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The exocyst component Sec5 is present on endocytic vesicles in the oocyte of *Drosophila melanogaster*

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The exocyst is an octameric complex required for polarized secretion. Some components of the exocyst are found on the plasma membrane, whereas others are recruited to Golgi membranes, suggesting that exocyst assembly tethers vesicles to their site of fusion. We have found that in *Drosophila melanogaster* oocytes the majority of the exocyst component Sec5 is unexpectedly present in clathrin-coated pits and vesicles at the plasma membrane. In oocytes, the major substrate for clathrin-dependent endocytosis is the vitellogenin receptor Yolkless. A truncation mutant of Sec5 (*sec5^{E13}*) allows the formation of normally sized oocytes but with greatly reduced yolk uptake. We find that in *sec5^{E13}* oocytes Yolkless accumulates aberrantly in late endocytic compartments, indicating a defect in the endocytic cycling of the receptor. An analogous truncation of the yeast *SEC5* gene results in normal secretion but a temperature-sensitive defect in endocytic recycling. Thus, the exocyst may act in both Golgi to plasma membrane traffic and endocytic cycling, and hence in oocytes is recruited to clathrin-coated pits to facilitate the rapid recycling of Yolkless.

Introduction

Accurate membrane trafficking requires mechanisms to ensure that transport vesicles and other carriers fuse only with the correct organelle. Although SNARE proteins mediate the final membrane fusion event, it now appears that "tethering factors" act before SNARE complex formation to attach carriers to their destination (Pfeffer, 1999; Whyte and Munro, 2002). Several such tethering complexes that appear to act before vesicle fusion have been identified. Of these, perhaps the best characterized is the exocyst (or sec6/8 complex), an evolutionarily conserved octameric complex that was initially identified in the budding yeast *Saccharomyces cerevisiae* as being required for secretion and in particular for the fusion of Golgi-derived vesicles to the plasma membrane (TerBush et al., 1996; Guo et al., 2000). Further aspects of the role of the complex in vesicle targeting have been uncovered by studies in mammalian cells. The complex was found to be required for basolateral protein targeting in epithelial cells, whereas apical delivery of membrane proteins appeared unaffected when exocyst function was impaired by antibody inhibition (Grindstaff et al., 1998; Hsu et al., 1999; Yeaman et al., 2001). In addition, the exocyst in neurons is associated with neurite tips and sites along the axon, but synaptic transmission seems independent of exocyst function (Hazuka et al., 1999; Murthy et al., 2003). These observations suggest that the exocyst may not be required for all vesicle fusion with the plasma membrane, but it seems to be particularly associated with polarized secretory events. The exocyst consists of eight different proteins, and these subunits are distantly related to those of two other vesicle-tethering complexes, the COG complex and GARP/VFT that are involved in vesicle fusion events at the Golgi apparatus (Whyte and Munro, 2001; Ram et al., 2002; Conibear et al., 2003). The existence of this family of "quatrefoil" complexes suggests that the exocyst serves a function shared with some other membrane traffic steps, although not all pathways appear to require a member of this family (Whyte and Munro, 2002).

The proposed mode of action of the exocyst is that a subcomplex of components associates with secretory vesicles, and that the other components localize to specific regions of the plasma membrane. Upon exocyst assembly, the vesicles would then be tethered to the target membrane. Several small GTPases of the Ras superfamily have been identified as being required for the membrane recruitment of various exocyst components. The yeast Rab family GTPase Sec4 recruits exocyst components to transport vesicles, and its putative mammalian homologue Rab11 shows similar interactions (Guo et al., 1999; Zhang et al., 2004). Studies in yeast have also uncovered interactions with several the Rho family GTPases (Rho1, Rho3, and Cdc42) that act at the plasma membrane to control cell polarity

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(Novick and Guo, 2002). In higher eukaryotes, components of the complex have been shown to bind RalA, an interaction that is thought to be important for secretion to the basolateral surface (Fukai et al., 2003).

Although it has become clear that the exocyst fulfils a spectrum of functions in exocytosis, it seems likely that other roles remain to be uncovered. The presence of eight subunits raises the possibility of many potential interactions, and indeed the octameric COG complex appears to act in at least two pathways, with different sets of subunits being required for intra-Golgi transport and endosome to Golgi transport (Whyte and Munro, 2001; Ram et al., 2002). The examination of different mammalian cell types has proven valuable for uncovering new aspects of the exocyst's function (Grindstaff et al., 1998; Hazuka et al., 1999). Therefore, we have chosen to investigate the complex in *Drosophila melanogaster*, which has a great diversity of cell types, many of which exhibit physiologically important differences in the rate and organization of secretion.

In this paper, we examine the localization of an exocyst subunit in the *Drosophila* female germline. *Drosophila* oogenesis starts out in the germarium, where a cyst of 16 interconnected germ cells is formed, and upon leaving the germarium this cyst is surrounded by a layer of somatic follicular epithelium (Huynh and St Johnston, 2004). The oocyte is specified and positioned at the posterior end of the egg chamber and remains connected to the 15 remaining germline cells, the nurse cells, via ring canals. Then, during stages 8–10 of oogenesis, the oocyte grows in size disproportionately compared with the nurse cells. Stages 8–10 are also the period of vitellogenesis during which yolk protein (or vitellogenin) is secreted by the fat body and the follicle cells and is taken up into the oocyte via receptor-mediated endocytosis (Roth and Porter, 1964; DiMario and Mahowald, 1987). The vitellogenin receptor Yolkless is translocated to the cell surface where it undergoes many rounds of ligand uptake and recycling (Tsuruhara et al., 1990; Schonbaum et al., 1995, 2000). The internalized yolk protein is stored in granules (thought to be equivalent to the lysosomal compartment in other cells) for later use during embryogenesis. *Drosophila* oogenesis thus involves extensive membrane trafficking, with exocytosis providing the membrane necessary for the massive increase in oocyte size, and endocytosis being extremely active to facilitate vitellogenesis.

Recent studies have used immunofluorescence to examine the distribution of the exocyst component Sec5 in *Drosophila* neurons and ovaries, and found that in the latter case the protein is located at the oocyte plasma membrane (Murthy et al., 2003; Murthy and Schwarz, 2004). In this paper, we examine the distribution of Sec5 by light and immunoelectron microscopy, and find that unexpectedly the clathrin-coated vesicles and pits that endocytose yolk contain a substantial proportion of the Sec5 in the oocyte. Although removal of all Sec5 activity blocks the formation of cysts of germ cells at an early stage, in a Sec5 hypomorphic allele oocytes develop to normal size but contain much less yolk. This defect appears to be caused by a loss of Yolkless from the plasma membrane and an accumulation in late endocytic compartments. This observation suggests that the exocyst is recruited to clathrin-coated vesicles to facilitate endocytic cycling processes, in addition to the role of the complex in Golgi to plasma membrane trafficking.

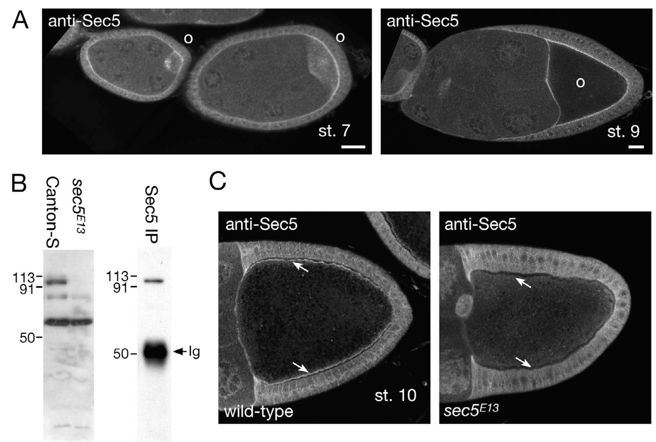

Figure 1. **Characterization of an anti-Sec5 antiserum.** (A) Confocal micrographs of wild-type ovaries labeled with the rabbit anti-Sec5 antibody. Sec5 is enriched in the oocyte (o) and from around stage 7 (st. 7) becomes localized to the plasma membrane and remains plasma membrane localized throughout vitellogenesis from stages 8–10. Bars, 20 μm. (B) Anti-Sec5 protein blots of total proteins prepared from wild-type (Canton-S) and *sec5*^{E13} eggs (left), or of an anti-Sec5 immunoprecipitation (IP; right) from wild-type ovary extract. The antibody recognizes two prominent bands, one of which is specific for native Sec5, whose predicted molecular mass is 100.7 kD. (C) As A, except anti-Sec5 was applied to stage 10 oocytes from wild-type or *sec5*^{E13} germline clones. Anti-Sec5 staining along the plasma membrane of the wild-type oocyte is indicated by arrows and is absent in the *sec5*^{E13} germline clone. The somatically derived border cells are positive for Sec5 in both wild type and mutant.

Results

Sec5 localizes to clathrin-coated pits and vesicles in *Drosophila* oocytes

Rabbit antisera were raised against the COOH-terminal 162 residues of *Drosophila* Sec5 and affinity purified. The specificity of antiserum binding in immunofluorescence and Western blotting was confirmed using a previously described hypomorphic allele of Sec5. *Sec5*^{E13} is an allele generated by EMS mutagenesis by Murthy et al. (2003). A premature stop codon is introduced at residue 361 (of 894) resulting in a truncated form of Sec5 that lacks the region of the protein against which the antiserum was raised. However, unlike a null allele, the *sec5*^{E13} homozygous germline can sustain oogenesis (Murthy and Schwarz, 2004).

When the anti-Sec5 serum was used to probe ovaries from wild-type females, labeling was found to be enriched in the oocyte compared with the nurse cells (Fig. 1 A). Upon initiation of vitellogenesis at stage 8, the oocyte labeling shifted from a distinctly cytoplasmic pool to a predominantly plasma membrane–associated distribution. This is consistent with previous analysis of Sec5 in egg chambers using a monoclonal antibody (Murthy and Schwarz, 2004). When the antiserum was used to probe blots of proteins from eggs, two prominent bands were detected, including a 100-kD band, which was absent from ovary extracts from *sec5*^{E13} germline clones (Fig. 1 B). Only this 100-kD band was present when the anti-Sec5 serum was used for precipita-

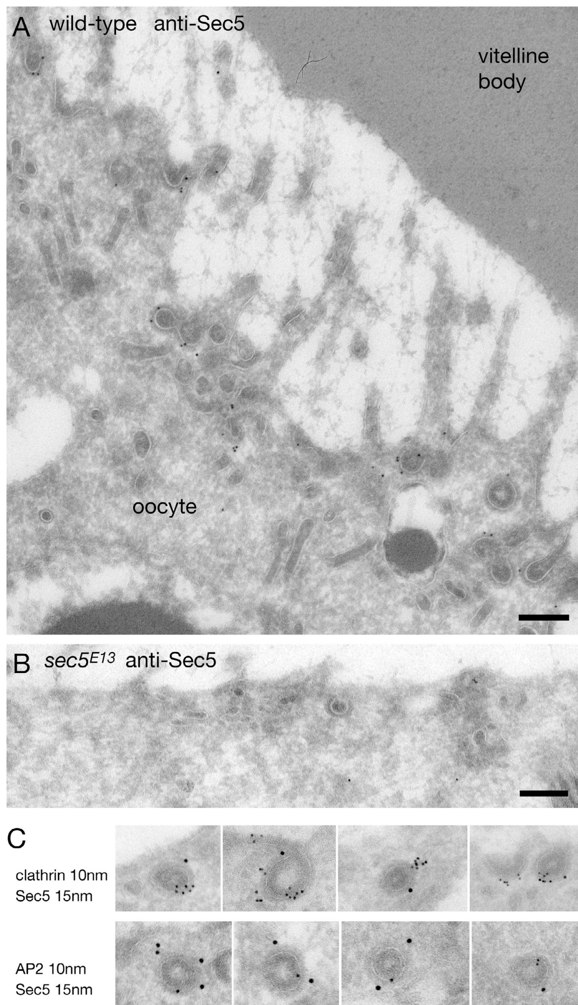

Figure 2. **Sec5 localizes to clathrin-coated pits and vesicles underneath the plasma membrane of *Drosophila* oocytes.** (A and B) Electron micrographs of cryosections of a wild-type oocyte or of an oocyte from a $sec5^{E13}$ germline labeled with anti-Sec5 followed by protein A conjugated to 10-nm gold particles. The gold particles decorate structures with electron-dense coats, and such labeling is absent from the section from $sec5^{E13}$. Bars, 200 nm. (C) Electron micrographs as in A of coated pits and vesicles labeled for Sec5, clathrin, and α-adaptin (AP2) as indicated.

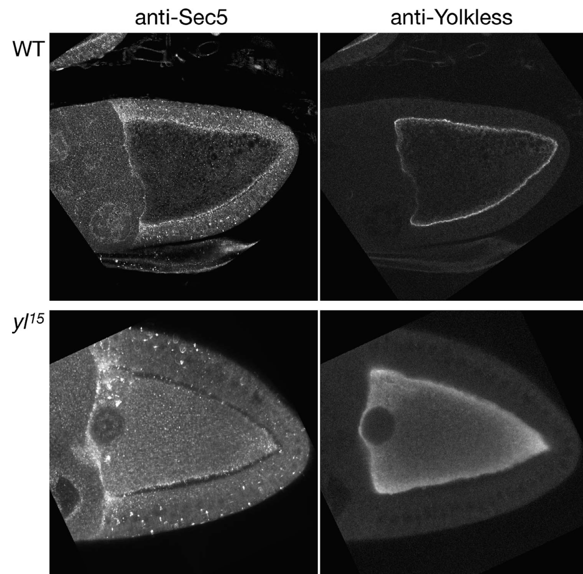

Figure 3. **Sec5 plasma membrane labeling is reduced in yl^{15} mutant oocytes.** (A) Confocal micrographs of oocytes from wild type (WT; Canton-S) or homozygous yl^{15} females labeled for both Sec5 and the vitellogenin receptor Yolkless. In the yl^{15} mutant, the receptor is still present but much accumulates internally, possibly in the ER, as seen for other *yolkless* alleles (Schonbaum et al., 2000).

tion from egg extracts, indicating that the other band represents cross reactivity with a protein that is only recognized when denatured. This hypothesis was confirmed by examining the ovaries from $sec5^{E13}$ germline clones, where the antibody labeling adjacent to the plasma membrane was now absent, indicating that it reflects recognition of endogenous Sec5 (Fig. 1 C).

To more precisely determine the localization of Sec5, immunoelectron microscopy was performed on ultrathin cryosections of stage 10 egg chambers. Surprisingly, a substantial amount of the anti-Sec5 labeling was found associated with coated pits and vesicles underneath the oocyte plasma membrane (Fig. 2 A). This labeling appears to reflect recognition of Sec5 as it was not present in sections from $sec5^{E13}$ germline clones (Fig. 2 B). Moreover, the coats were confirmed to be clathrin/AP2 by double-label immunoelectron microscopy (Fig. 2 C). Sec5 labeling of stage 10 oocytes was quantified by counting gold particles. 59.5 ± 2.5% of all clathrin-coated pits and vesicles were labeled with anti-Sec5 (n = 720), and this accounted for 41 ± 4% of the total Sec5 labeling. The remaining labeling was on noncoated plasma membrane or endocytic structures (30 ± 9%) or in the cytoplasm near these structures (29 ± 8%). The linear density of the Sec5 labeling was also examined and found to be 6.6-fold higher in the coated pits and vesicles (3.3 ± 1.2 gold/μm) than the rest of the plasma membrane and endocytic structures (0.5 ± 0.02 gold/μm).

Mutants affecting endocytosis affect the distribution of Sec5

During vitellogenic stages, the oocyte endocytoses large amounts of yolk proteins (vitellogenins) produced by the surrounding follicular epithelium. Uptake into the oocyte is mediated by the vitellogenin receptor Yolkless, a protein of the low density lipoprotein receptor superfamily (Schonbaum et al., 1995). Because endocytosis via Yolkless accounts for the bulk of endocytic events during vitellogenesis, mutating this receptor is an effective way of reducing total endocytosis, and in strong *yolkless* mutants the number of clathrin-coated pits and vesicles is reduced (DiMario and Mahowald, 1987). We made use of one such *yolkless* mutant, yl^{15}, to determine whether the distribution of Sec5 would be altered by the reduced amount of endocytic membrane trafficking. In oocytes from yl^{15} females, Yolkless accumulates in the cytoplasm, apparently due to a defect in exit from the endoplasmic reticulum (Schonbaum et al., 2000). Fig. 3 shows that Sec5 labeling is significantly reduced at the plasma membrane of stage 10 oocytes from yl^{15} females. Electron microscopic studies confirmed that the number of clathrin-coated pits and vesicles in the oocytes studied was reduced in the yl^{15} mutant (2.55-fold). The amount of Sec5 at the plasma membrane was concomitantly reduced, with those

clathrin-coated structures remaining showing a similar degree of labeling, 48.5 ± 5% of clathrin-coated structures being labeled in the mutant and 59.5 ± 2.5% in wild type.

Together, these results indicate that in developing oocytes the exocyst component Sec5 is enriched in clathrin-coated pits and vesicles at the plasma membrane. Moreover, the localization of Sec5 to the plasma membrane, at least in part, reflects the activity of clathrin-mediated endocytosis. The apparently normal growth of the yl^{15} mutant oocytes during development indicates that reduced levels of Sec5 at the plasma membrane are not incompatible with continued secretion and expansion of the oocyte plasma membrane.

Sec5 is predominantly assembled into the exocyst complex in *Drosophila* ovaries

Previous studies on the role of Sec5 in yeast and mammalian cells have shown that it plays a role in exocytosis by acting as a subunit of the exocyst complex (TerBush et al., 1996; Kee et al., 1997). The unexpected localization of Sec5 to clathrin-coated pits raised the possibility that this might reflect a specialized function of Sec5 acting independently of the exocyst. Antisera were also generated against three other *Drosophila* exocyst components, Sec3, Sec6, and Exo84, and these specifically recognized the respective proteins on protein blots, as determined by RNAi-induced knockdown of the proteins in tissue culture cells (Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200411053/DC1). Although these antisera did not prove suitable for immunofluorescence, we were able to use them to examine the state of complex assembly by protein blotting. Probing of anti-Sec5 immunoprecipitations from wild-type ovary extracts showed that Sec5 specifically interacts with all three exocyst components tested (Fig. 4 A). In addition, when ovary extracts were separated by glycerol gradient centrifugation, Sec5 clearly showed a single peak near the bottom of the gradient in a rapidly sedimenting fraction where it comigrated with Sec3 and Sec6 (Fig. 4 B). Exo84 also peaked in this fraction, but in addition was present in more slowly sedimenting fractions, which is consistent with a previous study on mammalian Exo84 that found it to be additionally present in a sub-complex (Moskalenko et al., 2003). Therefore, it appears that at least some Sec5 in ovaries is associated with other exocyst subunits, and the protein is not present in a detectable pool of smaller sub-complexes or monomers. Although this does not exclude the possibility that a pool of Sec5 might exist which is not assembled into the complex, it suggests that the exocyst complex itself could localize to clathrin-coated pits and vesicles in oocytes.

The distribution of the vitellogenin receptor Yolkless is altered in *sec5*^{E13} oocytes

The localization of an exocyst component to clathrin-coated pits raises the possibility that it plays a role in traffic through the endocytic system. In developing oocytes, much of the clathrin-mediated endocytosis is dedicated to the uptake of yolk proteins by the receptor Yolkless. It has been previously reported that oocytes derived from *sec5*^{E13} germline develop into eggs, but the uptake of yolk is defective (Murthy and

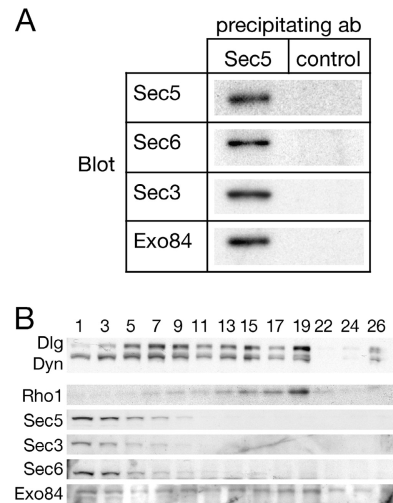

Figure 4. **Sec5 in ovaries is assembled into the exocyst complex.** (A) Protein blots with the indicated antibodies of immunoprecipitates from ovary extracts prepared from wild-type females. The precipitates were probed with sera against Sec5 or an irrelevant protein, Cog8 (Dor1), a component of the Golgi-localized COG complex (control). (B). Protein blots of fractions from a glycerol gradient separation of detergent-solubilized ovary extracts from wild-type females, probed with antisera to the indicated proteins. The four exocyst components examined peak toward the bottom of the gradient (fraction 1), with Exo84 being also present in less rapidly sedimenting fractions, which is consistent with a previous paper (Moskalenko et al., 2003). The small GTPase Rho1 (21.7 kD), Discs large (Dlg; 102.5 kD), and Shibire, the *Drosophila* homologue of the GTPase dynamin (Dyn; 97.8) sediment less rapidly.

Schwarz, 2004). Thus, the oocytes show a flaccid "yolkless" phenotype and often collapse upon ovoposition as a result of the lack of yolk granules that fill up the wild-type egg (Fig. 5 A). This phenotype has been previously attributed to a defect in the initial delivery of Yolkless to the cell surface, as by immunofluorescence Yolkless could be seen accumulating in the cytoplasm of *sec5*^{E13} oocytes, rather than translocating to the plasma membrane (Murthy and Schwarz, 2004). However, the site of Yolkless accumulation in these oocytes was not identified, and so we examined the distribution of Yolkless in wild-type and *sec5*^{E13} oocytes by immunoelectron microscopy.

The yolkless phenotype of the *sec5*^{E13} oocytes was clearly apparent in low magnification electron micrographs (Fig. 5 B), with the amount of yolk being reduced from 35 ± 3% of the cytoplasm to 4.1 ± 1%. In wild-type oocytes and consistent with previous studies, Yolkless was found to be mostly in coated and noncoated vesicles and tubules, in a region of the cytoplasm extending 500–800 nm below the plasma membrane (Fig. 5 C). A small percentage of Yolkless was found in small endosomal profiles where the yolk and receptor separate and from which Yolkless is recycled back to the plasma membrane. Relatively little of the receptor could be detected in later endocytic compartments (maturing and mature yolk granules) that are located at micrometer distances from the plasma membrane.

In *sec5*^{E13} oocytes, there was a net reduction in the level of Yolkless at the plasma membrane (Fig. 5 C). The reduction in Yolkless at the surface was accompanied by reduction in the number of vesicles and tubules beneath the surface, as well as a 7 ± 1-fold decrease in the number of clathrin-coated pits and ves-

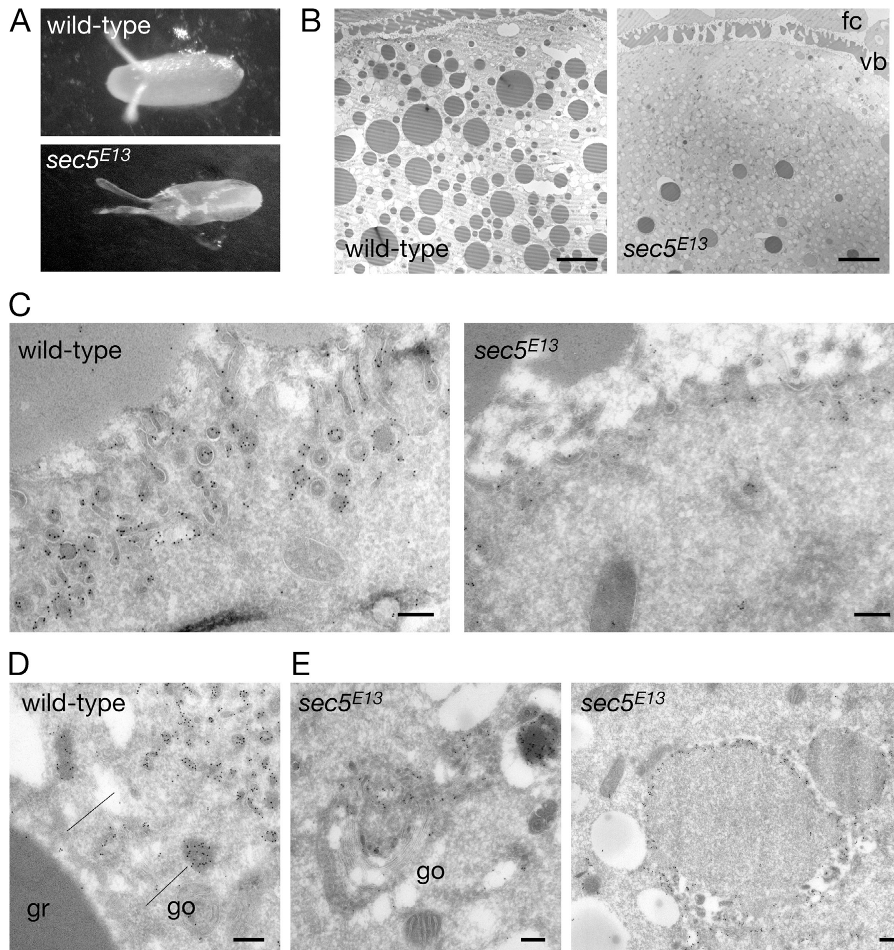

Figure 5. ***sec5^{E13}* oocytes have a defect in trafficking of the Yolkless receptor.** (A) Eggs derived from a *sec5^{E13}* germline have a yolkless phenotype, and are often collapsed due to the lack of yolk granules. The severity of the yolkless phenotype was indistinguishable between flies raised at 18, 25, or 30°C. (B) Low magnification electron micrographs of oocytes from wild-type or *sec5^{E13}* germlines. The vitelline bodies of the forming vitelline membrane (vb) lie between the oocyte and the follicle cells (fc). The small pale inclusions in the cytoplasm of both oocytes are lipid droplets. Bars, 5 μm. (C–E) Electron micrographs of cryosections of stage 10 oocytes labeled with an anti-Yolkless antibody followed by protein A gold (10 nm). The *sec5^{E13}* oocyte shows reduced Yolkless labeling on and below the plasma membrane and a reduction in endocytic structures (C). There is a two- to threefold increase in labeling in the Golgi (go; yolk granule, gr) (D) and a striking appearance of Yolkless in the rim of later endocytic compartments including large uncondensed yolk granules (E). Bars, 200 nm.

icles. There was also a small increase in levels of Yolkless in the trans-Golgi network. Both the Golgi stack and trans-Golgi network are approximately twofold larger, and exhibit a labeling density for Yolkless that is 2.7 ± 0.5-fold higher than wild-type Golgi areas, perhaps reflecting a delay in exocytosis (Fig. 5 D). However, the large majority of Yolkless was found in late endocytic compartments, in particular in immature or noncondensed yolk granules that are located toward the center of the oocyte (Fig. 5 E), as well as in small vacuoles and vesicles in the proximity of these later endocytic compartments, distributions not seen in the wild-type. To obtain an overview of the change in distribution of Yolkless in *sec5^{E13}* oocytes, the anti-Yolkless staining of cryosections was also visualized at lower resolution by using fluorescent secondary antibodies and confocal microscopy. Fig. 6 A shows that as expected Yolkless shifts from a plasma membrane distribution in the wild type to intracellular structures in the *sec5^{E13}* germline clone. Double labeling showed that in the mutant the overwhelming majority of this intracellular Yolkless colocalizes with yolk and hence has been transported beyond the Golgi and is accumulating in late endocytic compartments (Fig. 6 B).

Together, these results suggest that in the *sec5^{E13}* allele, the majority of Yolkless has translocated beyond the Golgi apparatus, as in wild type, but instead of being located at the surface it has accumulated in immature granules and other yolk-containing structures. The reduction in surface Yolkless has presumably resulted in a defect in yolk uptake and a reduction in the number of clathrin-coated pits and vesicles, similar to the phenotypes seen in mutants lacking Yolkless itself (DiMario and Mahowald, 1987).

Examination of exocytic and endocytic markers in *sec5^{E13}* oocytes

Several membrane traffic markers were examined to further characterize the defects in exocytosis and endocytosis in the *sec5^{E13}* allele. The plasma membrane SNARE syntaxin-1A is a membrane protein that must be trafficked to the oocyte surface, but its distribution in the mutant was found to be indistinguishable from wild type, indicating that its delivery to the plasma membrane is not significantly reduced (Fig. 7 A). The cell adhesion molecule E-cadherin is strongly expressed on the surface of both nurse cells and the oocyte up to stage 7 of oogenesis (Niewiadomska et al., 1999). This distribution was unchanged in the *sec5^{E13}* egg chambers with no detectable increase in intracellular material compared with wild type (Fig. 7 A).

To examine traffic along the endocytic route, we established an endocytosis assay using the membrane dye FM4-64. This dye readily partitions into membranes where it becomes fluorescent. Mutant and wild-type ovaries were allowed to internalize the dye for 30 min, after which the bulk of FM4-64 was present in the plasma membrane, although fainter internal staining could be seen in the wild type (Fig. 7 B). This internalized FM4-64 could be more clearly visualized after back-extraction of the cell surface dye at 4°C. As expected for the wild

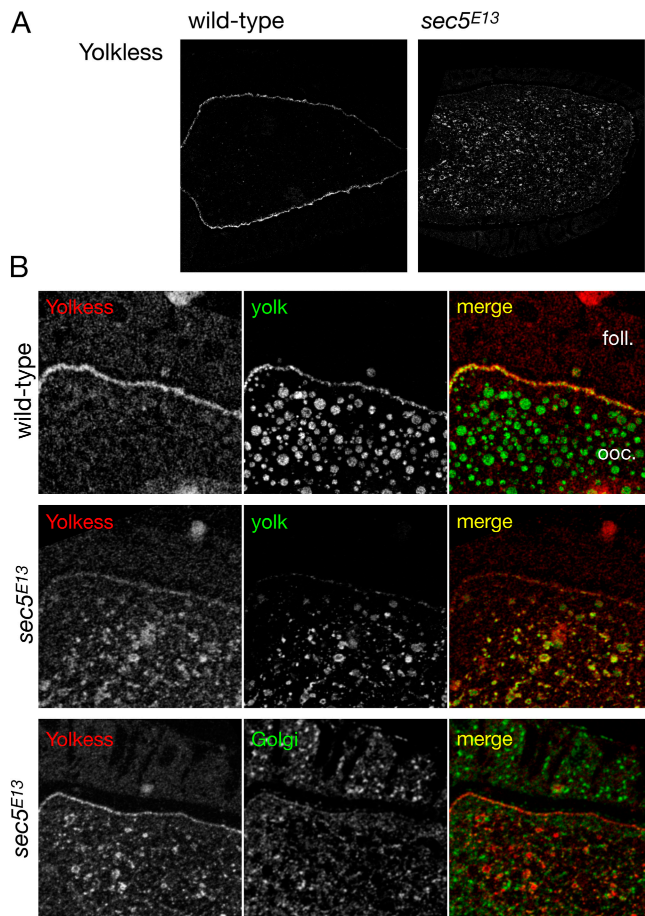

Figure 6. **Yolkless accumulates in endocytic compartments in *sec5^{E13}* oocytes.** (A) Confocal micrographs of 500-nm cryosections of egg chambers from wild-type or *sec5^{E13}* germlines labeled with antibodies to Yolkless and a fluorescent secondary antibody. (B) Confocal micrographs of cryosections as in A, double labeled with antibodies to Yolkless and either yolk or the Cog3 subunit of the *Drosophila* COG complex (Golgi), which recognizes the *Drosophila* Golgi (Fig. S1 B). A region near the surface of the oocyte (ooc.) is shown along with adjacent follicle cells (foll.).

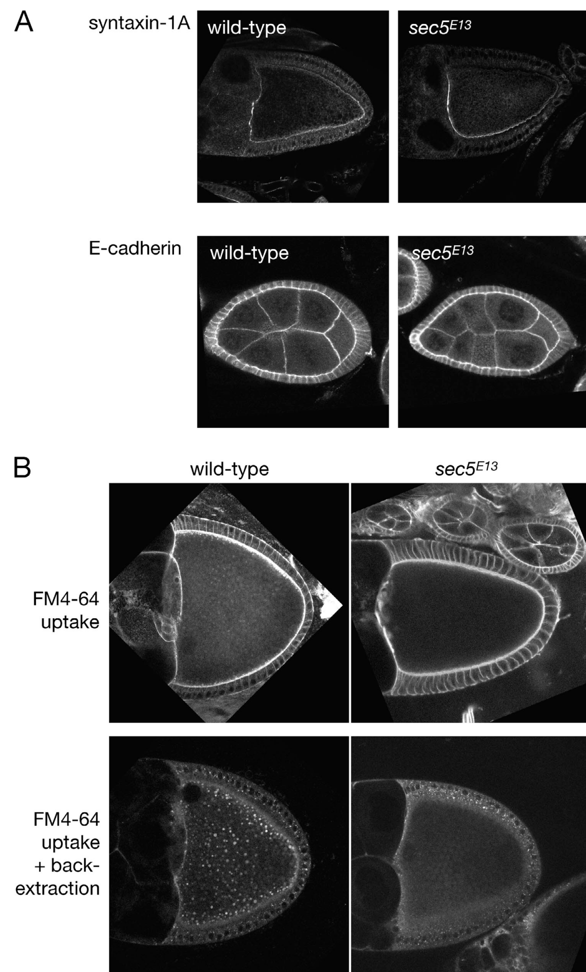

Figure 7. **Analysis of membrane-trafficking processes in *sec5^{E13}* oocytes.** (A) Confocal microscopic sections of egg chambers from wild-type or *sec5^{E13}* germlines labeled with antibodies to the plasma membrane SNARE syntaxin-1A or the adhesion molecule E-cadherin. (B) Confocal micrographs of unfixed ovaries incubated with FM4-64 for 30 min. Without back extraction, the bulk of the dye is in the plasma membrane, but after back extraction at 4°C only dye taken up into the endosomal system remains. Uptake into the oocyte from the *sec5^{E13}* germline clone is significantly reduced, whereas the surrounding, somatically derived follicle cells internalize dye at wild-type levels.

type, fluorescence was present in punctate structures in the cytoplasm of the oocyte and of the cells of the surrounding follicular epithelium. In the *sec5^{E13}* mutant, the level of such staining in the oocyte was substantially reduced, whereas the follicular epithelium, which was not part of the germline clone, and hence is heterozygous for the mutation, internalized FM4-64 at levels comparable to wild type (Fig. 7 B).

Together, these results suggest that the delivery of at least some proteins to the plasma membrane is relatively normal in the *sec5^{E13}* allele, which is consistent with this allele still showing the large increase in nurse cell and oocyte size that occurs during oogenesis. However, Yolkless shows internal accumulation and reduced levels at the cell surface, with the concomitant reduction in surface coated pits apparently leading to a reduction in bulk membrane uptake.

Localization of Sec5 to clathrin-coated pits in other cell types

The oocyte is a highly specialized cell that must perform an exceptional amount of endocytosis to accumulate all of the yolk that will nourish the developing embryo. Thus, aspects of its endocytic machinery may be altered or unusual, raising the question of whether exocyst components are recruited to cell surface coated pits in other cell types. Immunoelectron microscopic analysis of ovarian follicle cells and *Drosophila* S2 tissue culture cells probed with antibodies against Sec5 showed that a large portion (∼40%) of clathrin-coated pits in these cells were also labeled (Fig. 8 A). However, the number of coated pits in these cell types is a lot lower than in oocytes and the bulk of Sec5 is not found in these structures.

A yeast version of the *sec5^{E13}* mutant has a temperature-sensitive defect in endocytic recycling of the a-factor receptor

The phenotype of the *sec5^{E13}* allele suggests that the COOH-terminal portion of Sec5 may contribute to endocytic recycling rather than exocytosis. To examine this further we created the

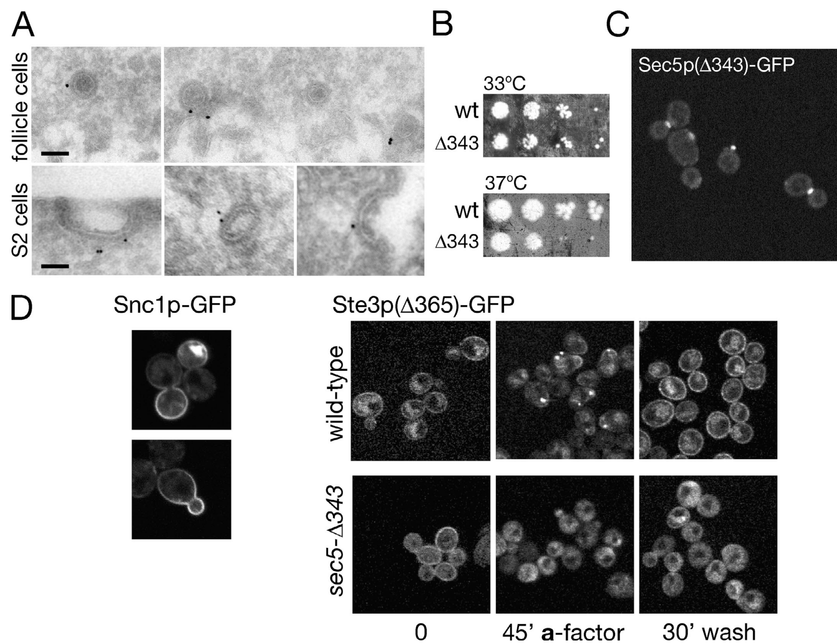

Figure 8. **Sec5 localization and function in other cell types.** (A) Electron micrographs of coated pits and vesicles labeled with anti-Sec5 in cryosections of follicular epithelial cells from wild-type ovaries and S2 cultured cells. Bars, 100 nm. (B) Growth at the indicated temperatures of wild-type yeast (BY4741; wt), or the same strain with *SEC5* truncated at residue 343 by insertion of a triple HA tag (*sec5-Δ343-3xHA*). (C) Confocal micrograph of live yeast in which the single copy of the *SEC5* gene is truncated at residue 343 by insertion of a GFP tag. (D) Confocal micrographs of *sec5-Δ343-3xHA* strain expressing Snc1p-GFP and imaged after 4 h at 37°C. (E) Confocal micrographs of live yeast expressing a truncated form of the **a**-factor receptor Ste3p(Δ365) tagged in the genome with GFP. Cells were imaged after incubation for 2 h at 37°C (0 min) and then 45 min after addition of **a**-factor, and 30 min after **a**-factor was removed by washing. The truncated **a**-factor receptor is competent for ligand-stimulated endocytic recycling and localizes to the plasma membrane in both wild-type and *sec5-Δ343-3xHA* mutant cells. 45 min after **a**-factor addition, the receptor is internalized and localizes to bright dots in the cytoplasm and to the plasma membrane at the emerging schmoos in wild-type cells. In *sec5-Δ343-3xHA* cells, the receptor is internalized but shows a diffuse distribution. 30 min after **a**-factor was washed away, wild-type cells restore the plasma membrane localization of the receptor, whereas in the mutant cells the receptor still localizes internally.

equivalent truncation mutation in the yeast *S. cerevisiae* by inserting a GFP or an epitope tag at the equivalent position in the *SEC5* gene (Fig. S1 C). This *sec5-Δ343* mutation leaves only the NH_2-terminal 343 residues of the 971-residue protein, but cells with this truncation are viable, with a slight reduction in growth at 37°C (Fig. 8 B). The GFP-tagged truncated Sec5 shows a similar subcellular distribution to the wild-type protein (Zhang et al., 2001), being present in the tip of emerging buds, and the neck of larger buds (Fig. 8 C). Not only were these cells able to grow but the v-SNARE Snc1p was found at the plasma membrane of the emerging daughter cell, even at 37°C, indicating that polarized secretion is not impaired in this *sec5-Δ343* mutant (Fig. 8 D).

To assess defects in endocytic membrane trafficking in the mutant, we devised an assay based on a truncated version of Ste3p, the **a**-factor receptor. This receptor has been shown to undergo two different modes of endocytosis: a constitutive and ubiquitin-dependent delivery to the vacuole and a ligand-dependent endocytic recycling (Chen and Davis, 2000). Deleting the COOH-terminal 105 amino acids of Ste3p removes the ubiquitination sites and thus abolishes constitutive endocytosis and vacuolar degradation, while leaving the receptor competent for ligand-stimulated recycling (Chen and Davis, 2000). We thus tagged Ste3Δ365 with a GFP tag in the genome, and found it to be predominantly plasma membrane associated, as expected, both in wild type and the *sec5-Δ343* mutant (Fig. 8 E). Upon **a**-factor addition to wild-type cells, most of the receptor was internalized and often accumulated in bright spots, which presumably correspond to localized recycling endosomes, whereas the remaining plasma membrane localized material polarized in the emerging schmoo. In the *sec5-Δ343* mutant at 37°C, the receptor was internalized but failed to accumulate in bright spots and showed hardly any plasma membrane localization. Moreover, after removal of **a**-factor, wild-type cells restored the initial localization of the receptor to the plasma membrane within 30 min, whereas in the mutant the receptor remained in a diffuse cytoplasmic distribution. These effects were not observed with *sec5-Δ343* at lower temperatures, indicating that at elevated temperatures the COOH terminus of Sec5p is more important for endocytic recycling than exocytosis, an effect comparable to the defects seen in the $sec5^{E13}$ oocyte.

Discussion

In this paper, we have used immunoelectron microscopy to examine the localization in *Drosophila* oocytes of the exocyst component Sec5. We find that the plasma membrane localization of Sec5 seen by light microscopy reflects a substantial amount of the protein being present in clathrin-coated pits and vesicles. This was unexpected as the exocyst had previously been shown to be required for fusion of Golgi-derived exocytic vesicles to the plasma membrane. It should be stressed that our observations do not question this role for the complex. In addition to the Sec5 present in coated structures, we could clearly detect Sec5 in uncoated areas of the plasma membrane, albeit at a lower linear density. Moreover, germline clones of a null allele of Sec5, $sec5^{E10}$, and of null mutants in other exocyst components, Sec6 and Sec8, arrest very early in germline cyst development with multinucleate cells containing ring canals detached from the cell surface, presumably reflecting a severe defect in the delivery of new plasma membrane that has prevented germ cell growth and division (Murthy and Schwarz, 2004; Murthy et al., 2005; unpublished data). However, our observations raise the possibility that Sec5, and potentially other exocyst components, not only acts in traffic from the Golgi to the plasma membrane but is also recruited to coated pits to act at some step in the membrane traffic events after endocytic internalization.

The primary cargo for clathrin-dependent endocytosis in the oocyte is the receptor Yolkless, which mediates the uptake of massive amounts of yolk during the 12-h period of vitellogenesis during developmental stages 8–10 (DiMario and Mahowald, 1987). Because Yolkless does not accumulate in the yolk granules, it must recycle many times. The oocyte endocytic pathway has been examined by using the *shibire* temperature-sensitive mutant in dynamin to reversibly block budding of clathrin-coated vesicles (Tsuruhara et al., 1990). This examination has shown that the network of tubes and small yolk-containing endosomes under the surface is lost in <10 min after a block in endocytosis and that these structures reform over 3–5 min when endocytosis is restored. Thus, it appears that endocytosed vesicles fuse with each other to continuously create early endosomes. Yolk condenses in these endosomes, and they emanate tubes that have been suggested to recycle the receptor back to the surface, whereas the residual yolk-containing core fuses with larger yolk granules deeper inside the oocyte (Fig. 9; Roth and Porter, 1964; Giorgi and Jacob, 1977; Tsuruhara et al., 1990). Thus, at least two membrane fusion events must occur to sustain the endocytic cycle of Yolkless—fusion of the endocytosed vesicles to other vesicles or nascent endosomes (Fig. 9, step 1), and then fusion of the recycling tubular carriers back to the plasma membrane (Fig. 9, step 2). The recruitment of Sec5 to the forming coated pits could allow it to participate in one or both of these fusion events.

Further suggestion that Sec5 acts in the endocytic cycling of Yolkless comes from the *sec5*^{E13} allele that expresses a truncated form of Sec5 lacking the COOH-terminal 534 of 894 residues. Germline clones for *sec5*^{E13} still develop oocytes, but these show a defect in yolk uptake (Murthy and Schwarz, 2004). In the initial characterization of this *sec5*^{E13} allele it was noted that Yolkless accumulated in cytoplasmic structures, which were suggested to reflect trapping of the protein in the secretory pathway due to a block in exocytosis (Murthy and Schwarz, 2004). However, by immunoelectron microscopy and double label immunofluorescence it appears that the bulk of Yolkless accumulates in late endosomal compartments.

We suggest that the *sec5*^{E13} allele might be defective in the fusion event mediated by the pool of Sec5 present in clathrin-coated pits. If the defective step is the fusion of uncoated vesicles with each other or with early endosomes, then the vesicles may fuse aberrantly with later endosomes. If these lacked the machinery for Yolkless recycling, the protein would be trapped. Alternatively, the *sec5*^{E13} allele could cause a defect in the fusion to the plasma membrane of the carriers that recycle Yolkless from endosomes back to the surface. Although there is not a massive accumulation of uncoated vesicles near the surface of the sort seen in yeast when exocyst function is inactivated, it may be that even a small amount of accumulation might sequester machinery for this transport step. Thus, the machinery for formation or consumption of endosome to plasma membrane carriers would be depleted from early endosomes, and hence Yolkless would be trapped and instead be delivered with the yolk to later endosomal compartments. In either case, the presence of Sec5 in clathrin-coated pits could be a means to ensure that it is ready to act as soon as vesicles uncoat, or postendosomal carriers are formed, so that the recycling of Yolkless can proceed as efficiently as possible.

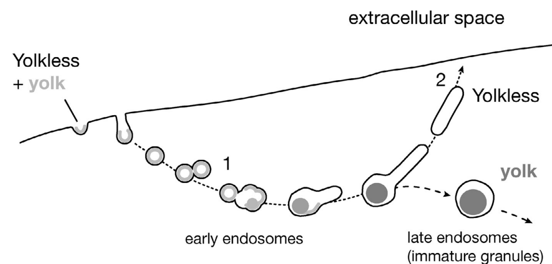

Figure 9. **Schematic illustration of the recycling route of Yolkless.** Yolkless binds yolk and both are endocytosed in clathrin-coated vesicles. Yolk condenses in early endosomes and is excluded from tubules that are thought to recycle Yolkless (dotted line). The condensing yolk (dashed line) remains in the late endosomes, which fuse together to form mature granules. Membrane fusion events that could involve Sec5 are between endocytosed vesicles (1) and between recycling tubules and the plasma membrane (2). Model based on previous microscopy and endocytosis experiments (Roth and Porter, 1964; Giorgi and Jacob, 1977; Tsuruhara et al., 1990).

It should be stressed that the aforementioned model is not proven, and alternative interpretations for the phenotype of the *sec5*^{E13} allele are possible. One alternative is that the initial delivery of newly synthesized Yolkless from the Golgi to the plasma membrane is defective. However, there does not appear to be a general block in Golgi to plasma membrane traffic in the *sec5*^{E13} allele. First, the plasma membranes of *sec5*^{E13} oocytes and nurse cells are able to expand to a near normal size (Murthy and Schwarz, 2004). In null alleles for Sec5 and other exocyst subunits, the germline cyst develops to a diameter of only 20–40 μm divided into two to three multinucleate cells, whereas by stage 10 of oogenesis the *sec5*^{E13} egg chamber has developed to a similar size to wild type (Murthy and Schwarz, 2004; Murthy et al., 2005). This development requires formation of 15 nurse cells, each of ~60 μm diameter, and an oocyte of ~150 μm diameter. Thus, the *sec5*^{E13} allele is able to sustain a very large increase in plasma membrane expansion beyond that seen when exocyst function is lost. This result is also in contrast to mutants with defects in other components of the exocytic machinery such as *syntaxin-1A*, *synaptotagmin*, and *rop* (*Drosophila* Sec1), which fail to develop oocytes or give miniature eggs, consistent with secretion being necessary for growth of the oocyte (Ruden et al., 2000). Moreover, we find that two plasma membrane proteins, syntaxin-1A and E-cadherin, are still delivered to the plasma membrane in *sec5*^{E13} germline cells and do not accumulate in internal structures. Thus it seems that in the *sec5*^{E13} allele the majority of traffic from Golgi to plasma membrane is normal. However, we cannot exclude the possibility that there are two routes from the Golgi to the plasma membrane with a subset of proteins, including Yolkless, being delivered to the surface via recycling endosomes, as has been suggested to occur in mammalian cells (Ang et al., 2004). In this case, the Yolkless-containing carriers in the *sec5*^{E13} allele might be unable to fuse with the early endosome and instead fuse with later endocytic structures from which Yolkless cannot escape.

The long time scale of vitellogenesis and the uncertainty about the precise steps involved means that it is not possible at present to be certain what step in the trafficking of Yolkless is defective in the *sec5*^{E13} allele. However, a specific defect in the postinternalization recycling route, rather than Golgi to plasma membrane transport, would correlate well with the presence of Sec5 in clathrin-coated pits and vesicles. Indeed, it is conceivable that the exocyst remains attached to membranes throughout the endocytic cycle. In mammalian cells, components of the exocyst have been localized to recycling endosomes and shown to be effectors for two small GTPases, Arf6 and Rab11 (Prigent et al., 2003; Zhang et al., 2004). Moreover, Exo70 was found to be recruited to a perinuclear compartment, possibly the recycling endosome, by the μ subunit of the clathrin adaptor complex AP1B (Folsch et al., 2003). We tested several possible interactions that might explain the recruitment of Sec5 to coated pits, but these proved negative. Thus a GST fusion to the cytoplasmic tail of Yolkless did not extract Sec5 or the endocytic adaptor Numb from ovary lysates. In addition, immunoprecipitated Sec5 did not coprecipitate Numb or the clathrin adaptor complex AP2 (unpublished data).

The COOH-terminal part of Sec5 that is missing in *sec5*^{E13} is well conserved in evolution (30% identical between humans and *Drosophila* vs. 44% for the part that remains). The suggestion that this region of Sec5 plays a role in exocyst function distinct from fusion of Golgi-derived vesicles is supported by the observation that an equivalent truncation of the yeast protein does not perturb growth, in contrast to deletion of the whole protein, which is lethal (TerBush et al., 1996). Although endocytic recycling also continues in these mutant yeast, it is temperature sensitive. At the nonpermissive temperature, endocytosed **a**-factor receptor accumulates in a cytoplasmic haze rather than endosomes, suggesting that a vesicle fusion step is blocked. It may be that at the permissive temperature other components of the exocyst are sufficient to sustain the endocytic function of the complex that involves the COOH terminus of Sec5. Interestingly, the yeast protein Rcy1p has been shown to act in recycling of endocytosed proteins and lipid markers (Wiederkehr et al., 2000; Galan et al., 2001), and this protein is related to the COOH-terminal part of the exocyst subunit Sec10. Of the other quatrefoil complexes in the cell, GARP/VFT has only four subunits, suggesting that this number may be sufficient for action at a single SNARE-dependent membrane traffic step (Conibear et al., 2003). The COG complex has eight subunits like the exocyst, but it appears to act in at least two steps in the Golgi apparatus (Wuestehube et al., 1996; Whyte and Munro, 2001; Ram et al., 2002). The central role that the exocyst plays in exocytosis means that deletion of subunits leads to a loss of cell viability, which may have masked other roles for the complex. Indeed, it has been reported recently that the requirement for a subset of exocyst subunits, including Sec5p, for growth of yeast can be by-passed by overexpressing the small GTPase Sec4p or the SNARE-binding protein Sec1p (Wiederkehr et al., 2004). This finding suggests that in yeast Sec5p is not obligatory for Golgi to plasma membrane transport. Moreover, a recent genome-wide RNAi screen in *Caenorhabditis elegans* identified Sec5, but not other exocyst components, as one of several genes whose knockdown causes defects in yolk granule formation in oocytes (Sonnichsen et al., 2005). In addition, a hairpin RNAi construct against *Drosophila* Sec10 was found to only show phenotypes in a small subset of tissues, implying that this subunit may be less critical for exocytosis (Andrews et al., 2002). We suggest that the exocyst acts in more than one membrane traffic step and that the *sec5*^{E13} allele uncouples the exocytic function of Sec5 from a role in the endocytic recycling pathway. Therefore, it may prove fruitful to look in more detail at the role the exocyst plays in postinternalization membrane traffic.

Materials and methods

Fly genetics

Fly crosses were performed at 25°C under standard laboratory conditions. Germ line clones of *sec5*^{E13} were generated using the female sterile technique. Male flies of the genotype *y w hsp70-FLP;FRT40 ovoD1/CyO* were crossed to *y,w, FRT40 s5*^{E13}/CyO (provided by T. Schwarz, Harvard Medical School, Boston, MA; Murthy and Schwarz, 2004). Larvae were heat-shocked at 36 ± 12 h and again 60 ± 12 h after egg laying for 1 h at 37°C. Ovaries were dissected from *y w/y w hsp70-FLP; FRT40 ovoD1/FRT40 s5*^{E13}. Yolkless stock (y^1 cv^1 v^1 yl^{15} f^1/FM0) was obtained from the Bloomington *Drosophila* Stock Center (DiMario and Mahowald, 1987).

Generation of antibodies

The COOH-terminal parts of Sec5 (192 COOH-terminal amino acids), Sec6 (197 aa), Sec3 (CG3885, 189 aa), and Exo84 (CG6095, 174 aa) were expressed in *Escherichia coli* as GST fusion proteins using vector pGEX-6P-2 (Amersham Biosciences). The fusion proteins were recovered in inclusion bodies that were washed twice in 0.5% (vol/vol) Triton X-100 and 1 mM EDTA in PBS, resuspended in sample buffer, and separated by SDS-PAGE. The gels were stained briefly in Coomassie blue, bands excised, and the protein eluted by electrophoresis and recovered by precipitation. Antigen was injected into rabbits (for Sec5 and Sec6) or rats (for Sec3 and Exo84) with Freund's complete adjuvant, boosted twice in Freund's incomplete adjuvant, and bled out. Rabbit sera were preabsorbed with GST-Sepharose, and then affinity purified using the corresponding antigen coupled to Sepharose and eluted with Na^+ citrate, pH 2.5. Rat antibodies were used for blotting without purification.

Immunofluorescence and antibodies

Ovaries were dissected in PBS and fixed in 4% PFA in PBS for 15 min. They were washed three times for 20 min in 0.3% Triton X-100 in PBS (PBS-T) and blocked for 1 h in 2% BSA in PBS-T. Antibody incubations were in 0.5% BSA in PBS-T overnight at 4°C, followed by washing several times in PBS-T. After mounting in Fluoromount-G (Southern Biotechnology Associates, Inc.), images were taken with a confocal microscope (model Radiance; Bio-Rad Laboratories). Primary antibodies were rabbit anti-Sec5 (1:3,000), rat anti-Yolkless and rabbit anti-yolk (provided by A. Mahowald, University of Chicago, Chicago, IL), rabbit anti–α-adaptin (provided by M. González-Gaitán, Max Planck Institute, Dresden, Germany; González-Gaitán and Jackle, 1997), mouse anti-rat clathrin (BD Biosciences), mouse anti-*Drosophila* Golgi (Stanley et al., 1997), mouse anti–Dsyntaxin-1A (1:10, 8C3; Developmental Studies Hybridoma Bank [DSHB] at the University of Iowa). Secondary antibodies were goat anti-mouse and anti-rabbit coupled to Alexa 488 and 546 (Molecular Probes) and Cy5-coupled goat anti-rat (Jackson ImmunoResearch Laboratories).

FM4-64 endocytosis assay

Wild-type ovaries from Canton-S flies and *Sec5*^{E13} germ line clone ovaries were dissected in Schneider's *Drosophila* medium (Sigma-Aldrich) with 10 μM FM4-64 (Molecular Probes) and incubated in the same medium at 25°C for 30 min. Ovaries were visualized directly, or, for the back-extraction step, washed in ice cold Schneider's medium over 15 min before mounting and confocal microscopy.

Immunoprecipitation and glycerol gradient analysis

Protein extracts were prepared from wild-type ovaries by homogenization in lysis buffer (50 mM Tris-HCl, pH 7.4, 110 mM NaCl, 1 mM EDTA, 1% NP-40, 1 mM DTT, and protease inhibitors). The homogenate was centrifuged at 16,000 *g* and the supernatant was used for immunoprecipitations and glycerol gradients. For immunoprecipitations, 1 μg of antibody

was added to the protein extract for a 1-h incubation at 4°C before protein A–Sepharose was added and incubated 1 h. Sepharose beads were then washed several times with lysis buffer and proteins were eluted with SDS sample buffer and analyzed by Western blotting.

For glycerol gradient analysis, the protein extract was loaded on top of a 5-ml 15–35% glycerol gradient, and centrifuged at 38,000 rpm in an SW60 rotor for 15 h at 4°C. 26 fractions were collected starting from the bottom of the gradient and proteins were recovered by precipitation with TCA. Fractions were analyzed by Western blotting; primary antibodies used were rabbit anti-Sec5 (1:5,000), rabbit anti-Sec6 (1:5,000), rat anti-Sec3 (1:200), rat anti-Exo84 (1:500), mouse anti-dynamin (1:200), mouse anti-Rho1 (1:50; 4F3, DSHB), and mouse anti-Discs large (1:2,000; p1D9, DSHB).

Immunoelectron microscopy and immunofluorescence of cryosections

Ovaries were fixed in 4% PFA in 0.1 M phosphate buffer, pH 7.4 (PB), for 3 h at RT followed by overnight at 4°C, and stored in 1% PFA in PB at 4°C. Individual egg chambers of appropriate stages were embedded in 12% gelatine (Liou et al., 1996), and mounted on pins so that cryosections could be cut along the long axis on an Ultracut S cryotome (Reichert; Herpers and Rabouille, 2004). For immunoelectron microscopy 60-nm ultrathin cryosections of stage 10 egg chambers were incubated with rabbit anti-Sec5p or rat anti-Yolkless, and then rabbit anti-rat, followed by protein A coupled to 10-nm gold (The Cell Microscopy Center). For immunofluorescence, 500-nm cryosections were deposited on silan-coated slides. After washing thoroughly with PBS, first at 37°C and then at RT, the sections were incubated with freshly prepared $NaBH_4$ (1 mg/ml in PBS, 5 min) followed by PBS washes. Labeling was the same as for immunoelectron microscopy except that the primary antibody incubation was for 1 h, followed by a fluorescent secondary for 45 min in the dark. The sections were rinsed with distilled water, mounted in Vectashield (Vector Laboratories) under a coverslip, and imaged with a confocal microscope (model TCS-NT; Leica) with a Plan-Apo 63× objective. Images were normalized in Adobe Photoshop 7.0 without altering the gamma, and denoised using a 0.7-pixel Gaussian blur filter.

Quantitation

The relative distribution of Sec5p was assessed on stage 10 oocyte cryosections by estimating how much of the gold particles in a region of 1 μm underneath the plasma membrane, at any part of the oocyte, decorated clathrin-coated structures, noncoated endocytic structures, and cytoplasm. Clathrin-coated structures were defined as flat pits, invaginations, and vesicular profiles where a thick and characteristic coat was observed, which was confirmed by a labeling with an anti-clathrin antibody (Fig. 2 C). The noncoated endocytic structures were characterized as such by labeling with an anti-Yolkless antibody that heavily labeled these structures (Fig. 5 C). The linear density of Sec5 on clathrin-coated structures and uncoated endocytic profiles was estimated on pictures at 90 K by the intersection method (Rabouille, 1999). More than 100 structures of each category were counted. The cytoplasmic content of yolk granules was estimated by point hit (Rabouille, 1999), using at least 10 pictures at 5 K.

Yeast methods

The *SEC5* and *STE3* genes were truncated and tagged with either GFP or a 3×HA tag using homologous recombination and kanMX selection (Wach et al., 1997). For **a**-factor recycling assays, cells were grown in YEPD medium at 30°C, diluted to an OD600 of 0.5, and shifted to 37°C for 2 h. **a**-Factor (Sigma-Aldrich) was added to 2 μM of final concentration and cells were incubated for 45 min. **a**-Factor was then washed away and cells were allowed to recover. At various time points, cells were visualized using a confocal microscope. Snc1p-GFP was expressed from a CEN plasmid as described previously (Lewis et al., 2000).

Online supplemental material

Fig. S1 shows validation of antisera to *Drosophila* exocyst and COG subunits and an alignment of Sec5 from *Drosophila*, yeast, and other species around the region mutated in *sec5*^{E13} allele. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200411053/DC1.

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