Wurst-Mediated Airway Clearance is Required for Postembryonic Development

Anna Lollies, Tamara Krsmanovic, Laura CA Jussen and Matthias Behr*

Life & Medical Sciences Institute (LIMES), Laboratory for Molecular Developmental Biology, University of Bonn, Carl-Troll-Strasse 31, 53115 Bonn, Germany

Abstract

Tubular organs are required for transport of liquids or gases. The *Drosophila* tracheal system and the human lungs need to undergo liquid to air transition to enable oxygen transport in the postembryonic or neonatal development respectively. In humans, a failure of airway liquid-clearance can lead to severe clinical syndromes, such as neonatal respiratory distress syndrome or transient tachypnea of newborn. However, the underlying molecular mechanisms are poorly understood. Previously, we identified the transmembrane J-domain protein Wurst as a key regulator of airway clearance in *Drosophila.* Wurst is involved in endocytosis and recruits clathrin and the associated Heat shock cognate protein 70 to the apical membrane. In *wurst* mutant embryos airways are oversized and airway liquid-clearance is impaired. Here we show that *wurst* expression in tracheal cells, but not in the respiratory openings, is sufficient to rescue airway clearance and air-filling. We found that only tracheal specific RNAi knock-down of *wurst* is essential for airway liquid-clearance in post embryonic stages resulting in severe growth arrest and early lethality. In the tracheal *wurst* knock-down, clathrin accumulated at the apical cell membrane. Cell culture assays identified Wurst co-localization with endocytic markers and co-clustering at the plasma membrane. In summary, our data characterize Wurst as a key regulator of airway liquid-clearance and physiology. Wurst is conserved and a single ortholog was identified in humans, termed DNAJC22. Thus, investigating the molecular function of Wurst/DNAJC22 in airway liquid-clearance will contribute to a better understanding of clinical relevant lung syndromes.

Keywords: Airways; Endocytosis; Extracellular matrix; Liquid-clearance; Respiratory distress; Tracheal system; Wurst/DNAJC22

Introduction

Lungs, the cardio-vascular system, kidneys and many other tube organs transport gases or fluids through a network of ramified tubular branches or vessels. Morphogenesis of branching and patterning of the respiratory tubes involves a number of genetic pathways [1]. Key signaling pathways and molecular mechanisms are evolutionarily conserved and are required for the development of respiratory systems among higher organisms [2,3]. The developing human lungs are net secreting organs where fluids in the tube lumina control organ size [4,5]. Before birth, the lung epithelium switches to net absorption, resulting in a fast process of airway liquid-clearance, which is essential for respiration of newborns. Defects in airway liquid-clearance have the potential to cause severe clinical syndromes, such as transient tachypnea of newborn (TTN) and the neonatal respiratory distress syndrome (nRDS) [4,5]. Furthermore, it has been discussed that edema liquid-clearance may require similar molecular mechanisms. However, little is known about molecular mechanisms underlying airway and edema liquid-clearance.

Drosophila melanogaster represents an ideal model organism for studying genetic and molecular mechanisms of a number of human diseases such as Alzheimer, diabetes and cancer [6,7]. The advantages of fast forward genetics, spatial and temporal specific over expression studies and a short generation time of the fly represent optimal preconditions to analyze conserved mechanisms and pathways involved in airway physiology. The *Drosophila* tracheal system serves as a prototypical model of genetically ordered branching [2,8]. It arises from epidermal cell clusters that develop a highly ordered stereotyped pattern of tubular branches during the second half of embryogenesis. Several conserved signaling pathways, like Fibroblast growth factor (FGF), Epidermal growth factor (EGF), wingless (wnt) and Notch signaling control tracheal branching [8-10]. In addition, extracellular matrix (ECM) components are secreted into the forming tube lumina. ECM components form a transient luminal cylinder in order to limit the tube size and to coordinate uniform tube lumen diameter expansion [10,11]. At the end of embryogenesis, *Drosophila* airways need to clear the luminal ECM cylinder. In addition, airway liquid-clearance enables accompanied air-filling [11,12]. Previously we showed that Wurst is required for embryonic airway protein and liquid clearance [13,14]. Wurst encodes for a transmembrane protein which contains a well-conserved DnaJ-domain and a clathrin binding motif at the C-terminus [13]. Single orthologs with a similar domain structure have been identified in vertebrates, referred to as DNAJC22 (DNAJC-domain at the C-terminus, number 22; [*H. sapiens*-NP_079178.2; *P. troglodytes*-XP_001158376.1; *C. lupus*-XP_543683.2; *B. teurus*-NP_001069169.1; *M. musculus*-NP_789805.1; *R. norvegicus*-NP_001014226.1; *D. rerio*-XP_001335380.1]) [15]. *Drosophila* Wurst interacts with clathrin and the clathrin coat component Heat shock cognate protein (Hsc) 70-4 and recruits both to the apical cell membrane [13,14]. RNAi-mediated Wurst knock-down resulted in inhibition of endocytosis in *Drosophila* Schneider (SL2) cells [13]. Genetic studies in epidermal tissues revealed a mutual dependency of Wurst and Clathrin heavy chain (Chc) [13], which is a key component of the clathrin coat [16]. Here we investigate

the role of Wurst in tracheal cells as a key modulator of airway liquid-clearance and its importance for postembryonic development.

Materials and Methods

Immunofluorescence studies, embryo fixation and air-filling assay

Embryos were dechorionated with 2.5% sodium hypochlorite (5 min) and fixed in 2ml 4% PFA (paraformaldehyde) and 3ml heptane for 20 minutes. Afterwards they were devitellinized (3ml heptane and 10ml methanol). Fluorescence immunolabeling and the RNA *in situ* hybridization studies were performed as described in [17]. The fluorescein-conjugated Cbp (1:100, New England Biolabs) is a recombinant fusion protein that binds specifically to chitin. We used the following antibodies: anti-clathrin (goat, 1:50; Sigma, [13]); anti-Chc (rat, 1:40 [17]); anti-Hsc70 (mouse, 1:100, Stressgene [13]); anti-RFP (rabbit, 1:50); anti-Wurst C (guinea pig, 1:40 [13,14]). For co-localization assays with anti-Chc a new anti-Wurst N antibody was generated (guinea pig) recognizing the N-terminus [GKDKTNDTSKGSPSKAKSNG]. After final bleeding, the anti-Wurst antibody was affinity-purified by using the N-terminal peptide and tested in *wild-type* and in Wurst over expression embryos (not shown) and by peptide competition (Supplementary Figure S1). The anti-Wurst N and the anti-Wurst C [13] antibodies detect an identical expression pattern in *wild-type* embryos. UAS-GAL4 mediated over expression of Wurst was detected by the anti-Wurst N antibody. In summary, our data show, that the antibody recognizes the N-terminus of the Wurst protein. The immune fluorescence studies were performed with a Zeiss Axiovert (SL2 cells and membrane sheets). Confocal images were achieved by using a Zeiss LSM710. Each fluorochrome was scanned in single optical sections. For confocal microscopy Pinhole "airy 1" Zeiss standard settings were employed using a Zeiss 63x Pan Neofluar objective. Images were analyzed using Axiovision and ZEN software and cut in Photoshop CS5. Figures were prepared with Illustrator CS5. For analyzing air-filling, dechorionated embryos at stage 17 were monitored by bright-field microscopy using a Zeiss Axiovert and the Axiovision software. The detailed procedure is described in [14,17].

Generation of the UAS-Wurst-*red fluorescent protein* (*rfp*) fusion construct

A UAS-*Wurst-rfp* transgene was generated as follows. The Wurst open reading frame was amplified by PCR using the primer pair [forward primer: GAATTCGCCTCTCTTTCTCCAATTCTTCGC; reverse primer: GGTACCTTAAGGACGACGATGGCCTCCTCCTC] and the cDNA LD21896.The PCR product was digested with EcoRI and KpnI and cloned in frame in front of an *rfp* open reading frame. PCR products were confirmed by sequencing. The resulting Wurst-*rfp* cassette was then digested with EcoRI and NotI before cloning it into the pUAST vector. Transgenic lines were established by germ line transformation and tested *in vivo* for RFP expression and for Wurst expression by immune fluorescent labeling studies using the anti-Wurst antibodies.

Fly stocks, rescue experiment and cell culture

Fly stocks were obtained from the Bloomington (http://flystocks.bio.indiana.edu/) and Vienna (www.vdrc.at) stock centers: w^{1118} (referred to as *wild-type*), *breathless* GAL4, *decapentaplegic* GAL4, *paired* GAL4; UAS-*WurstRNAi$^{#110270}$* [14]. *Wurst$^{l(1)G0162}$* is described in [13]. The Wurst gene locus is located at the first (X)-chromosome. The *Wurst$^{l(1)G0162}$*/Ymales are embryonic lethal and show impaired air-filling. For the rescue experiment, Wurst heterozygous females which
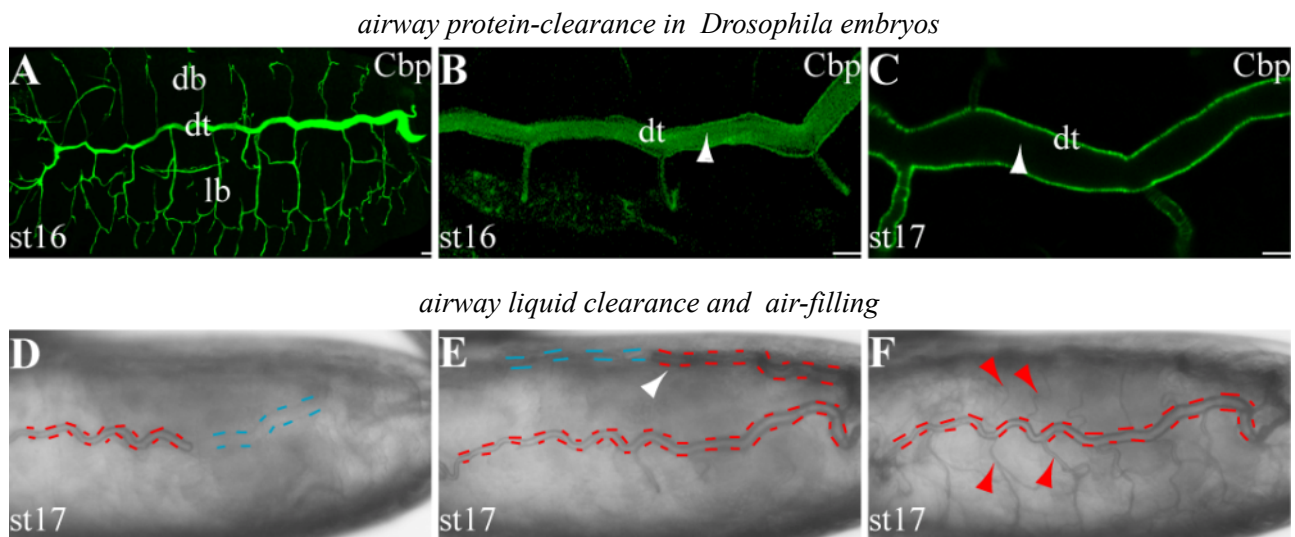

Figure 1: Airway clearance in *Drosophila* embryos

Confocal images (A-C) show whole mount (A) and magnifications of the trachea (B,C) at stage 16 and stage 17 embryos. For detecting tracheal tube lumina the fluorescein conjugated chitin-binding-probe (Cbp) was used. Cbp recognizes chitin. (A) Three-dimensional projection of confocal Z-stacks across the tracheal system visualizes chitinous tracheal tubes. The main airways are indicated as dorsal trunk (dt), dorsal branches (db), lateral branches (lb). (B) In stage 16 embryos single confocal sections show that chitin is localized in a transient luminal cylinder-like matrix (arrowheads), which controls tube expansion. (C) At the end of embryogenesis, at stage 17, airway lumina (arrowhead) are cleared from the chitin cylinder. Scale bars represent 10 μm. (D-F) At stage 17, embryos undergo airway liquid-clearance and air-filling. This process can be detected due to different light diffractions by bright-field microscopy. Red dashes indicate air-filled airways, blue dashes liquid-filled lumina. The air-filling process starts in the mid of one dorsal trunk (D), expands along the main airway including also the other hemisphere (E, white arrowhead) until all airways are aerated (F). Red arrowheads point to small dorsal and lateral tracheal tubes. All images show anterior to the left and dorsal to the top.

contain a UAS-*wurst-rfp* construct at the second chromosome were mated with males bearing the desired GAL4 driver construct. In the progeny *Wurst*[l(1)G0162]/Ymales were tested for Wurst-RFP expression and air-filling. For all experiments balancer strains (FM7) with GFP were used to identify individual genotypes.

For cell culture experiments *Drosophila* SL2 cells were weekly subcultured (3x10⁶ cells/ml) at 25°C in *Drosophila* Schneider Medium (Invitrogen), 10% fetal bovine serum (heat inactivated) and 1% penicillin/streptomycin (Sigma). For generating SL2 membrane sheets cells were grown on a Poly-L-lysine coated cover slip. For sonication, a petri dish was used as sonication chamber containing ice-cold sonication buffer. A sonication pulse was applied for 1 sec. The resulting membrane sheets were analyzed with TMA-DPH, a phospholipid membrane marker, and by fluorescence microscopy and were afterwards fixed in PFA. For immunofluorescent labeling studies

Figure 2: Wurst-mediated airway clearance is tracheal specific and independent of posterior spiracles
Bright-field *in vivo* microscopy (A,B,F,H) of late embryos at stage 17. Red dashes indicate air-filled, blue dashes liquid-filled airways. Confocal (C,D) and *in vivo* fluorescence microscopy (E,G) images of stage 16 embryos. (A) *Wild*-type embryos undergo air-filling (B) *wurst*[l(1)G0162] mutant embryos lack air-filling (C) RNA *in situ* hybridization reveals *wurst* transcript expression in the foregut (fg), hindgut (hg), epidermis (ep), tracheal system (ts) and posterior spiracles (psp). (D) Wurst and RFP were specifically expressed under the control of the UAS-GAL4 system. Immunofluorescence studies using anti-Wurst and anti-RFP antibodies reveal ectopic Wurst and RFP co-expression (orange, arrowheads) in the characteristic stripe pattern of the *paired* GAL4 driver line. Note the additional endogenous Wurst (green) expression in tracheal cells and posterior spiracles (arrows). (E,F) The failure of air-filling in *wurst*[l(1)G0162] mutants can be rescued by ectopically expressing *wurst* in tracheal cells, using the tracheal specific *btl*G4 driver line. Arrowheads in E point to RFP expression in the tracheal system (ts). (G,H) The failure of air-filling in *wurst*[l(1)G0162] mutants cannot be rescued by ectopically expressing *wurst* in posterior spiracle (psp) cells, using the *dpp*G4 driver line. Arrowheads in G point to the RFP expression in psp.

anti-Wurst C [13] was combined with aniti-clathrin or anti-Hsc70 antibodies and anti-Wurst N was combined with anti-Chc antibody.

Results

Wurst, a key regulator of airway clearance and air-filling

The tracheal tube lumina of *Drosophila wild-type* embryos contain a cylindrical extracellular chitinous matrix (Figure 1A,B). At the end of embryogenesis (stage 17), the chitinous-ECM and liquids are cleared from the tube lumina (Figure 1C) [13], enabling gas filling. The liquid-clearance and accompanied air-filling process can be monitored *in vivo* by bright-field microscopy due to differences in light diffraction caused by transition from liquid- to gas-filled airways [14]. During aeration, air expands from the mid of the dorsal main airway along the dorsal most posterior branch, enters the main airway of the other hemisphere and then extends into smaller airways (Figure 1D-F). Shortly after air-filling is completed, the larva hatches and conducts oxygen between the respiratory openings, the posterior spiracles and internal tissues. In a previous screen for regulators of airway physiology, we found that *Wurst*[l(1)G0162] (transposable P-element induced) [13] mutant embryos lack air-filling, when compared to *wild-type* embryos (Figure 2A,B) [13,14].

We tested the Wurst gene expression by antisense RNA hybridization. The Wurst transcript was strongly expressed in tracheal cells as well as in the posterior spiracles (Figure 2C) which are the only openings that connect the tracheal branches with the environment for gas exchange during early stages of post embryonic development. The *Wurst*[l(1)G0162] mutation affects all Wurst expressing organs, including trachea and posterior spiracles. In order to determine the importance of Wurst for airway clearance, we addressed the question whether organ specific Wurst expression is able to rescue *Wurst*[l(1)G0162] mutant air-filling defects. In *Drosophila*, the binary UAS-GAL4 system [18] offers ectopic gene expression in a spatial and temporal manner. In order to perform rescue experiments, we generated a transgenic fly line bearing a UAS-*Wurst-rfp* (*red fluorescence protein*) transgene. The expression of the transgene was tested *in vivo* by mating UAS-*Wurst-rfp* flies with transgenic *paired* GAL4 flies, which express GAL4 in a characteristic stripe-like pattern. Immunofluorescent confocal analysis of the offspring embryos demonstrated the efficiency of the ectopic Wurst and RFP expression in the expected paired pattern (Figure 2D). The driver line *breathless* GAL4 (*btl*G4) was sufficient to express the *Wurst-rfp* transgene specifically in all tracheal cells. Those embryos did not show obvious defects in tracheal morphology or air-filling (not shown). Next, we ectopically expressed the *Wurst-rfp* transgene with *btl*G4 in tracheal cells of *Wurst*[l(1)G0162] mutant embryos (Figure 2E). Strikingly, the resulting Wurst-RFP expression converted tracheal *Wurst*[l(1)G0162] mutant defects into *wild-type*-like air-filling (Figure 2F). In contrast, ectopic Wurst-RFP expression in cells of posterior spiracle in the *Wurst*[l(1)G0162] mutants did not rescue air-filling defects (Figure 2G,H). This demonstrates that only tracheal specific Wurst expression was able to rescue the airway liquid-clearance and air-filling defects in the *Wurst*[l(1)G0162] mutants.

Next, we tested organ specific Wurst knock-down for liquid-clearance and air-filling defects. We combined the UAS-GAL4 system with RNAi (interference) technology. For tracheal specific knock-down, we mated *btl*G4 flies with flies bearing a UAS-Wurst-RNAi transgene. In line with recent data [14], the offspring embryos showed strong Wurst reduction in tracheal cells and revealed severe air-filling defects when compared to *wild-type* (Figure 3A,B). Next we generated RNAi-mediated Wurst knock-down in cells of the posterior spiracle
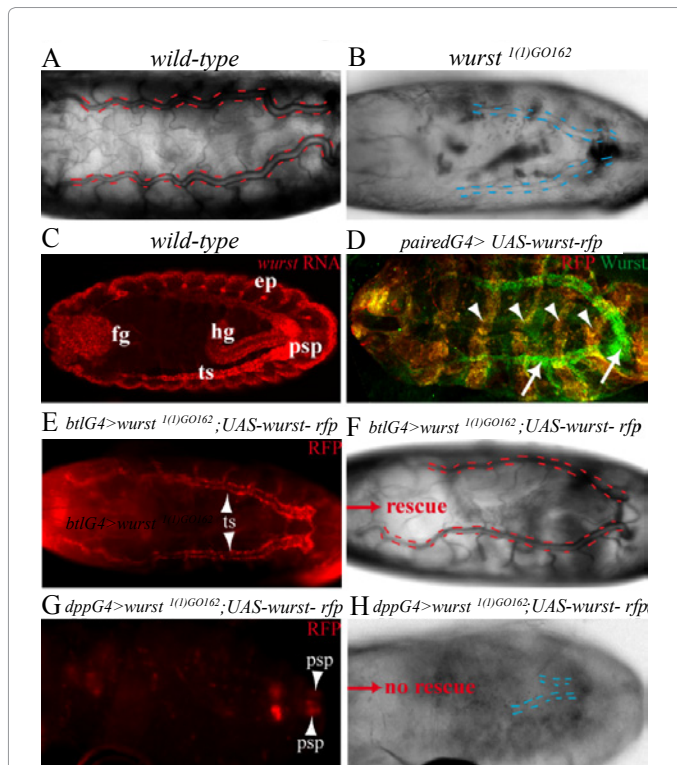
using the organ specific driver line *decapentaplegic* GAL4 (*dpp*G4) [19]. These embryos showed *wild-type* like air-filling (Figure 3C). In summary, we characterized Wurst as key regulator of tracheal airway physiology in *Drosophila*. Our findings show the importance of Wurst expression for airway liquid-clearance and air-filling. Wurst controlled liquid-clearance is restricted to tracheal cells, while its role in the posterior spiracles as respiratory openings remains elusive.

Wurst coordinated airway clearance is essential for growth control and survival

Wild-type embryos and larvae usually survive small periods of hypoxia by rapid and reversible responses [20]. In order to assess the importance of Wurst-mediated airway clearance for tracheal function as an oxygen delivery network, we monitored tracheal specific RNAi Wurst knock-down animals throughout development. *Wild-type* and animals with Wurst knock-down in posterior spiracles develop normally and finally hatch as adult flies (Figure 3D). In contrast, all tracheal specific RNAi-mediated Wurst knock-down animals died as larvae, in most cases even within the first larval stage (48 hours after egg laying [AEL], Figure 3D). These findings show that Wurst function in tracheal cells, enabling larval respiration, is essential for postembryonic development.

Hypoxic conditions have effects on larval mass gain and trigger the arrest of the developmental progress [20,21]. Next, we tested whether failure of air-filling in the tracheal Wurst knock-down mutants has any impact on larval growth and body size control (Figure 3E,F). We monitored freshly hatched *wild-type* and tracheal specific RNAi-mediated Wurst knock-down animals throughout larval development.

The *wild-type* animals dramatically increased their body size and showed darkened, strongly evaginated posterior spiracles until they reached the final larval stage (96 hours AEL; Figure 3E). The Wurst knock-down larvae showed no air-filling but crawled and ingested food like control animals (not shown). Although we observed food uptake, tracheal Wurst knock-down mutants failed to expand their body size. In addition, morphology and brightness of the posterior spiracles indicate the developmental arrest of Wurst knock-down mutants in early larval stages (Figure 3F). In summary, our findings show that embryonic airway liquid-clearance and air-filling is essential for post-embryonic development. The inhibition of larvae respiration in Wurst knock down animals causes developmental arrest and early lethality.

Wurst is required for clathrin-mediated endocytosis

Analysis of the transcript (Figure 2C) identified a broad Wurst expression pattern in epithelial organs which is similar to the recently identified expression pattern of Chc [17]. In co-labeling studies using specific antibodies, we found a strong overlap of Wurst with Clathrin and Hsc70 in *Drosophila* SL2 cells (Figure 4A,B). In order to analyze Wurst localization in the plasma membrane, we treated the SL2 cells by a previously described method [22], where cells can be grown on cover slips and subjected to brief sonication. This produces layers of plasma membranes, so called membrane sheets, which are devoid of cell constituents [22,23]. Immunofluorescent studies showed that Wurst appears small clusters in distinct regions of the SL2 membrane sheets (Figure 4C). Co-labeling studies identified overlap of Wurst clusters with Chc (Figure 4D). Next, we studied Wurst function in clathrin-mediated endocytosis in *Drosophila* embryos. In *wild-type* tracheal cells
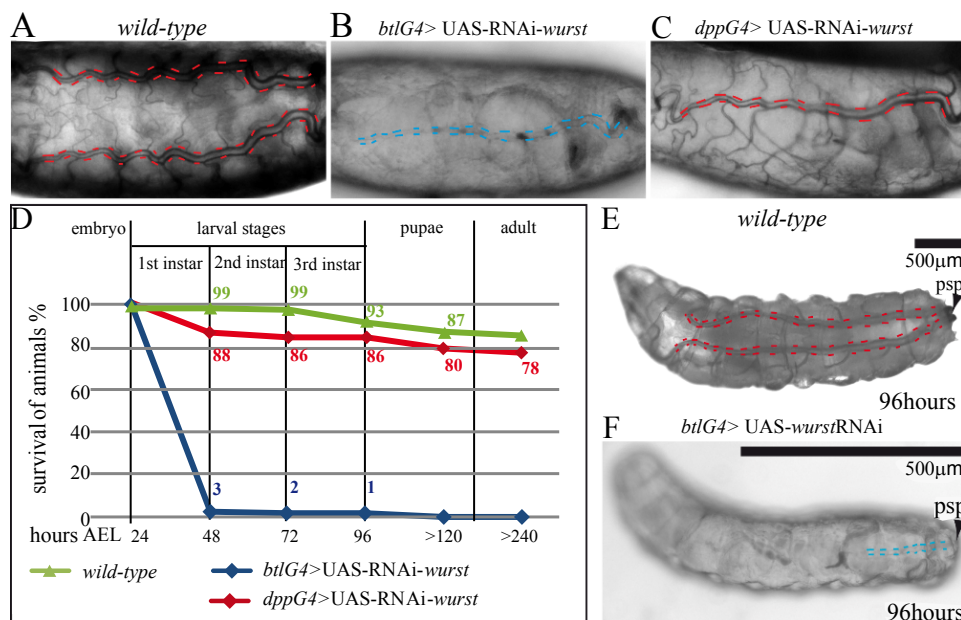

Figure 3: Wurst-mediated airway clearance is essential for developmental progress
Images show bright-field microscopy of living embryos at stage 17. Red dashes indicate air-filled, blue dashes liquid-filled airways. (A) *Wild-type* embryos undergo airway liquid-clearance and air-filling (B) Tracheal specific RNAi-mediated wurst knock-down resulted in severe air-filling defects (C) In contrast, posterior spiracles (respiratory openings) specific RNAi-mediated *wurst* knock-down did not lead to air-filling defects. (D) Histogram indicates survival in percentage of animals throughout development AEL. In contrast to *wild-type*, the tracheal specific RNAi-mediated *wurst* knock-down is lethal to early larvae. The RNAi-mediated *wurst* knock-down in posterior spiracles is not lethal, when compared to *wild-type*. Values in percentage are indicated in green, red and blue. Tested animals: *wild-type* n=300; *dpp*G4>UAS-wurst RNAi n=248; *btl*G4>UAS-wurstRNAi n=492. (E,F) Images display *wild-type* (E) and tracheal specific wurstRNAi knock-down (F) larvae about 96 hours AEL. Black bars indicate 500μm. *Wild-type*, which reaches the final larval stage, shows characteristic darkening and evagination of posterior spiracles. In contrast, the development of RNAi-*wurst* knock-down larvae is arrested. The body size is smaller, when compared to *wild-type*, and darkening of the posterior spiracles is not observed in the *wurst* knock-down mutants.
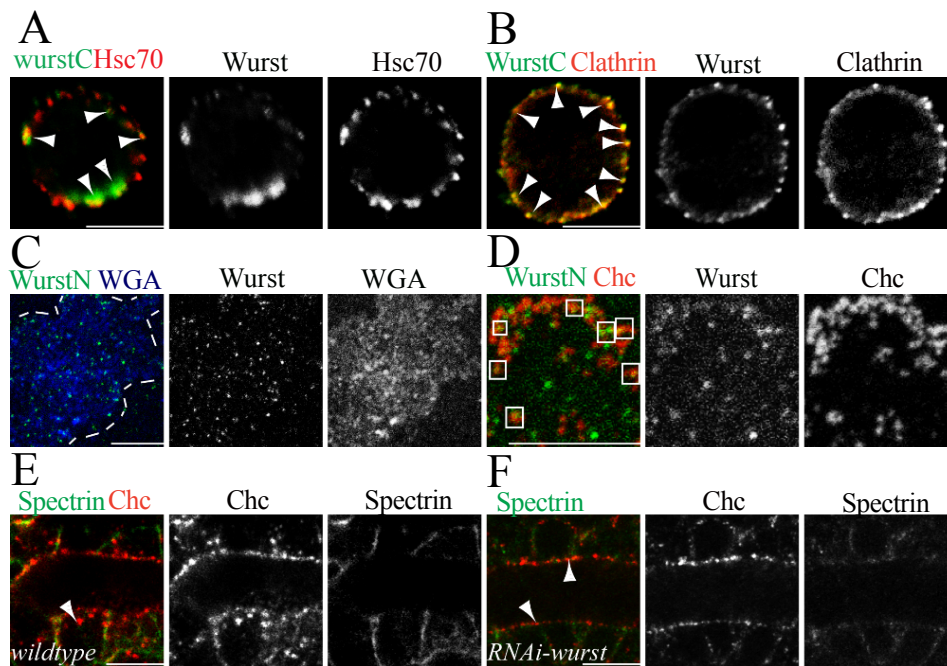
Figure 4: Wurst is involved in clathrin-mediated endocytosis
(A-D) Immunofluorescent analysis using anti-clathrin, anti-Chc, anti-Hsc70, anti-WurstC (recognizing the C-terminus [13]), and anti-WurstN (recognizing the N-termi-nus, see materials and methods) antibodies in *Drosophila* SL2 cells (A,B) and in membrane sheets of SL2 cells (C,D). The anti-clathrin antibody recognizes Chc and Clathrin light chain. (A,B) Wurst is localized in a vesicular like pattern in SL2 cells, which partially co-localize (arrowheads) with Hsc70 (A) and clathrin (B). (C) Wurst appears distributed in small clusters in the membrane sheet of a SL2 cell. Wheat germ agglutinin (WGA) selectively recognizes sugar residues at the plasma membrane surface. White dashes mark the membrane sheet. (D) Magnification of the membrane sheet reveals Wurst (green) and Chc (red) overlap (rectangles). (E,F) Confocal images using anti-Chc and anti-α-Spectrin antibodies in *Drosophila* stage 17 embryos. The α-Spectrin marks cell membranes. In *wild-type* tracheal cells Chc is found at the membrane and in addition in the cytoplasmic cell cortex (arrowhead in E). In contrast, in *btl*G4 driven UAS-RNAi-*wurst* tracheal specific knock-down embryos, Chc is accumulated at the apical plasma membrane of tracheal cells (arrowheads in F). Scale bars indicate 5µm.
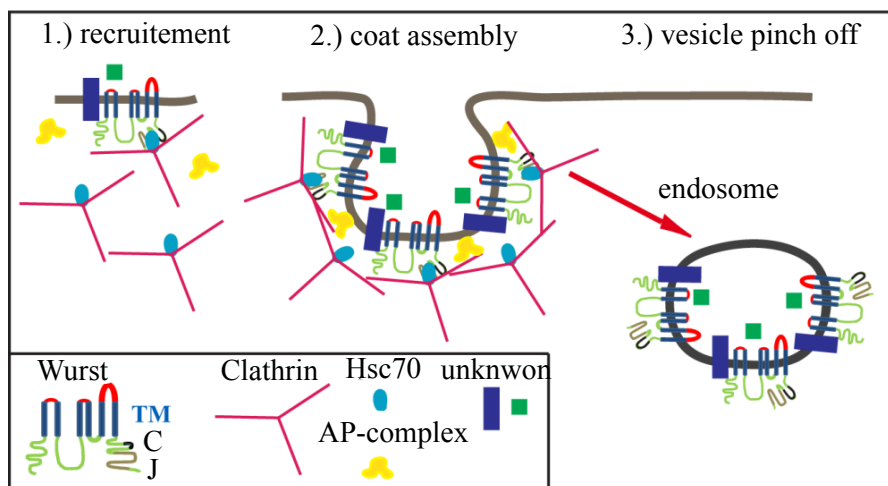

Figure 5: The evolutionarily conserved DnaJ-domain protein Wurst is involved in endocytosis
(A) The cartoon illustrates the putative role of Wurst in endocytosis of clathrin-coated vesicles. The predicted Wurst structure contains six transmembrane domains (TM; blue), a clathrin-binding motif (C; black) and a J-domain (J; grey).Wurst cytoplasmic parts are indicated in green, the extracellular in red. Clathrin assembles a coat-like structure around the nascent vesicle, aided by adaptor proteins (AP-complex). After vesicles scission, clathrin disassembles and can be reused for a new round of endocytosis. The uncoated vesicle pinches off and fuses with acceptor compartments such as early endosomes. Our data indicate that Wurst recruits clathrin and Hsc70-4 to the apical membrane. In contrast to clathrin and Hsc70-4, Wurst remains in the vesicle, which fuses to acceptor compartments, such as early endosomes. Thereby, Wurst may coordinate the endocytosis of unknown factors, which are involved in airway liquid-clearance.

Chc is predominantly localized at the apical plasma membrane as well as in the cortical cytoplasm (Figure 4E). In contrast, the organ specific Wurst knock-down in tracheal cells resulted in Chc accumulation only at the apical membrane (Figure 4F). In summary, these data identify

Wurst as an essential component of the clathrin-mediated endocytosis in tracheal cells.

Discussion

Our results demonstrate the importance of Wurst in airway clearance and air-filling. Tissue specific rescue and RNAi-mediated Wurst knock-down experiments provided evidence that Wurst is specifically required in the differentiated tracheal cells and not in the spiracles as putative openings for air-filling at the end of embryogenesis. Moreover, the knock-down experiments showed the specific tracheal importance of Wurst function also for postembryonic air-filling, for developmental growth control and finally for survival. Furthermore co-localization assays in SL2 cells are in line with supposed Wurst function for clathrin-mediated endocytosis [13]. In addition, membrane sheet assays indicate that Wurst appears in clusters in the cell membrane and overlaps with Chc. Tissue specific knock-down experiments finally demonstrate the dependency of Chc on Wurst function in tracheal cells at the end of embryogenesis, when airway clearance occurs. We propose a model (Figure 5) showing how Wurst may control the endocytosis of ECM matrix components for degradation thereby clearing the airway lumina at the end of embryogenesis. In addition, regarding its key function for airway liquid-clearance [14], we suggest that Wurst is involved in the endocytosis of channels, which are required for the liquid-clearance process. Epithelial Sodium Channels (ENaCs) are located at the apical membrane and establish a Sodium gradient, which regulates liquid absorption from airway lumina into the interstitium [5]. The activity of apical ENaCs is regulated by clathrin-mediated endocytosis [24]. Strikingly, apical Wurst localization in tracheal cells is essential for airway liquid-clearance [14]. Moreover, genetic studies demonstrate interaction of Wurst with *Drosophila* ENaCs [13], which are encoded by the *pickpocket* genes [25]. In summary, we assume that Wurst is required for regulating *Drosophila* ENaCs activity in order to control airway liquid-clearance. Wurst and its human ortholog DNAJC22 share conserved sequence and domain structure [15], suggesting that they could have similar function in clathrin-mediated endocytosis. Whether DNAJC22 plays a similar role in humans in endocytosis of ENaC is not yet known.

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