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The fatty acyl-CoA reductase Waterproof mediates airway clearance in *Drosophila*

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ABSTRACT

The transition from a liquid to a gas filled tubular network is the prerequisite for normal function of vertebrate lungs and invertebrate tracheal systems. However, the mechanisms underlying the process of gas filling remain obscure. Here we show that *waterproof*, encoding a fatty acyl-CoA reductase (FAR), is essential for the gas filling of the tracheal tubes during *Drosophila* embryogenesis, and does not affect branch network formation or key tracheal maturation processes. However, electron microscopic analysis reveals that in *waterproof* mutant embryos the formation of the outermost tracheal cuticle sublayer, the envelope, is disrupted and the hydrophobic tracheal coating is damaged. Genetic and gain-of-function experiments indicate a non-cell-autonomous *waterproof* function for the beginning of the tracheal gas filling process. Interestingly, Waterproof reduces very long chain fatty acids of 24 and 26 carbon atoms to fatty alcohols. Thus, we propose that Waterproof plays a key role in tracheal gas filling by providing very long chain fatty alcohols that serve as potential substrates for wax ester synthesis or related hydrophobic substances that ultimately coat the inner lining of the trachea. The hydrophobicity in turn reduces the tensile strength of the liquid inside the trachea, leading to the formation of a gas bubble, the focal point for subsequent gas filling. Waterproof represents the first enzyme described to date that is necessary for tracheal gas filling without affecting branch morphology. Considering its conservation throughout evolution, Waterproof orthologues may play a similar role in the vertebrate lung.

Introduction

The vertebrate lung and the tracheal system of insects are specialised organs for gas transport. Both systems develop during embryogenesis via a stereotypic branching pattern that gives rise to well-ordered three-dimensional tubular networks (Ghabrial et al., 2003; Metzger et al., 2008). During these maturation processes the tubes are liquid filled, a prerequisite for organ development. However, shortly before birth (vertebrates) or hatching of larvae (insects) the liquid is absorbed by the surrounding tissue and both organs, the lung and the tracheal system, become competent to mediate their physiological role, the transport of gases (Elias and O'brodovich, 2006; Förster and Woods, 2012; Hooper et al., 2007).

The tracheal system development of *Drosophila* is both an excellent system to study branching morphogenesis of tubular networks and also to analyse the maturation of networks to functional organs (reviewed in Affolter et al., 2009; Ghabrial et al., 2003; Uv et al., 2003). Tracheal system development is initiated by the differentiation of tracheal cell groups, the tracheal placodes, from ectodermal cells. These tracheal cells form tubes and branch out in a stereotyped pattern. Specific branches fuse and form the main anterior–posterior tubes, the dorsal trunks, one on either side of the embryo. Smaller tubes fuse at the lateral sides and the two halves of the tracheal system are interconnected by dorsal and ventral anastomoses. Thus, a three-dimensional liquid-filled tubular network is formed during embryogenesis. Normal tube maturation requires the formation of a chitinous luminal cable within the trachea, which is removed after branch morphogenesis during mid-stage 17 (Devine et al., 2005; Luschnig et al., 2006; Tonning et al., 2005). About two hours before the larvae hatch the liquid is cleared from the tubes and the trachea matures into functional gas-filled airways. Liquid clearance (LC) is initiated

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stochastically in either of the dorsal trunks by the formation of a gas bubble. The bubble extends within the dorsal trunk and reaches the contralateral dorsal trunk via the posterior anastomosis. After the dorsal trunks are cleared of liquid the residual tracheal tubes are gas filled within 20 min (Tsarouhas et al., 2007; Supplementary movie 1). Elaborate cuticle ridges facing the tracheal lumen prevent luminal collapse during the transition from liquid to gas filled tubes. These rigid cuticle structures, also called taenidial folds, are built from procuticular material that assembles perpendicular to the tube length and form annular rings surrounding the tracheal lumen (Moussian, 2010). Thus, a gas filled tubular system is established during the end of embryogenesis and the larvae hatch with a functional tracheal system.

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In the last 20 years extensive insight has been gained into the cellular and molecular mechanisms that lead to tracheal placode formation, guided branch outgrowth, branch interconnection and tracheole differentiation (for review see Affolter et al., 2003; Lubarsky and Krasnow, 2003; Neumann and Affolter, 2006). In contrast, only superficial knowledge is available about the molecular processes and molecules that are involved in tracheal gas filling. Preliminary results by RNA interference experiments show, that *pickpocket* (*ppk*) family members, which encode epithelial Na^+ channels (ENaCs), reveal defects in the LC of the tracheal tubes (Liu et al., 2003). Interestingly, ENaCs also seem to be crucial for LC of the vertebrate lung, since alpha-ENaC knockout mice despite exhibiting normal foetal lung development, die shortly after birth as they fail to clear their lungs of fluid (Hummler et al., 1996). In *Drosophila ppk11* may represent cargo for clathrin-mediated endocytosis, a process also essential for LC (Behr et al., 2007). ENaCs possibly participate in the establishment of an osmotic gradient between the tracheal lumen and the epithelial cells or the haemolymph. This gradient is similarly disrupted in mutants for septate junction (SJ) components, also leading to impaired gas filling (Behr et al., 2003). SJs are apicolateral membrane protein complexes in epithelial sheets and they establish an epithelial barrier function by controlling the transepithelial flow of solutes and ions (Wu and Beitel, 2004). Furthermore, exocytosis of the chitin deacetylases Serpentine (Serp) and Vermiform (Verm) and their chitin modifications within the tracheal lumen are also crucial for the subsequent normal gas filling of the tracheal tubes (Luschnig et al., 2006). However, defects in those genes essential for chitin modification, clathrin-mediated endocytosis or the transepithelial barrier reveal additional morphological defects of the tracheal system, *i.e.* tortuous and elongated branches, indicating that these processes participate in cellular mechanisms distinct from gas filling of the tubes (Behr et al., 2003, 2007; Luschnig et al., 2006; Wu and Beitel, 2004).

Here we describe the identification and functional characterisation of *waterproof* (*wat*; *CG1443*), which is involved in tracheal gas filling, without affecting tracheal network formation or morphology. *wat* Encodes a fatty acyl-CoA reductase and reduces very long chain, saturated fatty acids of 24 and 26 carbon atoms to fatty alcohols, which may serve as substrates for biosynthesis of wax or related hydrophobic substances. *wat* Is expressed in tracheal cells and it is essential for the formation of the outermost tracheal cuticle layer in a non-cell-autonomous manner.

Materials and methods

Gas chromatography/mass spectrometry

The Wat coding sequence was cloned in the yeast expression vector *pYES2/NTc* using the gene-specific primers 5′-ACGCTCGAGATGGATGATCCCAAAATAATGAACA-3′ (with attached *XhoI* restriction site) and 5′-ACGTCTAGACTATAAGAATAGCTTGAGCAGGGC-3′ (with attached *XbaI* restriction site). The resulting plasmid was named *pYES2/NT*-Wat. *Saccharomyces cerevisiae* strain *H1246* (Sandager et al., 2002), transformed with either *pYES2/NTc* as negative control or *pYES2/NT*-Wat were grown in 20 ml synthetic dropout (SD)-media containing 6.7 g/l yeast nitrogen base, 5.0 g/l $(NH_4)_2SO_4$, 2 g/l AS-dropout-powder lacking uracil and 2% (w/v) of galactose (for induction of Wat-expression) or 2% (w/v) of glucose (for non-inducing conditions). After five days of growth, twenty OD_{600} units of each culture were taken for analysis of fatty alcohols produced by the putative FAR. After centrifugation of respective amounts of cultures, 1 ml methanol was added to the pellets together with ~500 μl of glass beads. The samples were vortexed for 15 min at room temperature. Subsequently, 2 ml hexane-diethylether-acetic acid (80:20:2, per volume) were added, samples were vortexed for another 15 min and centrifuged for 15 min at 1500*g* in order to separate the organic from the aqueous phase. The organic phase was recovered, evaporated under a steam of nitrogen and resolved in 150 μl acetonitrile. Afterwards, 10μl of the samples were mixed with 10 μl BSTFA. The analysis was carried out using an Agilent 5973 mass selective detector connected to an Agilent 6890 gas chromatograph equipped with a capillary DB-23 column (30 m × 0.25 mm; 0.25 μm coating thickness; J&W Scientific, Agilent, Waldbronn, Germany). Helium was used as carrier gas at a flow rate of 1 ml/min. The temperature gradient was 150 °C for 1 min, 150–200 °C at 8 K/min, 200–250 °C at 25 K/min and 250 °C for 6 min. For the MS analysis an electron energy of 70 eV, an ion source temperature of 230 °C, and a temperature of 260 °C for the transfer line were used. The ions were detected in scan mode in a *m/z* range from 50 to 650 (Heilmann et al., 2012). Fatty alcohols were identified using the NIST MS Search 2.0 library.

Immunochemistry

Whole-mount immunostainings of fixed embryos were performed as described previously (Goldstein and Fyrberg, 1994). The following primary antibodies were used: guinea pig anti-Wat antibodies directed against the peptides CRNKYETPPIYNYVPD and CRFDNDNVRK LTEKLDDR were generated by PSL GmbH (Heidelberg, Germany) and used in a 1:50 dilution; mouse monoclonal anti-Mega antibody 1:20 dilution (Jaspers et al., 2012); anti-Verm 1:100 dilution and anti-Serp 1:200 dilution (Luschnig et al., 2006); anti-FasIII 1:50 dilution (Patel et al., 1987). The following secondary antibodies were used in 1:500 dilutions: goat anti-mouse IgG-Alexa568; goat anti-rabbit IgG-Alexa568; goat anti-guinea pig IgG-Alexa568; goat anti-mouse IgG-Alexa488; goat anti-guinea pig IgG-Alexa488; goat anti-rabbit IgGAlexa488 (Invitrogen). Image acquisitions were performed with a Leica TCS SP2 AOBS (Leica, Mannheim, Germany) confocal microscope. Each fluorochrome was scanned individually in sequential mode using standard settings and a HC PL APO 20 × 0.7 or a HCX PL APO CS 40 × 1.25 oil immersions objective.

Cell culture

The peroxisomal marker *tagRFP-SKL* was cloned via PCR by adding the targeting sequence to tagRFP. The PCR was performed with the template *tagRFP-T* and the oligos CCACCATGGTGTCTAAGGGCGAAGAGCTG and TCCTCTAGACTAGAGTTTTGACTTGTACAGCTCGTCCATGCC (Shaner et al., 2008). The PCR product was cloned into *pUbiP* (a gift from A. Herzig) using *NcoI* and *XbaI* to generate *pUbiTagRFP-SKL*. Wat-GFP was cloned by amplifying the open reading frame of *wat* via PCR using plasmid *LP06017* (BDGP; Berkeley Drosophila Genome Project) and the oligos AAAACTAGTATGGATGATCCCAAAATAATGAAC and AAAGCTAGCTAAGAATAGCTTGAGCAGGGCATAG. The

PCR product was cloned into *pUbi-C-EGFP* using *NheI* and *SpeI* to generate *pUbiWat-C-EGFP*. *pUbiKDELR-TdT* was a gift from M. Beller (Thiel et al., 2013). *pUbiTagRFP-SKL*, *pUbiKDELR-TdT* and *pUbiWat-C-EGFP* were cotransfected by Effectene (Qiagen, Hamburg, Germany) according to the manufacturer's protocol into S2 cells. 48 h after transfection cells were resuspended, seeded onto coverslips and fixed after 2 h of adhesion using 4% formaldehyde in PBS. After fixation cover slips were washed in PBS and mounted onto slides using ProLong Gold (Invitrogen, Darmstadt, Germany) as an embedding media. Cells were imaged on a Leica SP2 TCS-AOBS confocal microscope with a HCX PL APO CS 40.0 × 1.25 OIL immersion objective (Leica, Mannheim, Germany). Raw images were combined into RGB files using ImageJ. Intensity profiles were generated by the ImageJ plug in "RGB Profile Plot".

Dextran red permeability experiments

We crossed $wat^{\Delta R}$/*TM3, dfd-eYFP* flies with $wat^{\Delta G}$/*TM3*, *dfd-eYFP* flies and identified mutant $wat^{\Delta R}/wat^{\Delta G}$ embryos by the lack of YFP fluorescence mediated by the marked balancer chromosome (Le et al., 2006). Dechorionated $wat^{\Delta R}/wat^{\Delta G}$ mutant or wild-type embryos at stage 16 were covered with Voltalev 10S oil for injection. Rhodamine labelled dextran (MW 10,000; Molecular Probes, Eugene, OR) was purified and injected into the haemocoel of embryos as described (Lamb et al., 1998). The embryos were analysed by confocal microscopy.

Fly stocks

We used the viable P-element insertion line P{XP}d01446 for P-element jump-out experiments (Hartenstein and Jan, 1992). The generated DNA deletions of the *wat* alleles $wat^{\Delta R}$ and $wat^{\Delta G}$ were analysed by DNA sequencing after PCR amplification of genomic DNA. The Gal4 driver lines *sal*TE-Gal4 and *kni*TE-Gal4 were established by cloning *sal*TSE1000 (Kühnlein et al., 1997) and AE20 *kni* enhancer (Weiss et al., 2010), respectively, into the vector *pGal4*. The transgene constructs were used for P element-mediated germline transformation (Rubin and Spradling, 1982).

Electron microscopy

Drosophila embryos were mechanically dechorinated and placed on a 150 μm flat embedding specimen holder (Engineering Office M. Wohlwend GmbH, Sennwald, Switzerland) and frozen in a Leica HBM 100 high pressure freezer (Leica Microsystems, Wetzlar, Germany). The embedding of the vitrified samples was performed using an Automatic Freeze Substitution Unit (AFS) (Leica). Substitution was done at −90 °C in a solution containing anhydrous acetone, 0.1% tannic acid and 0.5% glutaraldehyde for 72 h and in anhydrous acetone, 2% OsO4, 0.5% glutaraldehyde for additional 8 h. After a further incubation for 18 h at −20 °C samples were warmed up to +4 °C and subsequently washed with anhydrous acetone. The samples were embedded at room temperature in Agar 100 (Epon 812 equivalent) and polymerised at 60 °C for 24 h. Images were taken in a Philips CM120 electron microscope (Philips Inc.) using a TemCam 224 A slow scan CCD camera (TVIPS, Gauting, Germany).

Results

waterproof is essential for liquid clearance of the tracheal tubes

We identified the gene *CG1443*, henceforth referred to as *waterproof* (*wat*), in an RNA interference (RNAi) screen for genes required for gas filling, *i.e.* liquid clearance (LC) of the tracheal tubes. Particularly, we found, in contrast to wild-type (Fig. 1A), that the UAS/Gal4 mediated (Brand and Perrimon, 1993) pan-tracheal expression of an RNAi-transgene targeting *wat* (*btl*-Gal4; UAS-RNAi-*VDRC1333*; (Dietzl et al., 2007) led to the complete lack of tracheal LC during embryonic development (Fig. 1B). Such embryos hatch but die during the first instar larval stage. In contrast, mesodermal (*mef2*-Gal4) or endodermal (Y48-Gal4) RNAi-mediated *wat* knock-down led to normal fertile flies (not shown). These results indicate that the RNAi-mediated tracheal phenotype is specific for the proper function of this organ.

The homozygous viable P-element insertion *P(XP)d01446*, which resides in the first intron of *wat* (Fig. 1C), was used to generate the lethal P-element jump out lines, *R4(d01446)* and *G4 (d01446)* (see Fig. 1C; Materials and methods). Both of these lines failed to complement each other or the chromosomal deletion *Df (3R)Exel6211*, which removes the region 98F5 that includes the *wat* gene. Furthermore, *R4(d01446)* and *G4(d01446)* mutant embryos lack the LC of the tracheal tubes in homozygous as well as in hemizygous conditions in the background of the *Df(3R)Exel6211* (Fig. 1D; not shown). This indicates that *R4(d01446)* and *G4 (d01446)* represent alleles of the *wat* gene and, thus, we refer to them $wat^{\Delta R}$ and $wat^{\Delta G}$. Both alleles were molecularly characterised and they contain deletions of the entire *wat* coding region (Fig. 1C). Ectopic tracheal *wat* expression by the UAS/Gal4 expression system in *wat* mutant embryos rescues the lack of LC, *i.e.* the tracheal system fills normally with gas (Fig. 1E). Moreover, such rescued embryos develop into adult and fertile flies. Thus, our results show that *wat* encodes an essential function for the normal gas filling of the tracheal tubes and that its activity is essential in tracheal cells.

The embryonic development of the *Drosophila* tracheal system is a sequential maturation process that ends with the replacement of luminal liquid by gas. Normal tracheal maturation is a pre-condition for the gas filling of the tracheal tubes (Tsarouhas et al., 2007). Thus, we next sought to determine the particular aspect of tracheal development that is affected in *wat* mutant embryos. Analysis of tracheal morphogenesis by visualisation of the luminal chitin matrix revealed normal tracheal morphogenesis of *wat* mutant embryos (compare Fig. 2A with B). The components of septate junctions, localised at the apicolateral membrane of tracheal cells, were also comparable between wild-type and *wat* mutant embryos (Fig. 2C and F). Furthermore, the transepithelial barrier mediated by septate junctions is not affected in *wat* mutant embryos as revealed by red dextran injection experiments (Fig. 2G and H) and exocytosis of the chitin deacetylases Serp and Verm (Luschnig et al., 2006) into the tracheal lumen of *wat* mutant embryos is also normal (Fig. 2I and L). These results therefore indicate that key processes of tracheal maturation are not affected in *wat* mutant embryos but rather suggest that *wat* activity is specifically required for the tracheal LC.

waterproof reveals a restricted expression pattern during embryogenesis

We then performed *in situ* hybridisation on whole mount embryos to visualise *wat* transcript expression during embryogenesis. *wat* RNA is first detectable during stage 13 in the developing tracheal system and the hindgut (Fig. 3A and B). During subsequent embryonic development *wat* transcripts accumulate apically in the cells of these organs (Fig. 3C and D). To analyse the Wat protein distribution we produced an antibody against Wat (Materials and methods). The antibody is specific for Wat as revealed by the lack of Wat staining in *wat* mutant embryos (compare Fig. 3E with F). Wat protein expression was found to coincide with the spatial aspects of *wat* transcription as revealed by the anti-Wat antibody stainings of wild-type embryos (not shown). The subcellular analysis reveals an

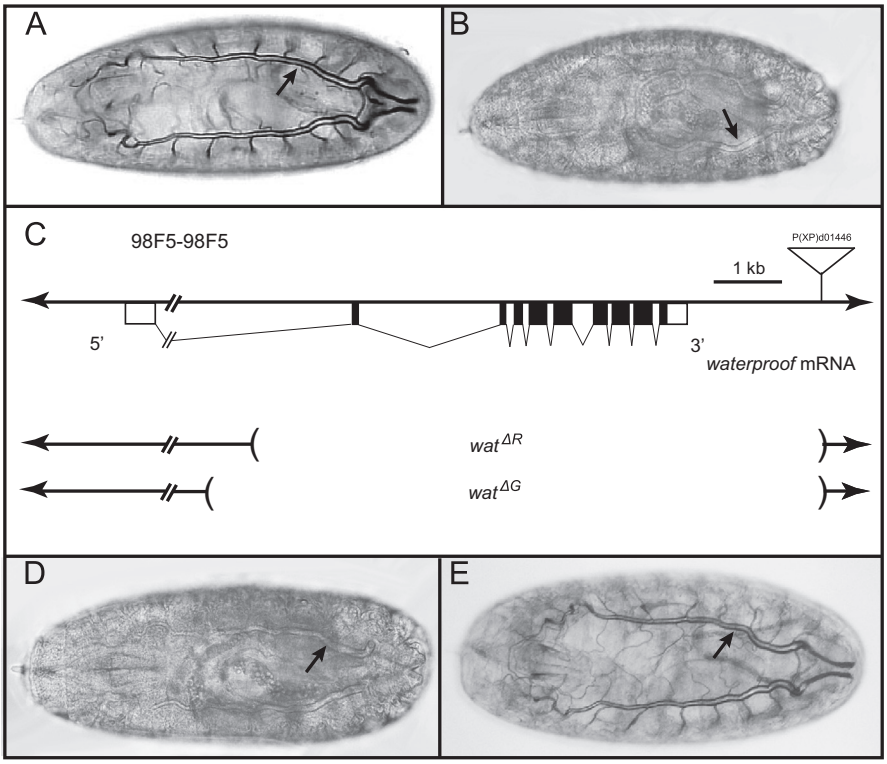

Fig. 1. The *waterproof* gene is essential for tracheal liquid clearance. Bright field light microscopic pictures of stage 17 wild-type (A) and *btl*-Gal4; UAS-RNAi-VDRC1333 (B) embryos. Wild-type embryos show gas filled tracheal tubes at the end of embryogenesis (arrow in A). In contrast, tracheal knock-down of the *wat* gene leads to a lack of tracheal gas filling (arrow in B). (C) Physical map of the genomic region 98F5 containing the *wat* gene (*CG1443*) according to McQuilton et al. (2012). The P-element insertion P(XP)d01446 was used for P-element jump out experiments (Materials and methods). The genomic DNA deletions (Materials and methods) of the *wat* alleles *R4(d01446)* ($wat^{\Delta R}$) and *G4(d01446)* ($wat^{\Delta G}$) are indicated. Bright field light microscopic pictures of stage 17 $wat^{\Delta R}/wat^{\Delta G}$ (D) and *btl*-Gal4/UAS-*wat*; $wat^{\Delta R}/wat^{\Delta G}$ (E) mutant embryos. *wat* mutant embryos lack gas filling (arrow in D) while *wat* mutant embryos that express *wat* in the tracheal cells have a normal gas filling of the tracheal system (arrow in E).

apically enriched cytoplasmic distribution of Wat (Fig. 3E), similar to that found for the *wat* transcript (Fig. 3D). Thus, *wat* expression and Wat protein localisation is restricted to the hindgut and the tracheal system during late embryonic development, consistent with a function of *wat* in LC of the tracheal tubes.

waterproof encodes a fatty acyl CoA reductase

The *wat* gene encodes a single 517 amino acid large protein and *in silico* analysis identified two human homologous proteins, the fatty acyl-CoA reductase1 (FAR1) and the fatty acyl-CoA reductase2 (FAR2; Supplementary Fig. 1). For the FAR enzymes from mouse it has been shown, that they reduce fatty acids to fatty alcohols by using the reducing equivalents of the NADPH cofactor (Cheng and Russell, 2004). Predicted binding sites for the NADPH cofactor are indicated in the sequence of FAR1, FAR2 and Wat (Supplementary Fig. 1). FAR1 from mouse reduces both saturated and unsaturated fatty acids of 16 or 18 carbon atoms as substrates, while FAR2 prefers saturated fatty acids of the same lengths. Both enzymes are localised to peroxisomes as shown by cell culture experiments (Cheng and Russell, 2004) and transient expression in onion epidermal cells (Heilmann et al., 2012). We expressed Wat and the peroxisomal marker mRFP-SKL in *Drosophila* S2 cells (see Materials and methods) to assess where these proteins were colocalised; these experiments revealed, in contrast to FAR1 and FAR2, no specific colocalisation of Wat with the peroxisomal marker *SKL* (Supplementary Fig. 2A and C). Rather, Wat appears similarly distributed as the endoplasmic reticulum marker, *KDELR* (Supplementary Fig. 2D and F).

To analyse the enzymatic activity of Wat, the putative *Drosophila* FAR protein, we expressed Wat in yeast cultures and analysed the fatty alcohols produced from the endogenous acyl-CoA pool by gas chromatography/mass spectrometry (GC/MS). In yeast cultures expressing Wat, tetracosanoyl-alcohol (24:0-OH) and hexacosanoyl-alcohol (26:0-OH) were identified by GC/MS analysis; unsaturated alcohols or alcohols of shorter chain-lengths are either absent or below the detection limit. Neither of these fatty alcohols was detected in the vector control yeast culture or the *wat* gene containing culture grown under non-inducing conditions (Table 1; Supplementary Fig. 3). These results indicate that Wat is a *Drosophila* FAR with a preference for very long chain, saturated fatty acids of 24 and 26 carbons.

Waterproof is essential for outer envelope formation of the tracheal cuticle

The *Drosophila* FAR protein, Wat, reduces very long chain fatty acids to fatty alcohols, which may be key components in the metabolic pathway of wax ester biosynthesis. Waxes are integral parts of the envelope, the outermost sublayer of the tracheal cuticle (Locke, 1961; Moussian, 2010; Moussian et al., 2006). The envelope faces the tracheal lumen and due to its hydrophobic nature this surface layer protects the fly from swelling and dehydration (Gibbs, 1998). To examine whether the lack of the FAR enzyme Wat affects cuticle formation we analysed the tracheal cuticle ultrastructure in wild-type and *wat* mutant embryos by transmission electron microscopy (Fig. 4). The tracheal lumen of wild-type embryos shows an unstructured uniform appearance (arrows in Fig. 4A). In contrast, *wat* mutants have membranous structures within the embryonic trachea (arrows in Fig. 4B). Furthermore, such structures are occasionally associated with the outermost cuticle sublayer of the tracheal taenidial folds (Fig. 4B′ and B″), suggesting that the membrane structures originate from the envelope. In contrast, the procuticle, the epicuticle (Moussian et al.,

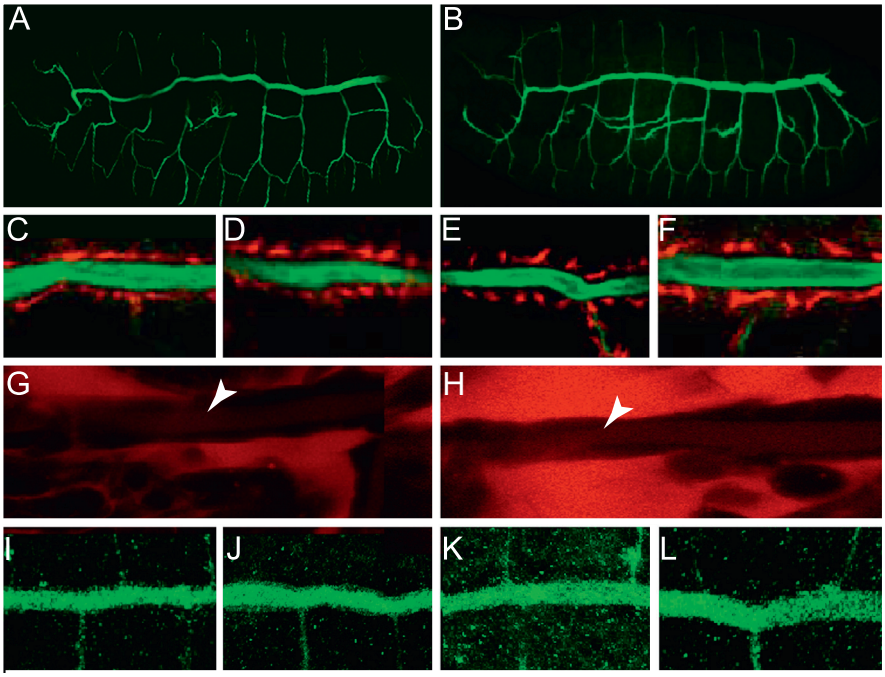

Fig. 2. Key processes for tracheal maturation are not affected in *waterproof* mutant embryos. Stage 17 wild-type (A) and $wat^{\Delta R}/wat^{\Delta G}$ mutant (B) embryos were stained with CY2 labelled chitin binding protein (CBP). CBP binds the luminal chitin matrix during tracheal development and outlines the tracheal network during embryogenesis. The tracheal morphogenesis of *wat* mutant embryos (B) is indistinguishable from wild-type embryos (A). Stage 17 wild-type (C, E) and $wat^{\Delta R}/wat^{\Delta G}$ mutant (D, F) embryos were stained with CBP (C–F, green) and anti-Mega (C, D) or anti-FasIII (E, F) antibodies. The septate junction markers Mega (red in C, D) and FasIII (red in E, F) exhibit normal apical-lateral membrane localisation in *wat* mutant embryos (D, F) as found in wild-type embryos (C, E). Confocal images of tracheal dorsal trunk branches of wild-type (G) and $wat^{\Delta R}/wat^{\Delta G}$ (F) stage 17 mutant embryos after rhodamine-dextran injection into the haemocoel (Materials and methods). Rhodamine-dextran is not found in the dorsal trunk lumen of wild-type (arrowhead in G) and $wat^{\Delta R}/wat^{\Delta G}$ mutant (arrowhead in H) embryos. Stage 17 wild-type (I, K) and $wat^{\Delta R}/wat^{\Delta G}$ mutant (J, L) embryos were stained with anti-Serp (I, J, green) or anti-Verm (K, L, green) antibodies. The exocytosis of Serp and Verm into the tracheal lumen of *wat* mutant embryos is indistinguishable from wild-type embryos (compare I and K with J and L).

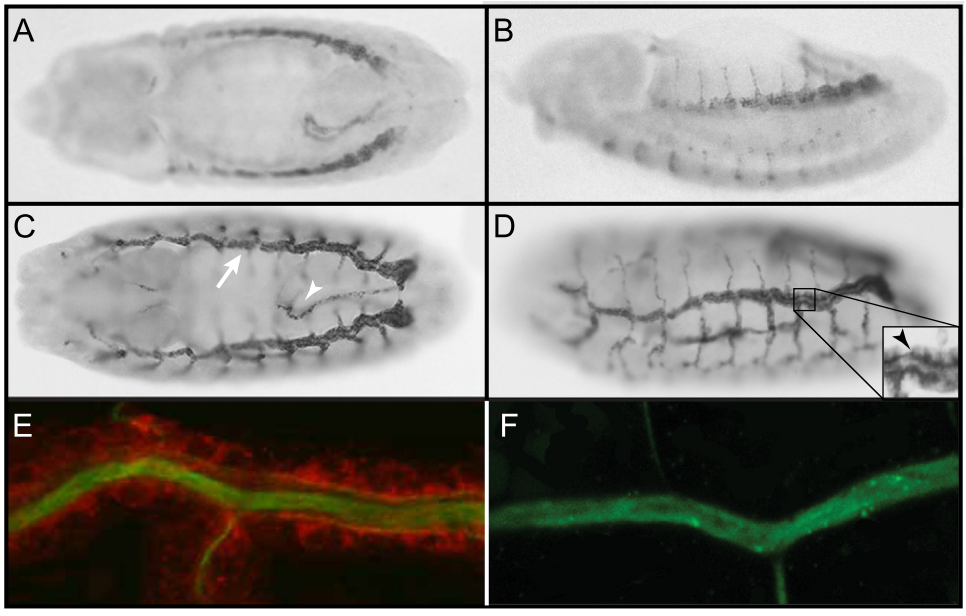

Fig. 3. Waterproof expression pattern during embryonic development. Whole-mount *in situ* hybridisation of wild-type embryos at stage 13 (A, B) and stage 16 (C, D) with a *wat* antisense RNA probe (Materials and methods); lateral view (B, D), dorsal view (A, C). Embryonic *wat* expression is restricted to the tracheal system (arrow in C) and the hindgut (arrowhead in C). *wat* Expression is not detectable before stage 13 (not shown). Arrowhead in inset D points to the apical cellular *wat* transcript accumulation. Stage 17 wild-type (E) and $wat^{\Delta R}/wat^{\Delta G}$ mutant (F) embryos were stained with CBP (green) and anti-Wat antibodies (red). The anti-Wat antibodies detect Wat protein specifically in the cytoplasm of wild-type tracheal cells (red in E), while no Wat protein is detectable in *wat* mutant embryos (F) using identical imaging parameters.

2006) and also taenidial fold morphology in *wat* mutant embryos is similar to wild-type (compare Fig. 4A with B). Our results suggest that Wat represents a central enzyme in the metabolic pathway of cuticle biosynthesis. In addition, our experiments provide evidence suggesting that *wat* is involved in the proper formation of the outermost tracheal sublayer, the cuticle envelope.

Table 1
Waterproof represents a fatty acyl CoA reductase.

Construct/condition	Fatty alcohols
pYES2/NT-empty (induced)	None detected
pYES2/NT-Wat (induced)	24:0-OH, 26:0-OH

pYES2/NT-empty vector control and *pYES2/NT*-Wat were transformed into *Saccharomyces cerevisiae H1246* and fatty alcohol production was measured in inducing conditions. Long chain saturated fatty alcohols (tetracosanoyl-alcohol (24:0-OH) and hexacosanoyl-alcohol (26:0-OH) could only be detected in cultures expressing the putative *Drosophila* FAR Wat, whereas cultures transformed with *pYES2/NT-empty* vector control growing under inducing conditions did not produce any detectable amounts of fatty alcohols (for details see Supplementary Fig. 3).

Waterproof acts in a non-cell-autonomous manner

To elucidate the function of *wat* for LC of the tracheal tubes in more detail we asked whether *wat* acts cell-autonomously or in a non-cell-autonomous fashion. It has been proposed that both of these different mechanisms mediating gene activity play a role in tube dilation processes. For example, ion-transport that results in increased luminal pressure followed by tube dilation is a non-cell-autonomous process (Bryant and Mostov, 2007; Jayaram et al., 2008), while Sec24-dependent secretion drives cell-autonomous tube expansion (Förster et al., 2010). To analyse whether locally restricted *wat* activity causes normal LC of the tracheal tubes we performed region restricted tracheal *wat* expression in *wat* mutants using the UAS/Gal4 system. To achieve region specific tracheal Gal4 expression we established transgenic fly lines, which express Gal4 controlled by the *spalt* (*sal*) (Kühnlein et al., 1997) and the *knirps* (*kni*) (Chen et al., 1998; Vincent et al., 1997) tracheal enhancers, respectively (Materials and methods). The *sal*-tracheal enhancer (*sal*TE) Gal4 fly line expresses Gal4 in the embryonic dorsal trunk, while the *kni*-tracheal enhancer (*kni*TE) Gal4 shows expression in dorsal branches and cells of the lateral trunk as expected (Supplementary Fig. 4). These region specific tracheal drivers and the well established pan-tracheal driver *btl*-Gal4 (Shiga et al., 1996) were used to achieve localised ectopic *wat* expression in *wat* mutant embryos (Fig. 5A and D). Pan-tracheal *wat* expression (*btl*-Gal4/UAS-*wat*) in *wat* mutant embryos results in normal LC in more than 90% of the embryos. Interestingly, wat expression limited to the dorsal trunk cells (*sal*TE-Gal4/UAS-*wat*) still gave rise to a normal wild-type like LC in about 50% of *wat* mutant embryos. Even more strikingly, low *wat* expression in the dorsal branch and lateral trunk cells of the tracheal system (*kni*TE-Gal4/UAS-*wat*) resulted in normal LC in about 4% of *wat* mutant embryos. In contrast, neither *wat* mutant embryos nor RNAi mediated pan-tracheal *wat* knock-down embryos (*btl*-Gal4/UAS-*wat*RNAi) produced gas filled tracheal systems at all (Fig. 5E). Also, restricted branch-specific RNAi mediated *wat* knock-down by the *sal*TE-Gal4 or *kni*TE-Gal4 driver results in normal gas filling and viable flies (not shown). Thus, these results indicate that region specific tracheal *wat* expression is sufficient to rescue the lack of tracheal gas-filling phenotype of *wat* mutant embryos.

At the beginning of the LC process of wild-type embryos bubble formation is initiated stochastically in one of the two dorsal trunk branches in the central metameres 4–6 (Tsarouhas et al., 2007; Supplementary movie 1). Also *wat* embryos rescued by *wat* expression in the dorsal trunk (*sal*TE-Gal4/UAS-*wat*) show initial bubble formation in dorsal trunk central metameres as found in wild-type embryos. More interestingly, even in the cases of *wat* mutant gas filling rescued by *wat* expression in dorsal branch and lateral trunk cells (*kni*TE-Gal4/UAS-*wat*), we observed initial bubble formation exclusively in the dorsal trunk (not shown). The spreading of the gas through the continuous tracheal system also followed the same stereotyped course as in wild-type embryos and we never observed partial gas filling after initiation of LC. From these results we conclude that *wat* acts in a non-cell-autonomous manner to mediate gas filling of the tracheal tubes during embryogenesis.

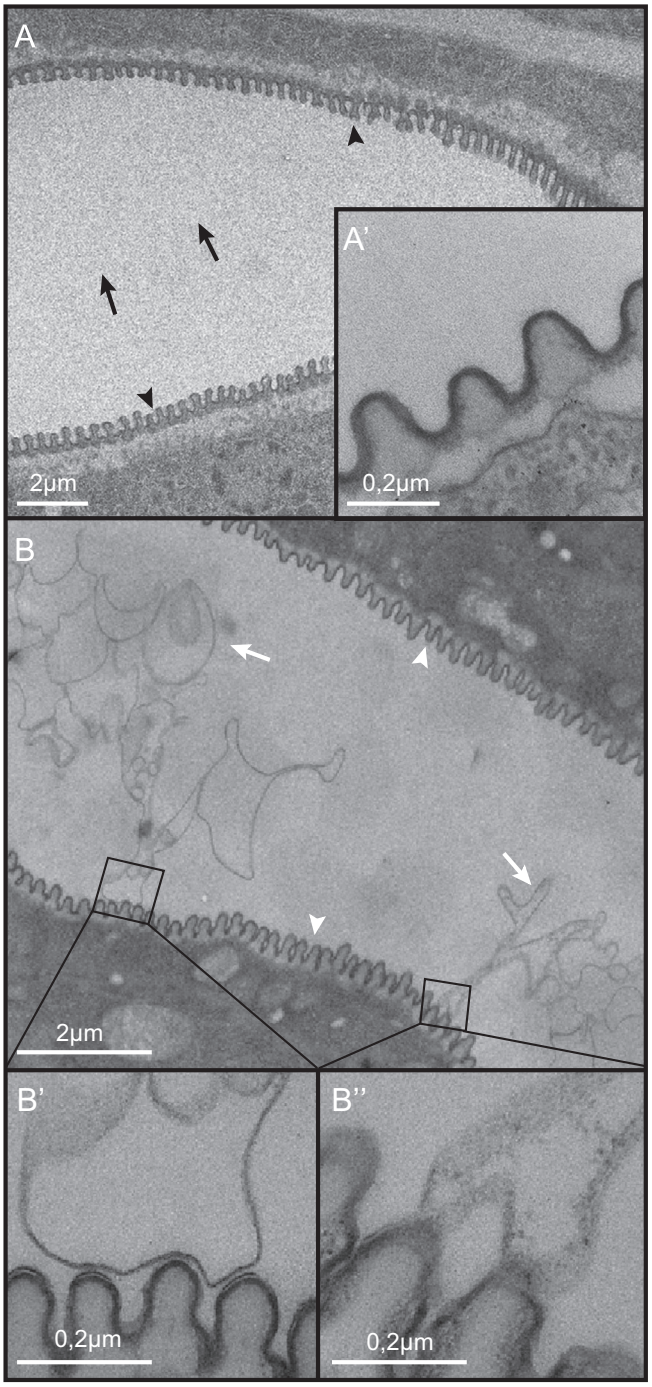

Fig. 4. *waterproof* mutant embryos display a disrupted envelope of the tracheal cuticle. Transmission electron microscopy of early stage 17 wild-type (A) and $wat^{\Delta R}/wat^{\Delta G}$ mutant (B) tracheal dorsal trunk branches. Arrowheads in A and B point to the taenidial folds lining the lumen of the tracheal system. Arrows point to the unstructured uniform tracheal lumen in wild-type trachea (A) and to the membrane-like structures in the tracheal lumen of *wat* mutant embryos. The insets (B′, B″) show the close association of taenidial folds and membranous structures in the *wat* mutant trachea.

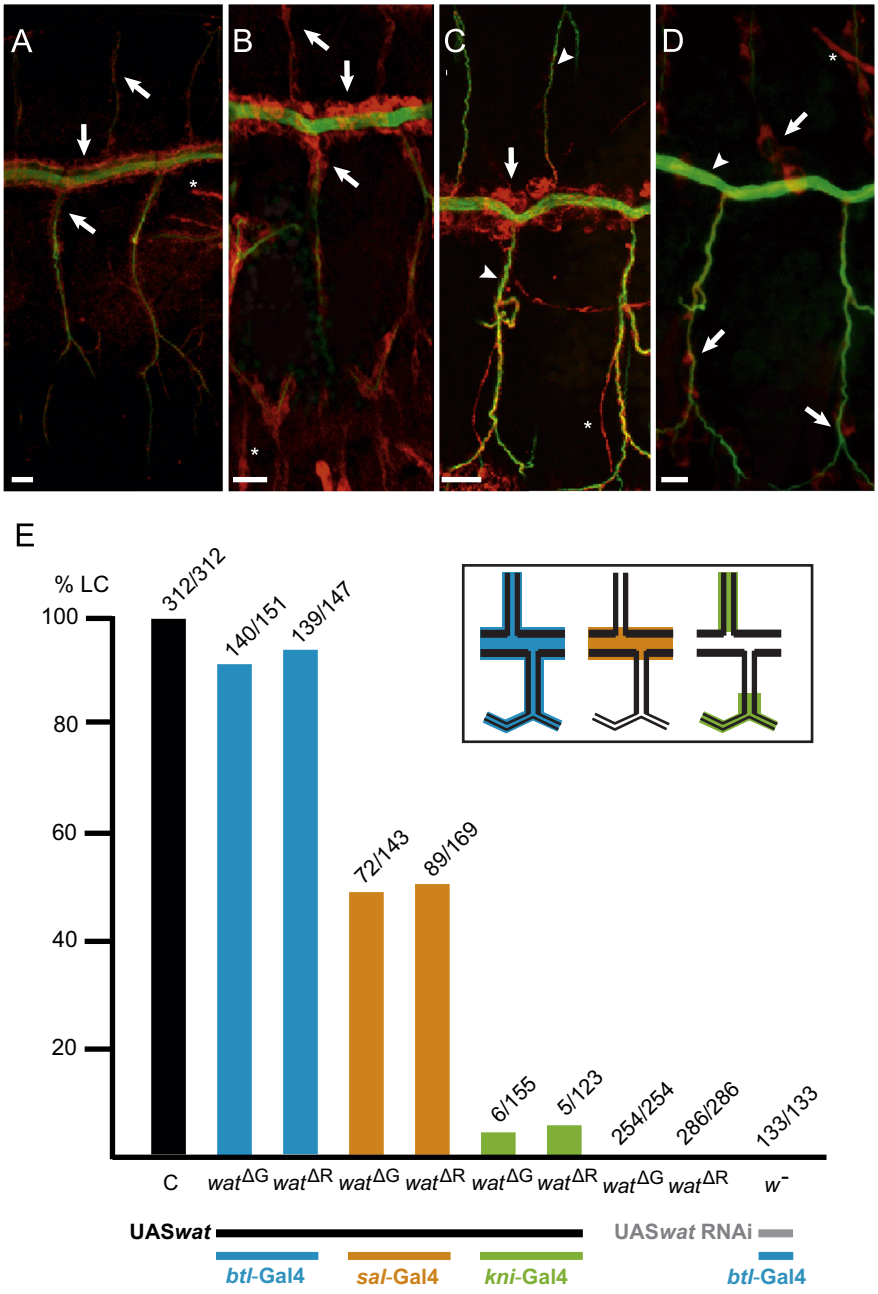

Fig. 5. Waterproof acts non-cell-autonomously. Stage 17 wild-type (A), *btl*-Gal4/UAS-*wat*; $wat^{\Delta R}/wat^{\Delta G}$ (B), *sal*TE-Gal4/UAS-*wat*; $wat^{\Delta R}/wat^{\Delta G}$ (C) and *kni*TE-Gal4/UAS-*wat*; $wat^{\Delta R}/wat^{\Delta G}$ (D) embryos were stained with CBP (green) and anti-Wat antibodies (red). Wat is expressed throughout the tracheal system in wild-type (arrows in A) and after pan-tracheal *wat* expression in *wat* mutant embryos (arrows in B). Wat is expressed exclusively in the dorsal trunk (arrow in C) after *sal*TE mediated *wat* expression in *wat* mutant embryos. Wat is not detectable in the dorsal branches and the lateral branches (arrowheads in C). Wat is expressed in the dorsal branches and the lateral branches (arrows in D) but not in the dorsal trunk (arrowhead in D) after *kni*TE mediated *wat* expression in *wat* mutant embryos. Note, that *kni*TE dependent expression is weaker than *btl* and *sal*TE dependent expression. Extracellular unspecific staining of the anti-Wat antiserum is indicated by asterisks. This staining is also present in *wat* mutant embryos, indicating unspecifity of the anti-Wat antiserum. (E) Quantification of the rescue efficiency. The diagram shows the tracheal liquid clearance (LC) of embryos bearing various genetic backgrounds as indicated. Numbers refer to: embryos with LC/total number of embryos. *Note*: we never observed partial LC, *i.e.* the tracheal systems were filled entirely with either gas or liquid at the end of embryogenesis. Schema in E illustrates the tracheal Gal4 expression of *btl*-Gal4 (blue), *sal*TE-Gal4 (orange) and *kni*TE-Gal4 (green).

Discussion

Airway liquid clearance is a critical developmental step to mature the liquid filled tubular branches of vertebrate lungs and invertebrate tracheal systems into functional organs. Here we present evidence that the *Drosophila* fatty acyl CoA reductase Waterproof mediates the hydrophobic surface coating of the outermost tracheal sublayer. This coating is essential for the initial gas bubble formation within the dorsal trunk of the tracheal system. The rupturing of the liquid column is the prerequisite for normal gas filling of the tracheal tubes and, thus, a functional tracheal system.

waterproof encodes an acyl CoA reductase

Wat shows sequence similarities to the vertebrate fatty acyl CoA reductases, FAR1 and FAR2. Both human and mouse enzymes are localised to peroxisomes distributed throughout the cytoplasm, as

revealed after transfection of these genes into Chinese hamster ovary-K1 cells and onion epidermal cells (Cheng and Russell, 2004; Heilmann et al., 2012). In contrast, Wat reveals an apically enriched cytoplasmic localisation in fly embryos and cell culture expression and localisation studies indicate no specific association of Wat with peroxisomes, but rather the endoplasmic reticulum. With regard to enzyme characteristics, FAR1 and FAR2 preferred fatty acids of 16 and 18 carbons as substrates (Cheng and Russell, 2004), but when FAR1 was expressed in the seeds of the plant *Arabidopsis thaliana* that harbour a different acyl-CoA pool, the enzyme also reduced unsaturated fatty acids of 18 and 20 carbons (Heilmann et al., 2012). We found that Wat expressed in yeast exclusively processes very long chain fatty acids of 24 and 26 carbons as substrates; Wat did not reduce fatty acids of 16 and 18 carbons even though these are present in large excess in the yeast system. Thus, while Wat is involved in lipid metabolism, it has distinct biochemical properties and a different intracellular localisation – indeed FAR1 and FAR2 do not rescue *wat* mutants (data not shown). Therefore Wat and the human FARs perform different functions in lipid metabolism. Wat localisation at the endoplasmic reticulum is consistent with the assumption that Wat is involved in wax biosynthesis, since the endoplasmic reticulum also associates with the wax synthase, which produces wax monoesters by trans-esterification of the fatty alcohol to a fatty acid (Cheng and Russell, 2004).

Waterproof mediates hydrophobic coating of the tracheal envelope

The cuticle of the tracheal system is composed of different horizontal layers with distinct biochemical and functional properties (Moussian, 2010). The hydrophobic nature of the tracheal envelope, the outermost layer of the cuticle, probably originates from the deposition of waxes. Here we present molecular and morphological evidence that the fatty acyl CoA reductase Wat is essential for the morphogenesis of the tracheal envelope. We further provide evidence that in embryos lacking *wat* the outermost layer of the tracheal envelope disintegrates and fragments of the layer are found detached from the cuticle inside the tracheal lumen. The observation that some fragments are still connected to the underlying cuticle suggests that the layer is initially formed but becomes disconnected from the cuticle during further development. The biochemical products of Wat activity are very long chain fatty alcohols, which occur naturally in free form as components of the cuticular lipids but more usually they are esterified into wax esters. Thus, our results indicate that the hydrophobic coating of the tracheal cuticle envelope with long chain alcohols and/or waxes is essential for normal tracheal morphogenesis and gas filling of the tracheal system at the end of embryogenesis.

How are these hydrophobic coating materials transported and deposited to their final target positions in the *Drosophila* cuticle? Larvae of the wax moth *Galleria mellonella* and the beetle *Tenebrio molitor* produce pore canals, which pass through the epidermal cuticle and might be the pathway by which the movement of wax precursors to the surface is achieved. Furthermore, the last step of wax ester biosynthesis is probably completed by an extracellular esterase activity close to the cuticle surface (Locke, 1959, 1961). Such canals and extracellular esterases have not been described in the *Drosophila* tracheal system. However, based on our results that *wat* mediates its function non-cell-autonomously, we also propose an extracellular wax assembly near its target positions. In our rescue experiments of *wat* mutant embryos by localised tracheal *wat* activity we exclusively observe initial bubble formation similar to wild-type embryos. Even when *wat* is merely expressed in dorsal branch and lateral trunk cells of the tracheal system, bubble formation is initiated stochastically in one of the two dorsal trunk branches of central metameres 4–6 as found in wild-type embryos. Thus, hydrophobic envelope formation is also established at the apical side of tracheal cells, which lack Wat expression. Since rapid diffusion of molecules has been described in the developing tracheal system (Förster et al., 2010) we propose diffusion of the wax precursors within the tracheal tubes. The insolubility, high molecular weight and inertness of the hydrophobic coating components suggest final assembly of these precursors at the site of deposition.

Hydrophobic coating of the tracheal system is essential for gas filling of the tubes

The initial gas bubble formation in the tracheal tube is very fast and, thus, it has been proposed that the underlying physical process of its formation is cavitation of the tracheal liquid *i.e.* generation of gas bubbles at constant temperature while pressure is decreased (Woods et al., 2009). Furthermore, the necessary pressure to cause cavitation, also called the tensile strength of the liquid, depends on several parameters of the liquid and its interaction with the solid wall (Brennen, 1995; Förster and Woods, 2012). An important parameter is the hydrophobicity of the wall: a more hydrophobic wall structure causes weaker tensile strength of the liquid. By this mechanism, air bubbles form on a wax coated glass rod held into water from cold trap, which is supersaturated with air. In contrast, no bubbles of gas are liberated from uncoated glass rods in such water (Wigglesworth, 1953). Thus, we suppose that *wat* activity generates a hydrophobic wax/lipid layer in the tracheal lumen, which decreases the tensile strength of the tracheal liquid and ultimately causes bubble formation by cavitation. We have no indication that the pressure inside the tracheal system of *wat* mutant embryos is different from wild-type embryos and, thus, cavitation may exclusively be caused by decreasing the tensile strength of the tracheal liquid. Indeed, the transepithelial barrier function of the tracheal epithelium is normal in *wat* embryos, a prerequisite for a wild-type like pressure inside the tubes of such embryos. After initiation of bubble formation the gas spreads throughout the whole tracheal system – no partial gas filling was observed in any of our experiments. Thus, initial bubble formation mediated by a hydrophobic layer invariably leads to a functional air filled tracheal system.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2013.10.022.

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