Supplemental material

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Provided online is a PDF of the complete donor vector sequence used for generation of the mKate2-*yki* knock-in allele. The sequence is represented as 5′ to 3′ and bears a modified (yet synonymous) gRNA sequence in the *yki* coding sequence and the 3xP3-dsRed cassette, flanked by TTAA, at the closest endogenous TTAA sequence. This repair template creates a mutant *yki* allele in the first instance, used for selection and counter-selection. The mutant allele is selectable for red fluorescence in the eyes. Upon establishment of the heterozygous mutant stock, it is crossed to tub-PiggyBac transposase flies, which seamlessly excises the TTAA-3xP3-dsRed cassette, and the excision is selected for the loss of red fluorescence. Finally the flies are interbred to create the homozygous viable mKate2-*yki* strain.

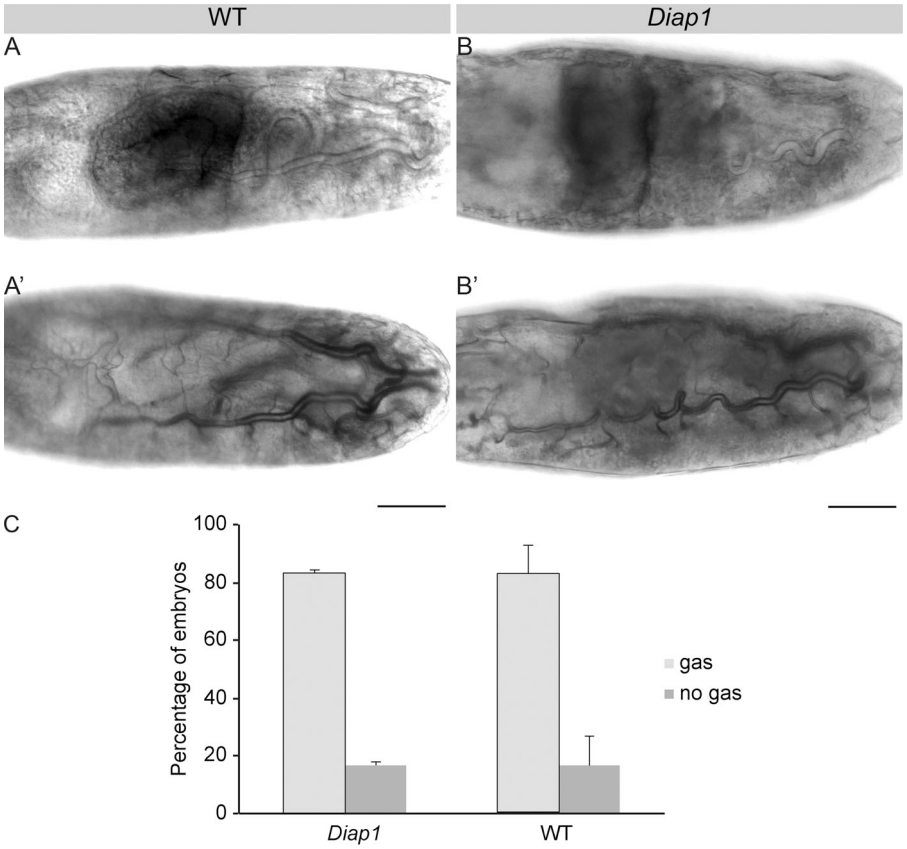


Figure S1. ***Diap1* mutant embryos do not exhibit gas-filling defects. (A–B′)** Bright-field images of WT (A and A′) and *Diap1* (B–B′) mutant embryos. Scale bars: 20 µm. **(C)** Diagram showing the percentage of embryos with gas-filled tubes. Diap1 (n = 50), WT (n = 50). Three biological replicates were performed per genotype. An unpaired two-tailed t test was performed and no significant difference was found between Diap1 and WT. Error bars represent SEM.

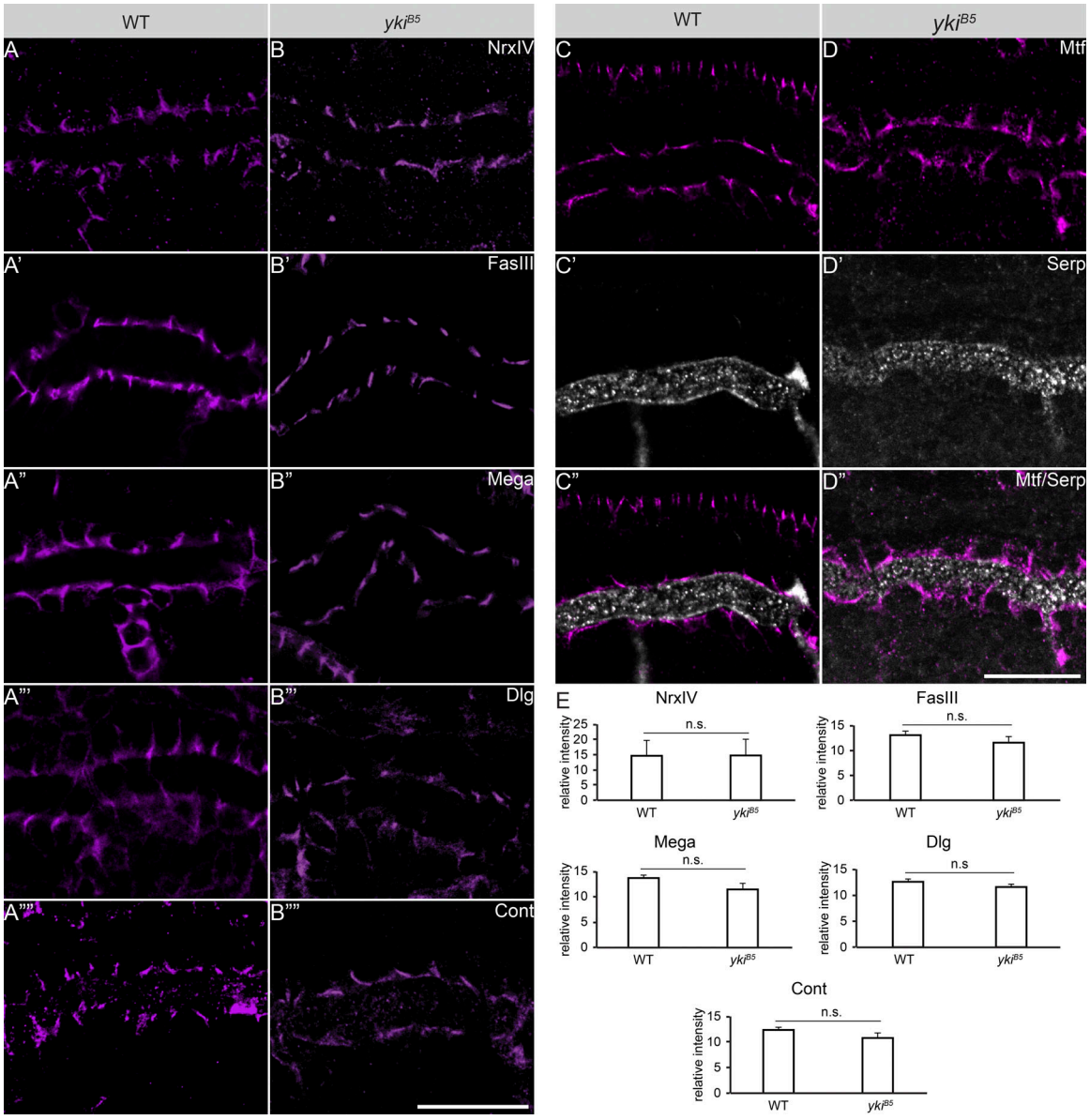


Figure S2. **Localization of SJ proteins is not compromised in the absence of Yki. (A–D″)** Single confocal sections of early stage 17 WT (A–A′′′′ and C–C″) and *yki*^{B5} (B–B′′′′ and D–D″) DTs stained for SJ proteins NrxIV (A and B), FasIII (A′ and B′), Mega (A″ and B″), Dlg (A′′′ and B′′′), Cont (A′′′′ and B′′′′), Mtf (C, C″, D, and D″), and Serp (C′, C″, D′, and D″). In *yki*^{B5} mutant embryos, SJ proteins and the luminal marker Serp do not change their expression or localization. Scale bars: 20 µm. **(E)** Quantification of the relative intensities of SJ proteins in WT and *yki*^{B5} mutant tracheal tubes. *n* = 5 per genotype per gene. An unpaired two-tailed *t* test was performed to obtain the indicated P value. Error bars represent SEM.

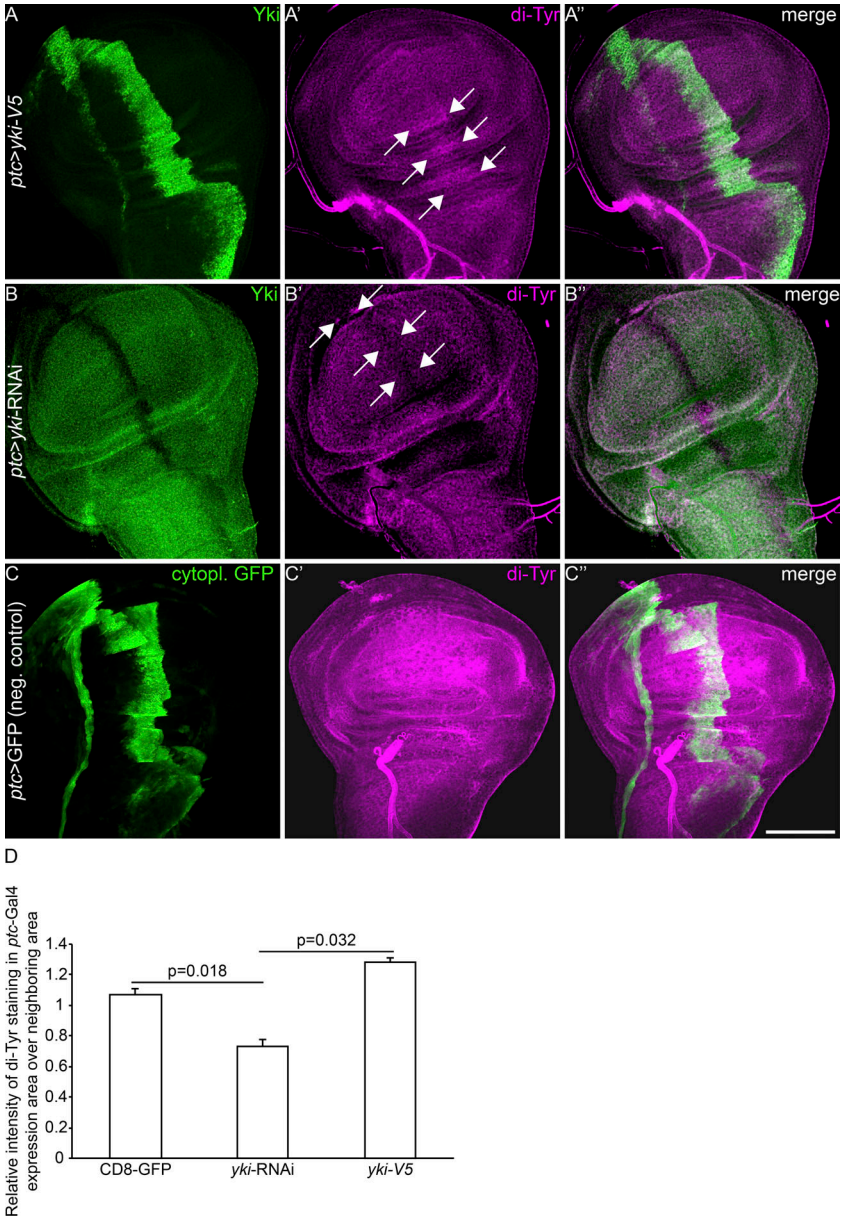
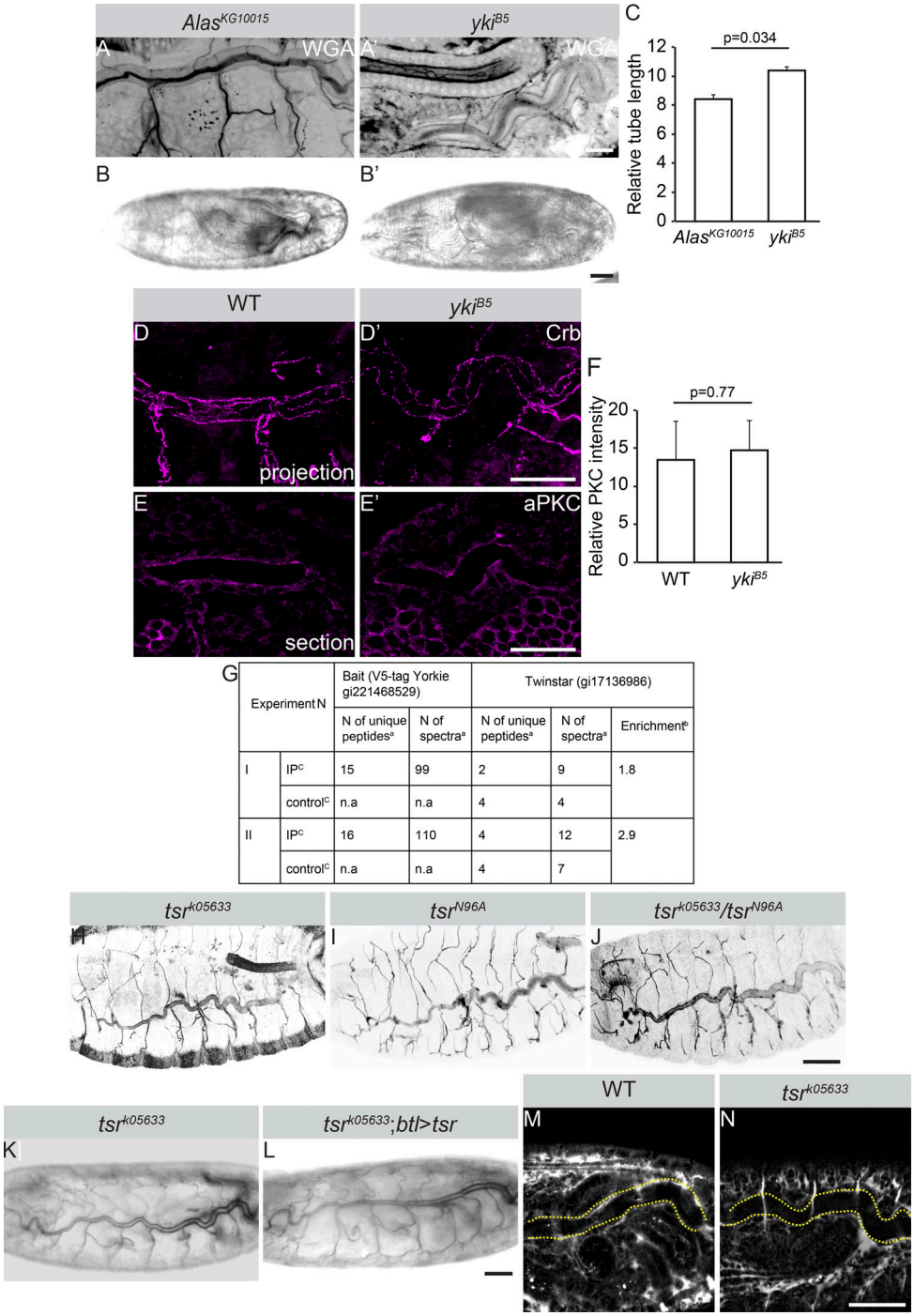


Figure S3. **Yki levels influence the dityrosine network in wing discs. (A–C″)** *yki* overexpression (A–A″) or knockdown (B–B″) by *ptc*-Gal4 leads to increased or decreased levels of dityrosine staining in the anterior–posterior boundary of third-instar wing discs, respectively. GFP (*ptc*>GFP) was used as a negative control (C–C″). White arrows indicate the region of *ptc*-Gal4 expression. Scale bar: 100 µm. **(D)** Quantification of relative intensities of dityrosine in the *ptc*-Gal4 domain. CD8-GFP (n = 30), *yki*-RNAi (n = 27), *yki*-V5 (n = 35). An unpaired two-tailed t test was performed to obtain the indicated P value. Error bars represent SEM.



Experiment N		Bait (V5-tag Yorkie gi221468529)		Twinstar (gi17136986)		
		N of unique peptides[a]	N of spectra[a]	N of unique peptides[a]	N of spectra[a]	Enrichment[b]
I	IP[c]	15	99	2	9	1.8
	control[c]	n.a	n.a	4	4	
II	IP[c]	16	110	4	12	2.9
	control[c]	n.a	n.a	4	7	

Figure S4. **Loss of *Alas* does not affect axial tube elongation; cell polarity is preserved in the absence of Yki; and *tsr* mutants exhibit defects in tube size, but not gas filling and paracellular barrier defects. (A–B′)** Lateral views of $Alas^{KG10015}$ (A) and yki^{B5} (A′) embryos stained with WGA and of live embryos (B and B′) showing that tubes in $alas^{KG10015}$ mutants are shorter than in embryos mutants for *yki*. Scale bars: 20 µm. **(C)** Bar graph showing the relative tube length of $Alas^{KG10015}$ (n = 10) and yki^{B5} (n = 11) embryos. An unpaired two-tailed t test was performed to obtain the indicated P value. Error bars represent SEM. **(D–F)** Confocal projections of the DT metamere 8 of early stage 17 WT and yki^{B5} embryos labeled for Crb (D and D′) and superficial sections of the DT metamere 8 of early stage 17 WT and yki^{B5} embryos labeled for aPKC (E and E′). Scale bars: 20 µm. **(F)** Quantification of relative intensities of aPKC in WT (n = 7) and yki^{B5} (n = 9) trachea tubes. An unpaired two-tailed t test was performed to obtain the indicated P value. Error bars represent SEM. **(G)** Mass spectrometric identification and relative quantification of Tsr protein in repetitive immunoprecipitations of V5-tagged Yki. [a]As reported by Scaffold software under settings described in Materials and methods. [b]Calculated as ratio between relative normalized intensities in immunoprecipitation and corresponding control; Tsr was not detected in the control of the experiment N1. [c]Immunoprecipitation of V5-tagged Yki and control experiments were performed as described in Materials and methods. **(H–J)** Tracheal phenotypes of two different alleles of *tsr*, tsr^{k05633}, and tsr^{N96A}, stained for Verm. Scale bar: 50 µm. **(K and L)** Overviews of tsr^{k05633} stage 17 embryos, showing proper tracheal gas filling in the absence (K) or presence (L) of a transgene expressing a WT *tsr*. Scale bar: 50 µm. **(M and N)** Fluorescent 10 kD Dextran injected into the body cavity of WT (M) and tsr^{k05633} (N) mutants does not leak into the tracheal lumen (dashed lines), indicating that the paracellular barrier in tsr^{k05633} mutants is intact.

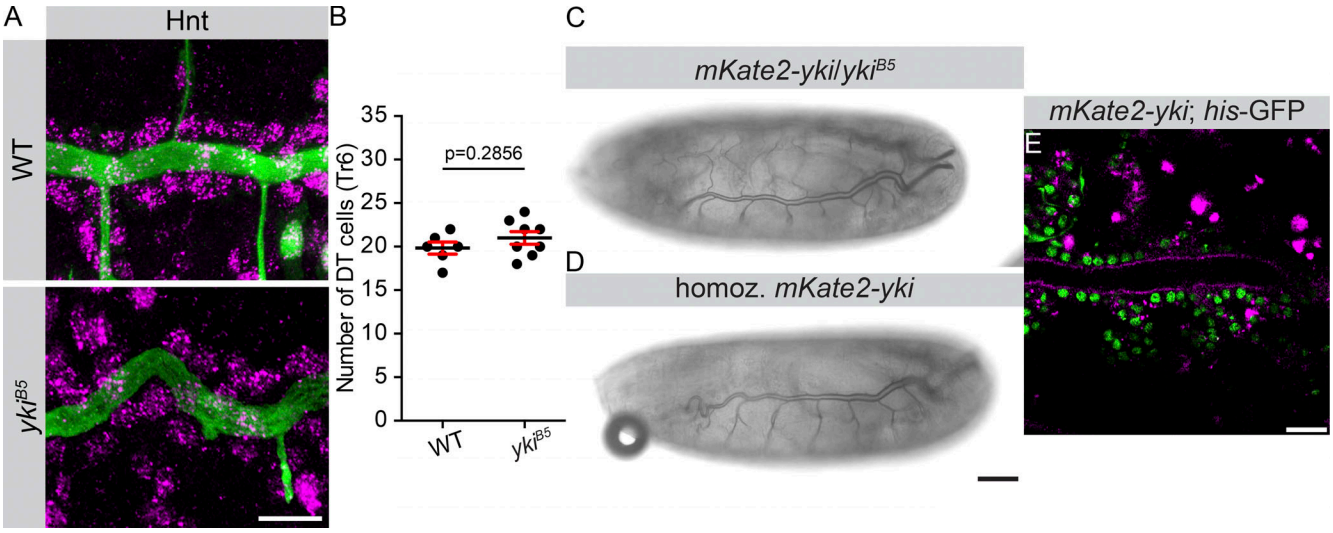


Figure S5. **Tracheal cell number does not change in *yki*^{B5} mutant embryos. (A)** Confocal projections showing the DT of stage 17 WT and *yki*^{B5} mutant embryos stained for the luminal protein Gasp (green) and the nuclear marker Hnt (Hindsight, magenta). Scale bar: 10 µm. **(B)** Plot showing the average number of cells, based on the number of marked nuclei, in WT (n = 6) and *yki*^{B5} (n = 8) mutant embryos of the tracheal metamere 6 (Tr6) of stage 17 embryos. Values were calculated based on a 3D Watershed-based image transformation (see details in Materials and methods). An unpaired two-tailed t test was performed to obtain the indicated P value. Error bars represent SEM. **(C and D)** Lateral views of an *mKate2-yki/yki*^{B5} (C) transheterozygous and an *mKate2-yki* homozygous (D) stage 17 embryo showing normal tube length and gas filling. Scale bar: 20 µm. **(E)** Confocal section of part of the DT of an *mKate2-yki* embryo of stage 17 expressing Histone-GFP (green). Yki (magenta) is localized apically in the tracheae but cannot be detected in the nuclei (marked by GFP). Scale bar: 50 µm.