

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

Nicholas Bogard, Lan Lan, Jiang Xu and Robert S. Cohen*

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 such 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 anterior cortex of the GSC. 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 are likely to reflect a role for Rab11 in E-cadherin trafficking as E-cadherin accumulates in Rab11-positive recycling endosomes (REs) and E-cadherin and Armadillo (β -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 this association polarizes the trafficking by Rab11 of E-cadherin and other cargoes toward the anterior cortex of the GSC, thus simultaneously fortifying GSC-niche junctions, fusome localization and asymmetric cell division. These studies bring into focus the important role of membrane trafficking in stem cell biology.

KEY WORDS: Asymmetric cell division, Membrane trafficking, Recycling endosomes, E-cadherin, Fusome

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 three morphologically distinct regions (Fig. 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- β -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 anterior cortex of the GSC (McGrail and Hays, 1997; Deng and Lin, 1997; de Cuevas and Spradling, 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 (encoded by *shotgun* – FlyBase)-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 thereby forming the stage 1 egg chamber, the basic unit of all subsequent steps of oogenesis (Huynh and St Johnston, 2004).

MATERIALS AND METHODS

Drosophila genetics

Fly culture and crosses were carried out according to standard procedures (Ashburner, 1989). The wild-type stock was *w*, or *w His2AV::GFP* (Morin et al., 2001). The *rab11* deletion (*rab11^{ΔFRT}*) 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 *rab11²¹⁴⁸* (Dollar et al., 2002) and subsequently confirmed by PCR. The *rab11^{ΔFRT}* allele complements a lethal allele of *rtet*, which lies just upstream of *rab11* and close to the FRT insertion of f05377, and produces no protein (Fig. 1I). Homozygous mutant clones were generated by crossing *w; rab11-null/FRT5377, Hrb98DE::GFP* or *w; rab11⁺/FRT5377, Hrb98DE::GFP* controls to *y w hsp::FLP*. The *FRT5377, Hrb98DE::GFP* chromosome was made by recombining the *Hrb98DE::GFP* transgene from line ZCL058 (Morin et al., 2001; Kelso et al., 2004) onto the FRT5377-containing chromosome and was verified by PCR. For most experiments, clones were induced in 2- to 5-day-old adults by heat shocking for 1 hour at 37°C on two 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 by Dollar et al. (Dollar et al., 2002), except for the omission of the N-terminal His tag.

University of Kansas, Department of Molecular Biosciences, 1200 Sunnyside Avenue, Lawrence, KS 66045, USA.

*Author for correspondence (e-mail: rcohen@ku.edu)

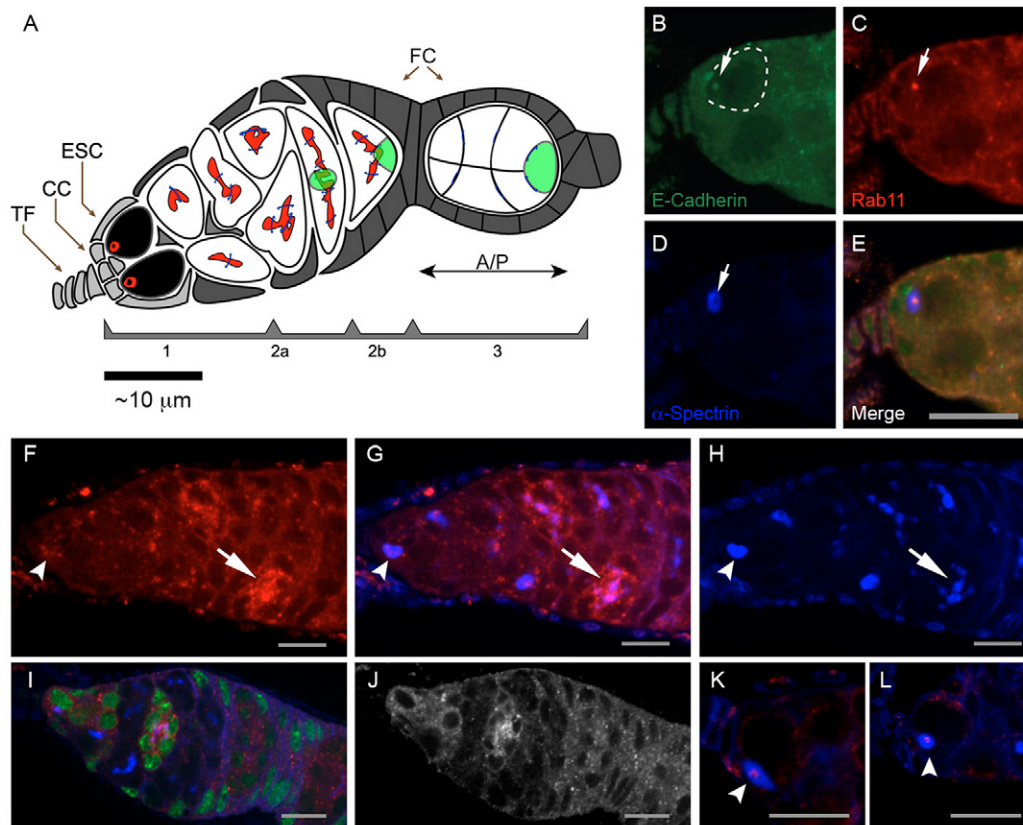


Fig. 1. Rab11 is enriched on the fusome of GSCs and germline cysts. (A) Diagram of the *Drosophila* ovarium. Niche terminal filament (TF), cap (CC), and escort stem (ESC) cells are shown in gray. The fusome (red) lies at the anterior cortex of germline stem cells (GSCs, black) and extends throughout the cytoplasm of germline cysts (white). Oocyte (green); somatic follicle cells (FC); ring canals (blue crescents). (B-E) Region 1 of a wild-type ovarium immunostained for E-cadherin (green), Rab11 (red), and the fusome marker α -Spectrin (blue). Merged image (E). Anterior is to the left in this and all subsequent figures. A single GSC is outlined in B, with the break in the tracing (dashed line) revealing strong E-cadherin staining at the GSC-cap cell interface. The smaller dot of E-cadherin staining (arrow) superimposes with Rab11 on the fusome (arrows in C and D). (F-H) Wild-type ovarial regions 1 and 2 immunostained for Rab11 (red) and α -Spectrin (blue). Merged image (G). Strong accumulation of Rab11 is apparent on the GSC (arrowhead) and fusome of the germline cyst (arrow). (I, J) Mosaic ovarium immunostained for nuclear GFP (nGFP, green), Rab11 (red) and α -Spectrin (blue). Note the absence of Rab11 protein in the *rab11*-null cells, which are marked by the absence of nGFP in this and all subsequent images. (J) Rab11 channel only. (K, L) Ovarial region 1 of *rab11::GFP* transgenic ovarium immunostained for GFP (red) and α -Spectrin (blue). Arrowheads indicate strong Rab11::GFP expression on the fusome. Scale bars: 10 μ m.

Immunocytochemistry and confocal microscopy

Ovaries were fixed and immunostained as previously described (Dollar et al., 2002), except that electron microscopy-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., 2005); E-cadherin (1:40; Hybridoma Bank); GFP (1:250; Invitrogen); α -Spectrin (1:10; Hybridoma Bank); Hts (1b1) (1:4; Hybridoma Bank); Orb (6H4) (1:20; Hybridoma Bank); Vasa (1:5000) (Williamson and Lehman, 1996); HtsRC (1:4; Hybridoma Bank); and BamC (1:500) (McKearin and Ohlstein, 1995). Secondary antibodies were purchased from The Jackson Laboratory and used at the manufacturer's recommended concentrations. Stained ovaries were mounted in 4% n-propyl gallate (Sigma) in 90% glycerol, 10% PBS. Images were collected on Olympus 3L Spinning Disc or Zeiss Meta 510 laser-scanning confocal microscopes.

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 in *Drosophila* came from immunostaining experiments that 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. 1). Strikingly, the proteins were concentrated as discrete dots on the fusome (Fig. 1E-L), which electron microscopy 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. 1B-E), which has been shown to transit through Rab11-positive recycling endosomes (REs) en route to the plasma membrane in some cells (Lock and Stow, 2005; Langevin et al., 2005). High-magnification images showed that the Rab11 (and, more rarely, E-cadherin) dots were often nestled into cavities within the fusome (Fig. 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. 1; data not shown). In view of the well-described enrichment of Rab11 in REs (Dollar et al., 2002; Emery et al., 2005; Lock and Stow, 2005; Riggs et al., 2003), we propose that these Rab11- and E-cadherin-harboring cavities are REs and will hereafter refer to them as FREs (fusome-associated REs).

Rab11 is required for maintenance of GSC identity

Previous studies of hypomorphic *rab11* alleles revealed a role for the gene in polarizing anterior-posterior axis of the mid-stage oocyte (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*^{ΔFRT}, deletes the *rab11* promoter and the first two exons of the gene, and produces no detectable protein (Fig. 1I).

Because *rab11*^{ΔFRT} 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 (n) GFP. Consistent with a role for Rab11 in the maintenance of GSCs, we recovered a disproportionately small number of *rab11*-null GSCs compared with *rab11*-null germline cysts. To determine the half-life of *rab11*-null GSCs, we calculated the percentage of GSCs that were *rab11*-null 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 *rab11*-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 (two or three) of wild-type GSCs. One apparent replacement event is shown in Fig. 2A,B, in which 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 (Fig. 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. 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 *rab11*-null GSCs examined (Fig. 2E). Consistent with the idea that such reductions reflect a loss of adherens junctions, we saw similar strong reductions of Armadillo (β-catenin) (data not shown). Concomitant with its reduction along the anterior surface of the GSC, increased amounts of E-cadherin (seen as discrete dots) were detected on the fusomes/FREs of *rab11*-null GSCs (Fig. 2E, yellow arrow). Thus, whereas 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 per 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 anterior surface of the GSC.

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

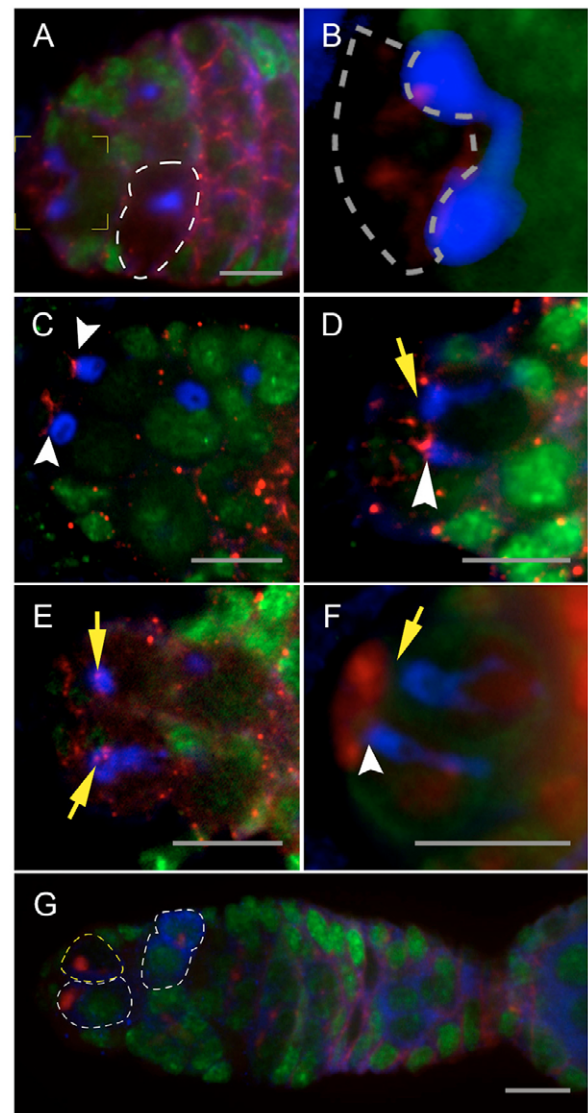


Fig. 2. Rab11 is required to maintain E-cadherin at the cap cell-GSC junction and to anchor the fusome to the anterior cortex of the GSC. (A,B) Region 1 of a mosaic *Drosophila* germline immunostained 8 days after clone induction (ACI) for E-cadherin (red), α-Spectrin (blue) and nGFP (green). (A) A 2-cell *rab11*-null germline cyst is outlined (dashed line). A dividing wild-type GSC (boxed) is shown to the left and magnified in B, where the dashed line outlines the cap cells. Note that the plane of division (evident by the stretched-out fusome) is such that both daughter cells remain in the niche, with one filling the vacancy created by a lost GSC. (C–E) Wild-type (C) and mosaic (D,E) germaria immunostained for nGFP (green), E-cadherin (red) and α-Spectrin (blue) 2 days ACI. Wild-type and *rab11*-null GSC-cap cell junctions are indicated with arrowheads and arrows, respectively. E-cadherin staining is reduced at the mutant junctions at the expense of increased fusome staining, especially in E, where several strong dots of staining are evident. (F) Dividing *rab11* (arrow) and wild-type (arrowhead) GSCs immunostained for α-Spectrin (blue), Vasa (cytoplasmic green, germline only) and nGFP (green) and stained with DAPI (red). The DAPI-stained nuclei at the left correspond to cap cells. The *rab11* GSC fusome is splayed and displaced from the anterior cortex. (G) Mosaic germline immunostained for nGFP (green), α-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. Scale bars: 10 μm.

Table 1. *rab11*-null GSCs have a 4-fold shorter half-life than *rab11*⁺ controls

Genotype of marked clones	Days after clone induction*			Half-life (days)
	4	8	12	
<i>FRT5377, rab11</i> -null	14.5 (186)	7.5 (240)	3.5 (144)	4.0
<i>FRT82B, rab11</i> ²¹⁴⁸	16.1 (87)	12.6 (238)	11.8 (144)	15.9 [†]
<i>FRT5377, rab11</i> ⁺	12.3 (81)	ND	9.4 (607)	16.2 [†]

ND, Not determined.

*Shown are the percentage of marked GSCs, with the total number of GSCs counted in parentheses, at 4, 8 and 12 days after clone induction.

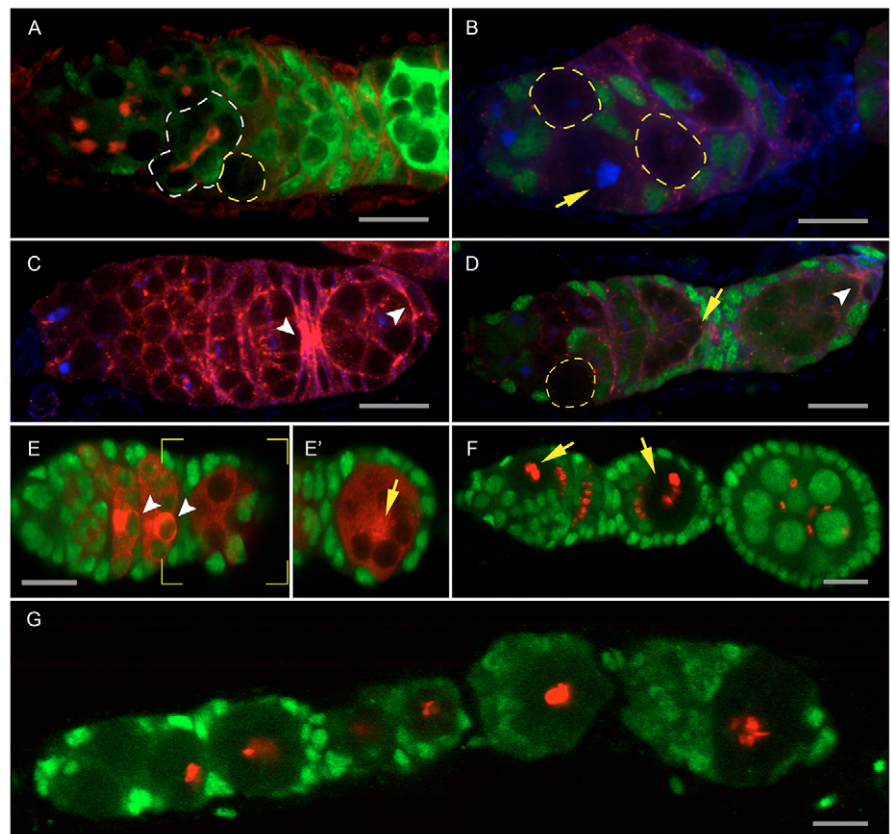
[†]Calculation based on the assumption that GSC loss occurs randomly and thus linearly over time.

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. 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 subcellular localization and behavior of the fusome. In wild-type GSCs (Fig. 2F, white arrowhead), the fusome is anchored to the anterior cortex and spreads out along the mitotic spindle such that about one-third of it extends into, and is ultimately donated to, the cystoblast (Deng and Lin, 1997). By contrast, the fusome of *rab11*-null GSCs (Fig. 2F, yellow arrow) was not anchored to the anterior cortex and, although 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 anterior cortex of the GSC, 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 might comprise a tripartite

Fig. 3. *rab11*-null germline cysts arrest early and display defects in fusome segregation and bulk membrane trafficking.

(A,B) Mosaic *Drosophila* germarium immunostained for (A) α -Spectrin (red), Vasa (cytoplasmic green) and nGFP (green) or (B) E-cadherin (red), α -Spectrin (blue) and nGFP (green). Severely affected *rab11*-null germline cysts with no detectable fusomes and reduced numbers of germline cells are outlined in yellow. A less affected *rab11*-null germline cyst with a normal fusome is outlined in white in A and indicated with an arrow in B. (C) Wild-type germarium immunostained for E-cadherin (red) and α -Spectrin (blue). The arrowheads point to the posterior of the oocyte, where enriched accumulation of E-cadherin is evident, especially in the region 2B oocyte (left arrowhead). (D) Mosaic germarium immunostained for E-cadherin (red), α -Spectrin (blue) and nGFP (green). The arrow points to a region 2B *rab11*-null oocyte with greatly reduced E-cadherin accumulation (compare with left arrowhead in 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, oocyte) and nGFP (green). The bracketed area in E is shown at a different focal plane in E'. Note that the oocyte is positioned at the posterior end of the wild-type germline cysts (arrowheads), but at the center of the *rab11*-null germline cyst (arrow). (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 the center of the cysts. Scale bars: 10 μ m.



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 the trafficking of E-cadherin by Rab11 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. 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 *α-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. 3A, white dashes), but contained clumped ring canals (Fig. 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, whereas the oocyte is positioned at the posterior end of wild-type germline cysts in germarial region 2B (Fig. 3E, white arrowhead), the oocytes of *rab11*-null germline cysts were often in the center (Fig. 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 posterior surface of the oocyte. 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. 3D, yellow arrow) compared with wild-type oocytes (Fig. 3C,D, white arrowheads) in region 2B and region 3 germline cysts. Nevertheless, it is difficult to conclude whether the observed defects in oocyte positioning in the *rab11*-null germline cysts reflects a role for Rab11 in E-cadherin trafficking, in bulk membrane trafficking, or both.

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. Although Rab11 has been implicated in the trafficking of E-cadherin in other cells, we know of no other cases in which 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 as Rab11, at least in *Drosophila*, is expressed in only a small subset of E-cadherin-expressing cells (J.X. and R.S.C., unpublished).

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