SUPPLEMENTAL DATA

A Drosophila model of Epidermolysis Bullosa Simplex

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Supplemental Methods

Immunohistochemistry and histology

Larval body wall muscles

Third instar larvae were collected and larval muscle preparations were performed according to published procedures (Burra *et al.*, 2013). Dissected body wall muscle preparations were fixed for 20 min in calcium-free saline (128 mM NaCl, 2 mM KCl, 4 mM $MgCl_2$, 5 mM HEPES, 35 mM sucrose, 5 mM EGTA) containing 4% formaldehyde, followed by three washes of 5 minutes each in TBS containing 0.5% Triton-X 100 and blocked in TBS containing 1% BSA and 0.5% Triton-X 100 for 1 hour. Fixed muscle preparations were stained with mouse anti-K14 1:200 (Santa Cruz Biotechnology, Heidelberg, Germany) or anti-K5 1:2000 overnight at 4°C. After three 10 minute washes with TBS containing 0.5% Triton-X 100, tissues were incubated for 2 hours with the relevant secondary antibody and DAPI in the dark at room temperature. Tissues

were washed three times for 10 minutes in TBS containing 0.5% Triton-X 100 and mounted. All antibodies were diluted in TBS containing 1% BSA and 0.5% Triton-X 100.

Larval tracheae

Tracheal branches from third instar larvae were dissected and fixed for 20 min in PBS containing 4% formaldehyde followed by three washes of 5 minutes each in TBS containing 0.1% Triton-X 100. Dissected tracheae were blocked in TBS containing 1% BSA and 0.1% Triton-X 100 for 1 hour. Muscle preparations were stained with mouse anti-K14 1:200 (Santa Cruz Biotechnology, Heidelberg, Germany) overnight at 4°C. After three 10 minute washes with TBS containing 0.1% Triton-X 100, tissues were incubated for 2 hours with secondary antibody and DAPI in the dark at room temperature. See Table S2 for antibodies. Tissues were washed three times for 10 minutes in TBS containing 0.1% Triton-X 100 and mounted. All antibodies were diluted in TBS containing 1% BSA and 0.1% Triton-X 100.

Larval epidermis

Third instar larvae were collected and larval muscle preparations were performed according to published procedures (Burra *et al.*, 2013). The tissues were fixed for 20 min in PBS containing 4% formaldehyde and washed three times for 5 minutes each in TBS containing 0.1% Triton-X 100. Then, most of the body wall muscles were removed from the epidermis using forceps and discarded. Epidermal preparations were blocked in TBS containing 1% BSA and 0.1% Triton-X 100 for 1 hour. Epidermis preparations were stained with primary antibodies overnight at 4°C. After three 10 minute washes with TBS containing 0.1% Triton-X 100, tissues were incubated for 2 hours with secondary antibodies and DAPI in the dark at room temperature. See Table S2 for antibodies. Tissues were washed three times for 10 minutes in TBS containing 0.1% Triton-X 100 and mounted. All antibodies were diluted in TBS containing 1% BSA and 0.1% Triton-X 100.

Wing hearts

Wing hearts were dissected as previously described (Togel *et al.*, 2013) and fixed 20 min in 4% formaldehyde followed by three washes of 5 minutes each in PBS. To stain actin, wing hearts were incubated 30 min with PromoFluor-488 Premium, phalloidin (Promokine, Heidelberg, Germany) 1:40 in TBS containing 1% BSA and 0.1% Triton-X 100, washed three times for 5 minutes in TBS containing 1% BSA and 0.1% Triton-X 100 and mounted.

Samples were analyzed using a laser-scanning confocal microscope (LSM 780, Carl Zeiss, Jena, Germany). Each fluorochrome was scanned individually in single optical sections ("sequential scan") to avoid fluorescence between channels. For confocal images, Pinhole "airy 1" Zeiss standard settings were used to receive signals only from the focal plane. Analysis and processing of acquired images were carried out using Zen software (Carl Zeiss, Jena, Germany).

Electron microscopy

Salivary glands

Salivary glands were dissected from third instar larvae and were placed in one ml of fixation solution (2% glutaraldehyde, 0.1 M Na cacodylate, ph 7.2) and left at 4°C overnight. Samples were then osmicated in 1% osmium in 0.1M cacodylate buffer for 30 minutes, dehydrated, embedded in Spurs resin and thin sectioned at ~80 nm. Images were collected on a JEOL JEM 1230 Transmission electron microscope (University of Iowa Center for Microscopy).

Wing hearts

Drosophila adults were placed for 4 h at room temperature and then overnight at 4 °C in fixation solution (1% glutaraldehyde, 4 % formaldehyde in PBS). For efficient penetration of the fixation solution, specimen were submerged in the fixative and cuts were made to remove the most ventral and anterior portion of the body. After fixation, the specimen was postfixed in 1% osmium tetroxide in PBS for 1-5 h at room temperature, dehydrated in a graded ethanol series and stored in 100 % ethanol for at the least 12 h at 4 °C. Then the tissue was incubated for 10 min in a mixture of 100 % ethanol and propylenoxide (1:1), 2 x 5 min in propylenoxide and

embedded in Epon 812. Poylmerization was carried out at 60 °C for 48 to 72 h. For light microscopy, the embedded probe was serially semi-thin sectioned (0.5 – 1.0 µm) with a diamond knife on a Leica Ultracut and stained with toluidine blue at 70 °C for 2 min. A Zeiss Axioskop 2 microscope equipped with a Zeiss Axiocam MRc5 camera was used to capture images. Ultra-thin sections (70 nm) were performed with a diamond knife on a Leica Ultracut. Sections were mounted on one slot grids, contrasted with 2 % uranyl acetate (30 min) and lead citrate (20 min) using Nanofilm Surface Analysis. A Zeiss 902 transmission electron microscope (60 kV) was used for imaging.

Documentation of GAL-4 expression in wing discs and adult wings

GFP was expressed by different wing disc and wing GAL4 drivers, respectively. Wing discs were dissected from third instar larvae and GFP epifluorescence was recorded using a Cell Observer SD system (Carl Zeiss, Jena, Germany). Analysis and processing of acquired images were carried out using Zen software (Carl Zeiss, Jena, Germany). Adult wings were cut off shortly after wing unfolding and GFP epifluorescence was recorded using a Nikon SMZ1500 stereomicroscope. To assure comparability of the GFP signal within each group same exposure settings were used for all wing discs respectively adult wings.

RNA preparation and Quantitative Real-time PCR

Adult flies were homogenized in TRIzol® (Invitrogen, Karlsruhe, Germany) supplemented with ribonucleoside-vanadyl complexes (5mM final concentration, New England BioLabs, Frankfurt am Main, Germany). Total RNA was phenol/chloroform extracted and precipitated followed by DNaseI treatment (Fisher Scientific, Schwerte, Germany). cDNA synthesis was carried out using RevertAid H Minus First Strand cDNA Synthesis kit (Fisher Scientific, Schwerte, Germany). Quantitative real-time PCR was performed with Maxima SYBR-Green®/ROX qPCR Master Mix (Fisher Scientific, Schwerte, Germany) and run on an Applied Biosystems 7500 real-time PCR system. α-tubulin and 60S ribosomal protein L32 were used for normalization. For oligonucleotide primers see Table S3.

Supplemental Figures

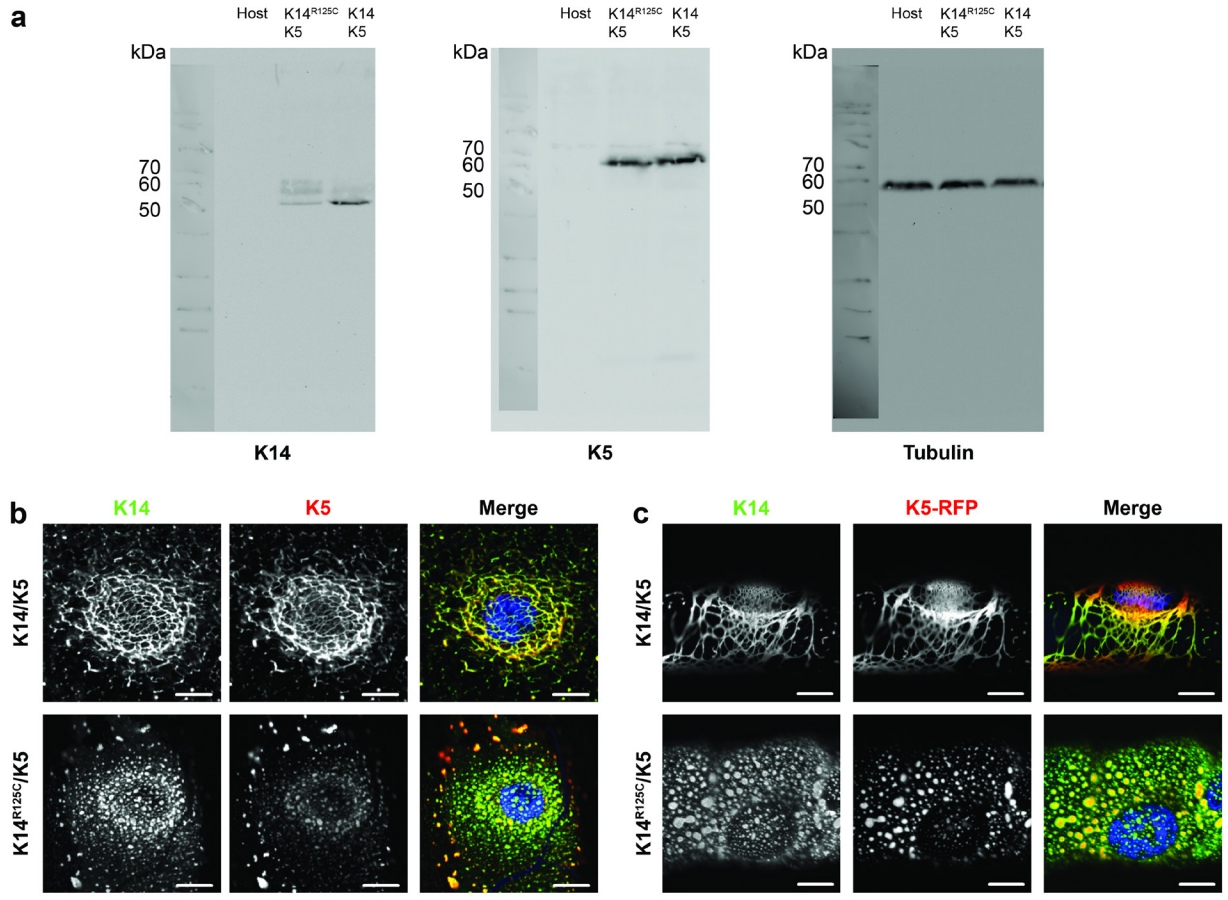

Figure S1.

Analysis of keratin expression. **a)** Whole membrane images of the western analysis shown in Figures 1 and 2. Western analysis of total protein extracts from adult flies expressing wt, mutant or no keratins (host stock) using the *Act5C-GAL4* driver. **b)** Confocal immunofluorescence images of muscle tissue from third instar larvae expressing either K14^{R125C}/K5 or K14/K5 using *Mef2-GAL4* muscle-specific driver. Double labeling of filaments respectively aggregates with Anti-K14 (green) and Anti-K5 (red). **c)** Confocal immunofluorescence images of tracheal tissue from third instar larvae expressing either K14^{R125C}/K5-RFP or K14/K5-RFP using the *Act5C-GAL4* driver. K14 is labeled with Anti-K14 (green) and K5 by K5-RFP (red) epifluorescence. DAPI-stained nuclei are blue, Scale bars: 10 µm

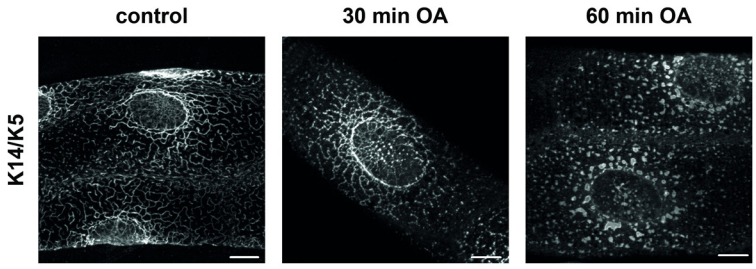

Figure S2.

Okadaic acid treatment. Maximum intensity projections of entire confocal stacks of tracheal cells from third instar larvae expressing K14 (white) and K5, using the ubiquitous *Act5C-GAL4* driver. Dissected tracheas were treated for 30 or 60 min with 0.1 µg/ml okadaic acid (OA) dissolved in PBS. The control tracheal cells were treated 60 min with PBS. Scale bars: 10 µm

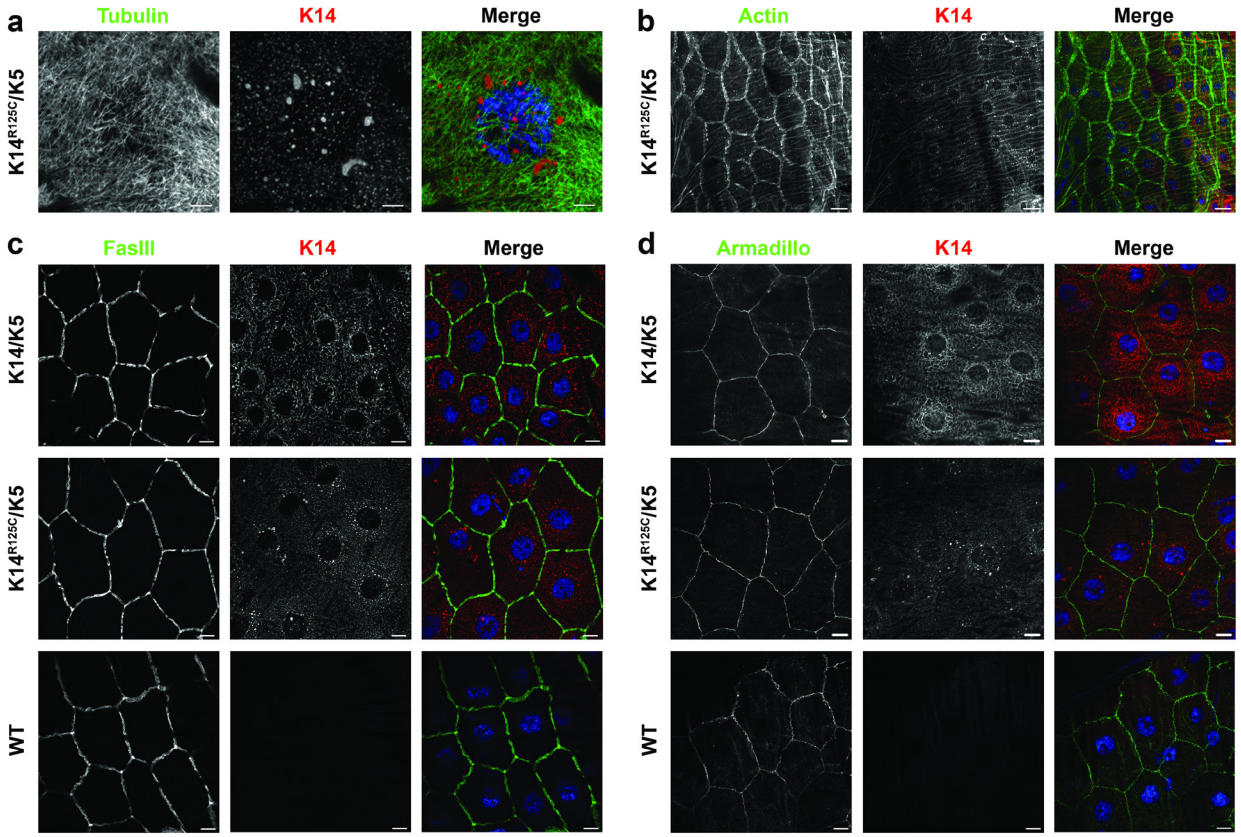

Figure S3.

Analysis of cytoskeletal structures and cellular junctions. **a-d)** Confocal immunofluorescence images of third instar larval epidermis. K14/K5 or $K14^{R125C}$/K5 were expressed by *Act5C-GAL4*. Double labeling for K14 with tubulin a), actin b), Fasciclin 3 (FasIII) c) or armadillo d). DAPI-stained nuclei are blue, Scale bars: a 5 µm; b 20 µm; c-d 10 µm

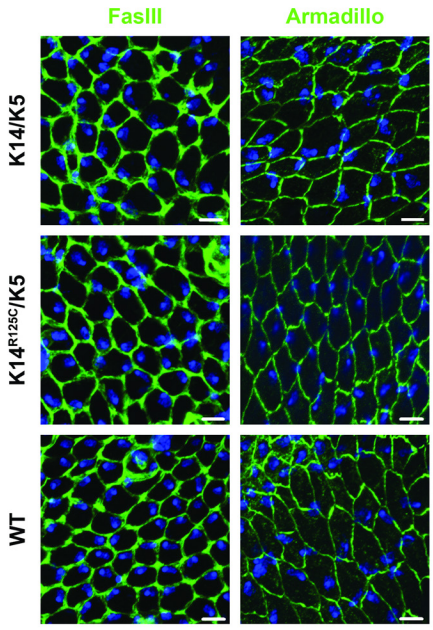

Figure S4.

Analysis of cellular junctions in pupal epidermis. Maximum intensity projections of confocal stacks of pupal epidermis from *Act5C-GAL4* expressed K14/K5 or $K14^{R125C}$/K5 flies. Fasciclin 3 (FasIII) or armadillo staining are indicated in green. DAPI-stained nuclei are blue, Scale bars: 5 µm

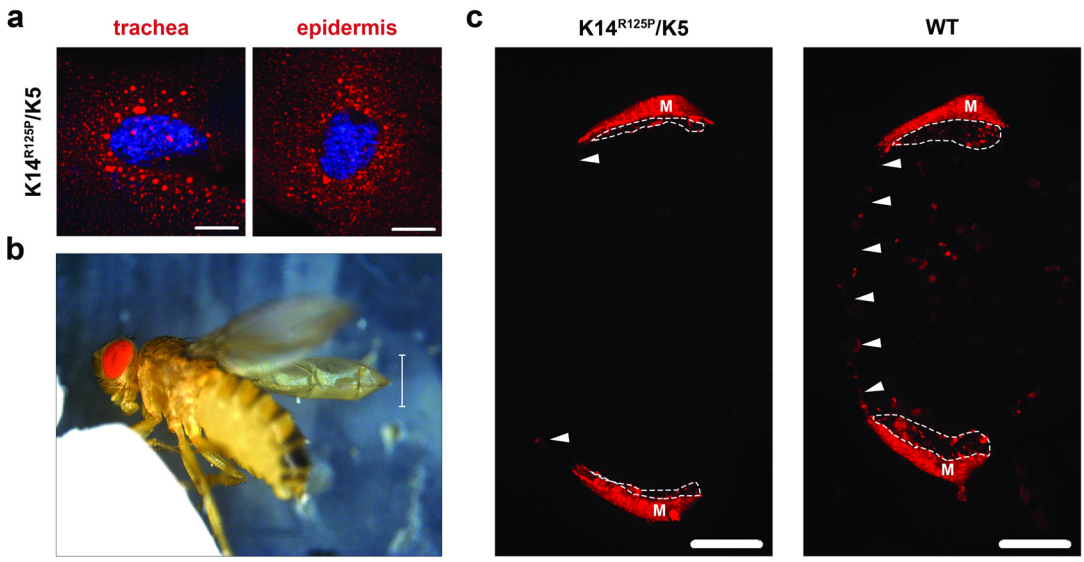

Figure S5.

Analysis of $K14^{R125P}$/K5 flies. $K14^{R125P}$/K5 were expressed by *Act5C-GAL4*. **a)** Confocal immunofluorescence images of tracheal and epidermal tissue from third instar larvae. Anti-K14 is labeled in red. DAPI in blue **b)** Wing blister phenotype in $K14^{R125P}$/K5 flies. **c)** Wing heart epithelial defect in $K14^{R125P}$/K5 flies. *In vivo* epifluorescence images of wing hearts and wing heart epithelium around 60 h APF using *Act5C-GAL4*,*handC mCherry*. Shown are maximum intensity projections of total confocal stacks. The wing heart muscle is marked by M. Dashed lines indicate the epithelial back-flow valves; arrowheads indicate wing heart epithelial elongation (sheet) of unknown function. Scale bars: a 10 µm, b 500 µm, c 50 µm

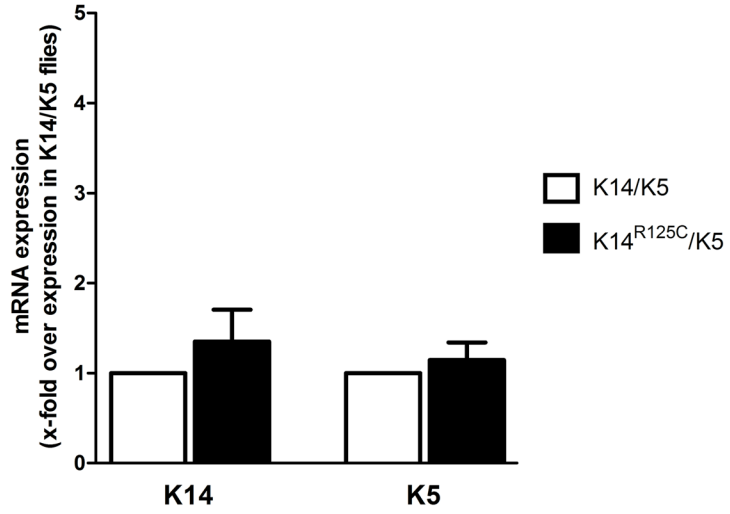

Figure S6.

Analysis of keratin mRNA expression.

Total RNA was isolated from adult flies. K14/K5 or $K14^{R125C}$/K5 were expressed by *Act5C-GAL4*. The K14 and K5 mRNA levels were quantified by SYBR-Green® real-time PCR assays relative to the house keeping genes α-tubulin and 60S ribosomal protein L32. K14 and K5 mRNA expression is presented as x-fold over K14 respectively K5 expression in K14/K5 flies. Data are means ± SD of three independent experiments performed in duplicates.

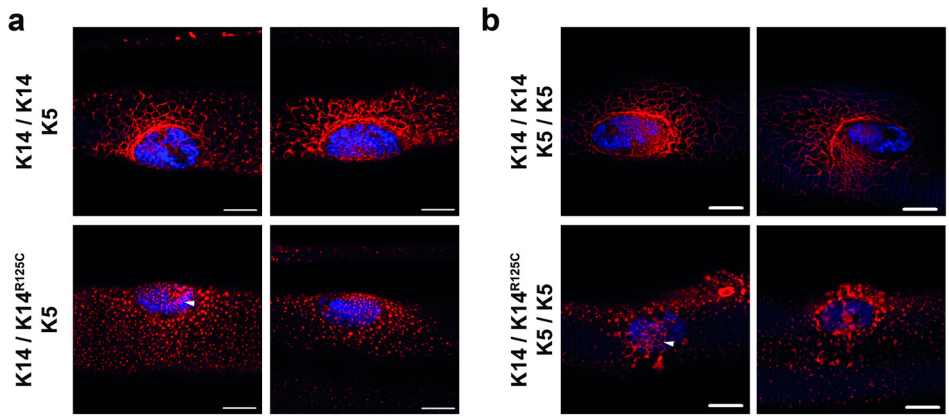

Figure S7.

Expression of multiple keratin copies. Confocal immunofluorescence images of tracheal tissue from third instar larvae. **a)** K14/K5 or K14R125C/K5 flies were crossed with K14,Act5C/CyO flies to express one copy of K5 along with one copy of wt K14 plus $K14^{R125C}$ respectively two copies of wt K14. **b)** K14/K5 or $K14^{R125C}$/K5 flies were crossed with *K14,Act5C-GAL4;K5*/*T(2;3)SM6a-TM6B* flies to express two copies of K5 along with one copy of wt K14 plus $K14^{R125C}$ respectively two copies of wt K14. Anti-K14 in red, DAPI-stained nuclei are blue, arrowheads indicate residual filamentary structures, Scale bars: 10 µm

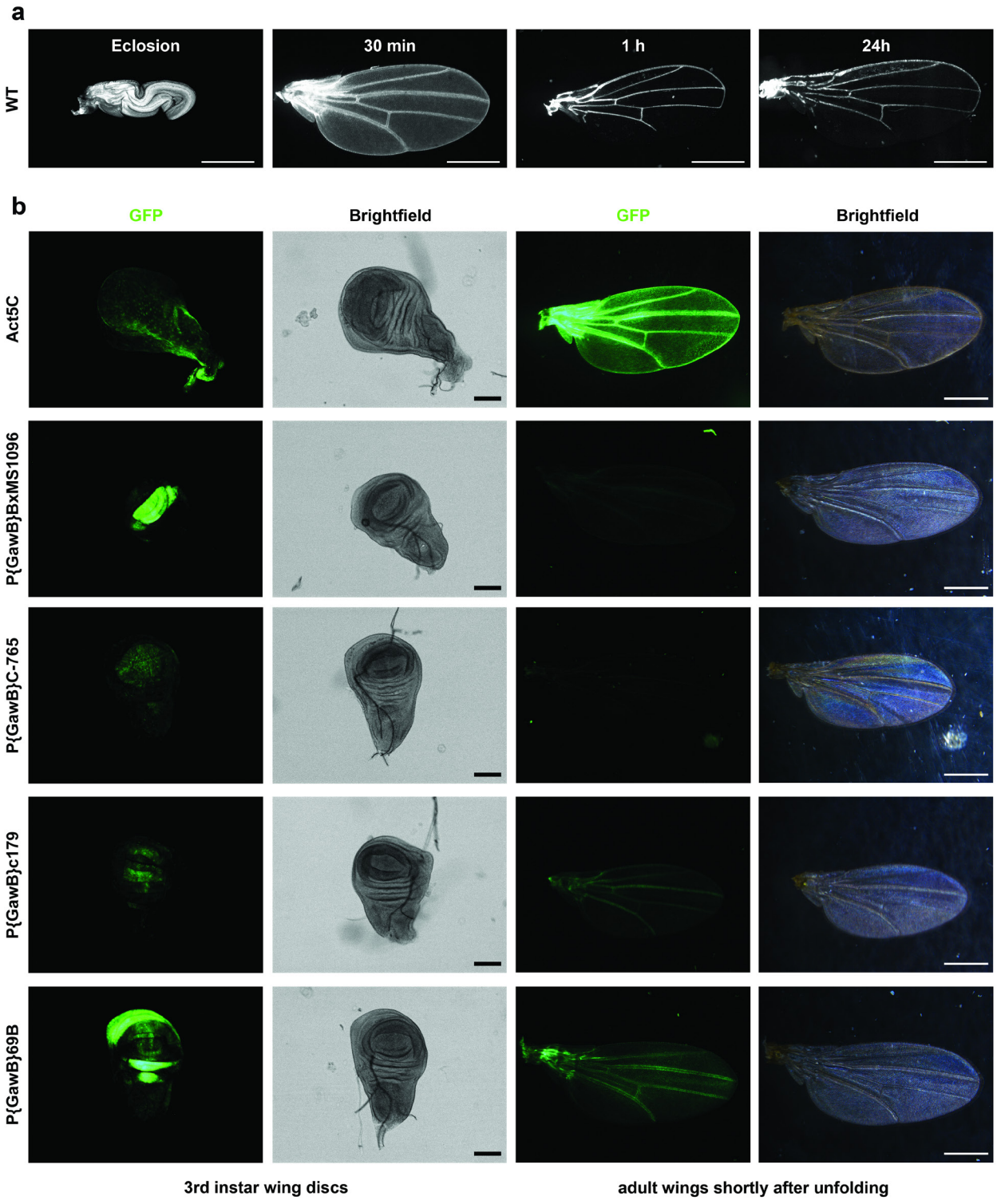

Figure S8.

Analysis of wings and wing discs. **a)** Intervein cell clearance in the wings of adult flies from the host stock expressing GFP by the *Act5C-GAL4* driver. GFP epifluorescence signal, indicated by white, in wings was recorded at eclosion and indicated time points after wing unfolding. **b)**

Expression of GFP in wing discs of third instar larvae and adult wings shortly after wing unfolding by using the indicated GAL4 divers. Shown are maximum intensity projections of confocal stacks of the GFP epifluorescence signal in wing discs and the corresponding brightfield signal. GFP epifluorescence signal in adult wings was recorded shortly after wing unfolding. The same exposure settings were used for analysis of wing discs respectively adult wings. Scale bars: a/b adult wings 500 µm, b wing discs 100 µm

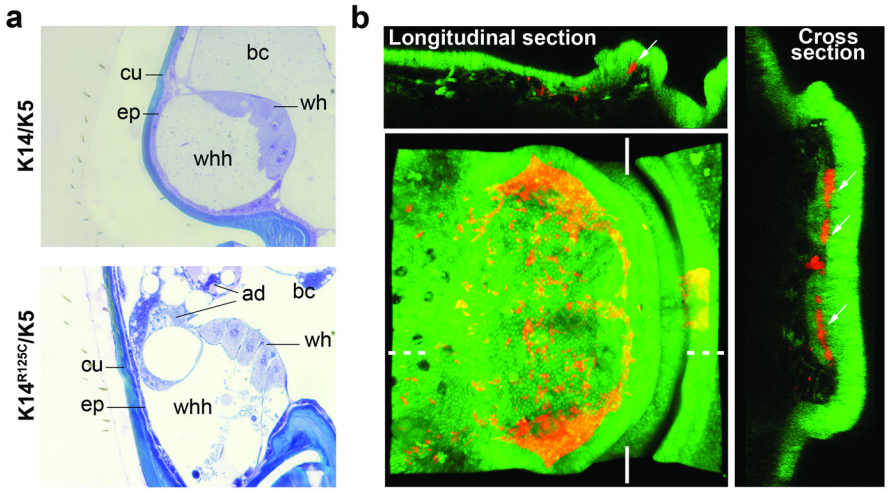

Figure S9.

a) Semi-thin sections of wing hearts of adult flies, stained with toluidine blue. K14/K5 or $K14^{R125C}$/K5 were expressed by *Act5C-GAL4.* ad: adipocyte, bc: body cavity, cu: cuticle, ep: epidermis, wh: wing heart, whh: wing heart hemocoel **b)** Maximum intensity projection of a confocal stack of developing wing hearts. K14/K5 flies were crossed with *Act5C-GAL4*,*handC mCherry/CyO; GFP* flies to visualize wing hearts and wing heart epithelial cells (red) and keratin expressing cells (green). Image was taken ~35 hours APF. White lines and dotted lines, as in Figure 3g. Arrows indicate tight contact of developing wing heart epithelium with overlaying epidermis.

Table S1.

Summary of GAL4-driver lines.

GAL4-driver	Expression
A58-GAL4	epidermal cells
Act5C-GAL4,handC-mCherry/CyO; UAS-GFP	ubiquitous
Act5C-GAL4,handC-mCherry/CyO-GFP	ubiquitous
Act5C-GAL4, UAS-GFP	ubiquitous
Act5C-GAL4/CyO-GFP	ubiquitous
C57-GAL4	in somatic muscle
e22c-GAL4	epidermal cells
Eyeless-GAL4	eye disc
handC-GAL4	heart and wing hearts
K14,Act5C/CyO	ubiquitous
K14,Act5C-GAL4;K5/T(2;3)SM6a-TM6B	ubiquitous
Sgs3-GAL4	salivary glands
Pnr-GAL4	dorsal pupal epidermis
btl-GAL4	in tracheal cells
P{Ubi-GAL4.U}2/CyO	expresses GAL4 in all cells
P{GawB}c754	in larval brain and fat body
P{GawB}T155	follicle cells
P{GAL4-Hsp70.PB}89-2-1	heat shock inducible / ubiquitous
P{GawB}how24B	in embryonic mesoderm
P{Lsp2-GAL4.H}3	in third instar fat body
P{GAL4-Mef2.R}3	in muscle cells
P{r4-GAL4}3	in the fat body and salivary glands
wing or wing disc	
P{GawB}BxMS1096	wing disc
P{GawB}apmd544	dorsal compartment of the wing disc

GAL4-driver	Expression
P{en2.4-GAL4}e16E	posterior compartment of the wing
P{GawB}C-765	embryonic salivary glands and larval wing and leg disc
P{GawB}69B	embryonic epidermis, and imaginal discs
P{GAL4}bs1348	most intervein cells of wing
P{GawB}c179	embryonic mesoderm, larval muscles and wing imaginal discs
P{GAL4}GugAGiR	wing peripodial membrane
P{GAL4-vg.M}2	pattern of vestigial gene in wing disc
P{GawB}C253	embryonic epidermal cells, and larval brain and wing disc
P{GawB}C855a	larval optic lobe, wing disc, leg and eye disc peripodial membranes and fat body, adult female post
P{GawB}MJ33a	amnioserosa, larval leg, wing and eye discs, salivary gland and gut, adult male testis pigment cells and cyst cells
P{GawB}T100	embryonic PNS and CNS, larval brain, wing discs and salivary glands, adult male accessory glands and spermatocytes
P{GawB}109-69	embryonic dorsal epidermis and in parts of the peripheral nervous system, in the larval wing imaginal disc and parts of the brain
P{GawB}Hr39c739	brain, ventral ganglion and wing and haltere discs
P{GawB}ushMD751	wing disc
P{GawB}srmd710	dorsal wing disc and tendon cells
P{cut-GAL4.B}3	at the wing margins in wing imaginal discs
P{GawB}c684	wing disc
P{GawB}OK10	in the developing wing blade

Table S2.

Summary of Antibodies.

Antibodies	Host	Source
Primary		
Anti-KRT5	Rabbit	Magin lab
Anti-KRT14	Rabbit	Magin lab
Anti-KRT14 (sc-53253)	Mouse	Santa Cruz Biotechnology, Inc.
Anti-KRT14 (ab7800)	Mouse	Abcam
Anti-Armadillo (N2 7A1)	Mouse	Hybridoma Bank, U of Iowa
Anti-Fasciclin III (7G10)	Mouse	Hybridoma Bank, U of Iowa
Anti-α-tubulin (AA4.3)	Mouse	Hybridoma Bank, U of Iowa
Anti-α-tubulin (T9026)	Mouse	Sigma-Aldrich
Secondary		
Anti-mouse-DL488, -HRP	Donkey	Dianova
Anti-rabbit-DL549, -HRP	Donkey	Dianova

Table S3.

Primers used for real-time PCR.

primer number	sequence	orientation	name
1	5'-TGGGCCCGTCTGGACCACAA-3'	s	αTub84B S
2	5'-TCGCCGTCACCGGAGTCCAT-3'	as	αTub84B AS
3	5'-AAGCGGCGACGCACTCTGTT-3'	s	RpL32 S
4	5'-GCCCAGCATACAGGCCCAAG-3'	as	RpL32 AS
5	5'-CATACTTGGTGCGGAAGTCA-3'	s	huK14 S
6	5'-GACCATTGAGGACCTGAGGA-3'	as	huK14 AS
7	5'-CTGGTCCAACTCCTTCTCCA-3'	s	huK5 S
8	5'-GGAGCTCATGAACACCAAGC-3'	as	huK5 AS

Video S1.

Movie illustrating proper delamination of intervein cells in a wing of a fly expressing $K14^{R125C}$, K5 and GFP with *Act5C-GAL4.* To illustrate correct delamination and free cell movement inside the wing, forceps were used to apply pressure to the wing at 1 s, 7 s and 13 s. Movie was recorded 1 hour after wing unfolding and is played real time.

Video S2.

Movie illustrating beating of a wing heart from a fly expressing $K14^{R125C}$, K5 and GFP with *Act5C-GAL4*,*handC mCherry*. Movie covers 1 min of beating and is played at real speed (5 fps). At ~18 s a zoom out shows an area directly on top of the wing heart. Wing heart in red, *Act5C* expressing cells in green, BC: body cavity, WV: wing vein.

Video S3.

Movie illustrating beating of a wing heart of a fly expressing K14, K5 and GFP with *Act5C-GAL4*,*handC mCherry*. Movie covers 1 min of beating and is played at real speed (5 fps). At ~20 s and 45 s a zoom shows an area directly on top of the wing heart. Wing heart in red, *Act5C* expressing cells in green, BC: body cavity, WV: wing vein.

Video S4.

Time lapse movie illustrating development of a wing heart in a fly expressing K14 and K5 with *Act5C-GAL4*,*handC mCherry*. Movie covers 24 hours of wing heart development starting 10 to 15 hours APF.

Video S5.

Time lapse movie illustrating development of a wing heart in a fly expressing $K14^{R125C}$ and K5 with *Act5C-GAL4*,*handC mCherry*. Movie covers 24 hours of wing heart development starting 10 to 15 hours APF.

Supplemental References

Burra S, Wang Y, Brock AR, Galko MJ (2013) Using Drosophila larvae to study epidermal wound closure and inflammation. *Methods Mol Biol* 1037:449-61.

Togel M, Meyer H, Lehmacher C, Heinisch JJ, Pass G, Paululat A (2013) The bHLH transcription factor hand is required for proper wing heart formation in Drosophila. *Dev Biol* 381:446-59.