Compensatory branching morphogenesis of stalk cells in the Drosophila trachea

Deanne Francis and Amin S. Ghabrial*

ABSTRACT
Tubes are essential for nutrient transport and gas exchange in multicellular eukaryotes, but how connections between different tube types are maintained over time is unknown. In the Drosophila tracheal system, mutations in oak gall (okg) and conjoined (cnj) confer identical defects, including late onset blockage near the terminal cell-stalk cell junction and the ectopic extension of autocellular, seamed tubes into the terminal cell. We determined that okg and cnj encode the E and G subunits of the vacuolar ATPase (vATPase) and showed that both the V0 and V1 domains are required for terminal cell morphogenesis. Remarkably, the ectopic seamed tubes running along vATPase-deficient terminal cells belonged to the neighboring stalk cells. All vATPase-deficient tracheal cells had reduced apical domains and terminal cells displayed mislocalized apical proteins. Consistent with recent reports that the mTOR and vATPase pathways intersect, we found that mTOR pathway mutants phenocopied okg and cnj. Furthermore, terminal cells depleted for the apical determinants Par6 or aPKC had identical ectopic seamed tube defects. We thus identify a novel mechanism of compensatory branching in which stalk cells extend autocellular tubes into neighboring terminal cells with undersized apical domains. This compensatory branching also occurs in response to injury, with damaged terminal cells being rapidly invaded by their stalk cell neighbor.

KEY WORDS: Tubulogenesis, Drosophila, vATPase, Tor, Apical polarity

INTRODUCTION
Tubular networks are essential for nutrient transport, enzyme secretion and ion homeostasis (Andrew and Ewald, 2010; Strilić et al., 2010). In many cases, organs are composed of distinct tube types, including multicellular, autocellular or seamless tubes (Andrew and Ewald, 2010). In the vascular system, for example, multicellular capillaries connect via seamless tubes (Blum et al., 2008; Herwig et al., 2011; Wolff and Bär, 1972). Likewise, the Drosophila tracheal system is composed of interconnected tubes of different types (Fig. 1A,B) (Ghabrial et al., 2003; Samakovlis et al., 1996). Prior work (Song et al., 2013) suggested that connections between tubes of different architectures requires ongoing maintenance, but the maintenance program is not well understood. Here we characterize mutants with late onset connection defects and uncover an unappreciated ability of autocellular seamed tubes to undergo compensatory growth and branching when terminal cells are unable to expand their apical membrane domain.

During fly development, ten pairs of epithelial sacs remodel into a tubular tracheal network in which large multicellular tubes connect to finer autocellular tubes, which in turn connect to intracellular seamless tubes that mediate gas exchange (Fig. 1A,B). Tracheal cells are epithelial, with their apical domain facing the tube lumen (Isaac and Andrew, 1996; Wodarz et al., 1995). Tip cells, located at the ends of primary branches, guide tube outgrowth and later differentiate into fusion cells or terminal cells and form intracellular seamless tubes. Long terminal cell seamless tubes branch extensively, whereas fusion cell tubes are short and unbranched. In dorsal branches, two tip cells connect to a Y-shaped stalk cell. This Y-shaped autocellular tube is bifurcated at the distal end to make independent connections to each tip cell (Samakovlis et al., 1996) (Fig. 1B). The interface between the stalk cell and terminal cell is simple, whereas the connection to the fusion cell is more complex: the stalk cell extends its seamed tube into the fusion cell, like “a finger poking into a balloon” (Gervais et al., 2012; Uv, 2003), such that stalk cell apical membrane surrounds almost the entire fusion cell lumen (Gervais et al., 2012). How the stalk cell makes and maintains these different connections, and what genetically and molecularly distinguishes them, remains undetermined.

To better understand seamed-to-seamless tube connections, we characterized mutations in oak gall (okg) and conjoined (cnj), which confer identical tube connection defects (Ghabrial et al., 2011). Sequence analysis revealed that okg and cnj carry mutations in Vha26 and Vha13, which encode the E and G subunits of the vacuolar ATPase (vATPase). Terminal cells deficient for vATPase showed defects in apical polarity and apical membrane domain size, and neighboring stalk cells extended seamed tubes into them. Depletion of the apical determinants atypical Protein kinase C (aPKC) or Par-6 reproduced key features of vATPase-deficient terminal cells. This implied that wild-type stalk cells compensate for neighboring terminal cells with undersized apical domains by extending branched autocellular tubes into the terminal cell lumen and displacing the terminal cell apical membrane distally. Compensatory stalk cell branching also occurs upon physiological challenge, not just in genetically compromised animals: we find that injured terminal cells are rapidly invaded by their neighboring stalk cell.

RESULTS
Ectopic branched autocellular tubes extend into okg and cnj terminal cells
We characterized the role of okg and cnj (Ghabrial et al., 2011) in tube architecture and connectivity in mosaic animals, with a focus on the connection between autocellular and seamless tubes. In wild-type larvae, a gas-filled autocellular tube connects the stalk cell to its terminal cell neighbor. Within the terminal cell, the seamless tube branches extensively (Fig. 1B,C). Cells mutant for okg or cnj exhibited identical tracheal defects. Mutant terminal cells showed a
gas-filling defect at the stalk to terminal cell connection (Fig. 1D, arrowhead). Strikingly, most gas-filling gaps were present in heterozygous stalk cell tubes adjacent to okg or cnj terminal cells (Fig. 1D′, D″, arrowhead). The gas-filling defect was 100% penetrant, but with late onset (appearing only at third larval instar), and was within the stalk cell rather than the terminal cell in 75% of cnj (n=24) and 70% of okg (n=30) terminal cell clones (Fig. 1D).

Examination of cell junctions within and between stalk cells and terminal cells revealed two classes of novel stalk cell-terminal cell interface defects. Control terminal cells (81%, n=84) had a single intercellular junction with a neighboring stalk cell, consisting of a ring of adherens junctions (AJs) (Fig. 1E′, E″, arrow) and a broader ring of septate junctions (SJs) (Fig. 1E′′, arrowhead). Terminal cells typically showed an unbranched seamless tube extending from the intercellular junction towards the nucleus (Fig. 1E, E′), distal to which extensive branching of the seamless tube occurred. A fraction of control terminal cells (15%, n=84) had a nucleus-proximal autocellular tube (<4 μm in length; supplementary material Fig. S1A), as previously described for embryonic (Samakovlis et al., 1996) and larval (Song et al., 2013) terminal cells. A comparable fraction of okg (18%, n=101) and cnj (25%, n=72) terminal cells had similar segments of short unbranched autocellular tube (Fig. 1H; supplementary material Fig. S1B).

In mutants, we identified a first class (class 1) of defects in which stalk cells formed two or more connections to a neighboring terminal cell (Fig. 1F-F″); this was evident in 24% of okg cells (n=101) and 25% of cnj cells (n=72), as compared with 4% of control cells (Fig. 1H). This topology has not previously been described for either wild-type or mutant terminal cells. In more severely affected terminal cells [9% (n=101) okg and 8% (n=72) cnj] we observed class 2 defects in which long, branched seams tubes extended well beyond the terminal cell nucleus (Fig. 1G, G′, H). Unlike class 1 seamed tubes,
class 2 tubes branched within the terminal cell and appeared to terminate at ring-like intercellular junctions distal to the terminal cell nucleus – phenotypes never observed in wild type (Fig. 1E,H). Beyond the ring-like structures, seamless tubes extended towards the terminal cell tips (Fig. 1G,G'). We could not detect any evidence of long autocellular tubes within homozygous mutant terminal cells (supplementary material Fig. S1C,D), suggesting that okg and cnj connection defects arise late.

**cnj and okg encode two subunits of the vacuolar H\(^+\) ATPase**

To understand how cnj and okg influence tube architecture, we mapped the mutations (Fig. 2A,B). Sequence analysis of candidate genes revealed a Vha13 mutation in cnj and a Vha26 mutation in okg (Fig. 2C,D). Vha13 and Vha26 encode the G (13 kDa) and E (26 kDa) subunits of vATPase. A Vha13 transgene rescued cnj tracheal defects, confirming gene identity and suggesting that the mutant allele is a loss of function (Fig. 2E-G'). The cnj\(^{356}\) allele carries a deletion that creates a frameshift predicted to encode a larger protein, all but 45 residues of which are novel. The okg\(^{406}\) allele is likely to be amorphic, with a nonsense mutation encoding a truncated Vha26 protein. Terminal cell-specific knockdown of Vha26 recapitulated the okg phenotype, with a higher incidence of class 2 defects (45%, n=67; Fig. 2H,H'). The severity of the knockdown phenotype suggested a maternal contribution of okg mRNA, early zygotic transcription prior to clone induction, or both. In addition, postembryonic knockdown was sufficient to confer the terminal cell defects (supplementary material Fig. S1E), consistent with a maintenance role of the vATPase.

**Loss of proton pumping results in ectopic autocellular tubes**

Vha13 and Vha26 function as a heterodimer within the V1 domain (Hildenbrand et al., 2010; Ohira et al., 2006; Okamoto-Terry et al., 2013; Smardon et al., 2002). Mutation of okg or cnj should compromise V1 and vATPase assembly, but not V0 function (Doherty and Kane, 1993; Tomashok et al., 1997). Since free V0 can bind SNAREs to promote membrane fusion (Bayer et al., 2003; Di Giovanni et al., 2010; Hiesinger et al., 2005; Liegeois, 2006; Strasser et al., 2011), it was important to test whether tracheal defects resulted from loss of vATPase function or gain of V0 membrane fusion activity. Terminal cells depleted for either V0d or c\(^{3-}\) (Fig. 3B-D') exhibited both class 1 and class 2 phenotypes (n=93 and n=42, respectively; see Fig. 3F for quantification); thus, loss of the holoenzyme is the underlying cause of the phenotype.

To test whether holoenzyme assembly (permitting interactions such as Pak binding (Lin et al., 2012)) is sufficient for tubulogenesis or if enzymatic function (acidification of intracellular compartments) is required, we used an allele of Vha100 (Vku) that specifically disrupts proton translocation (Kawasaki-Nishi et al., 2001; Williamson et al., 2010). Terminal cell expression of Vha100\(^{R755A}\) resulted in defects identical to those caused by loss of V1 or V0, with both class 1 (55%) and class 2 (5%) observed (n=58; Fig. 3E,F). This established that acidification is required for maintenance of tube architecture.

**Stalk cell autocellular tubes extend into okg and cnj terminal cells**

Why do vATPase mutant terminal cells have long, branched autocellular tubes? Does this reflect a failure to switch from autocellular to seamless tubulogenesis, altered spatial regulation of where this switch occurs, or does the sealed tube come from the neighboring stalk cell, as occurs in wild-type fusion cells? The latter seemed likely, as sealed tubes within terminal cells appeared to terminate at internal intercellular junctions. However, the organization of sealed-to-seamless tube transitions remains unclear for terminal cells: some evidence suggests the connection of sealed and seamless tubes within a terminal cell ‘transition zone’ (Samakovlis et al., 1996; Song et al., 2013), whereas other data suggest that terminal cells make exclusively seamless tubes (Gervais and Casanova, 2010; Samakovlis et al., 1996; Song et al., 2013; Uv,
extends a seamed tube into the fusion cell like (Uv, 2003). At wild-type stalk cell-fusion cell interfaces, the stalk cell
vATPase holoenzyme. (B-E) promote assembly/disassembly of the V1-V0 (membrane pore; lowercase) heterodimers are part of the V1 domain (ATPase; subunits capitalized), and vATPase holoenzyme function.

**Fig. 3. Maintenance of the stalk cell-terminal cell interface requires vATPase holoenzyme function.** (A) Okg (green) and Cnj (purple) heterodimers are part of the V1 domain (ATPase; subunits capitalized), and promote assembly/disassembly of the V1-V0 (membrane pore; lowercase) vATPase holoenzyme. (B-E) SRF-GAL4 drives RNAi knockdown and/or GFP expression (terminal cell is outlined by the dashed white line): (B,B′) control, (C,C′) V0 d (both VhaAC39-1 and VhaAC39-2 RNAi), (D,D′) VhaPPA1-1/ V0 c′ RNAi, (E,E′) dm-GAL4> V0 aR755A (pump-dead). (F) Quantification of phenotype frequency (see Fig. 1H for key). Scale bar: 10 µm.

2003). At wild-type stalk cell-fusion cell interfaces, the stalk cell extends a seamed tube into the fusion cell like “a finger poking into a balloon” (Uv, 2003) – note that these ‘fingers’ end in ring-like intercellular junctions; this has led to the suggestion that terminal cell autacellular tubes might also be stalk cell ‘fingers’. However, we and others see no evidence of this in wild-type terminal cells (Gervais and Casanova, 2010; Song et al., 2013).

To better understand terminal cell-stalk cell interfaces, we re-examined wild-type third instar larvae. We found that one cell membrane extends basolaterally from the intercellular AJ to ensheathe a portion of the neighboring cell; a broad collar of intercellular SJ staining correlated with this area (supplementary material Fig. S2A,B,C). In some instances, terminal cells ensheathe stalk cells (supplementary material Fig. S2A,D), but stalk cells also ensheathe terminal cells (supplementary material Fig. S2B,E). Within 15% of terminal cells, we find a short, unbranched autacellular tube extending from the terminal cell seamless tube to the intercellular junction (Fig. 1H; supplementary material Fig. S1A,B) (Song et al., 2013), whereas 85% of terminal cells had only seamless tubes. Importantly, wild-type terminal cell autacellular tubes lack the internal ring-like structures that mark the distal ends of the stalk cell tube within fusion cells.

To test if seamed tubes in okg and cnj terminal cells belong to the stalk cell, we knocked down the SJ protein Fasciclin 3 (Fas3) exclusively in cnj terminal cells. If Fas3-positive seamed tubes were detected in cnj, Fas3 RNAi terminal cells, then Fas3, and hence the tube, must belong to the stalk cell. As a control we depleted Fas3 from the stalk cell; we found that stalk cells were depleted for Fas3, but not other SJ or AJ proteins (supplementary material Fig. S3A,C). Knockdown in terminal cells was efficient, since control Fas3 RNAi terminal cells lost their Fas3 collar staining (n=12; supplementary material Fig. S3D,E; Fig. 4A,B). In cnj, Fas3 RNAi terminal cells, Fas3 was detected in seamed tubes running along the terminal cell, implying that the tubes belonged to the stalk cell (Fig. 4C).

As an independent test of seamed tube ownership, we utilized the Flybow system (Hadjieconomou et al., 2011) to differentially label neighboring stalk and terminal cells. In control larvae, terminal cell-stalk cell membranes were apposed at the site of initial cell-cell contact (Fig. 4D). In okg RNAi terminal cells, the autacellular tube was lined by stalk cell membrane that was partially or completely surrounded by terminal cell membrane (Fig. 4E,F). In some dramatic instances, terminal cell seamless tubes with no visible connection to each other were connected by stalk cell autacellular tube (Fig. 4F). These data suggested that okg and cnj apical membrane domains might be limiting, resulting in displacement of terminal cell seamless tubes distally, with compensatory branching and hypertrophy of stalk cell seamed tubes, the apical membrane domain of which is coupled to that of the terminal cell by AJs.

**okg and cnj multicellular and autacellular tubes have reduced apical domains**

If okg and cnj cells were unable to generate sufficient apical membrane, we hypothesized that it would compromise their ability to contribute to tubes of all architectures; therefore, we carefully tested okg and cnj dorsal trunk and stalk cell clones, which contribute to multicellular and autacellular tubes, respectively. In contrast to control dorsal trunk cells (Fig. 5A), okg and cnj dorsal trunk cells were not fully integrated into the epithelium and were oval, not hexagonal, in shape (Fig. 5B). Since the apical domain in epithelia lines the tube lumen, okg and cnj dorsal trunk clones had little or no apical domain. A statistically significant decrease in the ratio of apical membrane (area as defined by AJs) to cytoplasm (largest cross-sectional area as detected with cytoplasmic GFP) was observed in cnj dorsal trunk clones compared with control clones, which had a 1:1 ratio (Fig. 5C). In control autacellular tubes, stalk cells elongated along the long axis of the tube and connected to neighbors via ring-shaped intercellular junctions, with a single line of autacellular junctions running the length of the tube (Fig. 5D). By contrast, 89% (n=76) of cnj and 98% (n=95) of okg stalk cells did not have normal
autocellular or intercellular junctions (Fig. 5E); instead, these cells either detached from the epithelium or contributed a very short segment of autocellular tube, while the remaining cell cytoplasm bulged out of the tracheal epithelium (Fig. 5E′, E″). Interestingly, tubulogenesis is intact in cnj and okg embryos: we see no evidence of multicellular or autocellular tube defects in homozygous cnj embryos or in cnj stalk cell clones in first and second instar mosaic larvae, while dorsal trunk clones begin to round by second instar (supplementary material Fig. S1C,D,E,F-I). Thus, it is only over time, as the animal and its tracheal system increases dramatically in size, that tube architecture becomes disrupted.

**Mutations in the Tor pathway phenocopy okg and cnj**

Inhibition of the V-ATPase and Target of rapamycin (Tor) pathways have recently been reported to lead to reduced apical surface area (Gleixner et al., 2014). Thus, we sought to determine whether Tor-deficient terminal cells would phenocopy okg and cnj. Terminal cell-specific knockdown of Tor, expression of a dominant-negative Tor (Tor.TED) (Hennig and Neufeld, 2002), or knockdown of the Tor activator Rheb all lead to decreased terminal cell branching and a dramatic induction of class 2 defects (67% (n=26), 73% (n=24) and 48% (n=21), respectively) (Fig. 5F,G,H). These data are consistent with the Tor pathway acting through V-ATPase to expand the apical domain. Published data indicate that Tor regulates the expression of specific V-ATPase subunits (Gleixner et al., 2014) and, accordingly, we find that overexpression of Rheb, or of an activated form of the Tor downstream effector S6 kinase (S6kSDMTETE) (Barcelo and Stewart, 2002), does not suppress the cnj mutant terminal cell defects. These data imply that Tor acts genetically upstream of the V-ATPase (supplementary material Fig. S5) – although the pathway is more complex, as V-ATPase can also regulate mTOR (Jewell et al., 2013; Zoncu et al., 2011). Overall, these data support our hypothesis that limited apical membrane in okg and cnj cells leads to their displacement from the tube lumen by wild-type cells.

**Apical proteins accumulate in intracellular puncta in okg and cnj terminal cells**

Given that the V-ATPase and Tor pathways affect apical membrane surface area, we sought to determine whether apical
determinants were properly localized in vATPase- or Tor-deficient terminal cells. In wild type, Crumbs (Crb) localizes in foci at the terminal cell apical membrane (Fig. 6A) (Schottenfeld-Roames and Ghabrial, 2012); however, in vATPase-deficient cells, Crb levels were elevated but the steady-state localization was no longer to the lumenal membrane; rather, Crb was localized in large intracellular foci (Fig. 6B). We determined that aPKC, another apical polarity determinant (Wodarz et al., 2000), was similarly mislocalized in vATPase mutants (Fig. 6C,D). Likewise, we find that aPKC is mislocalized in Tor-DN and Rheb mutant terminal cells (Fig. 6E,F). Our results suggest that V-ATPase activity is required, downstream of Tor function, for localization of apical determinants.

To better understand how the transport of apical determinants is altered in the absence of V-ATPase function, we carried out extensive colocalization studies to determine which subcellular compartment(s) show an increased level of steady-state Crb. We found statistically significant changes in Crb colocalization with markers of the trans-Golgi (Galt) and early endosome (Rab5) (supplementary material Fig. S4A-K).

Cells deficient for apical determinants phenocopy okg and cnj

Another prediction of our model is that the loss of apical determinants alone should induce the stalk cell-terminal cell interface defects. We found that aPKC- or Par6-depleted terminal
cells showed class 1 [55% (n=53) and 61% (n=28)] and class 2 [38% (n=53) and 21% (n=28)] defects (Fig. 6G,H,I). Thus, wild-type stalk cells compensate for terminal cells with reduced apical domains by hypertrophy and branching of their autocellular tubes.

**Physiological induction of the stalk cell compensatory branching response**

We found that genetic reduction of the terminal cell apical membrane elicited a novel compensatory response in the neighboring stalk cell, and because we also noted the presence of class 1 defects at low incidence in wild type (4%, n=84), we next asked if the response could be physiologically induced. We found that in a pinch wound assay, in which second and early third instar control larvae were injured with forceps, within 24-48 h 27% (n=117) of injured terminal cells had class 1 defects and an additional 24% (n=117) had class 2 defects (Fig. 7A,B).

Next, we sought to determine whether a connection exists between terminal cell injury and maintenance of the apical domain. Injury to Crb-depleted (RNAi) terminal cells, which normally do not display junction defects, increased the frequency of class 2 defects (Fig. 7C). These data suggest that cells with a compromised ability to generate new apical membrane are sensitized for invasion by the stalk cell. Interestingly, we find that at least some aspects of the wound-healing response are induced by both genetic and physiological perturbations of terminal cells. In the larval epidermis, cells adjacent to a wound activate Jun kinase, leading to expression of *puckered* (*puc*), a negative-feedback component of the pathway (Galko and Krasnow, 2004). We observed increased *puc-lacZ* expression in stalk cells that displayed compensatory branching near injured terminal cells or near genetically comprised terminal cells (Fig. 7D). Taken together, these data suggest that the wounding-induced and the genetically induced compensatory stalk cell branching responses intersect.

**DISCUSSION**

Our data, together with previous indications in the literature, draw a strong connection between vATPase function, apical proteins and apical surface area. In some epithelia, vATPase itself is apical (Hurtado-Lorenzo et al., 2006; Liegeois, 2006), and Kanda et al. (2013) established a direct interaction between Atp6ap2, an accessory subunit of the vATPase, and the Par3 polarity protein in mice; furthermore, tissue-specific *Atp6ap2* knockout mice displayed severe retinal polarity defects. Recent work from the Simons laboratory (Gleixner et al., 2014) suggests that vATPase function, together with mTOR signaling, is essential for apical endocytosis and apical surface area growth. Our results in a distinct epithelium are consistent with a role of vATPase in the regulation of apical surface area. The Gleixner et al. study demonstrated a defect in apical endocytosis in V-ATPase mutant wing disc epithelia, with uptake of dextrans and avidin compromised by downregulation of Megalin and decreased apical surface area (Gleixner et al., 2014). Our studies
indicate that, in addition to affecting apical endocytosis, V-ATPase deficiency alters other transport pathways that are crucial for the regulation of apical surface area. Indeed, disruption of endocytosis specifically in the tracheal epithelium results in increased apical membrane surface area (Schottenfeld-Roames et al., 2014), whereas loss of V-ATPase function results in a reduction in apical surface area. In V-ATPase-deficient terminal cells we see an aberrant accumulation of Crb within the trans-Golgi, as well as within early endosomes. This suggests that there is a defect in the delivery of Crb to the apical membrane, as well as a defect in the delivery of endocytosed Crb to the lysosomal compartment. Indeed, although the lysosomal compartment appears dramatically enlarged in V-ATPase mutant cells – presumably owing to inefficient turnover of proteins, as V-ATPase function is required to acidify the lysosome – we could detect no significant increase of Crb within the lysosome. Thus, our data support a requirement for V-ATPase downstream of mTOR for the proper targeting of apical determinants to the plasma membrane.

Our results also point to a novel mechanism of compensatory tube branching. During larval life, branching morphogenesis was thought to be the exclusive purview of seamless tubes formed by terminal cells. Terminal cells are induced to branch by hypoxia, and normally compensate for terminal cells that are unable to sufficiently expand into undersupplied areas (Centanin et al., 2008; Jarecki et al., 2011). Our data uncover an unappreciated ability of autocellular tube-forming stalk cells to undergo ectopic growth and branching to compensate for terminal-healthy stalk cell interface defects. (D) Stalk cell branching adjacent to injured and VhaPPA1-1-depleted terminal cells induced puc expression. Pixel intensity is quantified in D. Error bars indicate s.d. Scale bars: 10 µm.

**Tissue-specific RNAi and misexpression experiments**

**Temperature shift experiments**

The following crosses were used: Tub-GAL80; Vha26[GD7112](okg) RNAi, VhaPPA1-1[GD16478] (V0 c′) RNAi, VhaAC39-1[GD9859], VhaAC39-2[GD10714] (V0 d), Par6 RNAi[HMS08129] RNAi and aPKC[FP01966] RNAi, Rheb[HMS00923] RNAi, UAS-Tor.TED, Tor[HMS00914] RNAi at 29°C. dnm-GAL4 drove expression of UAS-GFP and V0a R755A at 22°C.

**Determination of无缝管origin**

FasIII RNAi[GD80] , FRT72b (or FRT82b cnj) were crossed to btl-GAL4, UAS-GFP, FRT72b Tub-GAL80 to generate MARCM clones (Lee and Luo, 2001).

**Flybow experiments**

The following crosses were used: SRF-GAL4; hs-mFLP5 was crossed to UAS-flybow1; UAS-Vha26[GD7112]. Developing embryos and larvae were heat shocked three times and kept at 25°C until they were collected at third larval instar. Images were collected on a Zeiss 510 Meta confocal microscope and processed using Fiji (ImageJ) and Photoshop (Adobe) software.

**Colocalization experiments**

The following crosses were used: FRT82b cnj flies were crossed to UAS-Rab5:YFP, UAS-GFP;Sec15, UAS-golgi:RFP, UAS-Kdel:RFP or
UAS-Lamp1-GFP. Mosaic larvae were filleted and stained with anti-GFP, anti-RFP and anti-Crb antibodies (as described below). Images were collected on the Zeiss 510 Meta confocal microscope and processed using Fiji [Pearson’s colocalization coefficient (r) was determined using the Coloc2 plug-in] and Photoshop software.

Sequence analyses
okg genomic DNA, cnj genomic DNA and control genomic DNA from the parental strain on which okg and cnj mutations were induced were amplified by PCR and sequenced. Primer pairs are described in supplementary material Table S1.

Transgene rescue of cnj terminal cells
The following crosses were carried out: UAS-Vha13; FRT82B cnj(556)/TM6B flies were crossed to btl-GAL4, UAS-GFP/CyO; FRT102 tub-GAL80/TM6B. Mosaic larvae with GFP-marked clones were collected for immunostaining.

Immunohistochemistry
Third instar larvae were filleted, fixed in 4% paraformaldehyde (EMS) and washed in 1× PBS containing 0.3% Tween 20 and 0.3% Triton X-100. The following antibodies were used: rat anti-DE-cadherin (DCAD2; 1:20; DSHB), rabbit anti-Wkd peptide (1:750) (Schottenfeld-Roames and Ghabrial, 2012), chicken anti-anti (1:1000; Invitrogen, A10262), mouse anti-Fas3 (1:50; DSHB), mouse anti-Armadillo (1:200; DSHB), mouse anti-RFP (1:1000; Abcam, 65866), rabbit anti-Varicose (1:500; gift of E. Knust, Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany), rat anti-Crb (1:100; gift of E. Knust) and rabbit anti-PKceta (aPKC; 1:200; Santa Cruz, sc-216). Alexa Fluor-conjugated secondary antibodies were goat anti-chicken 488, goat anti-mouse 555, donkey anti-rabbit 647 and goat anti-rat 555 (Invitrogen). Larvae were mounted in Aqua Polymount (Polysciences) and images were acquired on Leica DM5500 and DM16000 B microscopes. z-stacks were captured and processed with Leica and Fiji software. Projected z-stacks are shown, unless noted otherwise. Statistical significance was determined using Fisher’s exact probability test and/or Student’s t-test (www.vassarstats.net).

puc-lacZ intensity
The following crosses were used: UAS-VhaPPA1-1/+; puc-lacZ females were crossed to SRF>GFP at 29°C. VhaPPA1-1; FRT82B males were crossed to SRF>GFP and sibling controls with puc-lacZ and SRF-GFP alone were collected. Third instar SRF-GFP/puc-lacZ larvae were subjected to a pinch assay as described below. All collected larvae were immunostained and images were captured on the Leica DM16000 B microscope. Exposure and intensity were kept constant. The corrected total cell fluorescence (CTCF) was measured as previously described Burgess et al., 2010. Statistical significance was determined using Student’s t-test (www.vassarstats.net).

Quantifying the ratio of apical to cytoplasmic area
The cyttoplasm (GFP) and apical domain (DE-cadherin) of control and cnj dorsal trunk cells were outlined and the area measured using Fiji. Standard deviation and statistical significance (Student’s t-test) were determined (Excel).

Mapping the mutations in okg and cnj
Meiotic recombination placed okg and cnj between FRT23B and the visible recessive marker curled (cu), or the visible recessive markers striped (sr) and ebony (e), respectively. Complementation tests against deficiency strains spanning the interval between the FRT and cu revealed that okg was uncovered by Df(3L)Exel6144, which deletes the segment of the chromosome between 83A6 to 83B6. Mutants within this region were tested against okg(566) for complementation.

Pinch assay
For double-labeled membranes: yw hsFLP122; btl-Gal4; FRT23B UAS-Td Tomato females were crossed to btl-Gal4; FRT23B UAS-myrGFP males and heat shocked for 1 h to induce either green, red or yellow tracheal cells. Quantification was performed on the larvae from yw FLP122, btl-Gal4,

UAS-GFP or UAS-RFP; FRT23A, FRT23B, SRF-GFP, SRF-GFP, UAS-crb RNAi and SRF-GFP/puc-lacZ strains. Late second and third instar larvae were identified based on spiracles and size. Larvae were pinched with forceps under the dissecting microscope. Disruption of terminal cell gas filling was used to monitor terminal cell injury. Larvae were incubated at room temperature and collected 24 h post injury for filet and immunostaining.

Epistasis experiments
The following crosses were used: UAS-Rheb.Pa, FRT23B cnj/TM6b and UAS-S6k SDTETE/UAS-TwDI::Mkate; FRT23B cnj/TM6b males were crossed to btl-mCD8GFP, FRT23B GFP RNAi virgins. Third instar larvae with clones were grown at 29°C, filleted and immunostained for clone markers and AJ’s as described.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
D.F. and A.S.G. conceived the experiments, D.F. carried them out, and D.F. and A.S.G. interpreted the data and wrote the manuscript.

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Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.119602/-/DC1

References


**Supplementary Figure 1:** The cytoplasm (btl/FGFR-UAS-GFP, green) and AJ (DE-cadherin, red) in 3rd instar larval filets. A bit of seamed tube extends from the intercellular AJ in WT (A, A’ arrow) and okg (B,B’ arrow) terminal cells. Fixed stage 15 embryos dorsal up, show tracheal nuclei (trachealess/Trh, cyan) and AJ (Armadillo, red). (C) A single intracellular AJ separates TCs from SCs and FCs in WT dorsal branches (C, C’,C”). (D) cnj in trans to a deficiency that uncovers cnj (cnj/Df) embryos are shown. A single intercellular junction is visible between TCs and SCs of cnj/Df dorsal branches (D, D’,D”). SRF>GFP, okg RNAi with Tubulin GAL80ts. AJ (red) and tube (UV, blue) in 3rd larval instar is shown. Branched autocellular defect is found in larvae shifted to 29 deg at hatching (E’), but not in unshifted (18 deg.) (E), and larvae shifted at 2nd instar (E”). (F-I) cnj dorsal trunk clones are normal at first instar (F), but round at 2nd instar (G). A normal configuration in cnj stalk cell clones at 1st and 2nd instar stages (H and I). Scale bar 10μm.
Supplementary Figure 2: The cytoplasm (Btl/FGFR,UAS-btl>GFP, green), SJ (Fas 3, white), AJ (DE-cadherin, red) and tube (UV, blue) in 3rd instar larval filets. (A-B) The intercellular AJs lie above the intercellular SJ (A’, A’’’), WT TC cytoplasm ends below the SJ (A”, A”’arrowhead). Intercellular SJ lie above intercellular AJ (B’, B”’), WT TC clone cytoplasm begins at the intercellular AJ (B”, arrowhead). Flybow marks overlapping TC and SC membranes (GFP and mCherry, respectively) separated by an intercellular SJ (Vari, white) (C,C”’arrow). (D and E) Schematic of possible TC:SC membrane interactions. Scale Bar 10 μm
Supplementary Figure 3: Cytoplasm (btl>GFP, green), AJ (DE-cadherin, red), SJ (Fas3, white), and the tube (UV, blue) in mosaic 3rd instar larval filets. A SC clone expresses Fas3 RNAi and retains its autocellular (A” arrow) and intercellular AJ (A”, A’’ arrowheads) in the absence of Fas3 staining (A”-A’’ arrow). Fas3 is absent from intercellular SJs in a Fas3RNAi expressing TC clone (B, B’ and B” arrowhead). The cytoplasm (btl>Btl/FGFR,UAS-GFP, green), Fas 3(white), Varicose (Vari, magenta) and tube (UV, blue) in mosaic 3rd instae larval filets. Fas3 co-localizes with Vari at WT TC and SC intercellular junctions (C, D ). (E) Fas3 is absent from autocellular junctions of a Fas3 RNAi expressing SC clone (C’’ arrow) and intercellular junctions of a Fas3 RNAi expressing TC clone (E’’’, arrowhead), while Vari staining is still present (C’’,E’’’). Scale Bar 10 μm.
Supplementary Figure 4: Slices from a Z stack of filleted 3rd instar larvae are shown. (A-J) WT and cnj terminal cells immunostained for Crb (yellow) express proteins of various intracellular compartments (magenta) (A-J) In contrast to a WT TC (A) where, small puncta of Golgi, Rab5, Lamp1, and Sec15 are found (A,C,E,I), these are abnormally enlarged in cnj TC (B,D,F,H,J). Crb localization in the golgi and early endosomes was significantly different in cnj TC (B, B’ and D, D’). Pearson’s co-efficient co-localization is shown (F). Scale Bar 10 μm.
Supplementary Figure 5: Quantified junction phenotypes of cnj TC expressing UAS-S6Kstede and UAS-Rheb were compared to cnj TC expressing UAS-Twdl D and heterozygous neighbors expressing UAS-Rheb or UAS-S6Kstede are shown. Expression of UAS-Rheb and UAS-S6Kstede fails to rescue junction defects in cnj TC.
Table S1. Primers (5'-3')

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