Arp2/3-mediated F-actin formation controls regulated exocytosis in vivo

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The actin cytoskeleton plays crucial roles in many cellular processes, including regulated secretion. However, the mechanisms controlling F-actin dynamics in this process are largely unknown. Through 3D time-lapse imaging in a secreting organ, we show that F-actin is actively disassembled along the apical plasma membrane at the site of secretory vesicle fusion and re-assembled directionally on vesicle membranes. Moreover, we show that fusion pore formation and PIP₂ redistribution precedes actin and myosin recruitment to secretory vesicle membranes. Finally, we show essential roles for the branched actin nucleators Arp2/3- and WASp in the process of secretory cargo expulsion and integration of vesicular membranes with the apical plasma membrane. Our results highlight previously unknown roles for branched actin in exocytosis and provide a genetically tractable system to image the temporal and spatial dynamics of polarized secretion in vivo.
Regulated exocytosis is a fundamental cellular process that involves the release of stored secretory cargo from membrane vesicles into the extracellular space. This type of secretion involves the formation of secretory vesicles and their subsequent docking and fusion with the plasma membrane (PM) in response to an external stimulus. Various tissues have different modes of vesicle formation and release, likely due to the different physical and functional properties of the secreted cargo as well as the size/geometry of the secretory vesicles. Regulated secretion is used by specialized tissues to produce and secrete bioactive molecules that mediate diverse functions including neurotransmission, immunity, reproduction and digestion (reviewed in refs 1–3).

Recent studies have highlighted essential, yet often disparate roles for the actin cytoskeleton in regulated exocytosis and in compensatory endocytosis. For example, actin along the cortex of cells acts as a physical barrier to prevent premature fusion of granules with the PM4–6. In Xenopus eggs, where secretory vesicles never integrate into the PM but are maintained as shells, actin and myosin recruitment enable vesicles to be retrieved via compensatory endocytosis7–10. Other studies have demonstrated that F-actin is required for proper vesicle compression and expulsion of cargo11–14 and to regulate the expansion of the fusion pore15. In addition, intravital imaging in rodent salivary glands (SGs) has shown that F-actin is required to regulate both the stabilization and collapse of secretory vesicles after fusion with the PM in vivo16. However, the molecular machinery controlling the formation of specific F-actin structures and the spatial kinetics of actin assembly in the process of cargo expulsion and membrane integration has not been fully defined17.

Actin exists as monomeric units (G-actin) and in a variety of filaments that can be linear, branched or bundled (F-actin). Linear actin filaments are initiated by the formin-family of actin nucleators/elongation factors that are activated by the concerted action of small Rho-GTPases and phosphoinositides18–20. Rho and formin influence actin coat formation on lamellar bodies during surfactant secretion12,13. Branched actin filaments, which are essential for cell polarity and migration21,22, are formed through the coordinated action of the Actin-related protein 2/Actin-related protein 3 (Arp2/3) complex and a variety of nucleation-promoting factors (NPFs), such as WASp, Wash, Whamy and suppressor of cAR (SCAR)23–25. Arp2/3 present on linear actin filaments is subsequently activated by NPFs, which bind Arp2/3 and induce a structural change. Branched actin is then formed at the site of Arp2/3 attachment at a 70° angle from the existing actin filament25,26. Actin nucleators influence glucose-induced biphasic insulin secretion, but their specific effects on actin remodelling in this process are unclear27. The role of branched actin assembly and structure is less well understood in other forms of secretion that involve large secretory vesicles containing bulky cargo3.

The factors that govern regulated secretion have been challenging to decipher, as many mammalian cells lose polarity and secretory capacity once excised and cultured ex vivo28. Additionally, in many systems, the small size of vesicular structures and the lack of markers for cargo impede the ability to image actin dynamics, secretory vesicles and secretion with sufficient resolution. Furthermore, limited genetic tools in many systems have restricted the ability to interrogate the function of specific genes involved in actin formation in vivo.

To investigate the role of actin dynamics and branched actin in secretion, we performed high-resolution, real-time imaging at single-granule resolution in Drosophila SGs, which are amenable to genetic manipulation and contain large secretory granules (3–8 μM in diameter) filled with high-molecular-weight cargo. Using Drosophila lines expressing fluorescently-labelled molecules, we are able to directly image secretory vesicle cargo, apical membrane dynamics and actin and myosin recruitment in real time to define the temporal and spatial sequence of events occurring during regulated exocytosis. By performing 4-dimensional (4D) imaging of actin dynamics during secretion, we show rearrangement of cortical actin at the point of vesicle fusion followed by coordinated recruitment of actin to the fused vesicle from the PM. We further demonstrate that fusion pore formation and redistribution of the phosphoinositide, PIP2, occurs before actin recruitment. By imaging cargo directly, we show that fusion pore formation and cargo secretion are temporally distinct events, with the latter being dependent on actin for completion. Finally, using in vivo RNA interference (RNAi), we identify essential roles for the branched actin nucleators Arp2, Arp3 and their activator, WASp, in mediating cargo expulsion and secretory granule membrane integration with the PM. This study demonstrates a previously unknown role for branched actin nucleators in exocytosis and offers a high-resolution, genetically tractable platform for interrogating the role of any factor involved in actin dynamics and regulated secretion in a living organ in real time.

Results
Spatial and temporal dynamics of regulated secretion. The Drosophila larval salivary system consists of two glands comprised of secretory epithelial cells that connect to a common duct (Fig. 1a). Secretory cells are filled with large secretory granules (3–8 μM in diameter) containing high molecular weight, highly glycosylated mucins (salivary gland secretion proteins or sggs proteins)29,30. Regulated secretion in Drosophila SGs begins with a small pulse of the steroid hormone 20-hydroxyecdysone (20E) during early third instar larval stage. This stimulus induces the expression of the sggs genes that encode the cargo that will be packaged into secretory vesicles; then, a second pulse of 20E during late third instar instructs these newly formed secretory granules to fuse with the apical PM to begin secretion31–33. To image secretion events in real time, we took advantage of Drosophila lines expressing fluorescent markers for secretory cargo (Sgs3-GFP)32, F-actin (Lifect-Ruby, Lifect-RFP or Lifect-GFP)34,35 and the apical membrane (Myr-tdTomato)36. SGs from Sgs3-GFP-expressing larvae were harvested during late third instar and cultured ex vivo. Cultured SGs maintain their polarity and respond to the exogenous addition of 20E, which triggers fusion of the secretory vesicles with the apical PM and release of their contents into the lumen (Fig. 1b). As secretion proceeds, the diameter of the lumen expands as it fills with the Sgs3-GFP cargo (Fig. 1b and Supplementary Movie 1), similar to what is seen in vivo (Supplementary Fig. 1a). (Real-time videos of all still images shown throughout this paper are in the Supplementary Movies section.) Imaging the dynamics of individual secretory granules using Drosophila lines expressing Sgs3-GFP cargo and the apical PM marker Myr-ttTomato revealed apical membrane deformations at the point of vesicle fusion (Fig. 1c). In addition, individual secretory vesicles undergo a slight expansion after fusion with the apical PM, likely due to hydration-related expansion of the mucin cargo. Real-time imaging of secretory cargo revealed that secretion proceeds with complete expulsion of cargo followed by integration of the vesicle membranes with the PM (Fig. 1c and Supplementary Movie 2).

We next imaged the dynamics of actin recruitment to individual secretory granules using Drosophila lines expressing both Sgs3-GFP and Lifeact-Ruby. Secretory vesicles in proximity to the apical membrane are rapidly coated with actin, which precedes vesicle compression and membrane integration (Fig. 1d...
and Supplementary Movie 3). To image actin recruitment around the entirety of the vesicle, we performed three-dimensional imaging in real time (4D imaging) in *Drosophila* lines expressing Lifeact-GFP. Interestingly, 4D reconstruction of actin dynamics showed that cortical actin present along the apical membrane is cleared or disrupted at the site of vesicle fusion, forming a small hole that continues to expand in size (Fig. 1e and Supplementary Movie 4, and 5). This actin clearance is followed by actin recruitment to the secretory vesicle, originating at the PM (Fig. 1e and Supplementary Fig. 1b, c and Supplementary Movie 4, and 5). These 4D reconstructions demonstrate dynamic actin clearance at the PM and subsequent directional actin recruitment to the secretory vesicle from the PM during SG secretion. Taken together, we show secretion of...
mucinous cargo at single granule resolution that involves dynamic rearrangement of both the cortical actin and the apical membrane at the site of secretory vesicle fusion. This process culminates in complete expulsion of cargo and integration of the granular membrane with the PM.

We next addressed the temporal order of membrane fusion (fusion pore formation) and actin recruitment to the secretory vesicles. By infusing a small molecular weight (MW) fluorescent dextran (A647-10 kDa dextran, stoke radius \( \sim 2 \text{ nm} \) (ref. 13)) into the lumens of SGs expressing Lifeact-GFP, we demonstrate that the fusion pore between the vesicle and the PMs forms before F-actin recruitment to the secretory vesicle (Fig. 2a, d and Supplementary Fig. 2a). Indeed, dextran diffusion from the SG lumen into the vesicle was detected before Lifeact-GFP (F-actin) on vesicle membranes (Supplementary Movie 6). The average time difference in the detection of dextran relative to secretory vesicles was 57 ± 18 s (n = 11; \( \pm \) refers to standard deviation). In addition, we did not see any evidence for compound exocytosis (for example, the sequential fusion of vesicles with one another at the PM), consistent with what was previously reported in mammalian SGs16.

Membrane mixing and the redistribution of phosphoinositides are known to regulate actin polymerization and membrane fusion events in other systems37. In particular, the membrane phosphoinositide PI(4, 5)P\(_2\) (PIP\(_2\)) is known to stimulate actin polymerization by regulating the activity of actin nucleators28,39. Therefore, we next examined whether redistribution of PIP\(_2\) occurs during SG secretion by using Drosophila lines expressing both the PIP\(_2\) reporter PLC\(_\alpha\)-PH-EGFP40 and Lifeact-RFP. Indeed, evidence for membrane mixing via PIP\(_2\) movement from the apical PM to secretory vesicle membranes is seen (Fig. 2b,e and Supplementary Movie 7). Interestingly, PIP\(_2\) recruitment precedes F-actin recruitment (by 51 ± 19 s (n = 12)), consistent with a role for PIP\(_2\) in F-actin formation along secretory vesicle membranes (Fig. 2b,e and Supplementary Fig. 2b and Supplementary Movie 7).

In rodent exocrine glands, it was shown that the actin motor non-muscle myosin II is recruited onto the secretory granules, but the temporal sequence of events is not known16. To determine the kinetics of myosin recruitment relative to actin recruitment, we performed time-lapse imaging in flies co-expressing the myosin marker non-muscle myosin II (Zip-GFP)41,42 and Lifeact-Ruby. RNAi to Arp3 substantially decreased Arp3 expression and resulted in granules that fused with the apical membrane (Fig. 3a,b and Supplementary Figs 2a,c and 2d). Arp3, WASp, Whamy and SCAR (but not Wash) each co-localized with phalloidin on granules that had fused with the apical PM, suggesting a role for these factors in secretion (Fig. 4a). We next examined the temporal order of recruitment of the Arp3 complex onto the secreting granules by creating a Drosophila line expressing both GFP-labelled Arp3 (Arp3-GFP) and Lifeact-Ruby. Real-time imaging of Lifeact-Ruby and Arp3-GFP during secretion revealed that F-actin is seen on granules before Arp3 recruitment (Fig. 4b,c, Supplementary Fig. 3b and Supplementary Movie 17). The average time difference in the detection of F-actin relative to Arp3 was 29 ± 14 s (n = 13). As the Arp3 complex is known to bind linear actin and is required for branched actin formation, our results suggest a model where fused vesicles are initially coated with linear actin, which is detected by Lifeact, and then subsequently bound by Arp2/3 to form a branched actin network.

To investigate whether branched actin is required for regulated exocytosis, we next performed in vivo knockdown of components of the Arp2/3 complex in flies expressing Sgs3-GFP or Lifeact-Ruby. RNAi to Arp3 substantially decreased Arp3 expression and resulted in granules that fused with the apical membrane, but failed to secrete cargo and failed to undergo membrane integration (Fig. 5a, Supplementary Figs 6a,b and 7a and Supplementary Movies 18 and 19). Upon loss of Arp3, fused granules continued to enlarge, similar to what was observed in SGs treated with LA. However, unlike LA treatment, no evidence of compound exocytosis was seen, suggesting that linear actin still present on granules may function to block compound fusion. Interestingly, some F-actin (likely linear actin) was still detected on fused granules in the Arp3 RNAi background, although no longer in a homogenous distribution (Supplementary Fig. 6c, d and Supplementary Movies 20 and 21). Likewise, RNAi to Arp2 (which forms a functional complex with Arp3) phenocopied the knockdown of Arp3, adding further support for the role of Arp2/3-mediated branched actin in proper cargo expulsion and membrane integration (Fig. 5b and Supplementary Figs 6a, b and 7b and Supplementary Movies 22 and 23). We next examined the role of the Arp2/3 activator, WASp. RNAi to WASp, an NPF that can activate Arp2/3, also resulted in granules that grew larger in size, failed to expel cargo and failed to undergo membrane integration; these granules also maintained limited and unevenly distributed staining for F-actin (likely detecting linear actin) and did not show evidence of compound fusion (Fig. 5c and Supplementary Figs 6a,b,c and 7c and Supplementary...
Movies 24–27). Finally, large, expanded granules could also be seen in the SGs in the intact pupae in Arp3 RNAi, Arp2 RNAi or WASp RNAi backgrounds (Supplementary Fig. 8), again indicating that our ex vivo SG system faithfully recapitulates what is occurring in vivo. Taken together, our studies demonstrate an essential role for the branched actin nucleators Arp2, Arp3 and WASp in secretory cargo expulsion and membrane integration during regulated exocytosis in vivo (Fig. 5d).

Discussion
Here, we show temporal and spatial dynamics of F-actin formation during regulated exocytosis and define essential roles for the branched actin nucleators Arp2, Arp3 and WASp in cargo secretion and membrane integration (Fig. 5d). By imaging actin dynamics in four dimensions at single granule resolution in a secreting organ, we demonstrate that actin along the PM is dynamically cleared at the point of vesicle fusion. This
observation supports a role for cortical actin acting as a barrier to premature vesicle fusion, as has been proposed for other modes of secretion. Shortly after this cortical rearrangement, actin begins to expand over the secretory vesicle from the site of PM contact. Previous studies imaging actin (as it is involved in exocytosis) in two dimensions suggest that recruitment occurs simultaneously over all regions of the vesicle. However, our 4D renderings provide spatial and temporal resolution that has not been seen previously and show that actin recruitment to the secretory vesicle begins at the apical PM. These dynamic changes in actin organization suggest a highly ordered process whereby factors on the PM and vesicle act in concert to remodel actin to mediate secretory vesicle docking/fusion as well as to reform the actomyosin machinery around the fused vesicle. This mode of actin recruitment may provide the continuity with cortical actin required to generate the appropriate forces needed for cargo expulsion/release, especially in cases of large, highly glycosylated cargo, such as mucins.

Actin recruitment to secretory vesicles was preceded by the formation of the fusion pore between the apical PM and the secretory vesicle membrane. In addition, membrane mixing and redistribution of the lipid membrane, LIDP2, also occurred before F-actin recruitment. As PIP2 is known to mediate actin formation in other systems, our results suggest that this membrane mixing may trigger actin polymerization on secretory granule membranes. Interestingly, actin polymerization appears to occur in two distinct steps, as the recruitment of Lifeact to secretory granules is detected before Arp3 recruitment. As the Arp2/3 complex is known to bind linear actin, this suggests that actin formation on fused secretory granules begins with linear actin formation (detected by Lifeact), which originates from the apical PM. Linear actin is then bound by the Arp2/3 complex, which catalyses the formation of branched actin. Myosin recruitment occurs after actin is present, as has been seen in cell-based systems. Future work will employ this system to temporally dissect the recruitment of other factors involved in secretion.

The ordered recruitment of linear and branched forms of actin suggests essential roles for each in regulated secretion. However, specific roles for branched actin in regulated secretion were previously unknown. Taking advantage of genetic tools, we directly interrogated the role of branched actin in regulated

Figure 3 | Actin is required for secretion and inhibition of compound vesicle fusion. (a) Secreting glands expressing Sgs3-GFP (white) were treated with latrunculin A and individual granule fusion events were imaged in real time. Red boxes denote areas shown as magnified images in b. (b) Drug treatment resulted in compound fusion events between granules that continue to expand and fail to collapse once fused with the apical membrane. Dashed red lines outline secretory granules undergoing expansion and compound fusion events. Representative times series from three independent experiments is shown. (c) Secreting glands expressing Lifeact-Ruby (red) and Zip-GFP (green) treated with latrunculin A show that actin is no longer recruited to granules that have fused with the apical membrane. A representative time series from four independent experiments is shown. All scale bars, 5 μm. Complete movies for each time series can be found in the Supplementary Movie section.

Figure 4 | Arp2/3 and WASp are recruited to granules undergoing secretion. (a) Secreting glands were fixed and stained for Arp3, WASp, Whamy, SCAR or Wash (green) along with phalloidin (red). Merged images are shown in yellow. Representative images from three independent experiments are shown. (b) Secreting glands expressing both Arp3-GFP (green) and Lifeact-Ruby (red) were imaged in real time to show that actin is detected on fused granules before Arp3, suggesting that linear actin first coats fused granules, followed by branched actin formation. A representative time series from 11 independent experiments is shown. (c) Quantification of actin recruitment relative to Arp3 recruitment to fused granules. Representative graph plotting percent fluorescent intensity of each marker as a function of time for an individual secretory event is shown. Quantification was performed on 13 individual secretory events from four independent experiments. Additional graphs are shown in Supplementary Fig. 3b. All scale bars, 5 μm. Complete movies for each time series can be found in the Supplementary Movie section.
secretion by performing RNAi to the genes that regulate its formation. In vivo knockdown of Arp2 or Arp3, both of which are involved in the synthesis of branched actin, resulted in similar phenotypes, where secretory vesicles fused with the PM but were unable to secrete their cargo and unable to undergo membrane integration. These vesicles grew very large and in some instances, separated from the PM and floated back into the cytoplasm. In addition, in vivo knockdown of the NPF WASp resulted in similar phenotypes, identifying it as an additional regulator of exocytosis in this system. Taken together, our results suggest that branched actin is required to generate the force necessary to complete expulsion of the secretory cargo and integration of vesicular membranes with the PM. In the absence of branched actin, the fused secretory vesicles continue to expand in size, likely due to the hydration and expansion of the highly glycosylated mucinous cargo present within. These results have implications for the importance of branched actin formation in other systems such as the digestive tract, where secretion of large, highly glycosylated cross-linked cargo is essential for extracellular matrix formation, microbial interactions and protection of epithelial cell surfaces.

Our results also suggest unique roles for linear actin in regulated exocytosis. LA and CD, which disrupt the formation of both linear and branched actin, resulted in compound fusion of secretory vesicles with one another, which was not seen upon knockdown of Arp2, Arp3 or WASp. This suggests that the linear actin first recruited to the fused secretory vesicle may act as a barrier to prevent the fusion of other secretory vesicles. However, we cannot rule out the possibility that residual branched actin present in our knockdown animals may be able to prevent compound fusion events. Future studies combining fluorescent imaging and electron microscopy of genetically manipulated SGs will aim to identify specific actin structures present on fused secretory granules and how they influence vesicle interactions.

Our study is unique in that we image secretory cargo as a direct indicator of secretion. Previous studies have used visualization of the fusion pore as evidence of cargo secretion. However, in our system, fusion pore formation and cargo expulsion are temporally distinct events, indicating that fusion pore formation alone is not sufficient for secretion of vesicle contents. In addition to the forces mediated by branded actin, other factors may be required for the secretion of large, glycosylated cargos. This system can be used to interrogate other genes that regulate the processing, packaging and release of complex cargos.

In summary, our study highlights coordinated actin clearance and directional recruitment from the PM as well as essential roles for branched actin nucleators in regulated exocytosis in a living, secreting organ. The powerful combination of Drosophila genetics with in vivo and ex vivo imaging allows one to rapidly interrogate the role of factors in many aspects of secretion, including vesicle biogenesis, vesicle movement, fusion with the PM, release of granule cargo and expansion of cargo once in the lumen. Given that many genes and mechanisms involved in secretion are conserved across species, this system can provide insight into the factors that regulate mammalian secretion.

Figure 5 | Arp2/3 and WASp are required for cargo secretion and membrane integration. (a) In vivo RNAi to Arp3 was performed in Drosophila lines that also express Sgs3-GFP (white) and secretion was imaged in real time. Knockdown of Arp3 resulted in granules that fused with the apical membrane but failed to collapse and secrete their contents. A representative time series from three independent experiments is shown. (b) In vivo RNAi to Arp2 was performed in Drosophila lines that also express Sgs3-GFP (white) and secretion was imaged in real time. Loss of Arp2 mimicked the secretion defects seen upon loss of Arp3. A representative time series from three independent experiments is shown. (c) In vivo RNAi to WASp was performed in Drosophila lines that also express Sgs3-GFP (white) and secretion was imaged in real time. Loss of WASp mimicked the secretion defects see upon loss of either Arp2 or Arp3. A representative time series from four independent experiments is shown. All scale bars, 5 μm. Complete movies for each time series can be found in the Supplementary Movie section. (d) Model of temporal and spatial events occurring during regulated exocytosis in Drosophila SGs. Fully formed secretory granules reside in the secretory cells of third instar larval SGs until secretion is triggered by a pulse of 20E. The early events of secretion involve cortical actin clearance along with fusion pore formation and redistribution of apical PIP2 to the vesicle membranes. The exact order of these events is not known. Following these early events, components of the actin cytoskeleton are assembled on fused granules. First, linear actin is recruited followed by the recruitment of the Arp2/3 protein complex, which forms branched actin structures from existing filamentous actin. Actin recruitment occurs in a directional manner originating from the PM. Actin recruitment is followed by recruitment of non-muscle myosin II. Finally, expulsion of cargo from secretory granules and integration of granular membrane with the apical PM is dependent on Arp2- Arp3- and WASp-mediated actin formation.
Methods

Fly strains and genetics. The following Drosophila lines were used: Bloomington #5884 (y w P{Sgs3-GFP}); #5885 (y w P{P3-GFP}); #35544 (y w P{UAS-Lifeact-GFP}); #35545 (y w P{UAS-Lifeact-Ruby}); VIE-19A, #7118 (y w P{UAS-myr-mRFP}); #33222 (w P{10XUAS-IVS-myr-cTnator}; #39723 (w P{UAS-Arp3-IR}); #39693 (y w P{UAS-PLC-BE}); #38562 (w P{UAS-Lifeact-RFP}). RNAi lines to generate expression of dsRNA were performed using flies SG culture and real-time imaging.

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Molecular markers. The prepupae were dissected in Schneider’s Drosophila medium (Gibco) and transferred to glass bottom culture dishes (MatTek) containing 50 μl medium. To enhance RNAi-mediated knockdown, VDRC #60009, which also expresses control of the UAS promoter, was crossed to VDRC #108951 and VDRC #108220. Bloomington stock #6978 (w1118; P{GawB}c135-IR) was used as a negative control. VDRC #60009 and Bloomington #3554 were recombined with Flytrap line #CC01626 (w1118; P{UAS-dicer2w4118}; VIE-19A, y1 w*; P{UAS-Lifeact-RFP}VIE-260B). Bloomington stock #6978 was recombined with Bloomington #3554 to generate Sgs3-GFP, UAS-Lifeact-Ruby. Sgs3-GFP was recombined with Bloomington #3554 to generate Sgs3-GFP, UAS-Lifeact-Ruby. The c135-Gal4 driver was recombined with Zip-GFP, UAS-Lifeact-Ruby to generate c135-Gal4->Zip-GFP, UAS-Lifeact-Ruby. Bloomington #3554 was recombined with Bloomington #39723 to generate UAS-Arg3-GFP, UAS-Lifeact-Ruby. The c135-Gal4 driver was recombined with Bloomington #5885 to generate c135-Gal4->UAS-Arg3-GFP. UAS-Lifeact-RFP crosses were kept on MM (KM Dical, Inc.) at 25°C unless specified otherwise. Crosses to generate expression of dsRNA were performed using flies from a UAS-RG transgenic line and the c135-Gal4->Sgs3-GFP or c135-Gal4->Zip-GFP, UAS-Lifeact-Ruby stocks.

Ex vivo SG culture and real-time imaging. SGs from third instar wandering larvae were dissected in Schneider’s Drosophila medium (Gibco) and transferred to glass bottom culture dishes (MatTek) containing 50 μl medium. To image intact gland images that were actively secreting, the majority of the media were then removed from the dish and a 13-mm, 0.05-μm polycarbonate membrane filter (Sterlitech) was gently placed on top of the gland. The media that were removed from the dish were then placed on top of the membrane filter and the sample was subsequently imaged. For imaging specimens longer than 2 h, 0.5% low melt agarose in Schneider’s Drosophila medium was layered on top of glands and the sample was subsequently imaged. To induce secretion in non-secreting glands, 20-hydroxyecdysone (Sigma) was added to glands in glass bottom dishes to a final concentration of 700 μM and plates were placed in a humidified chamber at room temperature for 90 min. The media from these dishes were then removed, and a glass bottom membrane was gently placed on top of the glands and media were added back to the top of the membrane. For experiments involving fluorescent dextran, SGs from non-secreting third instar wandering larvae were dissected and transferred to glass bottom culture dishes containing 50 μl medium. Alexa Fluor 647, Alexa Fluor 680 or Rhodamine Red-X Dextran (Invitrogen) was subsequently added to the media and incubated with secondary antibodies for 3 h at room temperature on a rocker. Alexa Fluor 488- or Alexa Fluor 647-conjugated secondary antibodies (Invitrogen, 1:50) and TRITC-conjugated phalloidin (Sigma, 1:100) were used. Samples were then washed three times for 15 min each in PBS and mounted in aqueous mounting medium (Electron Microscopy Sciences) and imaged using a Zeiss LSM 700 confocal microscope.

Quantitative RT–PCR. Quantitative RT–PCR was used to determine efficiency of gene silencing when RNAi was induced. cDNA prepared from SGs from third instar wandering larvae of c135-Gal4;Sgs3-GFP->IR crosses or controls (c135-Gal4;Sgs3-GFP->UAS-Arg3-GFP, UAS-Lifeact-RFP) was used in qPCR reactions. Briefly, RNA was isolated using the RNAqueous-Micro Total RNA Isolation Kit (Ambion). cDNA synthesis was performed using iScript cDNA Synthesis Kit (Bio-Rad). QRT–PCR was performed using SYBR-Green PCR Master Mix (Bio-Rad). RNA levels were normalized to 18S rRNA. Values represent mean value ± s.e.m. Significance values were calculated using two-tailed Student’s t-test.

Statistical analyses. Number of replicates used for each analysis is specified in figure legends. To measure percent fluorescence intensity for Lifeact/Dextran, Lifeact/PLC-PH, Lifeact/Zip or Lifeact/Arp3 experiments, Nikon Elements software was used. A rectangular or oval region of interest (ROI) was drawn around individual secreting granules and the Time Measurement analysis tool was used to measure average fluorescent intensity within the ROI for each channel. Values were exported to Microsoft Excel and percent fluorescence intensity was calculated. The percent fluorescence intensity of each channel at each time point was calculated as: % fluorescent intensity = (fluorescent intensity—Fluorescent intensity of T0)/(Fluorescent intensity of T0—Fluorescent intensity of Tmax) × 100. T0 is the time frame at which one of the two channels being measured begins to continually increase in fluorescent intensity. Tmax is the maximum fluorescent intensity value reached for each respective channel being measured. Each graph was plotted from T0 until at least two time points beyond Tmax for each channel. Percent fluorescence intensity values that were not measured were set to 0. Quantification was only performed on granules that did not move within or through the imaging plane during the entire process of secretion. To determine the average time difference in the detection of each component in the pairwise comparisons, we calculated the time between T0 and when fluorescent intensity of the second component began to continually increase and was at least 0.5% higher than the previous time point. Measurements were performed on 11 granules for Lifeact/Dextran, 12 granules for Lifeact/PLC-PH, 11 granules for Lifeact/Zip and 13 granules for Lifeact/Arp3. Averages were calculated for each pair and are expressed as mean values ± s.d. To measure fold change in size of secreting Sgs3-GFP granules, Fiji (http://fiji.sc/Fiji) was used to outline and measure the area of individual secreting granules as a function of time after fusion. Granule fusion with the apical membrane correlates with a change in fluorescent intensity of the fusing granule. Time 0 represents the frame immediately before a change in fluorescent intensity of a given granule is detected. Values were exported to Microsoft Excel and fold change in granule size was calculated. Graphs represent mean values of all granules measured ± s.d. Quantification was only performed on granules where fusion with the apical membrane could be detected and could be clearly visualized throughout the time frame of analysis. No statistical method was used to predetermine sample size.

Image analysis. Microscopy images were opened in Fiji and individual frames were exported as still images or Movies. Movies were subsequently formatted using Adobe Final Cut Pro, Adobe Media Encoder or Adobe Quicktime software. Images of the same frame software were then transferred to Photoshop for the final image experiment. Adobe Illustrator was used to generate figures from individual still images.
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