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# Auxilin is required for formation of Golgi-derived clathrin-coated vesicles during *Drosophila* spermatogenesis

**Xin Zhou[1], Lacramioara Fabian[2], Jennifer L. Bayraktar[1], Hong-Mei Ding[1], Julie A. Brill[2,3] and Henry C. Chang[1,*]**

## SUMMARY

Clathrin has previously been implicated in *Drosophila* male fertility and spermatid individualization. To understand further the role of membrane transport in this process, we analyzed the phenotypes of mutations in *Drosophila auxilin* (*aux*), a regulator of clathrin function, in spermatogenesis. Like partial loss-of-function *Clathrin heavy chain* (*Chc*) mutants, *aux* mutant males are sterile and produce no mature sperm. The reproductive defects of *aux* males were rescued by male germ cell-specific expression of *aux*, indicating that auxilin function is required autonomously in the germ cells. Furthermore, this rescue depends on both the clathrin-binding and J domains, suggesting that the ability of Aux to bind clathrin and the Hsc70 ATPase is essential for sperm formation. *aux* mutant spermatids show a deficit in formation of the plasma membrane during elongation, which probably disrupts the subsequent coordinated migration of investment cones during individualization. In wild-type germ cells, GFP-tagged clathrin localized to clusters of vesicular structures near the Golgi. These structures also contained the Golgi-associated clathrin adaptor AP-1, suggesting that they were Golgi-derived. By contrast, in *aux* mutant cells, clathrin localized to abnormal patches surrounding the Golgi and its colocalization with AP-1 was disrupted. Based on these results, we propose that Golgi-derived clathrin-positive vesicles are normally required for sustaining the plasma membrane increase necessary for spermatid differentiation. Our data suggest that Aux participates in forming these Golgi-derived clathrin-positive vesicles and that Aux, therefore, has a role in the secretory pathway.

**KEY WORDS: *Drosophila*, Spermatid individualization, Auxilin, Clathrin-mediated trafficking**

## INTRODUCTION

In *Drosophila* testes, male germ cells undergo drastic morphological changes before they mature into long, thread-like sperm. Cell morphogenesis is crucial for fertility, as genetic lesions disrupting this process result in absence or reduction of sperm formation (Wakimoto et al., 2004). The transformation from round to elongated spermatids is known to require extensive membrane biosynthesis and remodeling (Lindsley and Tokuyasu, 1980; Tokuyasu et al., 1972). However, the mechanism by which membrane addition during sperm development is achieved or regulated is not well understood.

*Drosophila* spermatogenesis is a multistage process (summarized in Fig. 1A). The spermatogonium, produced by an asymmetric germline stem cell (GSC) division at the apical tip of the testis, undergoes additional rounds of cell division with incomplete cytokinesis to generate 64 haploid spermatids (Gonczy and DiNardo, 1996; Hardy et al., 1979). These round spermatids, interconnected through cytoplasmic bridges, then elongate as a syncytium, reaching 1.8 mm in length (Lindsley and Tokuyasu, 1980). Elongation of syncytial cysts of spermatids also includes elongation of axonemes (microtubule-based cytoskeletal structures) and nuclei. Round nuclei first become canoe-shaped and then elongate to become almost needle-shaped. The interconnected, elongated spermatids are separated by 'individualization', a process characterized by coordinated movement of actin-based investment cones (ICs) along the axonemes, progressing from the nuclear end to the tail end. This synchronous movement of ICs removes most of the cytoplasmic content from the elongated spermatids and deposits it into a cystic bulge. In addition, each spermatid is encapsulated in a discrete membrane after IC passage (Tokuyasu et al., 1972). After individualization, bundles of mature sperm retract into basal coils and are deposited into the seminal vesicles.

Defects in phospholipid regulation and vesicle trafficking are known to perturb various aspects of sperm development. For example, mutations in *four wheel drive* (*fwd*), the *Drosophila* homolog of phosphatidylinositol (PI) 4-kinase, affect cytokinesis (Brill et al., 2000). Depletion of phosphatidylinositol 4,5-bisphosphate in germ cells causes defects in axoneme biogenesis, cytokinesis and cell polarity, suggesting that phospholipids have multiple distinct functions during spermatogenesis (Fabian et al., 2010; Wei et al., 2008; Wong et al., 2005). Studies on genes encoding Cog5 (Four Way Stop – FlyBase), a protein required for normal Golgi morphology and localization; Syntaxin 5, a Golgi-associated SNARE protein; and Brunelleschi, a subunit of the Golgi-associated TRAPP-II complex, suggest that Golgi is crucial for both cytokinesis and spermatid elongation (Farkas et al., 2003; Robinett et al., 2009; Xu et al., 2002). Mutations in *Rab11* and *Arf6* (*Arf51f* – FlyBase) GTPases, two of the proteins regulating recycling endosomes, disrupt cytokinesis (Dyer et al., 2007; Giansanti et al., 2007; Tiwari et al., 2008). Recent evidence shows that Rab11 localization during cytokinesis depends on *fwd*, providing a functional link between Rab11 and the PI 4-kinase (Giansanti et al., 2007; Polevoy et al., 2009). *Vps28*, a component of the endosomal sorting complex required for transport (ESCRT), participates in individualization (Sevrioukov et al., 2005). This genetic evidence suggests that secretory and endocytic pathways are utilized during spermatogenesis.

[1]Department of Biological Sciences, Purdue University, 915 West State Street, West Lafayette, IN 47907-2054, USA. [2]Program in Developmental and Stem Cell Biology, The Hospital for Sick Children, Toronto, ON M5G 1L7, Canada. [3]Department of Molecular Genetics, University of Toronto, Toronto, ON M4S 1A8, Canada.

*Author for correspondence (hcchang@purdue.edu)

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Clathrin has also been implicated in cell morphogenesis during spermatogenesis. In males mutant for *Chc*$^{4}$ (a partial loss-of-function mutation), the number of functional sperm is greatly reduced and spermatid individualization is disrupted (Fabrizio et al., 1998). However, *Chc*$^{4}$ flies have poor viability, indicating that other processes besides spermatogenesis are affected. This pleiotropy of clathrin function raises the question of whether disruption of clathrin in germ cells is the direct cause of male sterility in *Chc*$^{4}$ mutants. Thus, although the most apparent defect in *Chc*$^{4}$ mutant testes is the loss of IC synchrony during individualization (Fabrizio et al., 1998), the precise role of clathrin in spermatogenesis remains to be determined.

One important regulator of clathrin function is auxilin, first identified in mammals as a cofactor in Hsc70-mediated disassembly of clathrin coats from nascent clathrin-coated vesicles (CCVs) in vitro (Ungewickell et al., 1995). The mammalian genome contains two auxilin-related genes, auxilin and cyclin G-associated kinase (GAK). These differ in the presence of a N-terminal Ark (actin-related kinase) family kinase domain and in their respective tissue distributions (Umeda et al., 2000). GAK contains an Ark domain and is ubiquitously expressed, whereas auxilin lacks the kinase domain and is expressed predominantly in neural tissues. However, expression of auxilin in non-neural HeLa cells has recently been demonstrated (Hirst et al., 2008). Tissue-specific removal of GAK function in mice reveals that GAK is essential for the development of multiple tissues (Lee et al., 2008), whereas auxilin knockout mice show impaired synapse function (Yim et al., 2010). Within the cell, auxilin family proteins have been suggested to participate in the disassembly of clathrin coats (Gall et al., 2000; Greener et al., 2001; Massol et al., 2006; Pishvaee et al., 2000), recruitment of clathrin and adaptors to membranes (Lee et al., 2005), exchange of clathrin during coated-pit formation (Wu et al., 2001; Wu et al., 2003), constriction of coated-pits (Newmyer et al., 2003), and prevention of precipitous assembly of clathrin cages (Hirst et al., 2008; Jiang et al., 2000). GAK has also been implicated in mediating trafficking from the trans-Golgi network (TGN) via its interaction with the clathrin adaptor AP-1 (Kametaka et al., 2007).

*Drosophila* has only one auxilin ortholog (Aux – FlyBase), which is more similar to GAK, as Aux contains an Ark domain and is ubiquitously expressed (Hagedorn et al., 2006). Like other members of the auxilin protein family, Aux has a PTEN (phosphatase and tensin) homologous region, a clathrin-binding domain (CBD) and a C-terminal J-domain (Fig. 1B). Mutational analysis suggests that Aux participates in ligand endocytosis during Notch signaling in eye and wing discs (Eun et al., 2008; Hagedorn et al., 2006; Kandachar et al., 2008). In *aux* mutant cells, clathrin appears as abnormal aggregates, and formation of these aggregates is thought to deplete the level of functional clathrin in the cytosol, thereby inhibiting Notch ligand internalization (Eun et al., 2008; Kandachar et al., 2008). As localization of the epidermal growth factor (EGF) receptor also appears to be disrupted, Notch ligand is not the sole cargo of *aux*-dependent transport (Kandachar et al., 2008). Hence, it is likely that Aux has additional roles during fly development.

Here, we employ phenotypic analysis of testes from viable *aux* mutant males to further elucidate the role of clathrin-dependent transport in *Drosophila* spermatogenesis. Consistent with the notion that clathrin is crucial for male fertility (Fabrizio et al., 1998), *aux* mutant males contain asynchronous ICs and lack sperm in their seminal vesicles. Using the male germ cell-specific β2-*tubulin* promoter and fluorescently tagged subcellular markers, we provide evidence that Aux function is required in the germ cells for spermatogenesis, and that Aux participates in the formation of Golgi-derived clathrin-positive vesicles to generate sufficient plasma membrane for spermatid morphogenesis.

## MATERIALS AND METHODS

### Fly genetics and molecular biology

All fly crosses were carried out at 25°C in standard laboratory conditions. To determine if the males of a particular genotype were sterile, three males of the genotype in question were mated with five $w^{1118}$virgins. If no eggs hatched after seven days, the genotype was considered to be sterile. *dj-GFP* and *bam*$^{\Delta 86}$ flies were obtained from the Bloomington Stock Center (Bloomington, IN, USA).

The plasmids *pβ2tub-GFP-Clc* and *pβ2tub-myr-GFP* were generated by excising *GFP-Clc* and *myr-GFP* from *pUAS-GFP-Clc* and *pUAS-myr-GFP* (Chang et al., 2002) as *Eco*RI-*Xba*I fragments and cloning into *pβ2tub* (generous gift from Dr Bruce Hay, Caltech, CA, USA) (Huh et al., 2004). The *pβ2tub-dAux-mRFP* series was generated by excising *Eco*RI-*Not*I fragments from the corresponding *UAS-dAux-mRFP* series (Kandachar et al., 2008) and cloning into *pβ2tub*. To generate *pβ2tub-GFP-Rab11* and *pβ2tub-luciferase*, *GFP-Rab11* and *luciferase* were amplified by PCR and cloned into *pβ2tub*, respectively. Transgenic flies carrying these constructs were generated by P-element mediated transformation (Rubin and Spradling, 1982).

### S2 cell manipulation

S2 cells were maintained at 25°C in modified Schneider's medium supplemented with 10% fetal bovine serum and penicillin (50 IU/ml)/streptomycin (50 μg/ml). To determine Aux localization, cells were transiently transfected with *pRmHa3-GAL4* and *pUAS–dAux*$^{FL}$*-GFP* DNA using FuGENE HD transfection reagent (Roche). dAux$^{FL}$-GFP expression was induced with 0.5 mM $CuSO_4$ for 16 hours before staining.

### Immunofluorescence microscopy

Immunostaining of testes was performed according to Hime et al. (Hime et al., 1996). Primary antibodies against Lva (from Dr J. Sisson, University of Texas at Austin, TX, USA), FasIII (DSHB), Vasa (from Dr R. Lehmann, Skirball Institute, NY, USA), Myosin VI (from Dr K. Miller, Washington University, St Louis, MO, USA), AP-1 (Hirst et al., 2009) and GM130 (Abcam, USA) were used at 1:200, 1:200, 1:1000, 1:10, 1:3000 and 1:500 dilutions, respectively. AlexaFluor-conjugated secondary antibodies, Sytox Green and AlexaFluor-568 phalloidin (Molecular Probes) were used at 1:200, 1:50,000 and 1:1000, respectively. Fluorescence micrographs of cysts stained with αMyosin VI antibodies and spermatocytes expressing GFP-Rab11 were acquired with an Axiocam CCD camera on a Zeiss Axioplan 2 fluorescence microscope, using Axiovision acquisition software. Other fluorescence micrographs were acquired at 25°C with 4× (0.13) and 60× (1.25) lenses on a Disk Scanning Unit-enabled Olympus BX61 microscope equipped with a Hamamatsu DCAM-API camera.

### Electron microscopy

Scanning electron microscopy (SEM) was performed according to Wolff (Wolff, 2000). For transmission electron microscopy (TEM), testes were dissected and fixed with 2% glutaraldehyde in 0.1 M sodium cacoldylate (pH 7.2) for 1 hour at 4°C. Tissues were then intensified in 2% $OsO_4$ in 0.1 M sodium cacoldylate (pH 7.2) at 4°C for 1 hour, stained with 2% uranium acetate en bloc at room temperature overnight, dehydrated with a graded ethanol series, equilibrated with two 10-minute incubations in propylene oxide and incubated in 50% propylene oxide/50% epon resin mixture overnight. Testes were incubated in 100% epon resin for 4 hours, embedded, and baked overnight. TEM and SEM images were collected using Philips CM-10 transmission electron microscope and JSM-840 scanning electron microscope (JEOL), respectively.

### Luciferase assay

Luciferase activity was determined using a Luciferase Assay System kit (Promega), as described by the manufacturer. Briefly, a one-day-old fly was homogenized in 50 μl Passive Lysis Buffer, followed by a 15-minute incubation at room temperature. Lysates were then analyzed by a

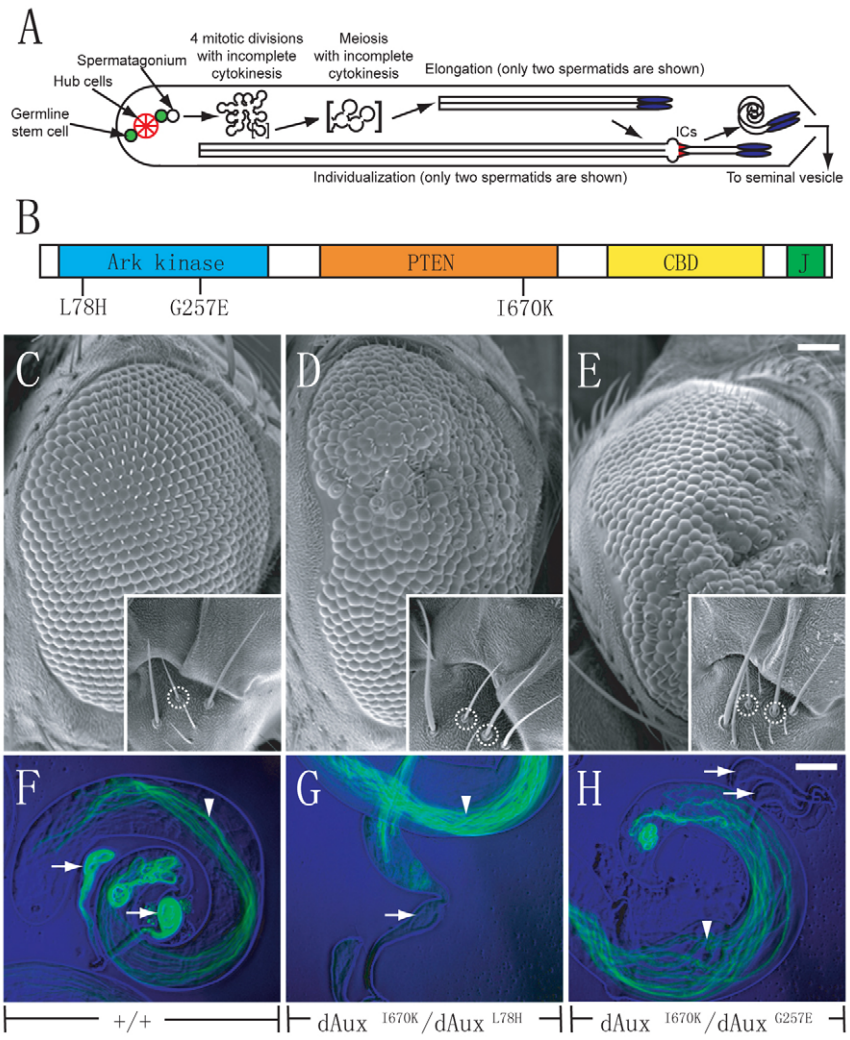


**Fig. 1. *aux* mutant males fail to produce mature sperm.** (**A**) Diagram of *Drosophila* spermatogenesis (see main text for description of various stages). IC, investment cone. (**B**) Schematic of the Aux protein, which contains an N-terminal Ark kinase domain (blue), a PTEN-related region (orange), a clathrin-binding domain (CBD, yellow) and a dnaJ domain (J, green). L78H, G257E and I670K denote the amino acid changes in *aux* mutants that permit viable adults. (**C-E**) Scanning electron micrographs (SEM) of adult eyes of $w^{1118}$ (C), $w^{1118}$; $dAux^{I670K/L78H}$ (D) and $w^{1118}$; $dAux^{I670K/G257E}$ (E) flies. Sternopleural bristles of corresponding genotypes are shown in the insets, and the middle sternopleural bristles are indicated by dashed circles. For all SEM images, anterior is to the right and dorsal is up. Scale bar: 100 μm. (**F-H**) Fluorescence micrographs of $w^{1118}$; *dj-GFP/+* (F), $w^{1118}$; *dj-GFP/+*; $dAux^{I670K/L78H}$ (G) and $w^{1118}$; *dj-GFP/+*; $dAux^{I670K/G257E}$ (H) testes. Cysts of Dj-GFP-positive (green) spermatids are indicated by arrowheads and seminal vesicles adjacent to the basal coiled regions of the testes are indicated by arrows. Scale bar: 100 μm.

Luminoskan Ascent luminometer (Thermo Electron), using substrates from the kit. Six flies of each genotype were assayed to account for experimental variability.

## RESULTS

### *aux* mutants exhibit phenotypes characteristic of defects in Notch signaling and male fertility

We previously isolated nine *aux* mutations from $F_2$ non-complementation screens (Kandachar et al., 2008). Although most of these are recessive lethal, certain combinations of weaker *aux* alleles allow occasional survivors, thereby permitting analysis of Aux function in adult animals. For instance, flies homozygous for $dAux^{I670K}$ (a point mutation disrupting the PTEN-related region) and those trans-heterozygous for $dAux^{L78H}/dAux^{G257E}$ (two point mutations disrupting highly conserved residues in sub-domains II and IX of the N-terminal kinase domain, respectively; Fig. 1B), $dAux^{I670K}/dAux^{G257E}$, or $dAux^{I670K}/dAux^{L78H}$ (hereafter referred to as $dAux^{L78H/G257E}$, $dAux^{I670K/G257E}$ and $dAux^{I670K/L78H}$, respectively) can survive to adulthood. Adults mutant for these partial loss-of-function alleles exhibited qualitatively similar phenotypes. Thus, for the sake of clarity, we will only present the phenotypic characterization of $dAux^{I670K/L78H}$ and $dAux^{I670K/G257E}$.

Consistent with the established role of Aux in Notch signaling, $dAux^{I670K/L78H}$ and $dAux^{I670K/G257E}$ adults exhibited rough eyes (Fig. 1C-E) with extra photoreceptor cells and occasional extra bristles on the notum, sternopleurum (Fig. 1C-E, insets) and scutellum (not shown). In addition, $dAux^{I670K/L78H}$ and $dAux^{I670K/G257E}$ males, when mated with $w^{1118}$ females, did not produce embryos that hatched, indicating that either they were sterile or they caused paternal-effect embryonic lethality. To distinguish between these two possibilities, we used Don juan-GFP (Dj-GFP), a fusion protein that decorates the elongated mitochondria (Santel et al., 1998; Santel et al., 1997), to visualize elongating spermatids in the testes and mature sperm in the seminal vesicles. In both wild-type and *aux* mutant testes, Dj-GFP-positive spermatids could be seen (Fig. 1F-H, arrowheads), indicating that differentiation of germ cells had proceeded to the elongation phase. By contrast, whereas seminal vesicles from wild-type males were filled with Dj-GFP-positive sperm (Fig. 1F, arrows), $dAux^{I670K/L78H}$ and $dAux^{I670K/G257E}$ mutant seminal vesicles were empty (Fig. 1G,H, arrows). These observations indicate that the cause of male sterility precedes fertilization and that Aux has a role in sperm production.

### Cytokinesis is mildly affected in *aux* mutant testes

Although Dj-GFP distribution showed that progression from elongated spermatids to mature sperm in *aux* mutants was abolished (Fig. 1F-H), it was unclear whether events prior to spermatid elongation were affected. We therefore examined earlier events to define further the role(s) of Aux during spermatogenesis.

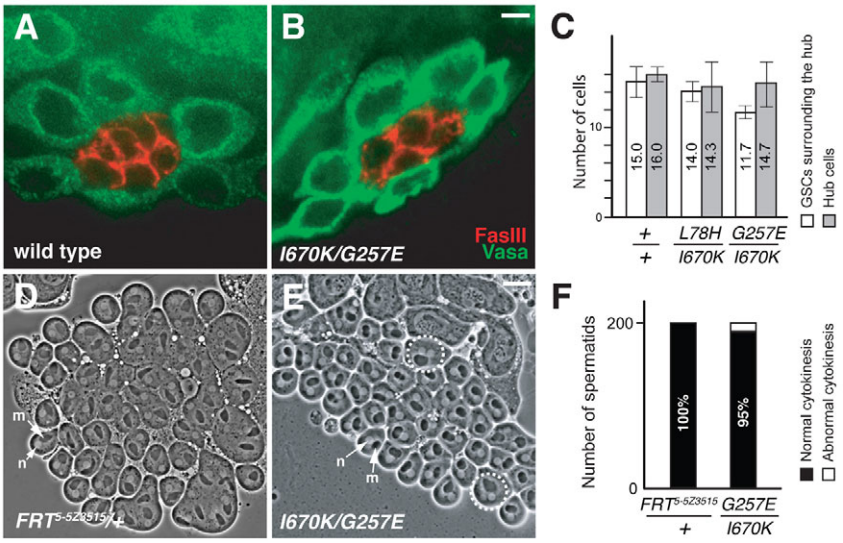


**Fig. 2. *aux* mutants have a mild defect in cytokinesis.** (**A**,**B**) Spinning-disk confocal micrographs of wild-type (A) and $dAux^{I670K/G257E}$ (B) testes stained with antibodies against FasIII (red) and Vasa (green), which label the hub and the germ cells, respectively. Scale bar: 5 μm. (**C**) Quantification of the number of germline stem cells (GSCs; Vasa-positive) contacting the hub and hub cells in wild-type and *aux* mutant testes. Error bars indicate s.d. (**D**,**E**) Phase-contrast micrographs of squashes of $FRT^{5\text{-}5Z3515}$/+ (D) and $dAux^{I670K/G257E}$ (E) testes. Nuclei (n) and mitochondria (m) can be recognized as light and dark circular structures, respectively, at this stage. *aux* mutant cells containing multiple nuclei are indicated by dashed circles. Scale bar: 20 μm. (**F**) Quantification of the defects in cytokinesis in $FRT^{5\text{-}5Z3515}$/+ and *aux* mutant testes.

To determine whether GSCs were disrupted, wild-type, $dAux^{I670K/L78H}$ and $dAux^{I670K/G257E}$ testes were stained with anti-FasIII (Fas3 – FlyBase) and anti-Vasa antibodies to visualize the hub and germ cells, respectively. The morphology and number of FasIII-positive cells at the apical tips of *aux* mutant testes appeared normal (Fig. 2A,B). The number of GSCs, recognized as Vasa-positive cells contacting the hub, was comparable in wild-type and $dAux^{I670K/L78H}$ testes, whereas the number of GSCs in $dAux^{I670K/G257E}$ was slightly reduced (Fig. 2C). These results suggest that defects in establishment and maintenance of GSCs are not responsible for the absence of sperm in viable *aux* mutant males.

As clathrin function has been implicated in cytokinesis in other systems (Niswonger and O'Halloran, 1997), and disruption of cytokinesis has been shown to block sperm formation in *Drosophila* (Giansanti et al., 2004), it seems possible that a cytokinesis defect during mitotic or meiotic divisions could be the primary defect leading to the absence of sperm in *aux* mutants. To test this, squashed preparations of live $FRT^{5\text{-}5Z3515}$/+ [parental chromosomes for generating *aux* alleles; Kandachar et al. (Kandachar et al., 2008)] and *aux* mutant testes were examined using phase-contrast microscopy, in which spermatid nuclei and mitochondrial derivatives appear as light and dark structures, respectively. In $FRT^{5\text{-}5Z3515}$/+ testes, each spermatid contained one mitochondrial derivative associated with one nucleus (Fig. 2D), indicating proper cytokinesis. Most of the $dAux^{I670K/G257E}$ spermatids had one mitochondrial derivative and one nucleus, although 5% of them contained an enlarged mitochondrial derivative associated with two nuclei (Fig. 2E,F), suggesting a cytokinesis defect during one of the meiotic divisions. However, the observed cytokinesis defects in our *aux* mutants were mild, and therefore unlikely to account for the complete absence of sperm.

## Migration of investment cones is disrupted in *aux* mutants

A partial loss-of-function *Chc* mutation has been shown to disrupt spermatid individualization (Fabrizio et al., 1998). To test whether *aux* has similar defects, wild-type and *aux* mutant testes were stained with Alexa-phalloidin and Sytox to label ICs and nuclei, respectively. In the posterior coiled region of wild-type testes, cysts of different stages could be seen (Fig. 3A, dashed box; shown in Fig. 3B at higher magnification). These include cysts containing bundles of elongated nuclei where ICs have not yet assembled (Fig. 3B, arrow), bundles of needle-shaped nuclei where ICs have assembled but have not yet begun their rostral migration (Fig. 3B, solid arrowheads) and bundles of nuclei where assembled ICs have already departed (Fig. 3B, open arrowhead). In the intermediate region, clusters of synchronously migrating ICs were easily detected (Fig. 3A, solid box, shown in Fig. 3C at a higher magnification). Faint Sytox staining was seen ahead of migrating ICs, presumably from the DNA of mitochondria deposited into the cystic bulges. On occasion, bundles of nuclei from caudally elongating cysts could also be detected in this region (not shown). In the more anterior part of the testis, IC clusters were being disassembled (Fig. 3A, dashed circle), marking the end of the individualization process. Using these descriptive criteria, we determined the number of cysts at various stages in testes from 1-day-old $w^{1118}$ males. Wild-type testes on average ($n$=3) contained 23.3±3.1 cysts with elongated nuclear bundles where ICs had not yet assembled; 21.0±1.7 cysts with ICs assembling at the nuclear bundles; 6.3±0.6 cysts with migrating ICs; and 4.6±1.2 cysts with disassembling ICs (Fig. 3J).

ICs were able to assemble on spermatid nuclei in *aux* mutants, as seen by light microscopy. In the posterior coiled region of $dAux^{I670K/L78H}$ and $dAux^{I670K/G257E}$ testes, the morphology (i.e. the dimensions) of the *aux* mutant ICs was similar to that of wild type (Fig. 3E,H, solid arrowheads). Furthermore, as in wild type, Myosin VI was present at the leading edges of these *aux* mutant ICs (Fig. 3K,L, arrowheads). Nuclear elongation, as revealed by Sytox staining, appeared normal, although some individual nuclei could be seen displaced from the bundles (Fig. 3E,H, asterisks). The number of cysts at various stages was slightly different in *aux* mutants (Fig. 3J). On average ($n$=3), 29.3±2.1 and 32.3±2.1 cysts with elongated nuclear bundles and no IC assembly were seen in $dAux^{I670K/L78H}$ and $dAux^{I670K/G257E}$ mutant testes, respectively. 12.3±1.5 and 16.0±2.0 cysts containing ICs assembling at the nuclear bundles were seen in $dAux^{I670K/L78H}$ and $dAux^{I670K/G257E}$ mutant testes. The increase in the number of cysts with elongated nuclei bundles without IC assembly and the concomitant decrease in the number of cysts containing ICs assembling at the nuclear bundles suggest that initiation of IC assembly was delayed by loss of *aux* function.

The average number of cysts with migrating ICs per $dAux^{I670K/L78H}$ and $dAux^{I670K/G257E}$ mutant testis ($n$=3) was 6.7±0.6, similar to the number observed in wild type. However, as in testes

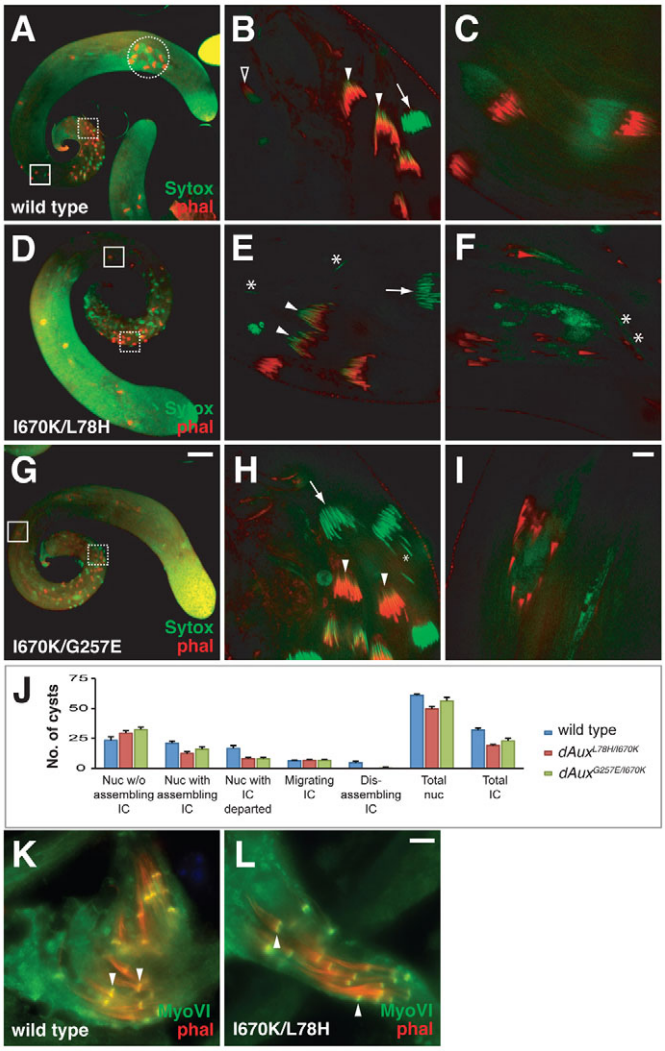


**Fig. 3. Spermatid individualization is disrupted in Aux mutant testes.** (**A-I**) Fluorescence micrographs of wild-type (A-C), *dAux*$^{I670K/L78H}$ (D-F) and *dAux*$^{I670K/G257E}$ (G-I) testes stained with Sytox (green) and Alexa-phalloidin (red). The boxed regions in A, D and G are shown at higher magnification in B, E and H (dashed) and C, F and I (solid), respectively. Images in A, D and G were acquired through wide field, whereas the rest were acquired through spinning-disk confocal microscopy. Arrows indicate clusters of nuclei where the ICs have not yet assembled; solid arrowheads indicate those that have just assembled; open arrowheads denote those that ICs have departed; and asterisks indicate stray nuclei. Scale bar: 100 μm in A, D and G; 10 μm in B, C, E, F, H and I. (**J**) Quantification of the number of cysts at various stages in wild-type and *aux* mutant testes. Error bars indicate s.d. (**K**,**L**) Fluorescence micrographs of wild-type (K) and *dAux*$^{I670K/L78H}$ (L) cysts stained with αMyosin VI antibody (green) and Alexa-phalloidin (red). Arrowheads indicate Myosin VI staining at the leading edges of the ICs. Scale bar: 5 μm. IC, investment cone.

mutant for *Chc*$^{4}$ (Fabrizio et al., 1998), these *aux* mutant ICs appeared scattered, suggesting that their coordinated migration was disrupted (Fig. 3D,G, solid boxes, shown at a higher magnification in Fig. 3F and 3I, respectively). This scattering probably hindered further IC procession, as disassembling IC clusters were rarely seen in the anterior region in *dAux*$^{I670K/L78H}$ and *dAux*$^{I670K/G257E}$ mutant testes. Whereas IC assembly in wild type usually appeared restricted to the posterior coiled region, single ICs were occasionally seen assembled on stray nuclei in the intermediate region of *aux* mutant testes (not shown). This suggests that maintenance of nuclear bundles and completion of cyst elongation are not obligatory for initiating IC assembly.

## Aux function is required in the germ cells for male fertility

As *dAux*$^{I670K/L78H}$ and *dAux*$^{I670K/G257E}$ flies were entirely mutant, it was unclear whether the observed sterility and IC defects were due to an autonomous defect in the germ cells or to a non-autonomous defect caused by disruption of Aux function elsewhere. To pinpoint the spatial and temporal requirement for Aux function in male fertility, we generated β*2tub-dAux*$^{FL}$*-mRFP*, which provides germ cell-specific expression of a functional mRFP-tagged full-length Aux (Kandachar et al., 2008). As the β*2-tubulin* promoter is active at the primary spermatocyte stage (Kemphues et al., 1982), rescue of *aux* male sterility by β*2tub-dAux*$^{FL}$*-mRFP* would suggest that the requirement for Aux function during spermatogenesis is autonomous. To ensure that the β*2tub* expression cassette (Huh et al., 2004) recapitulates the specificity of the β*2-tubulin* promoter (Hoyle et al., 1995), we made a β*2tub-luciferase* construct and showed that its activity was restricted to males and to the testes, and was absent in *bag of marbles* mutants, in which the differentiation of male germ cells is blocked (McKearin and Ohlstein, 1995; McKearin and Spradling, 1990) (see Fig. S1 in the supplementary material).

As β*2tub* is not active in other tissues, β*2tub-dAux*$^{FL}$*-mRFP, dAux*$^{I670K/G257E}$ male flies still exhibited morphological defects in eyes and bristles (data not shown). However, unlike *dAux*$^{I670K/G257E}$, these males were fertile when mated with *w*$^{1118}$ females. The seminal vesicles of β*2tub-dAux*$^{FL}$*-mRFP, dAux*$^{I670K/G257E}$ males were filled with Dj-GFP-positive sperm (Fig. 4G). Moreover, the defects in IC migration in *dAux*$^{I670K/G257E}$ were rescued (Fig. 4B; dashed box shown at higher magnification in 4B′). Identical results were obtained using the *dAux*$^{I670K/L78H}$ allelic combination, indicating that the rescue was not allele-specific (see Fig. S2 in the supplementary material). The observation that germ cell-specific Aux expression could rescue *aux*-associated sterility indicates that Aux function during sperm production is required in the germ cells.

## The CBD and the J-domain are indispensable for Aux function in male fertility

Deletion analysis has previously shown that the CBD and the J-domain, but not the kinase domain, are most crucial to the function of Aux in Notch signaling (Eun et al., 2008; Kandachar et al., 2008). To test whether the same Aux domains are required for spermatogenesis, we expressed versions deleted for the kinase domain (*dAux*$^{\Delta K}$*-mRFP*), the kinase and PTEN domains (*dAux*$^{CJ}$*-mRFP*), the CBD (*dAux*$^{\Delta C}$*-mRFP*) and the J-domain (*dAux*$^{\Delta J}$*-mRFP*) under control of the β*2tub* expression cassette (Fig. 4A), and tested their ability to rescue *aux* male sterility. Similar to full-length Aux, both β*2tub-dAux*$^{\Delta K}$*-mRFP* and β*2tub-dAux*$^{CJ}$*-mRFP* restored fertility to *dAux*$^{I670K/G257E}$ males. In addition, the seminal vesicles of β*2tub-dAux*$^{\Delta K}$*-mRFP*, *dAux*$^{I670K/G257E}$ and β*2tub-dAux*$^{CJ}$*-mRFP*, *dAux*$^{I670K/G257E}$ males were filled with Dj-GFP-positive sperm (Fig. 4H,I), and the ICs in their testes regained their normal appearance (Fig. 4C,D, dashed box; shown at higher magnification in 4C′ and 4D′). These results indicate that overexpression of Aux without its kinase and PTEN domains can

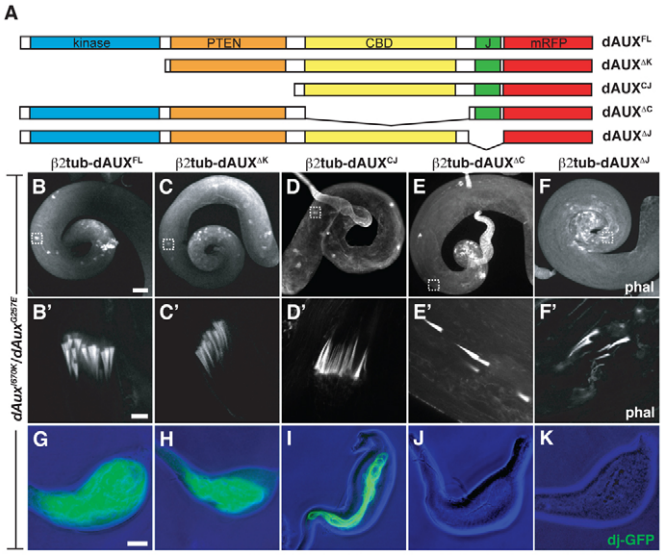


**Fig. 4. *aux*-associated male sterility can be rescued by germ cell-specific expression of functional Aux.** (**A**) Schematics of Aux constructs expressed under control of β*2tub* promoter for the rescue analysis. Colored boxes denote various domains, as in Fig. 1. (**B-K**) Fluorescence micrographs of testes stained with Alexa-phallodin to label the ICs (B-F′) and expressing Dj-GFP to visualize mature sperm in the seminal vesicles (G-K). The boxed regions in B-F are shown at higher magnification in B′-F′. The genotypes shown are β*2tub–dAux*$^{FL}$*-mRFP, dAux*$^{I670K/G257E}$ (B,B′,G), β*2tub–dAux*$^{\Delta K}$*-mRFP, dAux*$^{I670K/G257E}$ (C,C′,H), β*2tub–dAux*$^{CJ}$ *–mRFP, dAux*$^{I670K/G257E}$ (D,D′,I), β*2tub–dAux*$^{\Delta C}$*-mRFP, dAux*$^{I670K/G257E}$ (E,E′,J) and β*2tub–dAux*$^{\Delta J}$*-mRFP, dAux*$^{I670K/G257E}$ (F,F′,K). mRFP fluorescence from dAux-mRFP fusions were detected in testes of all abovementioned genotypes (not shown). Scale bars: 100 μm in B-F; 10 μm in B′-F′; 50 μm in G-K. IC, investment cone.

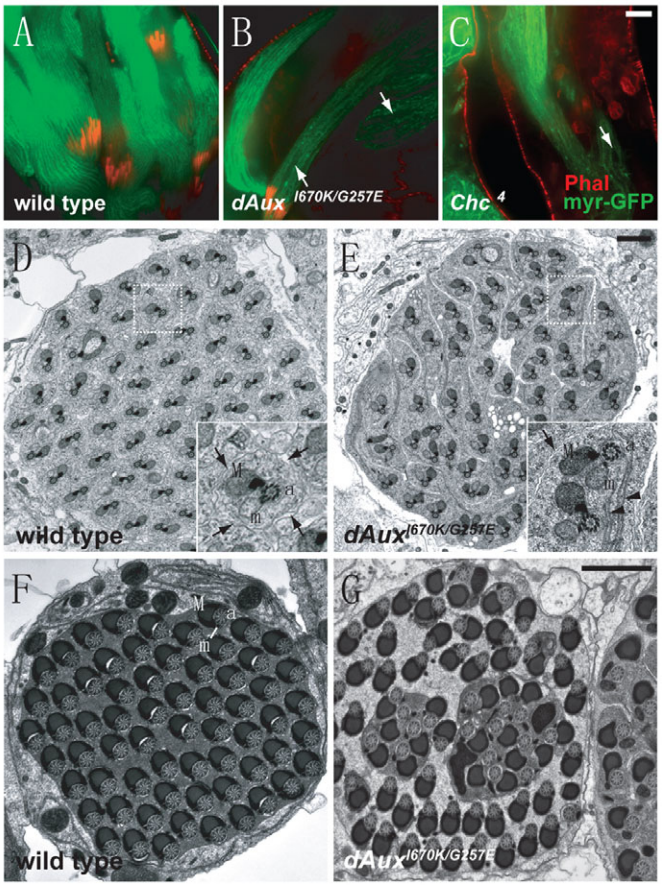


**Fig. 5. *aux* mutant germ cell cysts have deficient plasma membrane in the pre-individualized region.** (**A-C**) Spinning-disk confocal micrographs of wild-type (A), *dAux*$^{I670K/G257E}$ (B) and *Chc*$^{4}$*/Y* (C) testes expressing one copy of β*2tub-myr-GFP*. These testes were stained with Alexa-phalloidin (red) to reveal ICs. Arrows indicate the large gaps in myr-GFP localization in the *aux* and *Chc* mutants. Scale bar: 10 μm. (**D-G**) Transmission electron microscopy cross-sections of wild-type (D,F) and *dAux*$^{I670K/G257E}$ (E,G) testes. D and E represent pre-individualized regions, whereas F and G represent regions where ICs have passed. The dashed boxes in D and E are shown at higher magnification in the insets. Plasma membrane surrounding a single spermatid in wild type is indicated by arrows (D, inset). In the *aux* mutant, plasma membrane surrounding multiple spermatids is indicated by an arrow and tubular structures are indicated by arrowheads (E, inset). Scale bar: 1 μm. a, axoneme; m, mitochondria minor; M, mitochondria major; IC, investment cone.

support normal spermatogenesis. By contrast, β*2tub-dAux*$^{\Delta C}$*-mRFP, dAux*$^{I670K/G257E}$ and β*2tub-dAux*$^{\Delta J}$*-mRFP, dAux*$^{I670K/G257E}$ males were sterile. Moreover, their seminal vesicles were empty (Fig. 4J,K) and their IC morphology was abnormal (Fig. 4E,F, dashed boxes shown at a higher magnification in 4E′ and 4F′), suggesting that the CBD and the J-domain are essential for Aux function in male fertility. Identical results were obtained using *dAux*$^{I670K/L78H}$, indicating that this requirement for the CBD and the J-domain was not allele-specific (see Fig. S2 in the supplementary material).

## The plasma membrane is deficient in pre-individualized *dAux* mutant cysts

To understand the link between disruption of *aux* function and the uncoordinated IC phenotype, we made β*2tub-myr-GFP* to visualize the plasma membrane in *aux* male germ cells. Organized and continuous GFP localization was seen along the cell periphery in wild-type elongating cysts (Fig. 5A), indicating that plasma membrane is present around the spermatids before IC passage. By contrast, myr-GFP localization was disorganized and contained gaps in *dAux*$^{I670K/G257E}$ cysts (Fig. 5B, arrows), suggesting that the plasma membrane in *aux* male germ cells was abnormal. In *Chc*$^{4}$ spermatids, myr-GFP localization also appeared disorganized (Fig. 5C), further supporting the functional link between auxilin and clathrin.

To understand this defect further, wild-type and *dAux*$^{I670K/G257E}$ testes were processed for TEM analysis. In cross-sections of pre-individualized regions of wild-type cysts, arrays of axonemes and associated mitochondrial derivatives, along with ample cytoplasmic content, could be seen (Fig. 5D). Consistent with our observations with β*2tub-myr-GFP* and prior TEM analysis (Tokuyasu et al., 1972), the elongated spermatids were surrounded by plasma membrane (Fig. 5D, inset, arrows), although ICs had not passed through. After individualization, each spermatid was invested with discrete membrane and the cytoplasmic content was excluded (Fig. 5F). In pre-individualized regions of *dAux*$^{I670K/G257E}$ cysts, the number of spermatids (64.0±0.0 for wild type, *n*=3; 63.3±0.6 for *dAux*$^{I670K/L78H}$, *n*=3; and 62.5±2.1 for *dAux*$^{I670K/G257E}$, *n*=4) and the

configuration of axonemes with associated mitochondrial derivatives appeared normal, although their arrangement was disorganized (Fig. 5E). Unlike in wild type, most of the spermatids were not surrounded by their own plasma membrane. Furthermore, long tubular structures were abundant in the cytoplasm (Fig. 5E, inset, arrowheads). Some of these tubular structures were continuous with the double membrane surrounding the axonemes (see Fig. S3 in the supplementary material), suggesting that they were ER-related. In more anterior regions of $dAux^{I670K/G257E}$ cysts, a mixture of pre- and post-individualized spermatids was seen (Fig. 5G), indicative of the loss of synchrony in IC migration. Similar phenotypes were seen in $dAux^{I670K/L78H}$ (see Fig. S4 in the supplementary material) and $Chc^{4}$ mutant testes (Fabrizio et al., 1998). These results suggest that Aux, and probably clathrin also, has a role in forming plasma membrane during sperm production.

## The Golgi is enriched with Aux

In cultured mammalian cells, GAK has been shown to colocalize with Golgi markers in perinuclear regions (Kametaka et al., 2007; Lee et al., 2005). To determine whether Aux localizes to the Golgi in germ cells, *β2tub-dAux$^{FL}$-mRFP* testes were stained with anti-Lva (Lava lamp) and anti-AP-1 antibodies. Lva localizes to the Golgi (Sisson et al., 2000), and AP-1 is a clathrin adaptor known to associate with TGN and endosomes. In round spermatocytes, dAux$^{FL}$-mRFP localization was mostly cytosolic, but was abundant in Lva-positive structures (Fig. 6A-C), suggesting that Aux was present at the Golgi. In support of this, AP-1 staining was also seen in the vicinity of dAux$^{FL}$-mRFP-enriched structures (Fig. 6D-F). To confirm Aux enrichment at the Golgi, we examined colocalization of dAux$^{FL}$-mRFP and GFP-Four Way Stop (Fws) (Farkas et al., 2003). Similar to Lva, Fws showed extensive overlap with Aux (Fig. 6G-I). The presence of Aux at the Golgi is consistent with its role in forming AP-1-containing clathrin-positive structures during spermatogenesis (see below). To determine whether enrichment of the Golgi with Aux is specific to developing male germ cells, S2 cells were transfected with *pRmHa3-GAL4* and *pUAST-dAux$^{FL}$-GFP* and stained with anti-Lva antibody. Similar to *β2tub-dAux$^{FL}$-mRFP* spermatocytes, a higher level of Aux was seen in Lva-positive structures (Fig. 6J-L), indicating that Aux localization to the Golgi is not unique to germ cells.

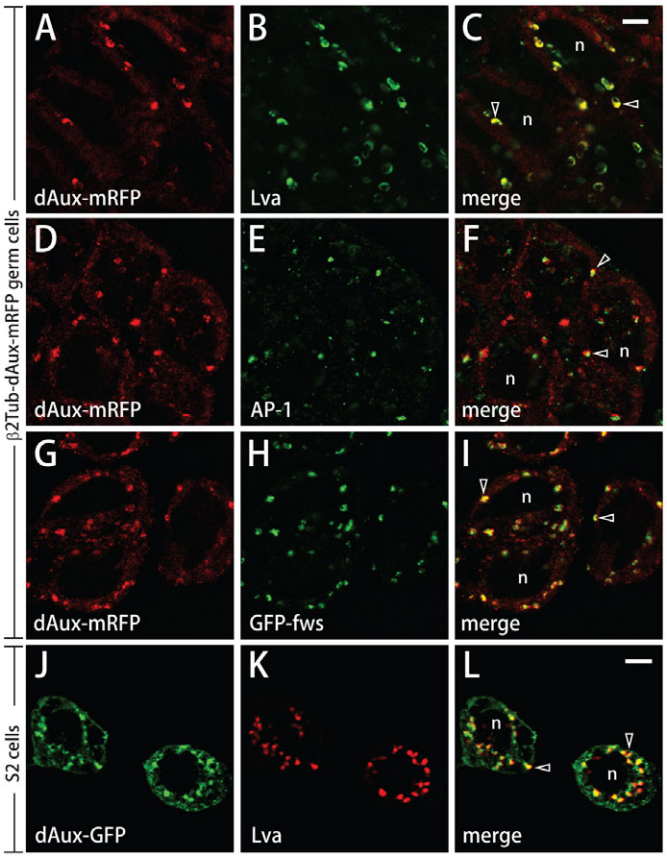


**Fig. 6. Aux is present at the Golgi.** (**A-I**) Laser-scanning confocal micrographs of *β2tub-dAux$^{FL}$-mRFP*/+ (A-F) and *β2tub-dAux$^{FL}$-mRFP*/+; *GFP-Fws*/+ (G-I) spermatocytes. *β2tub-dAux$^{FL}$-mRFP*/+ germ cells stained with anti-Lva antibody (green) to reveal the Golgi (A-C) and αAP-1 antibody (D-F), respectively. (**J-L**) Spinning-disk confocal micrographs of *Drosophila* S2 cells expressing dAux-GFP and stained with anti-Lva antibody (red). Open arrowheads indicate structures with elevated level of Aux. Scale bars: 5 μm.

## Mutations in *aux* disrupt clathrin-positive vesicular clusters localized near the Golgi

To understand how clathrin contributes to plasma membrane formation during spermatid development, we generated *β2tub-GFP-Clc* (encoding a GFP-tagged clathrin light chain) (Chang et al., 2002) to monitor clathrin distribution in the male germ cells. In round spermatocytes, puncta of clathrin-positive structures appeared around the cell periphery (Fig. 7A, solid arrowheads), probably representing endocytic sites. In addition, clusters of clathrin-positive vesicular structures were seen on the concave side of Lva-positive organelles (Fig. 7A, open arrowheads), suggesting that the clathrin-positive clusters were Golgi-derived. Indeed, some of these internal clathrin-positive structures colocalized with AP-1 (Fig. 7C, open arrowheads). The proximity of clathrin-positive structures to Lva-positive organelles persisted throughout the elongation phase (Fig. 7E,G,I). During individualization, clathrin-positive vesicles were seen ahead of ICs in the cystic bulge (Fig. 7K).

In round $dAux^{I670K/G257E}$ spermatocytes, puncta of Clc-GFP along the cell periphery were still seen, although their abundance appeared to be reduced (Fig. 7B, solid arrowheads). Clc-GFP localized to patch-like structures on the sides of Lva-positive organelles (Fig. 7B, open arrowheads). Although Clc localization was abnormal in $dAux^{I670K/G257E}$ cells, EM and immunostaining with an anti-GM130 (a Golgi marker) antibody showed that Golgi morphology was not disrupted (see Fig. S5 in the supplementary material). Furthermore, the localization of Rab11, which mediates trafficking from the Golgi and has been shown to colocalize with Lva (Giansanti et al., 2007), appeared normal in $dAux^{I670K/G257E}$ spermatocytes (see Fig. S5 in the supplementary material). The presence of aberrant clathrin-positive structures near the Golgi persisted throughout subsequent stages (Fig. 7F,H,J). Although these clathrin-positive structures remained close to the Golgi, they no longer colocalized with AP-1 (Fig. 7D, open arrowheads). Taken together, our results suggest that formation of AP-1-containing clathrin-positive structures requires Aux function.

## DISCUSSION

Using partial loss-of-function *aux* mutations, we showed that auxilin has an important role in *Drosophila* male fertility and sperm formation. As auxilin is a well-known regulator of clathrin function, the most direct explanation for the observed male sterility is that a clathrin-dependent event crucial for sperm production is disrupted in *aux* mutant testes. Indeed, the phenotypes of our viable

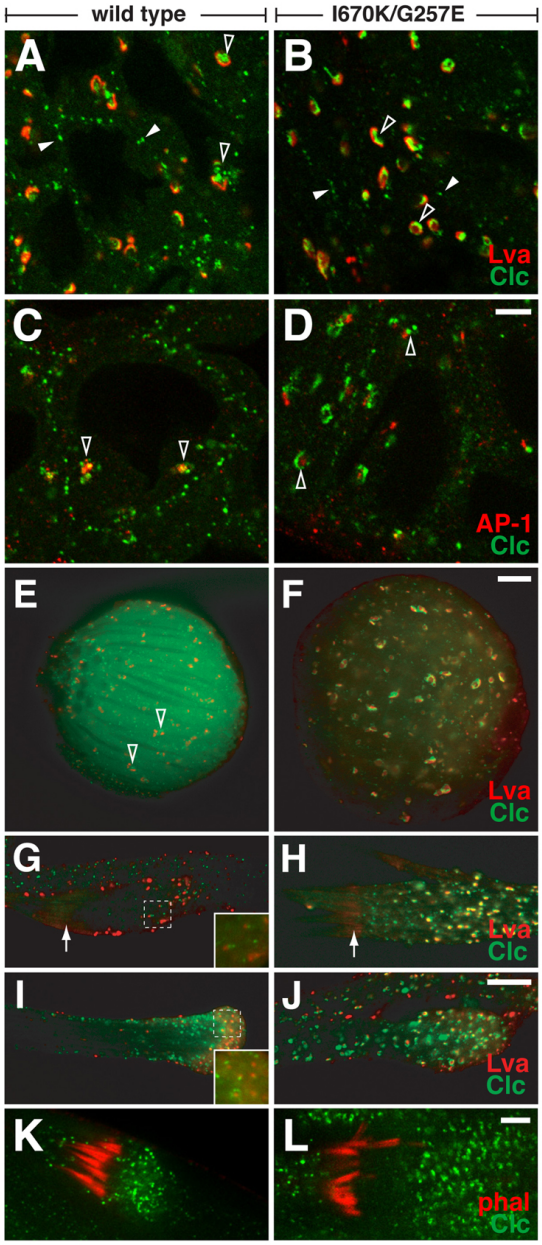


**Fig. 7. Clathrin-positive structures near the Golgi are disrupted in *aux* mutant cells.** (**A-D**) Laser-scanning confocal micrographs of wild-type (A,C) and *dAux*$^{I670K/G257E}$ (B,D) spermatids expressing one copy of β*2tub-GFP-Clc*. Note that these spermatids have not yet begun the process of elongation. (A,B) Cells stained with anti-Lva antibody (red) to reveal the Golgi. Solid arrowheads indicate clathrin-positive structures around the cell periphery, and open arrowheads indicate those near Lva-positive organelles. (C,D) Cells stained with anti-AP-1 antibody (red). Open arrowheads indicate the clathrin-positive structures that are colocalized with (C) or adjacent to (D) AP-1. (**E-L**) Spinning-disk confocal micrographs of wild-type (E,G,I,K) and *dAux*$^{I670K/G257E}$ (F,H,J,L) cysts expressing one copy of β*2tub-GFP-Clc*. (E-J) Cysts stained with anti-Lva antibody (red) to reveal the Golgi. (E,F) Spermatid cysts in an early phase of elongation. Open arrowheads indicate clathrin structures near the Lva-positive organelles. (G-J) The nuclear ends (G,H) and the tail ends (I,J) of cysts that have completed elongation. Newly assembled ICs are indicated by arrows. Boxed regions in G and I are shown at higher magnification in insets. (K-L) Spermatids with migrating ICs stained with Alexa-phalloidin (red). Scale bars: 5 μm in A-D; 10 μm in E-J; 5 μm in K,L.

*aux* allele combinations in spermatid individualization are similar to those of *Chc*$^{4}$. The finding that the CBD and J-domain are indispensable for rescue of the sterility of *aux* mutants by exogenously expressed Aux implies that its ability to bind clathrin and Hsc70 is necessary for male germ cell development. The disruption of clathrin distribution in *aux* mutant germ cells further emphasizes the importance of auxilin in regulating clathrin function. Thus, although GAK has been suggested to function in the nucleus (Sato et al., 2009), our data strongly argue that the sterility associated with *aux* mutant males is caused by a disruption of clathrin in the cytosol.

Using the β*2tub* promoter, we showed that male germ cell-specific expression of functional Aux at the primary spermatocyte stage could rescue all *aux*-associated male reproductive defects (i.e. sterility, absence of sperm in seminal vesicles and asynchronous IC movement). This result demonstrated that male sterility was indeed caused by a disruption of *aux* function and that Aux is required autonomously in the germ cells for successful spermatogenesis. This is in contrast to the Notch signaling pathway, where requirement for Aux function is non-cell-autonomous (Eun et al., 2008; Kandachar et al., 2008). As the β*2tub* promoter becomes active at the spermatocyte stage, rescue by β*2tub-dAux-mRFP* also implies that the cause for the sterility in *aux* males occurs at or after the spermatocyte stage. Consistent with this, processes prior to the spermatocyte stage (e.g. the morphology of the hub, the number of GSCs) appeared unaffected in *aux* mutant testes. Although Notch has recently been implicated in the maintenance of niche cells in *Drosophila* testes (Kitadate and Kobayashi, 2010), we did not observe significant reduction in hub cell number in *aux* mutant testes.

We have previously shown that overexpression of an Aux deletion consisting of just the CBD and the J-domains (dAux$^{CJ}$) could rescue the Notch signaling defect and lethality caused by *aux* (Kandachar et al., 2008). Similarly, expression of this construct from the β*2tub* promoter rescued *aux*-associated male sterility. By contrast, expression of Aux deletions missing either the CBD or the J-domain failed to rescue the sterility and IC defects. These results suggest that the CBD and J-domain are necessary for Aux function in spermatogenesis and that recruitment of Hsc70 to clathrin by Aux is a crucial event for spermatid differentiation. The kinase and PTEN-related regions also have a role in mediating Aux function in spermatogenesis, as the alleles used for generating the viable sterile *aux* males contain missense mutations in these domains. However, at least in the context of overexpressed rescue constructs, the kinase and PTEN-related domains are dispensable for the functions of auxilin family proteins.

Our analysis of *aux* mutants, in addition to confirming the importance of clathrin function in spermatogenesis, provides a plausible explanation for the observed male sterility. The phenotypes of *aux* mutant testes suggest that Aux participates in several processes during spermatid differentiation, including cytokinesis, formation of the plasma membrane and individualization. As the sterility of *aux* mutant males could be rescued by the expression of dAux$^{CJ}$, it is likely that all of these phenotypes are clathrin-related.

In mammals, clathrin is known to mediate vesicular trafficking from the plasma membrane, the TGN and endosomes (Bard and Malhotra, 2006). In addition to its role in endocytosis, GAK has been implicated in delivering lysosomal proteins from TGN (Hirst et al., 2008; Kametaka et al., 2007; Lee et al., 2005; Zhang et al., 2005). In *Drosophila*, although the importance of clathrin-mediated endocytosis in cell-cell signaling and cell morphogenesis

is well known, the roles of clathrin-dependent transport from organelles in development are less clear. Using Clc-GFP, we showed that in developing spermatids many clathrin-positive vesicular structures were localized in the vicinity of the Golgi. Furthermore, these structures contained AP-1 adaptors, suggesting that they were Golgi-derived CCVs. In *aux* mutant cells, the distribution of these clathrin structures and their colocalization with AP-1 were disrupted, implying that formation of these Golgi-derived clathrin-positive vesicles requires auxilin. This conclusion is further strengthened by localization of $dAux^{FL}$-mRFP at the Golgi. The presence of abnormal clathrin-positive structures, along with the deficit in plasma membrane formation, suggests that Golgi-derived CCVs act as intermediates to provide membrane for the cell surface during spermatid differentiation. In this scenario, the amount of membrane transported from the Golgi to the cell surface is expected to be reduced in *aux* mutants. Indeed, cytokinesis and spermatid elongation, two processes requiring significant increase in cell surface area (Tokuyasu, 1975), are affected in *aux* mutants.

The role of auxilin family proteins at the TGN remains unclear. Given the well-established role of auxilin in disassembling clathrin coats, *aux* mutations might block the transit of Golgi-derived vesicles by inhibiting removal of their clathrin coats. If this scenario is correct, the disrupted colocalization of clathrin and AP-1 in *aux* mutant cells would imply that disassembly of AP-1 from the newly formed vesicles does not require auxilin. Alternatively, this loss of colocalization of clathrin and AP-1 could suggest that Aux has a role in facilitating the interaction between clathrin and AP-1 at the TGN. In this scenario, its involvement could be direct (e.g. stabilizing binding between clathrin and AP-1), as Aux contains motifs capable of interacting with both clathrin and adaptors. Alternatively, as we have previously shown that clathrin forms aggregates in *aux* mutant cells (Kandachar et al., 2008), it is possible that clathrin in these aggregates is incapable of interacting with AP-1. Kametaka et al. (Kametaka et al., 2007) have shown that, in HeLa cells, AP-1 recruits GAK to TGN, and the presence of GAK at the TGN is required for lysosomal trafficking. It is possible that, in *Drosophila* germ cells, localization of Aux to the Golgi also relies on AP-1 and this recruitment is required for formation of CCVs.

The mechanistic link between clathrin-dependent trafficking and IC migration is less clear. As IC migration excludes cytoplasmic content during individualization, the plasma membrane of spermatids also becomes constricted (compare Fig. 5D with 5F). We originally thought that clathrin might mediate this decrease in the cell surface by endocytosis. However, we did not detect Clc-GFP within the ICs (Fig. 7K), where membrane constriction is expected to occur. Furthermore, genetic and pharmacological manipulations have previously shown that endocytosis and exocytosis have no direct role in IC migration during individualization (Noguchi and Miller, 2003). We thus speculate that, although the ICs were scattered, *aux* mutations might not have a direct role in IC organization or migration. Instead, *aux* mutations might have disrupted an event prior to individualization that would affect IC organization or migration later on. Indeed, we showed that, in *aux* mutant germ cells, the plasma membrane was not properly formed, even before IC assembly. We propose that this aberrant plasma membrane would impede subsequent IC movement, resulting in IC scattering.

It has long been appreciated that the cell surface of spermatids increases significantly during differentiation (Tokuyasu, 1975). We propose that auxilin-dependent membrane trafficking from the Golgi is required to sustain this expansion of the plasma membrane. As clathrin and Lva are also implicated in cellularization during embryogenesis (Sisson et al., 2000), this mechanism of clathrin-dependent membrane addition during cell separation is probably conserved. Given the large size of *Drosophila* male germ cells, differentiation of spermatids could be a useful paradigm to dissect genes required for these cell morphogenetic events.

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**Competing interests statement**

The authors declare no competing financial interests.

**Supplementary material**

Supplementary material for this article is available at
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