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Clathrin Heavy Chain Expression and Subcellular Distribution in Embryos of *Drosophila melanogaster*

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1. Introduction

Tubular organs are essential for organisms to establish transport systems for nutrients, liquids and gases. The development of tubes requires endocytosis of bound ligands, receptors and proteins at the plasma membrane (Bonifacino and Traub, 2003; Nelson, 2009). Clathrin coated vesicles (CCVs) organize major routes of cargo selective endocytosis in higher eukaryotic cells (Conner and Schmid, 2003). The formation of CCVs requires clathrin molecules. During CCV budding, clathrin molecules assemble to form a cage-like coat around the nascent vesicle membrane. Clathrin assembly is assisted by numerous adaptor proteins. After inward budding, CCV scission from the membrane is mediated by the large GTPase Dynamin. Released CCVs diffuse from the membrane and undergo uncoating, whereby Clathrin molecules disassemble from the vesicles. The uncoating process is mediated by the ATPase function of the Heat shock cognate protein (Hsc70), which interacts with Chc and DnaJ adaptor proteins. The released Clathrin molecules reassemble for subsequent rounds of endocytosis while vesicles fuse with acceptor compartments, such as early endosomes (Conner and Schmid, 2003; Kirchhausen, 2000; Ungewickell and Hinrichsen, 2007).

Clathrin is a three-dimensional array of so-called triskelia that possesses the intrinsic ability to form a cage-like lattice around the vesicles (Brodsky et al., 2001). The Clathrin triskelion, a three-legged structure, is composed of three clathrin-heavy chain (Chc) and three Clathrin-light chain (Clc) subunits. Thus, Chc provides a basic component of the Clathrin coat (ter Haar et al., 1998; Kirchhausen, 2000). Evolutionarily, Chc and Clc are highly conserved from yeast to human (Wakeham et al., 2005). In the human genome two isoforms of *chc* and *clc* have evolved by gene duplication (Wakeham et al., 2005). For example the human *clathrin heavy chain* comprises of *CHC17* (genomic location 17q23.2) and *CHC22* (genomic loccation 22q11.21), which show distinct expression patterns (Dodge et al., 1991; Sirotkin et al., 1996; Kedra et al., 1996; Long et al., 1996).

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Drosophila melanogaster is a well-established model organism to study gene and protein expression and function in tubular organs. During development of the *Drosophila* respiratory system, tracheal tube lumina undergo airway liquid-clearance, to enable liquid-air-transition at the end of embryogenesis. This occurs also in the vertebrate lung (Behr, 2010; Olver et al., 2004). Previously, we demonstrated in *Drosophila* the requirement of clathrin-mediated endocytosis for airway clearance and air-filling at the end of embryogenesis (Behr et al., 2007). However, though *Drosophila chc* gene function has been analyzed in a number of other genetic studies (reviewed in Fischer et al., 2006), the Chc expression, localization and dynamics remained elusive. Recently, we have characterized the *chc* mRNA and protein expression throughout *Drosophila* development (Wingen et al., 2009). In consistence with data of vertebrate Chc (Kirchhausen, 2000), we showed, using a specific purified anti-Chc antibody, Chc overlap with the trans-Golgi network, and co-localization with markers for early endocytosis (Wingen et al., 2009). In summary, the anti-Chc antibody is a new tool to analyze Clathrin heavy chain positive vesicles in *Drosophila.*

In order to analyze subcellular Clathrin distribution, we performed fluorescence labeling studies of endogenous Chc in *Drosophila* embryos. Immunofluorescent co-labeling studies demonstrate asymmetrical Chc distribution in epidermal cells and cells of tubular organs, such as the tracheal system, the salivary glands, and the gut. We show that Chc is enriched at the apical cell cortex and at the apical cell membrane, where it overlaps with the apical membrane organizer Crumbs (Crb). In consistence, we observed Chc mis-localization in airway cells of *crb* null and tracheal specific *crb* knock-down mutants. Furthermore, we show that the Crb-mediated apical membrane organization is involved in Chc-mediated airway-clearance at the end of embryogenesis. As Chc and Crb are highly conserved and broadly expressed in epithelial tissues (Wingen et al., 2009; Bulgakova and Knust, 2009), this new molecular mechanism of *crb* controlling apical Chc endocytosis is of general importance.

2. Results and discussion

In order to characterize Chc expression in *Drosophila* embryos, we used the anti-Chc antibody for immunofluorescent stainings on whole mount embryos. At late embryogenesis, stage 14 until stage 16, Chc was strongly enriched in the epidermis and tube forming organs, such as the foregut, the hindgut, the tracheal system and salivary glands (Fig. 1A-D).

At the end of embryogenesis, additional Chc enrichment was found in other organs, such as the midgut and secretory prothoracic glands (Fig. 1E,F). In *Drosophila,* foregut, hindgut, trachea, salivary glands and epidermis are of ectodermal origin. These organs are primary epithelia, which receive their epithelial character from the blastoderm epithelium (Tepass et al., 2001). Ectodermal epithelial cells display an asymmetric architecture of apical-basal polarity, where the apical cell membrane faces the tube lumen (Tepass et al., 2001).

In order to investigate subcellular Chc distribution, we analyzed immunofluorescent stainings, by using the anti-Chc antibody. In confocal sections of late *wild-type* embryos Chc was found in a vesicle-like punctuate pattern in the cell cortex as well as at distinct sites at the plasma membrane. This pattern was characteristic for cells of the foregut, hindgut, trachea, and salivary glands (Fig. 2A-D). Next, we generated confocal Z-stacks to generate three-dimensional projections of those organs. These projections revealed Chc accumulation at the apical cell cortex and plasma membrane (Figure 2A'-D'). In summary, the

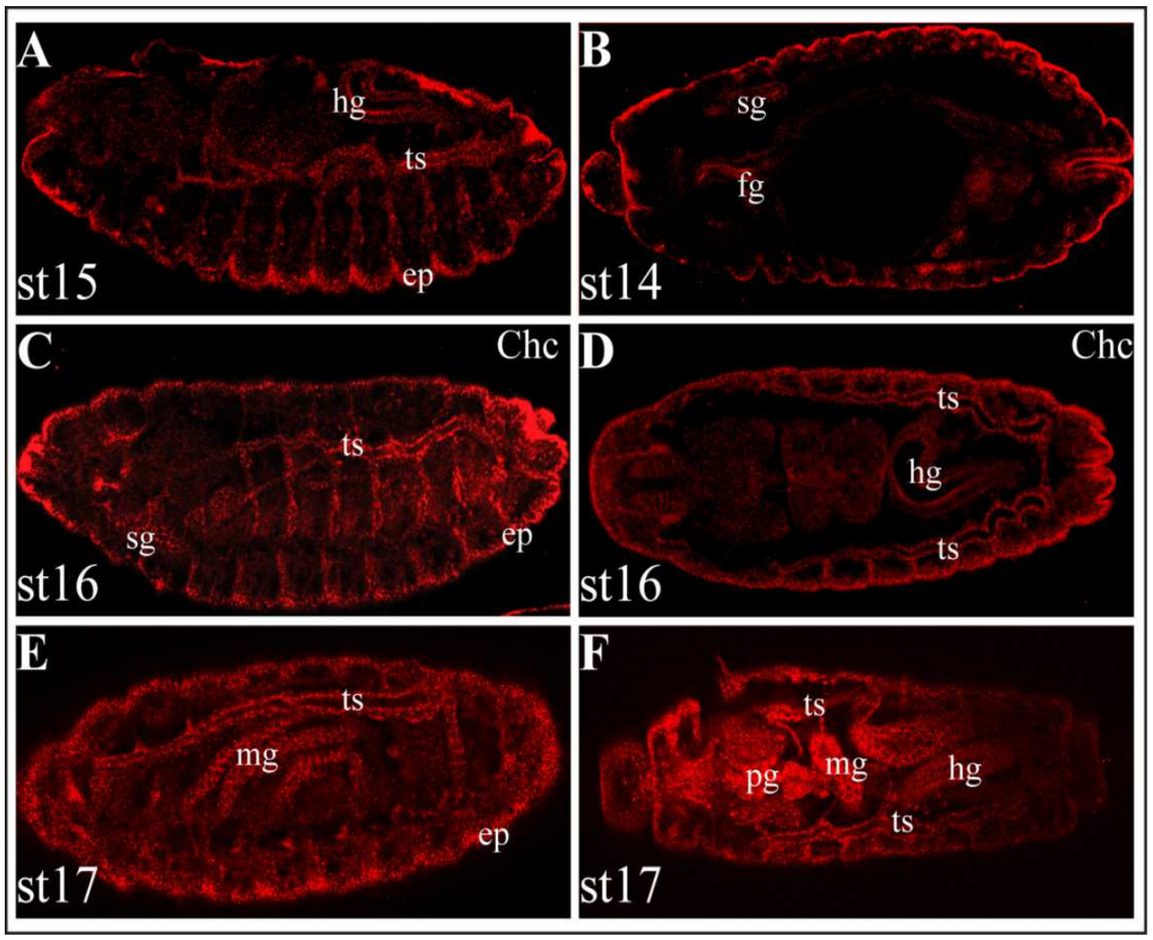

Fig. 1. **Chc is enriched in tube forming organs at late embryogenesis**.
(A-D) Confocal images of whole mount late embryos between stage 14 and stage 16. The left panels illustrate the lateral views, the right panels dorsal views of different embryos. All pictures here and in other Figures show anterior at the left. Immunofluorescent stainings using the anti-Chc antibody revealed strong Chc enrichment in ectodermally derived epidermis (ep) and tube forming epithelial organs, such as the tracheal system (ts), the hindgut (hg), the foregut (fg) and salivary glands (sg). **(E,F)** At the end of embryogenesis, at stage 17, additional Chc enrichment was detectable in the midgut (mg) and the prothoratic glands (pg).

asymmetrical distribution suggests that CCVs are most prominent at the apical membrane of tubular organs at late embryogenesis.

As Chc was apically enriched in tubular organs, we performed double immunofluorescent labeling studies, using anti-Chc together with an anti-Crb antibody, an apico-lateral cell membrane marker (Tepass and Knust, 1990; Wodarz et al., 1995). We analyzed single confocal sections of late *wild-type* embryos. In the cells of tubular organs, Chc accumulated adjacent to the Crb expressing apical cell membranes of foregut, hindgut, trachea, and salivary glands (Fig. 3A-D).

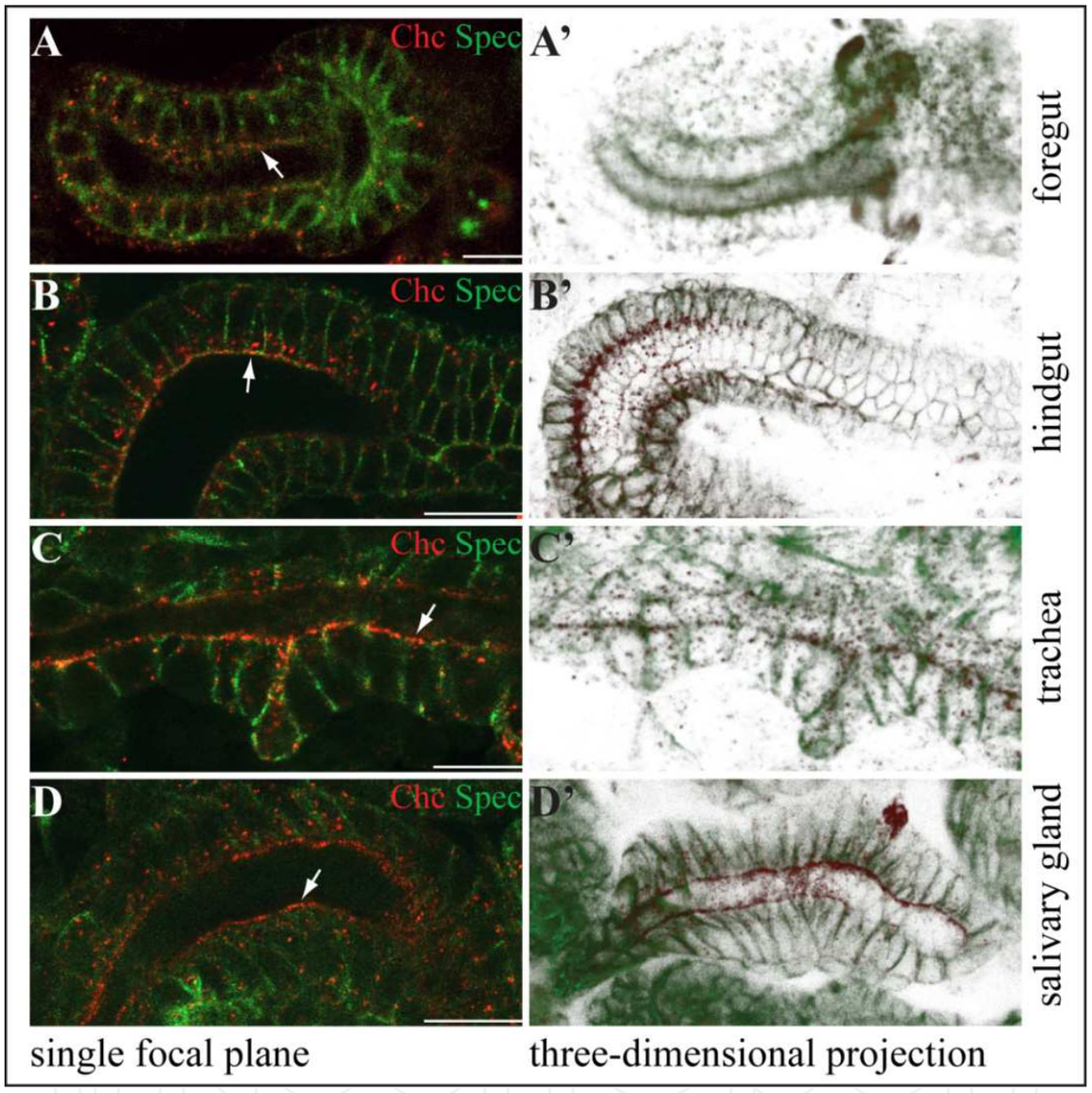

Fig. 2. **Chc vesicles are apically enriched in cells of tubular organs**.
(A-D) The left panels show confocal images of the tubes of foregut (A), hindgut (B), tracheal dorsal trunk (C), and salivary gland (D) of embryos at stage 15 (A,C) and 16 (B,D). **(A'-D')** The right panels illustrate three-dimensional projections of confocal Z-stacks across the tube of foregut (A'), hindgut (B'), tracheal dorsal main trunk (C'), and salivary gland (D'). Using anti-Chc (red) and anti-α-Spectrin (green; Pesacreta et al., 1989; cell membrane marker), images and projections show apical accumulation of Chc vesicles in the tubular organs. Arrows point to the apical membrane. Scale bar = 10μm.

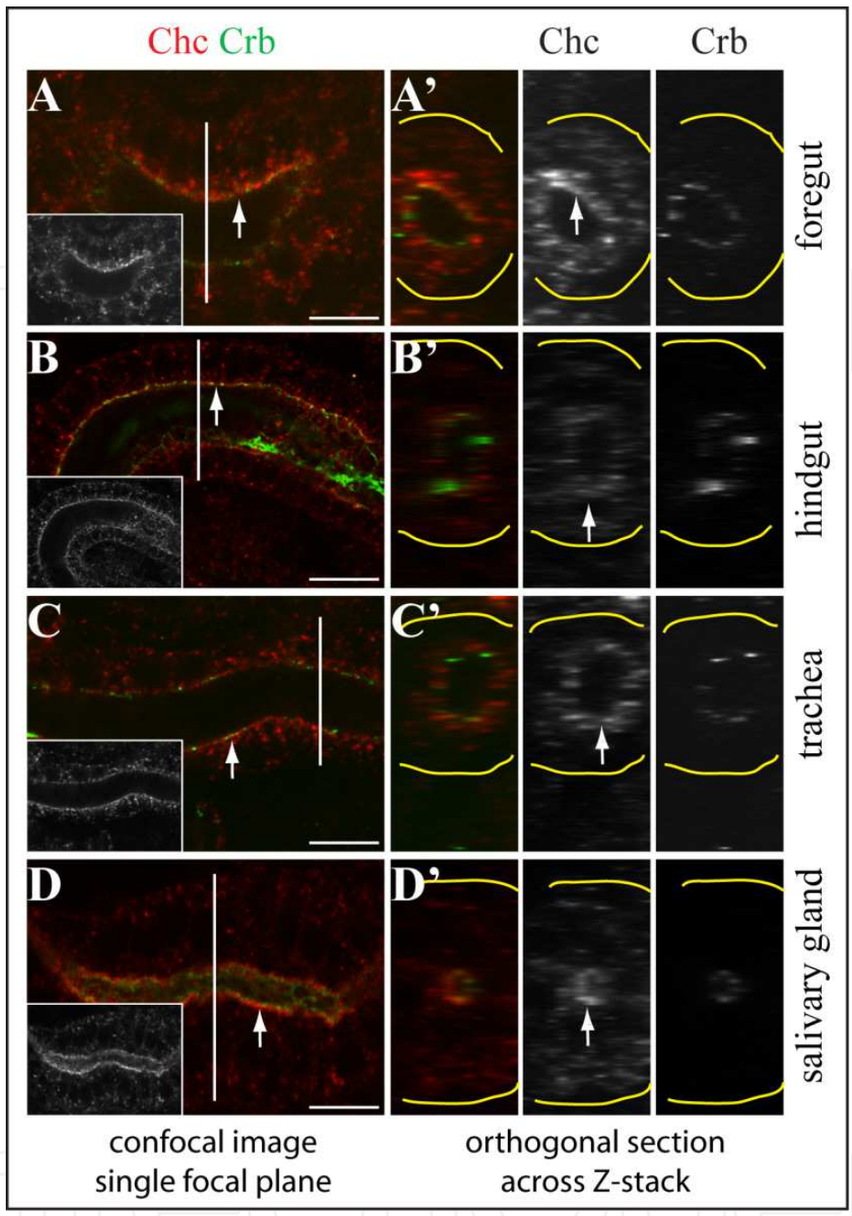

Fig. 3. **Chc vesicles are enriched at the apical cell membrane and apical cell cortex**. **(A-D)** The left panel shows confocal images across the tubes of foregut (A), hindgut (B), tracheal dorsal trunk (C), salivary gland (D) of embryos at stage 15. Arrows point to the apical membrane of tube lumina. The vertical white bars indicate the selected regions which were used for orthogonal projections across the entire tube. Inlays in A-D indicate single Chc stainings in grey. (A'-D') The right panel illustrates three-dimensional reconstructions of orthogonal section of confocal Z-stacks across the tube of foregut (A'), hindgut (B'), tracheal dorsal trunk (C'), salivary gland (D'). Using anti-Chc (red) and anti-Crb (green) antibodies, which mark the apical cell membrane, images and orthogonal projections, show apical accumulation of Chc positive vesicles (arrows) in the tubular organs. Yellow lines mark the basal cell membrane. Single orthogonal projections of Chc and Crb are indicated in grey in the right pannels. Scale bar = 10μm.

Next, we generated confocal Z-stacks, which were used for orthogonal projections and reconstruction of tube lumen and surrounding cells. Crb function during tracheal development has been recently studied. Crb is involved in determining apical polarity, apical membrane growth, cell-invagination, cell-intercalation, tube size control and airway liquid-clearance (Kerman et al., 2008; Laprise et al., 2010; Letizia et al., 2011; Stümpges and Behr, 2011). The orthogonal projection showed Chc enrichment at the Crb expressing membrane (Figure 3A'-D'). In summary, we have strong evidence, that Chc positive vesicles are asymmetrically distributed and accumulate at the apical cell cortex and cell membrane, which faces the tube lumen.

As *crb* null mutants show severe developmental defects of the tracheal system and other ectodermal tissues (Tepass and Knust, 1990), we tested tracheal specific *crb* knock-down embryos for Chc localization in tracheal cells. In *Drosophila,* organ specific expression experiments can be performed by the use of the UAS-GAL4 system (Brand and Perrimon, 1993).

In order to generate *crb* knock-down mutants, we mated flies bearing a UAS-RNAi-*crb* transgene with flies bearing the tracheal driver line *breathless*GAL4 (*btl*G4). This crossing resulted in a tracheal specific knock-down of *crb* (Stümpges and Behr, 2011) in the offspring. In *wild-type* embryos Chc staining is enriched at distinct sites towards the apical membrane of tracheal cells (Fig. 4A). In contrast, the tracheal *crb* knock-down, led to intracellular accumulation of the Chc staining in tracheal cells (Fig. 4B). Consistently, an intracellular accumulation of Chc staining was also observed in *crb* null mutant tracheal cells (Fig 4C). Next, we tested the Chc localization upon tracheal Crb overexpression, using the *btl*G4 driver and the UAS-*crb* full length transgene. Crb overexpression resulted in strong co-localization of Crb and Chc (Fig. 4D). These findings indicate that Crb is involved in the apical Chc localization.

As Chc and Crb are involved in airway liquid-clearance and air-filling (Behr et al., 2007; Stümpges and Behr, 2011), we tested whether they act together in this process. At the end of embryogenesis, airways undergo lumen clearance, which is accompanied by air-filling in order to enable respiration to conduct oxygen from spiracular openings to the internal tissues (Behr et al., 2007; Stümpges and Behr, 2011; Tsarouhas et al., 2007). Transition from liquid- to air-filled airways can be monitored *in vivo* by bright field microscopy in *wild-type* embryos (Fig. 5 A-A'''', Stümpges and Behr, 2011). In contrast, in *chc* and *crb* null mutants air-filling is defective (Fig. 5B; Behr et al., 2007; Behr et al., 2007; Stümpges and Behr, 2011). Next, we tested *chc* and *crb* genetic interaction for air-filling. One test for genetic interaction is the analysis of trans-heterozygous mutants. A 50% reduction of two genes, which interact in a common process, results in a phenotype, which cannot be observed in individual heterozygous animals. In contrast to *wild-type* and *chc* or *crb* heterozygous mutant embryos (Fig. 5A,D, not shown; Stümpges and Behr, 2011), severe air-filling defects were observed in the trans-heterozygous *chc* and *crb* mutants (Fig. 5 C,D). In summary, we provide evidence that *chc* and *crb* act in a common process for airway liquid-clearance and air-filling. Thus, the Crb-mediated Chc localization is involved in airway clearance and may result in air-filling defects upon mis-localization in *crb* mutants.

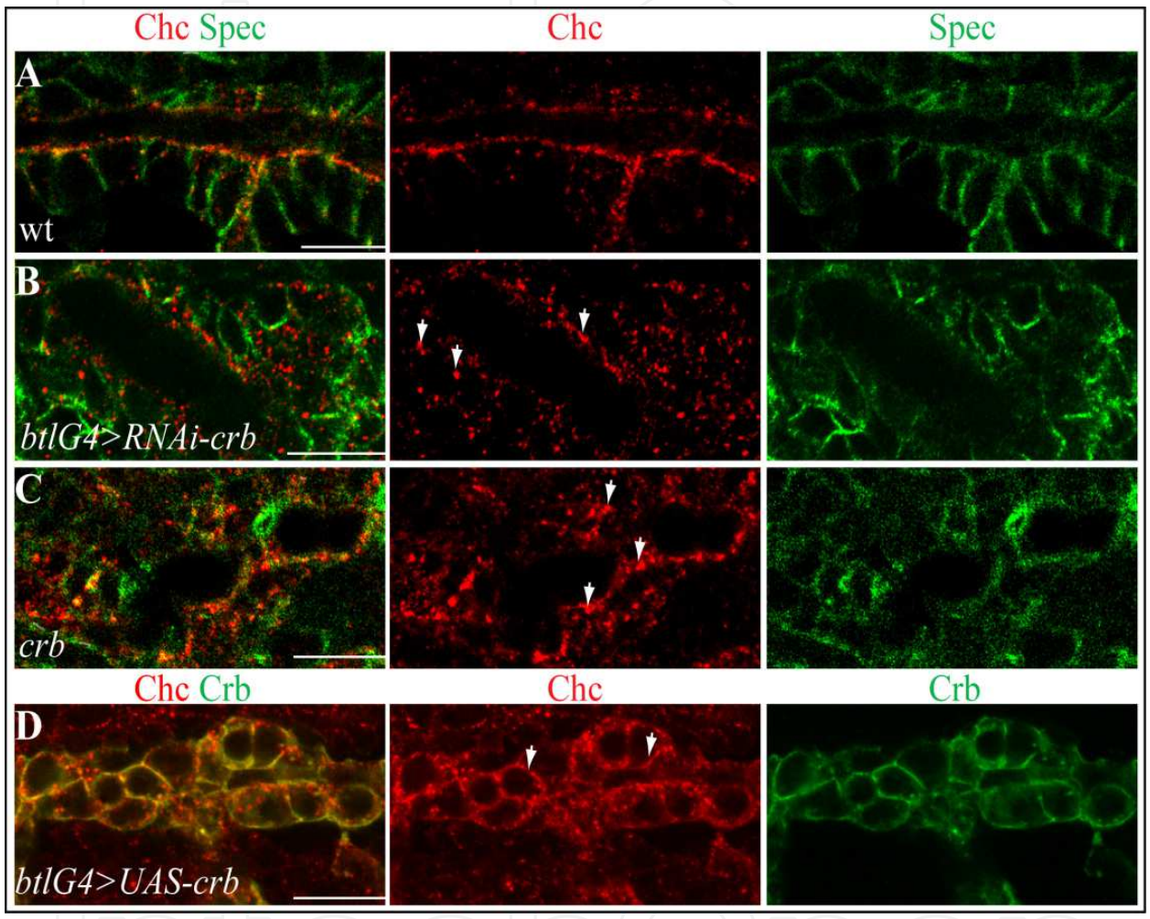

Fig. 4. **Chc mis-localization in *crb* knock-down and *crb* null mutants**.
Confocal immunofluorescent images of tracheal cells using the anti-Chc (red) and the anti α-Spectrin (green) and anti-Crb (green) antibodies. The α-Spectrin marks cell membranes and Crb indicates apical cell membranes. **(A)** In stage 17 *wild-type* embryos Chc (red) is distributed towards the apical cell membrane. **(B,C)** In stage 17 *btl*G4-driven UAS-RNAi-*crb* knock-down embryos and *crb* null mutant embryos, Chc showed intracellular mis-localization in tracheal cells (arrows). **(D)** Tracheal Crb overexpression led to intensive Chc co-localization with Crb (arrows). Scale bars=10μm.

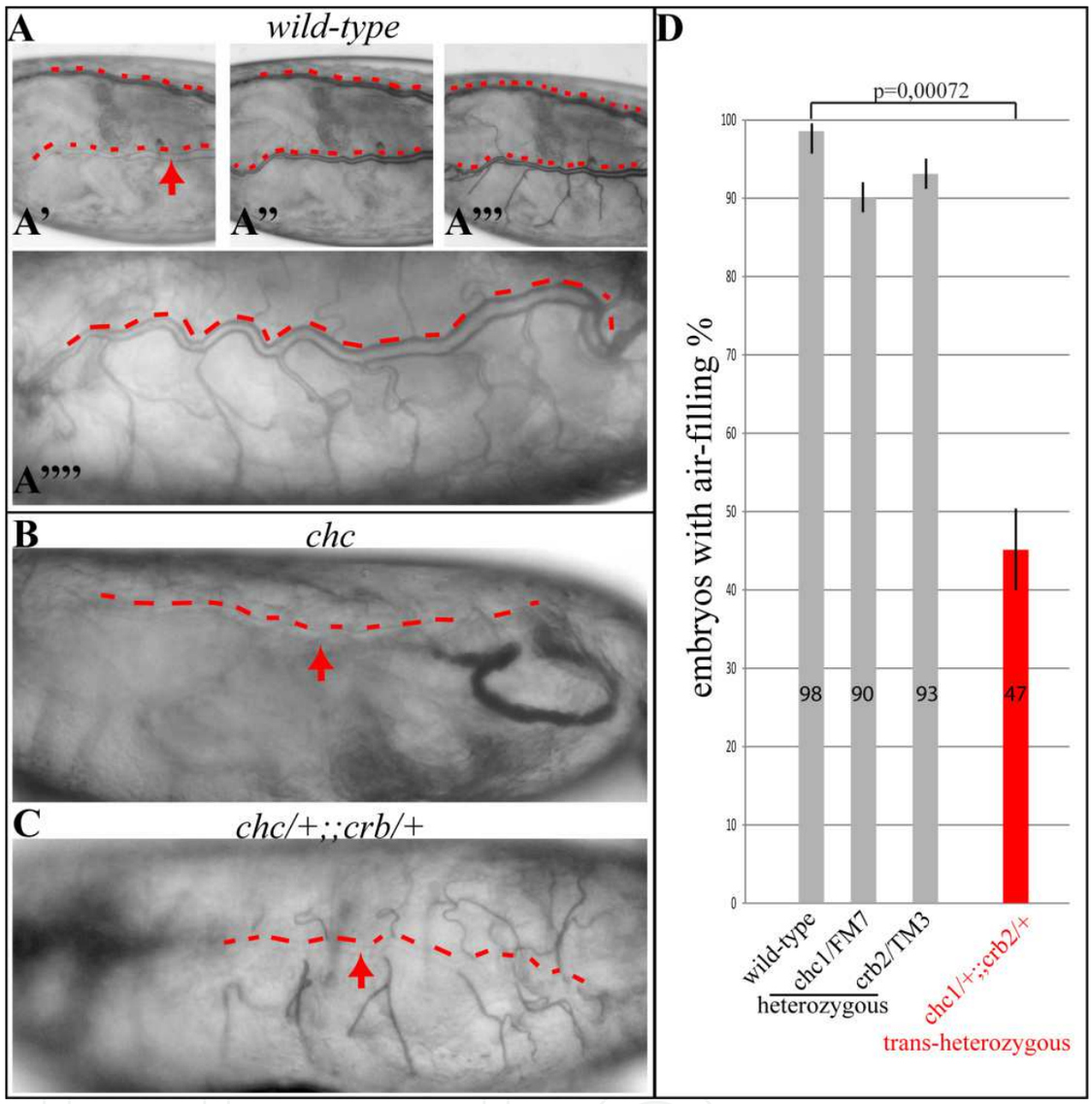

Fig. 5. ***chc* and *crb* trans-heterozygous embryos show defective air-filling.**
(A) Stage 17 *wild-type* embryos undergo airway liquid-clearance and accompanied air-filling. The liquid to air transition can be visualized due to different light diffractions by bright-field microscopy. Images A'-A''' show the air-filling process in a single embryo. Air-filling starts in the main dorsal trunks (indicated with red dashes) and spreads through the other airways. The arrow in A' points to a liquid-filled dorsal airway, where the air transition occurs later on (A''). **(B)** Stage 17 chc^{1} null mutant embryos failed for airway liquid-clearance and the accompanied air-filling. The main dorsal trunk is indicated with dashes, the arrow points to the liquid filled airway. **(C)** In stage 17 trans-heterozygous *chc* and *crb* mutant embryos airway liquid-clearance and accompanied air-filling is impaired. Red dashes indicate the main dorsal airways, the arrow points to the liquid filled airway. **(D)** The histogram shows the mean value of quantifications of air-filling in percentage. Standard deviations (bars) and important p-values (p) are indicated; analyzed embryos (n) = 601 (*wild-type*), 85 (*chc*/FM7), 296 (*crb*/TM3), 92 (trans-heterozygous *chc*/+;;*crb*/+).

3. Materials and methods

3.1 Antibodies

The following antibodies were used: anti-Chc (1:40, rat; Wingen et al., 2009), anti-Crumbs (1:10; mouse, Cq4; DSHB; Iowa, USA), anti-α-Spectrin (1:10, 3A9, DSHB; Iowa, USA). Primary antibodies were detected by secondary antibodies obtained from Molecular Probes (Alexa488- and Alexa546-conjugated).

3.2 Immunofluorescent labeling and confocal microscopy

For immunostainings embryos were dechorionated with 2.5% sodiumhypochloride (5 min) and fixed in 2ml 4% PFA (paraformaldehyde) and 3ml heptane for 20 min. Embryos were devitellinized in a mixture of 3ml heptane and 10ml methanol and stored in methanol at -20°C. Afterwards embryos were washed in PBT (PBS, Tween20). Primary antibodies were incubated at 4°C overnight and secondary antibodies were incubated at room temperature for two hours. Finally embryos were mounted in Vectashield (Vector Laboratories) and analyzed with a Zeiss LSM 710 confocal microscope (Zeiss MicroImaging GmbH, Jena, Germany). For confocal sections we used standard settings (Zeiss Zen software, pinhole airy 1 unit). Sequential scans of individual fluorochromes were performed to avoid cross-talk between the channels. Subcellular studies were analyzed by using a Zeiss 63x LCI Plan Neofluar objective. The confocal areas were scanned 16-times using a minimum scan time, suggested by the Zeiss-Zen software. Z-stacks were performed using the suggested optimized distance (between 0,5 - 1µm). The ZEN software was used for the projection of the orthogonal sectioning. Images were cropped and analyzed in Adobe Photoshop CS5; Figures were designed with Adobe Illustrator CS5.

3.3 Airway liquid-clearance and air-filling assay

Embryos were collected for 3 hours and grown at 25°C until stage 17. Embryos were dechorionated in 2.5% sodiumhypochloride for 5min, washed in distilled water and transferred to a thin apple-juice-agar layer. The living embryos were monitored for gas filling by brightfield-microscopy (Zeiss Axiovert) and documented with the Zeiss Axiovision software (release 7.1). The statistical analysis was performed with Microsoft Excel 2010. P-values were determined by using standard setting (2;2) in Excel 2010, which assume two data sets from distribution with same variants.

3.4 Fly stocks

The following fly stocks were obtained from the Bloomington stock center and are described in flybase (http://flybase.bio.indiana.edu/): w^{1118} (here referred to as *wild-type*), *btl*G4, chc^{1}, crb^{2}, UAS-$crb^{wt30.12e}$ were obtained by the Bloomington stock center. The *UAS-crbRNA*^{39178i} was obtained by the Vienna *Drosophila* RNAi stock center (Dietzl et al., 2007). For overexpression experiments, we used the Gal4/upstream activator sequence system and the tracheal specific *btl*G4 driver. For all experiments adequate balancer strains (FM7 and TM3) carrying a GFP transgene were used to recognize individual genotypes. For genetic interaction experiments, heterozygous *chc* mutant females, bearing the FM7-actinGFP, were mated with TM3-twistGFP balanced crb^{2} heterozygous males, in order to recognize the non GFP expressing trans-heterozygous animals.

4. Conclusion

We have analyzed the subcellular distribution of Chc in epithelial tube organs in *Drosophila* embryos. Our confocal analysis and three-dimensional reconstructions demonstrate the specific apical accumulation of Chc from stage 14 of embryogenesis onwards when the tracheal system, foregut, hindgut and salivary glands differentiate and mature for physiological functions. Genetic analysis shows that the apical membrane organizer Crb is involved in apical Chc distribution in tracheal cells and that normal Chc localization is required for airway liquid-clearance and air-filling at the end of embryogenesis. This is consistent with previous observations (Behr et al., 2007; Tsarouhas et al., 2007; Stümpges and Behr, 2011), suggesting that apical Clathrin-mediated endocytosis is essential for airway-clearance. Important roles of Clathrin-dependent endocytosis for the internalization of the cystic fibrosis transmembrane conductance regulator (CFTR) and for the activity of the epithelial sodium channels (ENaCs), which are involved in liquid-clearance in the vertebrate lung, have been shown (Lukacs et al., 1997; Shimkets et al., 1997). Thus, up-regulation and apical accumulation of Chc positive vesicles is essential for the development of the tracheal system and other tube forming organs. As Chc and Crb are highly conserved and broadly expressed in epithelial tissues (Wingen et al., 2009; Bulgakova and Knust, 2009), this new molecular mechanisms of *crb* controlled apical Chc endocytosis is of general importance.

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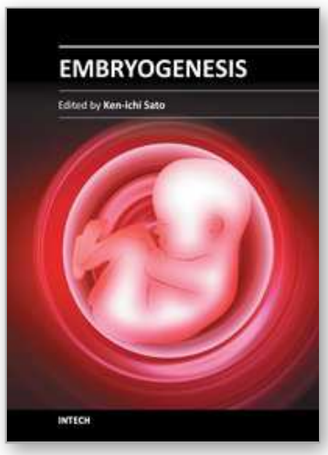

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