 Trafficking through COPII Stabilises Cell Polarity and Drives Secretion during Drosophila Epidermal Differentiation

Michaela Norum¹, Erika Tång², Tina Chavoshi², Heinz Schwarz³, Dirk Linke³, Anne Uv², Bernard Moussian¹*

¹Interfaculty Institute for Cell Biology, University of Tübingen, Tübingen, Germany, ²Institute of Biomedicine, Göteborg University, Göteborg, Sweden, ³Max-Planck Institute for Developmental Biology, Tübingen, Germany

Abstract

Background: The differentiation of an extracellular matrix (ECM) at the apical side of epithelial cells implies massive polarised secretion and membrane trafficking. An epithelial cell is hence engaged in coordinating secretion and cell polarity for a correct and efficient ECM formation.

Principal Findings: We are studying the molecular mechanisms that Drosophila tracheal and epidermal cells deploy to form their specific apical ECM during differentiation. In this work we demonstrate that the two genetically identified factors haunted and ghost are essential for polarity maintenance, membrane topology as well as for secretion of the tracheal luminal matrix and the cuticle. We show that they code for the Drosophila COPII vesicle-coating components Sec23 and Sec24, respectively, that organise vesicle transport from the ER to the Golgi apparatus.

Conclusion: Taken together, epithelial differentiation during Drosophila embryogenesis is a concerted action of ECM formation, plasma membrane remodelling and maintenance of cell polarity that all three rely mainly, if not absolutely, on the canonical secretory pathway from the ER over the Golgi apparatus to the plasma membrane. Our results indicate that COPII vesicles constitute a central hub for these processes.

Introduction

Epithelia produce apical extracellular matrices (aECM) that are essential for their function as barriers. For this purpose, epithelial aECMs often adopt a tissue-specific and elaborate architecture. A central element of aECM formation is the apical plasma membrane that serves as an interface of aECM material delivery and as a platform for aECM organisation. Hence, along with deposition of aECM components into the extracellular space, the apical plasma membrane has to be equipped with factors that mediate its function during aECM differentiation. Both processes conceivably require concerted and polarised secretion and membrane trafficking.

Generally, secretion and membrane trafficking engage the basic secretory route running from the ER via coatamer protein complex II (COPII) coated vesicles to the Golgi apparatus, and from the Golgi apparatus via adaptor protein (AP)-clathrin-coated vesicles to the plasma membrane. This anterograde transport is usually counterbalanced by the retrograde transport of membranes from the plasma membrane to endosomes and the Golgi apparatus back to the ER via COPII-coated vesicles. Selective docking of vesicles with their target membranes and their subsequent fusion both employ the activity of membrane-specific SNARE proteins [1]. These generic mechanisms are probably not sufficient to explain directionality of secretion. In polarised cells, directionality of vesicle transport depends on the cytoskeleton that is organised by subunits of protein complexes arranged along the apical and lateral plasma membrane [2,3]. The evolutionary conserved transmembrane protein Crumbs (Crb) has an influence on the organisation of the actin cytoskeleton at the apical portion of the cell through the interaction with the actin-binding factor β-heavy spectrin [4,5]. The stability of microtubules is regulated by the atypical protein kinase C (aPKC), which additionally manipulates the function of Crb [6,7]. The cytoskeleton in turn stabilises the protein complex that constitutes the adherens junctions, which being basal to the subapical Crb-complex contribute to the tautness of epithelia. Finally, positioning and function of the Crb-complex is also regulated by the exocyst complex subunit Exo84 and by membrane recycling driven by the endosomal small GTPase Rab11 [8,9].
While both the mechanisms of polarised secretion and the histology of various aECMs have been studied in detail, a link between polarised secretion in epithelia and aECM production is almost unexplored. An amenable tissue allowing detailed molecular and cellular analysis of aECM differentiation is the larval skin of the fruit fly Drosophila melanogaster. It consists of the epidermis and the apical cuticle that is an aECM deposited during embryogenesis. The Drosophila larval cuticle is a typical arthropod cuticle that adopts a stereotypic layered architecture composed of the polysaccharide chitin, lipids and proteins [10]. Several factors playing essential roles during Drosophila larval skin differentiation have been genetically identified and phenotypically characterised in the past few years. Most of these factors act within the apical plasma membrane. These are the Zona Pellucida (ZP) proteins Piopio (Pio) and Papilloto (Pot) that mediate the contact between the aECM and the surface of the epidermal cells [11], and Retroactive (Rtv) and Knickkopf (Knk) that are required for the organisation of the chitin microfibril in the aECM, the molecular functions of which, however, are unknown [12,13]. Mutations in the genes coding for these factors cause the detachment of the macroscopically normal-looking cuticle from the epidermis. A second group of mutations provokes a thin and pale cuticle suggesting a fundamental function of the affected genes in cuticle deposition. Some of these genes code for enzymes catalysing the synthesis of the steroid hormone ec dysone [14], while others encode factors involved in basic cellular processes related to secretion. One of these factors is Mummy (Mmy), the Drosophila UDP-GlcNAc pyrophosphorylase producing UDP-GlcNAc, which is an important component of N-glycans and the monomer of chitin [15,16,17]. Another genetically characterised factor that is crucial for secretion is the Drosophila Syntaxin1A (Syx1A), the SNARE of the apical plasma membrane [18]. Interestingly, mutations in syx1A abrogate the shape of the apical plasma membrane and the secretion of cuticle proteins, but not chitin synthesis that occurs at the plasma membrane suggesting that a second apical SNARE is needed for full aECM production.

Many of the same genes have been shown to be required for embryonic morphogenesis of the respiratory organ (trachea), a network of fine epithelial tubes that spans the organism. Newly formed tracheal tubes have a narrow lumen diameter that must expand several-fold before becoming functional. The widening of the tracheal lumen requires apical cell secretion [19,20], and uniform diameter expansion depends on a transient chitinous matrix [21,22]. Both Knk and Rtv are needed for the organization of luminal chitin matrix, as later of the tracheal cuticle, and loss of many abolishes cutin production for either matrix [13,15,16].

For an advanced understanding of the molecular mechanisms governing aECM differentiation in polarised epithelial cells, identification and characterisation of additional factors is needed. Two genetically defined genes ghost (gho) and haunted (hau) are excellent candidates for this goal, as larvae suffering mutations in these genes display a phenotype similar to that of syx1A and many mutant larvae [23,24]. In the present work, we show that hau and gho code for the Drosophila COPII components Sec24 and Sec23, respectively. We demonstrate that Hau and Gho function to maintain correct localisation of cell polarity markers in the tracheae and the epidermis while they support deposition of tracheal luminal and cuticle material. These findings underline that the secretory pathway is an important motor for the differentiation of the tracheae and the epidermis.

**Materials and Methods**

**Fly work**

Flies were kept in cages on apple juice agar plates at 25°C to collect. Embryos were staged according to the time of development at 25°C described in Hartenstein and Campos-Ortega [25]. Stocks used in this work are listed in table 1. Homozygous or transheterozygous mutant embryos or larvae were unambiguously and manually collected in a population of progeny segregating GFP-positive (Kr-Gal4 and UAS-GFP harbouring balancer [26]) and GFP-negative (mutant) embryos.

**Microscopy**

For light and fluorescence microscopy, embryos were fixed chemically (in 3.7% formaldehyde) or physically (by boiling) according to standard protocols [27]. For immunohistochemical detection of antigens, the following primary antibodies were used in this study: the tracheal luminal specific mouse IgM monoclonal antibody 2A12 (1:10, Developmental Studies Hybridoma Bank, DSHB), mouse IgG monoclonal anti-GM130 (1:500, Abcam), mouse IgG monoclonal anti-KDEL (1:400, Stressgen Bioreagents, Figure 9 or 1:500, KR-10, Abcam, Figure 8), rabbit anti-Rab5 (1:1000, [28]), rabbit anti-Rab11 (1:8000, [29]), rabbit polyclonal anti-Verm (1:300), and rabbi t polyclonal anti-Knk (1:1500, preabsorbed against wild-type embryos before use). A fluorescein-conjugated chitin-binding probe was used to detect chitin (CBP, 1:500, New England Biolabs). For visualisation, secondary fluorescent antibodies from Molecular Probes (1:500) were used: Alexa 488 goat anti-mouse IgG, Alexa 568 goat anti-mouse IgG, Alexa 568 goat anti-mouse IgG2a, Alexa 488 goat anti-rabbit IgG, Alexa 555 goat anti-rabbit IgG and Alexa 568 goat anti-mouse IgG1. A Nikon eclipse E1000 microscope was used for Nomarski and fluorescence imaging and Bio-Rad Radiance 2000 for confocal imaging.

Embryos and first instar larvae were prepared for electron microscopy following previously described protocols [29].

**Molecular biology**

For identification of hau and gho (PCR & sequencing), molecular experiments were performed following standard protocols for

| genotype | origin |
|----------|--------|
| Samarkand (wild-type) | Bloomington |
| hau<sup>2414</sup> | [23] |
| hau<sup>x</sup> | [35] |
| Df(3R)EDS187 | Bloomington |
| gho<sup>2104</sup> | [24] |
| gho<sup>2107</sup> | [24] |
| Df(2L)BSC688 | Bloomington |
| Df(2L)E17010 | Bloomington |
| sax<sup>1</sup> | [44] |

**Table 1. Fly stocks used in this work.**

**Fly stocks**

Stocks of flies segregating the hau<sup>2414</sup> and both gho mutations were obtained from the Tubingen Stock Collection (http://www.eb.tuebingen.mpg.de/departments/3-genetics/drosophila/drosophila-stock-collection/drosophila-stock-collection). The hau<sup>x</sup> segregating flies were generated by Kay Giesen in the laboratory of Christian Klambt (Münster University). The sax stock was a kind gift of Christos Samakovlis (Stockholm University). Flies that segregate deficiencies in the hau or gho chromosomic region were obtained from the Bloomington Stock Center (Indiana University, Bloomington, USA). All mutant stocks were kept over respective balancers carrying Kr-Gal4 and UAS-GFP [26], doi:10.1371/journal.pone.0010802.001
molecular biology. For Western blot analysis, larvae were homogenized for protein isolation in PLC buffer containing protease inhibitors. Protein amounts were estimated by spectrometry at 280 nm [30]. Following SDS-polyacrylamide gel electrophoresis (SDS-PAGE, 7.5%), proteins were transferred to a nitrocellulose membrane (Whatman) by the semi-dry method. Proteins were detected using the Odyssey infrared dual-colour detection system (LI-COR® Biosciences). For immunodetection of Knk and Serp on Western blots, specific primary antibodies were used at the dilutions of 1:1000 and 1:300, respectively.

Results

Mutations in hau and gho affect cuticle differentiation

The wild-type first instar larval cuticle lines the body of the animal, structures its head, and stabilizes the air-filled trachea (Figure 1A,A'). Embryos mutant for either haunted (hau) or ghost (gho) produce only a discontinuous or thin larval cuticle and the tracheae do not become air-filled and are barely visible, whereas their head skeleton albeit less melanised, has a normal morphology (Figure 1B,B' and C,C'). These animals die at the first instar larval stage within the egg case. Specialised cuticular structures such as the ventral denticles and the dorsal hairs are missing in hau and gho mutant larvae (Figure 1D–F). To test whether the epidermis and the trachea nevertheless produce chitin, we detected chitin with a FITC-coupled chitin-binding protein (CBP) in stage 17 wild-type and mutant embryos (Figure 1G–I). Mutations in either hau or gho cause reduced chitin in the epidermis and the trachea. Overall, the gho phenotype is stronger than the hau phenotype. Of note, larvae homozygous for any hau (HG14 and CK) or gho (IB104 and IP107) mutation or carrying the mutation in trans over each other or over respective deficiencies (see below) exhibit the same defects. We can therefore neglect the possibility that additional mutations on the chromosomes carrying hau or gho contribute to the strength of the

Figure 1. Larvae carrying mutations in hau and gho have a thin and pale cuticle. Wild-type larvae ready to hatch have a body cuticle that shrouds the inner organs (A). The head skeleton (*) and the air-filled dorsal trunk (arrow) are nevertheless discernable. By Nomarski microscopy, light refraction reveals the body cuticle of wild-type larvae within the egg case fixed in Hoyer’s medium (A’). Due to a thin cuticle, the inner organs, such as the Malpighian tubules and the digestive system (arrowheads) of larvae mutant for hau and gho are well visible (B and C). Probably due to the failure to air-fill, their dorsal trunk is not identifiable at this magnification (compare to Figure 7). As seen in Hoyer’s fixed hau and gho larvae, the mutant cuticle only weakly refracts light in Nomarski optics (B’ and C’). The head skeleton of these larvae seems to have a correct morphology but is less tanned. The ventral side of the wild-type larval body is decorated by belts of denticles, while more filigree hairs cover the dorsal side (D and D’). The ventral and dorsal sides of hau and gho mutant larvae, by contrast, are naked (E–F). The surface of both mutant larvae is, however, not smooth but wrinkly, and in gho mutant larvae, epidermal cells at both sides appear to round up and leave the epithelium (see Figure 3). The epidermis, the dorsal trunk (arrow) and the head skeleton of wild-type stage 17 embryos are lined by chitin as detected with the FITC-conjugated chitin-binding probe (green, G). The chitin signal in the head skeleton and the body of hau and gho mutant stage 17 embryos is weaker than in wild-type embryos (H and I). Moreover, their dorsal trunk (arrow) is narrower than the wild-type one. (A–F) Nomarski light microscopy of wild-type, hau and gho mutant larvae within the egg case. (G–I) Fluorescence microscopy of heat fixed stage 17 embryos. Scale bar in (D) is 50 μm and applies to (D–F).

doi:10.1371/journal.pone.0010802.g001
phenotypes described. Taken together, hau and gho are required for correct deployment of cuticle melanisation and chitin deposition.

To understand the cellular defects caused by mutations in hau and gho, we examined the ultrastructure of epidermal cells in hau and gho mutant embryos and larvae. In the wild-type larva the cuticle consists of three biochemically distinct horizontal layers (Figure 2A). The outermost layer is the envelope that is composed of alternating electron-dense and electron-lucid films. The middle epicuticle is a bipartite proteinaceous layer with an upper electron-lucid and a lower electron-dense sublayer. An ordered chitin-protein matrix constitutes the innermost procuticle. The cuticle of hau mutant larvae has a reduced epicuticle, and the chitin-protein organisation is lost in the procuticle, while the envelope has a normal appearance (Figure 2B). The cuticle of gho mutant larvae is very thin and fragmented and lacks the epicuticle (Figure 2C).

Organisation of the procuticle is supposedly controlled by the apical plasma membrane that forms longitudinal corrugations called apical undulae during chitin synthesis that run perpendicular to the anterior-posterior axis of the developing embryo (Figure 2D) [29]. The epidermal cells of hau and gho mutant embryos usually do not establish apical undulae (Figure 2E,F). In rare cases, the apical plasma membrane of the epidermis of hau mutant embryos forms apical undulae close to the apical cell-cell junctions. In summary, hau and gho are needed for cuticle formation and shaping the epidermal apical plasma membrane.

**Hau and gho are needed for epidermal cell shape and polarity**

The epidermis of larvae mutant for hau and gho eventually disintegrates and single cells leave the tissue (Figure 3A–C). To have a more detailed understanding of this defect, we studied the morphology of the epidermal cells by electron microscopy. The wild-type larval epidermal cells are flat and contact their neighbours via a meandering lateral membrane that is mainly characterised by the apical adherens junctions and the basolateral septate junctions (Figure 3D,F). The gho or hau larval epidermal cells are cuboidal and their lateral membrane does not meander (Figure 3E,G,H). The adherens junctions of hau and gho mutant larval epidermal cells look loose and the basolateral septate junctions appear to be less complex. Especially in gho mutant larvae, the lateral cell-cell contacts are effaced (Figure 3H). At the onset of cuticle differentiation, at stage 15 cell shapes in hau, gho and wild-type embryos are indistinguishable (data not shown).

As in many epithelia, the basal side of wild-type epidermal cells is covered by the basement membrane (Figure 4A). The basal side of hau and gho mutant epidermal cells is, by contrast, naked (Fig 4B,C). Defects at this side of hau and gho mutant epidermal cells have a more drastic effect in the muscle attachment sites, the apodemes (Figure 4D–F). Muscles contact the jagged basal plasma membrane of apodermal cells by specialised junctions, the basal hemidesmosomes in wild-type larvae and a complex intracellular ECM (Figure 4D). In hau and gho mutant apodemes the basal plasma membrane is smooth (Figure 4E–F), and the muscle often detaches from the epidermis (Figure 4F,F').

The ultrastructural studies indicate that cell polarity is compromised in hau and gho mutant larvae. To verify whether cell polarity may already be perturbed during the time of massive cuticle production, we performed immunohistochemical experiments in stage 15–17 embryos using antibodies against factors that mark different domains of the lateral plasma membrane. Crumbs (Crb) is a transmembrane protein and localises to a lateral position of the apical plasma membrane of epidermal cells in stage 15 and 16 wild-type embryos (Figure 5A,C) [4]. Crb localisation is normal in stage 15 hau mutant embryos (Figure 5B). In stage 16 gho mutant embryos, in addition to a variable but occasionally normal localisation at the apico-lateral membrane, the Crb signal accumulates within the cell (Figure 5D). Fasciclin 3 (Fas3) is a component of the lateral membrane strongly marking the apical position of the lateral membrane underneath the Crb-domain (Figure 5E). Fas3 amounts gradually decrease towards the basal end of the lateral membrane. In hau and gho mutant embryos the Fas3 localisation is not concentrated at the apico-lateral domain (Figure 5F,G). In conclusion, cell polarity is impaired only slightly during cuticle differentiation, and worsens until the end of embryogenesis.

---

**Figure 2. Hau and Gho are required for full cuticle differentiation.** The wild-type larval cuticle is a stratified extracellular matrix (A). Based on the molecular composition, it is subdivided into three layers. The envelope (env) is the outermost layer, separated by the bipartite epicuticle (epi) from the innermost procuticle (pro). The cuticle of hau mutant larvae is disorganised (B). The electron-dense basal sublayer of the epicuticle often contacts the envelope and the chitin matrix has lost its tight packaging. In gho mutant larvae, the cuticle is fragmented and thin (C). Between late stage 16 and mid-stage 17 the apical plasma membrane of epidermal cells forms regular corrugations called apical undulae (au), at the tip of which chitin synthesis takes place, while secretion occurs at the valley between the corrugations (D). The epidermal cells of hau and gho mutant embryos fail to form repeated corrugations (E and F). (A–F) Electronmicrographs of ultrathinsections. Scale bar in (A) is 500nm and applies to (B) and (C). Scale bar in (D) is 500nm and applies to (E) and (F).
doi:10.1371/journal.pone.0010802.g002
Both hau and gho mutations cause defects in membrane shape. To learn to what extent membrane trafficking is abrogated by these mutations, we investigated the behaviour of small GTPases such as Rab5 and Rab11 in stage 16 and 17 hau and gho mutant embryos. Rab11 is involved in endocytic membrane recycling, whereas Rab5 regulates the fusion of endocytic vesicles with early endosomes [31,32]. Both proteins are localised to the cytoplasm of wild-type epidermal cells with a slight accumulation to the apical portion of the cell (Figure 5A,C). In hau and gho mutant embryos the distribution of Rab5 and Rab11 is normal (Figure 5B,D).

The tracheae of hau and gho mutant larvae

The tracheal cuticle covers the apical (luminal) surface of the tracheal epithelium, and forms a specialized spiral-like structure called the taenidia. The taenidia are chitin-containing cuticular folds that are thought to support an open lumen while allowing flexibility along the tubular axis (Figure 6A,B). Detection of chitin by CBP highlights remnants of the taenidia in hau mutant embryos, whereas gho mutant embryos seem not to form these structures (Figure 6C and E). Ultrastructural analysis of the trachea confirms the presence of taenidia in hau mutant larvae (Figure 6D); however, their size is variable and their spacing is irregular. In gho mutant larvae traces of shallow taenidia can be distinguished at the ultrastructural level (Figure 6F).

Detection of cuticular chitin also reveals that the main tracheal branches, the dorsal trunks, of hau and gho mutant larvae have a much smaller lumen diameter than those of the wild-type (Figure 6A, C and E). Such narrow lumens are also obvious in living animals (Figure 7A,B). Since lumen diameter growth is found to depend on an intact secretory pathway, and substantial apical secretion is noted in tracheal tubes during stage 15 [19], we investigated whether the narrower dorsal trunks in hau and gho mutant embryos correlate with defects in secretion. The tracheal lumen-specific antibody 2A12, recognizes a secreted product that fills the tracheal lumen from stage 14. The 2A12 signal is detected in the lumen of hau and gho mutant embryos at late stage 15, but in the mutants, there is also a strong cytoplasmic signal in the perinuclear area (Figure 7C–E). Another luminal protein,
Vermiform (Verm), is a chitin-modifying enzyme detected in the dorsal trunk lumen from stage 13 [33,34]. In addition to its detection in the lumen, the Verm signal also accumulates within the mutant tracheal cells similar to the 2A12-signal (Figure 7C–H). The secretory pathway is also responsible for the correct localisation of membrane-inserted factors. To consider this aspect, we studied the membrane factors Fas3 and Crb in stage 16 embryos (Figure 7I–N). Localisation of the apical membrane marker Crb appears normal in tracheal cells of both mutants (Figure 7I–K), but the membrane staining for Fas3 is reduced in the mutants. In wild type tracheal cells, Fas3 accumulates within the apical third of the lateral membrane (Figure 7L), whereas in hau mutants, this apical Fas3-staining is reduced and Fas3 is also detected in the cytoplasm (Figure 7M). In gho mutants Fas3 levels appear even more reduced, and Fas3 is only weakly detected at the apical domain of the lateral membrane (Figure 7N).

In summary, Hau and Gho function is important for the secretion of some tracheal luminal factors and for the stable localisation of certain membrane-inserted proteins.

ER morphology is aberrant in the epidermal and tracheal cells of hau and gho mutant embryos

In electron micrographs, we noticed that the ER of hau and gho mutant larval epidermal cells consists of large spherical compartments instead of tubules as observed in respective wild-type cells (Figure 2, 3, 6 and 7). Figures 8A and B show a
The small GTPase Rab5 (green) is distributed in the cytoplasm. Localisation of Crb and Rab5 is normal in hau mutant embryonic epidermal cells (B). At stage 16, Crb continues to localise to the apico-lateral region of the lateral plasma membrane (C). The small GTPase Rab11 (green) is distributed in the cytoplasm with a considerable accumulation at the apical portion of the cell. The Crb signal is detected also in the cytoplasm of gho mutant embryonic cells, while Rab11 distribution is normal (D). The lateral plasma membrane of wild-type embryos – here stage 16 - is marked by proteins like Fas3 (red) constituting the septate junctions (E). The Fas3 signal gradually decreases from the apical to the basal end of the lateral plasma membrane. The nuclei (blue) of these cells locate to the basal side of the columnar cell. In hau and gho embryonic mutant epidermal cells the Fas3 signal is homogenously distributed along the lateral plasma membrane (F and G). The epidermal cells are cuboidal and the nuclei are lie in the middle of the cell. Images from confocal microscopy. doi:10.1371/journal.pone.0010802.g005

Figure 5. Hau and Gho stabilise epidermal cell polarity. The transmembrane protein Crb (red) localises to the apico-lateral region of wild-type stage 15 embryonic epidermal lateral plasma membrane (A). The small GTPase Rab5 (green) is distributed in the cytoplasm. Localisation of Crb and Rab5 is normal in hau mutant embryonic epidermal cells (B). At stage 16, Crb continues to localise to the apico-lateral region of the lateral plasma membrane (C). The small GTPase Rab11 (green) is distributed in the cytoplasm with a considerable accumulation at the apical portion of the cell. The Crb signal is detected also in the cytoplasm of gho mutant embryonic cells, while Rab11 distribution is normal (D). The lateral plasma membrane of wild-type embryos – here stage 16 - is marked by proteins like Fas3 (red) constituting the septate junctions (E). The Fas3 signal gradually decreases from the apical to the basal end of the lateral plasma membrane. The nuclei (blue) of these cells locate to the basal side of the columnar cell. In hau and gho embryonic mutant epidermal cells the Fas3 signal is homogenously distributed along the lateral plasma membrane (F and G). The epidermal cells are cuboidal and the nuclei are lie in the middle of the cell. Images from confocal microscopy. doi:10.1371/journal.pone.0010802.g005

To test whether a possible abrogated ER function may have an impact of mutant epidermal cells. In tracheal cells of stage 16 embryos, the Golgi organising protein GM130 to visualise the Golgi apparatus (Figure 9A–F). These experiments demonstrate that the organisation and identity of the ER and the Golgi apparatus are compromised in hau and gho embryos.

To test whether the disrupted organisation of the ER and the Golgi apparatus in hau and gho embryos may affect correct modification of proteins running through the secretory pathway, we performed Western blot experiments using antibodies against the membrane-bound Knickkopf (Knk) protein, or the secreted Serp protein (Figure 10). The size of Knk, that we have previously shown to be glycosylated at three sites [13,15], is unchanged in hau and gho mutant embryos (10A). The migration of the extracellular Serp protein with three putative N-glycosilation sites (Asp276, Asp298, Asp308) is also normal in these embryos (10B). Hence, ER and Golgi organisation but not their function as compartments of N-glycosilation are compromised by mutations in hau or gho.

hau and gho encode factors of the COPII complex

To understand the molecular roles of hau and gho in cuticle differentiation, we identified the genomic location and the sequence of both genes. Mutations in hau had previously been mapped to the right arm of chromosome 3 (cytological location 85D) [35], and mutations in gho had been localised to the right arm of chromosome 2 (recombination map position 68) [24]. By deficiency mapping, we localised hau to the cytological interval between 83B7 and 83B8 uncovered by the deficiency Df[3R]ED5187 on the right arm of chromosome 3 (Figure 11A), and gho to the cytological interval between 22D4 and 22D6 defined by the overlapping region of the deficiencies Df[2L]Exel7010 and Df[2L]BSC688 on the left arm of chromosome 2 (Figure 11C). Thus, the mapping data in [24] and [35] are inaccurate. The hau-containing interval harbours two genes, one of which is CG1250 that encodes the only Drosophila Sec23 ortholog, that, as a COPII component, is involved in vesicle budding from the ER [36]. We sequenced the sec23 genomic DNA of embryos homozygous mutant for hau and detected a point mutation in each of our alleles (Figure 11B). A transition of the C to T resulting in
a nonsense mutation changing Gln215 to amber (TAG) was detected in the hau9G14 allele. This mutation disrupts the Sec23/24 or von Willebrand factor type A (vWFA)-like domain in the first half of the protein and deletes all consecutive domains. The P-element induced allele [35] has a deletion of G2076 of the coding sequence causing a frame shift that changes the peptide sequence after Lys692. This mutation leads to the elimination of Arg722, which is essential for the interaction of Sec23 with Sar1, the GTPase that triggers vesicle budding from the ER [37,38]. The Drosophila Sec23 protein is over its entire length 73% identical and 85% similar to the human Sec23 protein (isoform A). A gene coding for a Drosophila Sec24-like protein, which interacts with Sec23 [38], is located in the gho-containing interval (CG10882) enclosing 16 genes (Figure 11C). Due to the phenotypic resemblance of hau and gho mutant embryos, we considered this gene as a candidate to be mutated in gho mutant embryos. We sequenced the respective genomic DNA and identified base-pair changes in both gho EMS alleles (Figure 11D). The 50th codon in IP107 (CAG) is mutated to an amber nonsense codon and the respective protein stops after Gln49. The mutation therefore results in a short protein that lacks the functional domain of the Sec24-like protein. Because of an amber nonsense mutation of codon 362 (CAG), the IB104 protein ends at residue Pro361. This protein lacks the complete domain set common to Sec23/Sec24 proteins, including the Sec23/24 (vWFA-like) domain, and only consists of a region which is specific to this Sec24 paralog and which is predicted to be unstructured. Besides CG10882 the Drosophila genome harbours another gene (CG1472) encoding a Sec24-like protein, which is actually annotated as the Drosophila Sec24 ortholog. The two Drosophila Sec24 proteins correspond to four human orthologs. The Drosophila Sec24 protein CG10882 is rather similar to the human orthologs Sec24C and D than to Sec24A and B, which share high similarity to CG1472 (Figure S2). Following these findings, we consider CG10882 as the Drosophila Sec24CD and CG1472 as the Drosophila Sec24AB paralogs.

The mutations in the Drosophila sec23 (hau) and sec24cd (gho) genes most probably disrupt protein function resulting in embryo lethality. We therefore conclude that hau and gho code for Sec23 and one of the Drosophila Sec24 paralogs, respectively, both being components of the COPII complex. In a recent article, Förster and colleagues reported on the role of CG10882, named Stenosis (Sten) during tracheal development [39]. Consistently, mutations in sten fail to complement mutations in gho (data not shown). Since mutations in gho were identified earlier [24], we continue denoting CG10882 gho. Despite the evident importance of both factors for cell viability, animals mutant for either factor die rather late during embryogenesis (Figure 1); this observation can be explained by the presence of maternally provided function supporting development until the end of embryogenesis [39,40].

Figure 6. Hau and Gho are needed for the formation of the tracheal cuticle. In the wild-type tracheal cuticle of the dorsal trunk and the primary branches of late stage 17 embryos, chitin is organised in a spiral running perpendicular to the length of the tube (A). Remnants of the luminal chitin are visible (arrow). These chitin cables constitute the taenial folds (tae), which are bulges of the larval cuticle (B). At the larval stage, the lumen (lum) of the tracheal tubes does not contain any solid material. In hau late stage 17 mutant embryos, the chitin cables of the dorsal trunk and the primary branches are properly formed (C). The tracheal lumen, however, is much narrower compared to the wild-type lumen. The hau larval tracheal cuticle dilates and the taenial folds are sloppy (D). The lumen of the hau mutant larval tracheae is not completely cleared. In gho stage 17 mutant embryos, chitin cables are largely disorganised and often absent (E). The tracheal tubes have an irregular diameter. The gho mutant larval tracheae have shallow taenidia and their lumen fails to be cleared (F). (B,D,F) Electronmicrographs. Scale bars are 500 nm. (A,C,E) Fluorescence microscopy. doi:10.1371/journal.pone.0010802.g006
Mutations in genes encoding COPII components cause similar phenotypes

Sec23 and Sec24 form a complex with Sar1, a GTPase that triggers membrane budding from the ER [41,42]. Mutations in the *Drosophila* sar1 have been reported to abrogate secretion in tracheal and epidermal cells [19,43]. Macroscopically, larvae with a deletion of the first four exons of the *sar1* gene [44] display a weaker phenotype than larvae mutant for *hau* and *gho* (Figs. 1 and 12). For a detailed comparison of *sar1*, *hau* and *gho* phenotypes, we examined the ultrastructure of the epidermis of *sar1* mutant larvae that are characterised by a pale cuticle (Figure 12A). A stratified cuticle is formed in *sar1* mutant larvae, which is however thinner than the wild-type cuticle (Figure 12B). As in *hau* and *gho* mutant larvae and consistent with recently published data [19], the ER of the epidermal cells of *sar1* mutant larvae is spherical. Apical undulae formation is unaffected by *sar1* mutations (Figure 12C). Moreover, the taenidia of the *sar1* mutant tracheae are normal (Figure 12D). In summary, the *sar1* mutation causes a weak COPII-deficient phenotype.

Figure 7. Apical secretion in tracheal cells utilizes Hau and Gho function. At early stage 17, the wild-type dorsal trunk has attained its final diameter of around 12 microns (white line, A). The early stage 17 dorsal trunks of *hau* and *gho* mutant embryos are narrower (B). During tracheal tube diameter expansion, the luminal marker 2A12 is secreted into the lumen of the tracheae (C). Most of the 2A12 signal remains within the tracheal cells of *hau* and *gho* mutant embryos during tube diameter expansion (D,E). The luminal chitin deacetylase Verm is involved in modifying the tracheal luminal chitin cable that participates in lumen diameter regulation (F). In *hau* and *gho* mutant tracheae large amounts of Verm fail to be secreted (G,H). Crb marks the apical plasma membrane in wild-type tracheal cells (I). In the tracheal cells of *hau* and *gho* mutant stage 16 embryos the localisation of Crb is unchanged (J,K). The membrane protein Fas3 lines the lateral membrane of wild-type stage 16 tracheal cells (L). In *hau* mutant embryos, some Fas3 signal is cytoplasmic (M). In *gho* mutant embryos, Fas3 localisation is as in the wild-type tracheal cells, the signal levels, however, seem to be reduced (N). Images from Confocal microscopy. doi:10.1371/journal.pone.0010802.g007
Discussion

Epithelia are composed of polarised cells and possess extracellular matrices at their apical and basal sides. In this article, we show that in the *Drosophila* embryonic epidermis and tracheal epithelium the COPII components Sec23 and Sec24 stabilise cell polarity, are needed for tracheal tube diameter growth and mediate formation of the apical and basal ECMs.

**hau** and **gho** code for the COPII components Sec23 and Sec24CD

Hau and Gho represent the only *Drosophila* Sec23 and one of the two *Drosophila* Sec24 proteins, respectively, which are components of the COPII complex that coats vesicles transporting ER products to the Golgi apparatus [45]. Sec24 is responsible for specific cargo recognition and binding. In yeast, for instance, several Sec24-like proteins such as Iss1p and Lst1p act in parallel to ensure correct delivery of cargo proteins to their destination [46]. Sec23 is a central molecule of the COPII complex [36]. Through the interaction with Sec24, Sec23 links cargo recognition to vesicle budding [47], and through binding to dynactin it links vesicle budding to vesicle transport [48]. Sec23 and Sec24 are essential proteins in eukaryotic cells from yeast to humans [49]. In yeast, depletion of Sec23 blocks protein transport [50]. A hypomorph mutation in the human Sec23A-coding gene has been shown to cause craniofacial morphological abnormalities termed Cranio- lenticulo-sutural dysplasia (CLSD) [49,51]. Consistently, zebrafish embryos carrying mutations in *bulldog* that codes for the Sec24D paralog, suffer skeletal dysmorphologies caused by the failure to secrete ECM material [52]. In a recent article, Förster and colleagues reported on the requirement of *Drosophila* Sec24CD, which they call Stenosis (Sten), for tracheal cell shape changes and tube expansion in a cell-autonomous manner [39].

Besides Sec23 and Sec24, the inner coat of COPII vesicles also harbours the small GTPase Sar1 that initiates vesicle formation. Mutations in *sar1* cause a similar albeit weaker, phenotype compared to those caused by *hau* and *gho* mutations. Indeed, regarding the cuticle, we observe a phenotypic series from strong defects in *gho* mutant larvae, over moderate defects in *hau* mutant larvae to weak defects seen in *sar1* mutant larvae. The differences in consequences of presumably null mutations in all three genes derive probably from the stability and endurance of the respective
maternally provided gene product. In this view, the virtual absence of the body cuticle in gho mutant larvae indicates that the function of the Sec24CD protein is almost completely eliminated in late embryonic stages and late secretion is utterly interrupted. One may conclude that the two Drosophila Sec24 paralogs do not have redundant functions in the epidermis and that the second Sec24-like protein, i.e. Sec24AB, does not play any role in epidermal cuticle formation.

Hau and Gho are needed for ER function and integrity

Blocking of COPII vesicle transport by mutations in sec23, sec24 and sar1 causes a stop of protein trafficking to the extracellular space or to the plasma membrane, and concomitantly, the ER loses its tubular shape and becomes spherical. This subcellular phenotype is also observed in human SEC23/2 fibroblasts and in HeLa cells deficient for Sec13, which is part of the COPII coat [36,53].

The loss of ER morphology may be due to the excess accumulation of not transported proteins that may physically destroy the molecular corset of the ER. This would imply that a mechanism controlling the amount of proteins in the ER does not exist. In other words, ER exit sites and ER loading complexes do not seem to communicate.

Overloading of the ER may downgrade ER physiology, and thereby interfere with the activity of ER specific enzymes. One may speculate that this in turn may induce ER stress that leads to the degradation of ER proteins [54]. Our data on this issue are conflicting. On the one hand, the amounts of Serp and ER residual proteins detected by the KDEL antibody seem to diminish in hau and gho mutant embryos, which on the other hand have

Figure 9. Tracheal ER identity and secretion depend on Hau and Gho function. In wild-type tracheal cells at late stage 16, the KDEL signal (red) is distributed within the cytoplasm (A,B). The membrane-associated protein Knk (green) localises to the apical plasma membrane in these cells (B). The green signal in the tracheal lumen is unspecific background. The KDEL signal in hau and gho mutant tracheal cells is strongly reduced (C–F). In both mutant tracheal cells, Knk fails to localise to the apical plasma membrane and accumulated within the cytoplasm (D,F). (A–F) Images from Confocal microscopy.

doi:10.1371/journal.pone.0010802.g009

Figure 10. ER function is not severely affected in hau and gho mutant embryos. The migration behaviour of the membrane-associated cuticle factor Knk (red, A) and the extracellular Serp (red, B) is normal in hau and gho mutant larvae in western blot experiments. The amount of Serp protein is reduced in the mutant protein extracts. By contrast, the amounts of Knk are comparable in mutant and wild-type protein extracts. Tubulin (green) was detected to control the amount of protein blotted. The tubulin signal allows comparing the signal intensities in wild-type and mutant protein extracts.

doi:10.1371/journal.pone.0010802.g010
normal amounts of Knk although it does not reach the plasma membrane. Hence, this type of ER dysfunction seems to elicit degradation or exhaustion of selective target proteins, at the same time sparing others.

**COPII function is necessary and sufficient to drive cuticle production**

The defects provoked by reduction or elimination of Sec24CD activity illustrate the COPII-deficient phenotype in the tracheae and the epidermis. Hence, structuring of all three layers of the cuticle depends on ER-to-Golgi transport via COPII coated vesicles. The Sec24CD dependent COPII vesicles, thus, carry cuticle components as diverse as for example the chitin synthase producing the bulk of the procuticle, factors of the extracellular melanisation pathway, and the yet unknown lipid-handling enzymes of the envelope and epicuticle. Of course, this does not exclude that COPII-independent routes are employed to modify layer architecture. For instance, transcytosis of phenoloxidases from the haemolymph to the cuticle [55] does not utilise the canonical secretory pathway and therefore is probably not directly affected by COPII-deficiency. It may well be, however, that disruption or weakening of cell polarity induced by gho mutations indirectly interferes also with alternative secretory routes [56,57].

How may a single secretory route accommodate asymmetric distribution of factors within the plasma membrane and within the extracellular space? From the conclusion that most, if not all, cuticle factors are recruited by the ER-COPII-Golgi path for delivery to the apical plasma membrane or extracellular space, it follows that cargo divergence has somehow to occur within the route itself. Indicated by the finding that the chitin synthase complex localises to the apical plasma membrane independently from the t-SNARE Syntaxin1A [18], one potential site of divergence is the apical plasma membrane itself, where different t-SNAREs may occupy different domains. Sorting is also a central task of the Golgi apparatus, where vesicles with distinct cargos are generated [58]. At the COPII level, cargo divergence possibly engages p24 proteins, which in yeast have been reported to recognise specific cargos [59]. A major endeavour in the near future will be to elucidate the sorting mechanisms in the secretory pathway that drive cuticle differentiation i.e. αECM formation.

---

**Figure 11. Molecular identification of hau.** The hau mutations were mapped to the deficiency Df(3R)ED5187 that uncovers two genes: sec23 and elm (A). Sec23 is a component of the COPII complex. The elm gene plays a role in memory formation and ethanol sensitivity, and mutations in this gene are not lethal [66]. A nonsense mutation was identified in the sec23-coding region of the 9G14 allele, and a frame shift mutation was identified in the same coding region of the CX allele (B). Sec23 is characterised by five motifs. From the N-terminus to the C-terminus, these are the Zn binding domain, the Sec23/24 domain, which belongs to the von Willebrand factor type A (vWFA) domain family, a β-sandwich domain, a helical domain and finally a Gelsolin domain. Molecular identification of gho. The gho mutations were mapped to the interval framed by the break points of the deficiencies Df(2L)BSC688 and Df(2L)Excel7010 (C). Among the 16 genes in this region, one codes for a Sec24-like protein, CG10882 (D). In the coding region of this gene, we identified one early nonsense mutation in each allele, IB104 and IP107. doi:10.1371/journal.pone.0010802.g011
Basement membrane formation requires COPII function

Concomitant with cuticle deposition and organisation the basement membrane is produced during Drosophila embryogenesis [29]. Hence, in parallel to the coordination of apical secretion, epithelial cells have to control basal secretion as well. In hau and gho mutant embryos, the basement membrane is practically missing, suggesting that a distinct population of COPII vesicles is charged with basal ECM formation. The handling of these vesicles is, however, not sufficient to form a functional basement membrane, as in addition to the epithelial contribution, some components of the basal ECM such as collagen IV are supplied by macrophages [60]. Nevertheless, epithelial cells appear to retain sovereignty over this process. For example, Scarface (Scarf), a serine-protease-like protein and Crag, a factor associated with the secretory pathway, have been proposed to regulate the localisation of basement membrane components and act within the epithelium itself [61,62]. The loss of basal factors in hau and gho mutant embryos, could, in agreement with the finding that the basal ECM is essential for epithelial cell polarity [63,64], at least partially explain the loss of epithelial character in these animals.

Plasma membrane topology in the epidermal cell depends on Hau and Gho function

The Drosophila COPII components Sec23 and Sec24CD are not only mediating cuticle deposition and basal ECM assembly, they are also important for shaping the apical plasma membrane. In contrast to the longitudinal corrugations, the apical undulac, that are presumably essential for organising the cuticle in the wild-type embryo [29], the apical plasma membrane of hau and gho mutant embryos fails to corrugate. The epidermal apical membrane is flat also in embryos mutant for the apical plasma membrane tSINARE Syx1A [18]. Hence, the topology of the apical plasma membrane requires the canonical secretory pathway running from the ER via COPII vesicles to the Golgi apparatus and from the Golgi apparatus to the apical plasma membrane where Syx1A is controlling incorporation of membrane material.

Likewise, during cuticle differentiation, Sec23 and Sec24CD serve for the maturation of the lateral plasma membrane from a straight to a meandering structure where different protein complexes assemble along its apico-basal domains, among others enforcing cell-cell contacts [65]. The lateral plasma membrane of hau and gho mutant larval remains straight and, as suggested by their less electron-dense appearance in electronmicrographs, the SJs are depleted from proteins arguing that cell polarity is perturbed. Weakened cell polarity supposedly corrupts the arrangement of the cytoskeleton, in turn affecting polarised secretion. At the end of such a vicious circle single naked epidermal cells round up and leave the epithelium as observed especially in gho mutant larvae. This finding is in agreement with the recently formulated notion that cell polarity is not a stable state of the cell but requires continuous recycling of Crb via a Rab11-dependent mechanism [9]. Taken together, the secretion pathway plays a central role during differentiation of the Drosophila larval epidermis coordinating its primary task of cargo transport and membrane trafficking with maintenance of cell polarity.

Supporting Information

Figure S1  Hau and Gho function contributes to the morphology of the ER of the tracheal cells. The ER of wild-type larval tracheal cells is tubular (A). By contrast, the ER of hau and gho larval tracheal cells is dilated (B,C). The ER enveloping the nucleus of the wild-type larval epidermal cell is tightly following the shape of the nucleus itself (D). In hau larvae, the perinuclear ER forms cysts (E). In gho larvae, this phenotype is similar (not shown). (A–E) Electron micrographs. Scale bar in (A,D,E) is 500 nm. The scale bar in (A) applies also to (B) and (C).

Found at: doi:10.1371/journal.pone.0010802.s001 (2.47 MB TIF)

Figure S2  Schematic sequence comparison between Drosophila and human Sec24 paralogs. Sequences were compared using pairwise BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The pairwise identity scores from BLAST are given in the colored bars; the size of the bars illustrates the alignable sequence regions. Alignable stretches of less than 30 residues were ignored. Clearly, Drosophila CG10882 (Gho) has higher local and global similarity to human Sec24G, Cb (a splice variant of C) and D, while
Acknowledgements

We are thankful to Anne Spang for insightful discussions about secretion.

References

1. Wickner W, Schekman R (2008) Membrane fusion. Nat Struct Mol Biol 15: 636–644.

2. Musch A (2004) Microtubule organization and function in epithelial cells. Traffic 5: 1–9.

3. Mellman I, Nelson WJ (2008) Coordinated protein sorting, targeting and distribution in polarized cells. Nat Rev Mol Cell Biol 9: 835–845.

4. Wodarz, A, Hinz U, Engelbert M, Knust E (1995) Expression of clathrin confers apical character on plasma membrane domains of ecdysial epithelia of Drosophila. Cell 82: 67–76.

5. Medina, E, Williams J, Klipfell E, Zarnescu D, Thomas G, et al. (2002) Crumbs interacts with moesin and beta(Hay)-specif in the apical membrane skeleton of Drosophila. J Cell Biol 158: 941–951.

6. Storey, S, Dazo-Mercado MT, Cammiso, M, Moscat J, Campuzano S (2004) DaPKC-dependent phosphorylation of Crumbs is required for epithelial cell polarity in Drosophila. J Cell Biol 166: 549–557.

7. Harris TJ, Peifer M (2007) aPKC controls microtubule organization to balance adherens junction symmetry and planar polarity during development. Dev Cell 12: 727–738.

8. Blankenship JT, Fuller MT, Zallen JA (2007) The Drosophila homolog of the Exo84 exocyst subunit promotes apical epithelial identity. J Cell Sci 120: 3089–3110.

9. Roeth, JF, Sawyer, JK, Wiener DA, Peifer M (2009) Rab11 helps maintain apical crumbs and adherens junctions in the Drosophila embryonic ectoderm. PLoS One 4: e7634.

10. Locke M (2001) The Wigglesworth Lecture: Insects for studying fundamental problems in biology. J Insect Physiol 47: 495–507.

11. Bokel C, Prekop A, Brown NH (2003) Papillote and Papiote: Drosophila ZP-domain proteins required for cell adhesion to the apical extracellular matrix and microtubule organization. J Cell Biol 161: 631–642.

12. Moussian B, Soding J, Schwarz H, Nusslein-Volhard C (2005) Retrotactin, a membrane-associated extracellular protein related to vertebrate snake neurotoxin-like proteins, is required for cuticle organization in the larva of Drosophila melanogaster. Dev Dyn 233: 1036–1045.

13. Moussian B, Tang E, Tonnong A, Helms S, Schwarz H, et al. (2006) Crumbs Knickkopf and Retroactin are needed for epithelial tube growth and cuticle differentiation through their specific requirement for cutin filament organization. J Cell Biol 173: 163–171.

14. Gilbert Li (2004) Halloween genes encode P450 enzymes that mediate steroid hormone biosynthesis in Drosophila melanogaster. Mol Cell Endocrinol 215: 1–10.

15. Tonnong A, Helms S, Schwarz H, Uy AE, Moussian B (2006) Hormonal regulation of mummy is needed for apical extracellular matrix formation and epithelial morphogenesis in Drosophila. Development 133: 331–341.

16. Arano SJ, Asham H, Tear E, Garas P, Casanova J (2005) mummy/cystic encodes an enzyme required for chitin and glycogen synthesis, involved in trachea, embryonic cuticle and CNS development-analysis of its role in Drosophila tracheal morphogenesis. Dev Biol 288: 179–193.

17. Schimmelplung K, Strunk M, Steck T, Klamb C (2006) Mummy encodes an UDP-N-acetylgalactosamine-6-sulfohydrolase and is required during Drosophila dorsal closure and nervous system development. Mech Dev 123: 487–499.

18. Moussian B, Veerkamp J, Muller U, Schwarz H (2007) Assembly of the Drosophila larval exocyst requires controlled secretion and shapping of the apical plasma membrane. Matrix Biol 26: 337–347.

19. Tsarouhas V, Senti KA, Jayaram SA, Tikhova K, Hemphala J, et al. (2007) Sequential pulses of apical epithelial secretion and endocytosis drive airway maturation in Drosophila. Dev Cell 13: 214–225.

20. Jayaram SA, Senti KA, Tikhova K, Tsarouhas V, Hemphala J, et al. (2008) COPI vesicle transport is a common requirement for tube expansion in Drosophila. PLoS One 3: e1964.

21. Devine WF, Lubarsky B, Shaw K, Luschnig S, Messina L, et al. (2005) Regulation of chitin biosynthesis in epithelial tube morphogenesis. Proc Natl Acad Sci U S A 102: 17014–17019.

22. Tonnong A, Hemphala J, Tang E, Nannmark U, Samakovlis C, et al. (2005) A transient luminal chitinous matrix is required to model epithelial tube diameter in the Drosophila trachea. Dev Cell 9: 423–430.

23. Jurges G, Wieschaus E, Nusslein-Volhard C, Kluding H (1984) Mutations affecting the pattern of the larval cuticle in Drosophila melanogaster. II. Zygotic loci in the third chromosome. Roux's Arch Dev Biol 193: 267–262.

24. Nüsslein-Volhard C, Wieschaus E, Kluding H (1984) Mutations affecting the pattern of the larval cuticle in Drosophila melanogaster. I. Zygotic loci on the second chromosome. Roux's Arch Dev Biol 193: 267–262.
53. Townley AK, Feng Y, Schmidt K, Carter DA, Porter R, et al. (2008) Efficient coupling of Sec23–Sec24 to Sec13–Sec31 drives COPII-dependent collagen secretion and is essential for normal craniofacial development. J Cell Sci 121: 3025–3034.

54. Malhotra JD, Kaufman RJ (2007) The endoplasmic reticulum and the unfolded protein response. Semin Cell Dev Biol 18: 716–731.

55. Asano T, Ashida M (2001) Cuticular pro-phenoloxidase of the silkworm, Bombyx mori. Purification and demonstration of its transport from hemolymph. J Biol Chem 276: 11100–11112.

56. Schotman H, Karhinen L, Rabouille C (2009) Integrins mediate their unconventional, mechanical-stress-induced secretion via RhoA and PINCH in Drosophila. J Cell Sci 122: 2662–2672.

57. Schotman H, Karhinen L, Rabouille C (2008) dGRASP-mediated noncanonical integrin secretion is required for Drosophila epithelial remodeling. Dev Cell 14: 171–182.

58. Jackson CL (2009) Mechanisms of transport through the Golgi complex. J Cell Sci 122: 443–452.

59. Strating JR, Hafmans TG, Martens GJ (2009) Functional diversity among p24 subfamily members. Biol Cell 101: 207–219.