# The exocyst component Sec5 is required for membrane traffic and polarity in the *Drosophila* ovary

**Mala Murthy[1,2] and Thomas L. Schwarz[1,*]**

[1]Division of Neuroscience, Children's Hospital, Harvard Medical School, Boston, MA 02115, USA
[2]Department of Molecular and Cellular Physiology, Stanford University, Stanford, CA 94305, USA
*Author for correspondence (e-mail: thomas.schwarz@tch.harvard.edu)

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## Summary

**The directed traffic of membrane proteins to the cell surface is crucial for many developmental events. We describe the role of Sec5, a member of the exocyst complex, in directed membrane traffic in the *Drosophila* oocyte. During oogenesis, we find that Sec5 localization undergoes dynamic changes, correlating with the sites at which it is required for the traffic of membrane proteins. Germline clones of *sec5* possess defects in membrane addition and the posterior positioning of the oocyte. Additionally, the impaired membrane trafficking of Gurken, the secreted ligand for the EGF receptor, and Yolkless, the vitellogenin receptor, results in defects in dorsal patterning and egg size. However, we find the cytoskeleton to be correctly oriented. We conclude that Sec5 is required for directed membrane traffic, and consequently for the establishment of polarity within the developing oocyte.**

Key words: *Drosophila*, Polarity, Trafficking

## Introduction

Directed membrane traffic is essential for many developmental processes, including cell growth, cytokinesis and signaling between cells. Such processes require membrane traffic to particular domains of the cell surface, in order to insert proteins at restricted regions of the membrane, to enlarge particular regions of the cell membrane, or to signal asymmetrically to neighboring cells. The identification, therefore, of the molecules required for directed membrane traffic will be important for understanding organismal development and cell-cell signaling.

The exocyst complex, a set of eight proteins first identified from secretory mutants in yeast (Novick et al., 1980), are attractive candidates for mediating directed traffic. Yeast cells use an anisotropic secretory apparatus for polarized growth at a selected bud site (Field and Schekman, 1980). While the bud is growing, there is almost no increase in the surface area of the mother cell (Waddle et al., 1996), indicating that all membrane addition occurs at the bud tip. Later, secretion is redirected to the neck between mother and bud (Tkacz and Lampen, 1972). The exocyst complex marks these areas of membrane addition, localizing to the bud tip of a growing daughter cell and the bud neck at the time of cytokinesis (Finger et al., 1998; Guo et al., 1999; Novick et al., 1995). Mutations in each member of the exocyst complex block the polarized trafficking that allows the bud to grow, but do not disrupt bud site selection. Thus, the exocyst complex in yeast may provide a model for the directed membrane traffic of developing cells in higher organisms.

The components of the exocyst complex, Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84, are conserved from yeast to mammals (Hsu et al., 1996; Novick et al., 1995). In multicellular organisms, though less extensively studied, these proteins are implicated in establishing cell polarity. In epithelial cells, E-cadherin mediated adhesion is sufficient to initiate the segregation of apical from basolateral membrane proteins (Vega-Salas et al., 1988; Wang et al., 1990). The exocyst localizes to these sites of adhesion and is required for the polarized transport of proteins to the basolateral domain (Grindstaff et al., 1998). Neurons generate their polarity by directing membrane traffic to growing neurites and growth cones, and by sorting proteins differentially between the axon and dendrites (Burack et al., 2000; Craig et al., 1995). The exocyst localizes to the tips of growing neurites and is required for neurite extension (Hazuka et al., 1999; Murthy et al., 2003). The distribution of the exocyst in some systems thus suggests that, as in yeast, it will play a necessary role in directing membrane traffic to subcellular domains.

Oogenesis in *Drosophila* requires the establishment and maintenance of cellular asymmetry within the developing oocyte, and provides a system in which to study directed membrane traffic. Within the egg chamber, which consists of 16 germline cells interconnected by ring canals and surrounded by somatic follicle cells, membrane ligands, adhesion proteins and transmembrane receptors are called upon to signal within particular domains of the cell surface. These signals allow the oocyte to migrate to the posterior end of the egg chamber, induce reorganization of the microtubule cytoskeleton, establish thereby asymmetries within the oocyte, and induce differentiations of the adjacent follicle cells (Queenan et al., 1999; van Eeden and St Johnston, 1999). These events all rely on the directed trafficking of proteins, including E-cadherin, Gurken and the EGFR, to the plasma membrane, so as to establish polarity within the oocyte and its surrounding cells. In addition, the localization of some non-membrane determinants of polarity, such as Oskar, may be indirectly

dependent on membrane trafficking (Bretscher, 1996; Dollar et al., 2002; Jankovics et al., 2001; Ruden et al., 2000).

We have used mutations in *sec5*, a core component of the exocyst complex, to investigate the role of the exocyst in membrane trafficking and in the generation of cell polarity within the *Drosophila* ovary. We find Sec5 is dynamically localized during oogenesis in a manner that corresponds with the changing needs of the egg chamber for directed membrane traffic. Furthermore, Sec5 is required both for growth of the germline cells and for membrane trafficking necessary for establishment of the anteroposterior axis and dorsoventral pattern.

## Materials and methods

### *Drosophila* stocks and phenotypic analysis

The following stocks were used in our experiments:

*y,w;Sp/Cyo;nanos-Gal4*
*FRT40 ovoD (2L)*
*y,w;FRT40 sec5E10/Cyo;UAS-FLP*
*y,w;FRT40 sec5E13/Cyo;UAS-FLP*
*y,w, hs-FLP;FRT40 sec5E13/Cyo*
*w;FRT40 Ubi-GFP;T155-Gal4, UAS-FLP* (a gift from D. Bilder)
*y,w;kinesin-lacZ/TM3,Sb* (Clark et al., 1994)
and *UAS-nod-GFP* (III) (Bolivar et al., 2001).

Germline clones of $sec5^{E10}$ and $sec5^{E13}$ were generated by crossing *y,w;FRT40 sec5/Cyo;UAS-FLP* female flies to *y,w;FRT40 ovoD/Cyo;nanos-Gal4/TM3*, Sb males. Ovaries were dissected from females of genotype *FRT40 sec5/FRT40 ovoD;UAS-FLP/nanos-Gal4*. $sec5^{E13}$ ovaries expressing *UAS-nod-GFP*, were dissected from females of genotype *y,w hs-FLP/+;FRT40* $sec5^{E13}$*/FRT40 ovoD;UAS-nod-GFP/nanos-Gal4* and heat shocked 1 hour at 37°C each day during larval and pupal development. Follicle cell clones of $sec5^{E10}$ or $sec5^{E13}$ were generated by crossing *y,w;FRT40 sec5/Cyo;UAS-FLP* females to *y,w, hs-FLP;FRT40 Ubi-GFP;T155-Gal4, UAS-FLP/+* males and heat shocking 1 hours at 37°C during larval development.

### Immunocytochemistry and microscopy

Ovaries from 1- to 4-day-old females were dissected in PBS, and kept on ice. Ovaries were fixed in 6:1 Heptane:FIX [FIX=4 vol $H_2O$, 1 vol Buffer B (100 mM potassium phosphate pH 6.8, 450 mM KCl, 150 mM NaCl, and 20 mM $MgCl_2$), and 1 vol 37% Formaldehyde] for 15 minutes. All antibody staining was carried out in PBS, containing 0.5% BSA, 0.1% Triton-X-100 and 5% normal goat or donkey serum. The following stains and primary antibodies were used: Texas Red-X phalloidin and Hoechst 33342 (Molecular Probes); fluorescein-*lycopersicon esculentum* lectin (Vector Laboratories); mouse anti-Gurken 1D12; mouse anti-Syntaxin 8C3; mouse anti-Orb 4H8 and mouse anti-FasIII 7G10 (Hybridoma bank); rabbit anti-βgal (ICN); mouse anti-Sec5 22A2 (Murthy et al., 2003); rat anti-DE-cadherin (Oda et al., 1994); rat anti-Yolkless (Schonbaum et al., 2000); rabbit anti-Oskar (Ephrussi et al., 1991); rabbit anti-Par-1 (Shulman et al., 2000); mouse anti-Dhc P1H4 (McGrail and Hays, 1997); and mouse anti-Bicaudal-D (Suter and Steward, 1991). Secondary antibodies used were: FITC-goat anti-mouse; FITC or TR-donkey anti-mouse; FITC or TR-donkey anti-rat; and FITC-goat anti-rabbit (Jackson Laboratories).

To disrupt microtubules, colcemid (50 μg/ml) in yeast paste was fed to adult females for 8-10 hours (Theurkauf and Hazelrigg, 1998).

Confocal data were acquired as single images or image stacks of multi-tracked, separate channels with a Zeiss LSM 510 microscope. Three-dimensional projections of image stacks were made with the 3D Zeiss software package. Nomarski images were captured on a Nikon Eclipse E800.

## Results

### Germlines homozygous for *sec5* possess defects in membrane addition and egg chamber polarity

We used the FLP-dominant female sterile technique (Chou and Perrimon, 1996) to generate maternal germlines homozygous for $sec5^{E10}$, a null allele that contains an early stop codon and is cell lethal when homozygous in the eye (Murthy et al., 2003). When homozygous in oocytes, $sec5^{E10}$ impaired development and no eggs were laid. These defects were entirely due to the loss of Sec5, because the viability and fertility of homozygous $sec5^{E10}$ females could be restored by a *sec5* transgene. The presence in the ovaries of sequentially staged homozygous egg chambers made it possible to investigate the timing and manner of the developmental arrest.

Wild-type development of the egg chamber has been subdivided into a series of 14 stages (King, 1970). During stages 2-6, after the egg chamber exits the germarium, the 15 nurse cells and one oocyte grow at similar rates. The oocyte occupies the posterior-most position among the group of sixteen cells (Fig. 1A). By stage 7, the microtubule cytoskeleton within the oocyte reorients and the nucleus moves to an anterior corner position, i.e. along the circular rim where the lateral and anterior surfaces of the oocyte meet, and thereby specifies this region as the dorsal side (Fig. 1B). Thereafter, the oocyte grows disproportionately to the nurse cells (Fig. 1C).

$sec5^{E10}$ mutant germlines arrest at approximately stage 6 with striking structural changes. Normally, F-actin resides just beneath the membrane of each of the 16 cells and in the actin-rich ring canals that provide cytoplasmic bridges between the germ cells. In $sec5^{E10}$ germlines, actin, visualized with rhodamine-phalloidin, no longer separates the nuclei and the ring canals cluster together in the center of the egg chambers (Fig. 1F). Defects are apparent much earlier, however, with many egg chambers failing to exit the germarium and ring canals clumping together by stage 2 (Fig. 1D,E). By stage 5 or 6, the follicle cell layer, which is heterozygous for the mutation, begins to disintegrate as well, a likely secondary consequence of the germline phenotype. The absence of phalloidin-staining suggested that the membranes that normally separate the cells of the cyst were absent. To test this hypothesis, we labeled egg chambers with a fluorescein-conjugated tomato lectin which binds to glycoproteins in membranes. In the control (Fig. 1G), the fluorescein-conjugated lectin labels the plasma membrane and therefore co-localizes with phalloidin. In $sec5^{E10}$ mutant egg chambers, either no plasma membrane was detected between nuclei, or a membrane was only observed between some nuclei and not others. Large dispersed pools of lectin-stained puncta resided within the cytoplasm of mutant, but not control, egg chambers (Fig. 1H). These puncta could either represent proteins that were not trafficked to the plasma membrane, or remnants of a disrupted plasma membrane. We also stained egg chambers for Syntaxin (Fig. 1I), an additional marker of the plasma membrane, where it is required for vesicle fusion (Burgess et al., 1997). In $sec5^{E10}$ germlines, Syntaxin-labeled membranes were frequently absent between nuclei and Syntaxin immunoreactivity was concentrated within the cytoplasm, surrounding the clumps of ring canals (Fig. 1J). These findings suggest that the primary defects in oogenesis are due to a block in membrane trafficking. As the heterozygous follicle cells

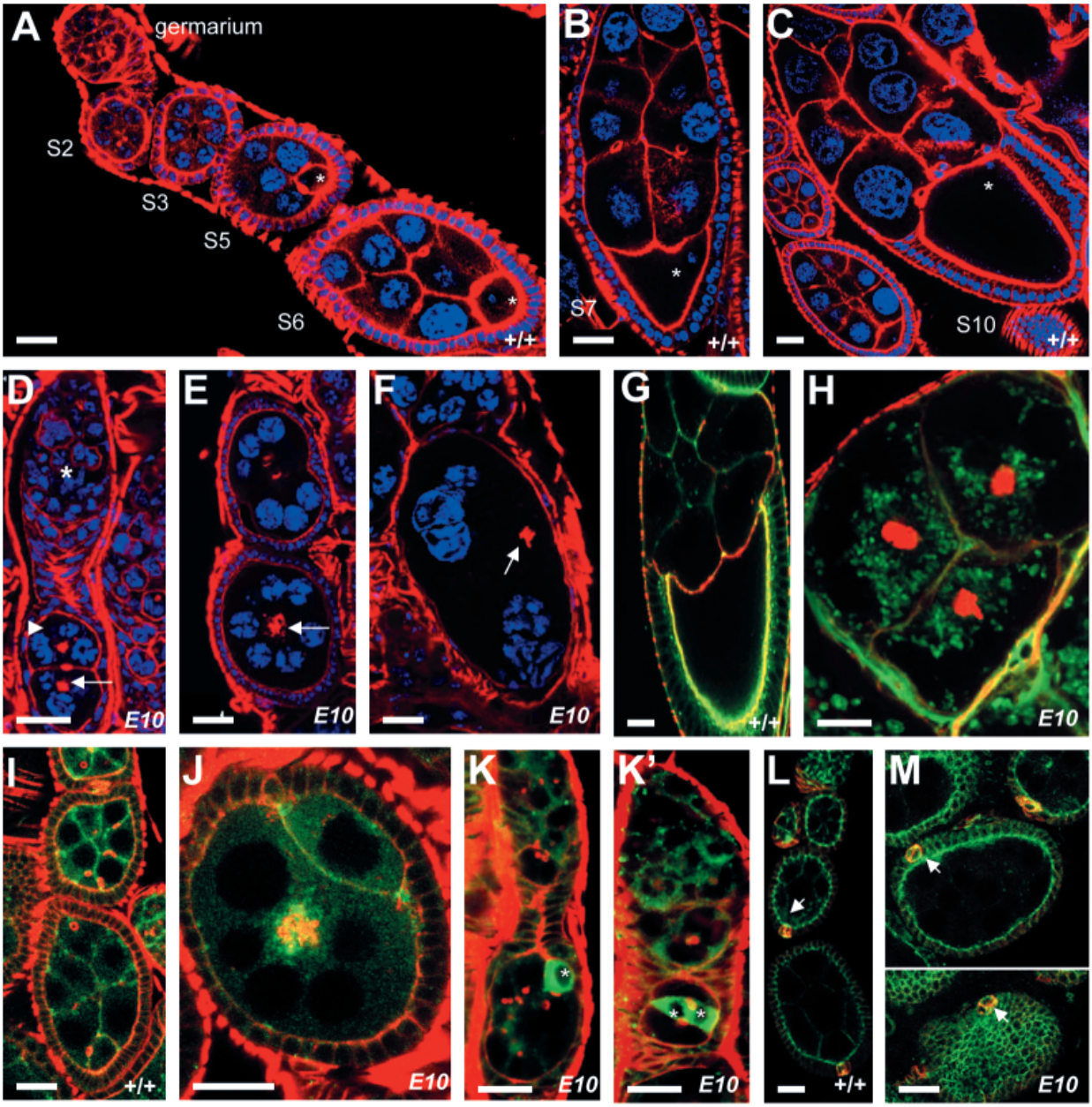



**Fig. 1.** *sec5*$^{E10}$ mutant germlines display defects in membrane addition and egg chamber polarity. (A-C) Wild-type development of the ovariole. Membranes are marked with phalloidin (red), which binds to F-actin, and nuclei with Hoechst 33342 (blue). The oocyte (*) and egg chamber stage are indicated. At stage 7 (B), the oocyte nucleus (*) has moved to an anterior corner of the oocyte. At stage 10 (C), the oocyte has grown disproportionately to the nurse cells while its nucleus (*) remains at the dorsal anterior corner. (D-F) Homozygous *sec5*$^{E10}$ germline clones. (D) Mutant egg chambers often fail to exit the germarium (*). Those that do possess multi-nucleate cells, lacking phalloidin-labeling between nuclei (arrowhead) and possessing aggregated ring canals (arrow). (E) By stage 4 or 5, all ring canals are clumped together in the multi-nucleate chamber (arrow). (F) Clones arrest by stage 6. Ring canals are aggregated (arrow) and follicle cells begin to die. Wild-type (G) and *sec5*$^{E10}$ (H) egg chambers labeled with fluorescein-conjugated tomato lectin (green) and phalloidin (red). Wild-type (I) and *sec5*$^{E10}$ (J) egg chambers stained for Syntaxin (green) and phalloidin (red). (K,K′) The oocyte (*), labeled with antibodies to Dynein Heavy Chain (green), is incorrectly positioned in *sec5*$^{E10}$ germline clones. Wild-type egg chambers (L) and two *z* sections through one *sec5*$^{E10}$ egg chamber (M). E-cadherin (green) concentrates at the adherens junctions between follicle cells in both mutant and wild type. Polar cells (arrows), labeled by antibodies to FasIII (red), are located at the posterior of the egg chamber in wild type, but are mispositioned in *sec5*$^{E10}$ germline clones. All images are single *z* sections of confocal image stacks. Scale bars: 20 μm.

expand, the surface area of the mutant germline cells probably fails to increase at an adequate pace, causing the membranes dividing these cells to fall apart. Consequently, ring canals clump together, while membrane fragments and possibly unincorporated transport vesicles remain within the cytosol of the large multinucleate cells.

Additionally, *sec5*$^{E10}$ mutant germlines displayed polarity defects. Normally, the oocyte occupies the posterior-most

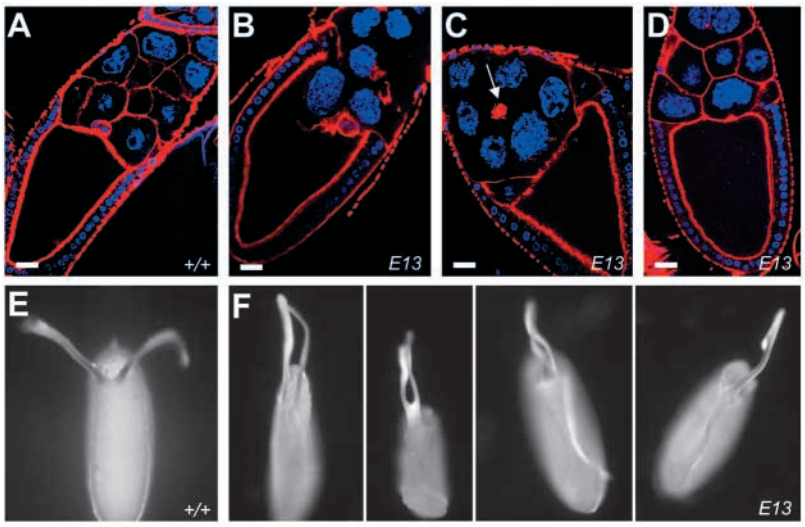



**Fig. 2.** Dorsal patterning defects in *sec5*$^{E13}$. Stage 10 egg chambers with wild-type (A) or *sec5*$^{E13}$ (B-D) germline clones. Some mutants lack phalloidin (red) between nuclei (blue) and have aggregated ring canals (C, arrow). However, many *sec5*$^{E13}$ germlines (D) more closely resemble wild type. Wild-type eggs (E) have two stereotypically spaced appendages on the dorsal side. All eggs laid by *sec5*$^{E13}$ germline mothers display fused or closely spaced dorsal appendages (F). Scale bars: 20μm.

position, owing to E-cadherin-based differential adhesion between the oocyte and the posterior follicle cells (Godt and Tepass, 1998; Gonzalez-Reyes and St Johnston, 1998). In *sec5*$^{E10}$ germlines, the oocyte, labeled with antibodies to Dynein Heavy Chain (Dhc), was often mispositioned (Fig. 1K,K′). In addition, the polar cells, which are important for establishing initial polarity cues within the egg chamber (Grammont and Irvine, 2002), are often mis-positioned within the heterozygous follicle epithelium (Fig. 1L,M). In *sec5*$^{E10}$ germline clones, we also observed the development of compound follicles with multiple germline cysts enclosed within a single follicle epithelium (data not shown), a phenotype common to mutants that cause a loss of polar cell identity. These phenotypes indicate that *sec5* is important for the initial establishment of anteroposterior polarity within the egg chamber.

## Females with germlines homozygous for *sec5*$^{E13}$ lay eggs with dorsal-ventral patterning defects

Because germline clones of *sec5*$^{E10}$ were lethal early in oogenesis, we were unable to assess the subsequent roles of Sec5 during cytoskeletal rearrangement and establishment of the anteroposterior and dorsoventral axes. However, germline clones of the hypomorphic allele *E13* (truncated at position 361) are not lethal, and these females lay eggs (Murthy et al., 2003).

*sec5*$^{E13}$ phenotypes in the germline were diverse, with some egg chambers resembling those of the control (Fig. 2D). Others possess defects similar to those of *sec5*$^{E10}$: phalloidin-marked membranes are missing between cells, nurse cell nuclei appear to fall into the oocyte where the membrane between them has broken down (Fig. 2B) and ring canals clump together (Fig. 2C). However, all eggs laid by *sec5*$^{E13}$ mothers show dorsoventral patterning defects, similar to those caused by hypomorphic mutations in the Gurken and EGF receptor signaling pathway (Nilson and Schupbach, 1999; Queenan et al., 1999; Roth et al., 1995). Dorsal appendages are either too closely spaced or are fused (Fig. 2F).

## Sec5 protein is enriched in the posterior follicle cells, and required there for the posterior positioning of the oocyte within the egg chamber

We used a monoclonal antibody (22A2) generated to the C-terminal end of Sec5 to study the localization of the protein during oogenesis. This antibody is specific for Sec5 on both western blots and immunocytochemistry (Murthy et al., 2003). The epitope recognized by the antibody is absent from the truncated Sec5 protein encoded by *sec5*$^{E13}$, so the antibody could also be used to mark homozygous clones in mosaic ovaries.

In early egg chambers, Sec5 localizes to the membranes between cells in the germline, and to the side of the follicle cells that contacts the germline (Fig. 3A). Sec5 also appeared consistently enriched at the border between the posterior follicle cells and the egg chamber (Fig. 3B). In egg chambers where only the germline is mutant for *sec5*$^{E13}$, this enrichment persists, indicating that it predominantly corresponds to the apical surface of the posterior follicle cells, particularly the two polar cells at the extreme posterior (Fig. 3C,D).

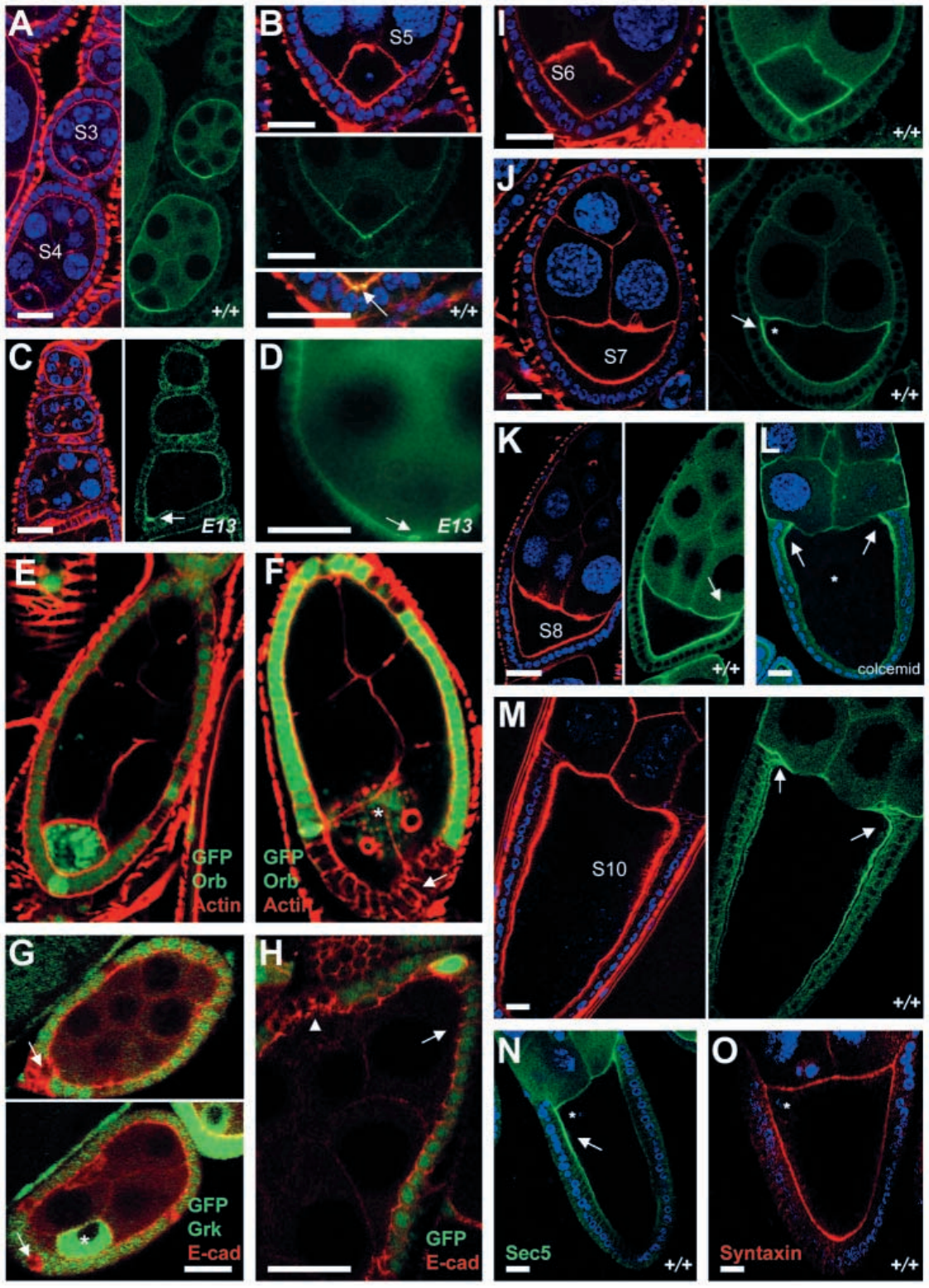
To determine the role of Sec5 in the posterior follicle cells, we generated mutant follicle cell clones, marked by an absence of green fluorescent protein (GFP). We were unable to generate clones of the cell-lethal *E10* allele, but the hypomorphic *E13* allele could be used. The follicle

**Fig. 3.** Sec5 localization and the establishment of the anteroposterior axis. (A-D,I-K,M) Egg chambers, stage and genotype indicated, were stained with phalloidin (red), Hoechst (blue) and anti-Sec5 monoclonal 22A2 (green). (A,B) Sec5 concentrates posteriorly, at the boundary between the follicle cells and the oocyte. Particularly at stage 5, sec5 resides at the apical end of the two posterior polar cells (arrow in merged image). (C,D) Sec5 persists in the posterior follicle cells (arrows) when the germline is homozygous for *sec5*$^{E13}$. (E-H) Follicle cell clones of *sec5*$^{E13}$ (marked by the absence of GFP). (E,F) The oocyte is labeled with anti-Orb (also green), and membranes are marked with phalloidin (red). When the posterior follicle cells are homozygous for *sec5*$^{E13}$ (F; arrow), the oocyte (*) no longer occupies the posterior-most position. (G) Two *z* sections through one egg chamber. Again, the *sec5*$^{E13}$ follicle cell clone at the posterior (arrows) causes the oocyte (*), labeled with anti-Gurken (green), to reside too anteriorly. Adherens junctions between follicle cells are labeled with anti-E-cadherin (red). (H) In larger *sec5*$^{E13}$ follicle cell clones (arrowhead), the epithelium is disordered compared with heterozygous cells (arrow). (I-K) Wild type. At stages 6-8, Sec5 appears increasingly at the anterior of the oocyte (arrows). (L) A colcemid-treated stage 10 egg chamber wherein the oocyte nucleus (*) did not migrate correctly. Sec5 (green) still concentrates at the anterior membranes of the oocyte. (M,N) At stage 10, Sec5 concentrates at the anterior corners (arrows), and is nearly absent from the posterior membrane. (O) Syntaxin (red) is uniformly localized on the oocyte membrane. The nucleus is marked with an asterisk. Scale bars: 20 μm.

epithelium appeared disorganized in large clones of *sec5*$^{E13}$ (Fig. 3H). However, even small homozygous clones induced a phenotype when they included posterior follicle cells; the oocyte no longer migrated to the posterior-most position among the germline cells (Fig. 3E-G), much as was seen when the oocyte was mutant for *sec5* (Fig. 1K,K′). Thus, mutations in sec5 in either posterior follicle cells or the germline prevented development of the proper anteroposterior axis.

### In late stage egg chambers, Sec5 protein alters its distribution within the oocyte membrane

At stage 6, Sec5 localization changes; although still enriched in the polar cells and expressed at low levels ubiquitously, Sec5



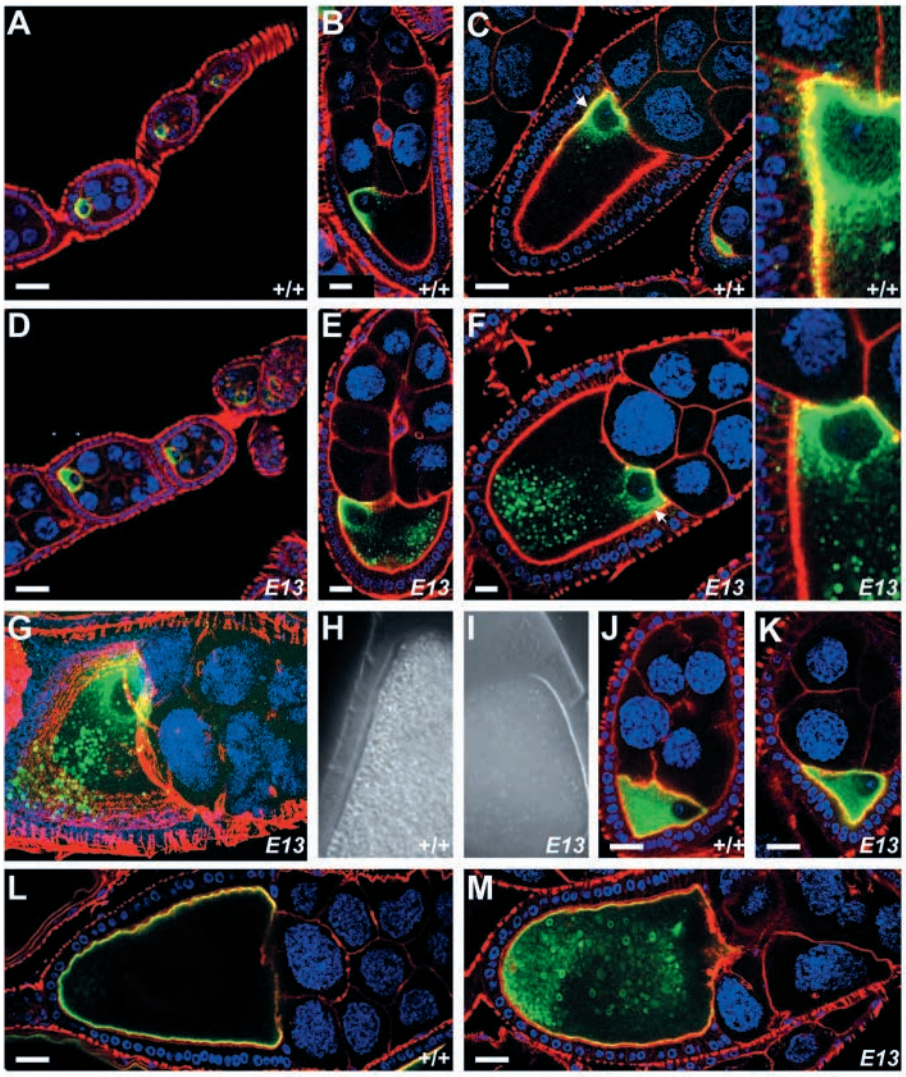



**Fig. 4.** Gurken and yolkless trafficking are disrupted in *sec5*$^{E13}$ oocytes. (A-G,J-M) Egg chambers, genotype indicated, were stained with phalloidin (red), Hoechst (blue), and anti-Gurken (green, A-G) or anti-Yolkless (green, J-M). (A,D) At early stages, Gurken accumulates in the oocyte in both wild-type and *sec5*$^{E13}$germlines. (B,E) At stage 8, Gurken is mislocalized within the cytoplasm of *sec5*$^{E13}$ oocytes. (C,F) Gurken localizes at the anterodorsal membrane in stage 10 wild-type oocytes (arrow and enlargement), but in *sec5*$^{E13}$ mutant oocytes accumulates in puncta within the ooplasm. Little Gurken is observed in the membrane (arrow and enlargement). (G) A compressed *z* stack of a *sec5*$^{E13}$ stage 10 egg chamber, displaying mislocalized Gurken protein throughout the ooplasm. (H,I) Oocyte yolk granules visualized by Nomarski optics. (J,K) Yolkless (green) accumulates in the cytosol of stage 7 oocytes. (L,M) By stage 10, Yolkless shifts to the plasma membrane of wild-type but not *sec5*$^{E13}$ oocytes. Scale bars: 20 μm.

concentrates at the oocyte membrane (Fig. 3I). At this time, the oocyte grows at a faster rate than the nurse cells and the enrichment in the oocyte membrane may reflect the greater need for membrane addition there. During stage 7, when the microtubule cytoskeleton reorients and the nucleus moves to the dorsoanterior corner of the oocyte, Sec5 appears enriched along this anterior rim, at the corners where the lateral and anterior membranes of the oocyte meet (Fig. 3J), although still expressed all along the membrane. This pattern continues through stage 8 (Fig. 3K). Finally, at stage 10, Sec5 is highly concentrated at the anterolateral margins of the oocyte, with less detectable towards the posterior end of the cell (Fig. 3M). To determine if this distribution was shared by other plasma membrane-associated components of the membrane-trafficking apparatus, we compared Sec5 labeling with that of the t-SNARE Syntaxin (Burgess et al., 1997). Syntaxin is present along the length of the oocyte membrane, including the posterior region (Fig. 3N,O).

Because Sec5 becomes enriched at the anterior membrane of the oocyte at the time when the microtubule cytoskeleton rearranges within the oocyte and because the exocyst has been shown to associate with the cytoskeleton (Vega and Hsu, 2001), we tested if the localization of Sec5 was dependent on the cytoskeleton. In oocytes from females treated with colcemid, a microtubule-depolymerizing drug, Sec5 continued to be concentrated at the stage-appropriate domains of the oocyte membrane, including the anterior corners at stage 10 (Fig. 3L).

## The anterior trafficking of Gurken is disrupted in *sec5*$^{E13}$ oocytes

The shift in Sec5 localization from the posterior of the oocyte to the anterior parallels a shift in the directed secretion of Gurken (Nilson and Schupbach, 1999). Secreted at the posterior margin before stage 7, Gurken thereafter signals from an anterior corner of the oocyte to adjacent follicle cells. Those cells that receive the highest levels of Gurken repress the differentiation of the dorsal lateral follicle cells, thus creating a space between two lateral patches of cells that will form the appendages (Morimoto et al., 1996; Neuman-Silberberg and Schupbach, 1994). Because females with *sec5*$^{E13}$ germlines lay eggs with fused dorsal appendages (Fig. 2), we hypothesized a role for Sec5 in Gurken signaling.

In early stages, both wild type and *sec5*$^{E13}$ germlines appropriately accumulated Gurken in the oocyte (Fig. 4A,D). After stage 7, however, Gurken was mislocalized in granules throughout the mutant oocytes (Fig. 4B,E). In stage 10 egg chambers, when Gurken is present at the dorsoanterior membrane of the oocyte in wild type (Fig. 4C), a substantial amount of Gurken is observed in granules scattered throughout the cytoplasm of *sec5*$^{E13}$ oocytes. Much Gurken remains in the vicinity of the nucleus, but very little is present in the membrane (Fig. 4F,G). The cytoplasmic Gurken in *sec5*$^{E13}$ oocytes is not coincident with a marker for the ER, Boca (data

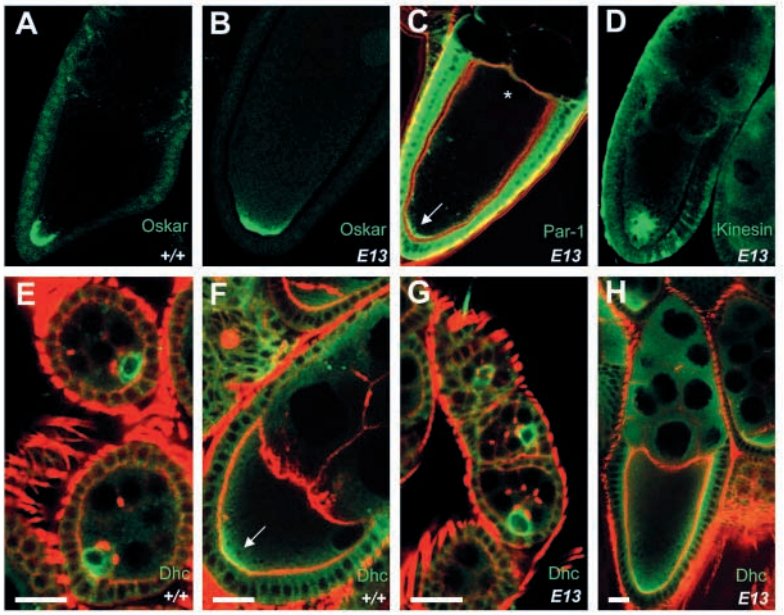



**Fig. 5.** Posterior markers are normal in *sec5*$^{E13}$ oocytes. (A) Wild-type and (B) *sec5*$^{E13}$ oocytes labeled with anti-Oskar (green). (C) Par-1 (green) localizes properly in *sec5*$^{E13}$ clones (arrow). Phalloidin (red) and the oocyte nucleus(*) are shown. (D) A Kinesin-βgal fusion (green) localizes to the posterior end of the *sec5*$^{E13}$ oocyte. In wild-type (E,F) and *sec5*$^{E13}$ (G,H) egg chambers, Dynein Heavy Chain (green) accumulates in the oocyte between stages 1-6 (E,G) and subsequently (F,H) at its posterior end (arrow). Scale bars: 20 μm.

not shown), indicating that the block in the directed trafficking of Gurken is at a later step of the pathway.

### The trafficking of the vitellogenin receptor is disturbed in *sec5*$^{E13}$ oocytes

Eggs derived from *sec5*$^{E13}$ homozygous germlines, were typically flaccid, small (Fig. 2F) and, by Nomarski optics, devoid of yolk granules (Fig. 4H,I). Yolk proteins, however, are synthesized in fat bodies and follicle cells (which were not homozygous for the mutation) and are subsequently imported into the oocyte by endocytosis after binding to the vitellogenin receptor, Yolkless (Schonbaum et al., 2000). A defect in the trafficking of Yolkless to the oocyte surface might therefore explain the decreased yolk content of the *sec5* oocytes.

In wild-type germlines, Yolkless is diffusely distributed until stage 8, whereupon, induced by an unknown signal, Yolkless translocates from the ooplasm to the cortex. At stage 7, Yolkless was detectable within both control and *sec5*$^{E13}$ oocytes (Fig. 4J,K). At stage 8 in the mutant, however, the majority of the receptor did not go to the surface, and remained cytoplasmic through stage 10 (Fig. 4L,M). The mistrafficking of Yolkless, like the general disruption of membranes in the *sec5* null allele, indicates that Sec5 is not only required for Gurken localization, but rather is of general significance for the membrane trafficking of many germline proteins.

### The orientation of the microtubule cytoskeleton is undisturbed in *sec5*$^{E13}$ oocytes

Although the Gurken and Yolkless mislocalizations were probably due to a defect in membrane trafficking, these phenotypes might be secondary to a defect in the concurrent reorganization of the oocyte, which includes the reorientation of the microtubule cytoskeleton (Theurkauf et al., 1992), the movement of the oocyte nucleus to the anterior cortex of the oocyte, and the localization of Gurken mRNA and protein near the nucleus.

To investigate this possibility, we examined the localization of several proteins restricted to the posterior pole of the oocyte: Oskar, Par-1 and a kinesin-β-gal fusion (Clark et al., 1994; Ephrussi and Lehmann, 1992; Shulman et al., 2000). In both control and *sec5*$^{E13}$ germlines, all three proteins accumulate properly at the posterior pole in stage 8-10 oocytes (Fig. 5A-D). Dynein Heavy Chain (Dhc), also localizes to the posterior end of late stage oocytes (Brendza et al., 2002). This marker also was normal in the mutants, accumulating first in the oocytes of early stage egg chambers (Fig. 5E,G) and after stage 8 at the posterior end of the oocyte (Fig. 5F,H).

To examine directly the polarity of the microtubules, we imaged *sec5*$^{E13}$ oocytes that expressed in the germline a marker for the minus ends of microtubules, a fusion of the head domain of Nod (no-distributive disjunction) to GFP (Bolivar et al., 2001; Clark et al., 1997; Hawley and Theurkauf, 1993). At stages 7 and 10, Nod-GFP was concentrated at the anterior end of the oocyte in both wild type and mutant (Fig. 6A-D). The correct positioning of the minus ends in *sec5*$^{E13}$ was also demonstrated with FITC-conjugated alpha-tubulin (data not shown). Thus, the defective trafficking of Gurken and Yolkless cannot be secondary to microtubule defects.

We observed, however, that the overexpression of Nod-GFP in *sec5*$^{E13}$ oocytes enhanced the phenotype of the *sec5*$^{E13}$ allele alone: the oocyte nucleus was often displaced from the cortex, membranes between cells were absent, the development of the follicle epithelium was disturbed and no eggs of this genotype were laid (Fig. 6E,F). These defects were never observed in Nod-GFP expressing lines that were not mutant for *sec5*. It is possible that the overexpression of the Nod motor domain impairs microtubule-based transport, thereby enhancing the *sec5*$^{E13}$ phenotype by further slowing the delivery of membrane to the cell surface.

### Defects in the membrane apposition of the nucleus in *sec5*$^{E13}$ oocytes

Examining *sec5*$^{E13}$ egg chambers, we noted that the oocyte nucleus was sometimes mislocalized (Fig. 5C; Fig. 4F,G). The nucleus invariably moved to the anterior, as in wild type, but was not closely associated with the dorsoanterior plasma membrane. A three dimensional composite image was assembled from individual *z* sections of stage 10 egg chambers and rotated to reveal the relationship of the nucleus to the plasma membrane. This analysis confirmed that the nucleus was not always adjacent to the dorsal membrane (Fig. 6I,J): eight out of 39 (21%) *sec5*$^{E13}$ oocytes had a mispositioned nucleus, but none of 40 wild-type oocytes.

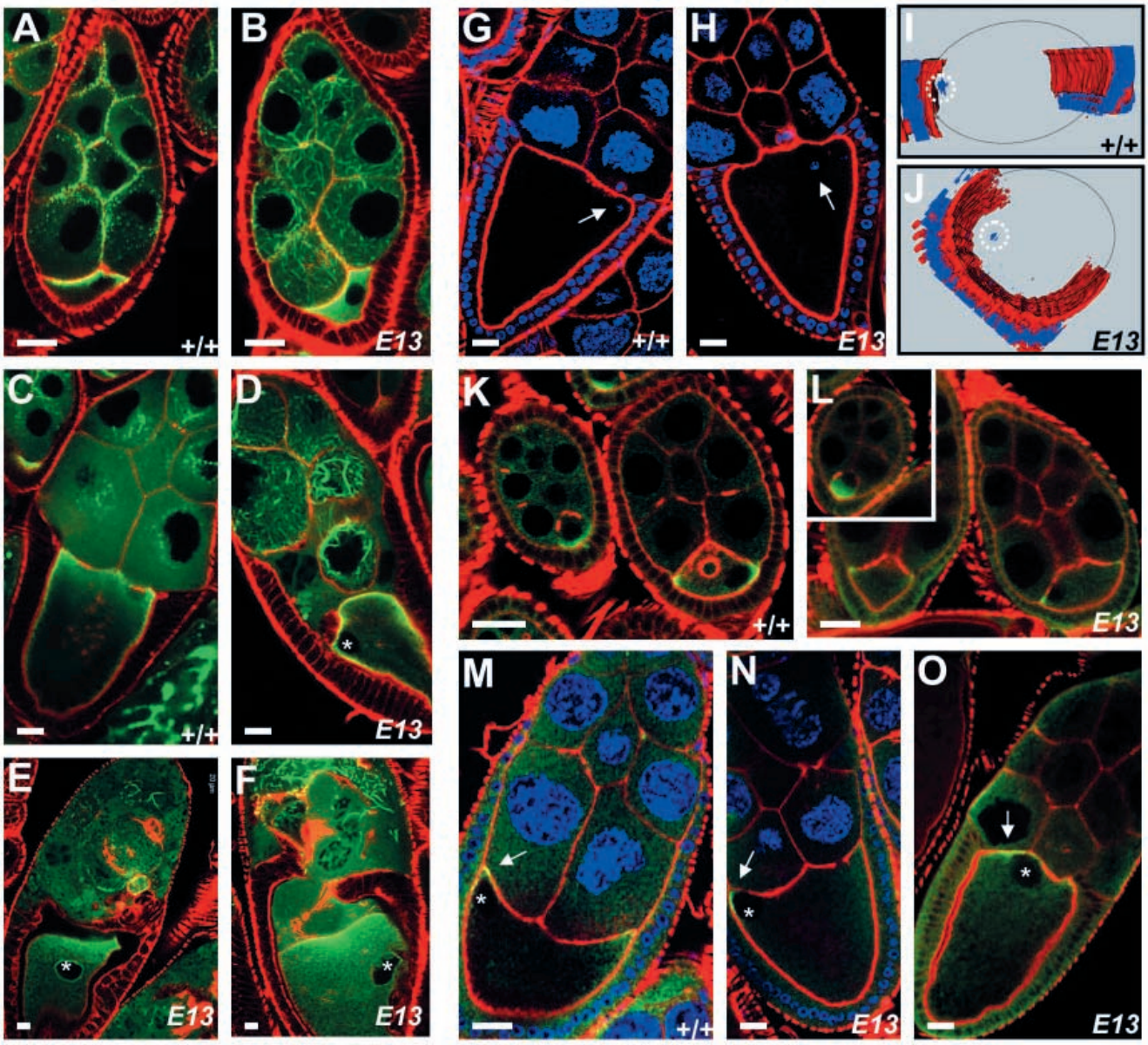



**Fig. 6.** The microtubule cytoskeleton is oriented correctly in *sec5*$^{E13}$ oocytes, although the oocyte nucleus is displaced from the cortex. (A) Wild-type and (B) *sec5*$^{E13}$ stage 7 egg chambers expressing Nod-GFP (green) under the control of nanos-Gal4 and stained with phalloidin (red). (C-F) Stage 10 egg chambers, stained as above. In *sec5*$^{E13}$ oocytes, Nod-GFP properly concentrates anteriorly (D). The oocyte nucleus (*) is also often at the dorsal anterior corner. However, the overexpression of Nod-GFP in the germline enhances the *sec5*$^{E13}$ phenotype (E,F). (G-H) Single *z* sections of stage 10 egg chambers labeled with phalloidin (red) and nuclei with Hoechst (blue), and the position of the oocyte nucleus shown with an arrow. (I,J) The full *z* stack of G and H were rotated in three dimensions and the nurse cells optically removed to reveal the close apposition of the nucleus to the surface in wild type (I), but not in the *sec5*$^{E13}$ chamber (J). (K-O) Egg chambers stained for Bicaudal-D (green), phalloidin (red) and with Hoechst (nuclei, blue), with genotypes indicated. (M-O) Bicaudal-D (arrows) remains associated with the nucleus (*) whether in wild type or *sec5*$^{E13}$ oocytes. Scale bars: 20 μm.

Bicaudal-D (Bic-D) is a cytosolic protein that interacts with the dynein-dynactin complex, and participates in the cortical anchoring of the nucleus (Matanis et al., 2002; Oh and Steward, 2001; Pare and Suter, 2000; Swan et al., 1999). Bic-D localized normally in *sec5*$^{E13}$ oocytes throughout oogenesis. In early stages, Bic-D was at the microtubule minus ends at the oocyte posterior, and by stage 6 relocalized to the anterior rim, preceding the arrival of the nucleus (Fig. 6K,L). Subsequently, Bic-D concentrated above the nucleus (Fig. 6M,N). Even when the oocyte nucleus was displaced from the dorsal cortex, Bic-D remained near the nucleus, indicating that its nuclear association was not sufficient to attach the nucleus to the dorsal cortex (Fig. 6O). Because we saw no alteration of the microtubule cytoskeleton in *sec5*$^{E13}$ germline clones, nor gross mislocalization of Bic-D, the lack of a tight association of the nucleus with the membrane must have other causes.

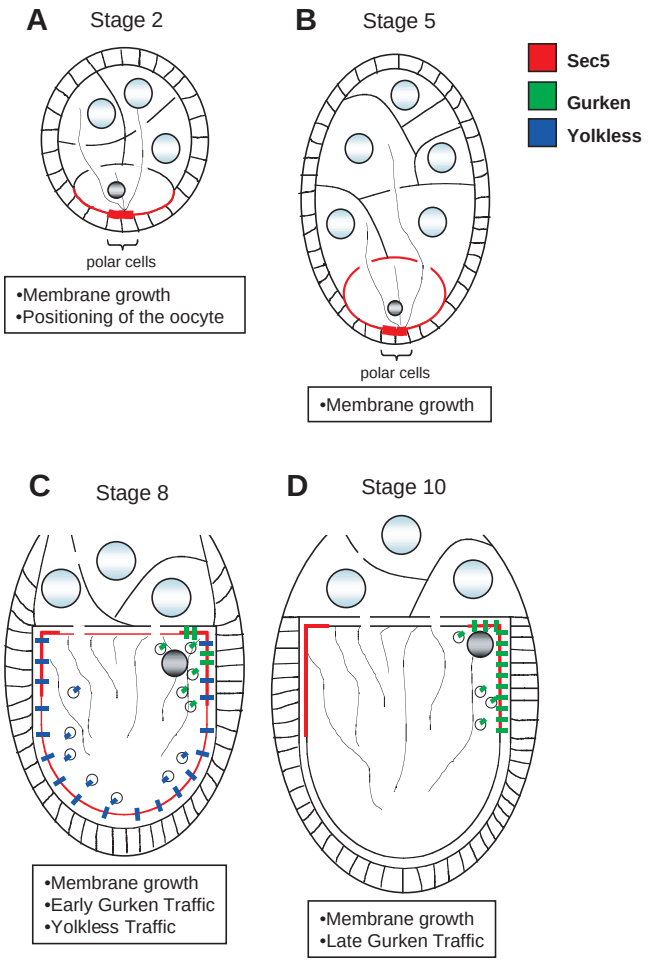



**Fig. 7.** Sec5 localization and function during oocyte development. (A) At stage 2, Sec5 is present on all egg chamber membranes at low levels, but concentrates between the posterior follicle cells and the oocyte, particularly at the apical membrane of the polar cells. Throughout the egg chamber, Sec5 is required to preserve the integrity of the growing cells. Sec5 in the posterior follicle cells and oocyte is essential to position the oocyte at the posterior of the egg chamber. (B) By stage 5, Sec5 concentrates also along the anterior oocyte membrane, at a time when this membrane grows disproportionately to the nurse cells. The enrichment of Sec5 in the polar cells persists, and is probably required there to maintain the posterior position of the oocyte. (C) At stage 8, Yolkless rapidly inserts in the oocyte membrane in a Sec5-dependent manner. At this time, Sec5 is present along the oocyte membrane. Sec5 is also moderately enriched at the anterior rim of the oocyte, and is required there for the initial trafficking of Gurken. (D) By stage 10, Sec5 is concentrated only at the anterior rim of the oocyte membrane. At this time, the majority of Gurken is trafficked to the dorsoanterior membrane to signal to the EGFR in the adjacent follicle cells. The enrichment of Sec5 along all the anterior rim of the oocyte indicates that Sec5 alone cannot specify to which region of that domain Gurken is targeted.

## Discussion

### Localization of Sec5 and correlation with directed membrane traffic

Oocyte polarity and egg chamber organization depend on the addition of particular proteins to subregions of the plasma membrane. The localization of these proteins is likely to involve multiple levels of regulation, including the localization of transcripts, the transport of proteins to particular cytoplasmic regions and the retention of proteins in defined membrane domains. If selective, localized insertion into the plasma membrane is to be a factor in the asymmetric distribution of membrane proteins, then a component of the exocytotic machinery must be appropriately localized. We propose such a role for Sec5 and the exocyst complex. No other protein involved in membrane trafficking is known to have a suitably regulated distribution within the oocyte. The cognate pairing of SNAREs is reported to be significant in distinguishing intracellular compartments for vesicle targeting (Chen and Scheller, 2001; Jahn and Sudhof, 1999). Syntaxin, however, is expressed all along the oocyte membrane and at constant levels (Fig. 1I,3O), and therefore cannot direct polarizing events. In contrast, by concentrating the exocyst complex at different regions of the membrane during oocyte development, the oocyte may target the trafficking of proteins (Fig. 7).

The distribution of Sec5 during oogenesis correlates with where it is required. Sec5 is initially present on all membranes within the egg chamber. At this stage, all the cells of the egg chamber are growing and removing *sec5* from the germline disrupts membranes. Glycoproteins and Syntaxin, which are normally found in the plasma membrane, accumulate instead within the cytosol of the egg chamber. At early stages, the exocyst thus appears to mediate general membrane traffic for cell growth. Sec5 is most abundant at this time at the boundary between the oocyte and posterior follicle cells, the site of reciprocal signaling that governs oocyte position. Making either the germline or the posterior follicle cells mutant for *sec5* results in an abnormally anterior position for the oocyte. In addition to its requirement in general membrane growth, Sec5 is therefore likely to be required at this cell boundary for signals and adhesion molecules on the oocyte and follicle cell surfaces (Godt and Tepass, 1998; van Eeden and St Johnston, 1999) and thereby for establishing the anteroposterior axis. At stage 8, its widespread distribution closely parallels the sites of Yolkless insertion, a Sec5-dependent process. Beginning at stage 7 and culminating at stage 10, Sec5 is increasingly concentrated at anterior corners, when Gurken is inserted at that site. Owing to an inability to traffic Gurken efficiently to the membrane, females with $sec5^{E13}$ germline clones lay eggs with dorsal patterning defects.

The trafficking of the Gurken protein provides at present the best example of an identified membrane protein whose selective, Sec5-dependent localization is crucial to proper development. The final deposition of Gurken is likely to arise from a combination of mechanisms, including the transport of the oocyte nucleus to an anterior corner, the nearby localization of Gurken mRNA, the microtubule-dependent transport of Gurken protein to the cortex, and the insertion of both pre-existing and newly synthesized Gurken into the plasma membrane by vesicle fusion. The presence of displaced Gurken protein in the posterior regions of the mutant ooplasm may be an indirect result of blocked membrane fusion after which Gurken-containing vesicles may drift away from their normal target. Gurken trafficking, however, also indicates that Sec5 and the exocyst cannot be the only cues that direct vesicle fusion: Sec5 localizes along the entire anterior lateral rim of

the oocyte, but Gurken is inserted only at that section adjacent to the nucleus. Furthermore, when the nucleus and Gurken transcripts are mislocalized by cytoskeletal changes, some Gurken signaling occurs ectopically, near the misplaced nucleus (Ghiglione et al., 1999), and away from the major concentration of Sec5 (Fig. 3L). Thus, the localization of Sec5 should be viewed as one of several layers of likely mechanisms for directing membrane proteins.

The phenotypes noted for *sec5$^{E13}$* oocytes were not due to defects in the cytoskeleton, as the microtubules were properly oriented and functional. Thus, *sec5* mutations can affect membrane trafficking without disrupting microtubule polarity or the localization of non-membrane proteins. In this manner, the role of the exocyst in the oocyte appears to parallel its role in yeast (Finger and Novick, 1998; Novick et al., 1980; TerBush and Novick, 1995). The oocyte findings are also consistent with proposed roles in epithelial cells for the targeted delivery of proteins to basolateral domains (Grindstaff et al., 1998).

### Sec5 and E-cadherin in follicle cell signaling

A comparison of the *sec5* phenotype in follicle cells to studies of mammalian epithelia suggests both similarities and differences. In MDCK cells, the exocyst has been shown to colocalize with E-cadherin at the tight junctions between cells (Grindstaff et al., 1998). In the ovaries, E-cadherin and Sec5 colocalize and mutations in them similarly disrupt oocyte positioning and axis formation (Godt and Tepass, 1998). They may therefore function in the same signaling pathway. Interestingly, however, the signaling in the egg chamber is from the apical surface of the follicle cells to the posterior surface of the oocyte. Heretofore, in polarized epithelial cells, the exocyst has been reported to be required exclusively for transport to the basolateral domain and to reside primarily at the tight junction. The involvement of Sec5 in follicle cell to oocyte signaling is thus surprising, as it implies a role for the exocyst in apical signaling. At present, this role could either be a direct requirement in apical protein insertion or an indirect role in establishing the adherens junctions and thereby epithelial polarity.

### Multiple membrane proteins depend on Sec5 for their traffic

In mediating the traffic of multiple membrane proteins, including both Gurken and Yolkless, Sec5 is clearly in a distinct category from Cornichon and Boca, proteins that act in the ER. These proteins are needed for the correct transport of individual proteins and appear to act at earlier trafficking steps. Gurken is retained inside the cell in *cornichon* mutants, although vitellogenesis proceeds normally (Roth et al., 1995). Boca, however, is required for the trafficking of Yolkless and other LDL receptor family proteins to the membrane, but does not influence Gurken traffic (Culi and Mann, 2003). These highly specific deficits, which are likely to occur upon exiting from the ER (Culi and Mann, 2003; Powers and Barlowe, 1998), are distinct from the more general disruption of traffic in *sec5* mutants.

Many forms of membrane traffic to the cell surface now appear to depend on the exocyst. In multicellular organisms, these include vesicles derived from the trans-Golgi network (TGN) carrying newly synthesized proteins or mediating neurite outgrowth (Grindstaff et al., 1998; Vega and Hsu, 2001; Murthy et al., 2003; Inoue et al., 2003; Sans et al., 2003). However, not all forms of exocytosis depend on the exocyst. We have previously shown that the fusion of synaptic vesicles at nerve terminals persists in *sec5* mutants in which other trafficking events are blocked (Murthy et al., 2003) and apical protein delivery in MDCK cells was resistant to a block by antibodies to exocyst components (Grindstaff et al., 1998). The essential differences between exocyst dependent and independent exocytotic events remain unclear.

### Is Sec5 required for earlier steps in protein transport?

In addition to an established role for the exocyst in targeting or fusion at the plasma membrane (Grote et al., 2000; Guo et al., 1999), there is also evidence to suggest a role at earlier stages of protein traffic. The exocyst may associate with microtubules and a septin protein, Nedd5, and thereby promote transport of post-Golgi vesicles to target membranes (Vega and Hsu, 2001; Vega and Hsu, 2003). Members of the complex have been observed on perinuclear compartments in the cell (Shin et al., 2000; Vega and Hsu, 2001), and Sec6 and Sec8 are recruited to budding vesicles in the TGN, where antibodies against these components interfere with the ability of cargo to exit the Golgi (Yeaman et al., 2001). Recently, the exocyst component Sec10 has been found associated with Sec61β, a component of the ER translocon complex (Lipschutz et al., 2003). Genetic interactions of *sec61beta* with members of the exocyst complex have also been found (Lipschutz et al., 2003; Toikkanen et al., 2003). Indeed, mutations in *sec61β* can cause abnormal dorsal appendages very similar to those observed here for *sec5*, probably owing to defects in Gurken translocation (Valcarcel et al., 1999).

In the present study, however, we have not detected exocyst functions at stages before exocytosis. Sec5 was concentrated only at the plasma membrane. Microtubule polarity and the polarized localization of cytosolic components were unaffected by the mutations. Perinuclear Gurken, a pool that is likely to represent protein in the ER and Golgi, was present in both wild-type and mutant stage 10 oocytes, but the mislocalized cytoplasmic granules of Gurken that characterize *sec5$^{E13}$* oocytes did not colocalize with the ER marker Boca. The early lethality of *E10* clones, however, required that the analysis of Gurken and Yolkless trafficking be performed on the hypomorphic allele, *E13*. It is therefore possible that residual Sec5 function was sufficient for transport through the ER but insufficient at the plasma membrane.

### The membrane association of the oocyte nucleus

Although some aspects of the *sec5* phenotype can be ascribed to defects in the transport of particular membrane proteins, such as Gurken and Yolkless, others cannot, and these phenotypes may imply the existence of as yet unidentified oocyte proteins. An example is the altered location of the nucleus in late stage oocytes: whereas control nuclei were inevitably tightly associated with the anterior membrane, in *sec5$^{E13}$* germline clones, the nucleus was frequently displaced (Fig. 6G-J). Members of the dynein-dynactin complex are probably important for the association (Swan et al., 1999). However, Bic-D, a component of the dynein-dynactin complex, is anteriorly transported and properly localized near the

nucleus in *sec5*$^{E13}$ clones. We hypothesize, therefore, the existence of an as yet unidentified membrane protein that tethers the oocyte nucleus to the cortex via the dynein-dynactin complex. If the directed membrane traffic of this unidentified protein is compromised in the *sec5* mutants, the displacement of the oocyte nucleus could be explained in a manner consistent with the other actions of Sec5 in the oocyte.

### Exocyst localization and the establishment of asymmetry

Owing to defects in directed traffic to the plasma membrane, aspects of the anteroposterior axis and dorsoventral axis develop incorrectly in *sec5* mutant germlines. The requirement for Sec5 in directed membrane traffic is consistent with previous studies in cells that use a polarized secretory apparatus for cell growth and the transport of certain cargoes, such as growing neurites (Hazuka et al., 1999; Murthy et al., 2003; Vega and Hsu, 2001), MDCK cells (Grindstaff et al., 1998; Lipschutz et al., 2000) and yeast (Finger et al., 1998).

The spatial correlation of membrane traffic with the position of the exocyst raises the crucial question of how the exocyst acquires its localization. This microtubule- and actin-independent mechanism remains elusive (Fig. 3L) (Finger et al., 1998). The membrane receptor for the exocyst is not currently known, but in yeast Sec3p may be the exocyst component closest to the membrane and its localization may be controlled by Rho1p and Cdc42p (Drees et al., 2001; Zhang et al., 2001). In the *Drosophila* oocyte, the localization mechanism must undergo developmental regulation to account for the shift in localization that we have observed between stages 5 and 10. The mechanism that targets the exocyst to the membrane and regulates the changes in its localization is likely to be crucial to patterning and polarization in the germline.

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