# Wunen, a *Drosophila* lipid phosphate phosphatase, is required for septate junction-mediated barrier function

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## SUMMARY

Lipid phosphate phosphatases (LPPs) are integral membrane enzymes that regulate the levels of bioactive lipids such as sphingosine 1-phosphate and lysophosphatidic acid. The *Drosophila* LPPs Wunen (Wun) and Wunen-2 (Wun2) have a well-established role in regulating the survival and migration of germ cells. We now show that *wun* has an essential tissue-autonomous role in development of the trachea: the catalytic activity of Wun is required to maintain septate junction (SJ) paracellular barrier function, loss of which causes failure to accumulate crucial luminal components, suggesting a role for phospholipids in SJ function. We find that the integrity of the blood-brain barrier is also lost in *wun* mutants, indicating that loss of SJ function is not restricted to the tracheal system. Furthermore, by comparing the rescue ability of different LPP homologs we show that *wun* function in the trachea is distinct from its role in germ cell migration.

KEY WORDS: *Drosophila*, Germ cell, Lipid phosphate phosphatase, Septate junction, Trachea, Wunen

## INTRODUCTION

Lipid phosphate phosphatases (LPPs) are integral membrane enzymes that regulate the levels of phosphorylated sphingolipids and glycerolipids. In vitro substrates include sphingosine 1-phosphate (S1P), ceramide 1-phosphate, phosphatidic acid (PA), lysophosphatidic acid (LPA), farnesyl diphosphate and geranylgeranyl diphosphate (reviewed by Morris et al., 2012). Phosphorylated forms of sphingolipids and glycerolipids are important metabolic intermediates and intra- and intercellular signaling molecules with a variety of effects on cells. S1P, for example, regulates the exit of T-lymphocytes from mouse lymph nodes (Mandala et al., 2002). The effects of LPA and S1P on vertebrate cells are mediated through related families of G protein-coupled receptors, including $S1P_{1-5}$ and $LPA_{1-5}$, but such receptors have not been identified in invertebrates.

Controlling the levels of such lipids both within and outside of the cell is crucial for regulating their effects. When expressed at the cell surface, the catalytic site of LPPs faces outside of the cell, and LPPs can indeed dephosphorylate extracellular substrates supplied to cells in culture (Roberts et al., 1998; Jasinska et al., 1999). In spite of their activity against a number of in vitro substrates, in vivo roles for LPPs have been more elusive. Mice null for *Lpp3* (*Ppap2b* – Mouse Genome Informatics) die at E10.5 with defects in extra-embryonic vascular development and axial patterning, indicating that LPP3 has a non-redundant role, presumably to regulate the level of an as yet unidentified lipid (Escalante-Alcalde et al., 2003).

The *Drosophila* LPPs *wun* and *wun2*, hereafter collectively referred to as the Wunens, are required for germ cell migration and survival during embryogenesis (Zhang et al., 1997; Starz-Gaiano

et al., 2001). Loss of *wun* and *wun2* in germ cells leads to germ cell death (Hanyu-Nakamura et al., 2004; Renault et al., 2004; Renault et al., 2010). By contrast, loss of *wun* and *wun2* from somatic cells leads to mis-migration of the germ cells, whereas overexpression of either gene in somatic cells leads to germ cell death (Zhang et al., 1997; Starz-Gaiano et al., 2001). Our current model is that spatially restricted expression of Wunens in particular somatic cells creates a gradient of a lysophospholipid through dephosphorylation. This lysophospholipid would act as a germ cell attractant and survival factor. Germ cell Wunens would be required to perceive the lysophospholipid gradient, possibly through concomitant lipid uptake (Renault et al., 2010).

In the course of our work on germ cells, we noticed that embryos lacking maternal and zygotic *wun* (hereafter termed *wun* M–Z– embryos) have trachea that do not fill with gas, prompting us to investigate the role of Wunens in tracheal development.

The tracheal system, which is the gaseous exchange network of the fly, is formed during mid-embryogenesis from ten placodes of cells on each side of the embryo that invaginate from lateral ectoderm. The cells in the placodes undergo cell shape changes and migrations, without further cell divisions, to form branching structures that eventually interconnect to form a continuous tubular network by late embryogenesis (reviewed by Affolter and Caussinus, 2008).

The largest tracheal vessels, the dorsal trunks, run longitudinally along the embryo. The dorsal trunk (DT) lumen at any particular point is surrounded by several tracheal cells with intercellular adherens junctions (AJs) (Samakovlis et al., 1996). The lumen is lined with a cuticle containing chitin, a polymer of N-acetyl-β-D-glucosamine, and is also filled with a transient intraluminal chitin filament during lumen expansion (Devine et al., 2005; Tonning et al., 2005), which is removed by tracheal cell endocytosis shortly before it fills with gas at the end of embryogenesis (Tsarouhas et al., 2007).

In mutants for genes involved in chitin synthesis or organization the trachea show severe tube dilations and cyst-like expansions (Devine et al., 2005; Tonning et al., 2005; Moussian et al., 2006b). Mutants of two luminal and predicted chitin-modifying enzymes, *serpentine* (*serp*) and *vermiform* (*verm*), also disrupt the

morphology of the luminal chitin filament but show a distinct tracheal phenotype in which the DT is excessively elongated and convoluted (Luschnig et al., 2006; Wang et al., 2006).

The impermeability of the DT is maintained by septate junctions (SJs). Mutants in SJ components show defects in paracellular barrier function and the majority show excessively elongated and convoluted DTs (reviewed by Wu and Beitel, 2004). Genetic data, however, suggest that these are independent phenotypes (Paul et al., 2003; Laprise et al., 2010). SJs also have an intriguing role in promoting the luminal accumulation of specific cargos: Serp and Verm do not accumulate in the lumen in many SJ component mutants, whereas the antigen recognized by the 2A12 antibody is unaffected (Wang et al., 2006; Wu et al., 2007; Laprise et al., 2010; Nelson et al., 2010). These data coupled with the appearance of intracellular Verm at stage 15 in some alleles has led to the proposal of an SJ-mediated secretion pathway specific for Serp and Verm (Wang et al., 2006).

In this work we show that embryos mutant for *wun* display defects in the tracheal system. In particular, such embryos have breaks in the DT and non-uniform deposition of luminal components. We further show that these defects are tissue autonomous and are caused, in part, through ineffective SJs leading to a failure to accumulate specific luminal components. Finally, we show that the blood-brain barrier is also defective in *wun* mutants, suggesting that LPP activity is required for SJ-mediated barrier integrity in multiple tissues.

## MATERIALS AND METHODS
### Fly stocks
The following *Drosophila* lines were described previously: *wun^{CE}* and *Df(2R)wun^{GL}* (Zhang et al., 1996), *FRT42B wun^{49} wun2^{EP2650ex34}*, *FRT42B wun^9* and *FRT42B wun^{23}* (Renault et al., 2004), *UAS wun2-myc* and *UAS wun2-myc H326K* (Starz-Gaiano et al., 2001), *UAS wun-GFP* (Burnett and Howard, 2003), *UAS ANF-GFP* (Rao et al., 2001) and *UAS verm-RFP* (Förster et al., 2010).

pENTR-D TOPO entry clones of C-terminal GFP-tagged mouse LPP2 (mLPP2) and mouse LPP3 (mLPP3) (provided by Andrew Morris, University of Kentucky College of Medicine, Lexington, KY, USA) were used with the destination vector pTW (Terence Murphy, Carnegie Institution of Washington, Baltimore, WA, USA) to create pUASt mLPP2-GFP and pUASt mLPP3-GFP using the Gateway reaction (Invitrogen). Standard P-element procedures were used to transform *Drosophila*.

*wun2* was amplified by PCR from a *UAS wun2-myc* plasmid (Starz-Gaiano et al., 2001) using the primers 5′-CACCATGAGCACCCTG-CGACCCGTC-3′ and 5′-CATAGCTTTAAATCGATGGGATCTCC-3′ and cloned into the pENTR-D TOPO vector (Invitrogen). The insert was transferred to the C-terminal GFP tag-containing destination vector pUASt-attB-WG (courtesy of Dr Saverio Brogna, University of Birmingham, UK) and phiC31 integrase-based procedures were used to transform *Drosophila*.

*wun wun2* M– embryos were made using the *FRT42B wun^{49} wun2^{EP2650ex34}* chromosome to generate females containing germ line clones using the dominant female sterile technique (Chou and Perrimon, 1996). To generate *wun wun2* M–Z– embryos these females were crossed to *Df(2R)wun^{GL}*- or *wun^{CE}*-containing males. To generate *wun* M– embryos we used either *FRT42B wun^9* or *FRT42B wun^{23}* chromosomes to generate germ line clone females. To generate *wun* M–Z– embryos these females were crossed to *wun^9*- or *wun^{23}*-containing males.

For tracheal rescue experiments, *UAS LPP-tag* transgenes were recombined with either *Df(2R)wun^{GL}* or *wun^{CE}* and made into stocks with Gal4 drivers on the third chromosome. Males from such stocks were mated to germ line clone females to generate *wun wun2* M–Z– *Gal4 UAS-LPP-tag* embryos. Because the *UAS LPP* recombinant lines were made with a chromosome that removes both *wun* and *wun2*, for consistency we used a *wun wun2* M–Z– background for the majority of experiments.

### Immunohistochemistry and electron microscopy (EM)
Embryos were laid at room temperature, dechorionated in 50% bleach for 3 minutes, and fixed for 20 minutes in 4% formaldehyde in PBS/heptane. For heat fixation, embryos were plunged into boiling 60 mM NaCl with 0.03% Triton X-100 then cooled on ice. Embryos were devitellinized using heptane/methanol and stained using standard protocols with the following antibodies: 2A12 (1:5), DCAD2 (1:20), Coracle (C566.9 and C515.16, 1:400), Armadillo (N27A1, 1:50), Crumbs (Cq4, 1:10), Fasciclin III (7G10, 1:50) and α-Spectrin (3A9, 1:10), all from the Developmental Studies Hybridoma Bank (DSHB); rabbit anti-Serp and anti-Verm (1:300, from Mark Krasnow, Stanford University, CA, USA); rabbit anti-Neurexin (1:1000, from Hugo Bellen, Baylor College of Medicine, Houston, TX, USA); and rabbit anti-Vasa (1:10,000, from Ruth Lehmann, New York University, NY, USA). Rhodamine-conjugated chitin-binding probe (New England Biolabs), Alexa Fluor 633-conjugated wheat germ agglutinin (WGA; Invitrogen), Alexa Fluor 488- (Invitrogen), Cy3-, Cy5- and biotin- (Jackson ImmunoResearch) conjugated secondary antibodies were used at 1:500. Fluorescently stained embryos were mounted in Aquamount (Polysciences) and visualized using an Olympus FV1000 or Leica SP2 microscope. Vasa-stained embryos were visualized using a Vectastain ABC Kit (Vector Labs) and 3,3′-diaminobenzidine, mounted in Epon resin and viewed on a Zeiss AxioImager.

Embryos were fixed and prepared for EM analysis as described (Moussian et al., 2006a).

### Dextran injection
Late stage 16 embryos were dechorionated in 50% bleach, lined up on apple juice agar slices, glued to a coverslip and dried for ~7 minutes; then 2.5 mM (for blood-brain barrier) or 0.5 mM (for tracheal barrier) 10 kDa dextran-tetramethylrhodamine (Invitrogen) was injected into the posterior and the embryos visualized using an Olympus FV1000 microscope.

### Rescue of *wun wun2* M–Z– lethality with *btl*-Gal4-driven LPP expression
Females containing germ line clones were made using the *FRT42B wun^{49} wun2^{EP2650ex34}* chromosome and mated to males containing chromosomes mutant for *wun* and *wun2* [*Df(2R)wun^{GL}* or *wun^{CE}*] in trans to a second chromosome balancer or recombinant chromosomes containing the deficiency with a UAS LPP-GFP or UAS *wun2-myc* transgene in trans to a second chromosome balancer and *btl*-Gal4 in trans to a third chromosome balancer. The resulting offspring were scored for the presence (*wun wun2* M–Z+) or absence (*wun wun2* M–Z–) of the second chromosome balancer. If *btl*-driven LPP expression was able to fully rescue the lethality of the *wun wun2* M–Z– animals, the theoretical ratio of offspring would be 2:1 *wun wun2* M–Z+ to M–Z–, as only 50% of offspring can inherit the *btl*-Gal4 chromosome.

## RESULTS
### Loss of maternal and zygotic expression of the *wun* LPP leads to tracheal defects
Wun is a *Drosophila* LPP that is expressed maternally and zygotically and we observed that the trachea of *wun* M–Z– embryos do not fill with gas (Fig. 1A,B). To examine this phenotype we stained embryos with fluorescently tagged chitin-binding probe (CBP), which detects chitin that is secreted into the tracheal lumen. Two tracheal phenotypes were observed in stage 16 *wun* M–Z– embryos: breaks in the DT (hereafter termed breaks; Fig. 1D,E) and bulbous chitin accumulation throughout the dorsal and lateral trunk and transverse connectives (hereafter termed bulbs; Fig. 1D,E,G,H). The prevalence of these bulbs was similar between the DT and other primary branches. The frequency of tracheal defects was scored in *wun* M–Z– mutants versus control embryos (Oregon R); 95% of the control embryos had normal-looking trachea without bulbs or breaks, whereas 92% of *wun* M–Z– embryos exhibited aberrant tracheal morphology (Fig. 1I, columns 1 and 2).
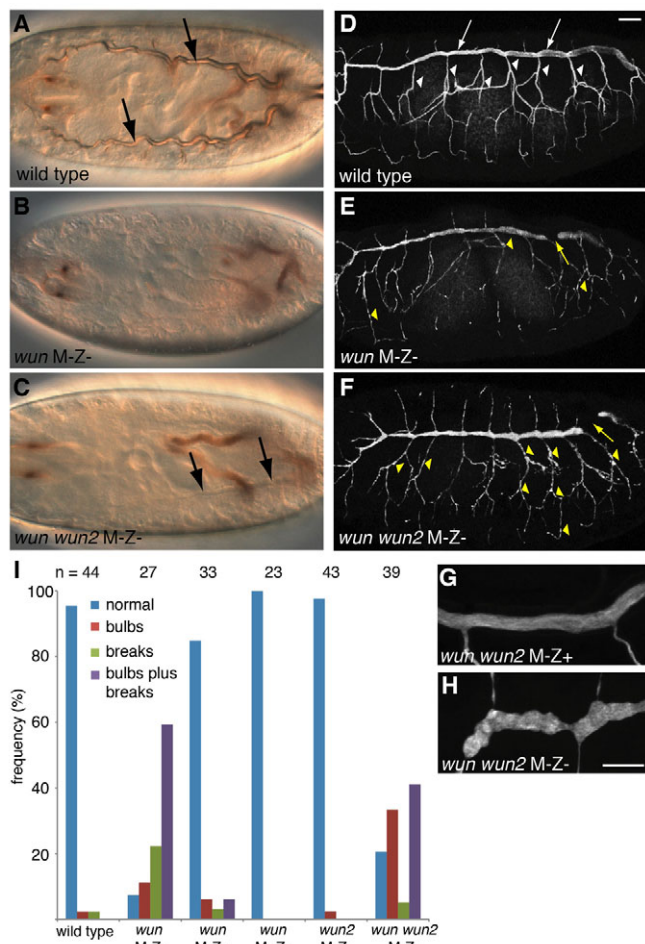
**Fig. 1. The LPP Wun is required for tracheal development.**
(**A-C**) Live stage 17 *Drosophila* embryos (anterior to the left) showing air filling in wild-type (A) but not in *wun²³* M– *wun⁹* Z– (B) or *wun wun2* M–Z– [*Df(2R)wunGL*] (C) embryos. Arrows indicate trachea.
(**D-F**) Confocal projections of stage 16 embryos stained with chitin-binding probe (CBP). In the wild type (D), the dorsal trunk (DT) (white arrows) and primary branches (white arrowheads) are indicated. *wun⁹* M–Z– (E) and *wun wun2* M–Z– [*Df(2R)wunGL*] embryos display chitin bulbs (yellow arrowheads) and breaks (yellow arrow) in the DT.
(**G,H**) Confocal projections of the DT of a stage 16 heat-fixed *wun wun2* M–Z– embryo (H) and sibling M–Z+ control embryo (G) stained with CBP showing disordered chitin and uneven luminal diameter.
(**I**) Frequency of tracheal defects in stage 15-17 *wun* and *wun2* maternal and zygotic embryos. *n*, number of embryos scored. Scale bars: 20 μm in D; 10 μm in H.

We analyzed whether embryos deficient in only maternal or only zygotic *wun* also displayed tracheal phenotypes. Such embryos had normal trachea morphology (Fig. 1I, columns 3 and 4, respectively), indicating that either maternal or zygotic *wun* contribution is sufficient for normal tracheal development, whereas embryos lacking both display tracheal defects.

## *wun2* is not required for normal tracheal formation

In germ cell migration, Wun functions redundantly with a second LPP called Wun2: severe migration defects are observed only when both genes are deleted zygotically in the soma (Starz-Gaiano et al., 2001) and germ cell death is most acute when both genes are removed maternally from the germ cells (Renault et al., 2010).

*wun2* is expressed both maternally and zygotically. *wun2* null alleles are viable and fertile indicating that *wun2* does not play an essential non-redundant role in tracheal development. To test whether *wun2* might nonetheless have an effect on trachea morphology, we examined *wun2* M–Z– embryos; 98% of *wun2* M–Z– embryos had wild-type trachea, indicating that deleting *wun2* alone does not affect tracheal development (Fig. 1I, column 5). To address whether *wun2* acts redundantly with *wun* we examined embryos lacking both *wun* and *wun2* maternally and zygotically; 79% of *wun wun2* M–Z– embryos exhibited aberrant tracheal morphology (Fig. 1I, column 6). Because the prevalence of abnormal trachea is not greater in *wun wun2* M–Z– versus *wun* M–Z– embryos, we conclude that *wun* is the crucial LPP involved in tracheal formation. However, for technical reasons, we have used *wun wun2* M–Z– embryos as a background for further experiments (see Materials and methods). The tracheal defects in *wun* M–Z– and *wun wun2* M–Z– embryos are not general morphogenesis defects, as cuticle preparations indicate wild-type patterning of such embryos (supplementary material Fig. S1; data not shown).

## LPP activity is required autonomously in tracheal cells

By in situ hybridization we have not detected *wun* RNA specifically in the trachea (Renault et al., 2002). This could indicate that either the gene is required autonomously in the trachea but is expressed below our detection limit or that it is expressed and required non-autonomously in neighboring tissues. To distinguish between these possibilities we asked whether expression of Wun in the trachea is sufficient to rescue the tracheal defects.

Wun-GFP, which is functional in germ cell assays (Burnett and Howard, 2003), was expressed in the trachea of *wun wun2* M–Z– embryos using the trachea-specific driver *btl*-Gal4. Expression of Wun-GFP rescued the mutant phenotype, with embryos showing trachea without bulbs or breaks (Fig. 2B). Quantification of tracheal phenotypes in Wun-GFP-expressing embryos indicates that the percentage of embryos with wild-type-looking trachea (100%) is similar to that of *wun wun2* M–Z+ sibling control embryos (83%) and much greater than that of *wun wun2* M–Z– sibling embryos (19%) (Fig. 2H, columns 1-3), indicating complete rescue.

Given that Wun and Wun2 act redundantly in germ cell migration, we tested whether Wun2 expression in the trachea could also rescue the tracheal phenotypes. Wun2-GFP and Wun2-myc, both of which are functional in germ cell assays (Fig. 3I) (Starz-Gaiano et al., 2001), fully rescued the *wun wun2* M–Z– phenotype, with 97% and 98% of the Wun2-GFP-expressing and Wun2-myc-expressing embryos, respectively, displaying normal tracheal phenotypes (Fig. 2C,D,H, columns 4 and 5).

Catalytically dead forms of mammalian LPP3 can have effects on cells (Escalante-Alcalde et al., 2003; Humtsoe et al., 2010); therefore, we asked whether catalytic activity is required to rescue the tracheal defects. A mutant form of Wun2 in which a predicted catalytic histidine has been converted to lysine, H326K, is incapable of affecting the survival or migration of germ cells and shows no activity in vitro against PA (Starz-Gaiano et al., 2001; Renault et al., 2004). Expression of Wun2-myc H326K under the *btl* driver did not rescue the tracheal defects of *wun wun2* M–Z– embryos (Fig. 2E,H, column 6), indicating that LPP catalytic function is indeed crucial for tracheal development.
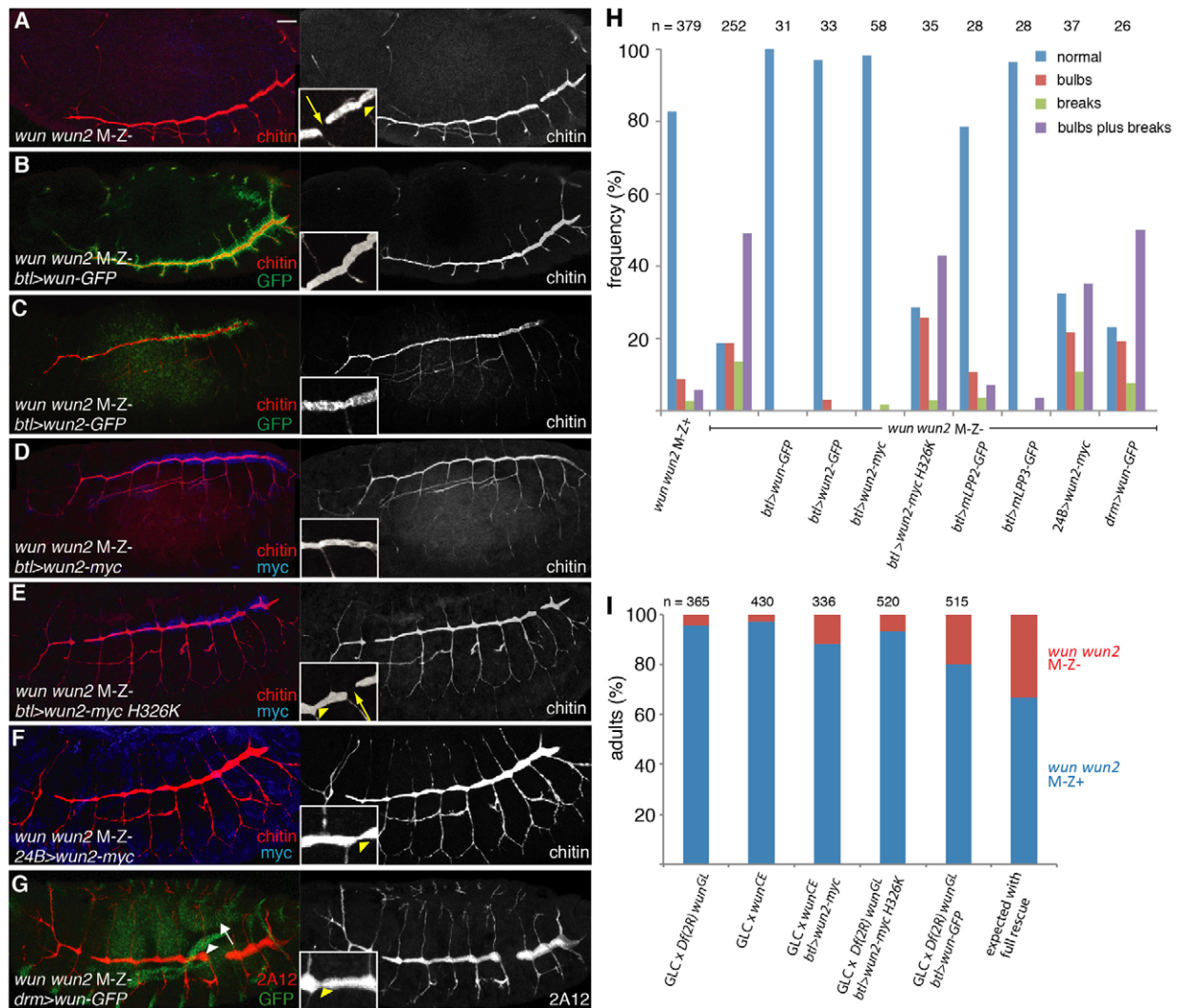
**Fig. 2. Tracheal defects in *wun wun2* embryos are rescued by Wun or Wun2 expression in the trachea but not in the mesoderm or hindgut.** (**A-G**) Tagged versions of Wun (B,G), Wun2 (C,D,F) or a catalytically dead form of Wun2 (E) were expressed in a *wun wun2* M–Z– background under *btl*-Gal4 (B-E), *24B*-Gal4 (F) or *drm*-Gal4 (G), which drive expression in the trachea, late mesoderm or hindgut/Malpighian tubules (white arrow and arrowhead, respectively, in G), respectively. Confocal projections of embryos stained for a luminal marker (CBP or 2A12) and Myc tag or endogenous GFP fluorescence. Insets with magnification of the DT are shown with breaks (yellow arrow) and bulbs (yellow arrowhead) indicated as appropriate. Scale bar: 20 µm. (**H**) Quantification of embryonic phenotypes described in A-G. The *wun wun2* M–Z+ and *wun wun2* M–Z– counts (columns 1 and 2, respectively) are from all of the sibling embryos from the experiments in the remaining columns. *n*, number of embryos scored. (**I**) Survival of *wun wun2* M–Z– animals when Wun or Wun2 is re-expressed in the trachea. *x*-axis shows parental cross, with mothers indicated first (GLC represents germ line clone mothers made using the *FRT42B wun^{49} wun2^{EP2650ex34}* chromosome) followed by fathers [containing either *Df(2R)wun^{GL}* or *wun^{CE}* over a balancer chromosome]. Very few *wun wun2* M–Z– null animals survive to adulthood compared with their M–Z+ siblings. When Wun or Wun2 is expressed in the trachea, an increased percentage of otherwise *wun wun2* M–Z– flies survive. The last column indicates the expected ratio of surviving adults if lethality were completely rescued by Wun or Wun2 expression; it is less than 50:50 because the *btl* driver is only heterozygous in the father and therefore is not present in all offspring. *n*, number of adults scored.

Our rescue analysis indicates that expression of Wun in tracheal cells is sufficient for tracheal development. However, our finding that maternal *wun* is also sufficient for tracheal development raises the possibility that Wun in neighboring cells could also be sufficient, perhaps by producing a lipid environment that is favorable for tracheal formation. To test this possibility, we expressed Wun2-myc under two drivers that are not expressed in the trachea: a mesodermal Gal4 driver, *24B*-Gal4 (*24B* is also known as *how* – FlyBase), that causes expression in muscle cells (Fig. 2F) and the *drm*-Gal4 driver, which is expressed in the hindgut epithelium (Fig. 2G). The *wun wun2* M–Z– tracheal

phenotype was not rescued by Wun2-myc, neither in the musculature nor hindgut, despite high levels of expression (Fig. 2F-H, columns 9 and 10). LPPs are active in mesodermal tissues, as overexpression of Wun2-myc using *24B*-Gal4 disrupts the migration of the germ cells (supplementary material Fig. S2). We conclude that LPP activity is required tissue autonomously in tracheal cells.

Both trachea formation and germ cell migration are dependent on the catalytic activity of Wunens, raising the question of whether Wunens are utilizing the same substrate in both cases. To address this issue, we attempted to rescue the *wun wun2* tracheal defects
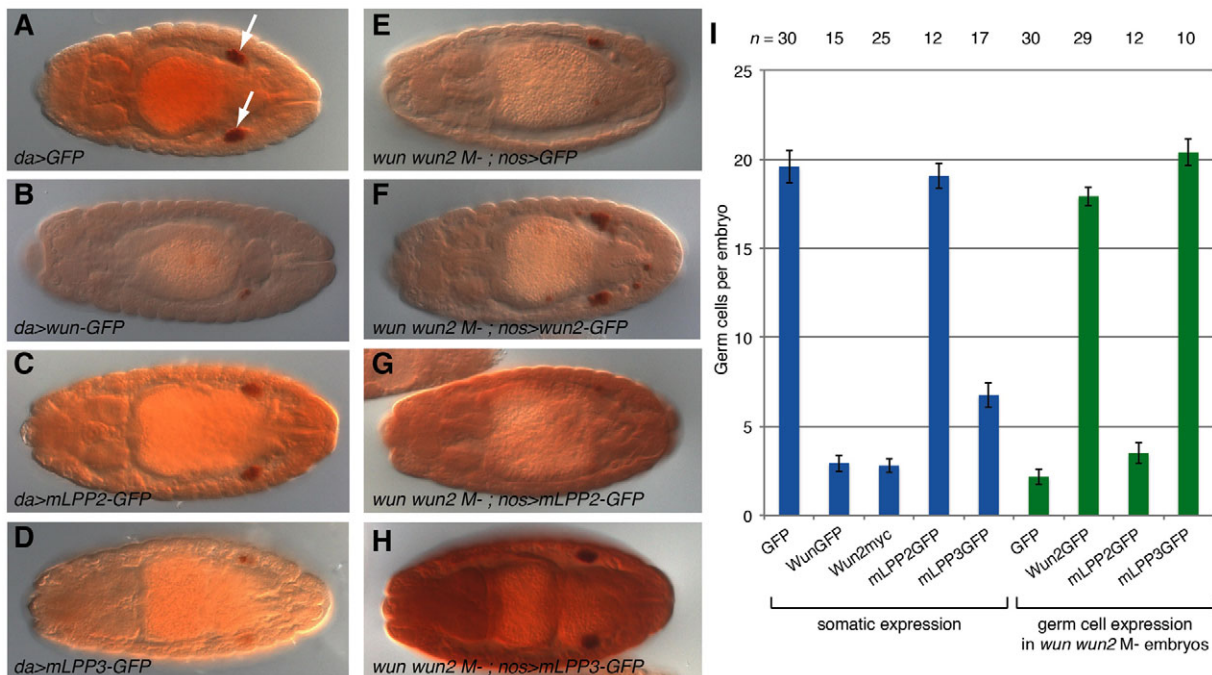
**Fig. 3. mLPP3 but not mLPP2 can replace Wunens in germ cell survival assays.** (**A-H**) Stage 14-15 *Drosophila* embryos stained for germ cells using an anti-Vasa antibody highlighting the germ cells contained within the embryonic gonads (arrows in A). (A-D) Embryos overexpressing Wun-GFP (B) or mLPP3-GFP (D) but not GFP (A) or mLPP2-GFP (C) in somatic cells using the ubiquitous driver *da*-Gal4 show reduced germ cell numbers due to germ cell death. (E-H) Germ cell death in embryos from *wun wun2* null mothers in which the germ cells lack Wunens and die can be rescued by expression of Wun2-GFP (F) or mLPP3-GFP (H) but not GFP (E) or mLPP2-GFP (G) in the germ cells using a *nos*-Gal4VP16 driver. (**I**) The average number of germ cells in stage 14-15 embryos from genotypes indicted in A-H. Blue bars indicate somatic overexpression with the *da*-Gal4 driver. Green bars represent germ cell expression in *wun wun2* M– embryos using the *nos*-Gal4VP16 driver. Error bars indicate s.e.m. *n*, number of embryos scored.

using mammalian LPPs. The rationale is that mammalian LPPs can differ in activity in vivo (Burnett and Howard, 2003; Renault et al., 2004). Mouse (m) LPP3-GFP, similar to Wun or Wun2, but not mLPP2-GFP (LPP2 is also known as Ppap2c – Mouse Genome Informatics) causes mis-migration and germ cell death upon overexpression in the soma (Fig. 3A-D,I, blue columns). Similarly, mLPP3-GFP but not mLPP2-GFP expression in germ cells can rescue the germ cell death caused by lack of germ cell *wun* and *wun2* (Fig. 3E-I, green columns). Identical results were obtained for untagged versions (data not shown). The difference in activity is not due to differences in protein levels as we observe strong expression of both mLPP2-GFP and mLPP3-GFP by immunofluorescence and western blotting (Fig. 4F,G; data not shown). To determine whether the same specificity is seen in the tracheal system, which would imply a similar mode of action, we expressed mLPP2-GFP or mLPP3-GFP in the trachea using the *btl*-Gal4 driver in a *wun wun2* M–Z– background. We found that both mLPP2-GFP and mLPP3-GFP expression rescued *wun wun2* M–Z– tracheal phenotypes to an almost identical extent to Wun-GFP or Wun2-GFP (Fig. 2H, columns 3, 4 and 7, 8). We conclude that the substrate for Wun differs between germ cell and tracheal development.

As the majority of *wun wun2* M–Z– animals die as late embryos, we asked whether the defects in trachea formation are solely responsible for this death. We assessed the ability of trachea-expressed Wun and Wun2 to rescue the lethality of *wun wun2* M–Z– mutants. Only a small percentage [4.1% when using *Df(2R)wunGL* zygotically] of embryos completely lacking maternal and zygotic *wun* and *wun2* survive to adulthood (Fig. 2I).

When Wun-GFP or Wun2-myc is expressed in the trachea, this percentage is raised to 19.8% or 11.6%, respectively. Thus, expression of Wun-GFP or Wun2-myc can only partially rescue the lethality of *wun wun2* M–Z– mutants, indicating that Wunens are required in additional developmental processes.

## Wunens localize to SJs and the apical side of tracheal cells

We addressed where in cells LPPs are localized, and are therefore likely to act, by examining the localization of tagged Wun, Wun2, mLPP2 and mLPP3. Despite the functional redundancy of Wun and Wun2, the localization of these proteins in the trachea is distinct. Wun2-GFP, mLPP2-GFP and mLPP3-GFP localize to the cell surface, as indicated by their colocalization with the plasma membrane-associated cytoskeletal protein α-Spectrin, with stronger accumulation at the apical surface (Fig. 4C,D,F,G). Wun-GFP, however, is localized only to the apical and apicolateral surfaces of the cell (Fig. 4A,B), the latter colocalizing with the SJ marker Fasciclin III, indicating that lateral Wun-GFP is restricted to SJs.

Using an anti-Myc antibody we found that the functional Wun2-myc protein is distributed throughout the cell, some of which colocalizes with Fasciclin III (Fig. 4E). Because both the myc- and the GFP-tagged proteins are capable of rescuing *wun wun2*-associated defects, we hypothesize that cell surface localization of LPPs is required for tracheal development, and that the additional cytoplasmic staining of Wun2-myc is a result of either myc cleavage or localization on intracellular membranes of the secretory pathway.
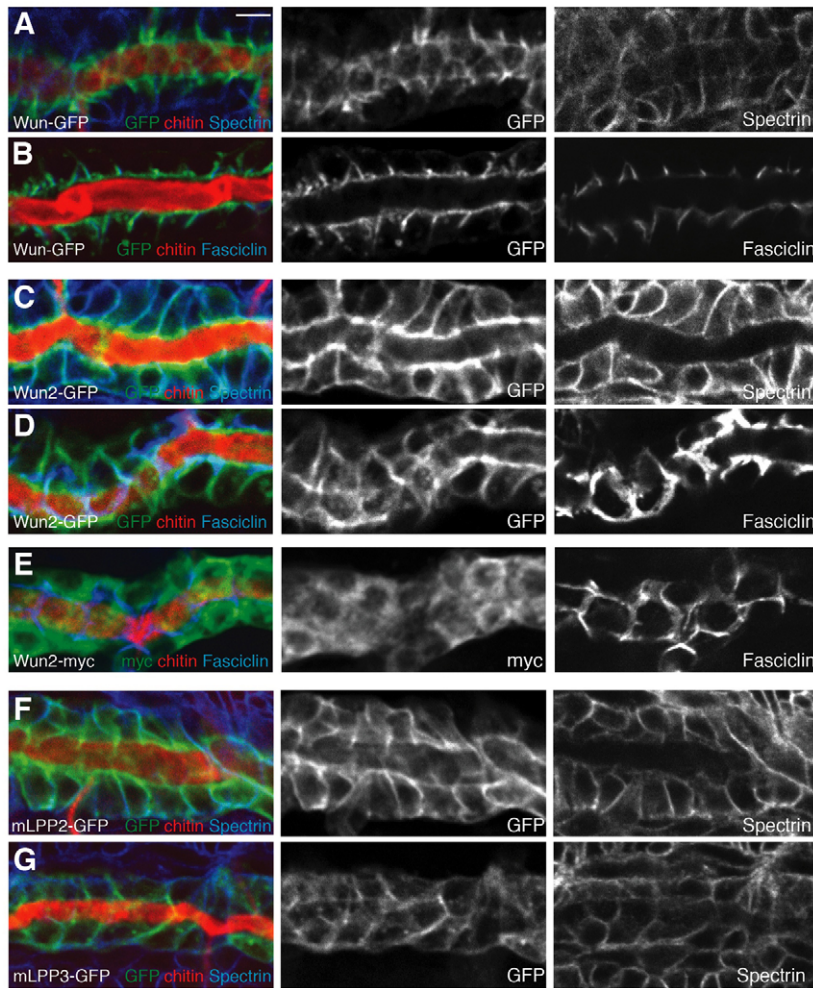
DEVELOPMENT

**Fig. 4. Localization of LPPs in tracheal cells.**
(**A-G**) Tagged versions of LPPs were expressed under the *btl*-Gal4 driver and visualized using endogenous GFP fluorescence (A-D,F,G) or antibodies against myc (E). Tracheal lumens were labeled using CBP and tracheal cells were stained using antibodies against α-Spectrin (A,C,F,G) or Fasciclin III (B,D,E). Single confocal sections are shown. Scale bar: 10 μm.

## Loss of Wunens does not affect cell polarity but leads to defects in AJs and SJs

To address whether loss of Wun affects cell polarity we examined whether various markers were localized correctly in *wun wun2* M–Z– mutants. The localization of the apical marker Crumbs is identical in wild type and *wun wun2* M–Z– mutants, indicating that overall tracheal cell polarity is correct in *wun wun2* mutants (Fig. 5A,B). Next, we examined the AJ protein DE-cadherin. In wild-type tracheal cells, AJ proteins localize to the apical surface of cell-cell contacts and at fusion sites of fusion tip cells. In *wun wun2* M–Z– mutants, DE-cadherin can still be detected, but staining at the apical surface is reduced compared with the wild type (Fig. 5C,D). Finally, we looked at SJs, which are junctions found on the lateral sides of tracheal cells. Electron microscopy (EM) analysis showed that SJs are present between tracheal cells in both wild-type and *wun wun2* M–Z– embryos (Fig. 5L,M). Immunofluorescence of SJ markers, however, revealed that, unlike wild-type embryos, in which SJ marker expression is restricted to the apical portion of the lateral membrane, *wun wun2* M–Z– embryos display SJ protein localization along the entire lateral membrane (Fig. 5C,D,F,G,I,J). Both the reduced apical staining of DE-cadherin and the mislocalization of SJ components are rescued by expression of Wun-GFP in the trachea (Fig. 5E,H,K).

To determine whether Wunens are required for the localization of SJs in other embryonic epithelia, we examined the localization of Neurexin, Coracle and Fasciclin III in the hindgut and salivary gland (SG). In the hindgut of *wun wun2* M–Z– embryos, these markers are restricted as in wild-type, even in embryos in which we can observe lateral spreading in tracheal cells (supplementary material Fig. S3). In the SG of *wun wun2* M–Z– embryos, Neurexin and Coracle show normal apicolateral restriction, but ~50% of embryos exhibit a loss of Fasciclin III restriction (supplementary material Fig. S3H,I for restricted and non-restricted, respectively). Thus, Wunens are not required for SJ localization in all tissues, but the SJs of the SG are partially affected in *wun wun2* M–Z– embryos.

## Loss of Wunens leads to defects in the accumulation of particular luminal proteins

The loss or mislocalization of SJ proteins is associated with decreased luminal levels of the predicted chitin-modifying proteins Verm and Serp (Wang et al., 2006; Wu et al., 2007; Nelson et al., 2010). To determine whether Serp and Verm secretion is affected in *wun wun2* M–Z– mutants, we assessed the localization of these proteins. At stage 13, before the formation of a continuous DT lumen, Serp and Verm are present in the tracheal cells of both mutant and wild-type embryos (Fig. 6A-D). However, by stage 15, when Serp and Verm are normally secreted into the lumen in wild type, little or no luminal staining was detectable in *wun wun2* M–Z– mutants (Fig. 6E,F,H,I and supplementary material Fig. S4B). Serp or Verm did not accumulate in the tracheal cells themselves. However, in the majority of embryos we detected low-level staining throughout the embryo
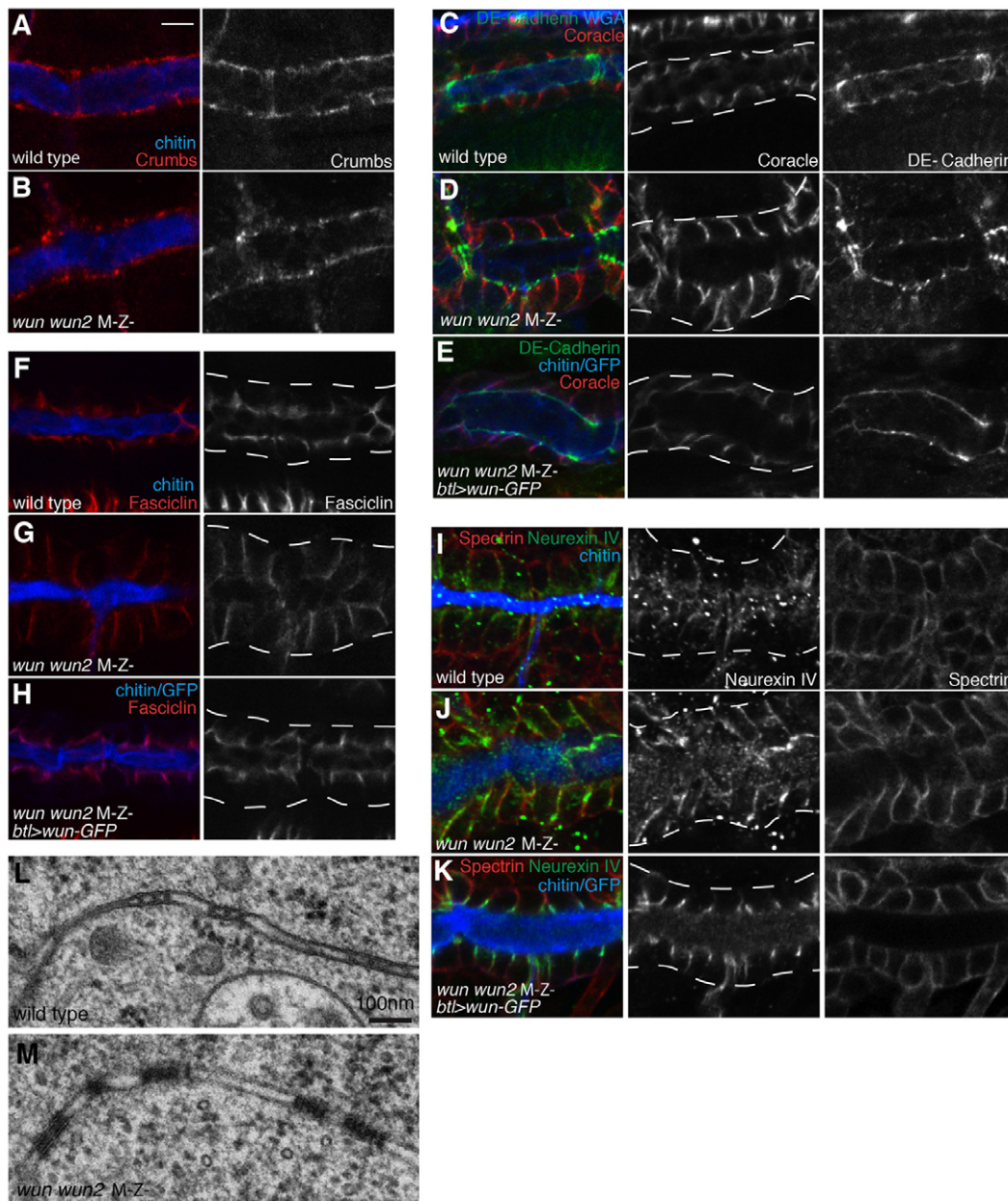
**Fig. 5. *wun wun2* mutant tracheal cells are polarized correctly but AJs and SJs are disrupted. (A-K)** Single confocal sections of stage 16 wild-type (A,C,F,I), *wun wun2* M–Z– (B,D,G,J) or *wun wun2* M–Z– *btl>wun-GFP* (E,H,K) *Drosophila* embryos stained for/with (A,B) Crumbs (red) and CBP (blue), (C-E) DE-cadherin (green), Coracle (red) and CBP or WGA (blue), (F-H) Fasciclin III (red) and CBP (blue), or (I-K) Neurexin IV (green), α-Spectrin (red) and CBP (blue). Punctate staining of Neurexin IV in the tracheal lumen is non-specific and is seen throughout the embryo. **(L,M)** EM sections of wild-type (L) and *wun wun2* M–Z– (M) embryos showing the presence of SJs. Scale bars: 5 μm in A; 100 nm in L.

(Fig. 6F,I and supplementary material Fig. S4B). This was not due to non-specific binding by the Serp antibody because no staining was observed in embryos deficient for *serp* and *verm* using identical staining and detection settings (supplementary material Fig. S4A-C). Western blot analysis confirmed that the Verm protein is still present in stage 15-17 *wun wun2* M–Z– embryos, despite its absence in the trachea (supplementary material Fig. S4D). Expression of Wun-GFP in the trachea restored normal Serp and Verm localization in otherwise *wun wun2* M–Z– embryos (Fig. 6G,J).

To confirm the distribution of Verm seen in fixed tissue we expressed Verm-RFP in the trachea and examined living embryos. In agreement with published data, Verm-RFP accumulated in the

tracheal lumen in stage 16 control embryos (Fig. 6K) (Förster et al., 2010). In *wun wun2* M–Z– embryos, however, we saw only weak fluorescence inside the lumen and, although we did see RFP puncta inside the tracheal cells, these were not brighter than in control embryos (Fig. 6K), indicating that Verm-RFP was not accumulating inside the cells.

The SJ-associated luminal accumulation defect of Serp and Verm is not a generalized secretion defect: another secreted tracheal protein, as detected by the 2A12 antibody, is secreted normally in SJ mutants (Wang et al., 2006). In *wun wun2* M–Z– mutants, the 2A12 antigen accumulates as in wild type, indicating that the secretion machinery in the tracheal cells is functional (Fig. 6E,F,H,I).
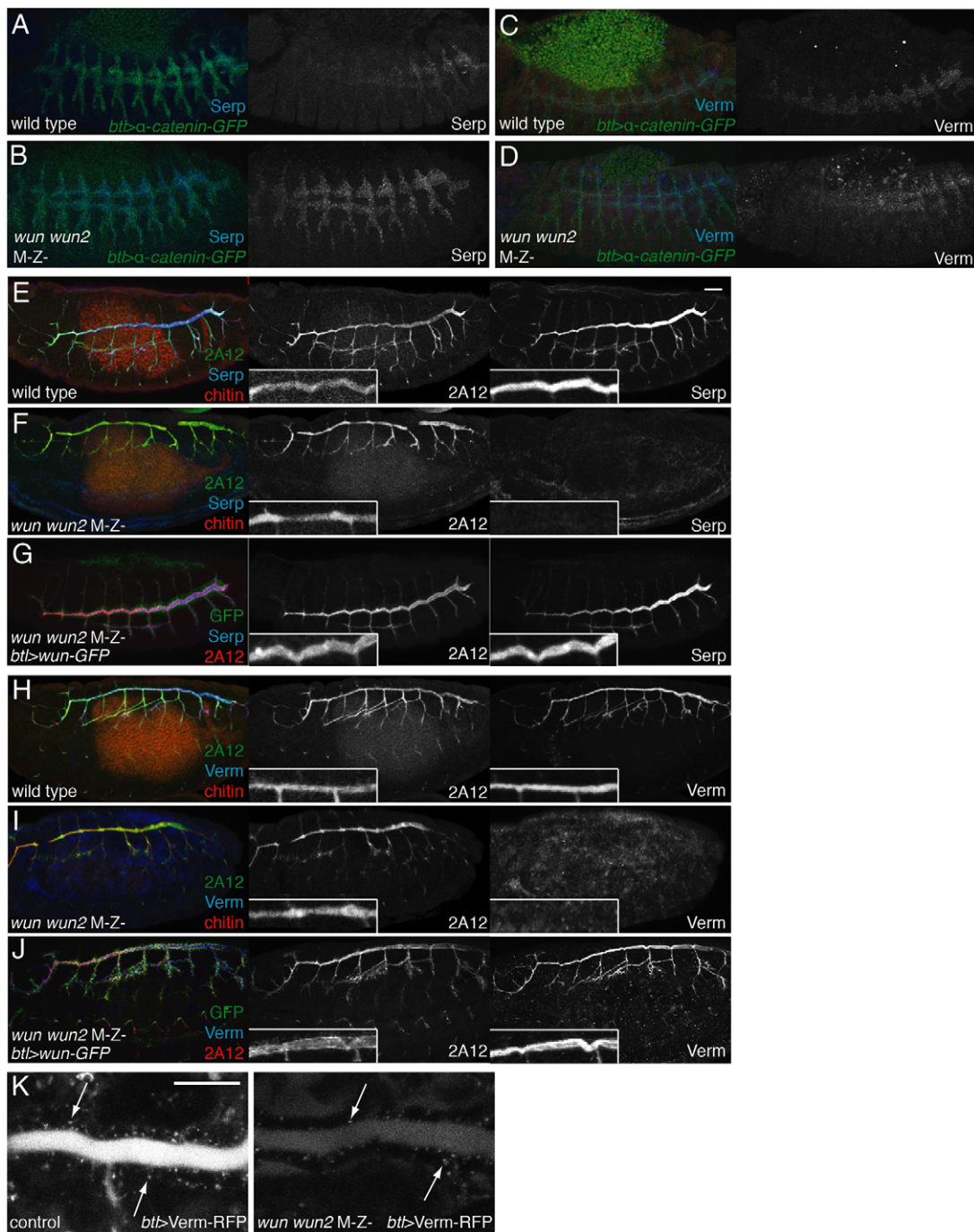
**Fig. 6. Luminal protein accumulation is disrupted in *wun wun2* mutants.** (**A-J**) Confocal projections of stage 13 (A-D) and stage 15 (E-J) wild-type (A,C,E,H), *wun wun2* M–Z– (B,D,F,I) and *wun wun2* M–Z– *btl>wun-GFP* (G,J) *Drosophila* embryos, stained for Serp (A,B,E-G) and Verm (C,D,H-J). Insets in E-J are a 3-fold magnification of a DT section. (**K**) The DT of *wun wun2* M–Z– (right) and sibling control *wun wun2* M–Z+ (left) stage 16 live embryos containing *btl-Gal4 UAS verm-RFP*. Verm-RFP puncta (arrows) are present in tracheal cells but there is no excessive accumulation in the *wun wun2* M–Z– tracheal cells. Scale bars: 20 μm.

Taken together, these data suggest that *wun wun2* M–Z– embryos do not have an absolute secretion defect and also that the failure to visualize Serp and Verm in the lumen is either because they are not properly secreted or because they leak out into the surrounding hemolymph. In support of the latter interpretation, we do not see accumulation of Serp, Verm or Verm-RFP in the tracheal cells (Fig. 6F,I,K); however, it remains possible that these proteins are degraded when not secreted.

To distinguish between these possibilities we examined the behavior of a GFP-tagged heterologous secretion marker, rat atrial natriuretic peptide (ANF) (Rao et al., 2001), that has previously been used to study tracheal maturation (Tsarouhas et al., 2007). Live imaging of *btl*-Gal4-driven UAS-ANF-GFP in a wild-type background shows GFP in the tracheal cells and in the lumen of the DT beginning at late stage 13. From stage 14, some GFP remained visible in the tracheal cells, while the luminal fluorescence increased
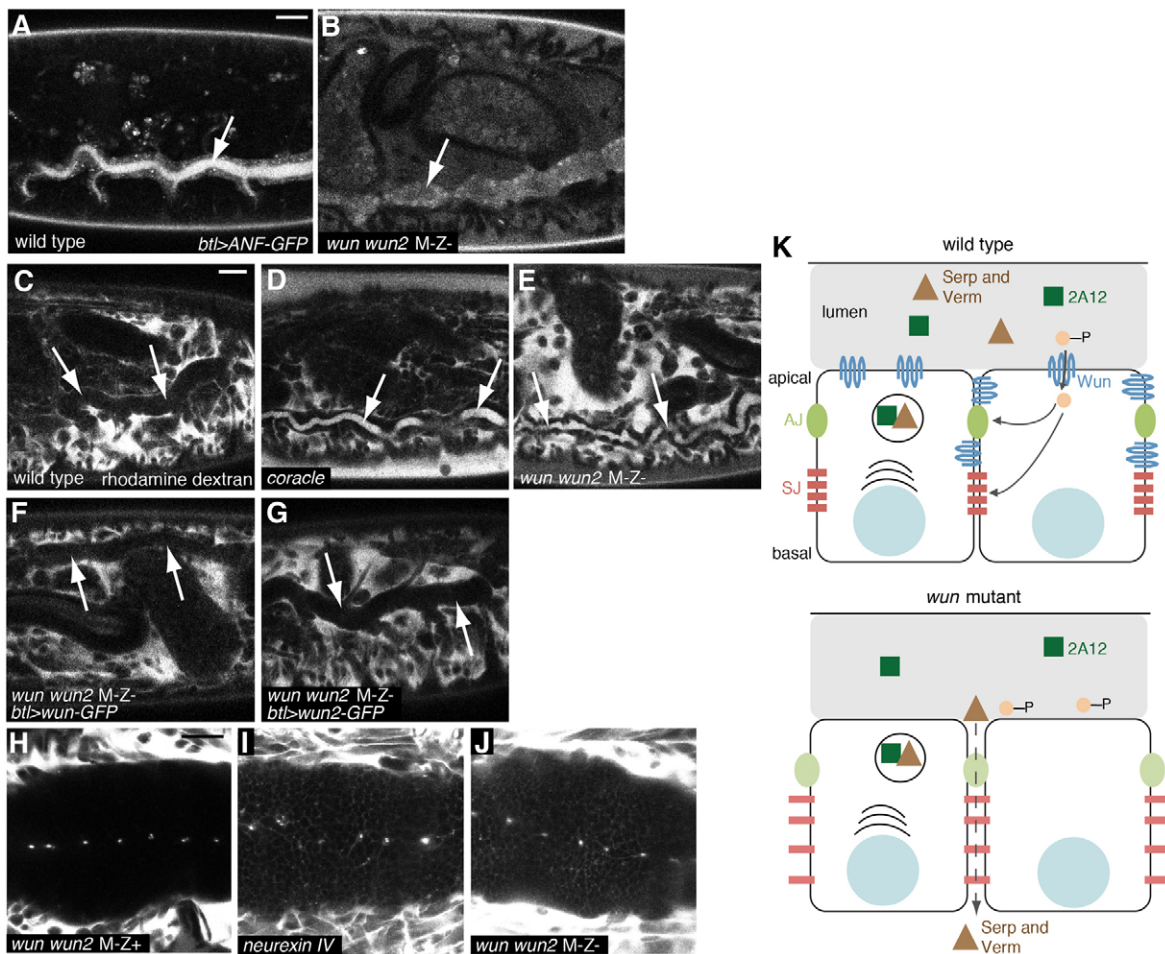
**Fig. 7. *wun wun2* embryos have paracellular barrier defects.** (**A**,**B**) In a wild-type stage 16 *Drosophila* embryo (A), ANF-GFP expressed under a *btl*-Gal4 driver is mostly located in the tracheal lumen. In a *wun wun2* M–Z– embryo (B), ANF-GFP does not accumulate in the lumen or the tracheal cells but instead enters the hemolymph. Arrows mark the DT. (**C-J**) 10 kDa rhodamine-labeled dextran was injected into late stage 16 embryos and visualized live in lateral view to examine the trachea (C-G, arrows mark the DT) and in ventral view for the ventral nerve cord (H-J). Dextran was excluded from the trachea in a wild-type (C), but not in a *coracle*[1] (D) or *wun wun2* M–Z– mutant (E) embryo. Barrier activity was restored by tracheal expression of Wun-GFP (F) or Wun2-GFP (G). The ventral nerve cord excluded the dextran in a sibling control *wun wun2* M–Z+ embryo (H) but not in a *Neurexin IV*[4304] (I) or a *wun wun2* M–Z– mutant embryo (J). The ventral nerve cord runs horizontally in H-J and is bounded above and below by the bright fluorescence of dextran in the hemolymph. Scale bars: 20 μm. (**K**) Model for the role of *wun* in trachea formation. Wun dephosphorylates an extracellular lipid substrate facilitating its transport into tracheal cells. The dephosphorylated lipid regulates the AJs and SJs that are necessary for formation of a tight barrier between tracheal cells. Without Wun, barriers between tracheal cells are weaker, allowing leakage of luminal proteins. AJ, adherens junction; SJ, septate junction; pink circle, LPP substrate and product [with and without phosphate (–P) group, respectively]; pale blue circle, nucleus.

(Fig. 7A). In a *wun wun2* M–Z– background, however, there was weak or no luminal accumulation from late stage 13 and, furthermore, GFP accumulation in the tracheal cells themselves did not exceed that seen in wild-type controls (Fig. 7B). However, there was an overall increase in fluorescence intensity of the hemolymph (Fig. 7B).

We conclude that tracheal cells are able to secrete all cargos tested and, although some components accumulate inside the tracheal lumen (2A12 antigen), other cargos (ANF-GFP, Serp, Verm) do not and instead become dispersed in the hemolymph.

### Loss of Wunens leads to defects in SJ-mediated luminal integrity

The appearance of Serp, Verm and ANF-GFP in the hemolymph of *wun wun2* M–Z– embryos instead of in the tracheal cell lumen prompted us to test the integrity of the SJs between the tracheal cells that would normally act as a barrier between hemolymph and lumen.

We injected fluorescently labeled dextran into the hemolymph of stage 16 embryos. In wild-type embryos the dextran was excluded from the tracheal lumen, but in embryos mutant for the SJ component Coracle the dextran diffused into the lumen (Fig. 7C,D). In *wun wun2* M–Z– embryos we also detected dextran inside the tracheal lumen (Fig. 7E), indicating that the SJ-mediated paracellular barrier between the hemolymph and trachea requires Wun. The luminal dextran is unlikely to have entered via breaks in the DT because these breaks are not continuous with the hemolymph but are surrounded by tracheal cells (supplementary material Fig. S5).

In *wun wun2* M–Z– embryos expressing Wun-GFP or Wun2-GFP in tracheal cells, the dextran was excluded from the tracheal lumen, indicating that autonomous LPP expression is sufficient to restore barrier function (Fig. 7F,G). LPP expression was also sufficient to restore gas filling: 86% of *wun wun2* M–Z+ control

embryos gas filled at the end of embryogenesis (n=36), as compared with 0% of *wun wun2* M–Z– embryos (n=40) and 84% of *wun wun2* M–Z– *btl>wun-GFP* embryos (n=19).

To determine whether the barrier defects were specific to the trachea we examined the ventral nerve cord, which is normally isolated from the hemolymph by SJ-mediated barriers between subperineurial glial cells, the so-called blood-brain barrier. We found that the blood-brain barrier was also compromised in *wun wun2* M–Z– embryos, similar to embryos mutant for an SJ component (Fig. 7H-J). We conclude that LPPs are of general significance for SJ function.

## DISCUSSION

Our study demonstrates a role for an LPP in development of the trachea. We have shown that, in the absence of *wun* function, the trachea suffers from breaks in the DT, non-uniform lumen diameter, and loss of luminal components resulting from ineffective paracellular barrier function. We have shown that *wun* functions tissue autonomously and that Wun activity can be replaced by that of the close paralog Wun2 and two mouse homologs, but not by a catalytically dead LPP.

We only see defects in the trachea when *wun* is removed both maternally and zygotically and this is likely to explain why *wun* has not been previously uncovered in screens performed to identify genes required for tracheal development. Our genetic data suggest that maternally provided Wun protein or the product of the reaction it catalyses lasts at least until the start of tracheal system formation and that zygotically expressed Wun in tracheal cells is sufficient to provide this activity.

In germ cells the Wunens function redundantly: the germ cell death caused by loss of both proteins from germ cells can be rescued by expression of either protein alone, indicating that the two proteins have overlapping substrate specificities (Renault et al., 2010). In the trachea the situation is similar in that overexpression of *wun2* in the trachea is able to substitute for loss of *wun*. To determine whether the roles of Wunens in germ cell migration and tracheal development are identical we used two mammalian LPPs with different activities. mLPP3 is able to substitute for Wunens in germ cell migration and survival assays, whereas mLPP2 is not, in spite of both proteins being highly expressed and localizing to the cell surface. As expected, we find that mLPP3-GFP is able to rescue the tracheal phenotypes of *wun wun2* M–Z– embryos; however, mLPP2-GFP is also able to do so. Thus, mLPP2 lacks an activity required for germ cell migration but possesses activity sufficient for tracheal development. We conclude that the crucial LPP substrate or substrates for germ cell and tracheal development are different. Wunens and mLPP3 show relatively little substrate specificity and can dephosphorylate the lipid essential for both germ cell and tracheal development. mLPP2, by contrast, shows more restrictive specificity and can only dephosphorylate the lipid that is crucial for tracheal development.

The localization of Wun-GFP to particular regions of the tracheal cell plasma membrane is intriguing. Mammalian LPPs have been demonstrated to localize to specific plasma membrane domains. Human (h) LPP1 (PPAP2A – Human Genome Nomenclature Committee) sorts apically, whereas hLPP3 colocalizes with E-cadherin in Madin-Darby canine kidney (MDCK) cells (Jia et al., 2003) and the C-terminal domain of hLPP3 has been shown to bind the AJ protein p120 catenin (catenin δ1) (Humtsoe et al., 2010). However, it remains to be confirmed whether specific Wun-GFP localization is crucial for activity. Wun2-myc can also rescue and, although the protein shows no specific localization, there might be

sufficient present at SJs or apical membranes to fulfill the requirement for LPP activity. What is clear is that the LPPs, just as in germ cell migration, are playing more than a structural role because Wun2-H326K showed no rescue ability.

How does loss of Wun affect the tracheal epithelial cells? Overall, the polarity of these cells is unaffected, but AJ proteins are weaker at the apical surface. In this respect the tracheal phenotype is reminiscent of weak alleles of *shotgun*, which encodes *Drosophila* E-cadherin. Such mutants exhibit incomplete fusion of the DT and uneven luminal diameter (Uemura et al., 1996). However, we do not see a genetic interaction between *wun wun2* and *shotgun* (data not shown), suggesting that reduced DE-cadherin is not the critical factor in causing the tracheal defects in *wun wun2* mutants.

SJs were also affected in the *wun wun2* M–Z– mutants: SJ components were not confined to the subapical region, as in wild type, and paracellular barrier function was lost. However, mutants for all essential SJ components reported to date display an abnormally elongated and convoluted DT (reviewed by Wu and Beitel, 2004). We do not see this phenotype in *wun wun2* M–Z– mutants, indicating that although barrier activity may be lost, SJs must still be present and indeed we do see them by EM. This situation is similar to that of *yrt* M–Z– embryos, which also show compromised paracellular barrier function despite a normal complement of septa when examined ultrastructurally (Laprise et al., 2009).

The lack of luminal accumulation of Serp and Verm in the *wun wun2* M–Z– animals is striking. Embryos mutant for *serp* and *verm* also display trachea with an abnormally elongated and convoluted DT (Luschnig et al., 2006; Wang et al., 2006). As we do not see this in *wun wun2* M–Z– animals, we suspect that the loss of luminal Serp and Verm is not absolute. Indeed, we do occasionally see extremely weak luminal staining (supplementary material Fig. S2B), but mostly we detect Serp and Verm in the hemolymph of late embryos (Fig. 6F,I and supplementary material Fig. S4B). Although we cannot exclude the possibility that Serp and Verm are incorrectly secreted at the basolateral surface, we favor the possibility that Serp and Verm are apically secreted but owing to defects in the SJ-mediated paracellular barrier they diffuse from the lumen into the hemolymph.

The differential accumulation of Serp and Verm versus the 2A12 antigen in the tracheal lumen in various mutant backgrounds has been interpreted to suggest that multiple secretory pathways exist (Massarwa et al., 2009). The first is actin dependent and is based on the observation that in *dia* mutants the 2A12 antigen is not present in the tracheal lumen whereas Verm is (Massarwa et al., 2009). The second is SJ dependent and is based on the fact that in mutants for the α subunit of the Na/K-ATPase, the 2A12 antigen accumulates in the lumen but Verm, which at stage 15 can been seen both in the lumen and in the tracheal cells, is undetectable in the lumen and tracheal cells by stage 16 (Wang et al., 2006). Similar results have been obtained with mutants for other SJ components, including those encoded by *sinuous*, *Lachesin* (Wang et al., 2006), *varicose* (Wu et al., 2007), *coracle* (Laprise et al., 2010) and *kune-kune* (Nelson et al., 2010). Based on our data, it is likely that the failure in Serp and Verm accumulation results, at least in part, from their diffusion out of the trachea. The difference in behavior between Serp, Verm and ANF-GFP versus the 2A12 antigen might depend more on the strength of their interaction with luminal components than on differences in their secretion.

We propose a model (Fig. 7K) in which Wun expression at the cell surface leads to changes in intracellular lipid levels, which affects both AJs and SJs. These changes result in paracellular

barrier defects and prevent particular luminal components from accumulating. Although the identification of which lipid or lipids are being affected and how changes in their levels and/or localization result in defects in specific tissues is ongoing, one potential Wun substrate, S1P, is known to increase barrier function in HUVEC cells via an $S1P_1$-dependent pathway (Lee et al., 2006).

What is particularly striking is that the role of Wun in barrier function for the trachea and ventral nerve cord in *Drosophila* appears to be representative of a more conserved aspect of LPP function. mLPP3 has an essential embryonic role in establishing vascular endothelial cell interactions during early development (Escalante-Alcalde et al., 2003). In addition, mice with postnatal inactivation of *Lpp3* specifically in the vascular endothelium are viable but have impaired vascular endothelial barrier function leading to vascular leakage, particularly in the lungs (M. Panchatcharam, A. J. Morris, D. Escalante-Alcade and S. S. Smyth, personal communication). Thus, it appears that mLPP3 is required in both the establishment and maintenance of vascular integrity in a tissue-autonomous fashion.

Recent studies have demonstrated crucial roles for lipids in establishing or maintaining epithelial cell plasma membrane identity. For example, phosphoinositides are central to establishing the apical surface during lumen formation in MDCK cells (Martin-Belmonte et al., 2007) and glycosphingolipids are needed to maintain apicobasal domain identity in *C. elegans* intestinal cells (Zhang et al., 2011). Modulation of lipid levels coupled with cell biological analyses in a developmental context will be invaluable in exploring this fascinating field further.

### Competing interests statement
The authors declare no competing financial interests.

### Supplementary material
Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.077289/-/DC1

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