rab11*$^{+/-}$). (C) Confocal image of an egg chamber that has one copy of P[*rab11*$^{38/42}$] and that are entire *rab11*-null follicle cell clone (GFP-negative). (D-D''') Enlarged views of the bracketed area in (C). The P[*rab11*$^{38/42}$]; *rab11-null* stalk cells (GFP-negative, LamC-positive; enclosed in the dashed white line) in this case did not intercalate, suggesting a lack or reduction of extracellular signals.

Figure 4.4

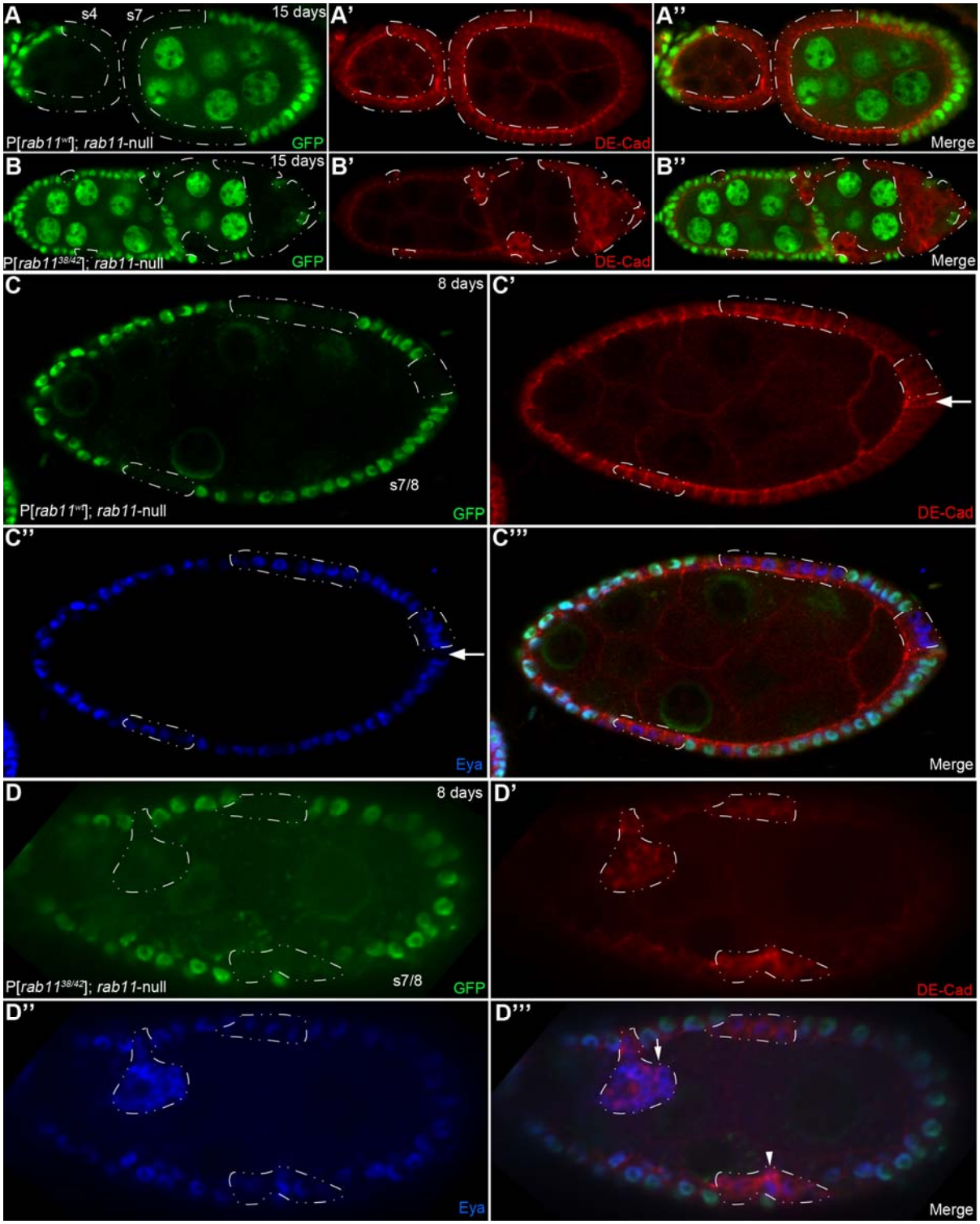
A
s4
s7
15 days
P[rab11wt]; rab11-null
GFP
A'
DE-Cad
A''
Merge
B
15 days
P[rab1138/42]; rab11-null
GFP
B'
DE-Cad
B''
Merge
C
8 days
s7/8
P[rab11wt]; rab11-null
GFP
C'
DE-Cad
C''
Eya
C'''
Merge
D
8 days
P[rab1138/42]; rab11-null
s7/8
GFP
D'
DE-Cad
D''
Eya
D'''
Merge

Figure 4.4 P[*rab11*$^{38/42}$] rescues *rab11*-null FECs lethality but not their nTSG-like behavior. (A-B’’’) Mosaic *rab11*-null egg chambers stained for nuclear GFP (green) and DE-Cadherin (DE-Cad; red) 15 days ACI, with one copy of the wildtype transgene P[*rab11*^{wt}] (A-A’’), or one copy of the chimeric transgene P[*rab11*$^{38/42}$] (B-B’’). (C-D’’’) Mosaic *rab11*-null egg chambers stained for nuclear GFP (green), DE-Cad (red), and Eyes-absent (Eya; blue) 8 days ACI, with one copy of the wildtype transgene P[*rab11*^{wt}] (C-C’’’), or one copy of the chimeric transgene P[*rab11*$^{38/42}$] (D-D’’’). Anterior is at left. Follicle epithelial cells (FECs) that have lost the endogenous *rab11* are marked by the absence of nuclear GFP and are enclosed with the white dashed lines in (A-D’’’). (A-A’’) Confocal images of two neighboring mosaic egg chambers. Note the size of the P[*rab11*^{wt}]; *rab11*-null FEC clone (GFP-negative; enclosed with the white dashed lines) is about the same as the wildtype FEC population (GFP-positive). Also note the expression of DE-Cad in these FEC clones is the same as in wildtype FECs. (B-B’’) Confocal images of two fused egg chambers. Note the P[*rab11*$^{38/42}$]; *rab11*-null FECs (GFP-negative; enclosed with the white dashed lines) can survive past stage 3 but have lost their polarity and formed multi-layers. (C-C’’’) Confocal images of a stage 7/8 egg chamber. Note the P[*rab11*^{wt}]; *rab11*-null FECs (GFP-negative, Eya-positive; enclosed with the white dashed lines) express DE-Cad normally like the wildtype FECs (GFP-positive; Eya-positive). Also note the wildtype polar cells do not express Eya (arrows in C’’) and up-regulate DE-Cad (arrows in C’). (D-D’’’) Confocal images of a stage 7/8 egg chamber. Note the P[*rab11*$^{38/42}$]; *rab11*-null FECs (GFP-negative, Eya-positive; enclosed with the white dashed lines)

accumulate DE-Cad in donut-shape pattern, and some of them have delaminated from the epithelium (arrowhead in D’’’) and invaded the germline cyst (arrow in D’’’).

Figure 4.5

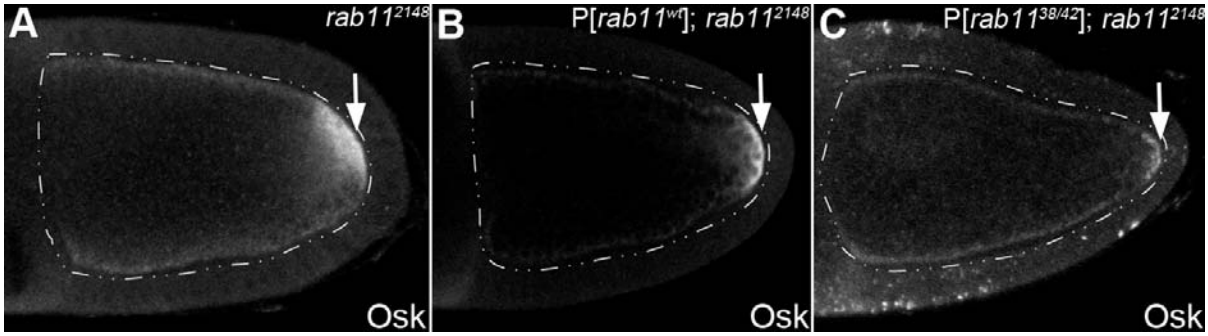
A
rab11^2148
Osk
B
P[rab11^wt]; rab11^2148
Osk
C
P[rab11^38/42]; rab11^2148
Osk

Figure 4.5 P[*rab11*$^{38/42}$] rescues Oskar localization defect in *rab11*^{2148} oocytes. (A-C) Stage 10 egg chambers with *rab11*^{2148} oocytes (enclosed in white dashed lines) stained for Oskar (Osk), with no transgene (A), one one copy of the wildtype transgene P[*rab11*^{wt}] (B), or one copy of the chimeric transgene P[*rab11*$^{38/42}$] (C). Anterior is at left. (A) Confocal image of the posterior half of a stage 10 egg chamber. Osk proteins (arrow) did not completely localize to the posterior pole in the *rab11*^{2148} oocyte (enclosed in the white dashed line), but diffused more to the anterior and the cortex, consistent with previous studies (DOLLAR *et al.* 2002; JANKOVICS *et al.* 2001). (B) Confocal image of the posterior half of a stage 10 egg chamber. Osk proteins (arrow) localized to the posterior pole in the P[*rab11*^{wt}]; *rab11*^{2148} oocyte (enclosed in the white dashed line). (C) Confocal image of the posterior half of a stage 10 egg chamber. Osk proteins (arrow) localized to the posterior pole in the P[*rab11*$^{38/42}$]; *rab11*^{2148} oocyte (enclosed in the white dashed line), just like in the P[*rab11*^{wt}]; *rab11*^{2148} oocyte in (B).

Appendix II

Rab11 maintains connections between germline stem cells and niche cells in the *Drosophila* ovary

5.1 SUMMARY

All stem cells have the ability to balance their production of self-renewing and differentiating daughter cells. The germline stem cells (GSCs) of the *Drosophila* ovary maintain this balance through physical attachment to anterior niche cap cells and stereotypic cell division, whereby only one daughter remains attached to the niche. GSCs are attached to cap cells via adherens junctions, which also appear to orient GSC division through capture of the fusome, a germline-specific organizer of mitotic spindles. Here we show that the Rab11 GTPase is required in the ovary to maintain GSC-cap cell junctions and to anchor the fusome to the GSC's anterior cortex. Thus *rab11*-null GSCs detach from niche cap cells, contain displaced fusomes, and undergo abnormal cell division, leading to an early arrest of GSC differentiation. Such defects likely reflect a role for Rab11 in E-cadherin trafficking as E-cadherin accumulates in Rab11-positive recycling endosomes (REs) and E-cadherin and Armadillo/b-catenin are both found in reduced amounts on the surface of *rab11*-null GSCs. The Rab11-positive REs through which E-cadherin transits are tightly associated with the fusome. We propose that such association polarizes Rab11's trafficking of E-cadherin and other cargoes toward the GSC's anterior cortex, thus simultaneously fortifying GSC-niche junctions, fusome localization, and asymmetric cell division. These studies bring to focus the important role of membrane trafficking in stem cell biology.

5.2 INTRODUCTION

Drosophila oogenesis is an excellent system in which to study stem cell maintenance and differentiation because all of the steps unfold in well-defined compartments. The initial steps occur within the germarium, which is divided along its anterior-posterior axis into 3 morphologically distinct regions (Fig. 5.1A). Two to three germline stem cells (GSCs) are attached by adherens junctions to niche cap cells at the extreme anterior end of germarial region 1 (Song et al., 2002; Kirilly and Xie, 2007). The cap cells and other neighboring niche cells continuously secrete Dpp and Gbb, short-range TGF-b-like signaling molecules that maintain GSC identity through repression of *bam* transcription (Xie and Spradling, 1998; Xie and Spradling, 2000; Song et al., 2004). Each GSC divides along its anterior-posterior axis to produce another GSC, which remains attached to the cap cells, and a posterior cystoblast, which is displaced from the niche and free to differentiate. The axis of GSC division is determined by the membrane- and spectrin-rich fusome, which anchors one pole of the mitotic spindle to the GSC's anterior cortex (McGrail and Hays, 1997; Deng and Lin, 1997; de Cuevas et al., 1998). A small amount of fusome is donated to the cystoblast where it guides four stereotypic rounds of incomplete cell division, resulting in a germline cyst of 16 cells interconnected by cytoplasmic bridges, called ring canals (Huynh and St Johnston, 2004). In germarial region 2A, Orb, BicD and other cell fate determinants become enriched in the cell with the most fusome material, committing it to differentiate as the oocyte, while each of the other 15 cells adopts a nurse cell fate (Huynh and St Johnston, 2004). The oocyte is positioned at the posterior end of the germline cyst in region 2B through

E-cadherin-mediated adhesion to neighboring somatic follicle cells (Gonzales-Reyes and St Johnston, 1998; Godt and Tepass, 1998). Finally, in germarial region 3, the germline cyst is encased in an epithelium of somatic follicle cells to give the stage 1 egg chamber, the basic unit of all subsequent steps of oogenesis (Huynh and St Johnston, 2004).

5.3 RESULTS AND DISCUSSION

Rab11 associates with the fusomes of GSCs and developing germline cysts

Our first clue that Rab11 plays important roles in early oogenesis came from immunostain experiments which revealed strong expression of endogenous Rab11 and a fully functional Rab11::GFP in GSCs, cystoblasts, and young (2- 4- and 8-cell) germline cysts (Fig. 5.1). Strikingly, the proteins were concentrated as discrete dots on the fusome (Fig. 5.1E-L), which electron microscope and photobleaching studies have shown is highly vesicular and rapidly exchanged with other membrane stores (Mahowald, 1972; Snapp et al., 2004). Triple stain experiments showed that some of these dots also contained E-cadherin (Fig. 5.1B-E), which has been shown to transit though Rab11-positive recycling endosomes (REs) en route to the plasma membrane in some cells (Lock and Stow, 2005; Langevin et al., 2005). High magnifications images showed that the Rab11 (and more rarely E-cadherin) dots were often nestled into cavities within the fusome (Fig. 5.1D-E). Such Rab11-harboring cavities were visible in the fusomes of all examined GSCs, cystoblasts, and young germline cysts, not only in the ovary but also in the testes (Fig. 5.1; data not shown). In view of Rab11's well-described enrichment in recycling endosomes (REs) (Dollar et al., 2002; Emery et al., 2005; Lock and Stow, 2005; Riggs et al., 2003; Zhang et al., 2004), we propose that these Rab11- and E-cadherin-harboring cavities are REs and will hereafter refer to them as FREs (fusome-associated recycling endosomes).

Rab11 is required for maintenance of GSC identity

Previous studies of hypomorphic *rab11* alleles revealed a role for the gene in

polarizing the mid-stage oocyte's anterior-posterior axis (Dollar et al., 2002; Jankovics et al., 2001). To investigate the role of Rab11 during early oogenesis, we set out to examine a *rab11* null allele. The one null allele available at the start of these studies proved to be tightly linked to a second site cell lethal mutation, so we made a new one using the FRT-flipase method (Parks et al., 2004). This new allele, called $rab11^{\Delta FRT}$, deletes the *rab11* promoter and the first two exons of the gene, and produces no detectable protein (Fig. 5.1I).

Because $rab11^{\Delta FRT}$ is homozygous lethal, we used the FRT-FLP system (Xu and Rubin, 1993) to generate homozygous *rab11-null* clones that were marked by loss of nuclear GFP. Consistent with a role for Rab11 in the maintenance of GSCs, we recovered a disproportionately small number of *rab11-null* GSCs compared to *rab11-null* germline cysts. To determine the half-life of *rab11*-null GSCs, we calculated the percentage of *rab11-null* GSCs to total GSCs as a function of days after clone induction (ACI). As a control, we made identical calculations for marked clones carrying the wild-type *rab11* allele. Such studies revealed a half-life of 4.0 days for r*ab11-null* GSCs, or ~4-fold less than wild-type (Table 1). We also made clones with the $rab11^{2148}$ hypomorphic allele and calculated a near wild-type half-life of 15.9 days (Table 1). This was the expected result as this allele, which contains a P element insertion in the first intron, produces apparently normal amounts of Rab11 protein during early oogenesis (Dollar et al., 2002). We conclude from these data that *rab11* is required to maintain GSC identity.

Consistent with previous findings that lost GSCs can be replaced (Kai and Spradling, 2003: Kai and Spradling, 2004), many of the germaria that had lost a *rab11-null* GSC

contained a full complement (2 or 3) of wild-type GSCs. One apparent replacement event is shown in Fig. 5.2A-B, where a wild-type GSC is dividing along an axis parallel to the niche and just anterior to a displaced *rab11-null* GSC.

Rab11 GSCs exhibit E-cadherin trafficking defects and have misplaced fusomes

To determine whether the observed defects in GSC maintenance reflect a requirement for Rab11 in E-cadherin trafficking, we compared the distribution of E-cadherin in wild-type and *rab11-null* GSCs. In contrast to wild-type GSCs (Figs. 5.2C-D, white arrows), we found little or no E-cadherin along the anterior surface (i.e., at the GSC-cap cell interface) of *rab11-null* GSCs (n=9) 8-10 days ACI (Fig. 5.2D, yellow arrow). Similar analyses of germaria 2.2 days ACI revealed reduced or no accumulation of E-cadherin along the anterior cortex of 16 of 22 examined *rab11-null* GSCs (Fig. 5.2E). Consistent with the idea that such reductions reflect a loss of adherens junctions, we saw similar strong reductions of Armadillo/b-catenin (data not shown). Concomitant with its reduction along the GSC's anterior surface, increased amounts of E-cadherin (seen as discrete dots) were detected on the fusomes/FREs of *rab11-null* GSCs (Fig. 5.2E, yellow arrow). Thus while wild-type GSCs contained an average of 0.16 dots of E-cadherin per fusome (n=31), *rab11-null* GSCs contained an average of 1.6 dots/fusome (n=17) (Table 1). We conclude that *rab11* is required for the maintenance of adherens junctions between cap cells and GSCs and propose that such maintenance involves the trafficking of intracellular E-cadherin, and possibly other cargoes, from the FRE to the GSC's anterior surface.

Although the simplest interpretation of the above data is that Rab11 maintains GSC

identity through E-cadherin trafficking, we cannot rule out the possibility that the primary role of Rab11 is that of recycling Dpp or other signals required for GSC maintenance and that the observed defects in E-cadherin trafficking are a secondary effect of insufficient signaling. To test this idea, we immunostained mosaic germaria for Bam, whose expression is negatively regulated by Dpp (Xie and Spradling, 1998; Xie and Spradling, 2000; Song et al., 2004). Such studies revealed a normal pattern of Bam expression; Bam was not detected in *rab11-null* GSCs or cystoblasts, but was detected in young (2- to 8-cell) germline cysts (Fig. 5.2G). These data argue strongly against the idea that the primary role of *rab11* is that of facilitating Dpp signaling, in which case *rab11-null* GSCs would be expected to move out of the niche only after they have activated Bam. We conclude from these findings that Rab11 does not affect GSC maintenance or E-cadherin trafficking through regulation of Dpp or other signals that maintain GSC identity via Bam repression.

The *rab11-null* clones also revealed a role for Rab11 in the sub-cellular localization and behavior of the fusome. In wild-type GSCs (Fig. 5.2F, white arrowhead), the fusome is anchored to the anterior cortex and spreads out along the mitotic spindle such that ~1/3 of it extends into, and is ultimately donated to, the cystoblast (Deng and Lin, 1997). In contrast, the fusome of *rab11-null* GSCs (Fig. 5.2F, yellow arrow) was not anchored to the anterior cortex and, while it spread out along the mitotic spindle during cell division, it was often splayed and generally much less organized. We suspect such splaying results from the detachment of the fusome from the cell cortex, but cannot rule out a more direct role for Rab11 in fusome segregation. Although it is not known how the fusome is attached to the

GSC's anterior cortex, it is likely to involve association with intracellular domains of the adherens junctions (Song et al., 2002). If so, Rab11, the fusome/FRE, and the adherens junctions may comprise a tripartite feedback loop whereby each reinforces the subcellular localization/behavior of the other. Specifically, we propose that the association of Rab11 with the fusome/FRE polarizes Rab11's trafficking of E-cadherin toward the GSC-cap cell interface, in turn reinforcing GSC-cap cell junctions, fusome localization, and asymmetric cell division.

Rab11 germline cysts arrest development early and exhibit defects in fusome segregation, oocyte positioning, and bulk membrane trafficking

All *rab11-null* germline cysts arrested development by stage 6 and were of two phenotypic classes. The rarer (~10%), more severely affected class arrested development in region 1 of the germarium, often contained less than 16 cells, and had little or no fusome (Fig. 5.3A-B, D, yellow outlines). Given the splayed fusome phenotype of dividing *rab11-null* GSCs described above, we speculate that this early arrest reflects a role for Rab11 in faithful segregation of the fusome to daughter cystoblasts. Consistent with this idea, mutations in *a-spectrin* and *hu-li tai shao*, which encode components of the fusome, cause a similar early arrest of cyst development (Lin et al., 1994; de Cuevas et al., 1996).

The less affected class of *rab11-null* germline cysts elaborated a normal fusome (Fig. 5.3A, white dashes), but contained clumped ring canals (Figs. 5.3F-G) and arrested development at ~stage 6. Clumped ring canals have also been reported for *sec5, sec6* and *rab6* mutations and have been interpreted to reflect a requirement for these genes in bulk

membrane trafficking to the cell surface (Murthy and Schwarz, 2003; Murthy et al., 2005; Coutelis and Ephrussi, 2007). A similar requirement for Rab11 is likely as many of the nuclei of *rab11-null* germline cysts were clumped together or otherwise poorly spaced (not shown). These cysts also exhibited defects in oocyte positioning. Thus while the oocyte is positioned at the posterior end of wild-type germline cysts in germarial region 2B (Fig. 5.3E, white arrowhead), the oocytes of *rab11-null* germline cysts were often in the center (Fig. 5.3E', yellow arrow). Previous studies (Godt and Tepass, 1998; Gonzales-Reyes and St Johnston, 1998) have shown that oocyte positioning is dependent on enriched accumulation of E-cadherin along the oocyte's posterior surface. Consistent with a role for Rab11 in such enrichment, we observed reduced accumulation of E-cadherin along the posterior surface of *rab11*-null oocytes (Fig. 5.3D, yellow arrow) compared to wild-type oocytes (Figs. 5.3C-D, white arrowheads) in region 2B and 3 germline cysts. Nevertheless it is difficult to know whether the observed defects in oocyte positioning in the *rab11-null* germline cysts reflects a role for Rab11 in E-cadherin trafficking, bulk membrane trafficking, or both.

5.4 **CONCLUSION**

Our studies indicate that Rab11 maintains GSC identity through polarized trafficking of E-cadherin and, possibly, other cargoes that reinforce essential GSC-niche contacts. Our studies further indicate that Rab11 is required for fusome localization and asymmetric GSC division and suggest a feedback linkage between these events and E-cadherin trafficking. While Rab11 has been implicated in the trafficking of E-cadherin in other cells, we know of no other cases where such trafficking has been correlated with a biological response. It will be of interest to determine whether Rab11 is required for the maintenance of stem cells in other systems and whether such maintenance involves E-cadherin trafficking or the trafficking of other adhesion molecules. It will also be of interest to determine the role of Rab11 in other E-cadherin-dependent cell behaviors, particularly since Rab11, at least in *Drosophila*, is expressed in only a small subset of E-cadherin-expressing cells (Xu and Cohen, unpublished).

5.5 EXPERIMENTAL PROCEDURES

Drosophila genetics

Fly culture and crosses were carried out according to standard procedures (Ashburner, 1989). The wild-type stock was *w*, or *w histone2::GFP* (Morin et al., 2001). The *rab11* deletion (*rab11*$^{\Delta FRT}$) was made by inducing recombination (Parks et al., 2004) between the FRT insertions (FRT5377 and FRT1994, respectively) of stocks f05377 and d01994 (Harvard Medical School Exelixis collection). The resulting deletion, which removes the Rab11 promoter and first two exons was initially identified by non-complementation with *rab11*^{2148} (Dollar et al., 2002) and subsequently confirmed by PCR. The *rab11*$^{\Delta FRT}$ allele complements a lethal allele of *rtet*, which lies just upstream of *rab11* and close to the FRT insertion of f05377, and produced no protein (Fig. 5.1I). Homozygous mutant clones were generated by crossing *w; rab11-null*/*FRT5377, Hrb98::GFP* or *w; rab11+*/*FRT5377, Hrb98::GFP* controls to *y w hsp::FLP*. The *FRT5377, Hrb98::GFP* chromosome was made by recombining the *Hrb98::GFP* transgene from line ZCL058 (Morin et al., 2001; Kelso et al., 2004) onto the FRT5377-containing chromosome and verified by PCR. For most experiments, clones were induced in 2- to 5-day old adults by heat-shocking for 1 hour at 37^{o}C on 2 consecutive days and examined 8 or more days ACI, thus ensuring that all examined *rab11-null* cells were derived from mutant GSCs; germline cysts normally clear the germarium within ~6 days (Song et al., 2002; Xie and Spradling, 1998). For half-life determination, a single large group of 2- to 3-day old adults were heat-shocked twice, 8 hours apart, at 37°C for 1 hour and the number of mutant

GSCs and germline cysts were counted 4, 8 or 12 days ACI. Homozygous *rab11-null* and *rab11+* control clones were identified by their lack of GFP staining. The fully functional *Rab11::GFP* transgene is identical to that described in Dollar et al (2002), except for the omission of the N-terminal His-tag.

Immunocytochemistry and confocal microscopy

Ovaries were fixed and immunostained as previously described (Dollar et al., 2002), except EM-grade formaldehyde was substituted for paraformaldehyde in the fixative. Primary antibodies were used at the following concentrations: Rat anti-Rab11 (1:500) (Dollar et al., 2002); Rabbit anti-Rab11 (1:250) (Satoh et al., 2004); E-cadherin (1:40; Hybridoma bank); GFP (1:250; Invitrogen); a-spectrin (1:10; Hybridoma Bank); Hts/1b1 (1:4; Hybridoma Bank); Orb (6H4) (1:20; Hybridoma Bank); Vasa (1:5000) (Williamson and Lehman); HtsRC (1:4; Hybridoma Bank); BamC (1:500;) (McKearin and Ohlstein, 1995). Secondary antibodies were purchased from Jackson labs and used at the manufacturer's recommended concentrations. Stained ovaries were mounted in 4% n-propyl gallate (Sigma) in 90%glycerol, 10% phosphate buffered saline. Images were collected on an Olympus 3L Spinning disc or Zeiss Meta 510 laser scanning confocal microscopes.

Figure 5.1 Rab11 is enriched on the fusome of GSCs and germline cysts.

(**A**) Diagram of the *Drosophila* germarium. Niche terminal filament (TF), cap (CC), and escort stem (ESC) cells are shown in gray. The fusome (red) is tightly associated with the anterior cortex of germline stem cells (GSCs) (black) and extends throughout the cytoplasm of germline cysts (white). Other symbols: oocyte (green); somatic follicle cells (FC); ring canals (blue crescents). (**B**-**E**) Region 1 of a wild-type germarium immunostained for E-cadherin (green), Rab11 (red), and the fusome marker, a-spectrin (blue). Merged image (**E**). Scale bar equals 10 microns and anterior is to the left in this and all subsequent figures. A single GSC is outlined in **B**, with the break in the tracing revealing strong E-cadherin staining at the GSC-cap cell interface. The smaller dot of E-cadherin staining (arrow) superimposes with Rab11 on the fusome (arrows in **C** and **D**, respectively). (**F**-**H**) Wild-type germarial regions 1 & 2 immunostained for Rab11 (red), and a-spectrin (blue). Merged image (**G**). Strong accumulation of Rab11 is apparent on the GSC (arrowhead) and germline cyst's (arrow) fusome. (**I**-**J**) Mosaic germarium immunostained for nGFP (green), Rab11 (red) and a-spectrin (blue) showing the absence of Rab11 protein in *rab11-null* (GFP negative) cells. (**J**) Rab11 channel only. (**K**-**L**) Rab11::GFP transgenic germarial region 1 immunostained for GFP (red) and a-spectrin (blue). Arrowheads denote strong Rab11::GFP expression on the fusome.

Figure 5.1

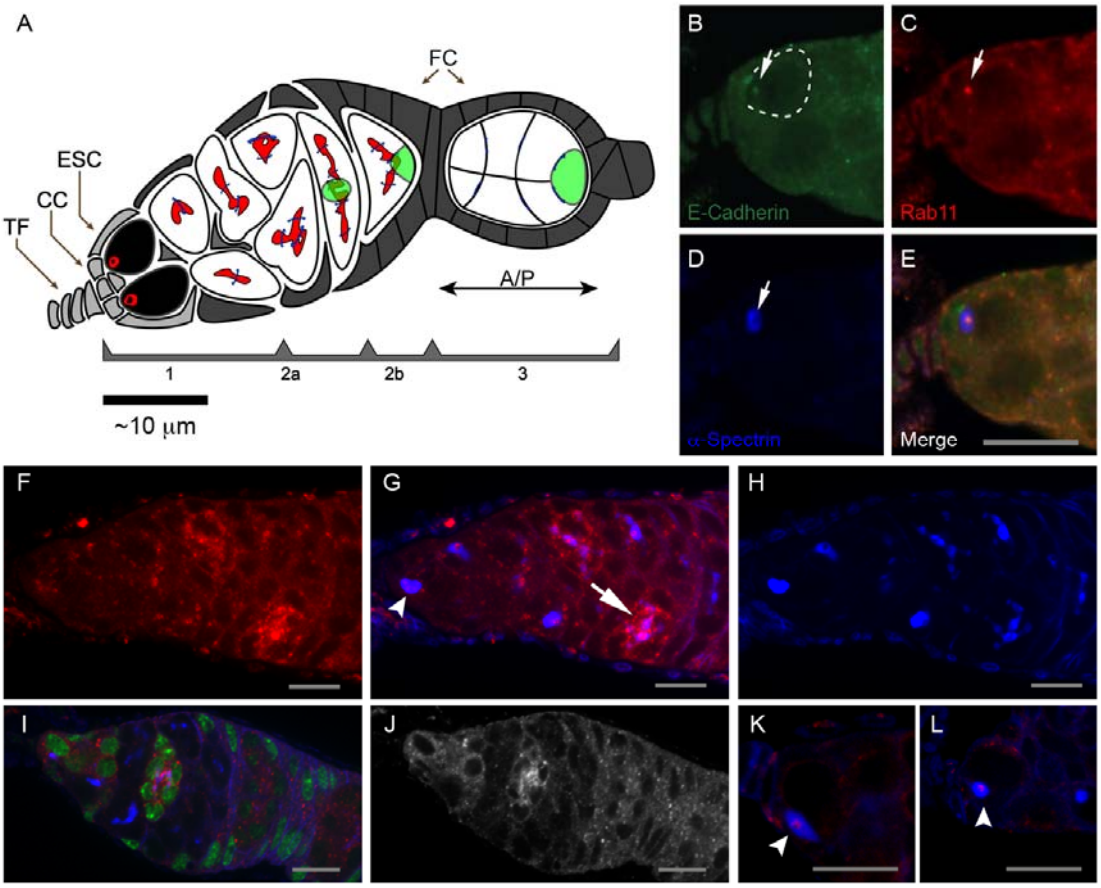
A
FC
ESC
CC
TF
A/P
1
2a
2b
3
~10 μm
B
E-Cadherin
C
Rab11
D
α-Spectrin
E
Merge
F
G
H
I
J
K
L

Figure 5.2 Rab11 is required to maintain E-cadherin at the cap cells-GSC junction and to anchor the fusome to the GSC's anterior cortex.

(**A**, **B**) Region 1 of a mosaic germarium immunostained 8 days after clone induction (ACI) for E-cadherin (red), a-spectrin (blue), and nuclear GFP (nGFP) (green) to mark *rab11-null* clones in this and all subsequent figures. (**A**) A 2-cell *rab11-null* germline cyst is outlined. This is the most anterior mutant cyst in the germarium, and thus, presumably derived from a displaced *rab11-null* GSC (see Text). A dividing wild-type GSC (boxed) is shown at the left and zoomed in (**B**), where the dashed line shows the position of the cap cells. Note that the plane of division (evident by the stretched out fusome) is orthogonal to the germarium's anterior-posterior axis such that both daughter cells remain in the niche, with one filling the vacancy created by the displaced *rab11-null* GSC. (**C**-**E**) Wild-type (**C**) and mosaic (**D**-**E**) germaria immunostained for nGFP (green), E-cadherin (red) and a-spectrin (blue) 2 days ACI. Wild-type and *rab11-null* GSC-cap cell junctions are indicated with arrowheads and arrows, respectively. Note the reduced E-cadherin staining at the three mutant junctions and the increased E-cadherin expression on the mutant fusome, especially in **E**, where three strong dots of staining are evident. (**F**) Dividing *rab11* (arrow) and wild-type (arrowhead) GSCs immunostained for a-spectrin (blue), DAPI (red), Vasa (cytoplasmic green, germline only), and nGFP (green). The DAPI-stained nuclei at the left correspond to cap cells. Note the splayed appearance of the *rab11* GSC fusome and its displacement from the anterior cortex. (**G**) Mosaic germarium immunostained for nGFP (green), a-spectrin (red) and Bam (blue) 2.2 days ACI. The *rab11-null* GSC (outlined in yellow) exhibits only background levels of

Bam expression. A wild-type GSC and a 2-cell germline cyst (outlined in white) are shown for comparison.

Figure 5.2

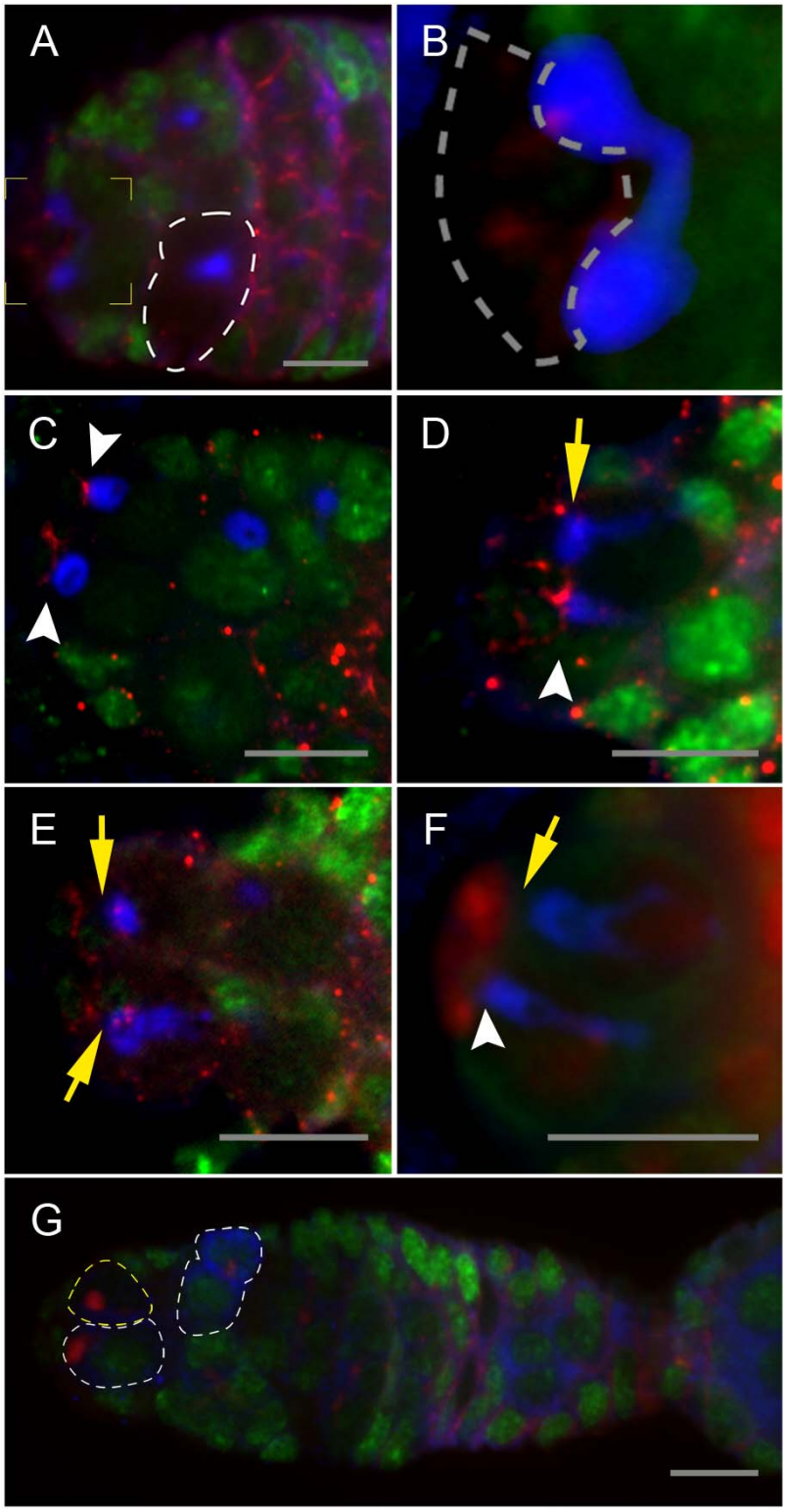

Figure 5.3 Rab11 germline cysts arrest early and display defects in fusome segregation and bulk membrane trafficking.

(**A**) Mosaic germarium immunostained for a-spectrin (red), Vasa (cytoplasmic green), and nGFP (nuclear green). A severely affected *rab11-null* germline cyst with no detectable fusome and a reduced number of germline cells is outlined in yellow. A less affected *rab11-null* germline cyst in which the fusome appears normal is outlined in white. (**B**) Mosaic germarium immunostained for E-cadherin (red), a-spectrin (blue), and nGFP (green). A 5 micron Z-stack is shown to capture all of the fusome. Two severely affected *rab11-null* germline cysts with no fusomes are outlined. A less affected *rab11-null* germline cyst is denoted with the arrow. (**C**) Wild-type germarium immunostained for E-cadherin (red) and a-spectrin (blue). The arrowheads point to the oocyte's posterior surface, where enriched accumulation of E-cadherin is evident, especially in the region 2B oocyte (left arrowhead). (**D**) Mosaic germarium immunostained for E-cadherin (red), a-spectrin (blue), and nGFP (green). The arrow points to a region 2B *rab11-null* oocyte, with greatly reduced E-cadherin accumulation (compare to left arrowhead in panel (**C**)). The arrowhead points to a wild-type region 3 oocyte, where enriched E-cadherin expression is still apparent. A severely affected, *rab11-null* germline cyst similar to those seen in (**B**) is outlined. (**E**, **E'**) Mosaic germarium immunostained for Orb (red) and nGFP (green). The bracketed area in (**E**) is shown at a different focal plane in (**E'**). Note the strong Orb expression in the wild-type region 2A and 2B oocytes (arrowheads). Strong Orb expression is also seen in the region 3 of *rab11-null* germline cyst (arrow), but it is concentrated at the center of the egg

chamber, rather than at the posterior pole. (**F**, **G**) Mosaic germarium immunostained for nGFP (green) and HtsRC (red) to label ring canals. (**F**) Left arrow points to a *rab11-null* germline cyst, with clumped ring canals. The right arrow points to a mosaic germline cyst, where the ring canals are only clumped in the *rab11-null* (GFP negative) portion. (**G**) Germarium with a completely *rab11-null* germline. All ring canals are clumped in center of the cysts.

Figure 5.3

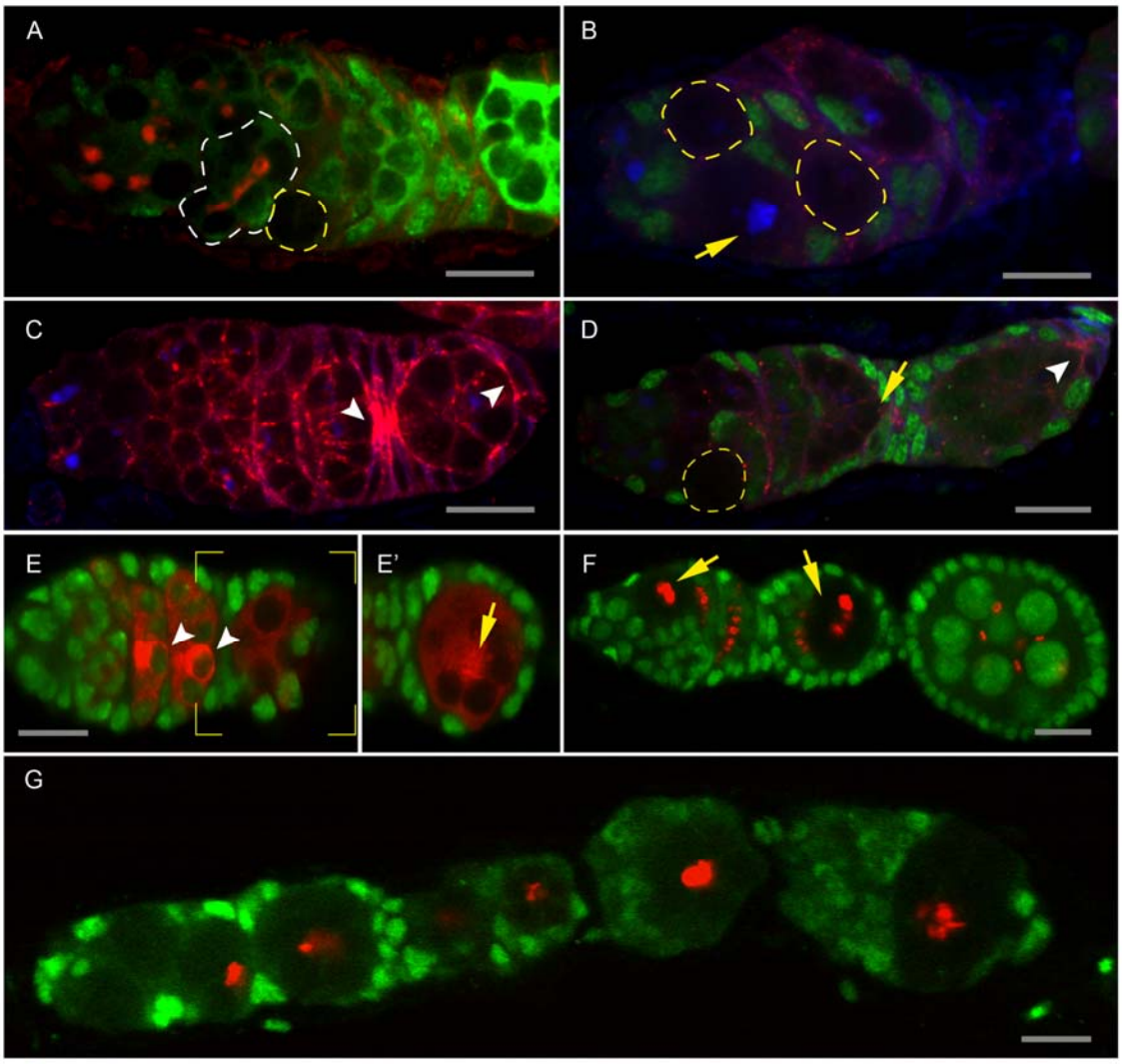

Table 5.1 *rab11-null* GSCs have a 4-fold shorter half-life than *rab11+* controls

	% marked GSCs (total number of GSCs counted)			
	Days after clone induction			
genotype of marked clones	4	8	12	Half-life
FRT5377, rab11-null	14.5(186)	7.5 (240)	3.5 (144)	4.0 days
FRT82B, $rab11^{2148}$	16.1 (87)	12.6 (238)	11.8 (144)	15.9 days*
FRT5377, rab11+	12.3 (81)	ND	9.4 (607)	16.2 days*

*calculation based on the assumption that GSC loss occurs randomly, and thus linearly over time.

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