

# **Male-female communication enhances release of extracellular vesicles leading to high fertility in *Drosophila***

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## **This PDF file includes:**

Supplementary Figures S1 to S7

## **Other supplementary materials for this manuscript include the following:**

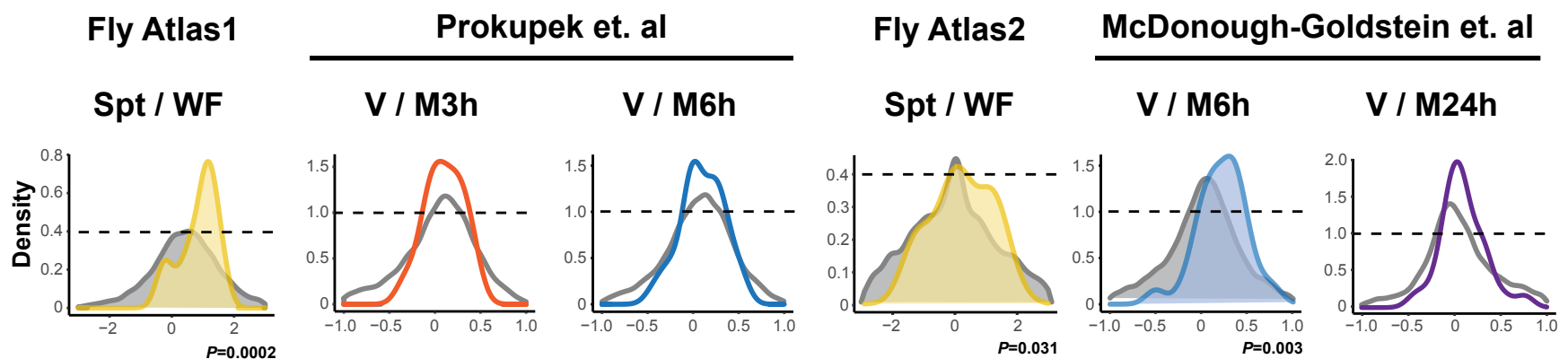
Supplementary Movies S1 to S6

Supplementary Data 1 and 2

## GO Term

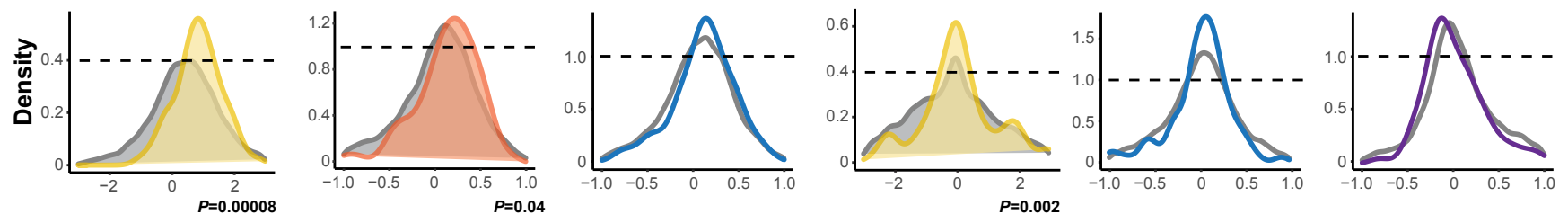
**Endosome transport via multivesicular body**

MA: 27 genes  
Seq: 27 genes



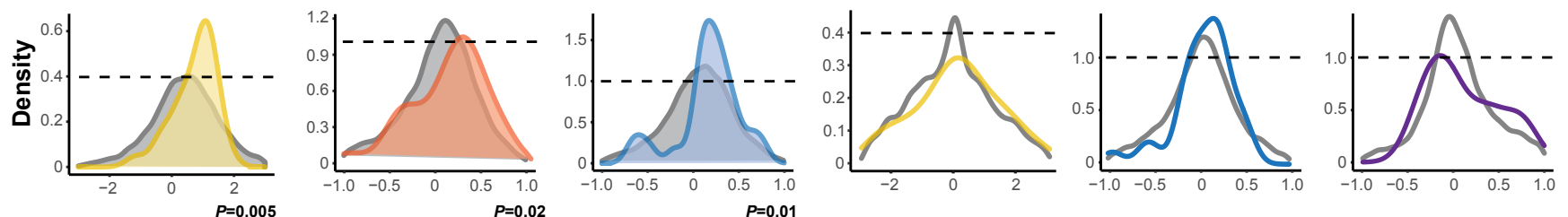
**Exocytosis**

MA: 72 genes  
Seq: 105 genes



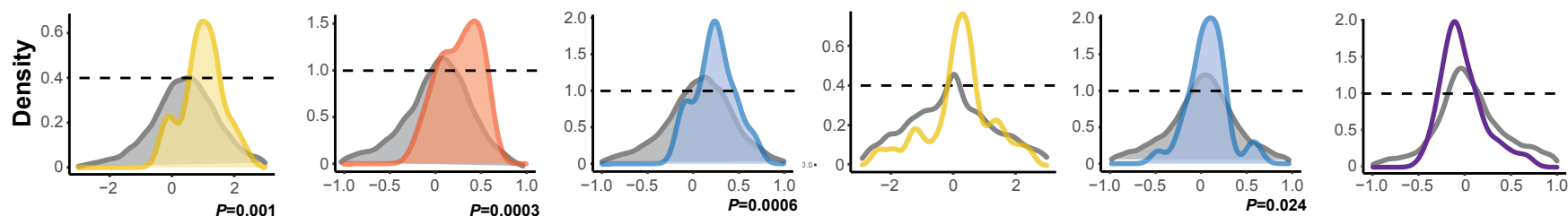
**Positive regulation of secretion**

MA: 29 genes  
Seq: 35 genes



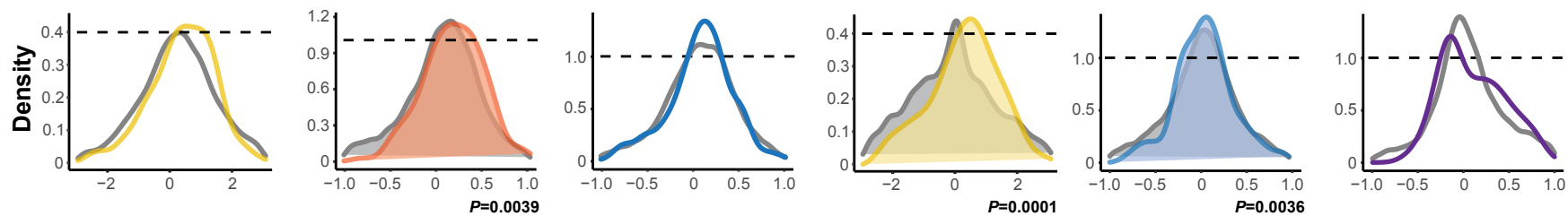
**Vesicle-mediated transport to the plasma membrane**

MA: 27 genes  
Seq: 33 genes



**Endocytosis**

MA: 139 genes  
Seq: 167 genes



Gene expression fold change (log2)

Gene expression fold change (log2)

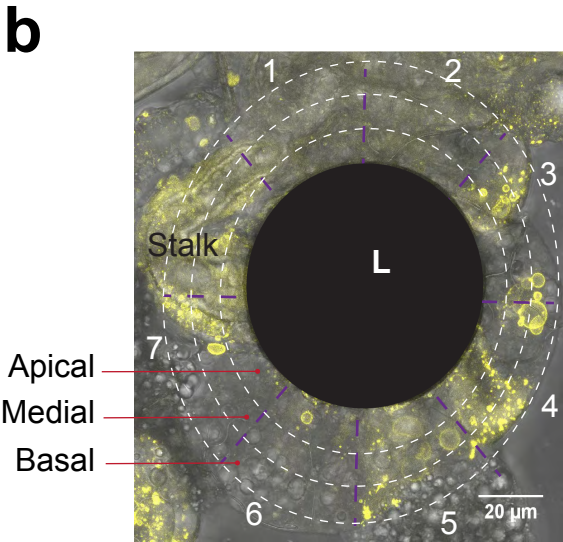
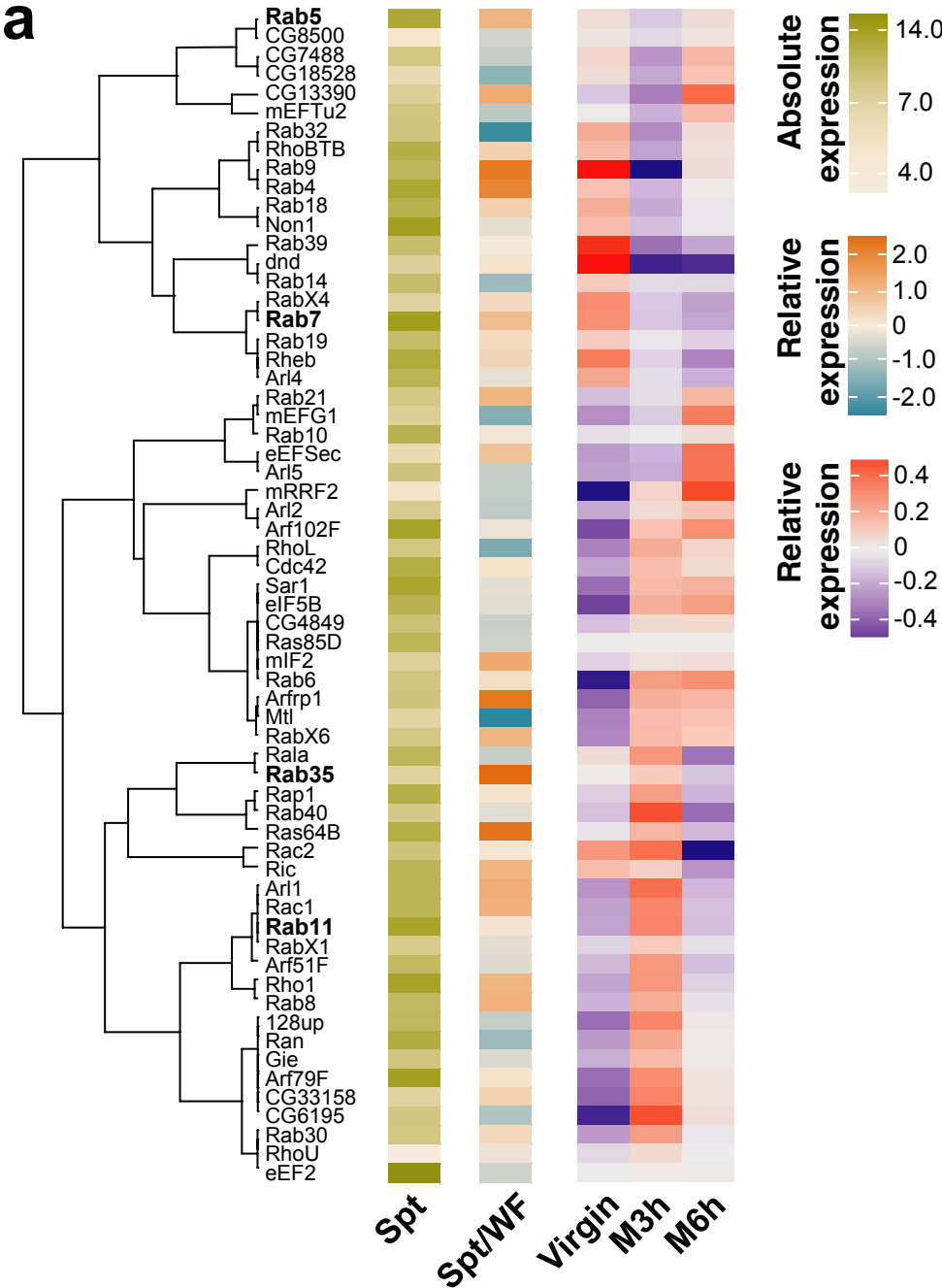
Fig. S1



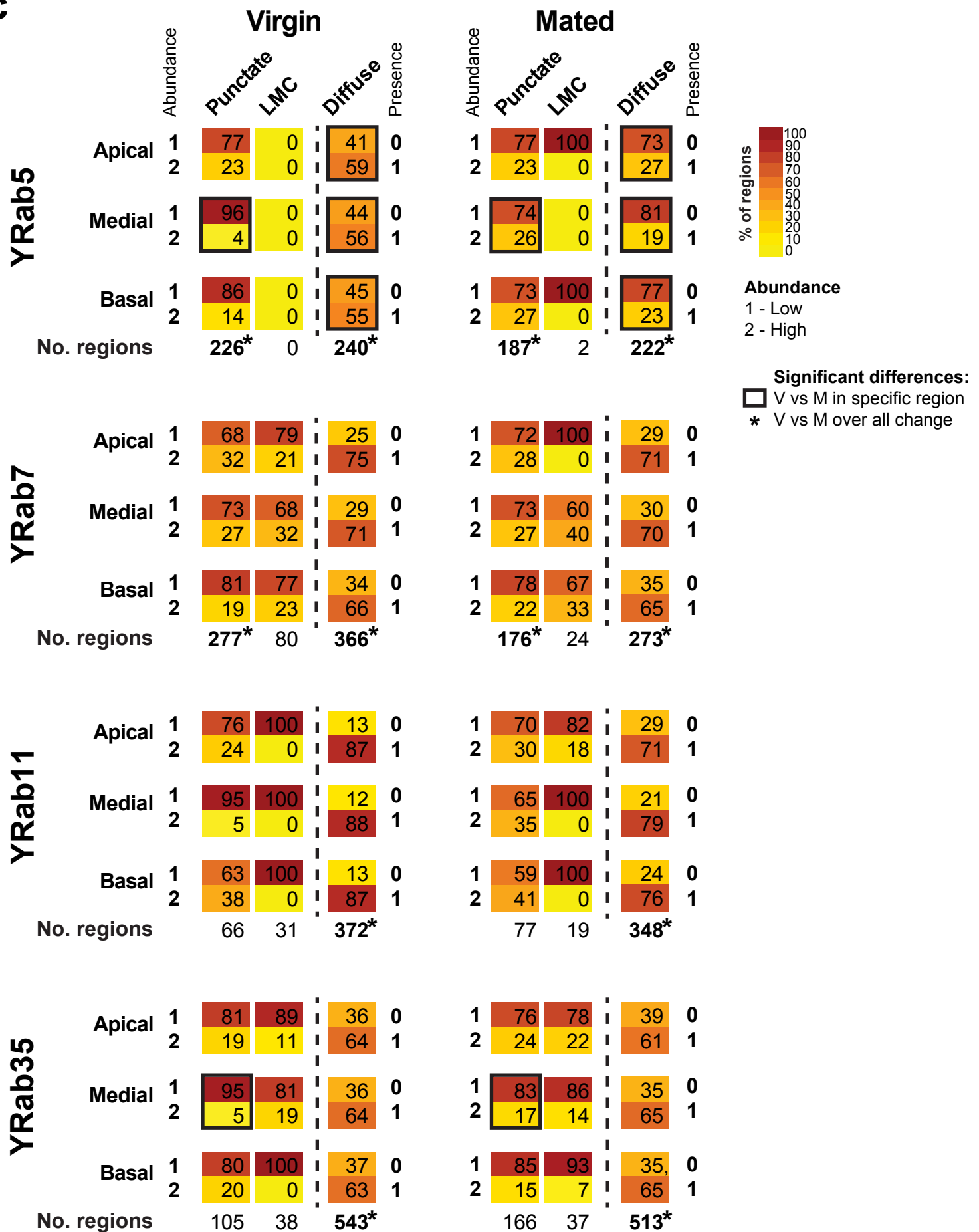
**Supplementary Fig. 1. Genes associated with GO terms related to EV exocytosis and endocytosis are enriched in the spermatheca and modulated by mating.**

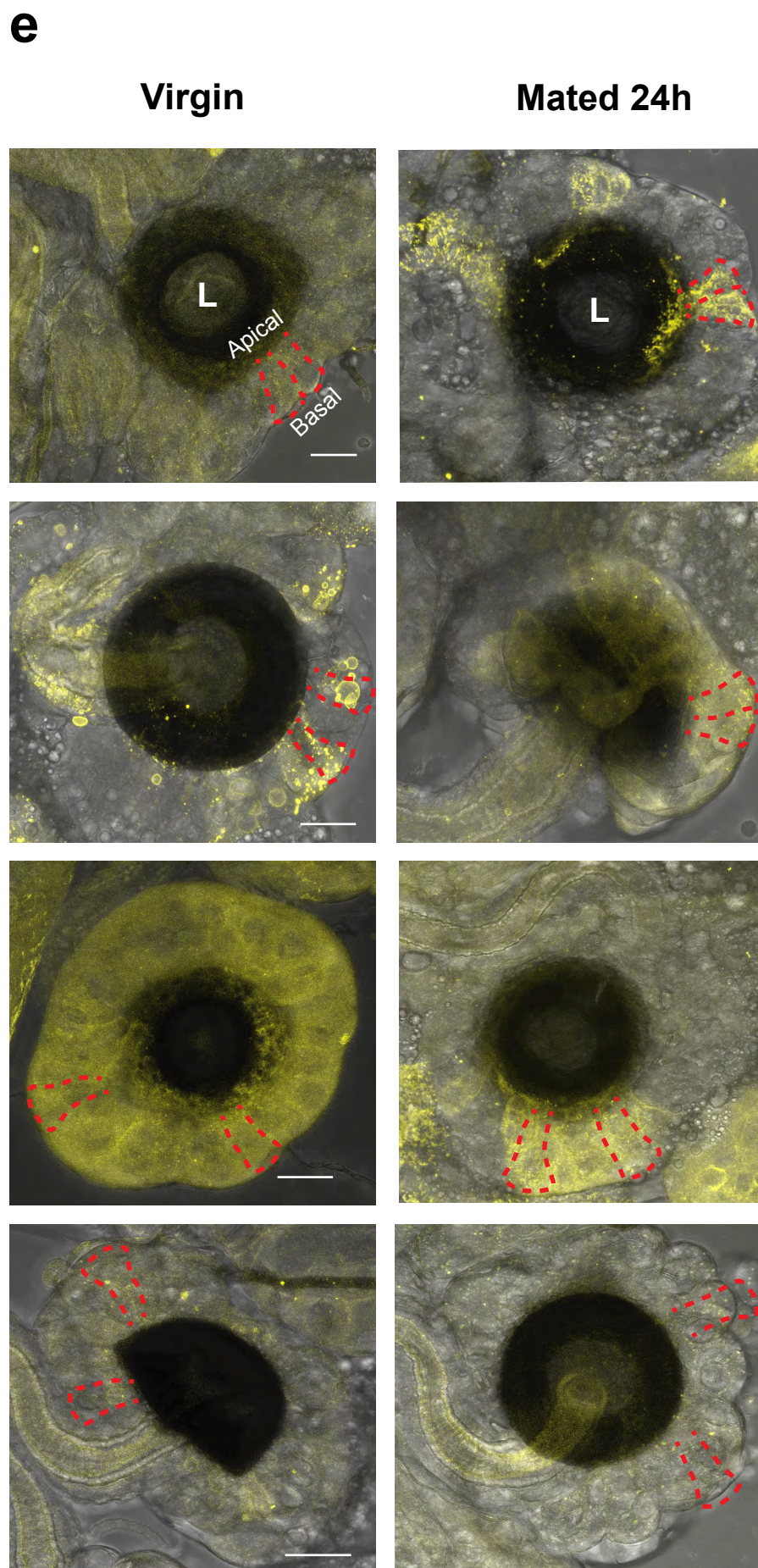
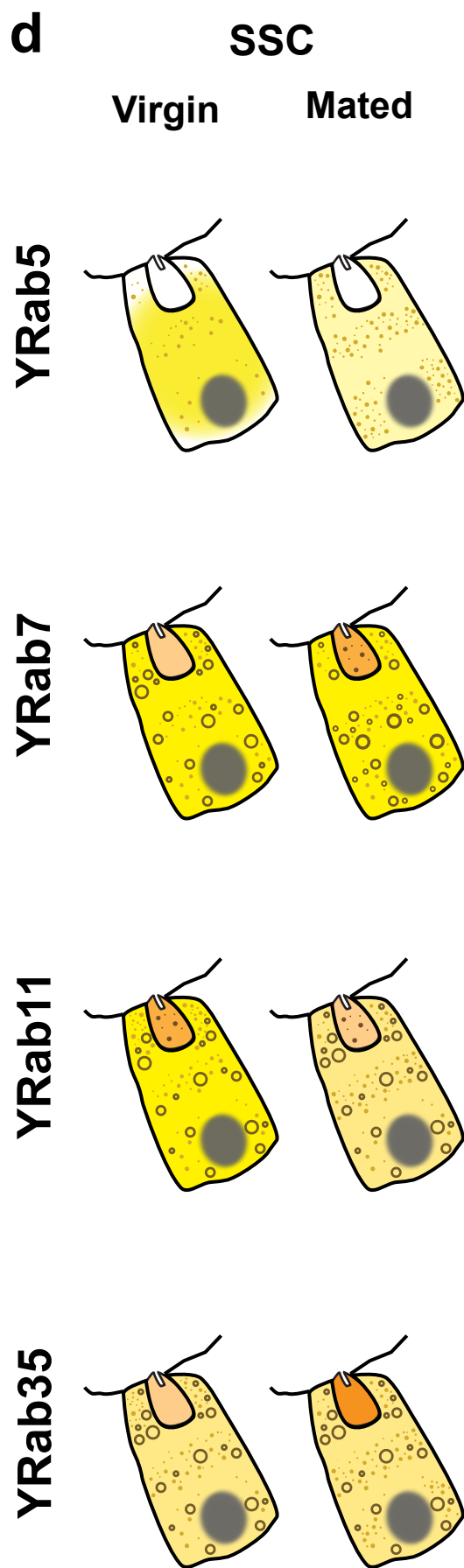
The presented GO terms were chosen based on their association with EV secretion and/or uptake. The density plots show the distribution of expression fold-change (log2) of genes associated with particular GO term (i.e. biological process; see Supplementary Data 1 for genes included in each term). The analysis is based on: FlyAtlas1 (expression microarray)<sup>22</sup>, and FlyAtlas2 (RNAseq)<sup>23</sup> datasets for **Spt/WF** [fold change of genes expressed in virgin spermatheca (Spt) versus their expression in the whole female (WF), contrasting genes associated with the designated GO-term (yellow) against all the genes expressed in the spermatheca (gray)]; Prokupek et al datasets for **V/M3h**, **V/M6h** [fold change of genes expressed in virgin spermatheca versus 3 and 6 hours post-mating (orange and blue respectively), against all genes expressed in the spermatheca (gray)]<sup>20</sup>; McDonough-Goldstein et al. **V/M6h**, **V/M24h** dataset for [fold change of genes expressed in virgin spermatheca versus 6 and 24 hours post-mating (blue and purple respectively), against all genes expressed in the spermatheca (gray)]<sup>21</sup>. Broken lines highlight the same reference density for each experimental contrast. The distributions of the contrasted experimental conditions were compared by the Kolmogorov–Smirnov test;  $p < 0.05$ , visually depicted as full colored curves (see also **Supplementary Data 1**).

Fig. S2



C

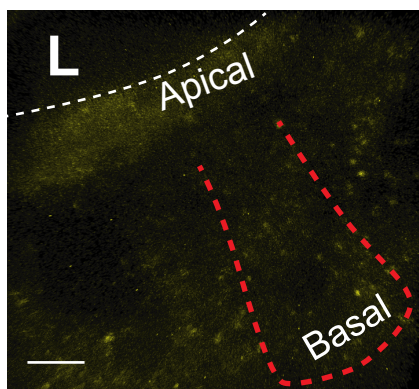
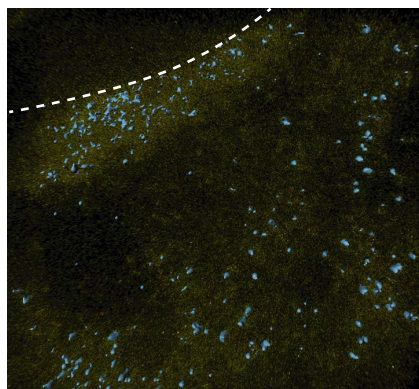




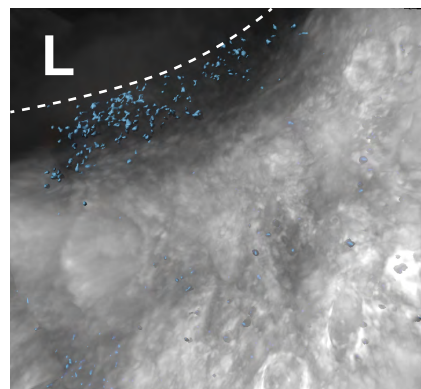


**f****YRab5**

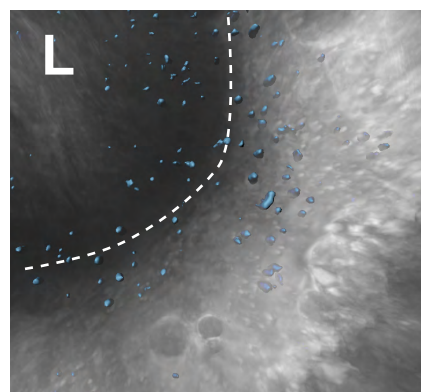
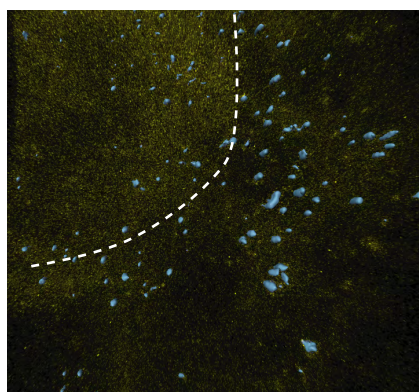
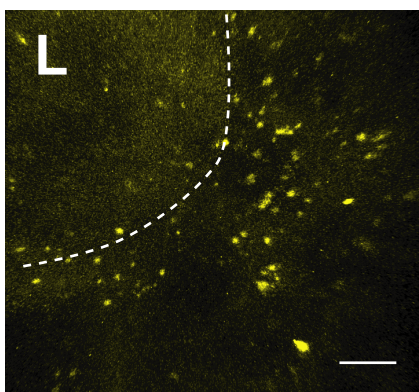
Virgin

*Yrab* + Surface

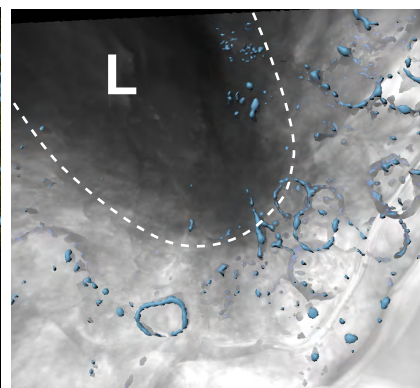
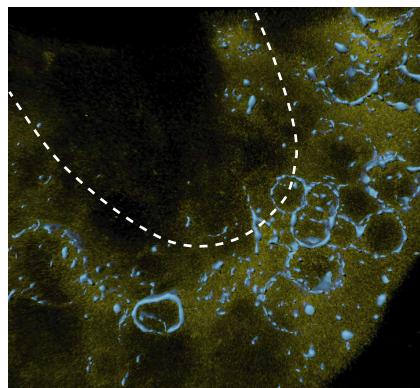
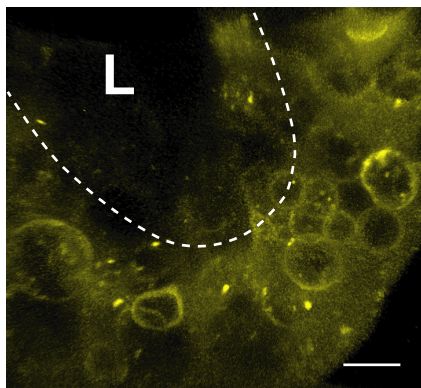
BF + Surface



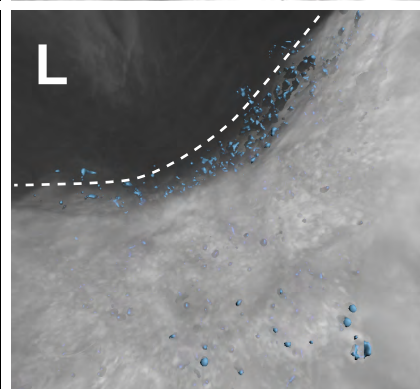
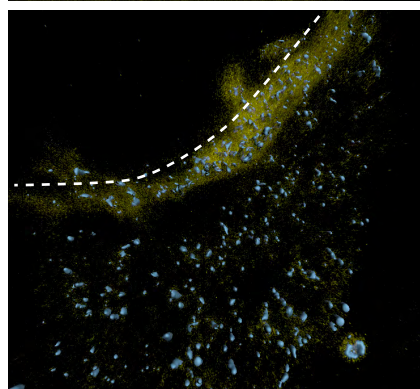
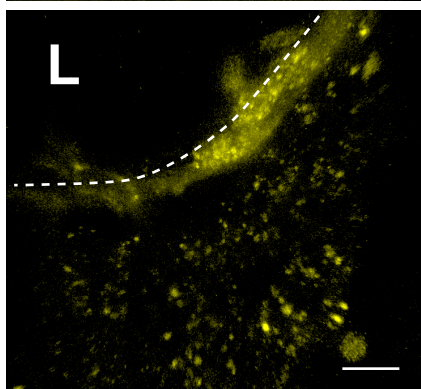
Mated 24h

*Rab5-YFP**Rab5* surfaces**YRab7**

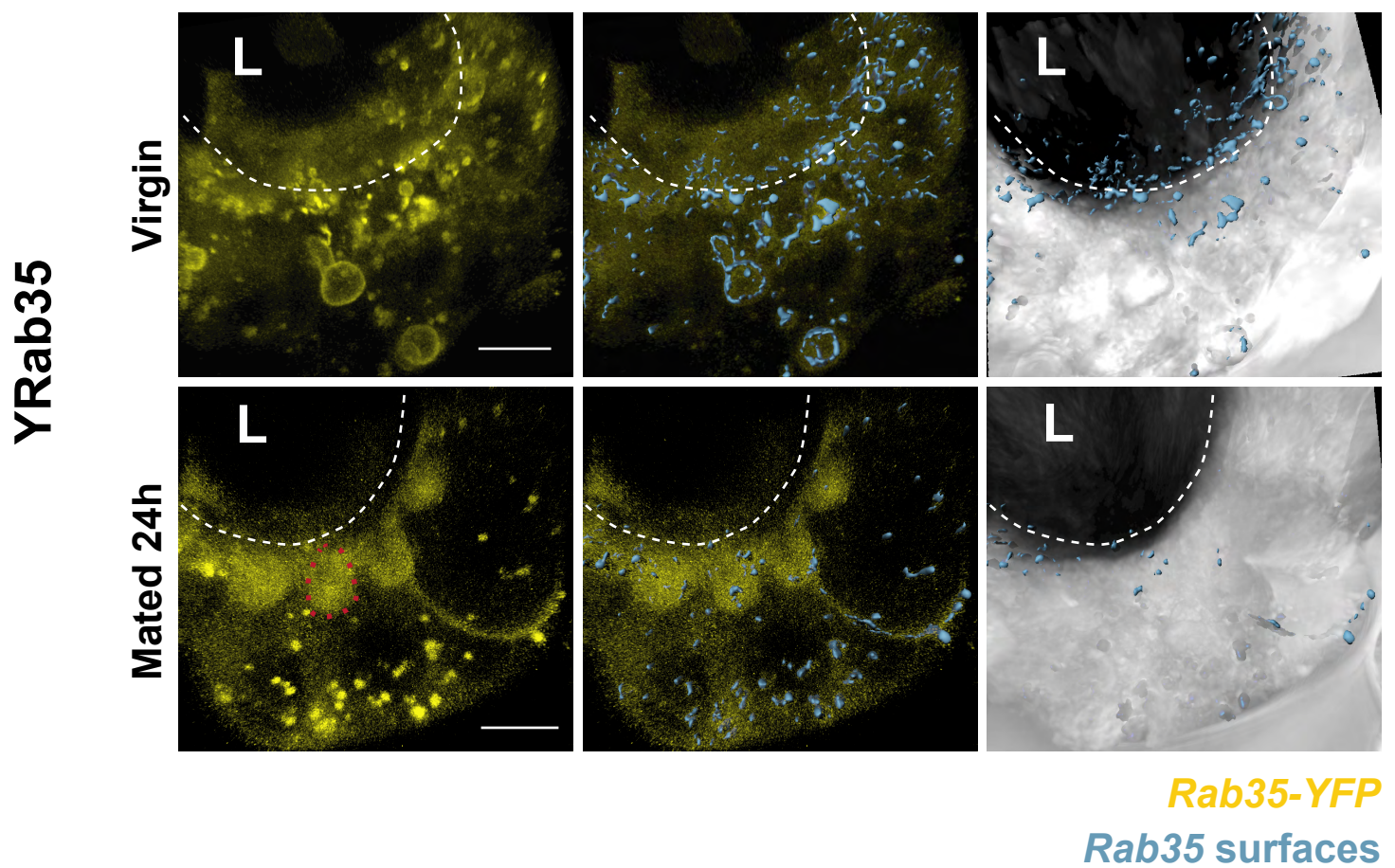
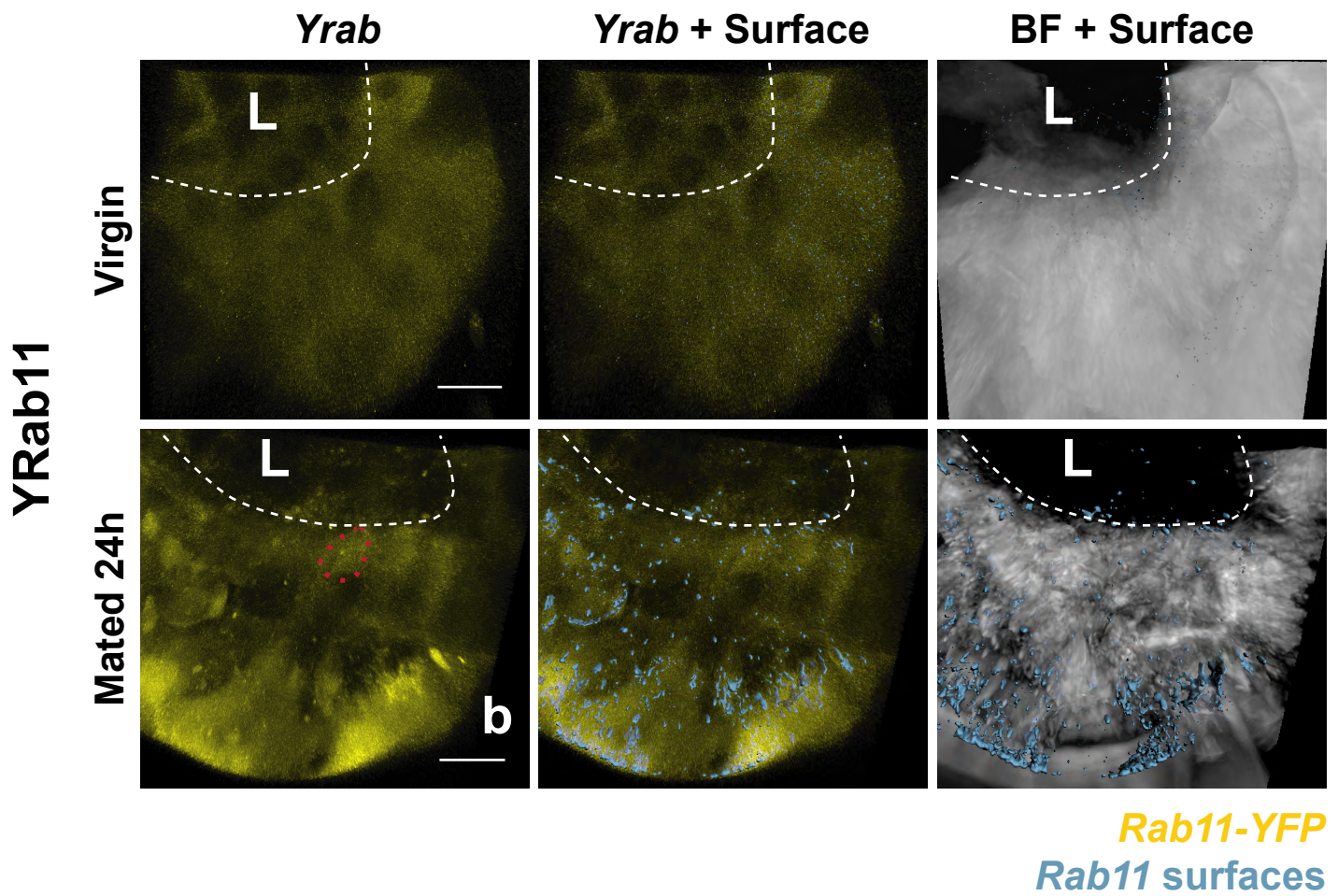
Virgin



Mated 24h

*Rab7-YFP**Rab7* surfaces

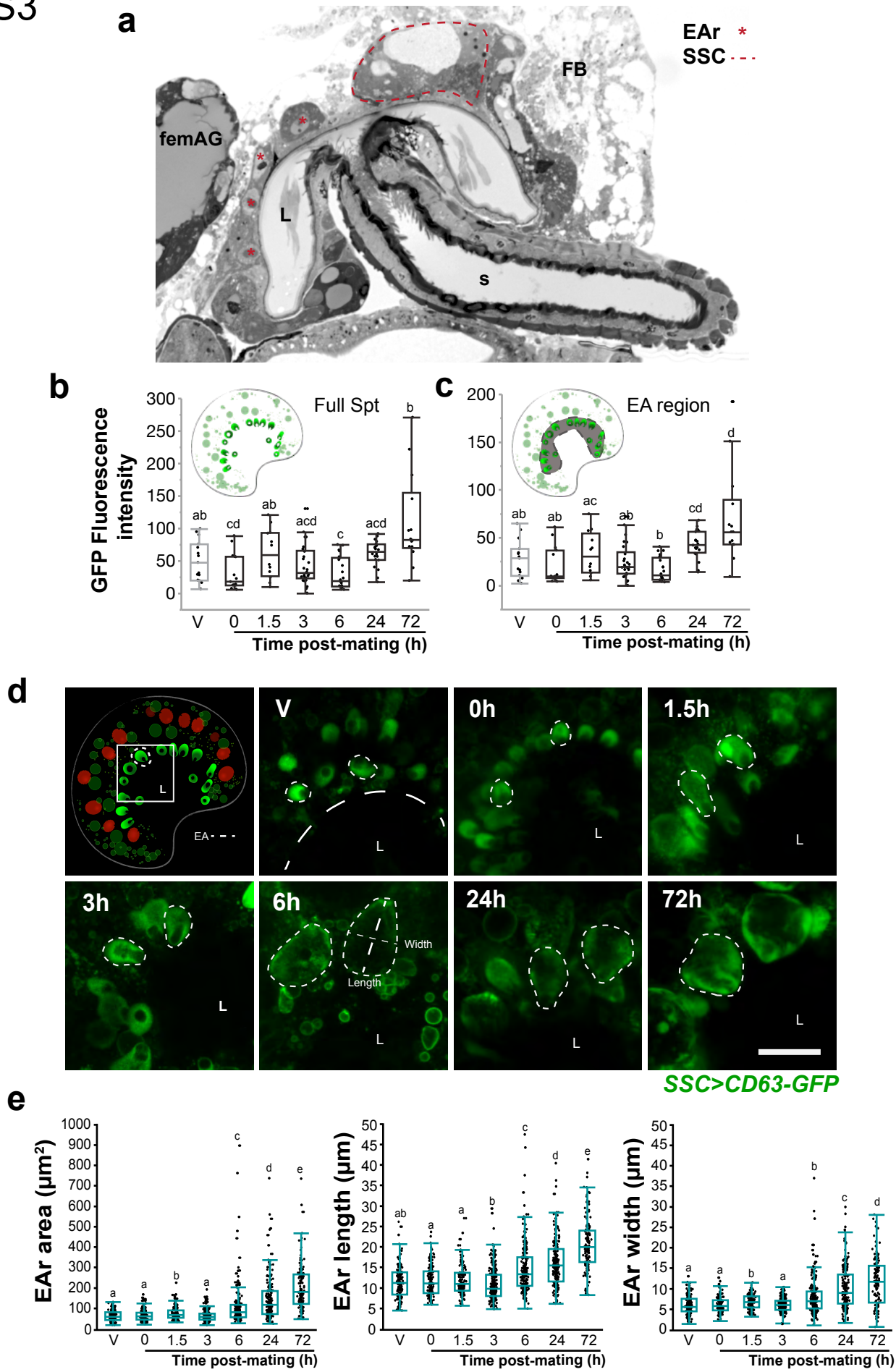




**Supplementary Fig. 2. SSC are enriched in Rab GTPases and their expression level and subcellular localization is modulated by mating.**

**a)** Expression of small GTPases (IPR001806) in the spermatheca. The heatmap presents (left to right): **Spt** - expression level of genes in virgin female spermatheca; **Spt/WF** - expression of genes in the spermatheca relative to the Whole female; expression level of genes in the spermatheca of **Virgin**, and mated females at 3 (**M3h**) and 6 hours post-mating (**M6h**) (mean subtracted; log2, based on Prokupek et al datasets<sup>20</sup>). **b)** Schematic that describes the analysis of YRab localization in the spermatheca. The grid was laid over the maximum projection images of the spermathecae, adjusting over the lumen and borders, and divided to 8 radial sections. The different subcellular regions (apical, medial and basal; see also **Fig. 2a**) were quantified to estimate the YRab patterns of expression for each section (7 sections, excluding the section of the stalk). **c)** Expression profiles of different YRab patterns in the subcellular regions of virgin and mated at 24h post-mating SSC (see also **Fig. 2a**). The heatmap color code represents the percentage of regions in the spermatheca where a specific YRab was detected in low (1) or high (2) abundance (for YRabs associated with punctate, and large membranal compartment LMC pools), or present (1) or absent (0) from the region of analysis (YRabs associated with diffuse pool), out of the total regions in which YRab was observed (the number of regions expressing YRabs is shown at the bottom of each YRab heatmap). To compare proportions, we applied the two-proportion comparison test corrected by the Benjamini-Hochberg correction for multiple comparisons<sup>77</sup> based on ranked *p*-values, in which the maximum allowed *p* was 0.20. **d)** Cartoon representations summarizing the YRab profiles found in **c** and **Fig. 2c**. **e)** Representative confocal maximum projections of whole spermathecae expressing the different YRabs; broken red line highlight the SSC; scale bar = 20µm. **f)** Magnification of spermathecae in which a specific YRab is visualized in the SSC. The YRab signal was rendered as blue surfaces in Imaris and overlaid on the bright field to highlight their subcellular localization within the SSC. Red broken lines indicate SSC, and white lines, the apical subcellular region lining the spermatheca lumen (L). In YRab11 and YRab35 the red spotted lines delineate end apparatus. (BF) – bright field; scale bar = 5µm.

Fig. S3

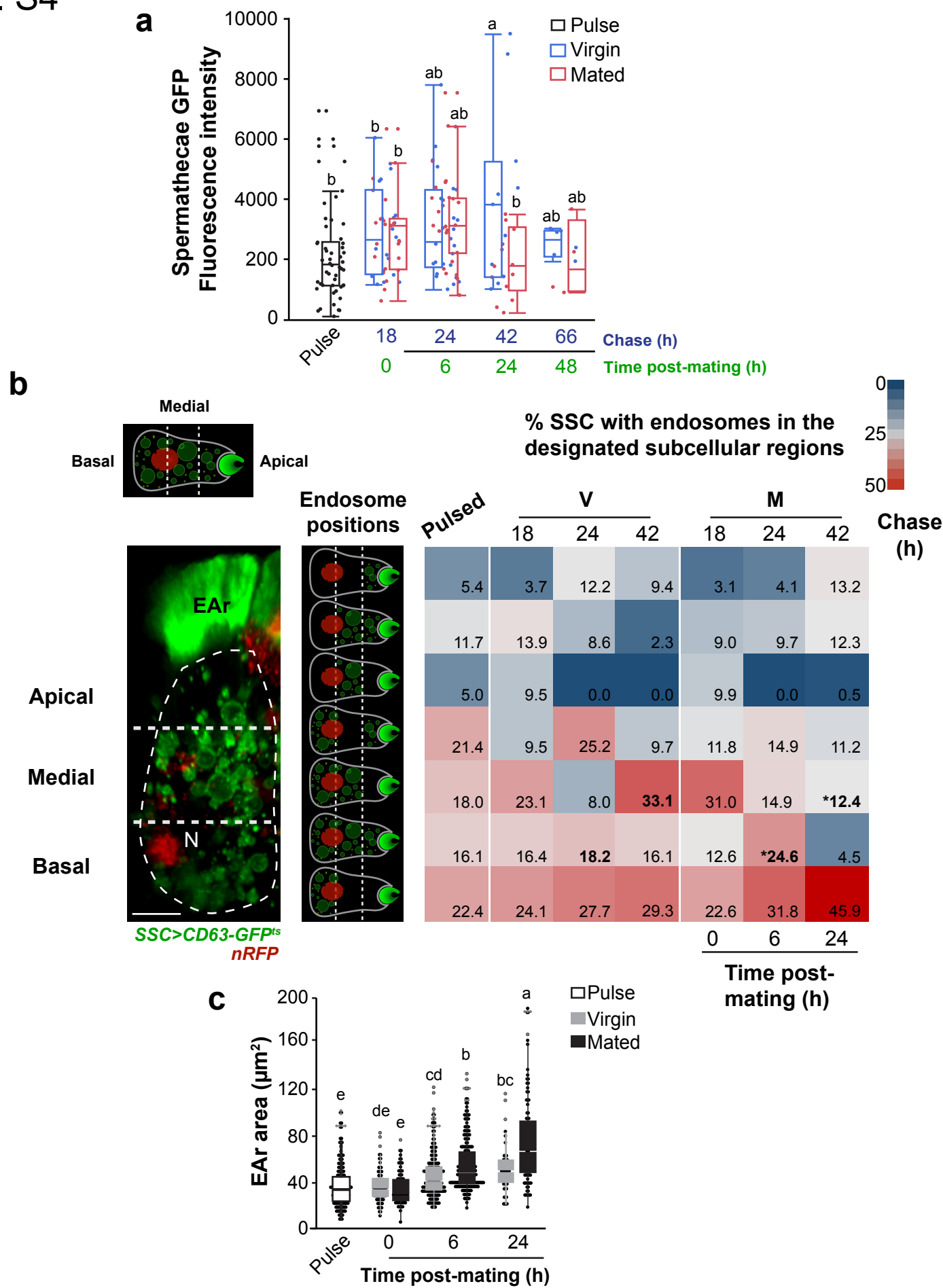




**Supplementary Fig. 3. The end apparatus undergoes changes post-mating plausibly to support secretory activity of the spermatheca.**

The end apparatus reservoir (EAr) collects the SSC secretions and it is formed by the invagination of the apical SSC membrane over a fibrillar cuticular meshwork that connects the secretory cavity to the lumen through a cuticular duct<sup>17,78</sup> (see **Fig. 1b**). **a**) Toluidine blue stained 1- $\mu\text{m}$  thick section of virgin female spermatheca: spermatheca secretory cell (SSC; broken line), EAr (star), lumen (L), stalk (s), female accessory gland (femAG), and fat body (FB) [imaged by Pat Rivlin, Anat Kapelnikov, Ron Hoy and Yael Heifetz]. **b**) CD63-GFP fluorescence intensity level of the whole spermatheca, and **c**) end apparatus (EA) region of the spermatheca as shown in the small schematics above (grey outline; in continuous to **Fig. 3a** and **b**). Box plots represent maximum, median and minimum values with outliers of 15-20 spermathecae per condition; letters denote significant differences (one-way ANOVA, multiple comparison post-hoc test,  $p < 0.05$ ). **d**) Representative confocal micrographs (from the region shown in the inset of the schematic) displaying morphological changes that the EA undergo in virgin and mated females (0, 1.5, 3, 6, 24 and 72h post-mating) SSC; L= lumen; scale bar= 5 $\mu\text{m}$  (see **Fig. 1b**). **e**) Morphological changes of EAr were evaluated by calculating the area ( $\mu\text{m}^2$ ), length and width ( $\mu\text{m}$ ) in each condition. Box plots of 10 different EAr from 30-40 spermathecae per condition, between 16-22 flies; boxes represent maximum, median and minimum values with outliers; letters denote significant differences (one-way ANOVA, multiple comparison post-hoc test,  $p < 0.05$ ).

Fig. S4

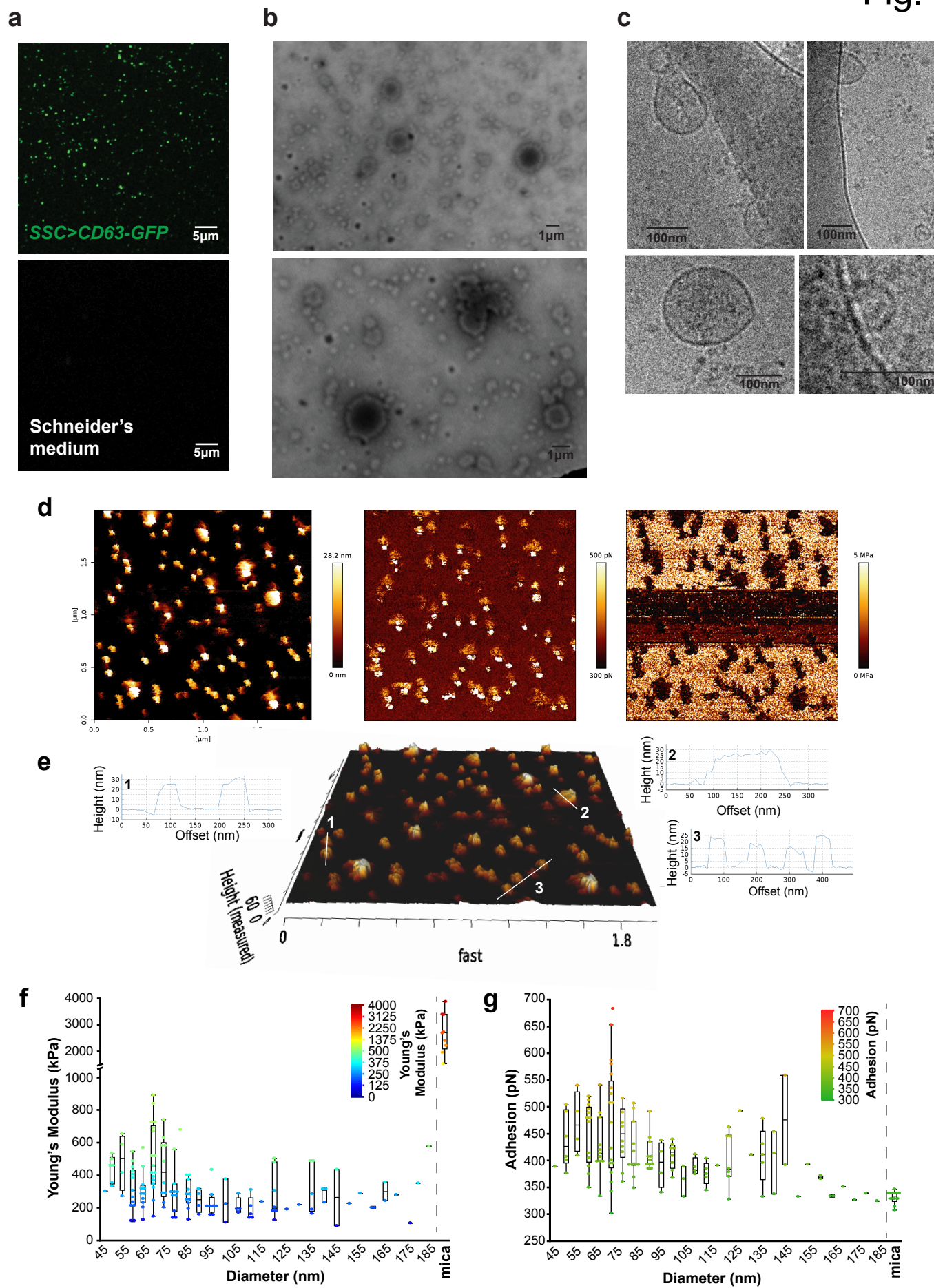


**Supplementary Fig. 4. The abundance of CD63-GFP-positive endosomes in the SSC increases post-mating.**

CD63-GFP was conditionally expressed in the SSC by using a temperature sensitive Gal80 (CD63-GFP). **a)** GFP fluorescence intensity level was measured in the whole spermatheca of virgin and mated females to detect the dynamics of CD63-GFP degradation throughout the chase (see schematic in **Fig. 4a**). **b)** Heatmap depicting the percentage of SSC with CD63-GFP-positive end apparatus reservoirs (EAr), and endosomes in the apical, medial or basal subcellular regions of the SSC across time and mating status. The schematic columns at the left show endosome spatial distribution in the SSC. Stars and bold numbers in the heatmap depict significant differences between virgin and mated at a given time. The left image is a representative lightning confocal micrograph of an SSC with endosomes distributed in the three regions of the cytoplasm, where green is CD63-GFP and red nRFP. Scale bar = 5 $\mu$ m. **c)** Mean EAr area ( $\mu$ m<sup>2</sup>) measured across time and mating status. Mating induced a progressive increase in the CD63-GFP-positive EAr area as observed in **Supplementary Fig 3b**. Box plots represent maximum, median and minimum values with outliers; letters denote significant differences (two-way ANOVA and multiple comparison Tukey HSD post-hoc test,  $p < 0.05$ ).

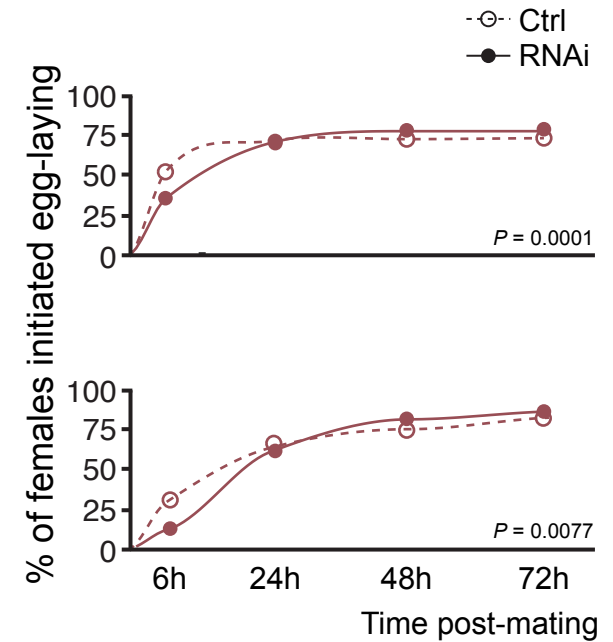
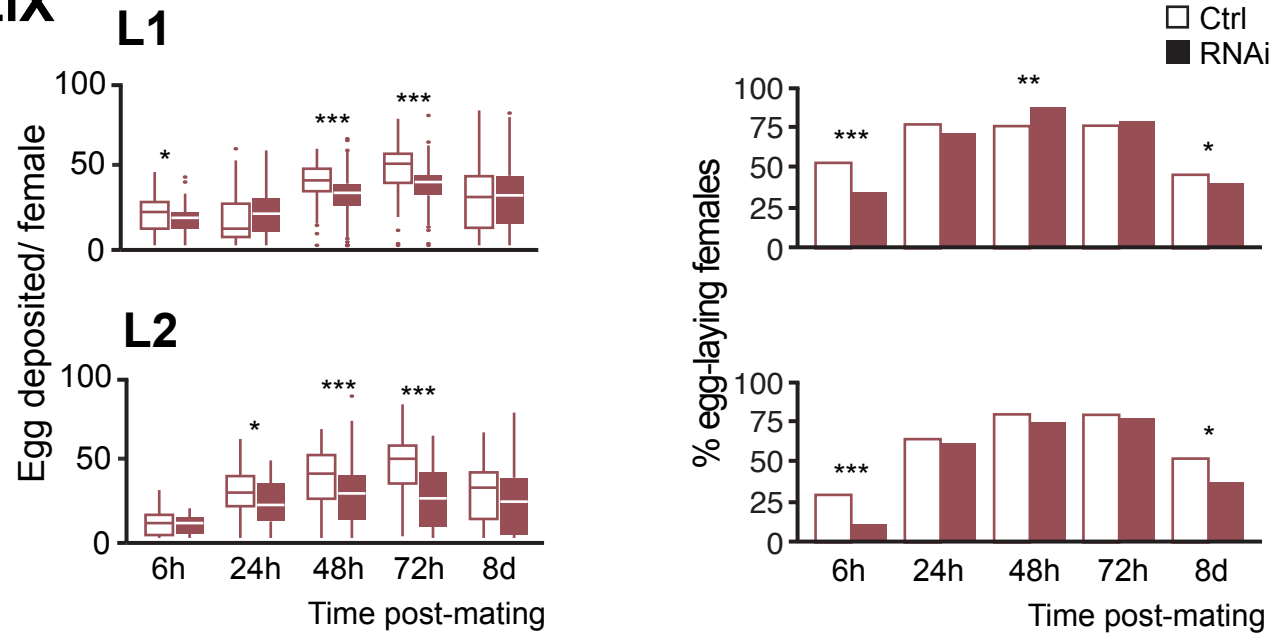
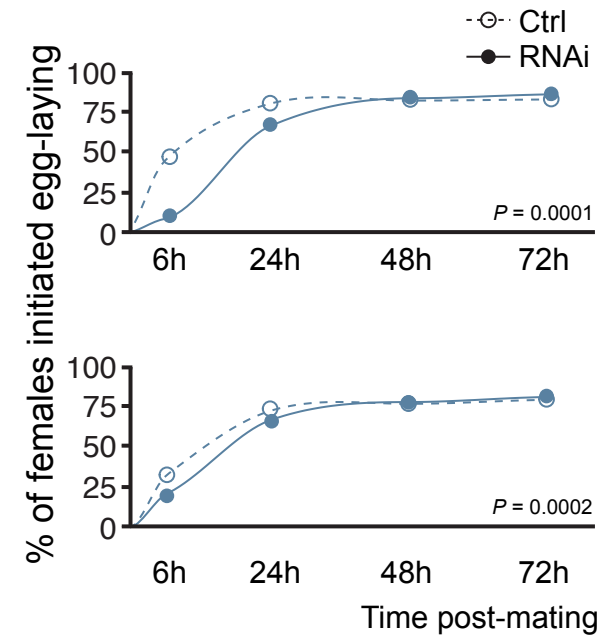
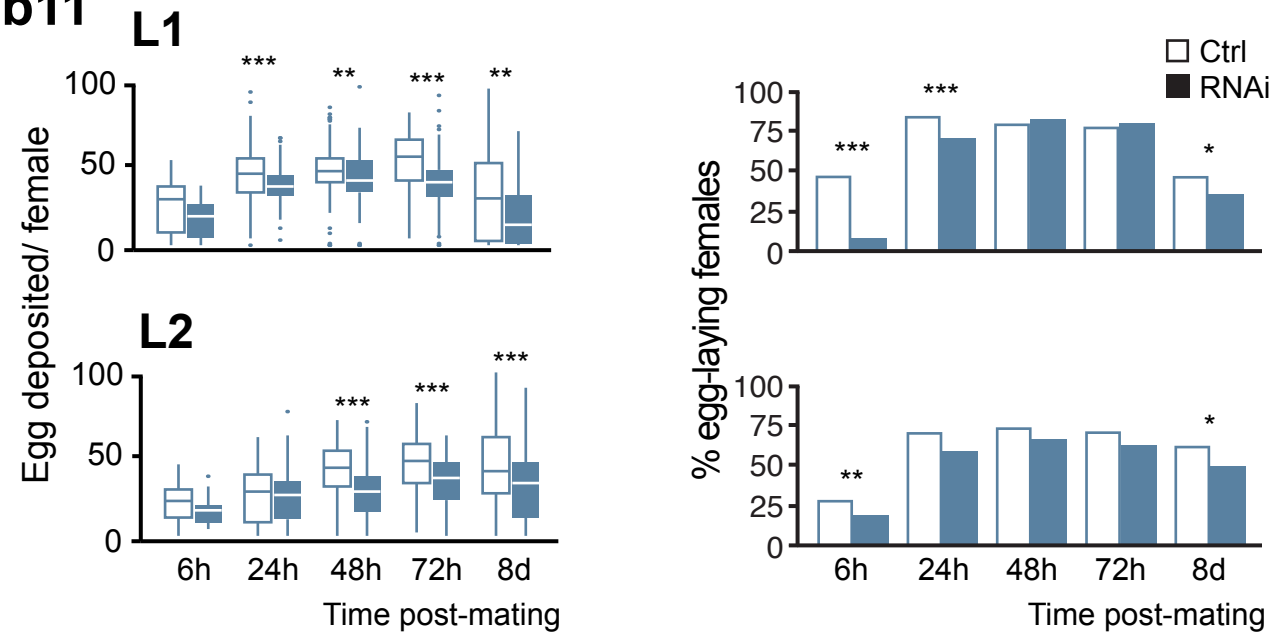


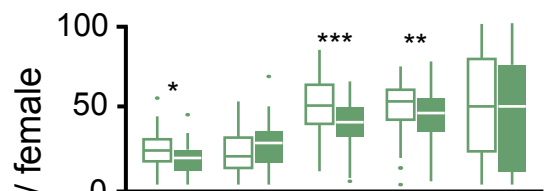
Fig. S5



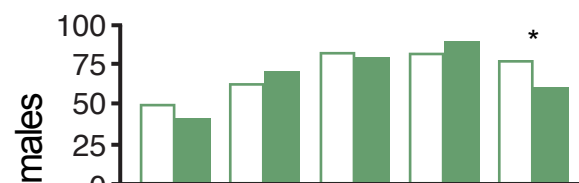
**Supplementary Fig. 5. Characteristics of CD63-GFP SSC and spermatheca-derived-EVs.** **a-c)** Spermathecae of females expressing CD63-GFP in the SSC were cultured *ex vivo* 72h post-mating and the spent media was assessed by different microscopy techniques (from 100 spermathecae and 52-55 flies): **a)** Spent or clean media were imaged by confocal microscopy using the Leica Lightning mode; scale bar = 5 $\mu$ m; **b)** The morphology of EVs in the spent media was observed by negative staining and STEM; scale bar = 1 $\mu$ m and 200 nm; and by **c)** CryoTEM; Scale bar = 100 nm. Shown are EVs of various sizes and morphology of the lipid bilayers. **d-g)** The spent media of *ex vivo* cultured spermathecae were imaged by AFM for the presence of EVs. **d)** Representative AFM scans (2x2 $\mu$ m) of EVs from spermathecae spent media (from 200 spermathecae from 105-110 flies) isolated by acoustic sorting (AcouSort). From left to right: height ( $\mu$ m), adhesion (pN) and Young's modulus (kPa) images. **e)** 3D profile of (**d**), exemplifying 3 cross sections of EVs (height and diameter in nm). Profiles of (**f**) Young's modulus and (**g**) adhesion force of EVs of different diameters and the mica substrate (see also **Fig. 5f** for examples of single EV AFM scans).

Fig. S6

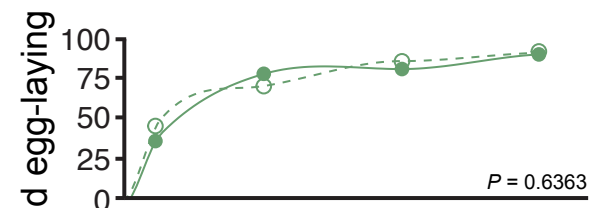
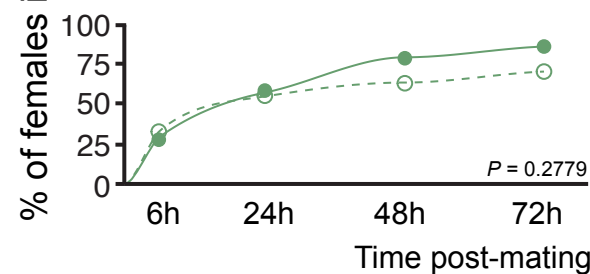
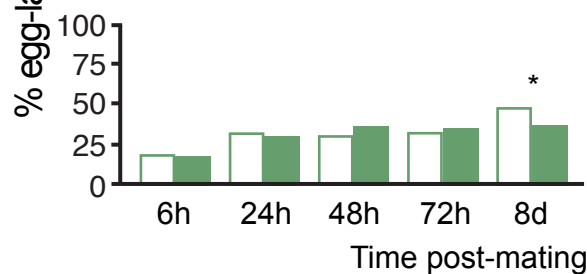
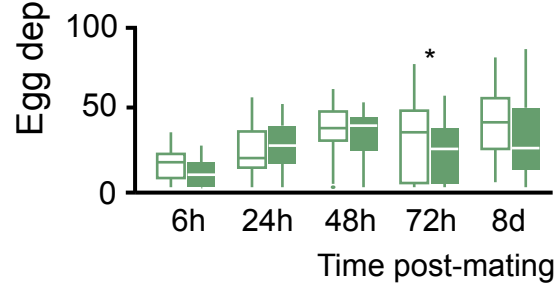
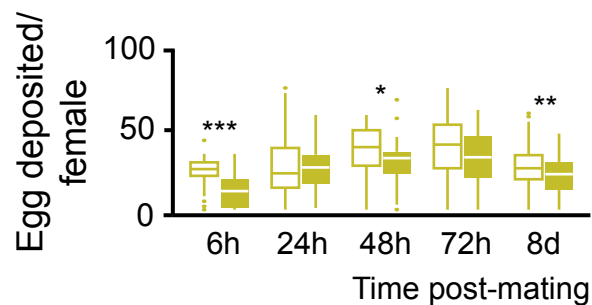
**ALiX****Rab11**

**Hrs****L1**

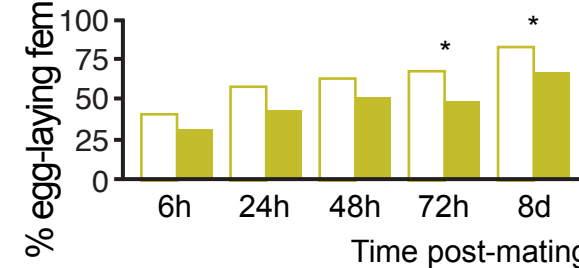
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■ RNAi



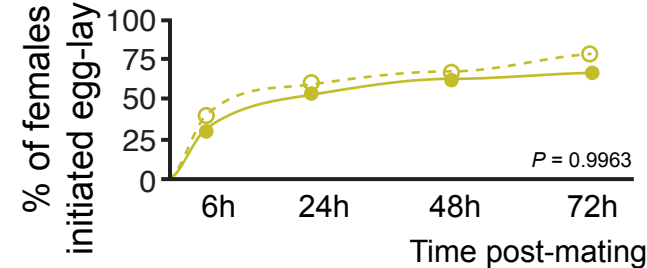
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**L2****Rab7**

□ Ctrl  
■ RNAi



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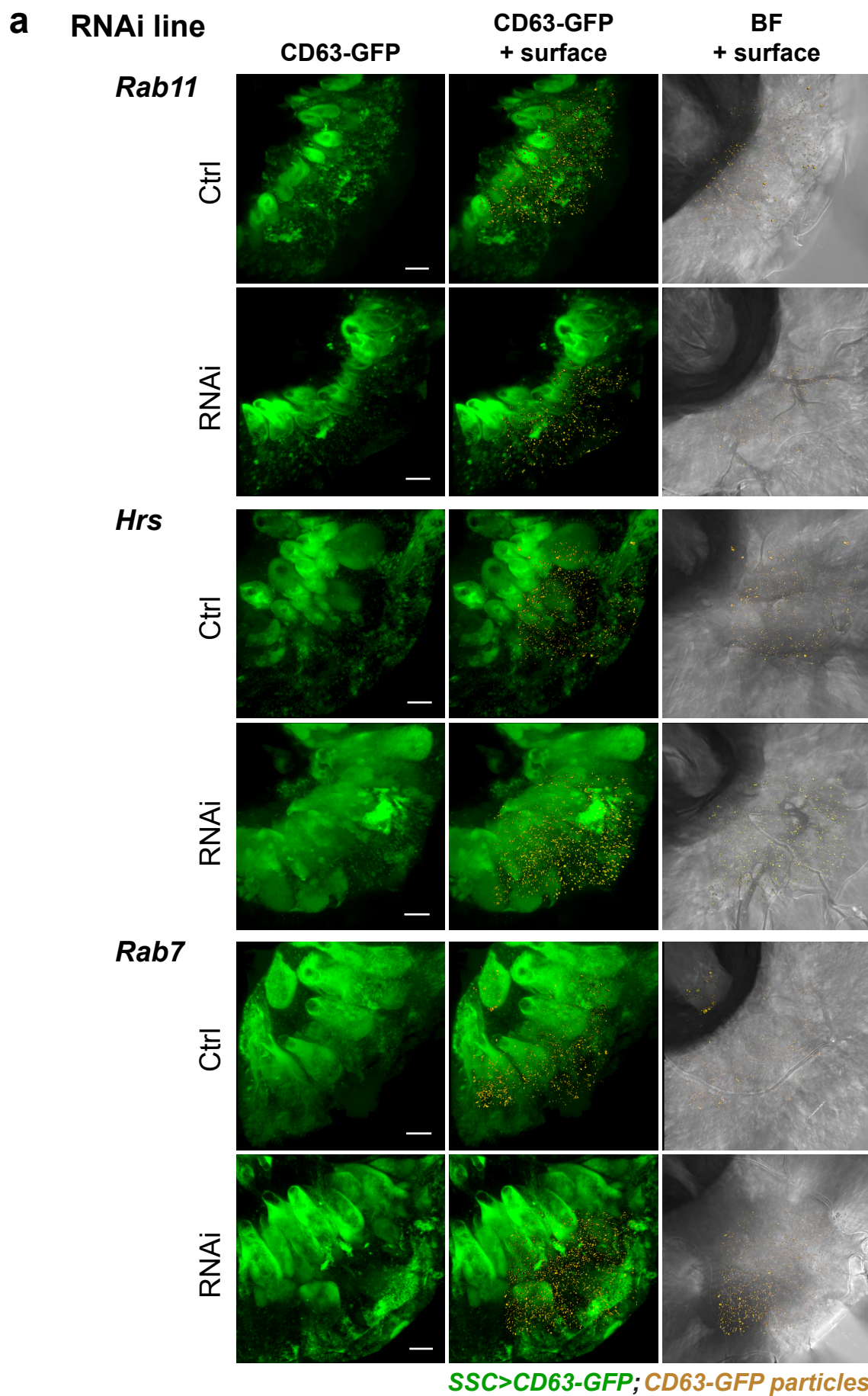




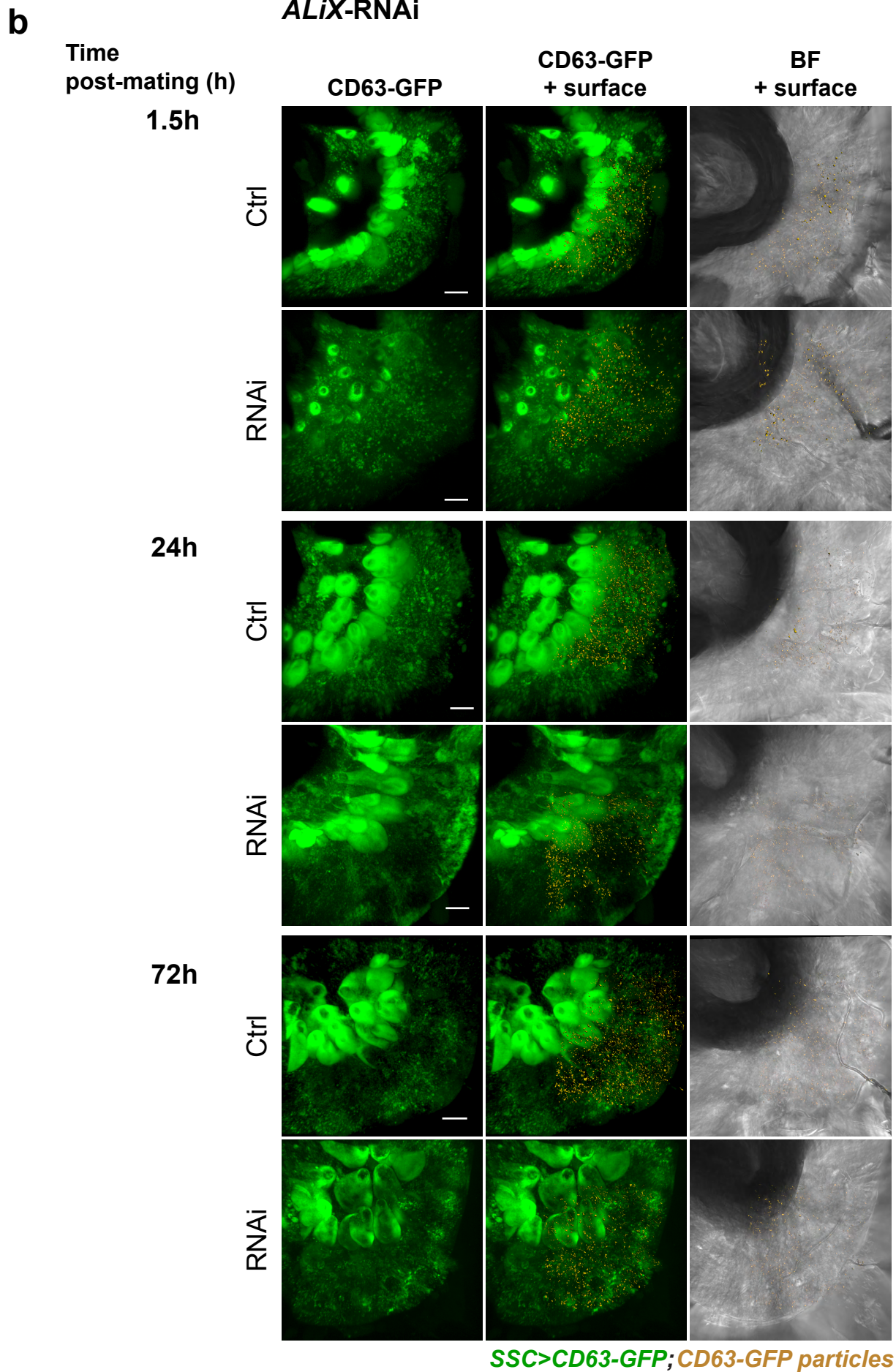
**Supplementary Fig. 6. Silencing of *ALiX*, *Rab11*, *Hrs* and *Rab7* in the SSC decreases egg-laying and delays the onset of deposition.**

Following silencing of *ALiX*, *Rab11*, *Hrs* and *Rab7*, specifically in the SSC we evaluated: the number of eggs deposited per female, the percentage of females that laid eggs at each time point, and the percentage of females that initiated egg-laying (time at which the female laid her first eggs) at 6, 24, 48, 72 h and 8 days post-mating. Control females (no RNAi, open bars); RNAi expressing females (full bars). The effects of silencing were evaluated in two independent different RNAi fly lines (L1, L2) for all the genes but *Rab7*. Box plots represent maximum, median and minimum values with outliers, and bars and points in the curves are the percentages of flies in each category. Sample sizes: *ALiX*-RNAi (n = 87-139), *Hrs*-RNAi (n = 52-97), *Rab11*-RNAi (n = 122-153), *Rab7*-RNAi (n = 71-77). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  (see supplementary methods for statistics).

Fig. S7



SSC>CD63-GFP; CD63-GFP particles



**Supplementary Fig. 7. The intracellular CD63-GFP in the SSC is affected by mating and silencing of *ALiX*, *Hrs*, *Rab11* and *Rab7*.**

Representative images of the analyzed regions of spermathecae that expressed CD63-GFP (control, Ctrl) or co-expressing RNAi and CD63-GFP for *ALiX*, *Hrs*, *Rab11* and *Rab7* (RNAi) (The sampled region is shown in the inset of **Fig. 3a**). **a)** Representative images of control, *Rab11*-, *Hrs*- and *Rab7*-RNAi, SSC at 24h post-mating. The left column demonstrates the pattern of GFP-positive puncta localized in the cytoplasm of the SSC, the middle column shows CD63-GFP particles rendered as yellow plastic surfaces using Imaris, and the right column depicts the distribution of CD63-GFP yellow surfaces in the spermatheca at brightfield (BF) for spatial reference. **b)** Representative images of the CD63-GFP expression in the spermathecae of control and *ALiX*-RNAi, at 1.5, 24 and 72h post-mating. See also **Fig. 6c and d** where the number and mean particle size are evaluated. Scale bar= 10µm.