

# Microsomal triacylglycerol transfer protein (MTP) is required to expand tracheal lumen in *Drosophila* in a cell-autonomous manner

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## Summary

The *Drosophila* tracheal system is a useful model for dissecting the molecular mechanisms controlling the assembly and expansion of tubular organs. We have identified microsomal triacylglycerol transfer protein (MTP) as a new player involved in the lumen expansion in unicellular tubes. MTP is an endoplasmic reticulum resident protein that can transfer triglycerides and phospholipids between membranes *in vitro*. MTP lipid transfer activity is crucial for the assembly and secretion of apoB family lipoproteins, which are carriers of lipids between different tissues. Here we describe an unexpected role of MTP in tracheal development, which we postulate to be independent of its known function in lipoprotein secretion. We propose that, in tracheal cells, MTP is involved in regulation of *de novo* apical membrane delivery to the existing lumen and thus promotes proper expansion of the larval tracheal system.

**Key words:** Tubulogenesis, Trachea, Lumen expansion, Unicellular tubes

## Introduction

Tubular epithelial structures are present in different animal organs and are involved in the distribution of liquids, gasses and nutrients throughout the body. One of the requirements for the efficient transport of substances in tubular networks is the correct size and shape of the individual tubes. Thus, in addition to the initial assembly of the tubular structures, their further growth and expansion also need to be regulated at the level of the single cell and the whole network. While many molecular and mechanistic aspects of tube formation have been identified (reviewed by Ghabrial et al., 2003; Affolter et al., 2009; Andrew and Ewald, 2010), our knowledge of the processes controlling lumen expansion is less advanced.

The tracheal (respiratory) system of *Drosophila melanogaster* is composed of tubes of different sizes and cellular architectures. It develops in a stereotypic manner and has served as a convenient model to study the mechanisms regulating tube outgrowth. The establishment of a functional embryonic and larval tracheal network involves cell migration, cell intercalation, cell shape changes, lumen formation and lumen expansion in length and diameter (reviewed by Affolter and Caussinus, 2008; Schottenfeld et al., 2010). Detailed studies of the expansion process revealed that, at least in the embryo, deposition of a luminal matrix, the COP I/II secretion apparatus and septate junction proteins contribute to tube size regulation. To expand the diameter of the lumen, tracheal cells require proper apical secretion (Tsarouhas et al., 2007; Jayaram et al., 2008; Forster et al., 2010; Norum et al., 2010; Schottenfeld et al., 2010; Armbruster and Luschnig, 2012; Wang et al., 2012). Over-dilation of the tubes is prevented by a

luminal matrix, formed by a chitin cable deposited into the growing luminal space and septate junction proteins. At the same time, these two components also restrict the length of the tubes (Swanson and Beitel, 2006; Schottenfeld et al., 2010).

Most studies on the control of the diameter of the lumen have used the multicellular tubes in the developing fly embryo. It is not known whether the same mechanisms also apply for later stages of development, when much more extensive growth occurs, or for unicellular tubes. Two types of unicellular tubes can be found in the tracheal system: tubes with autocellular junctions, in which the lumen is extracellular; and seamless tubes, in which a lumen is made within a cytoplasm of a single cell (Lubarsky and Krasnow, 2003; Uv et al., 2003; Baer et al., 2009).

The autocellular tubes are formed by cell intercalation along the preexisting lumen (Ribeiro et al., 2004) and seamless tubes undergo a cell hollowing process (Gervais and Casanova, 2010). Despite the differences between these mechanisms, balanced addition of apical membrane material is crucial for lumen growth and elongation in both kinds of tubes. Whether this addition is controlled by similar sets of molecules for both intracellular and extracellular lumen remains unclear. We have identified microsomal triacylglycerol transfer protein (MTP) as a molecule that is specifically required for shaping the lumen of all unicellular tubes in the larval trachea of *Drosophila*.

Mammalian MTP is needed for the formation of apolipoprotein B (apoB)-containing lipoproteins (chylomicrons and very low-density lipoproteins-VLDL). MTP possesses a Vitellogenin-N domain, which fulfills its three major functions: neutral lipid and

phospholipid transfer activity, binding to the Vitellogenin-N domain of apoB and association with the endoplasmic reticulum (ER) membrane (Hussain et al., 2003b; Hussain et al., 2003a). MTP has been proposed to be required for the assembly of lipoproteins at two steps (Gordon et al., 1996; Raabe et al., 1999): first, when apoB associates with phospholipids and some neutral lipids to form a primordial dense lipoprotein, and second, when these primordial lipoproteins fuse with neutral lipid droplets in the lumen of the secretory pathway to form a mature lipoprotein (Olofsson et al., 1999). In the second step, MTP is thought to be crucial for recruitment of the neutral lipids to the ER lumen rather than their direct transfer to the apoB (Raabe et al., 1999). Additionally, MTP was shown to transfer phospholipids to CD1d, one of the non-polymorphic major histocompatibility complex (MHC) class I-related CD1 proteins. CD1 presents endogenous and exogenous lipid antigens to T cells. MTP was shown to regulate loading of lipids both from intracellular and extracellular origins onto CD1 in mice and humans (Brozovic et al., 2004; Dougan et al., 2005; Sagiv et al., 2007; Kaser et al., 2008; Zeissig et al., 2010). This function of MTP is independent of any of the known lipoproteins, but the exact mechanism of action of MTP in this process remains unclear.

*Drosophila* possesses three apolipoproteins containing Vitellogenin-N domains, the apoB-family proteins ApoLipophorin (ApoLpp, CG11064) and Apo-Lipid Transfer Particle (apoLTP, CG15828), and a Vitellogenin-like protein, Crossveinless-d (Cv-d, CG31150). Proteins apoLpp and apoLTP are assembled into lipoproteins [called Lipophorin (Lpp) and Lipid Transfer Particle

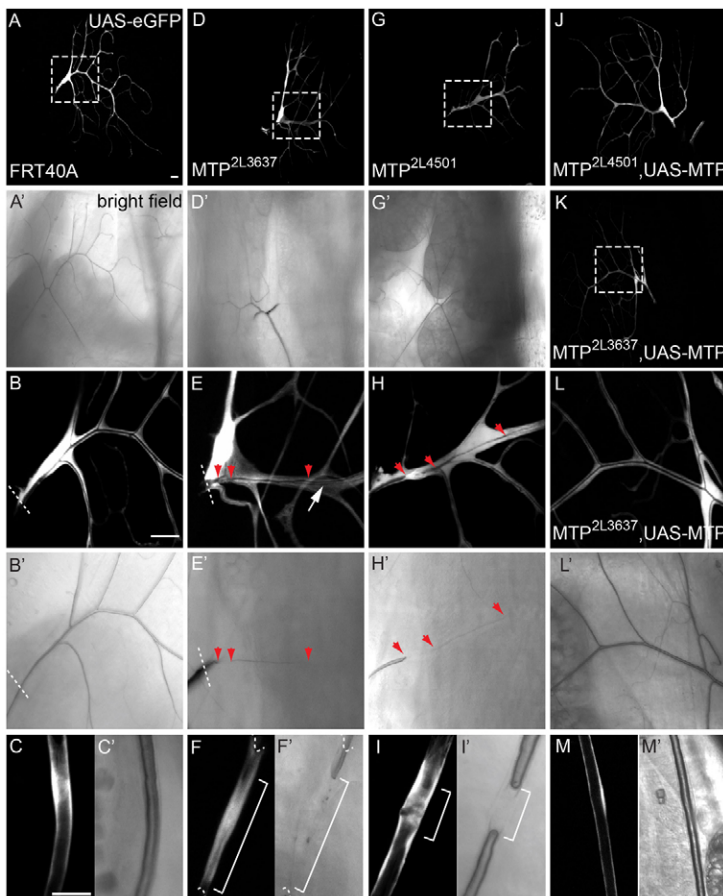
(LTP), respectively], and secreted from the fat body in an MTP-dependent process (Palm et al., 2012). The third fly apolipoprotein, Cv-d (Chen et al., 2012), does not require MTP for its secretion (Palm et al., 2012). In agreement with its role in the secretion of lipoproteins, *Drosophila* MTP is highly expressed in the fat body, both in the embryo and the larva. Furthermore, MTP transcript can be found also at low levels in the larval central nervous system, Malpighian tubules and the trachea (FlyAtlas) (Chintapalli et al., 2007). It is unknown whether the function of MTP in these tissues is related to lipoprotein secretion or to other processes.

Here we describe two new alleles of the gene encoding MTP and characterize a zygotic cell-autonomous role of MTP in the development of a lumen in unicellular tubes of the *Drosophila* tracheal system.

## Results

### Mutations affecting luminal structures of unicellular tubes

In a genetic mosaic screen (Baer et al., 2007) we identified two mutant lines, 2L3637 and 2L4501, which show defects in luminal structures of seamless terminal cells and in cells of branches with autocellular junctions. In wild-type larvae, the lumen of the tracheal system can be seen in brightfield microscopy because it is filled with gas, even in very fine terminal branches (Fig. 1A',B'). In MARCM-induced homozygous mutant cells in larvae heterozygous for either of the two mutant alleles, a gas-filled lumen of wild-type diameter was observed only in the most proximal part of terminal cells, while the more distal branches appeared to have no lumen (Fig. 1D',G'). Only the main branch



**Fig. 1. Mutations in the *MTP* gene cause defects in unicellular lumen structures.** (A–C') In wild-type cells, a gas-filled lumen can be detected throughout the whole terminal cells (A–B') and in autocellular branches (C,C'); the cell spans beyond the shown area of the branch); (B,B') shows a higher magnification of the region marked in A; dashed lines mark the start of the cell. (D–I') In mutant cells, only small portions of the cells are gas-filled. These fragments are often shorter and thinner than the luminal space detected by the absence of GFP (red arrowheads in E,E' and H,H'). Also, multiple lumens can be observed (arrow in E). The gaps in the cells in autocellular branches (brackets in F,F' and I,I') can span almost the whole cell (F,F'; cell borders are indicated by the dashed lines) or just a short fragment (I,I'; mutant cell spans beyond the shown branch area). E,E' and H,H' represent higher magnification of regions marked in D and G, respectively; dashed lines in E and E' mark the start of the cell. (J–M') Expression of *MTP* cDNA (UAS-MTP) in cells homozygous for the *MTP* mutation rescues the luminal phenotype completely. L,L' depicts higher magnification of region marked in K. UAS constructs were driven by *bt1-Gal4*. All images represent cells in 3rd instar dorsal branches (terminal or stalk cells). Scale bars: 20  $\mu$ m.

occasionally contained short fragments of thin lumen filled with gas (Fig. 1E',H'). Because the absence of gas does not necessarily imply the absence of a lumen, we used a UAS-eGFP transgene with the MARCM system, to mark the cytoplasm of mutant cells. In confocal optical sections we found that lumen was visible in some of the terminal branches in which no gas filling was detected (Fig. 1E,H). These lumens were thinner than the corresponding lumens in wild-type cells, their diameter was irregular and they did not form continuous networks. Occasionally, mutant cells contained multiple lumens within one branch (Fig. 1E and Fig. 2C). In autocellular branches, such as the dorsal branch or the lateral trunk, the presence of mutant cells resulted in gaps in the lumen, an event not observed in wild-type larvae (Fig. 1C',F',I'). The size of these breaks was variable and they either spanned the whole cell or small fragments of it. Similarly to terminal cells, the distribution of GFP in the cells indicated that lumen was partially present in these gaps, although again not filled with gas, and that the lumen had a variable diameter and irregular shape (Fig. 1F,I). All mutant terminal cells in mosaic larvae showed the described defects, whereas defects on the autocellular branches were not fully penetrant: in line 2L3637, 70% of mutant cells in autocellular branches showed gaps in the gas filling ( $n=470$ ) and in line 2L4501 73% ( $n=434$ ).

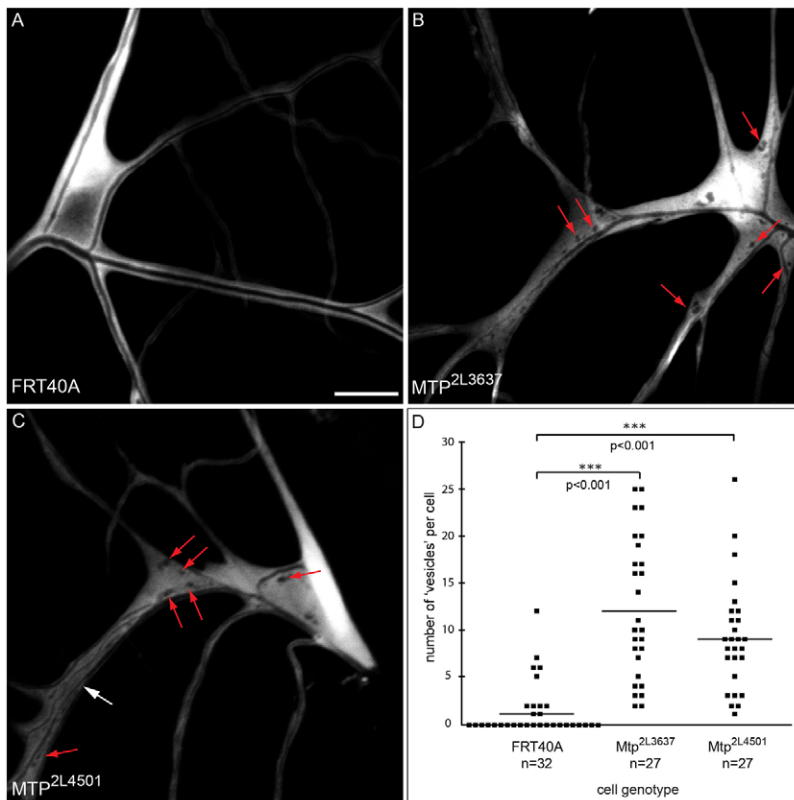
Additionally, terminal mutant cells contained GFP-negative vesicle-like structures along the existing lumen. These structures were found along both gas-filled and non-filled lumens (Fig. 2). Although such 'vesicles' can occasionally be seen in wild-type cells, the number of 'vesicles' found in mutant cells was significantly higher than in wild-type cells (Fig. 2D). 21 of 32 wild-type cells analyzed did not have any 'vesicles' and in the remaining 11 cells, we found 1 to maximally 12 such structures.

In both mutant lines, 'vesicles' were found in all mutant terminal cells; their number varied between individual cells and different genotypes (Fig. 2D). The average vesicle number per cell was 12 for line 2L3637 ( $n=27$ ) and 9 for line 2L4501 ( $n=27$ ). The vesicle size was variable, even within the same cell (Fig. 2B,C). In mutant cells in autocellular branches, we did not detect any vesicle-like structures.

### 2L3637 and 2L4501 are new alleles of the gene encoding MTP

The mutations *2L3637* and *2L4501* were members of one complementation group. Although both were homozygous lethal, they differed in their phase of lethality –*2L3637* was larval lethal and *2L4501* was embryonic semi-lethal (only 50% of homozygous embryos hatched,  $n=97$ ). This difference was likely due to two additional lethal hits that we identified on the left arm of the second chromosome of the chromosome carrying the allele *2L4501* (data not shown). When analyzed in trans, the mutants showed larval lethality: 60% of the transheterozygous larvae died within 12 hours of hatching, and 40% died within the next 24 hours, mostly still at the 1st instar stage. A few animals reached the 2nd instar stage.

To determine which gene is affected in lines 2L3637 and 2L4501, we used SNP mapping followed by complementation analysis with a set of chromosomal deficiencies (see Materials and Methods). Overlapping deficiencies defined a region containing only one candidate gene, *CG9342*, which encodes MTP. We sequenced the genomic region containing this gene in both lines and identified a single point mutation within the MTP open reading frame in each line. In line 2L3637 we found a G to A substitution in the splice consensus donor site at the first intron



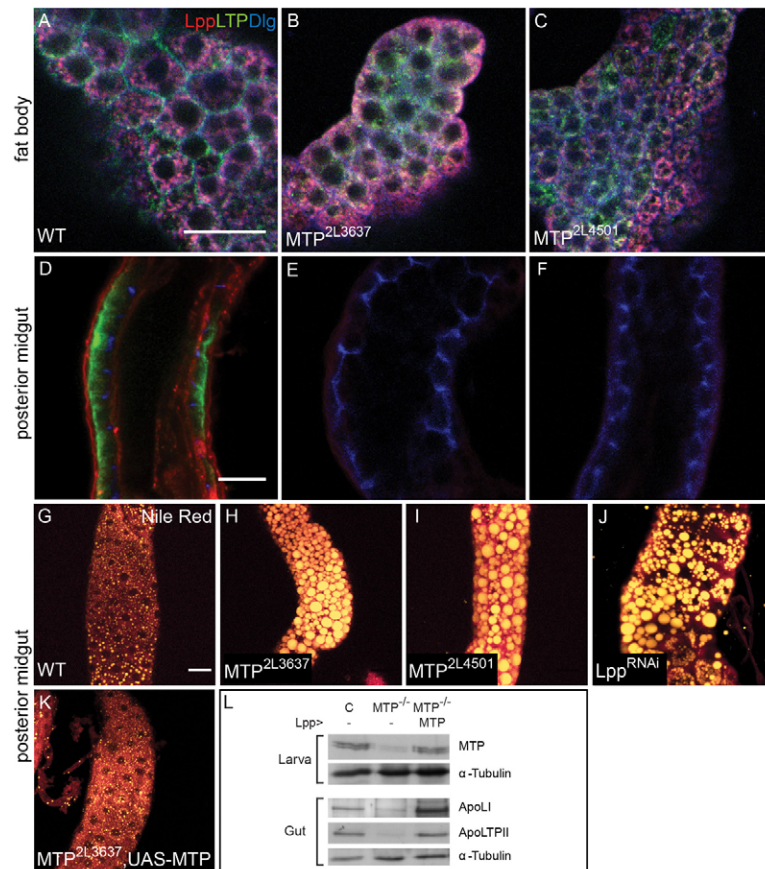
**Fig. 2. GFP negative 'vesicles' accumulate in the cell body of the mutant cells.** (A) In wild type, GFP signal is distributed evenly in the cell body and absent only from the luminal space. Sporadically, little GFP negative vesicle-like structures can be found in the cell body. (B,C) In cells missing MTP, these vesicle-like structures (arrows) can be found more frequently and are much bigger. They predominantly accumulate along the existing lumen. Note that in contrast to wild-type cells, multiple lumens can be observed in MTP mutant cells (white arrow in C). (D) The number of vesicles per cell varies strongly between cells, but is on average significantly higher than in wild-type cells. Each dot represents a single terminal cell; lines indicate average number for each genotype. All images represent dorsal terminal cells in 3rd instar larvae. Scale bar: 20  $\mu$ m.

(as predicted for transcript FBtr0081439). Translation of such a mis-spliced mRNA would yield a protein of 24 amino acids in length. Line 2L4501 carried a G to A substitution resulting in an amino acid residue change at position 134 from glycine to arginine (supplementary material Fig. S1A). This mutation falls within a conserved short  $\alpha$ -helix in the Vitellogenin-N domain of MTP (Smolenaars et al., 2007). MTP protein was detected as a double band on the western blot on wild-type larval extracts, although there are no recognizable cleavage sites or predicted alternative splice forms in the MTP sequence. These bands were dramatically reduced in extracts from both homozygous MTP mutant larvae (supplementary material Fig. S1B). Similarly, larvae hemizygous for either MTP mutant allele and a chromosomal deficiency spanning the genomic MTP region contained yet lower amounts of MTP protein. This may suggest that the residual MTP in mutant larvae represents maternally contributed protein.

The best described role of MTP is to facilitate the assembly and secretion of lipoproteins (Olofsson et al., 1999; Hussain et al., 2003a; Sellers et al., 2003; Rava et al., 2006). In larvae lacking MTP protein, the two *Drosophila* apoB-family lipoproteins Lpp and LTP cannot be secreted from the fat body and are concomitantly depleted in the gut, which normally recruits these lipoproteins from the circulation (Palm et al., 2012). To test whether our newly isolated MTP mutations also affect the secretion of lipoproteins, we stained 2nd instar larvae fat bodies and midgut with Lpp and LTP antibodies. Both lipoproteins were detected in the fat body (Fig. 3B,C; supplementary material S2)

but not in the midgut (Fig. 3E,F). The absence of MTP does not reduce lipoprotein levels in the fat body (supplementary material Fig. S2). However, loss of MTP causes mislocalization of LTP within cells; the prominent pool of cortical LTP is completely lost in MTP mutant fat bodies (supplementary material Fig. S2A''-C''). This is consistent with findings in mammals that MTP deficiency prevents exit of apoB from the ER to the secretory pathway.

Similar to mammals, *Drosophila* lipoproteins are required to export lipids from the gut to the hemolymph. Blocking lipoprotein production in the fat body by knock-down of either the apolipoproteins or MTP causes the formation of giant lipid droplets within gut cells (Fig. 3J) (Palm et al., 2012). To ask whether the MTP alleles we describe here prevented lipoprotein production, we analyzed the midgut of homozygous MTP mutant larvae for the presence of over-sized neutral lipid droplets using Nile Red staining. In wild-type midgut (Fig. 3G), only small neutral lipid droplets were observed, whereas in the midgut of larvae homozygous for both mutant MTP alleles, cells accumulated large droplets (Fig. 3H,I). Expression of MTP full-length cDNA in the fat body of the homozygous mutant animals restored normal levels of both neutral lipid (Fig. 3K) and lipoproteins (Fig. 3L) in the gut. Thus, the new MTP alleles we describe here strongly affect the established function of MTP – the secretion of lipoproteins. Altogether, these results demonstrate that the alleles 2L3637 and 2L4501 represent new loss of function alleles of MTP, which we named  $MTP^{2L3637}$  and  $MTP^{2L4501}$ .



**Fig. 3.  $MTP^{2L3637}$  and  $MTP^{2L4501}$  show defects in secretion of lipoproteins.** (A,D) In wild type, the lipoproteins Lpp and LTP can be detected both in the fat body (A), where they are assembled, and in the posterior midgut (D), where they get reabsorbed from the hemolymph. (B,C,E,F) In MTP mutants, apolipoproteins are still being translated in the fat body (B,C) but due to the secretion defect, they are depleted from the midgut (E,F). (G–J) The absence of lipoproteins in the midgut prevents the secretion of neutral lipids into the hemolymph, resulting in accumulation of giant lipid droplets in intestine cells (J). A similar phenotype can be observed in the midgut of MTP mutants (H,I) but not in the wild-type midgut (G). (K) The lipoprotein secretion defect and giant lipid droplet accumulation can be rescued by expression of MTP cDNA in the fat body of mutant animals with Lpp-Gal4 (Lpp >MTP). Dlg antibody in A–F was used to outline the cells. Fat bodies and midguts were isolated from 2nd instar larvae. (L) Western blot on 2nd instar whole larva (upper panel) or gut homogenates (lower panel) from wild-type (c),  $MTP^{2L3637}$  ( $MTP^{-/-}$ ) and  $MTP^{2L3637}$ ; UAS-MTP/Lpp-Gal4 ( $MTP^{-/-}$ ;MTP) animals; with antibodies against MTP, cleaved C-terminal part of apoLpp (ApoLI) and cleaved N-terminal part of apoLTP (ApoLTPII). Scale bars: 20  $\mu$ m.

### Zygotic MTP is required for proper tracheal development

To confirm that the tracheal defects observed in the mutants are caused by cell-autonomous loss of MTP, we expressed a full-length *MTP* cDNA in the mutant cells. This restored the wild-type luminal structures both in terminal cells (Fig. 1J–L') and in autocellular branches (Fig. 1M, M' and data not shown). The phenotype was rescued in 100% of mutant cells in line 2L4501 (TC  $n=93$ , AB  $n=106$ ) whereas in line 2L3637, 94% of analyzed terminal cells ( $n=85$ ) and 99% of mutant cells in autocellular branches ( $n=71$ ) were reverted to wild type with regard to lumen formation.

To further confirm the requirement of MTP for lumen formation, we removed *MTP* transcripts specifically in terminal cells by expressing MTP RNAi with an SRF-Gal4 driver, which is active only in this subset of tracheal cells. Knockdown of MTP caused defects in terminal cells similar to the phenotype observed in the mutant cells (Fig. 4B, B'). However, the strength of the defects was more variable than in mutant cells; 34% of the analyzed cells ( $n=273$ ) were missing the gas filled lumen only in very distal branches (data not shown), suggesting that MTP RNAi does not completely remove the protein from tracheal cells. Taken together, these results demonstrate that the observed tracheal defects result from mutations in the *MTP* gene and indicate a zygotic function of MTP in the tracheal system.

### Lipoproteins are not required for tracheal lumen development

Because a major function of MTP is to mediate lipoprotein production, we next asked whether the lumen defects observed in tracheal cells lacking MTP could be explained by the absence or altered secretion of lipoproteins. Since the phenotype was seen in clonal mutant cells, it must be due to a cell-autonomous requirement for MTP in tracheal cells. Expression profiling of larval tissues does not detect lipoprotein transcripts in tracheae above background levels (FlyAtlas) (Chintapalli et al., 2007). Nevertheless, we considered the possibility that tracheae might produce undetectable, but functionally important levels of lipoproteins. We knocked down apoLpp in the entire tracheal system via RNAi expression using the *btl*-Gal4 driver. The larvae

obtained did not show any defects (Fig. 5B, B') and they developed to adult flies. Similarly, inducing homozygous apoLTP mutant cells in the tracheal system did not cause any defects (Fig. 5D, D'). This suggests that the defects in lumen formation of MTP-deficient trachea are not caused by loss of tracheal lipoprotein production.

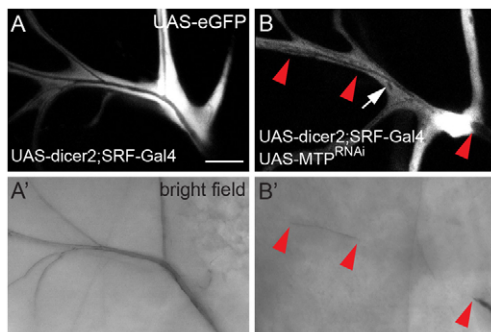
To ask whether lipoproteins recruited from the hemolymph might influence tracheal development, we analyzed the tracheal system of larvae in which lipoprotein production in the fat body was blocked. First, we depleted MTP specifically in the fat body by RNAi, which causes strong reduction of hemolymph lipoprotein levels (Palm et al., 2012). Although expression of RNAi against MTP in the fat body caused slight defects in larval growth and delay in development, the lumen of terminal cells was not affected (Fig. 5F). This indicates that normal levels of fat body-derived lipoproteins are not required for lumen formation. To further confirm that lipoproteins are dispensable for lumen formation, we examined the consequences of loss of apoLTP and apoLpp in the fat body. We analyzed larvae homozygous for a mutant apoLTP allele (*apoltp<sup>Δex1A</sup>*), which survive up to the 2nd instar stage (Fig. 5I). Since the apoLpp mutation causes embryonic lethality, we used fat body-specific RNAi to deplete the protein (Fig. 5G). We did not observe any tracheal defects in these animals. Lpp and LTP execute distinct functions in lipid transport – Lpp is the major carrier of hemolymph lipid, whereas LTP is a lipid transfer protein that mediates loading of Lpp with lipids in the gut (Palm et al., 2012). To confirm that there is also no redundancy between these lipoproteins in the trachea, we generated double knock-down in the larval fat body. Again, despite the delay in development, lumen in the tracheae was not affected (data not shown).

The third known fly apolipoprotein, Cv-d, does not require MTP for its secretion from the fat body. However, as tracheal functions of MTP and Cv-d had not been studied so far, we tested whether Cv-d was required for proper lumen formation by expressing RNAi against *cv-d* with the *btl*-Gal4 driver and by analyzing homozygous *cv-d* mutants. Similar to the results for other lipoproteins, we did not detect any defects (Fig. 5C, C', J). In summary, these results suggest that lipoprotein production in tracheal cells, or lipoprotein recruitment from the hemolymph, is not required for tracheal development. This points to a different substrate or pathway of MTP activity in the tracheal system.

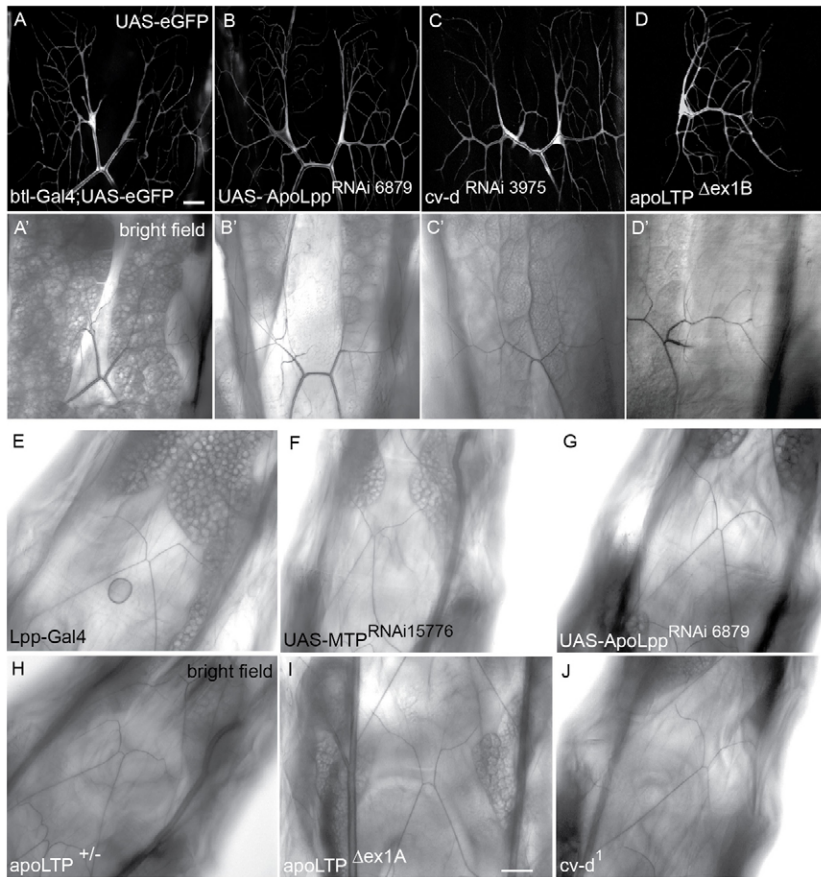
### The role of MTP in lumen expansion

To determine which step in lumen formation is affected by loss of MTP, we studied the phenotype of mutant trachea in more depth. First, we analyzed the initial lumen in the unicellular branches of the homozygous mutant embryos. Analysing tracheae stained with antibodies against the luminal antigen 2A12 and the chitin binding proteins Verm and Serp, which are secreted apically by tracheal cells (Luschnig et al., 2006), did not reveal any obvious defects (data not shown). Moreover, lumens of unicellular branches in homozygous first instar larvae were indistinguishable from wild-type controls (data not shown). This shows that the initial steps of lumen formation were not affected by zygotic loss of MTP activity; we cannot exclude the possibility that maternally provided wild-type MTP protein might contribute to proper lumen formation in the embryo.

Therefore, we focused on the morphology of mutant cells in unicellular branches of 3rd instar larva. The absence of cytoplasmic GFP in parts of the cells was the first indication



**Fig. 4. MTP RNAi knockdown in terminal cells mimics the *MTP* mutant phenotype.** (A, A') Expression of UAS-dicer only with the reporter construct, does not affect the lumen. (B, B') The expression of RNAi against MTP specifically in terminal cells causes phenotypes similar to the defects observed in mutant cells; gas-filled lumen is present only in short regions of the cell (red arrowheads). Also, GFP-negative vesicle-like structures can be observed (white arrow in B). Depicted cells are dorsal terminal cells. Scale bar: 20  $\mu$ m.



**Fig. 5. Lipoproteins are not required for tracheal lumen expansion.** (A–D') Removal of *Drosophila* apolipoproteins (Lpp, LTP, Cv-d) specifically from tracheal cells does not affect lumen formation in seamless tubes. Expression of UAS-eGFP only was used as a control (A,A').

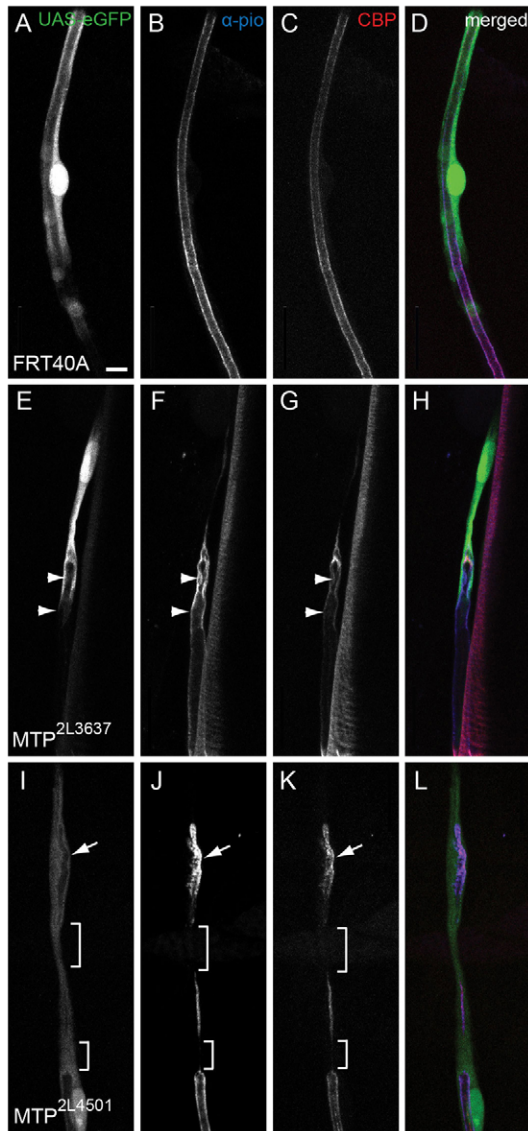
(E–G) Depletion of Lpp alone (G) or together with LTP (by removal of MTP; F) from the hemolymph by fat body-specific knockdown does not cause defects in the trachea. In control larvae (E) only the Lpp-Gal4 driver was expressed. (H–J) In agreement with this, larvae homozygous for mutant alleles of apoLTP (I) and *cv-d* (J) also show wild-type trachea and did not differ from the control animals (H). The apoLpp and Cv-d proteins were knocked down by RNAi expression in all tracheal cells (B–C'). For apoLTP, homozygous clonal mutant cells were generated in heterozygous larva (D,D'). For fat body knockdowns, RNAi lines were driven by Lpp-Gal4. All images represent dorsal terminal cells in 3rd (A–D') or 2nd (E–J) instar larvae. Scale bars: 50  $\mu$ m.

that at least some parts of the mutant cells have a lumen. To verify this further, we analyzed the outline of the luminal structures in mutant cells. We used an antibody against Piopio (Pio) – a Zona Pellucida domain containing protein that is secreted apically in tracheal cells – as a marker for the presence of luminal apical membrane and/or extracellular space (Jaźwińska et al., 2003) and an antibody against Uninflatable (Uif) – a transmembrane protein localizing to the apical plasma membrane in ectodermally derived epithelia (Zhang and Ward, 2009). Since tracheal tubes have an inner cuticle layer, we also used the chitin-binding probe (CBP) to detect the outline of this extracellular luminal structure. In wild-type cells, Pio, Uif and CBP can be detected as continuous lines along the apical surface of the cells in autocellular branches (Fig. 6A–D and data not shown). In cells mutant for either allele of *MTP*, all markers revealed irregular and discontinuous luminal structures (Fig. 6E–L and data not shown). The diameter of the lumen varied along the length of the cell, and often cyst-like structures were found (Fig. 6J,K). Pio and CBP staining was only present in those parts of the cells where the lumen was marked by a GFP-free central channel (Fig. 6E–H), suggesting that the other parts of the cell indeed contained no lumen.

We were not able to detect CBP either in wild-type or in mutant larval terminal cells, indicating that these cells do not possess the same cuticle structure as the rest of the tracheal system. The Uif and Pio staining, however, was detected even in the finest branches of the cell (Fig. 7 and data not shown). In wild-type terminal cells stained for Uif, two distinct lines were observed in longitudinal cross-sections in the most proximal and

thicker branches (Fig. 7A–C) and the signal was restricted to the apical membrane. As the cytoplasmic extensions become thinner, the two membranes were no longer distinguishable from each other and only single lines remained visible. In mutant terminal cells, the signal was more diffuse and present only where the lumen was properly formed (Fig. 7E,J–L). Although the staining was present in most of the remaining branches, it was discontinuous and irregular. Additionally, Uif staining revealed the presence of multiple lumens in some of the branches (Fig. 7D–L). These results indicate that the mutant cells do not completely lack a lumen, but cannot fill up with gas effectively due to breaks in the lumen.

The observed phenotype could be caused by defects in the maintenance of properly formed lumen or defects in the efficient expansion of pre-made luminal structures. To examine which of these two processes is affected in cells mutant for MTP, we analyzed mutant and wild-type cells at different developmental stages. Shortly before the first molt, only the main branch of the terminal cell had a gas-filled lumen both in wild-type and mutant cells (Fig. 8A–B'). In early 2nd instar larvae, side branches were also filled with gas in wild-type cells (Fig. 8C,C'). In contrast, despite formation of the side branches, only part of the main branch was filled with gas in mutant cells (Fig. 8D,D'). Also during the 3rd instar stage, when the lumen of branches in wild-type cells expanded in length and diameter (Fig. 8E,E',G,G'), the gas-filled lumen in mutant cells was thin and spanned only short fragments of the main branch, and the side branches showed thin lumens that were not filled with gas (Fig. 8F,F',H,H'). These observations suggest that in the absence of zygotic MTP, the



**Fig. 6. Mutant cells in autocellular branches have irregular and discontinuous lumen.** (A–D) The lumen of autocellular branches overall has an even diameter, as revealed by anti-pio ( $\alpha$ -pio) and chitin binding probe staining. (E–L) In cells mutated for *MTP*, the lumen is discontinuous (brackets in I–K) and has irregular shape and diameter (arrowheads in E–G). Also, cyst-like structures can be found (arrows in I–K). Images represent stalk cells in dorsal branches of 3rd instar larvae. Scale bar: 10  $\mu$ m.

lumen made in the embryo and 1st instar larva cannot expand properly. To confirm this interpretation further, we followed single mutant cells over time (supplementary material Fig. S3). Although the cells were increasing in size at similar rates to their neighbouring heterozygous cells, no expansion in gas filling was observed, suggesting that *MTP* is involved in lumen growth rather than maintenance.

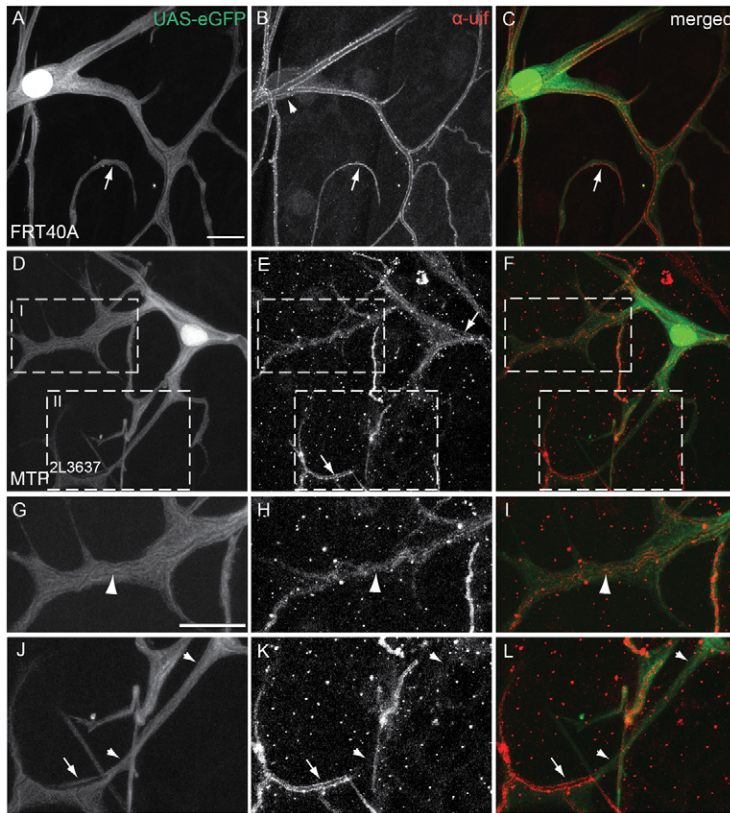
## Discussion

*MTP* protein function has so far almost exclusively been studied in the context of apoB family lipoprotein assembly and secretion, both in mammals and in *Drosophila* (Khatun et al., 2012; Hussain et al., 2003a; Sellers et al., 2003; Palm et al., 2012). Here we

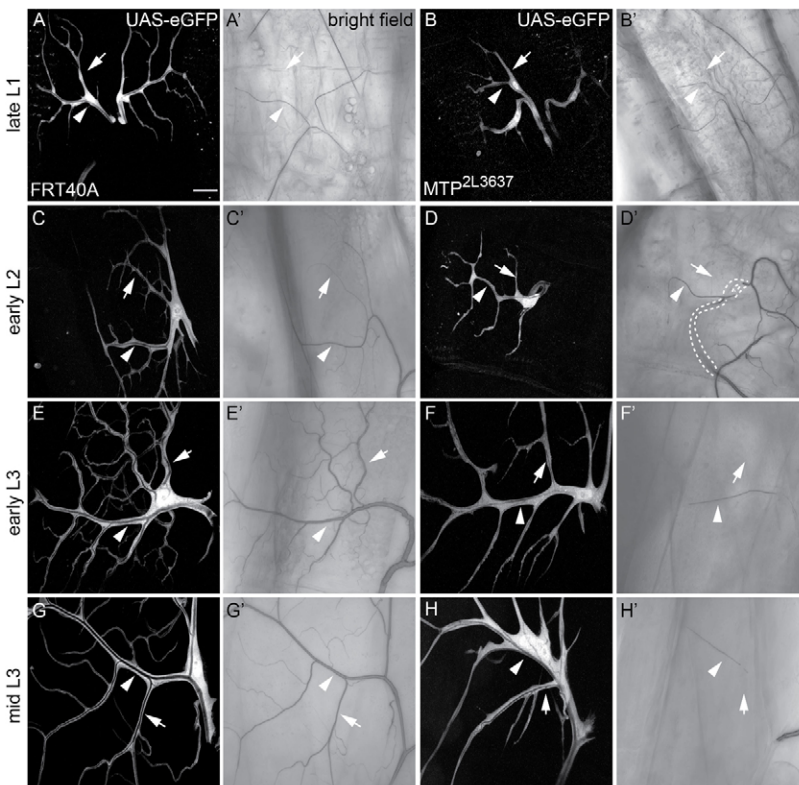
present results suggesting a function for this protein in a developmental process, in shaping the lumen of unicellular tubes in the *Drosophila* tracheal system. In the absence of *MTP*, the tracheal lumen does not expand properly, becomes irregular in shape and diameter and loses its continuity. Additionally, loss of *MTP* in terminal cells leads to the accumulation of vesicle-like structures and the formation of multiple ectopic lumens. Similar to the role of *MTP* in secretion of lipoproteins from fat body cells, the tracheal function of *MTP* is cell-autonomous, as we were able to rescue the mutant phenotype by expressing the mRNA in affected cells only. However, we were not able to detect any lipoproteins in tracheal cells. This, together with the observation that removal of *MTP* from fat body and lipoproteins depletion achieved by various approaches did not affect tracheal lumen development, suggests that the function of *MTP* in trachea is not related to lipoprotein secretion.

Studies in mice suggested that *MTP* has other functions in addition to its role in lipoprotein metabolism since it has been shown to be expressed in cells that do not produce lipoproteins – hematopoietic cells, including T cells, and antigen presenting cells such as dendritic cells (Dougan et al., 2005). In these cells, *MTP* is required for proper lipid antigen presentation by CD1 molecules (Brozovic et al., 2004; Dougan et al., 2005; Sagiv et al., 2007; Kaser et al., 2008). It has been proposed that *MTP* lipidates CD1 during its assembly in the ER. However, despite the ER localization of *MTP*, its removal also affects the presentation of the exogenous lipids that require CD1 recycling from the lysosome to the cell surface (Sagiv et al., 2007; Kaser et al., 2008). Although it remains unclear how ER lipid transfer protein affects lysosomal trafficking, Sagiv et al. (Sagiv et al., 2007) suggested that *MTP* might influence the lipid composition of some membranes or lipid storage that is important for the recycling pathway. Alternatively the impact of *MTP* on CD1 trafficking could be indirect via interaction with another unidentified protein involved in recycling (Sagiv et al., 2007).

The accumulation of GFP-excluding vesicles in terminal cells lacking *MTP* suggests that *MTP* could also affect cellular trafficking in tracheal cells – in this case, the delivery of new membrane material to allow lumen growth. An indication that intracellular trafficking and membrane fusion is important for proper lumen formation comes from a recent screen for tube morphogenesis and branching genes in the fly trachea (Ghabrial et al., 2011). One of the identified mutants, *moon cheese*, carries a mutation in the fly homolog of Membrin, an ER Golgi t-snare, and shows GFP negative vacuoles instead of continuous lumen in the cells of different branches – as if the newly made fragments of lumen were not able to fuse. Also, studies in the embryo have shown that both apical secretion and endocytosis are crucial for proper lumen expansion (Tsarouhas et al., 2007; Grieder et al., 2008; Jayaram et al., 2008; Forster et al., 2010; Norum et al., 2010; Armbruster and Luschnig, 2012; Wang et al., 2012). Interestingly, even in multicellular tubes the secretion that drives the enlargement of the apical membrane and leads to lumen expansion is regulated in a cell-autonomous manner (Forster et al., 2010). Since *MTP* can lipidate the CD1 molecules, one could speculate that also in flies there might be other *MTP* substrates in addition to apoLpp and apoLTP. *MTP* could be involved in the regulation of cellular trafficking through the lipidation of these unknown proteins. Alternatively, as *MTP* was found to be able to recruit phospholipids to specific locations within ER membranes (Khatun et al., 2012) and to transfer phospholipids between



**Fig. 7. Absence of MTP affects lumen diameter and continuity in terminal cells.** Uif ( $\alpha$ -Uif) localizes to the apical/luminal membrane of the terminal cell outlining the lumen. (A–C) In wild type, continuous signal can be detected in all, even very small branches (arrows). The diameter of the lumen decreases gradually, being the largest at the base of the cell (arrowhead). The signal is restricted to the apical surface. (D–L) In mutant cells, the signal is more diffuse. Only fragments where the lumen is formed show clear apical localization (arrows). The lumen diameter is irregular and branches with multiple (arrowheads in G–I) as well as no lumen can be found (arrowheads in J–L). G–I are higher magnification images of boxed regions I outlined in D–F, respectively. J–L are higher magnification images of boxed regions II in D–F, respectively. Images represent lateral terminal cells of 3rd instar larvae. Scale bars: 20  $\mu$ m.



**Fig. 8. Gas-filled lumen formed in first instar larvae does not expand during larval development in MTP mutant cells.** (A–B') In late 1st instar larvae, only the main branch of the terminal cells (arrowheads) is filled with gas, both in wild-type (A,A') and MTP mutant cells (B,B'). The side branches do not have a lumen yet (arrows). (C–D') After the first molt, branching of the cells continues, but in mutant cells the side branches are missing the gas-filled lumen (D,D', arrows), in contrast to wild-type cells (C,C', arrows). Also, parts of the main branch are not completely filled with gas (arrowheads in D,D'). (E,E',G,G') In 3rd instar larvae, the gas-filled lumen of wild-type terminal cells expands not only in length but also in diameter, and lumen can be seen in both main (arrowheads) and side branches (arrows). (F,F',H,H') In MTP mutant cells, the gas-filled lumen of the main branch does not expand in any of the directions (arrowheads). Also, side and newly made branches do not possess gas filling (arrows), despite a GFP-negative area, suggesting that the lumen is at least partially formed (arrow in H). All images depict dorsal terminal cells in different larvae, imaged at different developmental stages. Dashed lines in D' depict the gas-filled dorsal branch preceding the mutant terminal cell, being out of focus. Scale bar: 20  $\mu$ m.



membranes (Atzel and Wetterau, 1993; Atzel and Wetterau, 1994), MTP could affect, directly or indirectly, the lipid composition of the vesicles delivering new apical membrane to the expanding lumen. One could speculate that, in tracheal cells missing MTP, the altered membrane composition of these vesicles prevents them from fusing to already existing lumen or affects fusion efficiency and/or position.

We propose that only a subset of trafficking vesicles is affected by the absence of MTP, since mutant terminal cells have a similar number of branches as wild-type control (data not shown), indicating that there is no defect in addition of material to the basal membrane. Similarly, the outgrowth of dendritic arborization neurons that form elaborated networks is not affected by MTP knockdown in flies (data not shown). Furthermore, the deposition of Pio and Uif in almost all branches of mutant terminal cells suggests that initially the luminal material is correctly localized, but that tube expansion during the 2nd and 3rd instar larval stages is defective.

Finally, if our hypothesis of impaired delivery of apical material were correct, then we would have to wonder why expansion of diameter in multicellular tubes is not affected. Several mutants with defective lumen in unicellular tubes also show a phenotype in clonal cells in the dorsal trunk. For instance cells mutated for genes involved in chitin synthesis, *krotzkopf verkehrt* (*kkv*) and *knickkopf* (*knk*), cause narrowing or local collapse of lumen in the dorsal trunk, in addition to gaps in the lumen in autocellular branches and in terminal cells (Ghabrial et al., 2011 and data not shown). We never observed such a defect for *MTP* mutant cells. It is possible that neighbouring cells compensate for very slow growth of the apical surface of mutant cells by increasing their own surface or, alternatively, despite the large increase in tube diameter, the rate at which new membrane material is added to the dorsal trunk is slower than in terminal cells; in this case, slower trafficking might still be able to contribute to tube expansion. This last possibility would be in line with the observation that defects in lumen maturation in autocellular branches are less severe than those in terminal cells, where much more new membrane needs to be made.

Although the exact mechanism of MTP action in tracheal cells remains unclear we propose that zygotic MTP promotes lumen expansion in length and diameter during larval growth, possibly in an indirect manner that requires lipidation of a yet unknown protein, therefore influencing apical trafficking of new membrane material. In the absence of MTP, apical delivery might be disturbed, resulting in irregular diameter and a discontinuous lumen structure, which in turn would prevent gas filling of the already made lumen. A better understanding of MTP function in the tracheal system and, more general, in cell biology, requires the identification of this possible new target substrate and the characterization of its potential involvement in cellular trafficking. Little is known about lipoprotein-independent functions of MTP. This may in part be due to the severe metabolic perturbations that are caused by loss of MTP in lipoprotein-producing cells. The study we present here provides a framework for such an analysis and demonstrates that the *Drosophila* trachea can be used as a model to dissect novel roles of MTP.

## Materials and Methods

### *Drosophila* stocks

Flies were raised at 25°C in standard conditions. The mutant lines were isolated in an EMS genetic screen (Baer et al., 2007). The FRT40A stock used as a control in all experiments had the genotype  $y[d2] w[1118] P\{ey-FLP.N\}2 P\{GMR-lacZ.C(38.1)\}TPNI;P\{neoFRT\}40A$  (Berger et al., 2001). The line  $yw,$

$hsFlp1.22:tub-Gal80,FRT40A;btl-Gal4, UAS-eGFP$  (gift from S. Luschign, University of Zurich, Switzerland) was used for MARCM analysis. For analysis of homozygous animals, mutations were rebalanced with  $CyO, P\{w^{+mC}\}Df-EYFP\}2$  balancer chromosome (Le et al., 2006). The following lines were used for mapping procedure: 'EP-line':  $y[d2] w[1118] P\{ry[+7.2]=ey-FLP.N\}2 P\{GMR-lacZ.C(38.1)\}TPNI; P\{w[+mC]=EP\}EP511/CyO$  (Berger et al., 2001); and deficiencies:  $Df(2L)Exel6044, Df(2L)Exel6045, Df(2L)Exel7077, Df(2L)Exel6046, Df(2L)Exel7078, Df(2L)Exel7079, Df(2L)Exel7080, Df(2L)Exel6047, Df(2L)Exel6048, Df(2L)Exel7081, Df(2L)Exel6049$  (Exelixis, Inc.) and  $Df(2L)ED1315, Df(2L)ED1384, Df(2L)ED1455, Df(2L)ED1375, Df(2L)ED1378$ . The stocks used to drive UAS- RNAi constructs were  $UAS-dicer2; btl-Gal4,UAS-eGFP$  (kindly provided by N. JayaNandan, EMBL Heidelberg, Germany),  $UAS-dicer2; dSRF-Gal4, UAS-eGFP$  (dSRF-Gal4 line was a gift from J. Casanova, IBMB (CSIC)/IRBB, Barcelona, Spain) and  $hsFlp;Lpp-Gal4$  (Palm et al., 2012). For fat body double knockdown the larvae with following genotype were used:  $hsFlp;UAS-apoLTP^{inducibleRNAi}/+; UAS-apoLpp^{inducibleRNAi}, UAS-dicer2/Lpp-Gal4$ . The RNAi constructs were activated as described (Palm et al., 2012). The following RNAi lines were obtained from Vienna Drosophila RNAi Center:  $UAS-MTP^{RNAi 15775}, UAS-MTP^{RNAi 15776}, UAS-apoLpp^{RNAi 6879}, UAS-cv-d^{RNAi 3975}$ . To perform the MARCM analysis with apoLTP, an allele  $apoLTP^{Aex1B}$  (a P-element excision taking out exon 1) (Palm et al., 2012) was recombined onto FRT40A chromosome. For analysis of homozygous mutants, we used the apoLTP allele  $apoLTP^{Aex1A}$ , which is strongly hypomorphic, and allows analysis of 2nd instar larvae and  $cv-d^1$ . All stocks, unless mentioned otherwise, were obtained from the Bloomington Stock Center.

### Phenotype analysis

Males of the mutant stocks or wild-type control were crossed to the females carrying all components of the MARCM system, with UAS-eGFP as a marker and  $btl-Gal4$  to drive its expression in tracheal cells. The eggs were collected for 4–6 hours at 25°C and heat-shocked for 1 hour at 37°C to induce mitotic recombination, and left to develop at 25°C for 5–6 days. 3rd instar larvae were screened under a Leica fluorescence stereo microscope MZFLIII (Fluo-Combi), for presence of the clones. Positive larvae were placed on the slide with spacers in a drop of 70% glycerol and killed on a heat-block (20 seconds at 65°C). Next the larvae were arranged on the slides dorsal up, and immediately analyzed and imaged under the Leica TSC SP1 confocal microscope. The obtained data was processed with ImageJ (Rasband, W.S., ImageJ, NIH, Bethesda, MD, <http://imagej.nih.gov/ij/>, 1997–2011). The same imaging procedure was applied to 3rd instar larvae from the RNAi crosses. Fat body knock-downs were analyzed and documented under Leica DM6000B microscope.

### Vesicle counting

Confocal images used for quantitative analysis of vesicle-like structures in wild-type and mutant terminal cells were taken under the Leica TSC SP1 confocal microscope, with the same settings. Each image corresponded to the area of  $125 \times 125 \mu m$  covering base of the cell, including the nucleus. The vesicle-like structures in each documented cell were counted on averaged Z-projections with help of 'Cell counter' plugin in ImageJ.

### Analysis of cells over time

The crosses were set up as described in the 'Phenotype analysis' section. Eggs were collected for 1 hour at 25°C and heat-shocked 3 hours later for 1 hour at 37°C. 1st instar larvae positive for GFP clones were transferred to grape juice plates and left to develop at 25°C. Larvae were selected for analysis at 48 hours after egg lay (AEL) for late L1, 52 hours AEL for early L2, 74 hours AEL for early L3 and 96 hours AEL for mid-L3. After mounting as described above, they were imaged using a Zeiss LSM700 confocal microscope. For single cell analysis, larvae were anesthetized in a 1:1 mixture of ether and PBS and imaged under Leica DM6000B microscope.

### Mapping

The SNP mapping procedure was applied as described by Berger et al. (Berger et al., 2001). The recombinants were generated between the 2L3637 line and the reference EP line and analyzed for presence or absence of mutant phenotype. Genomic DNA isolated from the recombinant lines was genotyped for nine different SNP markers distributed along the left arm of the second chromosome (primers sequence, SNPs characteristics and protocols were obtained from FlySNP Database: <http://flysnp.imp.ac.at>). The mutant phenotype was mapped to a 2 Mb region between cytological bands 38A2 and 40A, limited by SNP marker 1104 (2L187) and FRT40A. Since in this region there were no suitable markers available at the time, a lethality complementation test was performed between both alleles and deficiency stocks covering this region, listed in the *Drosophila* stocks section. Both alleles were found to be lethal over  $Df(2L)Exel7080$ . Alignment of all deficiencies breakpoints compared with results of the complementation test led to the determination of a candidate gene. The genomic region of the identified

candidate gene was amplified by PCR on the DNA isolated from control and mutant homozygous single embryos and sequenced to identify potential mutations.

#### Rescue experiment

The *Drosophila* full-length MTP cDNA clone was obtained from the Drosophila Genomic Resource Center (clone no. SD01502). cDNA was cut out from the original pOT2 vector and inserted into MCS of pUASattB vector (Bischof et al., 2007) via *EcoRI/XhoI* restriction sites. The transgenic flies were generated using the phC31 mediated integration system (Bischof et al., 2007) in flies homozygous for the attP landing site on the third chromosome and the integrase on the fourth chromosome. The stable transgenic lines were established according to standard procedures and were crossed with the mutant lines. Males carrying the mutation and the rescue construct were crossed to the MARCM females and analyzed for the clonal phenotype as described above.

#### Immunostainings

For tracheal staining, 3rd instar larvae were filleted open along the ventral midline, dissected in 1×PBS and fixed in 4% paraformaldehyde in PBS for 20 minutes at room temperature, permeabilized with 0.3% Triton X-100 in PBS (PBTx), blocked for 30 minutes with 1% BSA and 2% normal donkey serum in PBTx. Incubation with the primary antibody in 1% BSA, 2% normal donkey serum in PBTx was conducted overnight at 4°C and after several washes the secondary antibody was applied for 2 hours at room temperature. Fat bodies and midguts were fixed with 4% paraformaldehyde in PBS for 20 minutes, permeabilized with 0.05% Triton X-100 in PBS (PBX) and blocked overnight with 10% normal goat serum in PBX. Tissues were incubated for 4 hours with the primary antibody, and after several washes for 2 hours with the secondary antibody; both steps in 10% normal goat serum in PBX at room temperature. All samples were mounted with Vectashield (Vector Labs). Antibodies used were: primary: rabbit anti-Pio (1:100) (Jaźwińska et al., 2003), guinea pig anti-Uif (1:250) (Zhang and Ward, 2009) guinea pig anti-Lpp (1:1000) (Eugster et al., 2007), rabbit anti-LTP (1:1000) (Palm et al., 2012), mouse anti-Dlg (1:500) (Developmental Studies Hybridoma Bank) and rhodamine-conjugated chitin binding probe (1:500) (New England BioLabs); secondary: goat anti-rabbit Alexa Fluor 488, goat anti-guinea pig Alexa Fluor 555, goat anti-guinea pig Alexa Fluor 568, goat anti-rabbit Alexa Fluor 633, goat anti-mouse Alexa Fluor 647 (Invitrogen).

#### Nile Red staining

Unfixed larval tissues were dissected in 1 µg/ml Nile Red (Molecular Probes) in PBS, mounted in the same solution, and imaged immediately. Phospholipids were visualized by excitation at 488 nm and detection between 530 and 600 nm, triacylglycerol by excitation at 543 nm and detection at >635 nm.

#### Biochemistry

UAS-MTP was specifically expressed in the fat body of mutant animals using Lpp-Gal4 driver. 2nd instar guts and larval homogenates were analyzed by SDS gel electrophoresis and immunoblotting. The following antibodies were used: rat anti-apoLI (1:1000) (Panáková et al., 2005), rabbit anti-apoLTP (1:1000) (Palm et al., 2012), rabbit anti-MTP (1:1000) (Palm et al., 2012), mouse anti-alpha-tubulin (1:2000) (Sigma), anti-rat HRP (Invitrogen), anti-rabbit HRP (Santa Cruz Biotechnology), anti-mouse HRP (Invitrogen).

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## Supplementary material

### **Microsomal triacylglycerol transfer protein (MTP) is required cell autonomously to expand tracheal lumen in *Drosophila***

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## Supplementary Figure Legends

### **Figure S1. *MTP* gene and protein in wild type and in *MTP*<sup>2L3637</sup> and *MTP*<sup>2L4501</sup>**

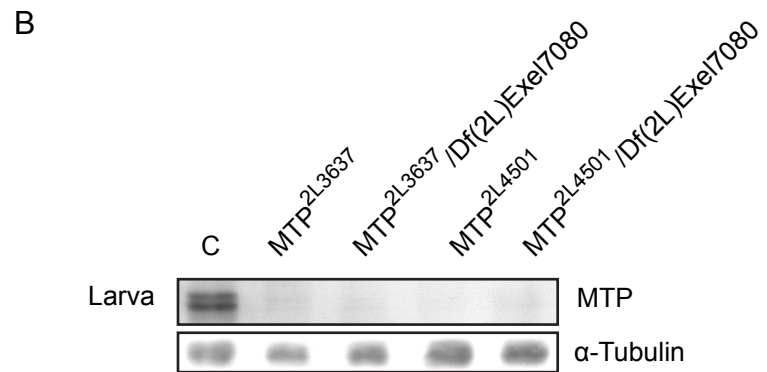
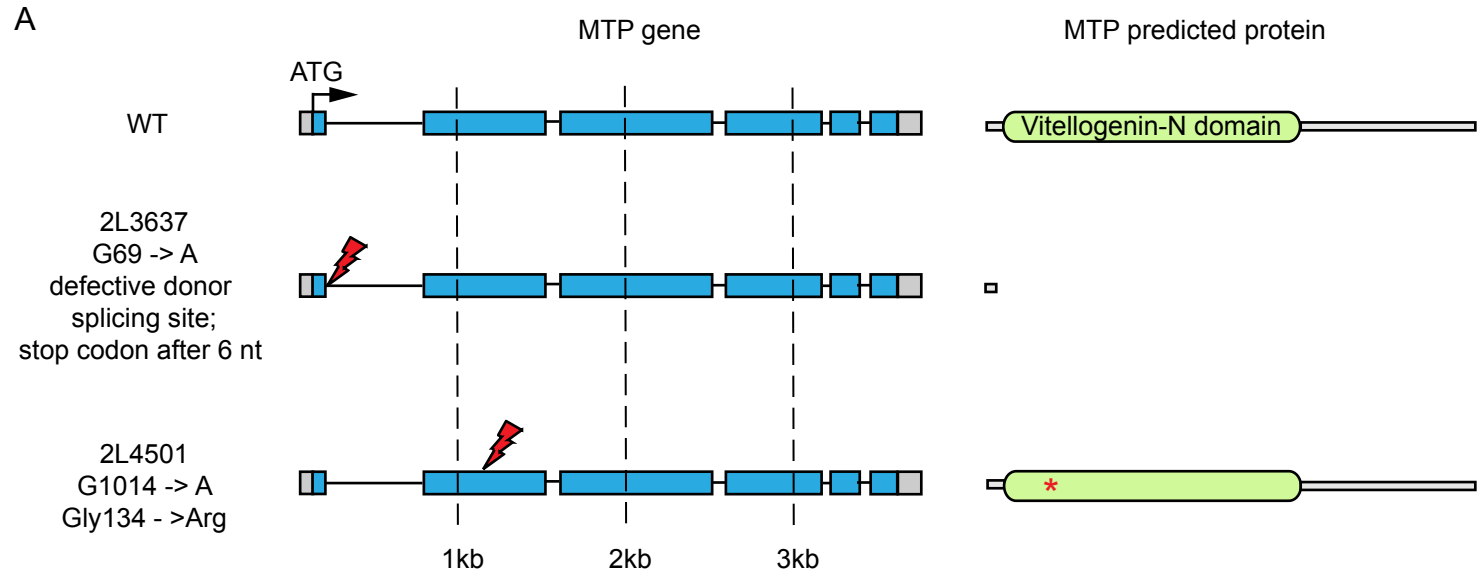
*MTP* gene consists of 6 exons (blue) and encodes a protein of 886 amino acids with N-terminal Vitellogenin-N domain (green). There is only one transcript and one polypeptide reported. In allele 2L3637 G nucleotide at position 69, counting from the ATG in the annotated transcript, is exchanged for A nucleotide – this leads to a defect in the donor splicing site of the first exon. As the result, a stop codon follows after 6 nucleotides. Translation of this transcript results in a truncated protein of 24 amino acids in length, missing the Vitellogenin-N domain. Allele 2L4501 carries a nucleotide substitution of G to A at position 1014, leading to an amino acid residue change at position 134 from Glycine to Arginine. This mutation falls within the Vitellogenin-N domain (asterisk) (A). In larvae homozygous for either of the lines or in combination with a deficiency removing the *MTP* coding region and neighbouring genes, only residual full length protein can be detected on Western blot (B). As there is no difference between the homozygous and hemizygous larvae, we assume that the observed band is due to the maternal contribution. Tubulin was used as a loading control.

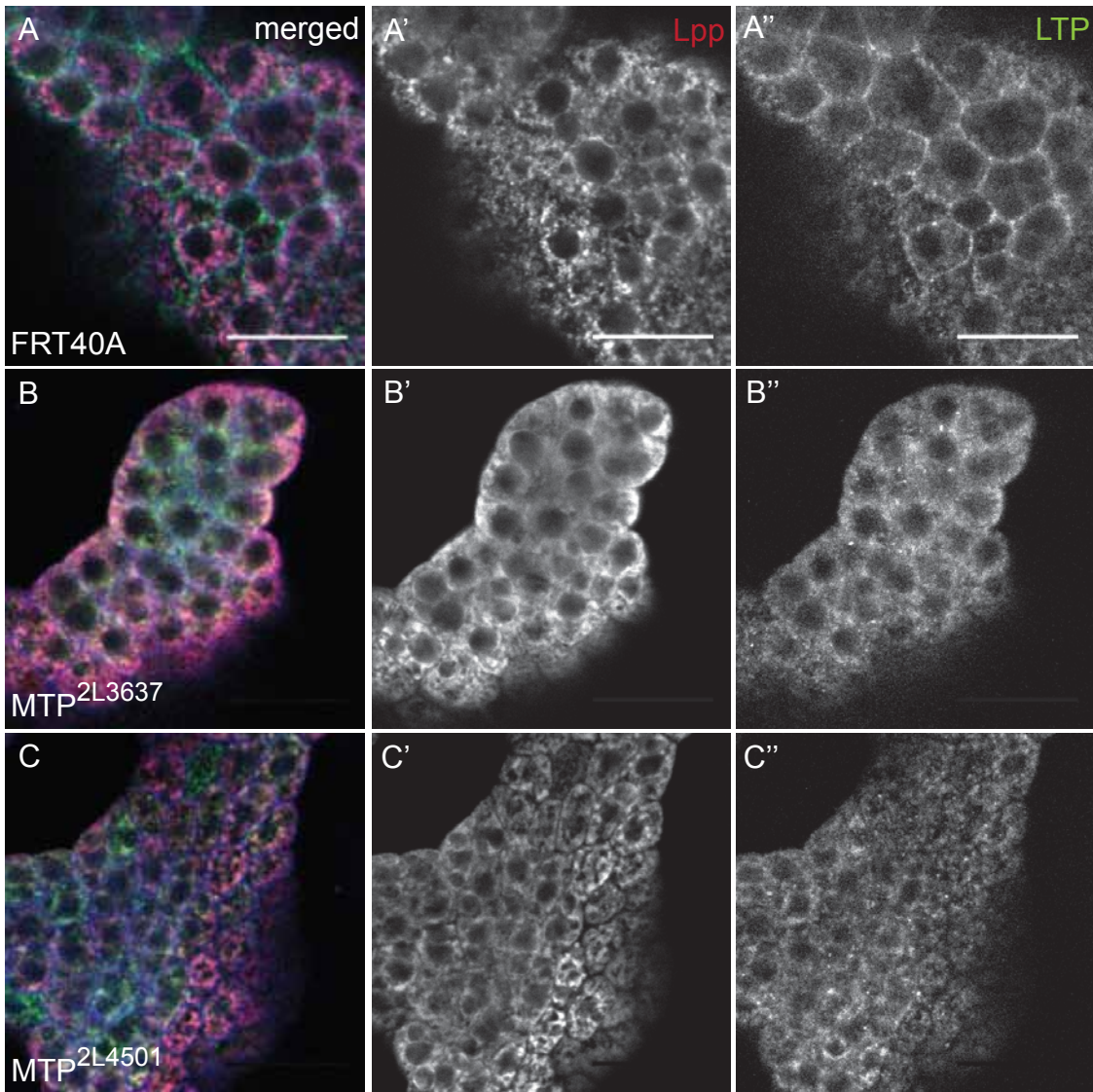
### **Figure S2. Lipoproteins in the fat bodies of wild type and *MTP* mutant larvae.**

Lipoproteins assemble in the cells of the fat body and show distinct cellular localization. In wild type (A'') LTP localizes at the cell membrane, whereas Lpp is distributed broadly in the cell body (A'). *In absence of MTP levels of both lipoproteins in fat body cells are not reduced (B-B'', C-C''). However, membrane localization of LTP is lost (B'' and C'') whereas Lpp preserves its broad cytoplasmic localization.* Scale bars: 20µm.

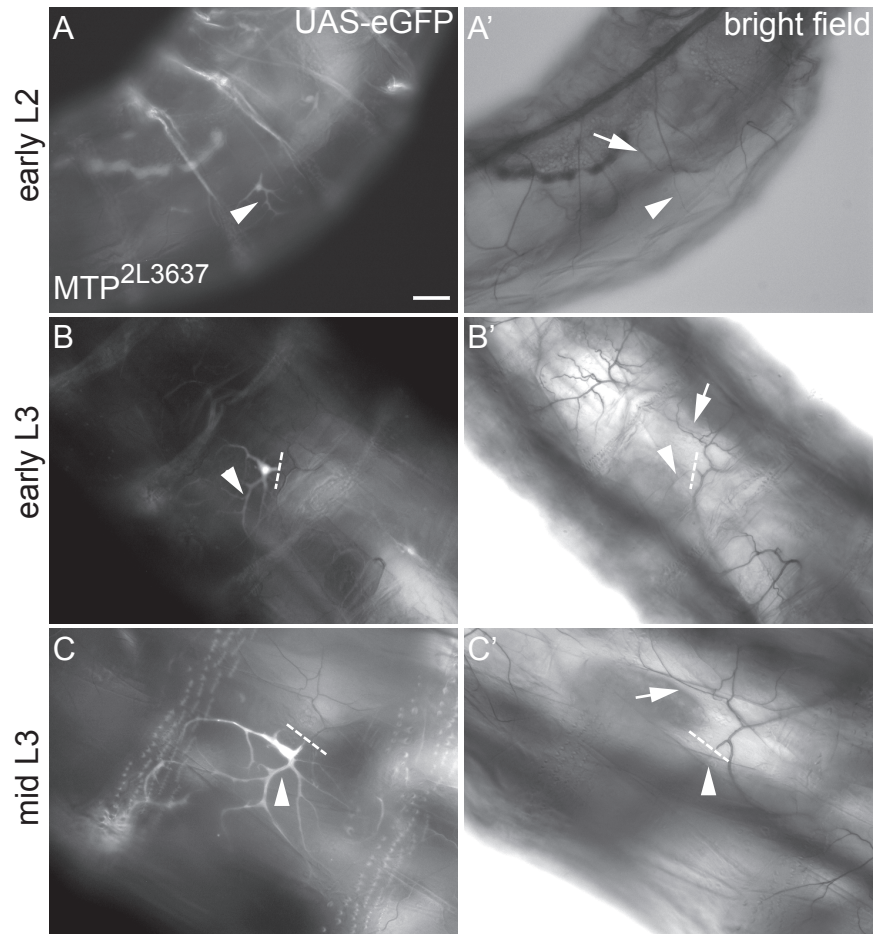
### **Figure S3. Phenotype analysis over time.**

Shortly after the L1 to L2 molt (A, A') there is no obvious difference in gas-filling of the lumen between the mutant (arrowhead) and the wild type (arrow) terminal cells. During next 24 hours and after the L2 to L3 molt (B, B') the wild type cell formed new gas-filled branches (arrow). The mutant cell also expanded its branches, but failed to fill with gas (arrowhead). Within the following 24 hours (C, C'), both wild type and mutant cells made new branches and elongated the existing ones, but the gas-filled lumen in the mutant cell did not expand. The images show the same larva with GFP-marked mutant cells at different developmental stages. The non-marked collateral cell was used as an internal control of lumen growth and gas filling. Dashed lines indicate the proximal end of the mutant cell. Scale bar: 50µm.





Baer et al., Figure S2



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