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Sec5, a member of the exocyst complex, mediates *Drosophila* embryo cellularization

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SUMMARY

Cellularization of the *Drosophila* embryo is the process by which a syncytium of ~6000 nuclei is subdivided into discrete cells. In order to individualize the cells, massive membrane addition needs to occur by a process that is not fully understood. The exocyst complex is required for some, but not all, forms of exocytosis and plays a role in directing vesicles to appropriate domains of the plasma membrane. Sec5 is a central component of this complex and we here report the isolation of a new allele of *sec5* that has a temperature-sensitive phenotype. Using this allele, we investigated whether the exocyst complex is required for cellularization. Embryos from germline clones of the $sec5^{ts1}$ allele progress normally through cycle 13. At cellularization, however, cleavage furrows do not invaginate between nuclei and consequently cells do not form. A zygotically translated membrane protein, Neurotactin, is not inserted into the plasma membrane and instead accumulates in cytoplasmic puncta. During cellularization, Sec5 becomes concentrated at the apical end of the lateral membranes, which is likely to be the major site of membrane addition. Subsequently, Sec5 concentrates at the sub-apical complex, indicating a role for Sec5 in the polarized epithelium. Thus, the exocyst is necessary for, and is likely to direct, the polarized addition of new membrane during this form of cytokinesis.

KEY WORDS: Rhodopsin, Cellularization, Exocyst, Membrane addition, Trafficking, *Drosophila*

INTRODUCTION

During cytokinesis, the directed traffic of new membrane acts in concert with a contractile system to divide cells (Albertson et al., 2005; Barr and Gruneberg, 2007; Finger and White, 2002). Cellularization in the *Drosophila* embryo is a specialized form of cytokinesis that gives rise to a polarized epithelium and thereby permits a genetic analysis of the mechanisms that govern cell division. After 14 rounds of nuclear replication, ~6000 nuclei at the periphery of the syncytial *Drosophila* embryo are segregated into individual cells by the invagination of plasma membrane between nuclei (Foe and Alberts, 1983). Both cytoskeletal rearrangements (Mazumdar and Mazumdar, 2002; Schejter et al., 1992; Sullivan et al., 1993) and membrane addition (Burgess et al., 1997; Lecuit and Wieschaus, 2000) are important for this process.

Two likely sources of the new membrane are Golgi-derived vesicles and the resorption of microvilli from the embryo surface (Burgess et al., 1997; Fullilove and Jacobson, 1971; Lecuit and Wieschaus, 2000; Pelissier et al., 2003; Sisson et al., 2000; Turner and Mahowald, 1976). The site of membrane addition during cellularization remains controversial. The observation of vesicles near the leading edge of the invaginations, termed the furrow canal, led to the hypothesis that new membrane was being added there (Loncar and Singer, 1995). In addition, Golgi bodies, which produce new membrane-bound vesicles, also associate with furrow canals (Sisson et al., 2000). Alternatively, membrane addition may occur towards the apical end of the plasma membrane. Lecuit and Wieschaus visualized the insertion of new membrane in living embryos by the displacement of a fluorescent lectin that marked existing membranes (Lecuit and Wieschaus, 2000). They concluded that membrane is added first apically, then apicolaterally as furrows advance past the nuclei, and finally predominantly laterally in the rapid, final phase of cellularization. Membrane addition away from the furrow canal would contrast with cytokinesis in yeast (Finger and Novick, 1998) and sea urchin zygotes (Shuster and Burgess, 2002).

In yeast, cytokinesis requires the exocyst, a protein complex required for the tethering and fusion of secretory vesicles at the plasma membrane. The complex marks the sites at which vesicles dock and fuse during growth and cytokinesis (Finger et al., 1998; Finger and Novick, 1998). Sec3p and Exo70 associate with the plasma membrane at these sites and recruit the other exocyst components that permit the docking of Golgi-derived vesicles (Finger et al., 1998; Haarer et al., 1996; He et al., 2007). The vesicle-associated GTPase Sec4p binds to the exocyst protein Sec15p, potentially linking vesicles to the complex and thereby to the fusion site (Guo et al., 1999). In higher organisms, the exocyst has also been shown to be required for late stages of cytokinesis (Cascone et al., 2008; Chen et al., 2006; Fielding et al., 2005; Gromley et al., 2005). Additionally, the exocyst complex defines areas of membrane addition in neuronal growth cones and developing *Drosophila* oocytes (Dupraz et al., 2009; Hazuka et al., 1999; Murthy and Schwarz, 2004). Previous evidence has demonstrated that the exocyst acts in post-Golgi transport (Hsu et al., 2004), but the exocyst is also involved in some steps of endocytosis (Sommer et al., 2005) and post-endocytic trafficking (Langevin et al., 2005; Oztan et al., 2007).

To address further the role of the exocyst in cell division and to clarify the zones of membrane insertion during embryo cellularization, we have examined the exocyst protein Sec5 in *Drosophila*. Using a newly isolated allele, we show that

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cellularization is arrested and newly synthesized membrane protein accumulates in cytoplasmic vesicular compartments instead of the plasma membrane. The exocyst is likely to provide a spatial cue at the plasma membrane for directed vesicle traffic, with membrane addition occurring primarily towards the apical end of the invaginating membranes.

MATERIALS AND METHODS

EMS mutagenesis and temperature-sensitive screen

w; FRT40iso; FRT82, GMR-hid, P[Sec5]/TM3 Sb males were starved for 6-10 hours, fed 25 mM EMS in 1% sucrose overnight, and transferred to fresh food for 12 hours, before mating to *w; sec5^{E10}, Sco, Ey-Gal4, UAS-FLP/CyO; FRT82* virgins. Progeny were raised at 29°C, and males were scored for eye morphology. Screening 270,000 chromosomes yielded 164 F1 males with reduced or ablated eyes, and these were pair-mated to *w; sec5^{E10}, Sco, Ey-Gal4, UAS-FLP/CyO; FRT82* virgins. In the F2 generation, raised at 29°C, eight lines reproduced the F1 phenotype. Eyeless males from each of these eight stocks were crossed to *w; sec5^{E10}, Sco, Ey-Gal4, UAS-FLP/CyO; FRT82* virgins. Progeny were raised at 18°C and assessed for eye phenotypes.

Fly stocks and egg collections

All control and wild-type embryos were of genotype *y,w*. To generate maternal germlines homozygous for *sec5^{ts1}*, we crossed males of genotype *y,w; FRT40, ovoD/CyO; nanos-Gal4/+* to *w; FRT40, sec5^{ts1}/CyO; UAS-FLP* virgins to generate *w; FRT40, ovoD/FRT40, sec5^{ts1}; nanos-Gal4/UAS-FLP* females. These females were crossed to either *y,w* or *y,w; FRT40, sec5^{E10}/CyO GFP* males at 15°C. Eggs were collected in three ways: (1) for 2 hours at 15°C and aged at 15°C for 0, 4, 6 or 8 hours; (2) for 2 hours at 15°C and aged at 25°C for 1.5 or 3.5 hours; or (3) for 1 hour at 15°C and aged at 15°C for 0, 1, 2 or 3 hours. Stages (cycles 1-14) were determined by counting nuclei in a 5000 μm^2 box drawn in the confocal image.

Immunocytochemistry

Eggs were dechorionated in bleach, transferred to heptane, and fixed in 7% formaldehyde in PBS that was mixed 1:3 with heptane for 30 minutes at room temperature. Eggs were hand-devitellinated in PBT (PBS plus 0.1% Triton X-100 and 0.5% BSA). For Nrt and Myosin II stainings, embryos were heat fixed (Miller et al., 1989). For Rh1 staining, adult brains were dissected in PBS and fixed in 4% formaldehyde for 30 minutes. Antibody staining was performed in PBT containing 5% normal goat or donkey serum with Texas Red-X-phalloidin (1:200; Molecular Probes), Hoechst 33342 (Molecular Probes), rabbit anti-Myosin II (1:2000; gift of C. Field, Harvard Medical School), mouse anti-Nrt (1:20; Developmental Studies Hybridoma Bank), mouse anti-α-Tubulin (1:500; Sigma), rabbit anti-Rab11 (1:1000; gift of D. Ready, Purdue University), rabbit anti-GM130 (1:500; Abcam), mouse anti-Sec5 22A2 (Murthy et al., 2003), rat anti-E-cadherin (Ripoche et al., 1994), rabbit anti-Patj and rat anti-Crumbs (Bhat et al., 1999) and rabbit anti-Dlg (gift of P. Bryant, University of Sussex). Secondary antibodies were FITC-conjugated goat anti-mouse, FITC- or Texas Red-conjugated donkey anti-mouse and anti-rat, and FITC-conjugated goat anti-rabbit (Jackson ImmunoResearch).

Quantification

To quantify Nrt intensity, anti-Nrt-stained images were acquired with constant settings and analyzed with ImageJ software (NIH) (four embryos per genotype and phase). Each zone analyzed extended 50 μm lengthwise. Intensity measurements, normalized for the area of the analyzed zone, were made on single sections. Rab11 and GM130 puncta were quantified in a 40×15 μm rectangle in five embryos per genotype in individual confocal sections. After thresholding, counts and colocalization of puncta were performed in ImageJ with the Image Calculator function AND. Significance between pairs was determined using a two-tailed unpaired Student's *t*-test.

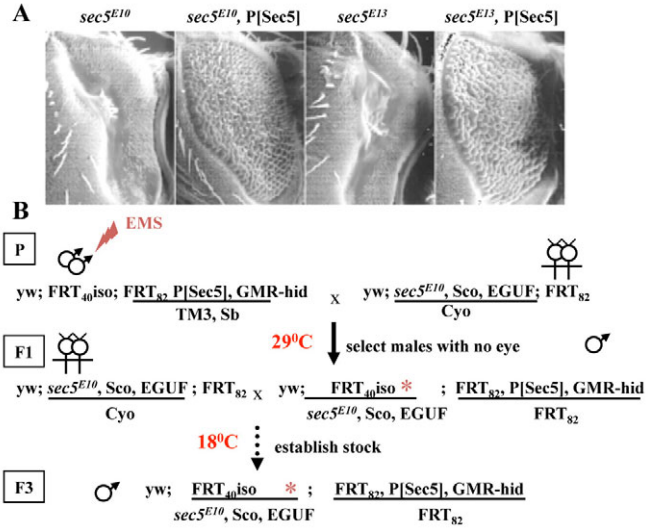

Fig. 1. An F1 screen for a temperature-sensitive allele of *sec5*. (**A**) Scanning electron micrographs of *Drosophila* eyes made homozygous for *sec5^{E10}* or *sec5^{E13}*, with or without the presence of the *P[Sec5]* transgene. (**B**) To isolate *sec5^{ts1}*, F1 males were selected based on the absence of eyes and retested at 29°C in the F2 generation and at 18°C in the F3. EGUF is *ey-Gal4, UAS-FLP*.

RESULTS

Identification of a *sec5* allele with a temperature-sensitive phenotype

The existing *sec5* alleles, *E10* and *E13*, could not be used to address the role of the exocyst complex in cellularization because larvae homozygous for these alleles survive up to 96 hours after egg laying (AEL) due to maternal contribution (Murthy et al., 2003) and germlines homozygous for either allele do not produce fertilizable eggs (Murthy and Schwarz, 2004). We therefore conducted an EMS screen for new alleles of *sec5*, in particular those with temperature sensitivity. Temperature-sensitive mutations are typically scarce (Grigliatti et al., 1973) and few screens are large enough to make their recovery in a particular gene likely. Therefore, a novel and efficient F1 screen for *sec5* alleles was undertaken.

Eyes composed exclusively of cells homozygous for *sec5^{E10}* or *sec5^{E13}* can be generated in an otherwise heterozygous animal by means of mitotic recombination during eye development (Stowers and Schwarz, 1999) and result in the ablation of the eye (Fig. 1A). In *sec5^{E10}*, the eye is completely ablated at both 25°C and 18°C, whereas *sec5^{E13}* shows some temperature sensitivity and forms a partial eye at 18°C (data not shown). A *Sec5* transgene restored eye development, demonstrating that the cell lethality was not due to second-site mutations (Fig. 1A). We adapted the use of whole-eye clones to perform an F1 screen of 270,000 chromosomes for new alleles of *sec5* (Fig. 1B).

The genotype of F1 flies (in all tissues except the eye) is shown in Fig. 1B. Induced mutations in other genes were heterozygous and did not impair the viability of the fly. Induced mutations in *sec5* were lethal over *sec5^{E10}* but were rescued by the *P[Sec5]* transgene. Thus, new alleles of *sec5* were viable in the F1 fly. However, selectively in the eye and antennal discs, FLP-mediated mitotic recombination driven by the *eyeless* promoter generated clones lacking *P[Sec5]*. Any photoreceptor clones that continued to carry the *P[Sec5]* chromosome died due to the eye-specific

apoptosis transgene *GMR-hid* (*hid* is also known as *Wrinkled* – FlyBase). Thus, the eye consisted only of cells in which the mutant phenotype was expressed. Because mutations induced in *sec5* should ablate the eye in combination with $sec5^{E10}$, new mutations could be recognized by the absence of eyes.

We raised the F1 generation at 29°C (non-permissive temperature). Because the germline of the F1 fly is mosaic for the mutation of interest, we rescreened 164 individual positives at 29°C in the F2 generation (not shown). The F3 generation was raised at 18°C and screened for alleles that produced an eye at the permissive temperature. From this screen we were able to recover one new allele with a strongly temperature-sensitive phenotype; $sec5^{ts1}$ produced no eyes when homozygous at 29°C, and a full eye, similar to the recombinant control, at 18°C (Fig. 2A). The *P[Sec5]* transgene rescued both the $sec5^{ts1}$ phenotype in eye clones and the lethality of the allele in homozygous larvae.

To test the $sec5^{ts1}$ allele in a membrane traffic assay, we examined Rhodopsin (Rh1; NinaE – FlyBase) trafficking to the rhabdomere, a specialized apical membrane of the photoreceptors. Rh1 localization to the rhabdomere is dependent on the exocyst component Sec6 (Beronja et al., 2005). To determine whether Sec5 was also required and whether $sec5^{ts1}$ has a temperature-dependent trafficking defect, we made eyes homozygous for $sec5^{ts1}$ and raised the flies at different temperatures. As expected, at 25°C the eye did not form (Fig. 2A), making it impossible to analyze Rh1 trafficking. At 18°C, however, the external eye appeared morphologically almost indistinguishable from control eyes (Fig. 2A) and photoreceptors were viable. Nevertheless, whereas wild-type photoreceptors have elongated cell bodies, $sec5^{ts1}$ photoreceptors were short. Moreover, Rh1 was not present in the plasma membrane, accumulating instead in large cytoplasmic structures (Fig. 2Bii). By contrast, at 15°C, $sec5^{ts1}$ photoreceptors were of normal size and some Rh1 was detected at the rhabdomeres (Fig. 2Biii). Thus, like Sec6 (Beronja et al., 2005; Satoh et al., 2005), Sec5 is required for Rh1 trafficking. Additionally, the temperature sensitivity of $sec5^{ts1}$ was apparent in the gross morphology of the eye and in an assay that explicitly involves membrane addition.

The penetrance of $sec5^{ts1}$ is a graded phenomenon rather than a binary switch: 18°C allowed enough trafficking for a complete external eye to form with viable, but stunted, photoreceptors, but 15°C permitted greater function. Even at 15°C, however, the truncated protein of $sec5^{ts1}$ was not equivalent to that of wild type: Rh1 trafficking was only partial and homozygous $sec5^{ts1}$ larvae did not survive past the first instar. A stop codon at amino acid 443 was found in $sec5^{ts1}$ (Fig. 2C). We also determined that the temperature-sensitive yeast allele *sec5-24* (Novick et al., 1980) contains a nonsense mutation, truncating Sec5p at amino acid 300. Thus, $sec5^{ts1}$ allows for some function at lower temperatures, which is likely to be due to increased stability of the truncated protein, but this allele is not equivalent to the wild type even at the permissive temperature.

Cellularization defects in $sec5^{ts1}$ embryos

Flies with homozygous $sec5^{ts1}$ clones in the female germline laid few eggs at 25°C and 18°C, but at 15°C laid some fertilized eggs, although still far fewer than would arise from a wild-type female. Because 15°C permitted partial exocyst function, it revealed a cellularization phenotype: $sec5^{ts1}$ embryos were severely defective during cellularization. Their phenotypes were identical whether fertilized by *y,w* or $sec5^{E10}$/*CyO* males; zygotic transcription of *sec5* is likely to occur too late to ameliorate the defects. Control embryos were also raised at 15°C to permit a direct comparison.

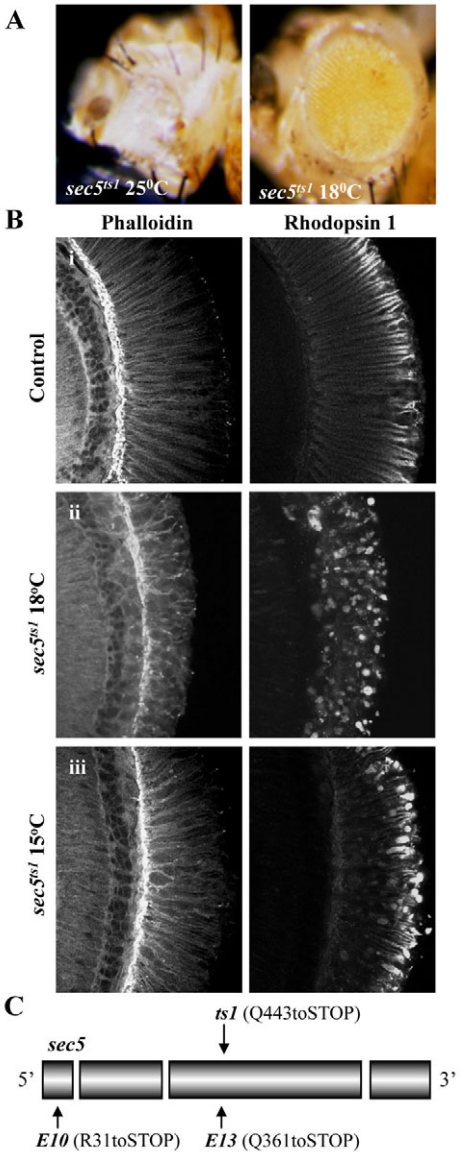

Fig. 2. Characterization of the temperature-sensitive allele $sec5^{ts1}$. (**A**) Owing to mitotic recombination, $sec5^{ts1}$ became homozygous in the developing eye and ablated the eye at 25°C but not 18°C. (**B**) Photoreceptors made homozygous for $sec5^{ts1}$ in flies raised at 18°C (**ii**) or at 15°C (**iii**), versus wild-type control (**i**), stained with Texas Red-phalloidin and antibodies to Rhodopsin 1. Both photoreceptor size and Rhodopsin 1 localization were partially restored at 15°C. (**C**) Location of mutations in *Drosophila sec5* alleles, including the nonsense mutation in the third exon of $sec5^{ts1}$.

To examine development prior to cellularization, we used hourly egg collections between 0 and 4 hours at 15°C. The first nine rounds of nuclear division occur in the center of the embryo, whereupon most nuclei migrate to the cortex. Nuclear divisions 10-13 occur in the periphery, prior to cellularization during round 14. At this stage, although the embryo is still a syncytium, the surface membrane becomes segregated into domains that limit the lateral diffusion of membrane proteins (Frescas et al., 2006). During interphase of divisions 10-13, actin caps form above nuclei and are required for these mitoses. When centrosomes divide and separate during these rounds, transient invaginations of the membrane, called metaphase furrows, arise between nuclei. Concurrently, actin caps disassemble

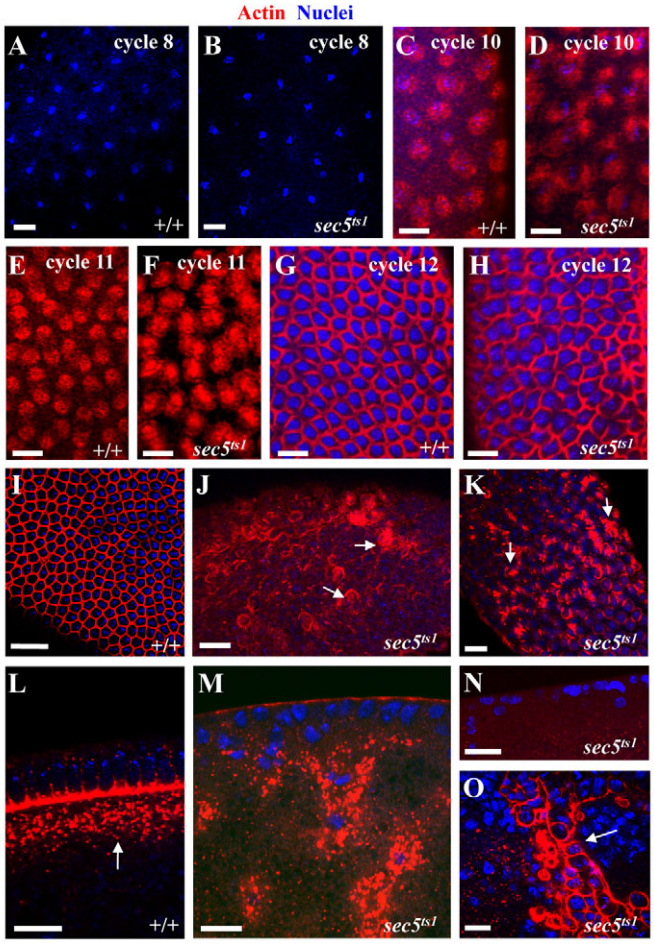

Fig. 3. F-actin localization indicates an abrupt failure of cellularization in *sec5*^{ts1} embryos. (**A-H**) Control and mutant *Drosophila* embryos, aged at 15°C, prior to cellularization. (A,B) Cycle 8. Control and mutant embryos possessed similar densities of nuclei (blue), which had not yet migrated to the cortex. (C,D) Cycle 10. Nuclei migrated to the cortex in both mutant and wild type and, during interphase, had Texas Red-phalloidin-labeled actin caps (red). (E,F) Actin caps during interphase of cycle 11 were present in both control and *sec5*^{ts1} embryos. (G,H). During metaphase of cycle 12, actin-rich furrows (red) surrounding nuclei formed in both *sec5*^{ts1} and control embryos. (**I**) Control embryo, 4-6 hours AEL at 15°C. Actin properly concentrated in the furrow canal, forming a hexagon around each nucleus. (**J**,**K**) *sec5*^{ts1} embryos, 4-6 hours AEL at 15°C. The density of cortical nuclei resembled that of the control (A), but apical actin aggregates were still present (arrows) and actin was disorganized rather than concentrated at furrow canals. (**L**,**M**) Control (L) and mutant (M) embryos 6-8 hours AEL at 15°C. The control phase III-IV embryo contained a reservoir of F-actin (arrow) below the furrow canals. In the mutant, this reservoir extended far into the embryo, surrounding some of the displaced nuclei. (**N**,**O**) *sec5*^{ts1} embryos were collected for 2 hours at 15°C and aged at 25°C for 1.5 (N) or 3.5 (O) hours. Most embryos lacked detectable F-actin at the periphery (N), and their nuclei were misshapen and disordered. The few embryos that underwent gastrulation (O) possessed some F-actin (arrow) but were primarily acellular. Scale bars: 20 μm.

and actin concentrates at metaphase furrows. Embryonic development during these pre-cellular stages can therefore be assessed by examining nuclear divisions and actin reorganization with Hoechst stain and Texas Red-conjugated phalloidin.

At 15°C, most wild-type and *sec5*^{ts1} embryos were undergoing nuclear divisions 8-11 between 2 and 3 hours AEL and divisions 11-13 between 3 and 4 hours AEL. These events appeared normal in *sec5*^{ts1} embryos (Fig. 3A-H). Prior to stage 10, their nuclear density was comparable to that of wild type (Fig. 3A,B), nuclei appropriately migrated to the cortex (Fig. 3C-F) and metaphase furrows formed correctly between nuclei (Fig. 3G,H). During subsequent interphases, actin caps formed, as in wild type (Fig. 3C-F), although these caps might be subtly different in morphology. Thus, development prior to cellularization was normal in *sec5*^{ts1} embryos.

Lecuit and Wieschaus (Lecuit and Wieschaus, 2000) divided wild-type cellularization as follows (see Fig. 7). In phase I, nuclei are arranged in a syncytium at the cortex. In phase II, during cycle 14, membranes grow in between the nuclei. The speed of membrane invagination is slow and the furrow canal remains ~5 μm basal to the embryo surface. Nuclei start to elongate. In phase III, the furrow canal reaches the basal part of the nucleus, which has completed its elongation. Finally, in phase IV, the invagination rate increases twofold and cellularization is completed. During these phases, Patj accumulates in the furrow canals and thus serves as a marker for the extent of membrane invagination (Bhat et al., 1999). Patj is the fly homolog of a vertebrate tight junction PDZ protein and was formerly thought to correspond to the *discs lost* (*dlt*) locus (Pielage et al., 2003).

At 15°C, control embryos began to enter phase II of cellularization within 4 hours AEL (Fig. 4A), but mutant embryos, even when 4-6 hours old, had not (Fig. 4B). Although the density of nuclei at the periphery was comparable to that of control embryos in phase II, indicating that cycle 14 had commenced, furrows rarely descended between nuclei and nuclei did not elongate. At the surface of control embryos, Patj was hexagonally arrayed around each nucleus (Fig. 4E). In the mutant, much of the Patj remained above the nuclei and hexagonal arrays did not form properly (Fig. 4F). In some cases, a broken hexagonal network remained, with several nuclei enclosed by short Patj-labeled furrows (Fig. 4G).

Within 6 hours at 15°C, most embryos from wild-type females had advanced to phase III or IV (Fig. 4C). This never occurred in embryos from *sec5*^{ts1} germlines, although in some cases Patj-labeled furrow canals grew past the nuclei. These nuclei, however, remained round rather than elongated and the furrows below the nuclei often appeared fused (Fig. 4D). By 8-10 hours AEL at 15°C, most control embryos began gastrulation (Fig. 4H). Several equivalently aged *sec5*^{ts1} embryos also began gastrulation, despite the absence of proper cellularization (Fig. 4I). Most regions of the gastrulating mutant embryos were acellular, but some patches contained misshapen, disorganized cells.

The disrupted cellularization observed with anti-Patj was also evident in the distribution of actin because actin associates with furrow canals (Fig. 3I) and, during phase IV, is in a pool just basal to the furrows (Fig. 3L). In *sec5*^{ts1}, actin was apical to the nucleus in 4- to 6-hour-old embryos (Fig. 3J,K), when controls were advanced in cellularization and all actin had moved into furrow canals. Actin was disordered in the mutant embryos and, unlike Patj, did not accumulate in furrow canals. At 6-8 hours, when cellularization in controls was complete, some actin remained inappropriately at the apical surface of the mutant embryo. Although most of the actin in these embryos was located below the nuclei, it extended abnormally far into the center of the embryo, often surrounding misplaced nuclei (Fig. 3M).

Thus, at 15°C, development departed precipitously from the normal sequence at the onset of membrane invagination in phase II. Some extension of short membranes, however, occasionally

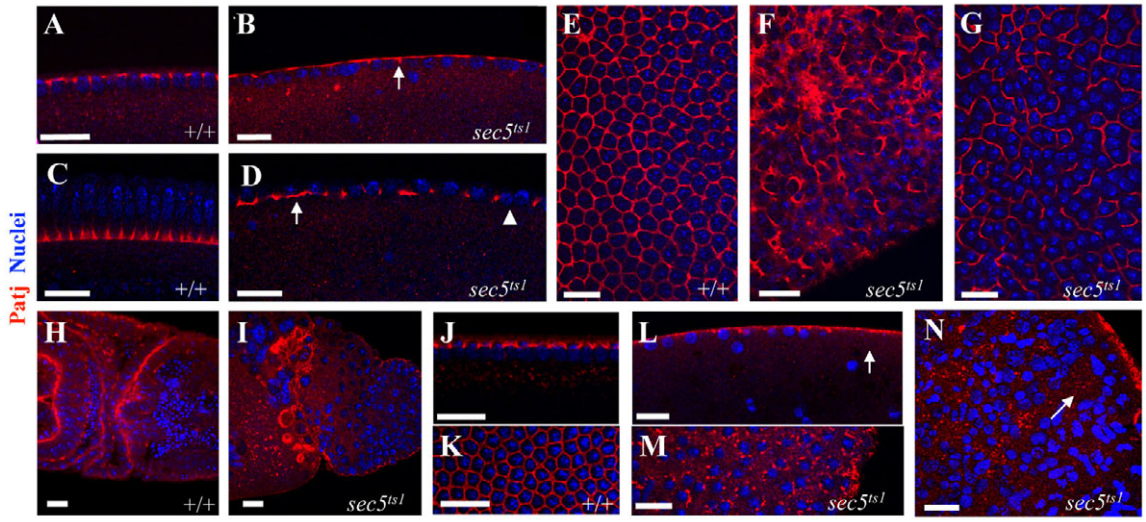

Fig. 4. *sec5*^{ts1} embryos show defects in furrow growth. (**A**) Control phase II *Drosophila* embryo from a 0- to 2-hour collection at 15°C. Patj (red) was concentrated ~5 μm from the embryo surface and between elongating nuclei (blue). (**B**) Mutant embryo, 4-6 hours AEL at 15°C, with a similar density of nuclei as in A. Patj was chiefly apical to nuclei, although it was occasionally seen on short furrows (arrow). Nuclei were not elongated. (**C**) Control embryo, 4-6 hours AEL at 15°C, that had advanced to phase IV. Furrow canals containing Patj immunoreactivity had moved basally. (**D**) *sec5*^{ts1} embryo, 4-6 hours AEL at 15°C, in which nuclei had not elongated, but some Patj was basal to the nuclei (arrow). In places, short Patj-containing furrows surrounded multiple nuclei (arrowhead). (**E-G**) Surface views of control (E) and *sec5*^{ts1} (F,G) embryos raised at 15°C. Although their nuclear densities were comparable, in mutants either the hexagonal array was completely disorganized (F, a surface view of B), or a broken array formed around multiple nuclei (G, a surface view of D). (**H**,**I**) Wild-type and *sec5*^{ts1} embryos raised at 15°C and going through gastrulation. (**J-N**) Temperature-shifted embryos, with genotypes indicated, were collected for 2 hours at 15°C and then aged at 25°C for 1.5 hours. Control embryos developed normally (J,K, the same embryo), whereas the *sec5*^{ts1} phenotype was intensified [L,M (the same embryo) and N], with almost no membrane invagination or hexagonal arrays of Patj, and many nuclei were displaced from the surface (arrows in L,N). Scale bars: 20 μm.

enclosed nuclei (Fig. 4C,D). Because there is residual Sec5 function at 15°C, we shifted the temperature at which the embryos were raised. Embryos were collected for 2 hours at 15°C and then aged for 1.5 hours at 25°C. All control embryos were in phase I or II of cellularization, and no defects were seen to result from the temperature shift (Fig. 4J,K). The *sec5*^{ts1} phenotype after the temperature shift was, however, more severe than that at 15°C (Fig. 4L-N). Membrane invagination did not occur and, in the absence of membranes, the nuclei were often disorganized and not retained at the embryo surface (Fig. 4L,N). Although Patj was present near the surface, it formed irregular (Fig. 4M) or no (Fig. 4N) arrays. Little actin was detected in the periphery (Fig. 3N). Some embryos were collected for 2 hours at 15°C and then aged for 3.5 hours at 25°C; most of these did not advance to gastrulation, although those that did appeared highly disordered (Fig. 3O), again showing that no membrane addition occurred at the non-permissive temperature.

Post-Golgi trafficking of Neurotactin is blocked in *sec5*^{ts1} mutants

Neurotactin (Nrt) is a transmembrane protein that is absent in early embryos but synthesized beginning in phase I of cycle 14. At this stage, little Nrt is present, but during phases II-IV Nrt levels increase and it is rapidly trafficked through the cytosol and inserted into the plasma membrane; some Nrt puncta are seen en route to the plasma membrane, but they are largely absent from the cytoplasm by the end of cellularization (Fig. 5A-C).

In *sec5*^{ts1} embryos, Nrt was synthesized but was not inserted into the plasma membrane: instead, it remained cytoplasmic and basal to the nuclei (Fig. 5D,E). To better understand this trafficking defect, we quantified the levels of Nrt in control and *sec5*^{ts1} embryos. We divided the embryos into three zones (as indicated in Fig. 5): (1) Nrt intensity apical to nuclei (0-3 μm); (2) Nrt within the next 10 μm, which encompasses the zone containing nuclei during phase III (3-13 μm); and (3) Nrt basal to nuclei (13-33 μm). During phase I and II of control embryos, Nrt was synthesized, trafficked and inserted into the plasma membrane, but was also found en route and in the basal cytoplasm. However, during phase III, Nrt was predominantly in the plasma membrane (in zones 1 and 2; Fig. 5B,F). In *sec5*^{ts1} embryos, however, Nrt was present but not enriched apically (Fig. 5D,F). Even when embryos were older and nuclei were falling away from the apical surface, Nrt density remained higher in zone 3, basal to the nuclear layer, than in the apical zones and was almost undetectable apical to the nucleus (Fig. 5E,F). Nrt was never observed in the plasma membrane of the mutant embryos. The lack of apical localization suggests that, besides being required for membrane addition, Sec5 is also required for transport of the newly synthesized protein through the cytosol. Because the exocyst has been reported to regulate microtubule dynamics (Wang et al., 2004), we examined whether changes to the microtubule cytoskeleton could account for the cellularization defects. Microtubule structure, however, appeared unaltered in *sec5*^{ts1} embryos (Fig. 5G,H).

To characterize the stage of Nrt trafficking that requires *sec5*, we determined the relationship of the accumulated Nrt to the Golgi marker GM130 and the endosomal marker Rab11 (Fig. 6). Overall, the total numbers of Golgi and Rab11 puncta were statistically identical in control and *sec5*^{ts1} embryos (P=0.71 and P=0.75, respectively), but there were significantly (P=0.007) fewer Nrt puncta in the mutants (Fig. 6I). Post-Golgi Nrt traffic is dependent on Rab11,

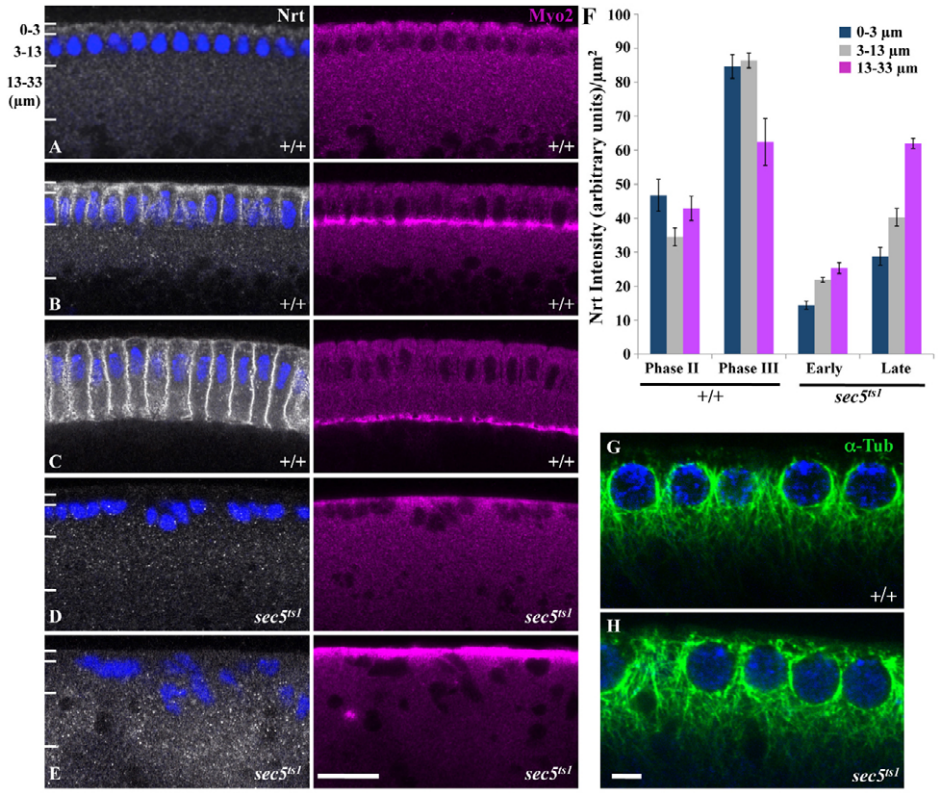

Fig. 5. Neurotactin trafficking is defective in *sec5^ts1^* embryos. (**A-E**) *Drosophila* embryos immunostained for Nrt and Myosin II (Myo2; Zipper – FlyBase) to mark progression of the furrow canal (Royou et al., 2004). (A) In phase I control embryos, Nrt was synthesized and inserted into the plasma membrane. (B) In phase III control embryos, Nrt was more abundant and chiefly in the plasma membrane, although some cytoplasmic puncta remained. (C) In phase IV control embryos, Nrt localized to lateral plasma membranes. (D) In *sec5^ts1^* embryos (stage equivalent to that shown in Fig. 4B), Nrt was synthesized but not trafficked to the plasma membrane, instead remaining cytoplasmic. (E) In older *sec5^ts1^* embryos (stage equivalent to that shown in Fig. 4D), Nrt levels had increased, but the protein remained cytosolic and basal to the nuclear layer. (**F**) Nrt intensity per μm^2 in control (phases II and III) and *sec5^ts1^* (early and late) embryos in three defined zones of the embryo spanning 0-3 μm, 3-13 μm and 13-33 μm from the embryo surface, as indicated by the white bars in A,B,D,E. All images were taken with the same settings. (**G**,**H**) Microtubule organization appears normal in mutant (H) versus wild-type (G) embryos stained with anti-α-Tubulin (α-Tub, green). Throughout, nuclei are labeled with Hoechst 33342 (blue). Scale bars: 20 μm in A-E; 5 μm in G,H.

and in control embryos many Nrt puncta are Rab11 positive (Pelissier et al., 2003) (Fig. 6A,B). Likewise, in *sec5^ts1^* embryos, there was extensive colocalization of Nrt and Rab11 (Fig. 6C,D). Quantification of Nrt and Rab11 puncta was undertaken in a region basal to the nuclei in order to determine whether loss of Sec5 impaired the trafficking of Nrt to the Rab11 compartment. The percentage of Nrt puncta that colocalized with Rab11 was not statistically different between control and *sec5^ts1^* embryos (27.2% versus 26.6%; P=0.84) (Fig. 6I,J). Nrt and GM130 also partially colocalized in control and *sec5^ts1^* embryos (Fig. 6E-H,J), but to a lesser extent than Nrt and Rab11: 8.8% of Nrt puncta were GM130 positive in controls and 7.1% in *sec5^ts1^* (P=0.11) (Fig. 6I,J). These values are significantly different from those for colocalization of Nrt with Rab11 (control, $P=1.15\times10^{-6}$; *sec5^ts1^*, $P=6.95\times10^{-6}$), but were not significantly different between the genotypes. In the *sec5^ts1^* embryos, Nrt therefore appears capable of exiting the Golgi to a Rab11 compartment, but it does not progress to the plasma membrane.

The localization of Sec5 during cellularization

Because cellularization requires Sec5 and exocyst proteins mark sites of exocytosis in dividing yeast and developing *Drosophila* oocytes (Finger and Novick, 1998; Murthy and Schwarz, 2004), we examined Sec5 localization in early embryos. Using a previously characterized Sec5-specific antibody (Murthy et al., 2003; Murthy and Schwarz, 2004) we examined wild-type embryos raised at 25°C and related the localization of Sec5 to the phases of cellularization. During phase I, Sec5 immunoreactivity was cytoplasmic, punctate and absent from the furrows (Fig. 7B). If there is Sec5 on the plasma membrane at this stage, it might have been obscured by the intense immunoreactivity in the cytoplasm, perhaps representing transport vesicles en route to the surface. During phase II, Sec5 was still largely cytoplasmic, although there appeared to be more apical to the nucleus and perhaps concentrated on the apical plasma membrane (Fig. 7C). Because the *sec5* phenotype is apparent at this stage, the Sec5 present in phase II must have an essential role in membrane invagination. In phase III, although cytoplasmic Sec5 persisted, it was enriched on the apicolateral membrane (Fig. 7D). Finally, during the fast phase of cellularization, i.e. phase IV, Sec5 was highly concentrated on the lateral membrane, apical to the nucleus (Fig. 7E). Sec5 immunoreactivity, including the apical concentration in phases I and II, was absent in *sec5^ts1^* embryos, confirming the specificity of the antibody (Fig. 7G). Throughout this process, Sec5 did not concentrate at the furrow canal.

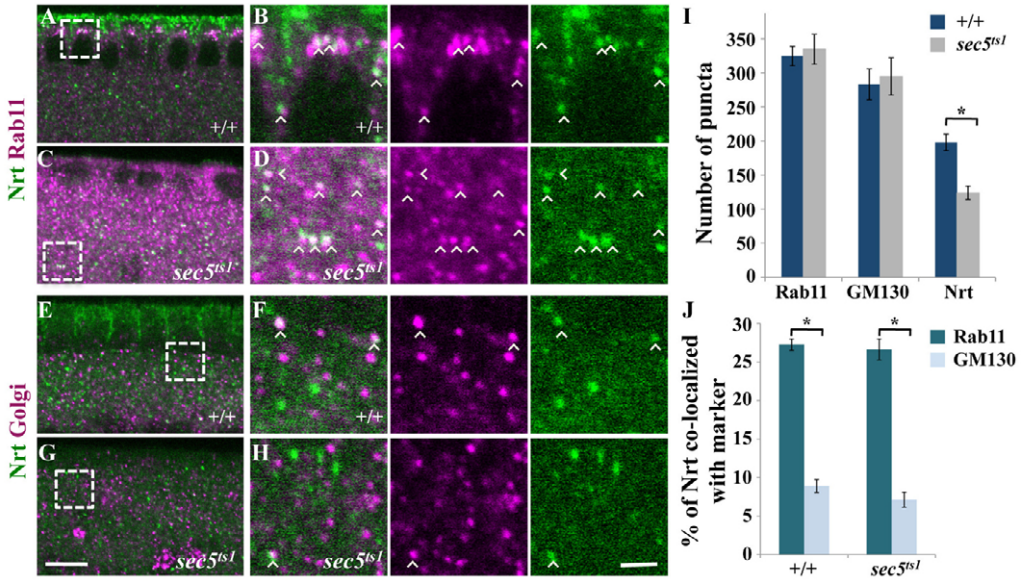

Fig. 6. Neurotactin colocalization with Rab11 and GM130 is normal in *sec5^{ts1}* embryos. (**A-D**) Wild-type (A,B) and *sec5^{ts1}* (C,D) embryos stained with anti-Nrt (green) and anti-Rab11 (magenta). B and D are enlargements of the boxed areas in A and C, respectively. During phase II-III, Nrt and Rab11 were highly colocalized (arrowheads), but apical to nuclei in wild-type and basal to nuclei in *sec5^{ts1}* embryos. (**E-H**) Wild-type and *sec5^{ts1}* embryos stained for Nrt (green) and the Golgi marker GM130 (magenta). F and H are enlargements of the boxed areas of E and G, respectively. Although the two proteins partially colocalized (arrowheads), most Nrt puncta were GM130 negative. (**I**) Counts of Rab11, GM130 and Nrt puncta in a 40×15 μm region immediately basal to the nuclear layer. Rab11, P=0.71; GM130, P=0.75; Nrt, P=0.007. (**J**) The percentage of Nrt puncta that colocalized with Rab11 and GM130. Control, $P=1.15\times10^{-6}$; *sec5^{ts1}*, $P=6.95\times10^{-6}$. Scale bars: 10 μm in A,C,E,G; 2 μm in B,D,F,H.

The pattern of Sec5 concentration at the membrane corresponds well with the model for membrane addition proposed by Lecuit and Wieschaus (Lecuit and Wieschaus, 2000). In that model, addition occurs first apically (phases I and II), then apicolaterally (phase III), and finally mostly laterally (phase IV). Our finding of an apicolateral concentration in phases III and IV corroborates the model. We cannot rule out the possibility that some membrane addition occurs at the furrow canal, either via an exocyst-independent mode or by low levels of exocyst; however, even in the final stages of cellularization, when furrow canals spread laterally for basal closure, Sec5 remained apicolateral (Fig. 7E and Fig. 8D). Presently, there are no other known proteins that are distributed like Sec5, marking the sites of membrane addition. Syntaxin 1, the plasma membrane t-SNARE, is required for cellularization. However, in contrast to Sec5, Syntaxin 1 is uniformly present at the plasma membrane (Burgess et al., 1997) (Fig. 7F).

Sec5 colocalizes with markers of the sub-apical complex

The basal adherens junctions (BAJs), which form at the onset of cellularization, separate furrow canals from lateral membranes (Hunter and Wieschaus, 2000; Muller and Wieschaus, 1996). As cellularization concludes and the blastoderm becomes a polarized epithelium, the BAJs disintegrate and apical adherens junctions (AAJs) form. These consist of three distinct sub-regions: the septate and adherens junctions and the sub-apical complex (Lecuit et al., 2002) (Fig. 8A). Furrow canal components reside in the mature sub-apical complex, whereas proteins of the BAJ localize to mature adherens and septate junctions.

How does the distribution of Sec5 compare with that of these junctional components? E-cadherin (also known as DE-cadherin or Shotgun – FlyBase), which is part of the mature adherens junction, was present in the BAJ and at the apical end of the plasma membrane during phase IV (Fig. 8B). This apical E-cadherin, although overlapping with the distribution of Sec5, is mostly basal to where Sec5 is enriched. Discs large (Dlg; Dlg1 – FlyBase), which concentrates at mature septate junctions and is required for the integrity of the AAJ (Woods et al., 1996), was concentrated along the lateral membrane during phase IV. Like E-cadherin, this Dlg is chiefly basal to Sec5 (Fig. 8C). Patj, although mostly concentrated in the furrow canals during phase IV, begins to accumulate at the apical end of the plasma membrane in the forming AAJ. This apical Patj colocalized with Sec5 (Fig. 8D).

Similarly, in mature polarized epithelia, Sec5, although broadly distributed in the cytoplasm, was enriched at the apical end of the lateral membrane (Fig. 8E). This Sec5 was consistently apical to the concentration of E-cadherin at the mature adherens junction. The mature sub-apical complex consists of Patj, its transmembrane binding partner Crumbs (Crb) and additional cytoplasmic proteins (Bhat et al., 1999). Sec5 colocalized with Patj and Crb (Fig. 8F,G).

DISCUSSION

The exocyst complex was identified in yeast as essential for post-Golgi secretion and for the separation of the daughter and mother cells (Novick et al., 1980; TerBush et al., 1996). In higher organisms, unlike yeast, some forms of exocytosis appear to be independent of exocyst components: the transport of vesicles to the apical surface of MDCK cells is not blocked by antibodies to Sec6 and Sec8 (Grindstaff et al., 1998) and the release of neurotransmitter at the synapse persists in *Drosophila sec5* mutants (Murthy et al., 2003). In yeast, the exocyst is required for septum growth and separation during cytokinesis (Wang et al., 2002), whereas in higher organisms RNAi studies indicate that the exocyst is only needed for the resolution of the intracellular bridge during late stages of cytokinesis

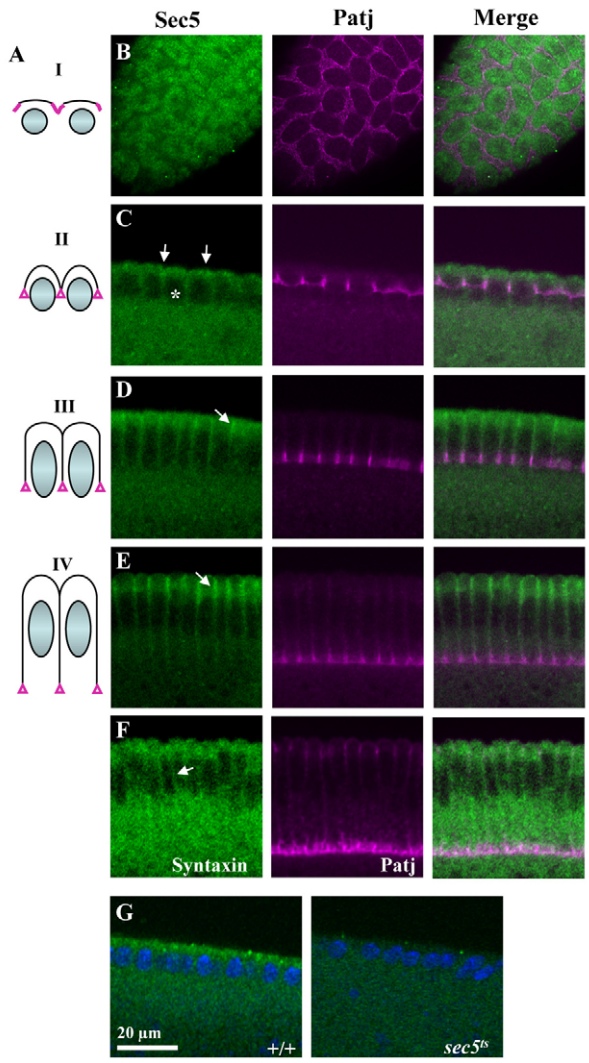

Fig. 7. Sec5 localization during cellularization. (**A**) The four phases of cellularization, as defined by Lecuit and Wieschaus (Lecuit and Wieschaus, 2000). In phase I, the furrow canal (magenta) forms between nuclei. In phase II, nuclei begin to elongate and the furrow canal moves 5 μm basal to the embryo surface. In phase III, the furrow canal moves basal to the fully elongated nuclei. The 'fast phase', phase IV, involves rapid membrane addition and advancement of the furrow, and ends in the formation of a polarized epithelium (see Fig. 5). (**B**) Surface view of a wild-type embryo prior to membrane invagination. Sec5 (green) was concentrated around each nucleus, but did not substantially overlap with the forming furrow, which is labeled with anti-Patj (magenta). (**C**) Side view of a phase II embryo. Sec5 was concentrated in the cytoplasm, with a slight enrichment apical to the nuclei and furrow canal (arrows). Nuclei (asterisk) lacked Sec5. (**D**) In phase III, Sec5 was concentrated at the apical ends of the growing membranes (arrow). (**E**) In phase IV, Sec5 was enriched on the lateral membranes in a zone apical to the nuclei (arrow). (**F**) Anti-syntaxin 1 staining (green) is uniformly distributed on the plasma membrane. (**G**) Anti-Sec5 staining (green) during phase II of cellularization. The truncated *sec5^{ts1}* protein is not recognized by the antibody, confirming the staining specificity.

(Cascone et al., 2008; Chen et al., 2006; Echard et al., 2004; Fielding et al., 2005; Gromley et al., 2005). These studies, however, are constrained because complete loss of exocyst function is lethal to cells and so earlier phases of cytokinesis may persist due to residual exocyst function. In the present study, we have circumvented the requirement for the exocyst for cell viability by isolating a *sec5* allele that gives rise to embryos with minimal Sec5 function.

We have found that the cellularization of the *Drosophila* blastoderm is clearly dependent on the exocyst component Sec5 and, as in yeast, the exocyst marks the locations of membrane addition. In embryos containing only the truncated *sec5^{ts1}*-encoded protein, embryo development arrested when membrane addition should have begun. The arrest was abrupt and specific; prior stages of embryogenesis, including fertilization, nuclear divisions, nuclear migration to the cortex, nuclear divisions in the periphery, metaphase furrow formation and actin cap formation, occurred properly. Although the density of peripheral nuclei was normal relative to the control and the microtubule network appeared normal, the mutant embryos failed to cellularize. Nuclei did not elongate, furrow canals did not invaginate correctly, F-actin became disorganized, and nuclei fell away from the periphery. Because the complete lack of Sec5 is cell-lethal in the germline and arrests the development of the oocyte (Murthy and Schwarz, 2004), it would appear that this new allele of *sec5* permits a degree of exocytosis that at 15°C is only enough for oocyte formation but, at either 15 or 25°C, is inadequate to support the massive membrane addition during cellularization. Even in the absence of most cell membranes, many mutant embryos attempted gastrulation. The defect thus appears to be focused on membrane invagination itself.

During the slow phase of membrane growth, the cellularization front progresses ~7 μm basal to the surface of the embryo. The front never progressed this far in *sec5^{ts1}* embryos at 15°C, although shorter furrows were seen in patches. Defects were also apparent in the persistence of apical actin in 4- to 6-hour-old embryos, with a failure of nuclei to elongate and the inclusion of several nuclei within a partially formed cell. The hallmarks of the fast phase of cellularization, i.e. the transition of F-actin from hexagons to rings and the rapid invagination that results in cells of 35 μm, never occurred in *sec5^{ts1}* embryos. We conclude that during both the slow and fast phases of cellularization, the residual function of *sec5^{ts1}* cannot support proper membrane growth. The small degree of membrane invagination sometimes observed at 15°C could be completely prevented by shifting the embryos to 25°C, whereas that shift did not change cellularization in control embryos. These defects appear to be more severe than those observed in conventional cytokinesis in higher organisms, in which the exocyst is only required for late stages (Cascone et al., 2008; Chen et al., 2006; Echard et al., 2004; Fielding et al., 2005; Gromley et al., 2005). This discrepancy could be explained by the greater amount of membrane addition needed in cellularization compared with other types of cytokinesis or because the allele we have used more completely disrupts exocyst function than the RNAi constructs used previously.

Membrane addition during cellularization is supported by Golgi-derived vesicles and the exocyst is needed for post-Golgi trafficking to the plasma membrane (Novick et al., 1980; TerBush et al., 1996). The failure of newly synthesized Nrt to be trafficked and inserted into the plasma membrane in *sec5^{ts1}* embryos is consistent with this role. Because significantly more Nrt in the mutant embryos was found in Rab11-positive endosomes than in GM130-positive Golgi membranes, a direct movement of Golgi proteins to Rab11 endosomes, without previous plasma membrane insertion and endocytosis, is likely. This direct route from the Golgi to recycling endosomes has been suggested previously (Ang et al., 2004; Pelissier et al., 2003; Satoh et al., 2005) and our data provide further support for the existence of this pathway.

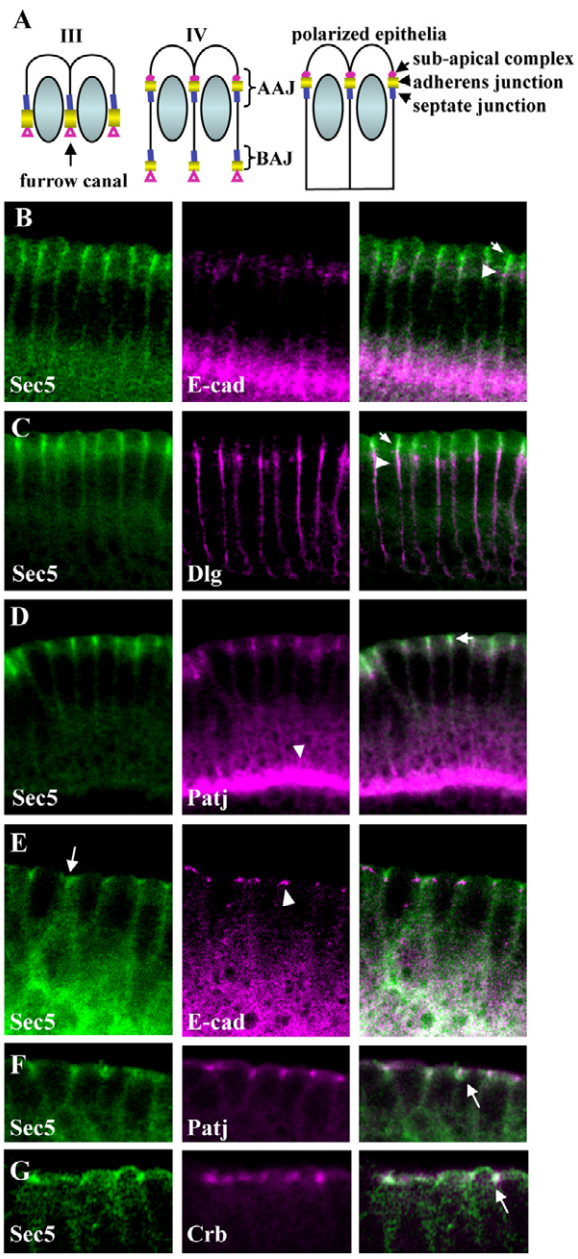

Fig. 8. Sec5 colocalizes with members of the sub-apical complex (SAC). (**A**) In the transition from phase III to IV, the components of the furrow canal and basal adherens junctions (BAJs) reappeared at the SAC, apical adherens junctions (AAJs) and septate junctions, but with an inverted apical-basal order. The BAJ disintegrated at the end of phase IV, leaving the three domains of the AAJ in the now polarized epithelium. (**B**,**C**) During phase IV, E-cadherin and Dlg were concentrated at the apical ends of the lateral plasma membranes (arrowhead), near, but largely basal to, the lateral membrane enrichment of Sec5 (arrow). (**D**) Patj was concentrated both at the furrow canal (arrowhead) and in apical zones of lateral membranes that overlapped with the apicolateral Sec5 enrichment during phase IV (arrow). Sec5 was not detected in the furrow canal. (**E-G**) In polarized epithelia, Sec5 was concentrated at the apical end of the lateral membrane (arrows) but was also abundant throughout the cell. The localization of Sec5 was distinct from that of E-cadherin at adherens junctions (arrowhead), but overlapped with Patj and Crb in the SAC (arrows).

The phenotype of *sec5*^{ts1} embryos is distinct from those of several other early embryonic mutations. Cellularization proceeds normally in *shotgun*, *armadillo* (β-*catenin*), *bazooka* (*par-3*), *stardust* and *crb* mutants, but the formation of the AAJ is disrupted and the epithelium is transformed into a multilayered sheet of cells (Muller and Wieschaus, 1996; Sokac and Wieschaus, 2008; Tepass, 1996; Tepass et al., 1990; Uemura et al., 1996). In *slow as molasses* (*slam*) embryos, membrane invagination does not occur in the slow phase, Nrt is not inserted into the plasma membrane, and the furrow canal remains at the apical end (Lecuit et al., 2002), much like *sec5*^{ts1} embryos. However, *slam* embryos are able to initiate the fast phase of cellularization and *slam* nuclei elongate. Also, in *slam* mutants, Nrt is still transported through the cytoplasm and accumulates just under the apical membrane (Lecuit et al., 2002), whereas in *sec5*^{ts1} embryos Nrt remains mostly in the basal cytoplasm.

Although Syntaxin and Sec5 localize differently during cellularization, the *Syntaxin 1* phenotype (Burgess et al., 1997) is most similar to that of *sec5*. Ovaries homozygous for the hypomorphic allele *Syntaxin*^{L266} yield embryos that complete nuclear divisions but fail to cellularize correctly, producing large acellular patches. Because Syntaxin is essential for vesicle fusion, the similar phenotypes suggest that Sec5 also mediates vesicle fusion at the embryo plasma membrane.

Although it acts in post-Golgi transport (Hsu et al., 2004), the exocyst is also required for some steps of endocytosis (Sommer et al., 2005) and post-endocytic traffic (Langevin et al., 2005; Oztan et al., 2007). During cellularization, apical membranes, which begin cellularization with microvilli, are internalized, pass through an endosome and are then reinserted to form the growing lateral membranes (Pelissier et al., 2003). Because of the extreme failure of membrane growth in *sec5* mutant embryos, it seems likely that the addition of endosomally trafficked, apically derived membrane is blocked. Thus, Sec5 might also be involved in the fusion of endosomally derived vesicles.

Three *sec5* truncations give rise to temperature-sensitive phenotypes: (1) *sec5*^{ts1} as reported here; (2) *sec5*^{E13}, which we found to have modest temperature sensitivity but less activity at permissive temperatures than *sec5*^{ts1}; and (3) the yeast allele *sec5-24*. We speculate that this temperature sensitivity might arise from thermal instability of exocyst complexes that incorporate the truncated proteins, rather than from the temperature-dependent unfolding of the Sec5 protein itself. The novel F1 screen that yielded *sec5*^{ts1} should be of wide utility for obtaining new alleles of a gene for which there is a transgenic rescue construct.

The *sec5*^{ts1} phenotype does not preclude functions for Sec5 in addition to membrane addition. In particular, we observed that Nrt-containing membranes were not transported to the apical region and nuclei failed to elongate in *sec5*^{ts1}. These phenotypes could reflect cytoskeletal defects involving microtubule organization (Foe and Alberts, 1983; Lecuit et al., 2002) and prior studies have found biochemical interactions between exocyst components and microtubules (Vega and Hsu, 2001; Vega and Hsu, 2003; Wang et al., 2004). However, the failure in cellularization is unlikely to be due to a defect in microtubules because the microtubule network remains intact in *sec5*^{ts1} embryos (Fig. 5G,H) and other microtubule-dependent processes, such as nuclear migration to the cortex, are likewise intact. Actin localization was normal in *sec5*^{ts1} embryos prior to the arrest of cellularization; there was no apparent defect in actin cycling between caps and metaphase furrows during mitotic divisions preceding cellularization. The late actin phenotypes are therefore likely to arise as an indirect consequence of a membrane traffic defect. However, exocyst interactions with

actin-regulating Rho family GTPases (Lipschutz and Mostov, 2002; Novick and Guo, 2002; Wu et al., 2008; Zuo et al., 2006) might contribute to the phenotypes.

The exocyst appears to correlate with, and is likely to define, particular domains of the plasma membrane at which vesicle fusion occurs (Dupraz et al., 2009; Hazuka et al., 1999; Murthy et al., 2003; Murthy and Schwarz, 2004; Vega and Hsu, 2001). Once established as requisite for membrane addition in cellularization, Sec5 localization could serve as an index of the site of membrane insertion. Of the two prevalent models for cellularization, i.e. addition at the tip of the furrow canal (Loncar and Singer, 1995; Sisson et al., 2000) or addition at the apicolateral membrane (Lecuit and Wieschaus, 2000), our data clearly favor the latter. Indeed, the correspondence is striking between the dynamic localization of Sec5 and the sites of membrane addition identified by Lecuit and Wieschaus. The redistribution of exocyst proteins to correlate with changing sites of membrane fusion has precedent (Finger et al., 1998; Murthy and Schwarz, 2004) and our study offers a further example of the exocyst complex providing a spatial cue for directing post-Golgi vesicles to the plasma membrane. The resulting membrane addition is crucial to the cellularization of the embryo. The identification of the molecules that are responsible for the localization and relocalization of the exocyst will be key to understanding the mechanism of localized membrane addition.

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Competing interests statement

The authors declare no competing financial interests.

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