

# *Drosophila* Sec10 is Required for Hormone Secretion but not General Exocytosis or Neurotransmission

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The sec6/8, or exocyst, complex is implicated in trafficking of secretory vesicles to fusion sites in the plasma membrane. Genetic analyses have been done primarily in yeast, where mutation of the eight protein subunits similarly disrupts polarized vesicle fusion. The goal of this study was to assay the sec6/8 complex in *Drosophila*, and specifically to test its widely hypothesized functions in synaptogenesis and neurotransmission. We used a transgenic RNAi approach to remove the most highly conserved complex component, *Drosophila* sec10 (dSec10). Ubiquitous dSec10 RNAi resulted in early postembryonic lethality, demonstrating that dSec10 is essential. Surprisingly, tissue-specific dSec10 RNAi revealed no essential requirement in nervous system, musculature, gut or epidermis. Assays of polarized secretion in all these tissues failed to reveal any role for dSec10. In particular, the neuromuscular synapse showed no defects in morphogenesis or vesicle trafficking/fusion underlying neurotransmission. The essential requirement for dSec10 was restricted to the ring gland, the *Drosophila* organ specialized for endocrine function. The developmental arrest of dSec10 RNAi animals was partially rescued by feeding ecdysone, suggesting dSec10 mediates steroid hormone secretion. We conclude that dSec10 has no detectable role in most forms of polarized trafficking/exocytosis, including neurotransmission, but rather is essential for endocrine secretion.

**Key words:** dSec10 ecdysone, exocyst, neurotransmission, sec6/8, sec10, secretion

**Received 18 July 2002, revised and accepted for publication 28 August 2002**

Polarized vesicle trafficking and fusion is essential for many cellular processes, including the membrane addition driving directional cell growth, the establishment and function of polarized epithelial cell layers, and the highly evolved secretory function of neurons mediating synaptic transmission. Golgi-derived vesicles destined for the plasma membranes in polarized cells are first transported along the cytoskeleton with directional motor proteins (1). At the target membrane, the trimeric SNARE complex, which couples a transmembrane

vesicular SNARE and its specific target membrane SNARE, has been proposed to mediate late-stage vesicle targeting specificity and subsequent fusion (2). Although the SNAREs are essential for vesicle fusion (3–5), they are at least singly dispensable for targeting vesicles to fusion sites (6, 7), suggesting that other classes of proteins may define fusion sites and/or actively direct polarized vesicle trafficking to these sites.

One group of proteins strongly implicated in these vesicle-targeting events is the sec6/8 complex (also known as the exocyst complex in yeast), which consists of 8 members: sec3, sec5, sec6, sec8, sec10, sec15, exo70 and exo84 (8). In yeast, the exocyst complex localizes to the plasma membrane bud tip of developing daughter cells, and mutations in any of the subunits similarly block the fusion of Golgi vesicles to the plasma membrane and cause an accumulation of vesicles in the cytoplasm (9–11). In polarized mammalian epithelial MDCK cells, the sec6/8 complex is associated with the plasma membrane at sites of cell–cell contact, specifically at the apex of the basolateral membrane (12). When antibodies directed against rat sec8 are introduced into these cells, vesicle transport to the basal-lateral membrane is reduced 2.5-fold compared to controls. In PC12 cells and cultured hippocampal neurons, the complex is localized to growth cones during neurite outgrowth (13, 14) and, during synaptogenesis, to periodic domains along axons preceding the arrival of synaptic markers, including Synapsin (synaptic vesicle associated) and FM1-43 dye uptake (evidence of localized vesicular cycling) (13). In mature mammalian neurons, the sec6/8 complex may be down-regulated at synapses, suggesting that its role may be restricted to the establishment of synaptic fusion sites (13).

Taken together, these studies strongly suggest that the sec6/8 complex plays a generalized role in vesicular exocytosis, for example in polarized epithelial cells, and a more specialized function in the establishment and/or function of synaptic fusion sites in neurons. However, functional tests of these hypotheses are lacking. We therefore implemented a genetic study of sec6/8 complex function in *Drosophila* by targeting the best-conserved complex component in flies, *Drosophila* sec10 (dSec10). We used a transgenic RNA interference (RNAi) strategy coupled to the UAS-GAL4 system (15) to disrupt dSec10 function in a tissue-specific manner, and then assayed polarized secretion in each of these tissues. We report here that dSec10 is essential for postembryonic viability, but, surprisingly, plays no detectable function in the nervous system, musculature, gut or epidermis. Polarized secretion in all these tissues is unperturbed in the absence of

detectable dSec10, including the massive, high-fidelity exocytosis mediating synaptic transmission. The only tissue that showed an essential requirement for dSec10 is the ring gland, which mediates endocrine functions in *Drosophila* (16,17). Supplementing hormone to animals lacking dSec10 partially rescues the lethal defect. These studies show that dSec10, an essential exocyst component in yeast, plays no detectable role in polarized vesicular trafficking/secretion in multiple *Drosophila* tissues, but could be required for an undefined subset of vesicular trafficking underlying endocrine signaling functions.

## Results

### **Molecular characterization of *Drosophila sec10***

The sec6/8, or exocyst, complex is composed of 8 protein subunits: sec3, sec5, sec6, sec8, sec10, sec15, exo70 and exo84 (8). Orthologous genes encoding 7 of these proteins are present in the *Drosophila* genome, but no significant ortholog of rSec3 is detectable (18). dSec5 is located on the second chromosome at chromosomal position 23F3 and shares 33% amino acid identity to rSec5. dSec6 is located on the second chromosome at position 55E11 and is 36% identical to rSec6. dSec15 is located on the third chromosome at 93B12-13 and shares 40% identity to rSec15. dExo70 is located on the third chromosome at position 66C11-12 and shares 31% identity to rExo70. The *Drosophila* genome contains dSec8 at 60E and dExo84 at 69F on the third chromosome, but their sequences have not been determined. The *Drosophila* homolog of the rat sec6/8 complex component with the highest homology is dSec10, which is 43% identical and 65% similar to rSec10. This high degree of homology made dSec10 ideal for genetic and molecular analyses of sec6/8 complex function *in vivo*.

Southern blot analysis and polytene chromosome *in situ* hybridization revealed a single dSec10 gene located on chromosome 3R at position 95E5-6 (Figure 1B). The genomic sequence was obtained using PCR-based intron-exon mapping with dSec10 cDNA plasmid and P1 colonies as templates (Figure 1A). The dSec10 genomic DNA sequence contains 5 small introns, making the total genomic length 2619bp (GenBank accession number 242993). The dSec10 cDNA sequence (GenBank accession number AJ292992) of 2313bp was determined by sequencing three EST clones obtained from the Berkley *Drosophila* Genome Project EST database (<http://www.fruitfly.org/>): LD11464, LD21465 and HL02545. The predicted 710 amino acid protein contains a coiled-coil domain at the N-terminus, which presumably functions in protein-protein interactions.

### **Expression of *Drosophila sec10***

*Drosophila sec10* EST sequences were isolated from all developmental stages, suggesting that the dSec10 gene is expressed throughout the entire life cycle of *Drosophila*. Seven EST clones containing full or partial dSec10 sequence were isolated from cDNA libraries from embryos, larvae, pupae,

and adult head: LD21465, LD11464, LP08321, GH20209, GH05719, GH019049 and HL02545. To examine temporal and spatial distribution of the dSec10 message during embryonic development, RNA *in situ* hybridization was performed (Figure 2). There was no detectable expression before stage 8. Beginning at stage 9, dSec10 RNA was expressed at moderate levels, and appeared to be widely present in most/all tissues, including the epidermis, musculature, nervous system, and gut. There was particularly enriched dSec10 RNA expression in the midgut and neuroblasts/neurons in the brain/ventral nerve cord (VNC) (Figure 2A). Both tissues have high levels of vesicle cycling activity. In later developmental stages (stages 10–16), persistent dSec10 expression was noted in most/all tissues, including continued expression in the brain and ventral nerve cord, and strong expression in the midgut.

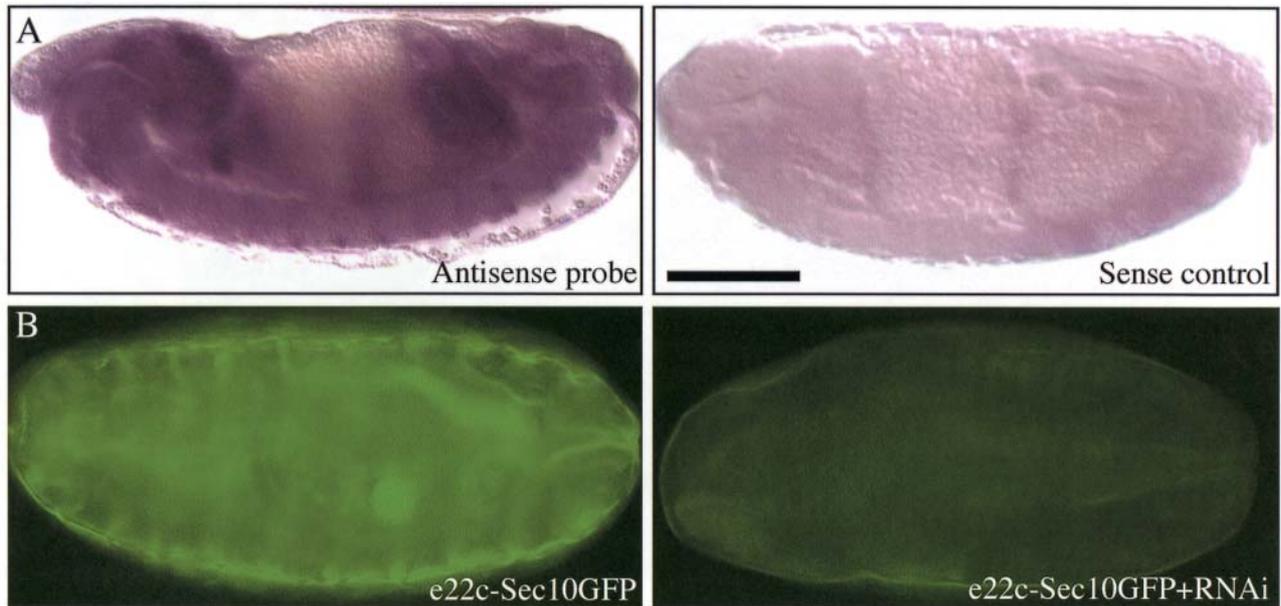
To examine the subcellular distribution of dSec10, a UAS-dSec10GFP c-terminal fusion construct was produced. Expression of this transgene was driven in specific tissues using the UAS-GAL4 transcriptional control system (15). Sec6/8 function was presumed to be essential in polarized cells with extensive secretory activity. Therefore, expression of the UAS-dSec10GFP construct was activated in animals which express GAL4 in the nervous system [ELAV-GAL-4 and 4G-GAL4 (19)], musculature [MHC-GAL4 and G7-GAL4 (20)], salivary glands (multiple drivers), or gut [e22c-GAL4 (21)]. The transgenic protein was efficiently driven in all of these tissues. Please note that a transgenic dSec10 fusion protein was used in these studies, which may not perfectly mimic the localization of the endogenous protein.

At the well-characterized larval neuromuscular junction (NMJ), dSec10GFP colocalized with synaptic vesicle markers such as Cysteine String Protein (CSP) (22) (Figure 3A). GFP was also detected at lower levels in neuronal axons, although without any specific localization pattern. In muscle, dSec10GFP did not preferentially localize to the postsynaptic compartment, but was readily detected throughout the cytoplasm, filling the sarcomeric striations (Figure 3B). In other highly polarized secretory cells, specifically the epidermis, salivary gland and gut, dSec10GFP was detected throughout the cytoplasm, but was excluded from the nucleus (Figure 3C,D). However, in none of these tissues did dSec10GFP show a polarized distribution. Specifically, dSec10 did not localize to periplasmic domains specialized for polarized vesicular secretion. Given the observations in yeast and mammalian cells, the finding that no polarized dSec10GFP was detected in tissues where polarized secretion is essential was a surprising finding.

### **Tissue-specific removal of dSec10 using transgenic RNA interference**

To assay dSec10 function *in vivo*, the UAS-GAL4 system was employed in conjunction with RNA interference (RNAi), which has proven to be an effective method to degrade specific endogenous mRNA in *Drosophila* (23,24). Recent studies by Kalidas and Smith (25), utilizing a genomic-cDNA





**Figure 2: The expression of dSec10 RNA and dSec10 GFP fusion protein in the *Drosophila* embryo.** (A) A representative dSec10 RNA *in situ* hybridization in whole embryo. Left panel: the antisense probe shows that dSec10 RNA is expressed ubiquitously in the embryo. Right panel: sense control demonstrating staining specificity. (B) Transgenic expression of UAS-dSec10RNAi eliminates detectable dSec10GFP protein in all tissues. Left panel: the ubiquitous e22c-GAL4 line driving UAS-dSec10GFP expression in all tissues of a *Drosophila* embryo. Right panel: coexpression of a single copy of the UAS-dSec10RNAi transgene eliminated detectable dSec10GFP protein expression. Scale bar equals 90  $\mu\text{m}$ .

studies on other genes (25). These results suggest that the transgenic RNAi approach provides a 'knockdown' (strong hypomorph) rather than a complete elimination (null) of the dSec10 protein.

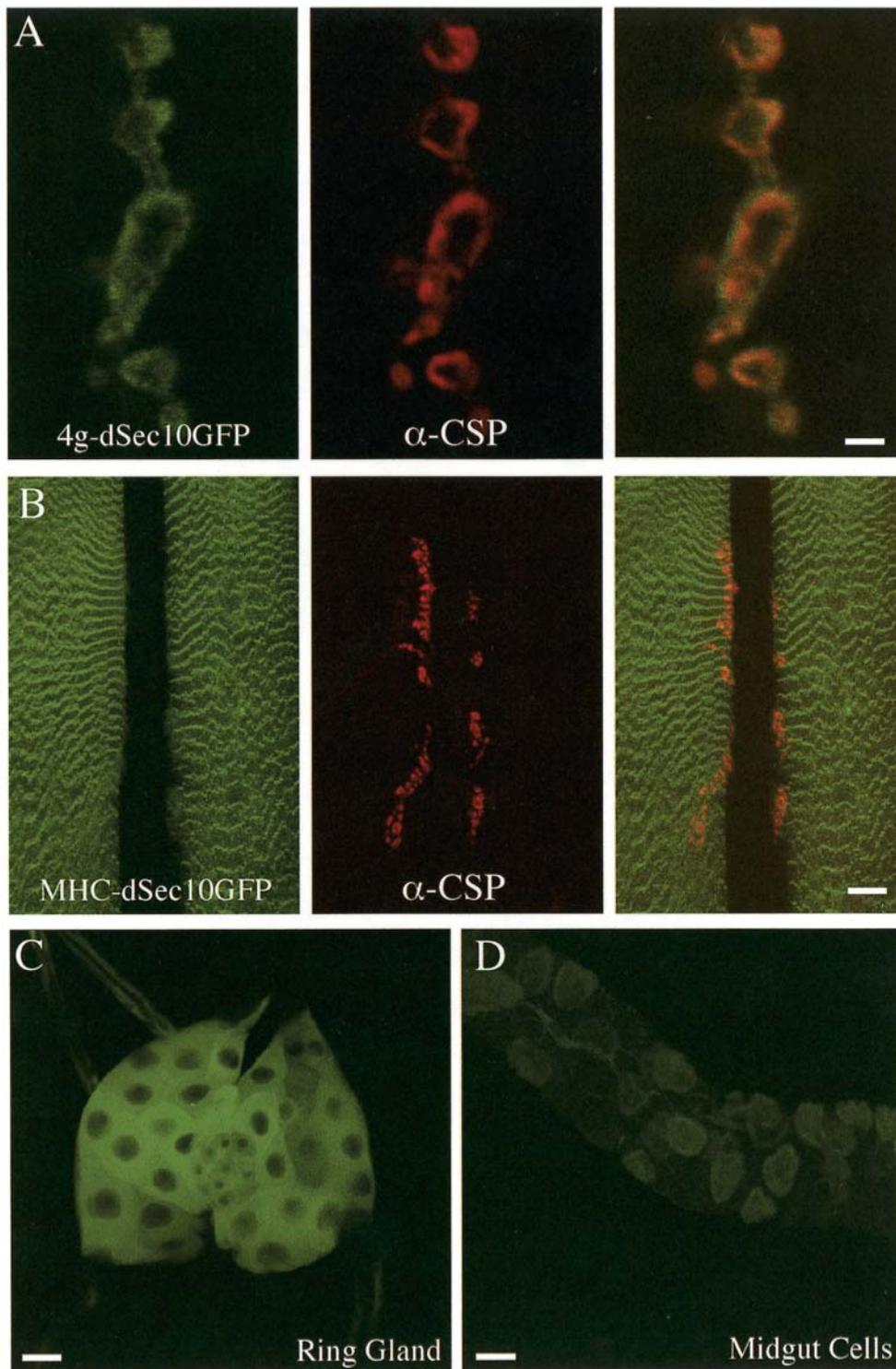
#### ***Drosophila sec10* is essential for postembryonic viability**

To elucidate the function of the sec6/8 complex in *Drosophila*, the UAS-dSec10RNAi line was crossed to a series of GAL4 drivers in different tissues. A summary of these results is presented in Table 1. We anticipated that the complex would be required in a variety of tissues based on its predicted function and widespread expression (Figure 2), and so an essential first experiment would be to determine what would happen following ubiquitous knockdown of dSec10. RNAi animals were crossed to various ubiquitous GAL4 drivers, including e22c-GAL4 (21) and tubulin-GAL4 (Bloomington stock center). Global loss of dSec10 resulted in 100% lethality at the second instar (L2) larval developmental stage (Table 1). However, some animals arrested in L2 but continued to live up to 5 days after egg laying. It was surprising that these animals did not die during embryogenesis, considering the need for coordinated exocytosis throughout development; this lack of embryonic lethality may be attributed to incomplete elimination of all dSec10 (but see Figures 2 and 4) or an undetectable maternal contribution of protein.

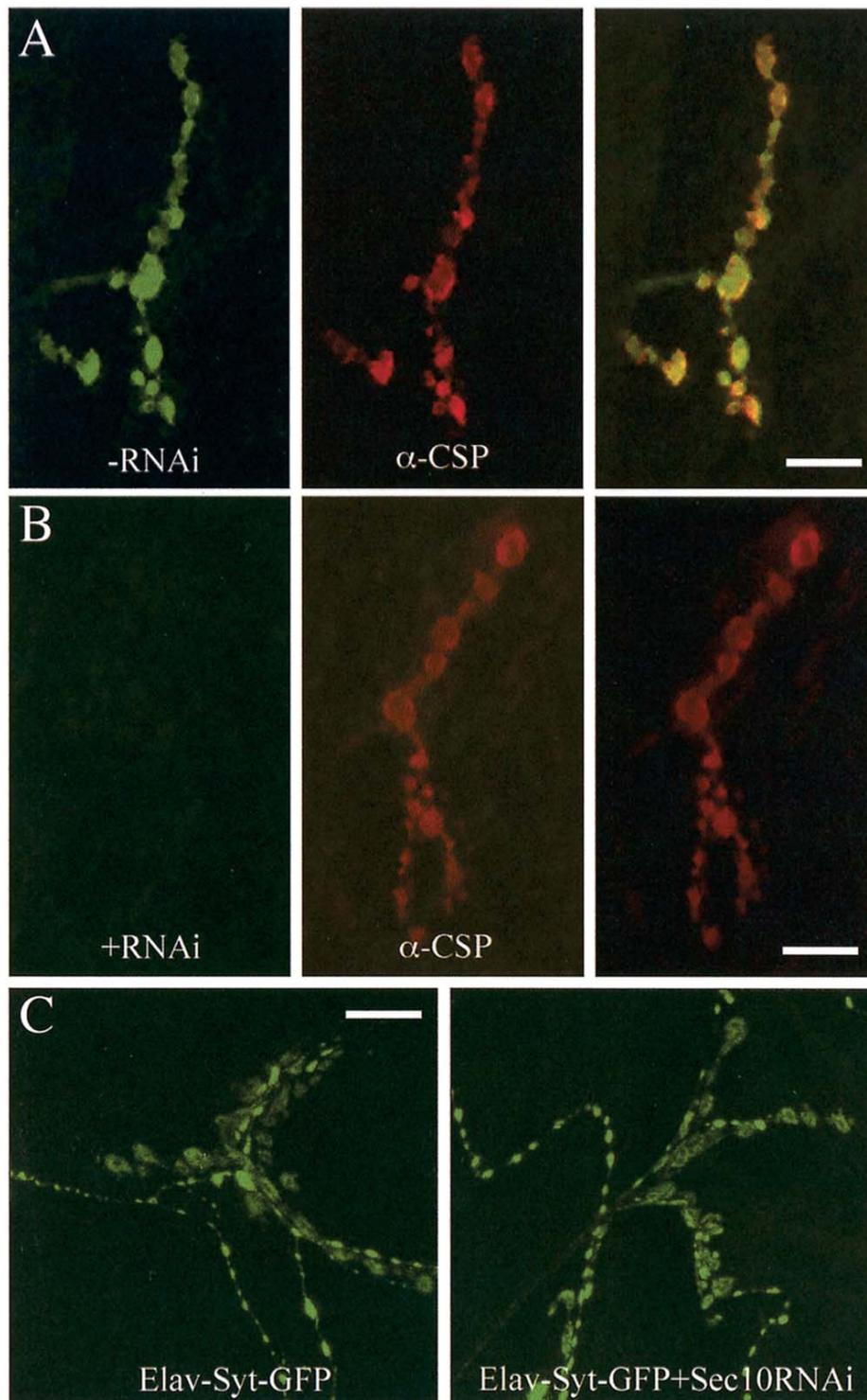
To determine the tissue or tissues that are responsible for the essential requirement of dSec10, GAL4 lines driving tissue-specific RNAi expression were employed. The role of dSec10

in the nervous system, including both interneurons and motor neurons, was examined using GAL4 drivers such as Elav-GAL4 (20) and 4G-GAL4 (19). Expression of dSec10RNAi throughout the nervous system, with multiple drivers, failed to produce a lethal phenotype, suggesting that there is no essential function for dSec10 in nervous system morphogenesis (embryo or pupal) or synaptic function (Table 1; Figure 4C). To investigate its role in muscle, specifically in the post-synaptic field of the NMJ, dSec10RNAi expression was driven using multiple muscle-specific drivers [e.g. pan-muscle MHC-GAL4 (20)]. Knockdown of dSec10 in muscle did not produce any detectable phenotypes (Table 1). Although no essential requirement was found using drivers pre- or postsynaptically at the NMJ, the possibility that there is an essential requirement earlier in nervous system development was examined. Work done in cultured hippocampal neurons demonstrated that the sec6/8 complex is localized to growth cones during neurite outgrowth (13) where it is hypothesized to be required for neurite outgrowth. In *Drosophila*, however, expression of dSec10RNAi throughout the neural ectoderm or in neuroblasts also failed to produce any detectable phenotypes (Table 1). Thus, we conclude that there is no detectable essential requirement for dSec10 throughout the neuromusculature.

dSec10 expression is also robust in the gut, epidermis and other tissues where active polarized secretion is necessary for function (Figure 2). Therefore, GAL4 lines that specifically express in these different tissues were studied. The epidermis shows polarized secretion of cuticle, to form mouth-hook and



**Figure 3: Subcellular localization of dSec10 GFP fusion protein in multiple tissues.** (A) In the neuromuscular junction (NMJ) dSec10-GFP (green) colocalizes with the synaptic vesicle marker Cysteine String Protein (CSP; red). The transgene is driven by the 4G-GAL4 neuronal driver. Scale bar equals 2 $\mu$ m. (B) In the muscle, dSec10-GFP does not localize to the postsynaptic NMJ field, but rather is distributed throughout the cytoplasm in sarcomeric striations. The transgene is driven by the MHC-GAL4 muscle driver. Scale bar equals 20 $\mu$ m. (C) In the ring gland, dSec10 GFP expresses throughout the corpora allata and prothoracic regions. The transgene is driven by Feb36-GAL4. Scale bar equals 40 $\mu$ m. (D) In the midgut, dSec10-GFP does not express in a polarized fashion in the copper cells. Transgene is driven by e22c-GAL4. Scale bar equals 40 $\mu$ m.



**Figure 4: dSec10 RNAi eliminates dSec10GFP protein in neuromuscular synapses without perturbing morphology and vesicle pool distribution.** (A) The expression of dSec10GFP fusion protein (green) in an NMJ terminal marked with an antibody against synaptic vesicle associated CSP (red). The transgene is driven by the 4G-GAL4 neuronal driver. Scale bar equals 10  $\mu$ m. (B) A single copy of UAS-dSec10RNAi eliminates detectable dSec10GFP protein. Note that the CSP-positive NMJ boutons have normal morphology and synaptic vesicle distribution. Scale bar equals 10  $\mu$ m. (C) A Synaptotagmin-GFP fusion protein driven by Elav-GAL4 in the presynaptic NMJ terminal (left) is unperturbed by the coexpression of dSec10RNAi (right). Note that the Syt-GFP-positive NMJ boutons have normal morphology and synaptic vesicle distribution. Scale bar equals 20  $\mu$ m.

denticle belts essential for viability. Driving dSec10RNAi with GAL4 lines specific for the epidermis failed to show any essential role. Three GAL4 lines that express in the epidermis, as well as some other tissues, were used to check if there is a requirement for dSec10: 69B, V49, and 76-D (Table 1). No detectable phenotypes were produced with either V49 or 76-D drivers, but early larval lethality was produced with the 69B driver. The gut shows polarized secretion necessary for food digestion. Driving dSec10RNAi with a GAL4 line specific for the endoderm failed to show any essential role. Three lines that express in the gut, as well as a few other tissues, were used to check if dSec10 is essential for gut function: 5053a, 34B, and c323a (Table 1). No detectable phenotypes were produced with gut drivers 5053a or 34B, but early larval lethality was observed with c323a.

The fact that no lethal phenotype was produced with two of three drivers in both the gut and epidermal drivers presented an enigma. To confirm published expression data, all GAL4 lines were crossed to UAS-GFP to assay temporal and spatial expression patterns. Every GAL4 line that produced a lethal phenotype displayed unreported expression in the ring gland, the fly endocrine organ. In order to determine if expression of dSec10RNAi in this tissue was truly the cause of lethality, drivers that express in the ring gland, and in as few other tissues as possible were obtained (Table 1). Ring gland-specific GAL4 drivers (e.g. GAL4 2–286), as well as all lines showing ring-gland expression, resulted in larval lethality. These results show that the only tissue displaying a detectable requirement for dSec10 is the ring gland.

### ***dSec10 has no detectable role at the synapse***

The nervous system is highly specialized for vesicular trafficking and secretion. Work in mammalian cell culture shows that the sec6/8 complex may regulate the late stages of exocytosis between the *trans*-Golgi network and the plasma membrane (28) and that the complex may associate with microtubules to promote neurite outgrowth (29). To investigate the role of dSec10 in nervous system development and function, we used the *Drosophila* neuromuscular junction (NMJ) as a model. This is an ideal system to conduct structural and electrophysiological experiments because of the high resolution studies permitted with the wide array of tools available.

Studies were done following UAS-dSec10RNAi expression in the presynaptic terminal (using the pan-neuronal driver Elav-GAL4) and postsynaptic muscle (using pan-muscle MHC-GAL4). Both GAL4 drivers initiate expression during mid-embryogenesis, prior to axonal outgrowth from the CNS. If dSec10 is necessary for synaptic development in *Drosophila*, eliminating dSec10 from the NMJ would be expected to affect pathfinding and synaptic morphology. To investigate this possibility, third instar NMJs were stained with antibodies to examine synaptic architecture. Comparison of the number of boutons per synaptic terminal at the muscle 6/7 NMJ of MHC-GAL4 or Elav-GAL4 driven RNAi animals compared to wild-type (OR) showed no significant difference (RNAi/Elav  $79 \pm 16$ , RNAi; MHC  $86 \pm 13$ , OR  $80 \pm 16$ ;  $n = 7$ ). Native synaptic vesicle proteins (e.g. CSP) and transgenic fusion proteins (e.g. Synaptotagmin-GFP), localized properly to synap-

**Table 1:** Targeted expression of UAS-dSec10RNAi to specific *Drosophila* tissues

Gal4 driver	Tissue specificity	Lethality/defects	Reference
e22c	Ubiquitous	Lethal at L2	Lawrence 1995
Tubulin	Ubiquitous	Lethal at L2	Flybase
UH1	Ubiquitous	Lethal at L2	Flybase
c323a	Salivary glands, some cells in gut, ring gland	Lethal at L2	Flybase
69B	Embryonic epiderm, imaginal discs, brain, ring gland	Lethal at L2	Flybase
Feb36	Ring gland, trachea, some cells in midgut, malpighian tubules, salivary glands	Lethal at L3	Siegmund 2001
2–286	Ring gland, salivary glands	Lethal at L3	Timmons 1997
48Y	Embryonic endoderm	Fully viable	Flybase
108.4	Embryonic mesoderm	Fully viable	Flybase
V49	Embryonic epiderm	Fully viable	Flybase
1	Neural ectoderm	Fully viable	Flybase
Elav	Pan neuronal, salivary glands	Fully viable	Zhang 2001
31–1	Pan neuronal	Fully viable	Flybase
V85	Pan neuronal	Fully viable	Flybase
c698a	All central neurons	Fully viable	Flybase
4G	Subset of neurons	Fully viable	Rohrbough 2000
386	All peptidergic neurons	Fully viable	Siekhaus 1999
(3)31–1[31–1]	Neuroblasts and neurons	Fully viable	Flybase
MHC	All muscles	Fully viable	Zhang 2001
5053a	Longitudinal muscles, some gut cells	Fully viable	Flybase
34B	Posterior midgut, imaginal discs, salivary glands	Fully viable	Flybase
76-D	Epidermal stripes	Fully viable	Flybase

Transgenic animals bearing UAS-dSec10RNAi were crossed to fly lines bearing the indicated GAL4 insertion. The expression pattern, lethal stage and GAL4 references are noted.

tic boutons in characteristic synaptic vesicle domains (Figure 4B,C), indicating that synaptic vesicles are localized properly in the absence of detectable dSec10. Since glutamate receptors are inserted into the muscle through a polarized vesicle fusion process, glutamate receptor insertion/localization in the postsynaptic membrane were examined following muscle expression of dSec10RNAi. Immunocytochemical studies with anti-gluRIIA antibodies (30) revealed glutamate receptor fields which were indistinguishable from those in wild-type (data not shown).

Electrophysiological analyses at the third instar NMJ were employed to assay synaptic function. Using two-electrode voltage-clamp (TEVC) recording, both stimulation-evoked and spontaneous synaptic events were monitored following removal of dSec10 from the pre- or postsynaptic compartments (Figure 4B). Electrophysiological measurements were performed using reduced  $[Ca^{2+}]$  (0.4 mM) standard *Drosophila* saline, to examine synaptic vesicle exocytosis under nonsaturating conditions (19). Excitatory junctional current (EJC) recordings from the wild-type strain Oregon-R exhibit amplitudes of  $38.83 \pm 8.06$  nA under these conditions, comparable to control larvae carrying a single copy of the UAS-dSec10RNAi transgene ( $42.81 \pm 7.01$  nA), the pan-neural Elav-GAL4 driver ( $42.93 \pm 8.04$ ) or the muscle-specific MHC-GAL4 ( $44.53 \pm 11.45$ ) (Figure 5A,B). Surprisingly, no significant impairment of transmission was observed following the constitutive removal of either pre- or postsynaptic dSec10 from mid-embryogenesis through mature 3rd instar (~5 days). Quite contrary to expectations, a small increase in synaptic current amplitude was observed in Elav-GAL4/UAS-dSec10RNAi ( $53.01 \pm 7.26$  nA vs.  $38.83 \pm 8.06$  nA), although this change was not statistically significant ( $p = 0.097$ ). Moreover, EJC amplitudes were comparable in MHC-GAL4 heterozygotes ( $44.53 \pm 11.46$  nA) compared to MHC-GAL4/UAS-dSec10RNAi larvae ( $47.70 \pm 5.71$ ). Thus, dSec10 does not play any detectable role in either pre- or postsynaptic neurotransmission (Figure 5A,B).

Miniature EJCs (mEJCs) were next measured for all genotypes to assay for spontaneous vesicle fusion (mEJC frequency) and postsynaptic receptor function/distribution (mEJC amplitude). The frequency of mEJCs was unchanged in all genotypes with the exception of Elav-GAL4/UAS-dSec10RNAi, which showed a statistically significant ( $p < 0.03$ ) reduction in spontaneous vesicle fusion rate ( $1.09 \pm 0.12$  Hz compared to  $2.12 \pm 0.26$  Hz; Figure 5C). mEJC amplitudes were unchanged in all genotypes assayed (Figure 5D). The finding that Elav-GAL4, dSec10RNAi displays unchanged/enhanced stimulus evoked EJCs, but a reduced likelihood for spontaneous release events was surprising. Therefore, we repeated the physiological analysis with an independent pan-neural driver (4G-GAL4), and found no significant changes in either EJC amplitude, or mEJC frequency (data not shown). Taken together, these results indicate that dSec10 does not play any obvious role in exocytosis underlying synaptic development or neurotransmitter release. It should be noted, however, that a low level of persisting

dSec10 under these transgenic RNAi conditions may be masking some role for the protein in neurotransmission.

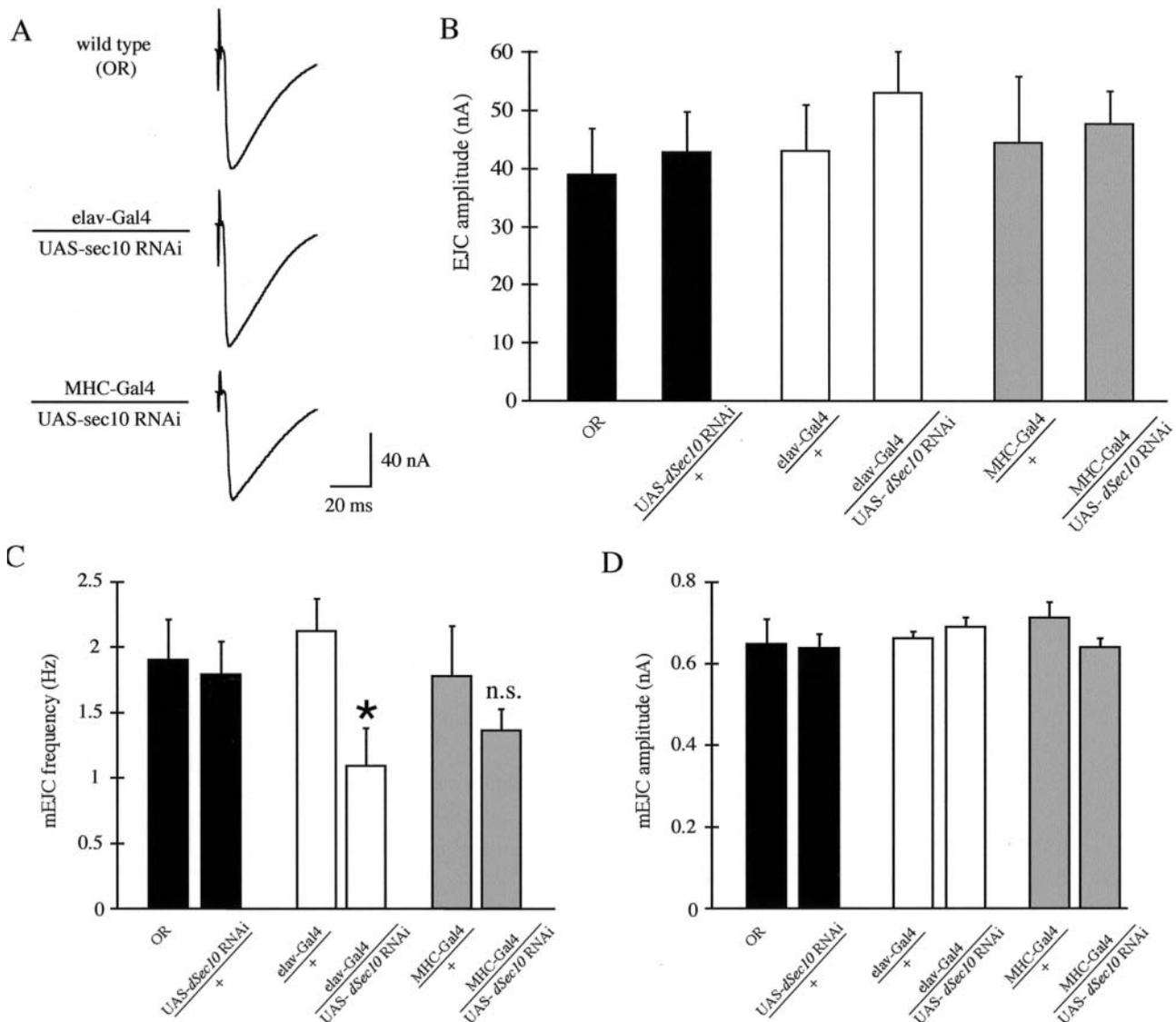
#### ***dSec10 has no detectable role in other cell types specialized for polarized secretion***

Although UAS-dSec10RNAi driven by GAL4 lines that express in the gut, salivary glands and epidermis (which are all highly polarized) does not produce lethality, it is possible that dSec10 plays a nonessential role in vesicle trafficking in these tissues. The salivary gland is a polarized organ that is needed for secreting the glue protein that holds the pupal case to a substrate during metamorphosis (31). Based on analyses using numerous GAL4 drivers, many of which express in the salivary gland (Table 1), there is no indication of a glue protein secretion defect in the salivary glands (data not shown). The epidermis must have polarized vesicle fusion in order to secrete the cuticular denticle belt structures and mouth-hooks. When dSec10RNAi was expressed throughout the epidermis from any early stage (Figure 2B), there were no detectable morphological defects in denticle belt or mouth-hook structures, which are easily visualized under a compound microscope (data not shown). The midgut must have polarized secretion in order to secrete the acid and enzymes needed to digest food. To assay for secretory defects in the gut, UAS-dSec10RNAi; e22c-GAL4 animals were fed bromophenol blue-supplemented yeast paste according to Dubreuil et al. (32) to determine if there was a lack of midgut acidification. Defects in polarized secretion are detected if the midgut region does not turn yellow after feeding. No detectable difference was observed between mutant and control animals (data not shown), so no quantifiable experiments were performed. These results indicate that dSec10 does not appear to function in polarized secretion in the salivary glands, epidermis, or gut, in addition to not having a detectable role in the polarized secretion underlying neurotransmission.

#### ***dSec10 is essential for ring-gland function***

From investigations using tissue-specific GAL4 drivers, the only essential requirement for dSec10 was found in the ring gland (Table 1; Figure 6A). The ring gland is the primary endocrine organ in *Drosophila*, and is responsible for synthesis and secretion of the steroid hormone ecdysone (from the prothoracic gland region) and the peptide hormone juvenile hormone (from the corpora allata) (17), among other hormones. These observations, together with previous studies of exocyst function, therefore suggest that dSec10 may be required for a specialized type of vesicular transport and/or polarized secretion necessary for the release of ring-gland hormones, which leads to lethality if disrupted. If lack of dSec10 causes disruption of hormone secretion required for pupal metamorphosis and subsequent viability, one might predict that the requirement could be bypassed by providing a hormonal supplement.

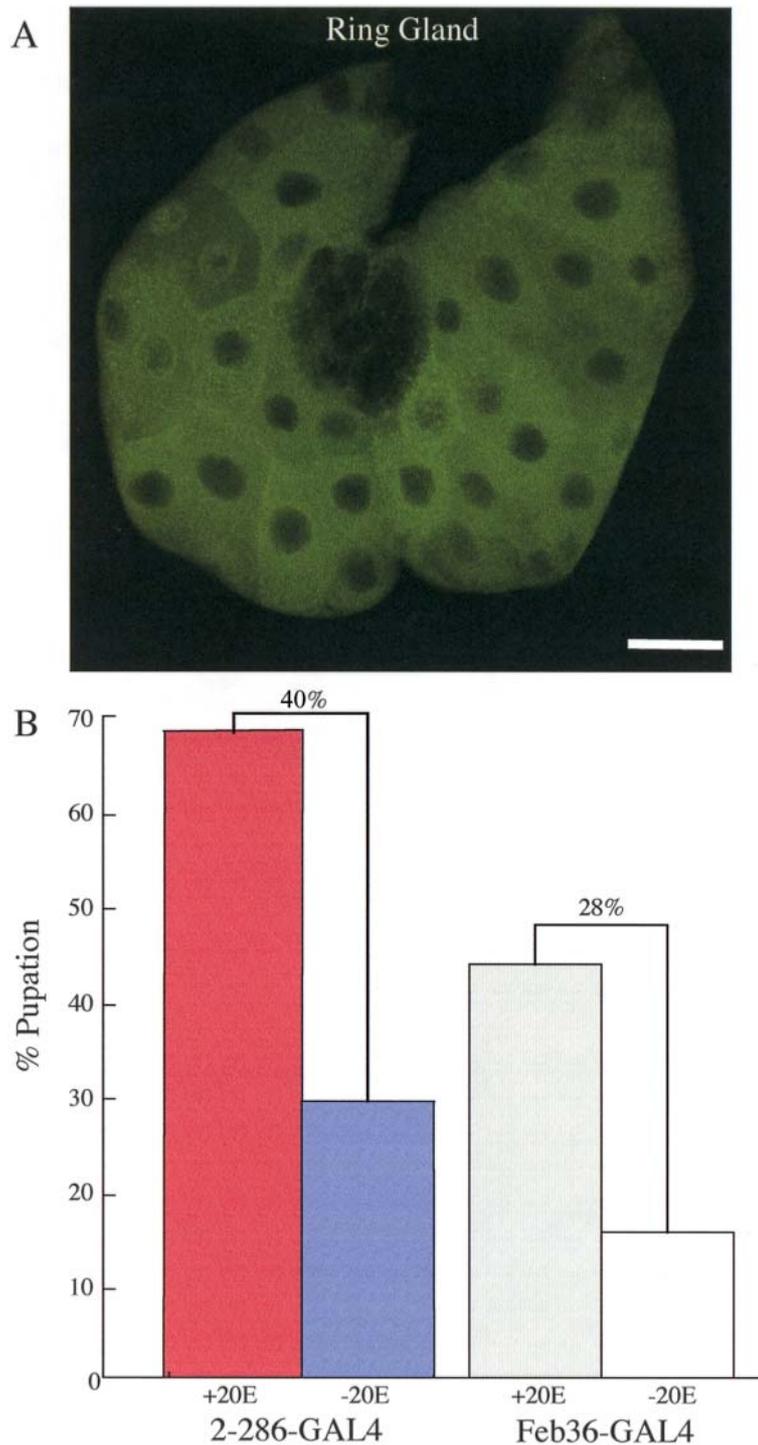
The prepupal lethal stage of dSec10 RNAi mutants is most consistent with an impairment in ecdysone secretion, which is required to trigger the pupal molt and metamorphosis. Therefore, 20-hydroxyecdysone (20E), the active form of the



**Figure 5: Electrophysiological analyses at the NMJ of dSec10 RNAi mutants show that the protein is not required for synaptogenesis or synaptic function.** (A) Representative excitatory junctional current (EJC) traces in 0.4 mM  $[Ca^{2+}]_i$  in wild-type (OR), animals expressing dSec10 RNAi presynaptically (elav-GAL4; UAS-sec10 RNAi), or postsynaptically (MHC-GAL4; UAS-sec10 RNAi). The shock artifact indicates the time of nerve stimulation. (B) Mean EJC amplitudes from controls and pre- or postsynaptic dSec10 RNAi lines show no detectable differences. The RNAi transgene alone (UAS-dSec10 RNAi/+) or the GAL4 drivers alone (elav-GAL4 or MHC-GAL4) act as genetic controls. Pre- or postsynaptic expression of dSec10 RNAi permits EJC responses comparable to both wild-type and genetic controls. (C) The frequency of miniature EJC (mEJC) currents in 0.4 mM  $[Ca^{2+}]_i$  in all genotypes. The average frequency is not significantly (n.s.) different in any genotypes except elav-GAL4; UAS-dSec10 RNAi which is significantly (\* $p < 0.05$ ) reduced compared only to the elav-GAL4/+ control. (D) The mean amplitude of mEJC currents in 0.4 mM  $[Ca^{2+}]_i$  in all genotypes. The indistinguishable amplitudes show that postsynaptic function does not differ between any genotypes.

molting hormone ecdysone, was fed to mutants in an attempt to rescue larval lethality caused by driving dSec10RNAi with ring-gland drivers (Figure 6A). A similar experiment has been shown to rescue ecdysteroid-deficient mutants (33). The specific assay was a rescue to puparium formation, as would be predicted if the absence of secreted ecdysone is the cause of dSec10 mutant larval lethality. As in all other tissues examined, coexpression of dSec10RNAi and dSec10GFP in the ring gland resulted in suppression of

the dSec10GFP protein to undetectable levels (data not shown). Larvae expressing dSec10RNAi in the ring gland were fed a diet supplemented with 20E at a final concentration of 0.33 mg/ml. Compared to controls, animals fed 20E were significantly rescued from larval lethality and to puparium formation (Figure 6B). We utilized two different GAL4 drivers: (i) 40% more larvae survived to pupation following 20E feeding in the 2-286-GAL4 line [69.4% (+ 20E) compared to 29.3% (- 20E)]; and (ii) 28% more larvae survived



**Figure 6: Supplementing ecdysone partially rescues mutants lacking dSec10 in the ring gland.** Two drivers were used to drive UAS-dSec10RNAi in the ring gland: Feb36-GAL4 and 2-286-GAL4. The image in (A) shows 2-286-GAL4 driving UAS-dSec10GFP expression. Scale bar equals 40  $\mu$ m. (B) Ecdysone (20E) was either fed (+20E; experimental) or not fed (-20E; control) during larval development and the percentage of animals pupating quantified. With the 2-286-GAL4 line, 69.4% pupated when fed 20E compared to 29.3% without the supplement, a 40% improvement in pupation rate. With the Feb36-GAL4 line, 43.8% pupated with 20E compared to 15.4% without 20E, a 28% improvement. These data show that loss of ecdysone secretion is a primary cause of larval lethality in the absence of dSec10.

following 20E feeding in the Feb36-GAL4 line [43.8% (+ 20E) compared to 15.4% (– 20E)]. Wild-type (OR) larvae treated under the same conditions pupated at 97.2% in the same period. The fact that some of the –20E control lines pupated was a density-dependent phenomenon, compared to the L3 lethality observed under normal rearing conditions (Table 1). Although the rescue was incomplete, these results demonstrate the larvae lacking dSec10 specifically in the ring gland are defective in ecdysone secretion. It is not surprising that the rescue was incomplete, since the ring gland mediates a number of essential hormonal release functions, of which ecdysone secretion is only one. However, note that dSec10 is not generally required for the secretion of peptide hormones, since expression of dSec10RNAi in all neuronal peptidergic neurons with GAL4 driver 386 (34) did not result in lethality (Table 1). We conclude therefore that dSec10 plays a specialized function in vesicular exocytosis underlying the secretion of steroid hormones and possibly other hormones in the endocrine system, although further experiments to test this hypothesis must be performed.

## Discussion

The goal of this study was to assay hypothesized roles of the sec6/8 exocyst complex in vesicle trafficking and exocytosis during polarized membrane addition and secretion. We took a genetic approach in *Drosophila* by targeting the most highly conserved complex member in the *Drosophila* genome, dSec10, with an RNA interference (RNAi) strategy that allows dissection of temporal and tissue-specific requirements. We show here that dSec10 is essential for viability as ubiquitous expression of dSec10RNAi results in early post-embryonic lethality. However, there were no detectable anatomical abnormalities associated with this essential requirement. We therefore next systematically removed dSec10 from specific tissues and, surprisingly, found the essential requirement restricted to the ring gland, a small organ specialized for endocrine function. There was no essential role for dSec10 in a host of other tissues specialized for polarized secretion including the epidermis, salivary glands, gut and nervous system. All of these tissues displayed apparently normal polarized membrane addition and secretion in the absence of detectable dSec10. Most particularly, the extensive, continuous membrane addition required for neuronal axon pathfinding and subsequent synapse elaboration was unaffected, as well as the high-frequency, highly polarized synaptic vesicle exocytosis underlying neurotransmission. We conclude from these studies that dSec10 plays no detectable role in a wide range of polarized vesicle trafficking and exocytotic events. Rather, dSec10 appears to play a highly selective role in a specialized form of exocytosis associated with the ring gland, although more experiments are clearly required to rigorously test this hypothesis. This putative exocytotic mechanism drives, in part, the secretion of the steroid hormone ecdysone, since feeding ecdysone to animals lacking dSec10 in the ring gland significantly rescues their developmental arrest and larval lethality. These results suggest that

dSec10 is likely required only for a highly specialized form of endocrine secretory trafficking, which has not previously been identified.

There are two important caveats to these conclusions. First, note that the assumption that dSec10 is an essential component of the *Drosophila* sec6/8 exocyst complex is based on the essential requirement of the orthologous yeast protein in the yeast exocyst (see below). Another possible interpretation of these data is that dSec10 is not an essential component of the *Drosophila* sec6/8 complex. Therefore, while the conclusions of this study are presumed to reflect the role of the entire *Drosophila* sec6/8 complex, this study was strictly limited to assaying the function of dSec10. Second, the transgenic RNAi approach employed here does not produce a condition equivalent to a genetic null mutant. Although expression of the dSec10GFP reporter protein was reduced to undetectable levels in all tissues expressing dSec10RNAi, RT-PCR analyses revealed the persistence of some endogenous dSec10 message. These results resemble recent studies using a similar approach with other genes, which show that the native message is reduced up to 100-fold, but is not eliminated by transgenic RNAi (25). Therefore, these studies represent a strong suppression of dSec10 expression but not a complete elimination of the protein. It is possible that low levels of dSec10 are sufficient to provide the requirement in vesicle trafficking/secretion in either neuronal or non-neuronal cell types.

### **Analyses of *Drosophila* sec10 do not support hypothesized functions for the sec6/8 complex in multicellular animals**

Members of the yeast exocyst complex were discovered based on a group of temperature sensitive mutants with defects in secretion (35, 36). Mutations in any of the eight subunits in yeast promote cytoplasmic accumulation of secretory vesicles at the tip of the growing daughter cell and defects in polarized growth, which result in lethality (11, 37). Specifically, analyses of the yeast sec10 mutant show a cytoplasmic accumulation of vesicles, indicating that the mutant causes a block in either vesicle trafficking or exocytosis (38). Based on phenotypic comparisons, the complex is proposed to be required after the formation of vesicles and their delivery to target membrane, but before assembly of the SNARE complex and vesicle fusion with the plasma membrane (39). One subunit, sec3, localizes to exocytotic sites, even in the absence of membrane traffic, suggesting that it is a spatial landmark which defines the site of exocytosis (37). Another subunit, sec15, binds the vesicle-associated sec4 (a Rab GTPase) when in its GTP-bound state (40). These interactions are consistent with a role in vesicle tethering, where sec15 on the vesicle could associate with the plasma membrane through an interaction with sec3. Mutant alleles of sec3 also result in accumulation of ER and Golgi vesicles, and block carboxypeptidase Y transport from ER to Golgi and from Golgi to vacuole (41). Thus, the exocyst complex has been firmly implicated in both late-stage plasma membrane trafficking and intracellular vesicle trafficking.

Similar to yeast, the mammalian sec6/8 complex has been proposed to be essential in polarized vesicle trafficking and exocytosis. In mice, the complex is expressed ubiquitously in all tissues (14, 42–44), and mice that lack Sec8 die during early embryogenesis, showing that the complex has some essential function (45). These results agree well with our analyses of dSec10, excepting only the latter lethal stage in the absence of the *Drosophila* protein. This different lethal stage is neither unusual nor surprising, given the brief (<24h) embryogenesis in flies. In the rat epithelial MDCK cell line, rSec8 antibody perturbation experiments suggest that the complex is required for the continuous delivery of Golgi-derived vesicles to the plasma membrane (12). Following rSec8 antibody introduction, delivery of vesicles to the basolateral, but not the apical, membrane is blocked, indicating that the complex is required for the polarized recruitment of vesicles to specific sites on the plasma membrane. In contrast, dSec10 plays no discernible role in polarized vesicle trafficking in any tissue or any stage of development. Following dSec10 RNAi expression in the epidermis, we detected no defect in the polarized secretion to form cuticular structures. Likewise, we detected no role for dSec10 in the polarized secretion in salivary gland secretory cells, midgut secretory cells, or muscle. Therefore, dSec10, and, by extension presumably the *Drosophila* sec6/8 complex, is not detectably required for polarized vesicle trafficking or exocytosis in a range of tissues, in contrast to pharmacological results obtained in mammalian culture cells.

The most extreme examples of cell polarity and polarized secretion are in the nervous system. rSec6/8 immunoreactivity is detected in the cell bodies, axons, and in dendritic and axonal growth cones in cultured hippocampal neurons (13). In differentiated PC12 cells and cultured hippocampal neurons, the sec6/8 complex and microtubules extend to the growing neurite and colocalize at the growth cone, and the sec6/8 complex coimmunoprecipitates with microtubules from total rat brain lysate (29). Overexpression of a c-terminal sec10 deletion construct into these PC12 cells has been reported to abolish neurite outgrowth. Contrary to this observation, our findings suggest that there is no essential function for dSec10 in nervous system development. When the dSec10RNAi transgenic line is driven throughout the neural ectoderm, in all neuroblasts, pan-neuronally, or in subsets of neurons, we detected no essential requirement and no morphological defects in neurite outgrowth. Moreover, there is also no defect observed following the delayed pupal metamorphosis, ruling out any possibility of an embryonic rescue by maternally contributed protein. Following outgrowth, the mammalian sec6/8 complex localizes to periodic domains along axons, colocalizing with synaptic vesicle associated synapsin and domains of FM1-43 dye recycling (13). Along with the previous results, these findings have led to the hypothesis that the sec6/8 complex plays a role in establishing fusion sites during synaptogenesis, or mediating polarized vesicle trafficking/secretion underlying synaptic transmission. Our results do not support either hypothesized function, assuming dSec10 is an essential component of the *Drosophila*

sec6/8 complex. Removal of dSec10 does not detectably perturb synaptic development or function at the well-characterized *Drosophila* NMJ synapse. The electrophysiology assays employed are the most sensitive, quantifiable means to assay polarized vesicle secretion. Therefore, the absence of any hint of defect in dSec10RNAi mutants suggests that the dSec10 protein does not play even a mild facilitatory function in synaptic processes. Although a small level of endogenous dSec10 protein likely remains in the RNAi conditions employed here, it would be surprising if this could completely mask a requirement in the high-frequency secretion underlying neurotransmission.

Our results from non-neuronal and neuronal tissues suggest that *Drosophila* sec10 does not play the generalized role in polarized vesicle trafficking/fusion attributed to sec10 and the exocyst complex in yeast, or putative roles for the sec6/8 complex derived from work in mammalian cell culture. Although dSec10 is widely expressed in tissues ranging from the epidermis to musculature to gut to nervous system, our analyses have failed to detect any role for the protein in any of these tissues. Removal of detectable dSec10GFP reporter from any of these tissues allows full viability with no observed trafficking or secretion defects. Given that, as in yeast, all complex members are similarly required for complex function, we conclude that the *Drosophila* sec6/8 complex has no clear role in either polarized vesicle trafficking or exocytosis in any of the tissues examined.

#### ***The specialized role of dSec10 in the endocrine ring gland***

The only tissue with a detectable requirement for dSec10 is the endocrine organ of *Drosophila*, the ring gland. All transgenic drivers that cause lethality when driving the dSec10 RNAi transgene share expression in the ring gland, and exclusive expression of dSec10RNAi in the ring gland causes similar lethality. We note, however, that exclusive expression of dSec10 RNAi in the ring gland causes lethality in the third larval instar, compared to second instar lethality following ubiquitous expression (Table 1). This result could be due to a coordinate role for dSec10 in multiple tissues (causing a synergistic lethal effect), or an essential requirement for dSec10 in some small, unidentified tissue at an earlier developmental stage, in addition to its essential requirement in the ring gland.

Three tissues join to form the ring gland: the prothoracic gland, the corpora allata and the corpora cardiaca (17). The prothoracic gland is the site of the steroid hormone ecdysone synthesis and secretion (16). Pulses of 20-hydroxyecdysone (ecdysone) trigger each of the major developmental transitions in *Drosophila*, including larval molting and pupal metamorphosis (46). The corpora allata is responsible for the synthesis and secretion of the peptide hormone juvenile hormone (47), which is necessary at the time of ecdysone release to allow larval molting and prevent metamorphosis. Another peptide hormone important for ecdysis is eclosion hormone, which is expressed in one pair of ventromedial

neurosecretory cells in the larval, pupal and pharate adult brain (48). Axons leading from these cells synapse on the corpora cardiaca to provide for release of eclosion hormone into the hemolymph (16). Peptide hormones are secreted via fusion of dense-core vesicles. Although little is known about the mechanism of steroid hormone secretion, there is some evidence that these hormones are also secreted through polarized vesicular exocytosis, rather than simple diffusion (49). One study from the waxmoth using electron microscopy showed immunocytochemical staining of ecdysone in secretory vesicles arrested during fusion with the plasma membrane, indicating the ecdysone is being secreted via vesicular exocytosis (50). These studies indicate that steroid hormone secretion, specifically ecdysone secretion, may be dependent on a vesicle-mediated process.

The late larval lethality caused by dSec10RNAi expression only in the ring gland is most consistent with the loss of the ecdysone metamorphosis signal (51, 52). Therefore, it was possible that a supplement of exogenous ecdysone might bypass this developmental arrest and permit viability into the pupal stage. We have shown that providing ecdysone does indeed provide a significant rescue of larval lethality, indicating that this lethality is caused by the loss of ecdysone secretion. Given everything we know about the sec6/8 or exocyst complex (see above), we conclude that it is most likely that dSec10 plays a specialized role in polarized vesicle fusion underlying secretion of this steroid hormone. We note, however, that the rescue provided by ecdysone is incomplete. This is most likely due to the fact that we are only (crudely) supplying one hormone and it is likely that the loss of dSec10 in the ring gland impairs the secretion of multiple, interacting, hormones.

Our findings suggest that dSec10 may play a role in a specialized subset of vesicular transport and/or secretion. There is clear evidence that multiple mechanistic forms of secretion exist. The most striking example of a difference is small, clear vesicles compared to large, dense-core vesicles. Small, clear vesicles, which contain, for example, classical neurotransmitters, and dense-core vesicles, which contain peptides and protein hormones, are both released by Ca<sup>2+</sup>-dependent, SNARE-dependent processes (53, 54). However, these different types of vesicles also require different machinery in order to secrete their contents; for example, dense-core vesicles specifically require the CAPS protein (55). *Drosophila* dSec10 does not appear to be essential in either of these vesicular pathways, since both small, clear vesicle exocytosis mediating glutamatergic neurotransmission and large, dense-core vesicle exocytosis in peptidergic neurosecretory cells [386-GAL4 driver, see Table 1 (55)] continue in the absence of detectable dSec10. However, a different mechanistic form of vesicular trafficking/exocytosis may mediate steroid hormone secretion. Our results suggest that dSec10 plays a role only in this putative specialized subset of vesicular trafficking. Although the experiments shown here suggest a role for dSec10 in ring-gland secretion, additional experiments are clearly required to demonstrate the mechanism by which

dSec10 acts in the ring gland. We will need finer definitions of different classes of vesicular trafficking, and more tools with which to dissect these mechanisms, in order to fully elucidate the function of the sec6/8 complex in *Drosophila*.

## Materials and Methods

### *Drosophila sec10* genomic and cDNA sequence determination

The Berkeley *Drosophila* Genome Project (BDGP) EST database (<http://www.fruitfly.org/>) was searched for clones with homology to all of the yeast and mammalian sec6/8 complex genes. The dSec10 gene showed the highest homology and was selected for characterization. Three fly EST clones with high homology to rat sec10 (LD11464, LD21465 and HL02545) were used to determine the dSec10 cDNA sequence. These clones share identical coding sequences and 3' ends, but LD21465 has the longest 5' untranslated region, followed by LD11464 and then HL02545. Five P1 clones mapped to 95E1-E6 were obtained from BDGP to determine the intron-exon structure of dSec10: DS06648, DS00620, DS04347, DS06876 and DS08055. The last four P1 clones overlap and form the contig Dm0249. PCR product size difference from cDNA template and from P1 genomic DNA clones indicated the presence of introns. Primers used for PCR and sequencing of the dSec10 gene were primer 1: CTGGCCCTGTGATTGTCAAC; primer 2: CAGTGGCACTGCTTGCAGTC; primer 3: GCTATCCCAATACATGGAGG; primer 4: TGGTCTGCAGGTGATC-GTAG; primer 5: TGGTGCGCCAAGTCAGACGC; primer 6: TGGAACCTCGGT-GCACCGAG; primer 7: TGCAGCCCGTATTGTGCAG. Primers 1-4 are within the gene and primers 5-7 are for sequencing the ends of the gene: primer 5 towards 5' end and primer 6 and primer 7 towards 3' end.

### *In situ* hybridization to polytene chromosomes

*In situ* hybridization to polytene chromosomes was performed essentially according to Ashburner (56), except that the ABC kit from Vector Laboratories (Burlingame, CA, USA) was used in the post-hybridization detection step. The dSec10 EST clone LD11464 was biotin-labeled by nick translation and used as the hybridization probe.

### Whole-mount *in situ* hybridization

RNA probes were labeled with digoxigenin-11-dUTP using DIG RNA labeling kit (Roche: Indianapolis, IN, USA) by following the product protocol. The EST clone LD11464 with full-length insert of dSec10 was linearized with BamHI for synthesizing the antisense RNA probe with T7 polymerase, and with KpnI for synthesizing the sense RNA probe with T3 polymerase. The newly generated RNA probes were hydrolyzed in alkaline condition at 60°C for 20 min to reduce their lengths for better probe penetration. *In situ* hybridization was performed essentially according to Tautz and Pfeifle (57), except proteinase K treatment was omitted in the pretreatment step and levamisole treatment was omitted in the staining step. The nervous system specific expression gene *embryonic lethal abnormal visual system* (*elav*) was used as a positive control (58). The *elav* clone (HL03451) was ordered from the same source as the dSec10 clones and its identity was confirmed by end sequencing. The clone was linearized with KpnI and SacI to make antisense and sense probes with T7 polymerase and T3 polymerase, respectively.

### Transgenic constructs

Two transgenic constructs were used in this work. dSec10 cDNA was amplified using PCR with primers containing unique overhanging restriction sites. For fusion of dSec10 to Enhanced Green Fluorescent Protein (EGFP) coding sequence, the coding region of dSec10 (with the stop codon changed to GCT) was cloned into the EcoRI and XhoI site of pUAST-EGFP (27), followed by sequencing to confirm that dSec10 is in frame with EGFP at the c-terminus of the dSec10 gene. For UAS-dSec10RNAi, exon

two and intron two were amplified from a dSec10 positive P1 clone using PCR with primers containing the unique overhanging restriction sites EcoR1 and Not1. This fragment was cloned into pBluescript. The antisense fragment of exon two was amplified from the same P1 clone using PCR with primers containing the unique restriction sites Not1 and Xba1. This fragment was also cloned into pBluescript. Once positive clones were selected, the fragments were digested out of pBluescript and ligated in a trimolecular reaction into pUAST. This created a construct which contains a sense exon, a sense intron spacer, and the antisense of the same exon which, when transcribed, will create a double-stranded RNAi molecule (Figure 1A). All constructs were transformed into the embryonic germ line using standard *Drosophila* transformation techniques (59).

#### Immunohistochemistry

Immunohistological experiments were performed as described previously (60). Preparations were incubated overnight at 4°C with mouse anti-Cysteine String Protein (CSP) (1 : 500) (22) to label synaptic vesicle domains or with mouse anti-GluRIIA (1 : 10) (30) to label the postsynaptic glutamate receptor field. All primary antibodies were visualized using Alexa 488. Fluorescent images were obtained on a Bio-Rad MRC 600 laser scanning confocal microscope (Hercules, CA, USA). All images presented were processed with Adobe Photoshop 6.0.1 software.

#### Polarized secretion assays in the epidermis, salivary glands and gut

To assay subcellular localization of dSec10 protein, UAS-dSec10GFP was expressed using tissue-specific GAL4 drivers, either alone or in combination with the UAS-dSec10RNAi construct. Tissues were dissected and imaged using a Bio-Rad MRC 600 laser scanning confocal microscope. To assay for defects in polarized cuticle secretion in the epidermis, the denticle belts and mouth-hook structures were imaged with Nomarski (DIC) optics with a Zeiss Axiophot microscope. To detect gut secretion function, mutant and control animals were fed yeast paste supplemented with bromophenol blue for 2h, and assayed for midgut acidification based on color reaction (32).

#### Electrophysiology

Third instar larval dissections and recordings were done in modified standard *Drosophila* saline containing (in mM): 128 NaCl, 2 KCl, 4 MgCl<sub>2</sub>, 70 sucrose and 5 HEPES. Saline was stored in 50-ml aliquots at 4° and, before use, received appropriate volumes of 100mM Ca<sub>2</sub>Cl stock solution to make the final Ca<sup>2+</sup> concentration 0.4mM. All recordings were made at 18° from muscle 6 in abdominal segments A3–A4 as previously described (61). Two-electrode voltage-clamp (TEVC) recordings of excitatory junctional currents (EJCs) were made at a holding potential of –60mV using an AxoClamp 2B amplifier (Axon Instruments: Burlingame, CA, USA). Miniature EJCs (mEJCs) were acquired from at least 6 animals per genotype and consisted of at least 3 min of continuous recording per animal. All mEJCs recordings were done in the same 0.4mM Ca<sup>2+</sup> standard saline as was used for evoked EJC recording. mEJC amplitude and frequency were analyzed using Mini Analysis software 3.0 (Jaejin Software: Englewood, NJ, USA).

#### Ecdysone rescue

UAS-dSec10RNAi animals were crossed to Feb36-GAL4 and 2–286-GAL4 (ring-gland drivers) in independent laying pots with agar apple-juice plates and allowed to lay eggs for 6h. After the laying period, embryos were collected, divided into two groups and each put on a new agar apple-juice plate (200 embryos per plate). One group was fed yeast paste with a final concentration of 3.3% ethanol (Sigma: St. Louis, MO, USA) as a control, and the other group was fed yeast paste supplemented with 20-hydroxyecdysone dissolved in 3.3% ethanol at a final concentration of 0.33mg/ml. The wild-type Oregon-R was used as a developmental control. The number of animals that hatched after 24h was counted to exclude

unfertilized embryos, and then the number that had pupated within the given time period was scored. The percentage of animals that pupated was calculated by dividing the total number of animals pupated by the total number of animals that hatched.

## Acknowledgments

The authors are grateful to Fude Huang for helpful discussions about RNAi construct design; Carl Thummel for informative discussions regarding the ring-gland, ecdysone rescue experiments, and providing the ring-gland drivers used in this study; the Bloomington *Drosophila* stock center for GAL4 drivers; Glen Andrews and Clayton Morrison for careful examination of the manuscript; Kabilar Gunalan for his help cloning the GFP fusion construct; and members of the Broadie lab for discussion. This work was supported by NIH grant RO1 GM54544 to K.B.

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