BMP-regulated exosomes from Drosophila male reproductive glands reprogram female behavior

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ale reproductive glands secrete signals into seminal fluid to facilitate reproductive success. In Drosophila melanogaster, these signals are generated by a variety of seminal peptides, many produced by the accessory glands (AGs). One epithelial cell type in the adult male AGs, the secondary cell (SC), grows selectively in response to bone morphogenetic protein (BMP) signaling. This signaling is involved in blocking the rapid remating of mated females, which contributes to the reproductive advantage of the first male to mate. In this paper, we show that SCs secrete exosomes, membrane-bound vesicles generated inside late endosomal multivesicular bodies (MVBs). After mating, exosomes fuse with sperm (as also seen in vitro for human prostate-derived exosomes and sperm) and interact with female reproductive tract epithelia. Exosome release was required to inhibit female remating behavior, suggesting that exosomes are downstream effectors of BMP signaling. Indeed, when BMP signaling was reduced in SCs, vesicles were still formed in MVBs but not secreted as exosomes. These results demonstrate a new function for the MVB–exosome pathway in the reproductive tract that appears to be conserved across evolution.

Introduction

In addition to sperm, seminal fluid contains multiple factors secreted by male reproductive glands that activate sperm (Rodríguez-Martínez et al., 2011) and can influence female physiology and behavior (Kershaw-Young et al., 2012). In Drosophila melanogaster, the paired accessory glands (AGs) secrete peptides that perform such functions (Wolfner, 1997). Sex peptide (SP) is one key product that in females can induce enhanced egg laying (Chapman et al., 2003; Kubli, 1997). Scattered among MCs in the distal tip of the gland are roughly 40 secondary cells (SCs). SCs are unusually large, vacuole-filled cells, ∼25 µm in diameter, whose cell biology remains relatively uncharacterized (Rylett et al., 2007; Leiblich et al., 2012). SCs have an important modulatory influence on the maintenance of postmating female responses (Minami et al., 2012; Gligorov et al., 2013). One aspect of this, reduced receptivity to remating, is regulated by SC-specific bone morphogenetic protein (BMP) signaling (Leiblich et al., 2012) via an unknown mechanism.

Exosomes are secreted membrane-bound, nanosized (40–100 nm) vesicles generated in late endosomal multivesicular bodies (MVBs; Raposo and Stoorvogel, 2013; Christianson et al., 2014). They are thought to mediate short- and long-range intercellular signaling events in biological contexts, such as immune (Théry et al., 2009) and tumor cell signaling (Ge et al., 2011). Exosomes are released from various cell types, including both professional and nonprofessional secretory cells, and carry a variety of genetic, epigenetic, and secreted factors as well as organelles (Bostock et al., 2014; Celniker et al., 2014). They are also known to interact with immune cells and contribute to immune responses (Coppe et al., 2008; Iwasa et al., 2014).

Exosome release is required for BMP signaling in SCs

To determine whether exosomes were involved in BMP signaling in SCs, we first examined the effect of BMP on exosome release from SCs. We cultured SCs in vitro and added recombinant BMP to the media. Exosome release was reduced in SCs, vesicles were still formed in MVBs but not secreted as exosomes. These results demonstrate a new function for the MVB–exosome pathway in the reproductive tract that appears to be conserved across evolution.

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ably representing intraluminal vesicles (ILVs), were also observed (ANCE; Fig. 1, E and F). CD63-GFP–positive puncta, presumably containing the secreted protease angiotensin-converting enzyme I (ANCE; Fig. 1, E and F). CD63-GFP–positive puncta, presumably representing intraluminal vesicles (ILVs), were also observed inside many vacuoles, especially in live specimens.

Not all vacuoles were identical, however; the majority appeared to fuse with sperm in vivo in females after mating, phenocopying reported properties of human prostasomes in vitro (Arienì et al., 1997; Park et al., 2011). Furthermore, SC-derived exosomes interact with epithelial cells in the female reproductive tract and are required to induce changes in female remating behavior controlled by BMP signaling. Secretion, but not formation, of SC-derived exosomes requires BMP signaling, highlighting endolysosomal trafficking as a key regulator of SC function. Thus, regulated secretion of exosomes in the AG provides an essential part of the armory promoting male-specific interests during Drosophila reproduction.

Results

SCs secrete CD63-GFP-marked puncta into the AG lumen

Each AG secretes into a large lumen, which opens at its proximal end into the ejaculatory duct (Fig. 1, A–C). We expressed a GFP-tagged tetraspanin, CD63, a mammalian transmembrane exosome marker used previously to mark fly exosomes (Panáková et al., 2005; Gross, et al., 2012), independently in MCs and SCs (Figs. 1, A–C; and S1, A and B) using the GAL4/upstream activating sequence (UAS) system (Brand and Perrimon, 1993). In SCs of 3-d-old males, CD63-GFP localized to the limiting membrane of all large (>2-µm diameter) vacuoles and, in some cells, to the apical plasma membrane (Fig. 1 F).

These puncta were typically most prominent in at least one acidic compartment, stained with the vital acid-sensitive dye LysoTracker red (Fig. 1, G and H).

The AG lumen contained marked puncta (Figs. 1, C and D; and S1, A and F), which were not observed when CD63-GFP was expressed in the large number of MCs in the AG (Fig. S1 B), suggesting that only SCs secrete this tagged membrane protein. Overexpressed cytoplasmic GFP was sometimes incorporated into puncta inside SC vacuoles (Fig. S1 C), indicating that the puncta are not induced by CD63-GFP expression. Furthermore, another tagged, nonexosomal membrane protein, CD8-RFP, which labels all vacuolar compartments and is internalized in some of them, was not secreted (Fig. S1, D and E), suggesting that CD63-GFP is packed into intraluminal structures and selectively secreted. In support of this, luminal vesicles ∼40 nm in diameter, as well as other particulate material, were observed by transmission EM in the AG lumen of wild-type males (Fig. S2 G).

CD63-GFP-positive puncta are located inside late endosomes and lysosomes

We analyzed the large SC compartments in more detail using LysoTracker red–stained living glands. Counting vacuoles by scanning through multiple focal planes revealed remarkably consistent numbers of large (>2 µm) and small (<2 µm) CD63-positive acidic compartments and large nonacidic CD63-positive compartments in each SC of 6-d-old adults (Fig. 1 I). Both classes of acidic compartments were also visible in non-CD63-GFP–expressing SCs (Figs. S1, G versus H; and 2, A–C) and therefore were not induced by CD63-GFP expression. We hypothesized that the smaller apical acidic compartments were immature late endosomes (iLEs; CD63-GFP+/LysoTracker+ and <2 µm; Fig. 1 I), and the large acidic compartments, at least one of which contained small CD63-GFP–positive puncta, were unusually large (∼10 µm in 6-d-old SCs) maturing MVBs or lysosomes (mMVBLs; CD63-GFP+/LysoTracker+ and >2 µm; Fig. 1 I). In addition, some, but not all, nonacidic SV compartments (CD63-GFP+/LysoTracker− and >2 µm; Fig. 1 I) contained larger CD63-GFP–positive intraluminal structures.

In each SC, an anti-CD63 antibody, which cross-reacted with fluorescent GFP puncta in the AG lumen (Fig. S2, B and C), also stained most of the lumen of either one or two compartments, which contained only sporadic fluorescent GFP puncta (Fig. S2 A). These compartments were ANCE negative and therefore not SVs. We reasoned that they were acidic compartments in which the microenvironment must partially inhibit fluorescence of internalized GFP. To confirm that SCs have giant vesicle-containing compartments, we analyzed SC ultrastructure by transmission EM (Fig. S2, E–I). In addition to the SV compartments previously reported by Rylett et al. (2007), SCs possessed one or two less electron-dense compartments containing vesicles ∼40 nm in diameter, an appropriate size to be precursors of secreted exosomes (Fig. S2 I). The ultrastructure of these large compartments was difficult to preserve in fixed, sectioned material, but because they are not filament- and ANCE core-containing SVs, we conclude that they must represent the acidic vesicle-containing compartments we observe in live specimens.
We performed more detailed live-imaging analysis of these large acidic and nonacidic SC compartments to confirm their identity, using selected ubiquitously expressed YFP-tagged Rab GTPases (Marois et al., 2006) that regulate endocytic trafficking and exosome secretion (Savina et al., 2002; Fader et al., 2005; Stenmark, 2009; Koles et al., 2012; Beckett et al., 2013). SCs typically contained two or three large vacuoles lined with Rab7-YFP, most of which were acidic (Fig. 2 A). Importantly, because all large acidic compartments were Rab7 positive (n > 50 cells), this confirms that the acidic compartments with CD63-GFP–positive puncta are either MVBs or lysosomes containing undegraded GFP. In support of this, these large acidic compartments were also lined by a GFP fusion with type II phosphatidylinositol kinase (PI4KII-GFP; Fig. S2 D), which marks late endosomes and lysosomes in other Drosophila secretory cells (Burgess et al., 2012).

Rab11-YFP, a marker for exocytic and recycling endocytic membranes, which regulates exosome secretion in flies (Chen et al., 1998; Koles et al., 2012; Beckett et al., 2013), localized to the limiting membrane of nonacidic SVs (Fig. 2 B). However, expression of this marker induced loss of large mMVBLs in most SCs (10/13 cells); overexpression of wild-type Rab11 therefore

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<td>mMVBL</td>
<td>2.8 +/- 0.2 (22)</td>
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<td>iLEs</td>
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with iLEs. In support of this, we found Rab7 colocalizes with Rab11-YFP at the limiting membrane of a subpopulation of Rab11-YFP–lined SVs (Fig. 2 D). In conclusion, SCs contain unusually large secretory and acidic vacuoles, permitting the in vivo analysis of these compartments at high spatial resolution. At least one large acidic compartment in each SC contains CD63-positive puncta (Fig. 1, G and H), and equivalent large vesicle–containing structures are also observed by EM (Fig. S2).

Figure 2. Rab GTPase signatures define different SC subcellular compartments. (A–D) SCs from 3-d-old males expressing different Rab-YFP constructs and stained with LysoTracker red. SCs have 5–10 small acidic Rab7-YFP–positive iLEs [A, arrowheads; most of these are usually located more apically than this confocal section; see Fig. 3] and 2.4 ± 0.9 (n = 11) Rab7-YFP–positive large vacuoles, 85% of which are acidic mMVBLs (A, asterisks; similar numbers [2.9 ± 0.5, n = 9] of large acidic compartments are seen in CD63-GFP–expressing SCs at this stage). Other Rab-YFP lines reveal Rab11-YFP–positive, nonacidic SVs [B, +; and D], many Rab5-YFP–positive small nonacidic compartments [C, arrowheads], and more rarely (4/16 SCs), a Rab5-YFP–positive small acidic compartment (C, arrows). In some fixed tub>rab11-YFP SCs stained for Rab7, Rab7 colocalizes with Rab11-YFP in parts of the limiting membrane (D, arrow) of a single SV (D, #). DAPI stains nuclei (blue). Approximate outline of SC is marked in all images. Bars, 5 µm.
Strikingly, real-time confocal imaging of SCs revealed nonacidic SVs fusing with mMVBLs and rapid exchange of limiting membrane components (Fig. 4 A and Video 1). Although the internal contents of these compartments did not mix freely, these findings suggest a surprising level of exchange between SVs and mMVBLs and might explain how CD63-GFP–positive structures accumulate in SVs (Fig. 1, E–H). As discussed previously, anti-CD63 staining indicates that GFP in many CD63-positive ILVs does not fluoresce (Fig. S2 A). However, inward invaginations of the CD63-GFP–labeled limiting membrane into the mMVBL lumen were observed in time-lapse videos, and occasionally, it appeared that new fluorescent ILVs might be formed from these (Fig. 4 B and Video 2). Therefore, some steps in ILV formation can be visualized by confocal microscopy in the large mMVBLs of living SCs, allowing us to screen for genetic manipulations that block these processes.

Figure 3. CD63-GFP traffics from secretory to endocytic compartments in SCs. (A–G) An 8-h pulse (at 28.5°C) of CD63-GFP expression was chased at 18°C for 0–60 h in virgin males, and proportions of cells with one or more LysoTracker red–positive iLEs (arrows in A, C, and E in apical sections; GFP positive in C and E) and mMVBLs (asterisks in B, D, and F in more basal views) that were CD63-GFP–positive were scored (G). Data shown are from a single representative experiment out of two repeats. The images in A–F are shown again in Fig. S3 alongside the corresponding single color channel images. Approximate outline of SC is marked in all images. *, P < 0.01; **, P < 0.001; n > 24, pairwise comparisons, Fisher’s exact test. Bars, 5 µm.

Endolysosomal and secretory compartments in SCs are highly dynamic

We followed CD63-GFP trafficking through the secretory and endolysosomal systems using a pulse–chase approach. Induction of a short (8 h) pulse of CD63-GFP expression, by inhibiting the temperature-sensitive GAL4 transcriptional repressor GAL80<sup>ts</sup> at 28.5°C, followed by a chase period at 18°C of 0–60 h, revealed rapid labeling of two (1.9 ± 0.2, n = 17) nonacidic SVs, presumably receiving newly synthesized CD63-GFP from the Golgi (Figs. 3, A and B; and S3, A and B). Some iLEs were also GFP positive immediately after the pulse. During the early part of the time course, a greater proportion of SCs contained labeled iLEs than labeled mMVBLs (P = 0.001, n > 27), and this trend continued at later time points (Figs. 3, A–G; and S3, A–F), supporting our model in which CD63-GFP traffics first to the iLEs and subsequently to mMVBLs.
upon mating (Fig. 5 K), consistent with the reduced secretion of this exosome marker. An alternative explanation for these results is that ESCRT knockdown affects all secretion by SCs. However, the SC-specific production (Rylett et al., 2007) of the secreted, nonexosomal protease ANCE (Figs. 5 J and S4, B–D), its secretion into the AG lumen (Fig. S4, E–G), and its transfer into females (Fig. 5 K) were not affected by ALiX or Hrs knockdown, showing that these treatments selectively affect the exosome biogenesis pathway in SCs.

To provide further evidence that SCs produce CD63-positive exosomes, we modulated the activities of three Rabs previously implicated in exosome secretion: Rab11 (Savina et al., 2002; Koles et al., 2012; Beckett et al., 2013), Rab35 (Hsu et al., 2010), and Rab27 (Ostrowski et al., 2010). Expression of dominant-negative Rab11-YFP (Rab11DN; Zhang et al., 2007), Rab11-RNAi, Rab35-RNAi, and one of two Rab27-RNAi’s significantly reduced the number of secreted GFP-positive puncta (Figs. 5 A and S5, A and B). Previous studies have indicated that Rab35 and Rab27 do not regulate secretion of exosomes carrying the cargo receptor Evenness Interrupted (Evi) in Drosophila S2 cells (Koles et al., 2012; Beckett et al., 2013), suggesting multiple exosome secretion routes in flies as well as mammals. Indeed, expression of two different RNAi’s targeting Evi, which is required to produce Wingless-containing exosomes (Beckett et al., 2013) but is not required for CD63-GFP-positive exosome secretion in flies (Gross et al., 2012), did not affect the number of secreted puncta in the gland lumen (Fig. 5 A). Finally, the number of secreted puncta was also significantly reduced when Rab7-RNAi or dominant-negative Rab7-YFP (Rab7DN; Zhang et al., 2007) was expressed in SCs (Fig. 5 A), implicating late endolysosomal trafficking events in secretion. Based on these extensive genetic data, Endosomal sorting complex required for transport (ESCRT)-dependent secretion of exosomes in SCs

To assess whether CD63-GFP-positive puncta in the AG lumen are exosomes derived from mMVBLs of SCs, we specifically blocked expression or function of a broad range of molecules in SCs, which in other systems are implicated in exosome biogenesis. The ESCRT complex has well-characterized roles in ILV formation, although ESCRT-independent mechanisms have also been reported (Trajkovic et al., 2008). Hrs is a component of the ESCRT-0 complex directly involved in protein sorting into ILVs and ILV formation through subsequent recruitment of downstream ESCRTs (Lloyd et al., 2002; Katzmann et al., 2003). Several recent studies demonstrate a role for Hrs in ESCRT-dependent exosome secretion in mammals and flies (Tama et al., 2010; Gross et al., 2012; Colombo et al., 2013). ALiX (ALG-2–interacting protein X) interacts with several ESCRT complex proteins (Matsuo et al., 2004; Odorizzi, 2006). Recent knockdown studies in mammalian cells indicate that it is required in exosome biogenesis (Matsuo et al., 2004; Baietti et al., 2012), but this has not been investigated in flies. Fluorescent ILVs inside mMVBLs were almost completely absent in SCs expressing either Hrs-RNAi or ALiX-RNAi constructs, unlike controls (Fig. 5, B–D). The number of CD63-GFP-positive puncta secreted into the AG lumen was also drastically reduced (P < 0.001, n > 9; Fig. 5 A). mMVBLs in ALIX-RNAi– and Hrs-RNAi–expressing SCs were smaller and larger than controls, respectively (Fig. 5 I). These genotypes had normal numbers of iLEs (Fig. 5 H), but Hrs-RNAi–expressing SCs had significantly fewer mMVBLs (Fig. 5 G). Importantly, these ESCRT knockdown SCs still synthesized equivalent levels of the SC-specific CD63-GFP fusion protein by Western blot analysis of whole glands (Fig. 5 J) but transferred much less protein to females upon mating (Fig. 5 K), consistent with the reduced secretion of this exosome marker.

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Figure 5. ESCRTs, Rab GTPases, and BMP signaling regulate the exosome biogenesis pathway. (A) The numbers of CD63-GFP-positive puncta secreted into the AG lumen were significantly reduced in SC>Hrs-RNAi, SC>ALiX-RNAi, SC>Rab11DN, SC>Rab11-RNAi, SC>Dad, SC>Rab7-DN, SC>Rab7-RNAi, SC>Rab27-RNAi1, and SC>Rab35-RNAi males compared with control (number of glands above or in the bars) but not SC>Rab27-RNAi2 and
we conclude that SCs secrete CD63-GFP–positive exosomes whose biogenesis and secretion are regulated by evolutionarily conserved molecular mechanisms.

Interestingly, despite the large number of CD63-GFP–positive exosomes in the AG lumen, they did not seem to fuse with MCs in most of the gland (Fig. S5 C). However, we did observe occasional GFP puncta inside MC neighbors of SCs at the distal tip of each AG (Fig. S5 D). The regionalized nature of this uptake indicates that it may be mediated by contact-dependent transfer between SCs and MCs, rather than transfer via the AG lumen.

**SC exosomes fuse with sperm and interact with epithelial cells in the female reproductive tract**

We investigated whether the interaction between SC exosomes and target cells primarily takes place after mating. SC>CD63-GFP males transferred fluorescent puncta into the lumen of the female reproductive tract during mating (Fig. 6, A–D). Multiply mated males of 11 d and older can sporadically transfer SCs to females (Leiblich et al., 2012). Importantly, all mating experiments performed in our current study were with 4-d-old males; we never observed transfer of fluorescent SCs in >50 mated females that we imaged, indicating that SC transfer is not involved in the phenotypes observed.

Several seminal peptides target specific regions of the female reproductive tract and/or sperm after mating (Peng et al., 2005; Ravi Ram et al., 2005). Isolated human prostasomes fuse with the sperm midpiece in vitro, delivering Ca^{2+} signaling molecules implicated in sperm motility (Fabiani et al., 1994; Park et al., 2011). We also detected colocalization of CD63-GFP and sperm heads in the female reproductive tract after mating (Fig. 6, D and E). Analysis of confocal z series images revealed narrow rings of GFP surrounding the sperm head (Fig. 6 F), as might be expected after exosome–sperm fusion. These events were most commonly observed at 20 min after the start of mating (88% [14/16] mated females; Fig. 6 H) in the anterior portion of the female reproductive tract where sperm and exosomes accumulated, either in the anterior uterus (9/16) or the oviduct (5/16). This fusion frequency is almost certainly underestimated: first, because CD63-GFP is likely to rapidly diffuse and dissipate over the sperm surface after fusion, so the GFP fluorescence signal will rapidly become undetectable, and second, because there may be additional fusion to sperm tails. Although tools were not available to fluorescently colabel exosomes and sperm tails, pairs of short parallel fluorescent lines or extended irregular lines of GFP fluorescence were always seen in the female reproductive tract lumen (100%, n = 8; Fig. 6, F and G; and Video 3), which might represent such events. We did not observe CD63-GFP in sperm storage organs, though as discussed previously in this paper, GFP may dissipate after fusion to sperm before entering these structures.

Interestingly, we noted accumulation of CD63-GFP exosomes at the surface of the reproductive tract either in the uterus or oviduct in 40% of females (Fig. 7; n = 34), suggesting that SC exosomes also interact with female epithelial cells. In some cases, fluorescent aggregates made contact with the epithelium (Fig. 7 C and Video 4), whereas in others, dispersed puncta aligned along the epithelial surface (Fig. 7 D). These interactions may reflect docking to female cell targets, either before internalization via endocytosis (Morelli et al., 2004) or possibly as part of a cell surface signaling mechanism. In summary, SC-derived exosomes do not seem to fuse to the AG epithelium in virgin males but do fuse to sperm and interact with female epithelial cells after mating.

**SC exosomes modulate female receptivity to remating**

In contrast to virgin females, recently mated *Drosophila* females will reject a courting male (Wolfner, 1997). We have previously shown that BMP signaling in SCs is required for full induction of this female postmating behavior (Leiblich et al., 2012). We hypothesized that SC-derived exosomes might be involved in this function. Remarkably, blocking exosome production, using SC-specific expression of either *Alix-RNAi* or dominant-negative Rab11, reduced the ability of males to prevent female remating (Fig. 8 A). Importantly, and similar to our observations when BMP signaling is blocked in SCs (Leiblich et al., 2012), we did not see any significant effect on egg laying, fecundity, or mating behavior for SC>ALix-RNAi males (mating times, but not egg laying or fecundity, were reduced for Rab11DN; Fig. 8, B–F). Therefore, exosome secretion from SCs appears to be required to fully induce long-term postmating reprogramming of female behavior.

**BMP signaling regulates endolysosomal trafficking and is required for exosome secretion**

Blocking SC function and development affects multiple aspects of fecundity (Minami et al., 2012, Gligorov et al., 2013), but reduced BMP signaling and loss of exosome secretion in...
Figure 6. SC exosomes interact with sperm in females. (A) Schematic of female reproductive tract in D showing mating plug (mp), uterus (ut), oviduct (ovi), and sperm storage organs (spermatheca [spt] and seminal receptacle [sr]) as well as the autofluorescent hindgut (HG). (B and C) w^{1118} female reproductive tract dissected 20 min after mating to either a control w^{1118} (B) or SC>CD63-GFP (C) male, imaged under identical confocal settings. Images show lumen of the female uterus (asterisks) containing sperm whose heads are visible with DAPI staining (arrowheads). (D and E) Female reproductive tracts 20 min after mating to a protamineB-RFP; SC>CD63-GFP male. (D) ProtamineB-RFP (prot-RFP), which marks sperm heads, accumulates in the anterior uterus (red arrowhead) and oviduct (red arrow). Most CD63-GFP–positive exosomes localize to the posterior uterus (green asterisk), but they can also be seen in the anterior uterus (green arrowhead) and oviduct (green arrow). The autofluorescent mating plug (closed asterisk) is shown. (E) CD63-GFP–positive exosomes colocalize with sperm heads marked by protamineB-RFP (open arrows). (F) Orthogonal view of a z stack through near-complete ring of CD63-GFP fused to ProtamineB-RFP sperm head (arrows). The confocal image below the blue line shows part of the female reproductive tract lumen. The two images above the blue line, separated by a white line, are green (top) and red/green (bottom) z stacks captured in the z plane marked in the bottom image. Note multiple pairs of parallel GFP-positive lines (arrowheads), potentially produced by sperm tail fusion. (G) These (arrowheads) and irregular extended lines of fluorescence (arrow) are also seen in G, which shows a maximum 3D projection image of a z stack from a female anterior uterus and oviduct where the epithelium is marked by actin>CD8-RFP (also shown in Video 3). (H) Female reproductive tracts were dissected and fixed at specific times after the start of mating to SC>CD63-GFP males. The frequency of exosome–sperm interaction events within the female reproductive tract was analyzed (n = 8 for each time point after the start of mating [ASM]). Only fusions to the sperm heads are included. Bars: (B and C) 50 µm; (D) 200 µm; (E and F) 5 µm; (G) 20 µm.
these cells specifically modulate female remating behavior. One possible explanation is that BMP signaling controls exosome production. Remarkably, antagonizing BMP signaling by expression of the negative regulator Dad almost completely blocked CD63-GFP–labeled exosome secretion (Fig. 5 A), strongly supporting this idea. Unfortunately, it was not possible to test the effect of elevated BMP signaling on exosome secretion by expression of a constitutively activated form of the BMP type I receptor Thickveins (Tkv ACT, containing a Q199D substitution; Hoodless et al., 1996). The hypertrophic SCs induced by expression of this construct protrude into the AG lumen and shed large amounts of GFP-positive cellular debris (Leiblich et al., 2012), interfering with exosome counting.

We tested whether BMP signaling might modulate exosome secretion by altering endolysosomal trafficking in SCs, using live imaging of CD63-GFP–expressing cells. In contrast to the effect of ALiX and Hrs knockdown (Fig. 5, C and D), mMVBLs in Dad-expressing SCs contained unusually high levels of internalized CD63-GFP (Fig. 5 D), indicating that BMP signaling is not required for ILV formation but must control a key trafficking and/or maturation event from the mMVBL to the plasma membrane and lysosomes, where GFP fluorescence would be lost. Indeed, SCs overexpressing Tkv ACT had significantly fewer, but much larger, mMVBLs (P = 0.04; Fig. 5, G and I) with no intraluminal fluorescence, supporting this model. Numbers of iLEs were elevated (Fig. 5 H), and more of these cells than control cells (17/19 versus 16/31; P = 0.003, Fisher’s exact test) contained multiple SVs clustered around the enlarged mMVBL, consistent with the idea that there is increased bulk trafficking into the large acidic compartment. We therefore conclude that BMP signaling controls a key endolysosomal trafficking step in SCs required for exosome secretion, and this potentially explains the role of SC-specific BMP signaling in modulating female postmating behavior.

Because BMP signaling drives SC growth, as measured by relative size of SC versus neighboring MC nuclei (Leiblich et al., 2012), we tested whether exosome formation or secretion might be involved in this. Interestingly, although nuclei of SCs expressing either ALiX-RNAi or Rab11DN were reduced in size, nuclear size was increased in Hrs-RNAi–expressing SCs (Fig. 9). This suggests that ESCRT-dependent ILV formation is not required for SC growth.

Figure 7. **SC exosomes interact with female epithelial cells.** (A) Schematic of female reproductive tract shown in B. mp, posterior mating plug (autofluorescent); ut, uterus; sr, seminal receptacle; spt, spermathecae; ovi, oviduct. (B–D) actin>CD8-RFP–expressing (B and C) or wild-type (D) females mated to SC>CD63-GFP males. (B) CD63-GFP exosomes are found in the uterus (green arrow) and oviduct (green asterisk). (C and D) These exosomes accumulate at the apical surface of female reproductive tract epithelial cells (arrows), which are either marked with CD8-RFP (C, Video 4) or unmarked (D). DAPI stains nuclei. In D, the reproductive tract is stained with an antibody against human CD63 to confirm that GFP and CD63 colocalize. Specific spermathecal cells exhibit weak autofluorescence. Bars: (B) 200 µm; (C) 20 µm; (D) 5 µm.
**Discussion**

Seminal fluid synthesized by male reproductive glands has a powerful influence on fertility, affecting multiple sperm activities and altering female behavior, in some cases directly conflicting with female reproductive interests. Several previous studies have revealed an important function for seminal peptides in *Drosophila* (Chen et al., 1988; Wolfner, 1997) and mammals (Park et al., 2011; Kershaw-Young et al., 2012) in these processes. However, in this study, we present the first in vivo evidence that exosomes also play a key role and identify a completely novel role for BMP signaling in regulating this process.

**SCs secrete exosomes: A new in vivo model to study exosome biogenesis**

Exosome biogenesis, secretion, and uptake have been previously studied in *Drosophila* (Gross et al., 2012; Koles et al., 2012; Beckett et al., 2013). However, the small size of exosomes, MVBs, and fly tissues makes these processes difficult to analyze in vivo. The AG contains only nanoliter volumes of secretions, making it impractical to use standard exosome analysis techniques, such as ultracentrifugation and Nanosight Tracking Analysis (Vlassov et al., 2012). Like other studies in flies, we used genetic and imaging approaches to test the identity of SC-specific CD63-positive puncta. In addition, we used Western blot analysis of transferred seminal fluid and live imaging of giant MVBs in SCs to test our hypothesis that SCs produce exosomes.

We primarily used the human CD63-GFP tetraspanin marker in our analysis. However, GFP-positive puncta were also observed in large secretory compartments of SCs expressing cytosolic GFP, and exosome-sized vesicles in MVBs and the AG lumen were observed in EM analysis of wild-type glands (Fig. S2), confirming their presence in nontransgenic flies. Because exosomes can be loaded with many cellular components, our findings provide a potential explanation for the observation that AGs of several insects, including *Drosophila*, secrete intracellular proteins (Walker et al., 2006; Sirot et al., 2011).

Other evidence strongly supports the idea that CD63-positive puncta secreted from SCs are exosomes and not vesicles shed from the plasma membrane. This includes the observation that CD63-positive puncta are found inside both acidic Rab7-positive MVB-like compartments as well as nonacidic Rab11-positive vacuoles and require the ESCRT and ESCRT-associated proteins Hrs and ALiX, as well as several Rabs linked to mammalian exosome secretion, to be formed and secreted. Secreted puncta counts have been used previously in flies to study genetic control of exosome secretion (Gross et al., 2012). A criticism of this approach is that reduced puncta numbers may merely reflect aggregation. However, the transfer of CD63-GFP to females was drastically reduced in mutant backgrounds, arguing...
et al., 2012 versus Colombo et al., 2013), Hrs (Edgar et al., 2014), and Evi (Beckett et al., 2013). If different exosome subtypes are made in SCs, these cells should offer an ideal system to study their differential regulation.

The remarkably large size of endosomal compartments in SCs provides new opportunities to study exosome biogenesis in vivo. To date, many studies of the intracellular exosome against a simple aggregation model. Furthermore, because genetic manipulation of ESCRT function does not alter other secretory processes in SCs (Figs. 5 K and S4), this strongly implicates the endocytic pathway in secretion of tagged CD63.

Studies of exosomes in Drosophila as well as mammals already suggest that multiple exosome subtypes exist and may be regulated differently, e.g., different roles for ALiX (Baietti et al., 2012 versus Colombo et al., 2013), Hrs (Edgar et al., 2014), and Evi (Beckett et al., 2013). If different exosome subtypes are made in SCs, these cells should offer an ideal system to study their differential regulation.

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Figure 9. **ALiX and Hrs have different effects on SC growth.** (A) Growth of SCs relative to MCs in 6-d-old males expressing different transgenes in SCs, as measured by the ratio of SC to MC nuclear size and normalized to controls. Values inside bars show number of males tested (n). DN, dominant negative. (B–D) SCs in 6-d-old males expressing either GFP linked to a nuclear localization sequence (GFP<sup>NLS</sup>) alone (B) or in combination with either Hrs-RNAi (C) or ALiX-RNAi (D), under the control of the *esgf/O*<sup>+</sup> driver. Control SC nuclei are smaller than MC nuclei at eclosion but grow to be larger by 6 d (B). Data for A were analyzed using the Student’s t test or Mann–Whitney U test (*, P < 0.05; **, P < 0.01; ***, P < 0.001, n = 10) after a Shapiro–Wilk test for normality. Error bars indicate ±SE. Bars, 10 µm.

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biogenesis machinery and endolysosomal trafficking in higher eukaryotes have relied on expressing an activated form of Rab5 (Stenmark et al., 1994; Hirota et al., 2007; Wegner et al., 2010; Dores et al., 2012; Schroeder et al., 2012) or addition of the ionophore monensin (Savina et al., 2002) in cell culture to artificially enlarge the endolysosomal compartments, disrupting normal trafficking events. Hence, our new SC in vivo model should allow us to reinvestigate previously reported regulators of exosome biogenesis and identify functional differences in trafficking phenotypes, as we have seen for Hrs and ALIX.

Our study has already revealed a surprisingly dynamic interaction between the secretory and endolysosomal systems in SCs. Communication between these compartments using vesicular transport and tubulation processes has been reported in other cell types in flies and mammals (Savina et al., 2005; Minogue et al., 2006; Burgess et al., 2012), but our study suggests that direct fusion can also be involved. Indeed, our data are also consistent with mMVBLs forming after fusion between SVs and iLs (Fig. 2 D), suggesting that fusion events may play a critical role in establishing distinct compartments within SCs. In light of this dynamic flux between compartments, it remains unclear whether CD63-GFP–labeled exosomes might be released by the classical route involving mMVBL fusion to the plasma membrane or via an intermediate secretory compartment.

**SC exosomes have complex physiological functions**

Although most analysis of the fly AG has highlighted roles for MC products, such as SP (Chen et al., 1988; Chapman et al., 2010; Dores et al., 2012; Schroeder et al., 2012) or addition of the ionophore monensin (Savina et al., 2002) in cell culture to artificially enlarge the endolysosomal compartments, disrupting normal trafficking events. Hence, our new SC in vivo model should allow us to reinvestigate previously reported regulators of exosome biogenesis and identify functional differences in trafficking phenotypes, as we have seen for Hrs and ALIX.

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**SC exosomes have complex physiological functions**

Although most analysis of the fly AG has highlighted roles for MC products, such as SP (Chen et al., 1988; Chapman et al., 2010), in reprogramming female postmating responses, several recent studies have also suggested a central but poorly defined function for SCs (Leiblisch et al., 2012; Minami et al., 2012; Gligorov et al., 2013). A transcriptional program regulated by the Hox gene Abd-B controls vacuole formation in SCs (Gligorov et al., 2013). Our findings now indicate that at least one of the effects mediated by SCs, altered receptivity to remating, requires exosome secretion.

It is difficult to accurately estimate the frequency of SC exosome–sperm fusion events in each female fly because they can probably only be visualized transiently, and many may involve fusion to the very long sperm tail. Sperm play an essential role as mediators of SP-dependent postmating effects in females (Liu and Kubli, 2003; Peng et al., 2005), so it is plausible that exosome fusion to sperm may modulate specific SP functions. Another appealing hypothesis is that SC exosomes also interact with the female reproductive tract to influence female behavior. However, whatever the target tissues, our data clearly demonstrate a role for SC exosomes in female reprogramming. Furthermore, like human prostasomes (Arienti et al., 1997; Park et al., 2011), SC exosomes fuse with sperm, highlighting possible conserved roles for exosomes in male reproductive biology. In prostate cancer, prostasomes are inappropriately secreted into the bloodstream (Sahlén et al., 2004; Tavosidiana et al., 2011), so that other cells in the body may be subjected to these powerful reprogramming functions, potentially supporting tumor–stroma interactions and metastasis (Ronquist et al., 2010; Ge et al., 2012).

**BMP-dependent regulation of endolysosomal trafficking and exosome secretion**

Reducing BMP signaling in SCs inhibits exosome secretion and leads to the formation of a novel mMVBL compartment that is filled with fluorescent CD63-GFP. A simple interpretation of this result is that MVBL compartments in these cells do not mature properly, blocking exosome secretion. Consistent with this, increasing BMP signaling in these cells produces a highly enlarged acidic compartment.

Previous studies have shown that blocking endosomal maturation by knockdown of the early ESCRT component Hrs increases the size of immature endosomal class E compartments lacking ILVs and also results in increased BMP signaling (Piper et al., 1995; Jékely and Rørth, 2003; Chanut-Delalande et al., 2010). Our data demonstrate that elevated BMP signaling increases mMVBL size, suggesting that there is a complex bidirectional interaction between mMVBL maturation and size and the level of BMP signaling in SCs.

Our findings are consistent with a model in which BMP signaling also controls SC growth by driving endolysosomal trafficking and maturation events. Late endosomes and lysosomes have previously been shown to house major nutrient sensors and cell growth machinery, including the mTORC1 complex (Goberdhan, 2010; Ögmundsdóttir et al., 2012), which is activated by intraluminal amino acids. Interestingly, the growth rate of knockdown cells with reduced ESCRT function appears to correlate with mMVBL size (Fig. 5 I) rather than exosome secretion rate. We now need to test whether growth in these cells is mTORC1 dependent.

Whatever the explanation for the growth defects in SCs, our data very clearly implicate BMP signaling in the regulation of endolysosomal trafficking and exosome secretion. It will now be important to test whether BMP signaling plays a similar role in mammalian glands that secrete exosomes, such as prostate and breast, and determine whether this role is affected in diseases such as cancer.

**Materials and methods**

**Flies stocks**

The following stocks were used: esgf/"O" (esg-GAL4 tub-GAL80° UAS-FLP/CyO; UAS-GFP.0, actin>FRT>CD2>FRT>GAL4/TM6 (Jiang et al., 2009); a gift from B. Edgar, Center for Molecular Biology, University of Heidelberg, Heidelberg, Germany; esg-GAL48797, tubP-GAL80°, P(UAS-FP1.D), P(UAS-GFP.nls), and actin>FRT>CD2>FRT>GAL4/TM6 are described in FlyBase), UAS–CD63-GFP (Panáková et al., 2005; a C-terminal fusion of full-length human CD63 with eGFP-C1), which was then inserted into the pUAST vector [Brand and Perrimon, 1993]; see FlyBase; a gift from S. Eaton, Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany), tубrab11-YFP, tubrab17-YFP and tубrab15-YFP (gifts from J.-P. Vincent, Medical Research Council National Institute for Medical Research, London, England, UK), Acp26Aa-GAL4 (Chapman et al., 2003; ~1.4kb fragment upstream of the Acp26Aa coding region fused to GAL4; a gift from M. Wollner, Cornell University, Ithaca, NY), dsx-GAL4 (Rideout et al., 2010; a GAL4 gene trap inserted into the first codon of the dix gene; a gift from S. Goodwin, University of Oxford, England, UK), sp-GAL4 (Hayashi et al., 2002; sp[^2][2010]; see FlyBase), UAS–ALIXRNAi [RNAi1, v32047 [Vienna Drosophila Resource Center]; RNAi2, HMS00298 [Bloomington Drosophila Stock Center] containing nonoverlapping target sequences; Dietz et al., 2007; Ni et al., 2008), UAS–HrsRNAi [RNAi1, HMS00841 [Bloomington Drosophila Stock Center]; RNAi2, HMS00842 [Bloomington Drosophila Stock Center]; Ni et al., 2008], UAS–YFP.Rab11.S25N (Zhang et al., 2007; see FlyBase),
which expresses only in SCs within the AG, was used in all experiments
Vienna Drosophila Resource Center. A UAS-TkvQD
and analysis of ANCE staining in the AG lumen (Figs. 5 and S4, respec-
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polyclonal rabbit anti-Rab7 (1:3,000; Tanaka and Nakamura, 2008; a gift from A. Nakamura, RIKEN Center for Developmental Biology, Kobe, Japan), and rat anti-ANCE (1:2,000; Rylott et al., 2007; a gift from E. Isaac, University of Leeds, Leeds, England, UK). All washes were performed at room temperature (22°C). Fluorescent GFP puncta were more readily observed in the female reproductive tract, perhaps because the luminal microenvironment (e.g., pH) affects fluorescence levels.

Western blot analysis
Protein extraction. For male AG samples, 3-d-old virgin dsx-GAL4; tubGal80°, UAS-CD63-GFP males (incubated at 29°C from eclosion) expressing UAS-
ALiX-RNAi; UAS-Hrs-RNAi, or UAS-Rab11DN constructs were dissected in ice-cold PBS. w1118 virgin males were used as a negative control.

For female samples, these males were introduced to single w1118 virgin females, and matings were observed. The whole female reproductive tracts with the ovaries removed were dissected in ice-cold PBS immediately after the end of mating. w1118 virgin females and w1118 females mated to w1118 males were used as a negative controls.

AG proteins were extracted by homogenizing the glands using a polycarbonate pestle on ice in 100 µl lysis buffer (radioimmunoprecipitation assay buffer; Sigma-Aldrich) supplemented with anti-protease and anti-
tiphosphatase cocktails (Sigma-Aldrich). The crude lysates were centrifuged briefly (300 g; 5 min), and the protein-containing supernatant was extracted. Proteins were quantified using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific).

Blotting. Blotting of AG proteins was performed as previously described (Rylott et al., 2007), with minor alterations; the protocol is described in this paragraph. Samples containing 2.5 µg homogenized protein (made up to 15 µl) were added to 5 µl of 4x sampling buffer (0.2 M Tris-HCl, pH 6.8, 12% SDS, 40% glycerol, 20% β-mercaptoethanol, and 0.008% bromophenol blue) and heated to 95°C for 5 min. Proteins were separated using 10% mini-Protean TGX precast gels (Bio-Rad Laboratories) for 30 min along with a prestained ladder (Precision Plus Protein Dual Color Standards). Separated proteins were transferred to a polyvinylidene fluoride membrane (Immobilon-P, EMD Millipore) at 100 V for 1 h. The polyvinylidene fluoride membrane was then blocked for 1 h in 5% skimmed milk in TBS with Tween 20 (TBST) and then incubated with the anti-ANCE antibody (Rylott et al. 2007) at a 1:1,000 dilution, anti-GFP at 1:500 (rabbit; abcam, Abcam), or anti-actin 1:10,000 (rabbit; 04-
1040; EMD Millipore) in 5% milk in TBST overnight at 4°C. The membrane was washed in TBST for 3 × 5 min, incubated at room temperature for 1 h with a HRP-conjugated anti-rabbit secondary antibody (W4018; Promega) at 1:20,000 in 5% milk in TBST, and then washed in TBST for 3 × 5 min. Bound anti-ANCE, anti-GFP, or anti-actin antibody was detected using a SuperSignal West Fico Chemiluminescent Substrate kit (Thermo Fisher Scientific) as described in the manufacturer's instructions. Photographic films were exposed to the membrane for 30 s to 1 min before film development. Band intensities were calculated using ImageJ (National Institutes of Health).

Imaging
Live and fixed samples were imaged on a scanning confocal microscope (LSM 510 Meta [Axioplan 2]; Carl Zeiss), using LSM 510 Meta software. Low magnification images (Figs. 1, A and B; 6 D; and 7 B) were obtained using the 10x dry objective (0.45 NA Plan Apochromat); all other confocal images were obtained using the 63x objective (1.4 NA oil differential interference contrast Plan Apochromat) with immersion oil (Carl Zeiss). All live imaging was performed at 16°C. Fixed imaging was performed at 21°C. Details of fluorochromes used in individual experiments are described in the immunostaining procedure and figure legends.

Largest mMVBL diameter was measured on ImageJ by selecting the widest diameter of the largest compartment in a confocal z series. Quantification of CD63-GFP puncta (Figs. 5 A and S4 A) were performed using ImageJ particle analysis. Fluorescence intensity analysis of content (Fig. S4 G) was performed using Photoshop (Adobe) software (gray intensity mean). Time series Videos 1 and 2 were recorded using LSM Image Browser after cropping video frames. Maximal projection image processing was performed on ImageJ, and rotating 3D projections shown in Videos 2 and 4 were recorded using the ImageJ 3D Viewer to process confocal series images. All other confocal images were processed using LSM Image Browser.

For exosome lumen counts and ANCE staining intensity quantification, three stacked confocal images of the lumen were obtained at 5-µm intervals within the central third of each gland at a 1.7x zoom, using the 63x objective. Glands were imaged at gain settings to minimize background noise for exosome staining, for exosome control, and for nuclear size, were repeated at a lower gain setting (Fig. S4 A) to confirm that decreased numbers of puncta did not represent a reduced number of more intensely fluorescent structures.

Video 2 depicts a live-imaging sequence showing an acidic mMVBL fusing to a nonacidic SV in SCs (Fig. 4 A). Video 2 depicts a live-imaging sequence showing invagination of a CD63-GFP–positive membrane from the mMVBL limiting membrane (Fig. 4 B). Video 3 shows a rotating 3D projection of the image shown in Video 4. Videos 3 and 4 show a rotating 3D projection of the image in Fig. 7 C. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201401072/DC1. Additional data are available in the JCB DataViewer at http://dx.doi.org/10.1083/jcb.201401072.dv.

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Figure S1. SCs and not MCs secrete CD63-positive puncta. (A and B) Transverse sections of AG (in an equivalent focal plane to Fig. 1 C) showing that CD63-GFP is secreted in puncta (arrows; see F for higher magnification image at higher gain setting) into the AG lumen (asterisks) when expressed by SCs (A) but not MCs, imaged here in the middle of the gland where no SCs are present (B). (C) Puncta containing cytoplasmic GFP can also occasionally be seen inside SC vacuoles (arrows) in the surface section. (D and E) CD8-RFP localizes either within (asterisk in D) or at the limiting membrane of large vacuoles (surface section in D) but is not secreted into the AG lumen (asterisk, transverse section in E). (F) Many CD63-GFP-positive puncta secreted by SCs into the AG lumen are detected when the sensitivity and resolution of confocal imaging is increased. (G and H) The w^{1118} (non-GFP marked) control SC (G), imaged live, contains two large (≥2 µm, asterisks) and several small (<2 µm, arrowheads) acidic compartments, which stain strongly with LysoTracker red, similar to CD63-GFP-expressing SCs (H). Accurately counting these compartments is not possible because their limiting membranes are not marked. All images show SCs from 3-d-old males incubated at 28.5°C after eclosion. Fixed cell nuclei are stained with DAPI. Approximate outline of cell is marked in D, G, and H. Bars: (A, B, and F) 10 µm; (C–E, G, and H) 5 µm.
Figure S2. SCs have large acidic compartments containing internalized CD63-GFP. (A) A CD63-GFP–expressing SC stained with anti-ANCE and anti-CD63 antibodies reveals one compartment that is filled with CD63 but has low GFP fluorescence (arrows; insets show higher magnification images of boxed regions that include this compartment). This compartment does not contain a dense ANCE core (arrows). (B and C) Transverse section through the lumen of an AG containing CD63-GFP–expressing SCs stained with anti-CD63 and anti-GFP antibodies. Many CD63-GFP fluorescent puncta in the AG lumen colocalize with the anti-CD63 antibody (e.g., arrows in C). C is a higher magnification image of the square outlined in B. (D) Live image of an SC from a tub-PI4KII-GFP fly stained with LysoTracker. The late endosome and lysosome marker PI4KII localizes to the limiting membrane of large acidic compartments (asterisks) as well as tubulations extending between them (arrows), which have been observed previously in Drosophila salivary gland cells (Burgess et al., 2012). (E–I) Electron micrographs of virgin w^{1118} male AGs. MCs (apical membranes marked with red arrowheads in E) make up the majority of the monolayered epithelium; SCs (boxed in E) are dispersed between MCs, bulge into the lumen, and can be seen in transverse (e.g., cell in H) or surface (e.g., cell protruding into lumen in F) section. SCs contain SVs, comprising an electron-dense core and fibrillar material (F and H, green arrows) and one or two large and less electron-dense, non-SV compartments (F and H, asterisks; H shown at high magnification in I), which by a process of elimination, must represent the large acidic compartments seen in live confocal imaging. The AG lumen contains a range of secreted structures (E), including putative membrane-bound vesicles (G, closed arrows and arrowheads; red arrowheads mark apical surface of epithelium), some of which (G, closed arrows; boxed area enlarged in inset) resemble the ∼40-nm vesicles (I, arrows; enlarged in inset) observed inside the large multivesicular compartments in SCs (I, asterisk). These compartments therefore appear to be the counterparts of much smaller mammalian MVBs. F and H are higher magnification images of the boxed regions in E; G and I show higher magnification images of the regions boxed in F and H, respectively. All images show SCs from 3-d-old males incubated at 28.5°C after eclosion. Note that despite attempting several fixation protocols, we were unable to prevent spaces forming at high frequency between SC and MC plasma membranes in the epithelium (# in H). Preservation of compartment membranes was also not optimal (e.g., blue arrowhead in I). Approximate outline of cell is marked in A and D. Bars: (A [main images and insets] and D) 5 µm; (B) 50 µm; (C) 10 µm; (E, F, and H) 2 µm; (G and I, main images) 200 nm; (G and I, insets) 100 nm.
Figure S3. Analysis of CD63-GFP trafficking and internalization in mMVBs. Images from Fig. 3 showing individual color channels: an 8-h pulse of CD63-GFP (induced by inhibition of GAL80 at 28.5°C) was chased at 18°C for 0–60 h, and proportions of cells with one or more LysoTracker red–positive iLEs (arrows in A, C, and E) and mMVBs (asterisks in B, D, and F), which were CD63-GFP positive, were determined. The rate of CD63 traffic into endosomes is not identical in all cells (Fig. 3 G). Note that when the temperature is shifted down at the end of the pulse, transcripts encoding CD63-GFP are not immediately degraded, so new protein will continue to be made, potentially explaining the persistence of GFP fluorescence in SVs and iLEs. Approximate outline of cell is marked. Bars, 5 µm.
Figure S4. **Blocking ESCRT function in SCs does not affect ANCE secretion.** (A) Relative numbers of SC-expressed CD63-GFP–positive puncta in the AG lumen were quantified for SC>Hrs-RNAi1, SC>ALIX-RNAi1, and SC>Dad males compared with control males at two confocal gain settings that altered absolute counts by approximately twofold. No effect on relative levels was observed. (B–E) ANCE, which normally localizes to the dense core of control SC SVs (B, arrows) and is secreted into the gland lumen (E, asterisks) but is not detectably expressed in MCs, does not show altered subcellular localization in Hrs-RNAi– or ALIX-RNAi–expressing SCs (C and D, respectively, arrows). DAPI stains nuclei (blue). (E–G) There is no significant difference in the level of diffuse staining for ANCE in the AG lumen (E and F, asterisks) when ALIX is knocked down in SCs (F and G), as measured by calculating the mean signal intensities of three stacked confocal images from the AG lumen taken at 5-µm intervals. Control and SC>ALIX-RNAi males were subjected to the same mating protocol as in A, whereas B–D were obtained from virgin males. Data in A and G were analyzed using an unpaired two-tailed Student’s t test (***, P < 0.001, n > 11) after the Shapiro–Wilk test to confirm normality. Error bars indicate ±SE. Bars: (B–D) 5 µm; (E and F) 20 µm.
Figure S5. SC-specific CD63-GFP is transferred to neighboring MCs, but Rab11-YFP is not secreted. (A and B) Analysis of Rab11-YFP and CD63-GFP secretion when expressed in SCs. Unlike CD63-GFP (A, arrows), YFP-tagged Rab11 is not secreted by SCs, even when highly overexpressed under GAL4 control (B). Images show transverse sections including the lumen (A and B, asterisks) and SCs from 3-d-old males. (C) Transverse section showing no obvious interaction can be seen between CD63-GFP-positive exosomes and the apical surface of MCs (arrow; the asterisk marks the lumen) in most of the AG at 3 d. (D) However, GFP is internalized by the MCs surrounding SCs (arrows). discs large (Dlg) marks apically positioned lateral septate junctions between MCs (junctions between MCs and larger SCs are more apical). DAPI stains nuclei. Bars, 10 μm.
Video 1. **Acidic mMVBLs fuse to nonacidic SVs in SCs.** SC from a 2-d-old male expressing CD63-GFP (green) and stained with LysoTracker red was imaged by time-lapse confocal microscopy using a laser-scanning confocal microscope (LSM 510; Carl Zeiss). Frames were taken every 13.6 s. Stills from this video are shown in Fig. 4 A. Video was produced over a 5-min period. Note how CD63-GFP appears to be transferred to the limiting membrane of the mMVBL at the moment of fusion.

Video 2. **mMVBLs contain CD63-GFP–positive invaginations and acidic microdomains inside mMVBLs.** SC from a 2-d-old male expressing CD63-GFP (green) and stained with LysoTracker red was imaged by time-lapse confocal microscopy using a laser-scanning confocal microscope (LSM 510; Carl Zeiss). Frames were taken every 16.3 s. Stills from this video are shown in Fig. 4 B. Note one CD63-GFP–positive invagination appears to form an ILV-like punctum. A line of peripheral acidic microdomains appears after this event either de novo or through imaging a slightly different plane of the section in the mMVBL. The video was produced over a 5-min period.

Video 3. **CD63-GFP exosomes may fuse with unmarked sperm tails in females.** Rotating 3D projection of a confocal z stack including the image shown in Fig. 6 D. A z series was collected using a laser-scanning confocal microscope (LSM 510; Carl Zeiss) and the rotating 3D projection movie was recorded using ImageJ 3D Viewer. Note the presence of extended strands of GFP fluorescence and some parallel pairs of short GFP-positive lines that presumably surround sperm tails to which exosomes have recently fused (marked in Fig. 6 D). The female reproductive tract epithelium is marked with CD8-RFP.

Video 4. **Accumulation of CD63-GFP exosomes along the surface of the female reproductive tract epithelium.** Rotating 3D projection of a confocal z stack including the image shown in Fig. 7 B, highlighting a single GFP-positive interaction site at the epithelial surface that is marked in this figure. A z series was collected using a laser-scanning confocal microscope (LSM 510; Carl Zeiss), and the rotating 3D projection video was recorded using ImageJ 3D Viewer. The female reproductive tract epithelium is marked with CD8-RFP.

**Reference**