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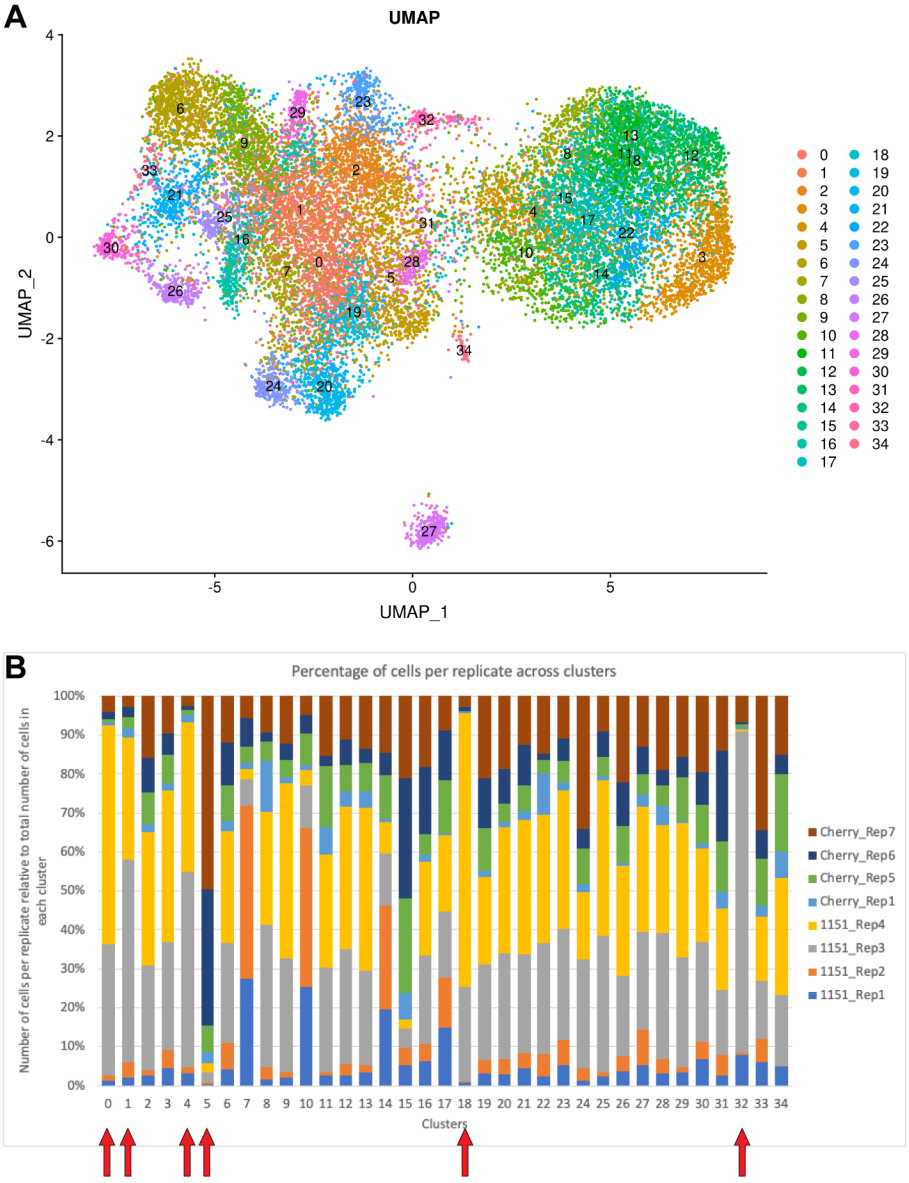
Appendix Figures

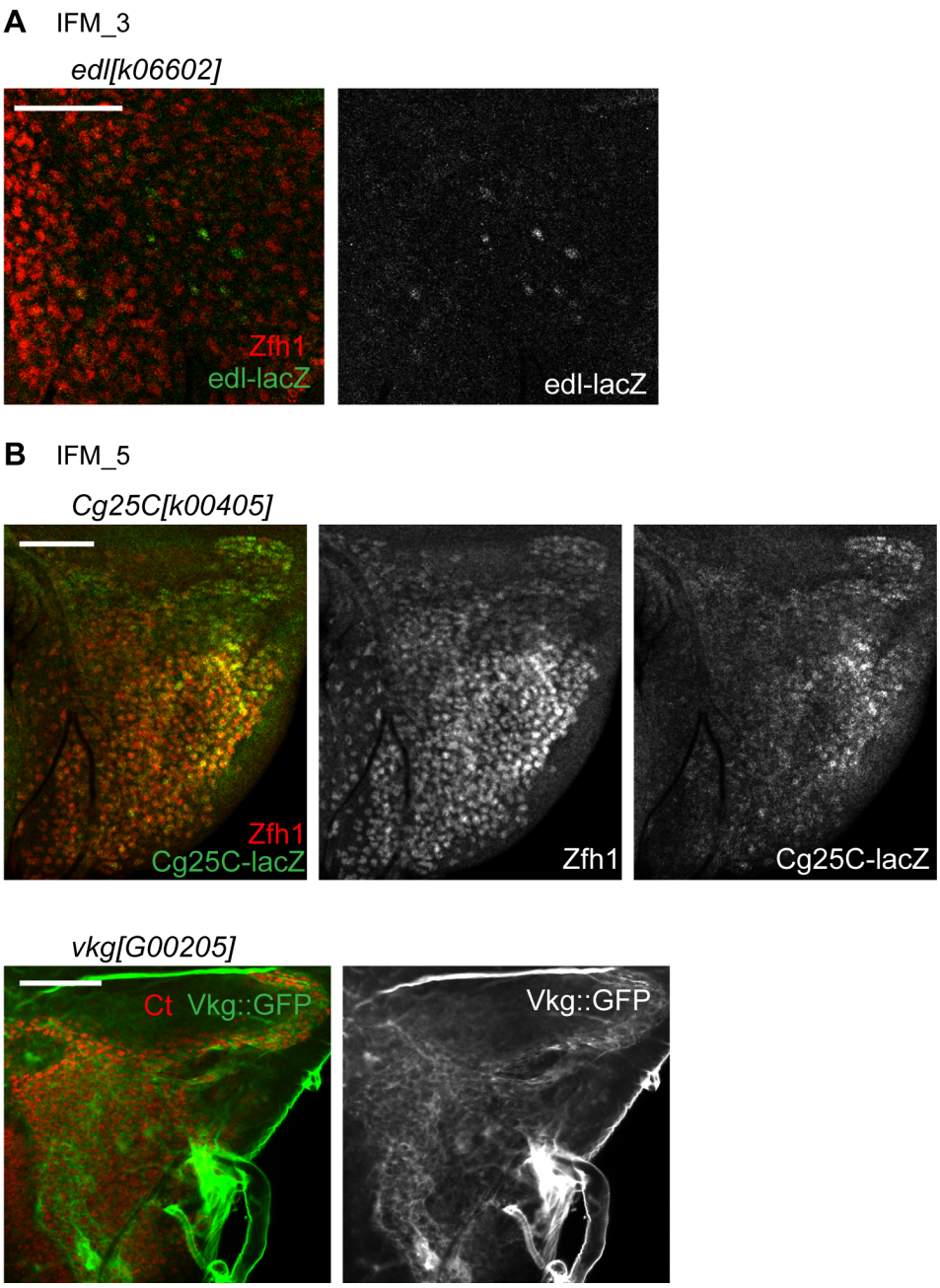


Appendix Figure S1. Clusters unevenly distributed across replicates. Related to Figure 1.

A. UMAP plot of the wild type dataset generated from wandering third instar larval wing discs prior to removal of unevenly represented clusters across replicates.

B. Percentage of cells in each replicate across clusters relative to the total number of cells per cluster. Four replicates *1151-GAL4* are indicated as 1151_Rep1, 1151_Rep2, 1151_Rep3, 1151_Rep4 and four replicates *1151>mCherry-RNAi* as Cherry_Rep1, Cherry_Rep5,

Cherry_Rep6, and Cherry_Rep7. Red arrows: clusters unevenly represented across replicates that were removed in downstream analysis.



Appendix Figure S2. Spatial localization over the wing discs of gene markers for IFM clusters.

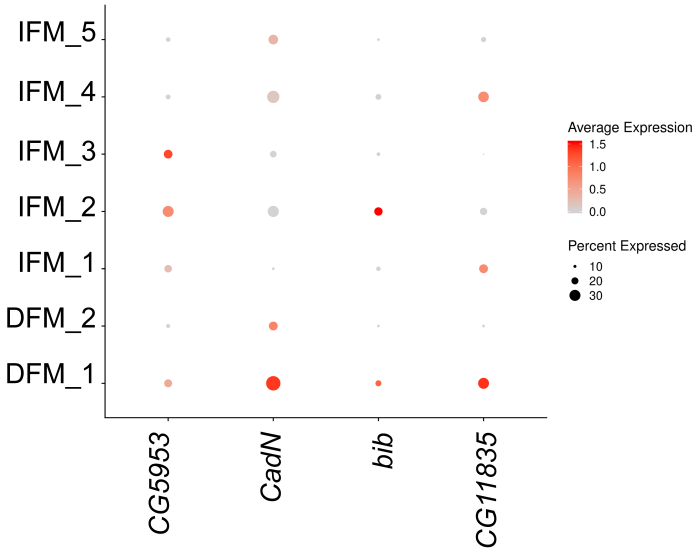
Related to Figure 5.

Confocal single plane images of third instar larval wing discs:

A. *edl[k06602]-lacZ* stained with anti-β-gal (green) and anti-Zfh1 (red).

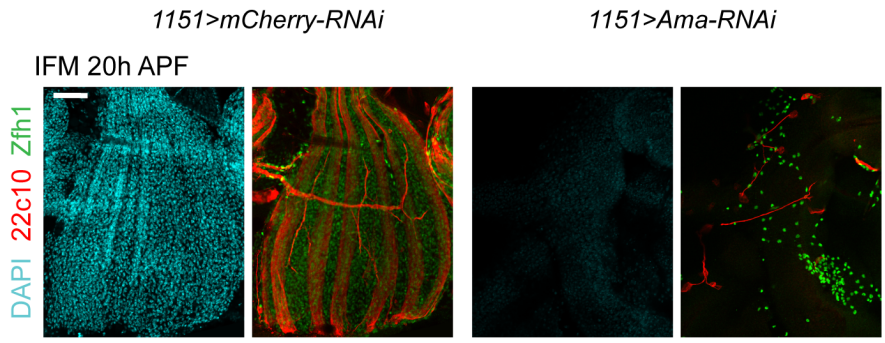
B. *Cg25C[k00405]-lacZ* stained with anti-β-gal (green) and anti-Zfh1 (red) and *vgk[G00205]-GFP* (green) stained with anti-Ct (red).

Scale bars: 50 μm. Full genotypes: (A) *y-, w-; edl[k06602]; +*, (B) *y-, w-; Col4a1[k00405]-lacZ; +* and *w-*; *vkg-GFP[G00205]*;+.



Appendix Figure S3. Additional gene markers for myoblast clusters. Related to Figure 6.

Dot plot showing the expression of the differentially expressed genes across myoblast clusters of the reference single cell atlas. Color intensity represents the average normalized expression level. Dot diameter represents the fraction of cells expressing each gene in each cluster.



Appendix Figure S4. Loss of *Ama* in myoblasts blocks myoblast fusion in developing pupa.

Related to Figure 8.

Confocal single plane images of *1151>mCherry-RNAi* and *1151>Ama-RNAi* forming IFM (DLM) at 20 h APF stained with anti-Zfh1 (green), anti-Futsch/22c10 (red) and DAPI (cyan).

Scale bar: 50 µm. Anterior up. Full genotypes: *1151-GAL4; +; UAS-mCherry-RNAi* and *1151-GAL4; +; UAS-Ama[HMS00297]-RNAi.*

Appendix Tables

Gene	RNAi -Stock Number	Lethality	Flight defect	Insertion	Source
Act57B	31551	no	none	P{TRiP.JF01113}attP2	BDSC
Alk	27518	no	none	P{TRiP.JF02668}attP2	BDSC
Ama	33416	yes (early)	N/A	P{TRiP.HMS00297}attP2	BDSC
Ama	22944	yes (mild, early)	N/A	P{GD12733}v22944	VDRC
Argk	41697	no	none	P{TRiP.HMS02262}attP2	BDSC
Argk	31951	yes (pharate)	N/A	P{TRiP.JF02243}attP2	BDSC
bib	27691	no	none	P{TRiP.JF02771}attP2	BDSC
CadN	41982	no	none	P{TRiP.HMS02380}attP2	BDSC
CadN	27503	no	none	P{TRiP.JF02653}attP2	BDSC
CG11835	41725	no	flight defect	P{TRiP.HMS02291}attP2	BDSC
CG5953	57287	no	none	P{TRiP.HMJ21225}attP40	BDSC
CG5953	57543	no	none	P{TRiP.HMC04860}attP40	BDSC
CG9650	58323	no	none	P{TRiP.HMJ22453}attP40	BDSC
CG9650	26713	no	none	P{TRiP.JF02253}attP2	BDSC
chinmo	33638	yes (early)	N/A	P{TRiP.HMS00036}attP2	BDSC
chinmo	31738	yes (pupa)	N/A	P{TRiP.HM04048}attP2	BDSC
chinmo	62873	yes (early)	N/A	P{TRiP.HMC05346}attP40	BDSC
chinmo	26777	no	none	P{TRiP.JF02341}attP2	BDSC
Col4a1	44520	no	none	P{TRiP.HMC02910}attP2	BDSC
con	28967	no	none	P{TRiP.HM05178}attP2	BDSC
E(spl)m3-HLH	55302	no	none	P{TRiP.HMC03989}attP2	BDSC

E(spl)m3-HLH	25977	no	none	P{TRiP.JF01999}attP2	BDSC
E(spl)m6-BFM	66950	no	none	P{TRiP.HMS05416}attP40	BDSC
E(spl)m7-HLH	29327	no	none	P{TRiP.JF02489}attP2	BDSC
E(spl)malpha-BFM	66949	no	none	P{TRiP.HMS05415}attP40	BDSC
E(spl)mdelta-HLH	26203	no	mild flight defect	P{TRiP.JF02101}attP2	BDSC
E(spl)mdelta-HLH	65129	no	none	P{TRiP.HMC05943}attP2	BDSC
edl	57552	no	none	P{TRiP.HMC04869}attP40	BDSC
eEF1alpha2	64659	no	flight defect	P{TRiP.HMC05694}attP40	BDSC
h	34326	no	none	P{TRiP.HMS01313}attP2	BDSC
hoip	21752	no	none	P{GD11169}v21752	VDRC
LamC	31621	yes (early)	N/A	P{TRiP.JF01406}attP2	BDSC
mamo	60111	yes (early)	N/A	P{TRiP.HMC05105}attP2	BDSC
mamo	26766	no	none	P{TRiP.JF02330}attP2	BDSC
mamo	51770	no	mild flight defect	P{TRiP.HMC03325}attP40	BDSC
mid	50681	no	none	P{TRiP.HMC03082}attP2	BDSC
nkd	67788	no	flight defect	P{TRiP.HMS05702}attP40	BDSC
nop5	55262	yes (early)	N/A	P{TRiP.HMC03949}attP40	BDSC
Nrt	28742	no	none	P{TRiP.JF03170}attP2	BDSC
side	50642	no	none	P{TRiP.HMC03042}attP2	BDSC
sp1	35777	no	mild flight defect	P{TRiP.HMS01526}attP2	BDSC
SPARC	40885	yes (early)	N/A	P{TRiP.HMS02133}attP40	BDSC
stg	34831	mild (pharate)	flight defect	P{TRiP.HMS00146}attP2	BDSC
stg	29556	no	none	P{TRiP.JF03235}attP2	BDSC

Ten-a	29439	no	none	P{TRiP.JF03375}attP2	BDSC
vkg	50895	yes (early)	N/A	P{TRiP.HMC02400}attP2	BDSC
wb	29559	no	none	P{TRiP.JF03238}attP2	BDSC

Appendix Table S1. Functional analysis of myoblast markers by screening muscle-related phenotype using *Mef2-GAL4* driver. Related to Figure 6A-B.

The *UAS-RNAi* lines were crossed to *Mef2-GAL4*. List of stocks and genes used in the screen.

Both viability and flight ability were assessed unless phenotype was lethal.

Gene	RNAi -Stock Number	Lethality	Flight defect	Insertion	Source
Ama	33416	yes (pharate)	NA	P{TRiP.HMS00297}attP2	BDSC
SPARC	40885	no	none	P{TRiP.HMS02133}attP40	BDSC
chinmo	33638	yes (pharate)	NA	P{TRiP.HMS00036}attP2	BDSC
chinmo	31738	Yes (mild, pharate)	NA	P{TRiP.HM04048}attP2	BDSC
chinmo	62873	no	NA	P{TRiP.HMC05346}attP40	BDSC
nop5	55262	yes (pharate)	N/A	P{TRiP.HMC03949}attP40	BDSC
mamo	60111	yes (early)	N/A	P{TRiP.HMC05105}attP2	BDSC
LamC	31621	no	none	P{TRiP.JF01406}attP2	BDSC
vkg	50895	no	none	P{TRiP.HMC02400}attP2	BDSC

Appendix Table S2. Functional analysis of myoblast markers by screening muscle-related phenotype using *1151-GAL4* driver. Related to Figure 6C-D.

The *UAS-RNAi* lines were crossed to *1151-GAL4* drivers if cross to *Mef2-GAL4* showed early lethality. List of stocks and genes used in the screen. Both viability and flight ability were assessed unless phenotype was lethal.

Gene	Stock	Type	Source	Insertion
UAS-G-TRACE		cell lineage system	Evans et al 2019, Nat Methods	P{UAS-RedStinger} 4, P{UAS-FLP1.D}JD1, pUbi-63E-STOP-Stinger insert 9F6
UAS-Ama^{OE} (II)		Overexpression (UAS-cDNA)	This work	UAS-Ama-FLAG-HA (DGRC 1621050)
	9723	PhiC31 integrase-mediated transgenesis	BDSC	PBac{y[+]-attP-3B}VK00002
1151		GAL4	Cripps	
Mef2	27390	GAL4	BDSC	P{GAL4-Mef2.R}3
Ama	103970	GAL4	KYOTO Stock Center (DGRC)	P{GawB}Ama[NP1297]
SPARC	77473	GAL4	BDSC	Mi{GT-GAL4}SPARC[MI00329-GAL4]
E(spl)m3-HLH	46517	GAL4	BDSC-Janelia	P{GMR10E12-GAL4}attP2
E(spl)m6-BFM		GAL4	Jagla (Aradhya et al. eLife 2015;4:e08497)	m6gap-GAL4
E(spl)m6-BFM		GFP reporter	Jagla (Aradhya et al. eLife 2015;4:e08497)	m6gapGFP
E(spl)m7-HLH	55839	GFP tag	BDSC	PBac{E(spl)m7-HLH-GFP.FPTB}VK00037
E(spl)mbeta-HLH	65294	GFP tag	BDSC	E(spl)mbeta-HLH-GFP.FPTB}attP40
grh	42272	GFP tag	BDSC	PBac{grh-GFP.FPTB}VK00033
vkg	110692	GFP tag	KYOTO Stock Center (DGRC)	vkg-GFP{PTT-un1}G00454
vkg	110626	GFP tag	KYOTO Stock Center (DGRC)	vkg-GFP{PTT-un1}G00205
Argk	51522	GFP trap	BDSC	P{PTT-GB}Argk[CB03789]
nkd	30664	GFP trap	BDSC	Mi{MIC}nkd[MI00209]
Ten-a	60541	GFP trap	BDSC	Mi{PT-GFSTF.2}Ten-a[MI04411-GFSTF.2]
Col4a1/Cg25C	499	lacZ	BDSC	P{lArB}Col4a1[A109.1F2]
Col4a1/Cg25C	10479	lacZ	BDSC	P{lacW}Col4a1[k00405]
Dad	10305	lacZ	BDSC	P{w[+mC]=lacW}Dad[j1E4]
dpp	12379	lacZ	BDSC	P{ry[+t7.2]=PZ}dpp[10638]
edl	10633	lacZ	BDSC	P{lacW}edl[k06602]

edl	102845	lacZ	KYOTO Stock Center (DGRC)	P{lacW}edl[k12907]
twi		lacZ	Cripps	
kirre	rp298	nlacZ	Cripps	
UAS-GFP		Reporter	Frolov	P{UAS-nGFP}2
UAS-mCD8::GFP	32185	Reporter	BDSC	P{10XUAS-IVS-mCD8::GFP}attP2
UAS-mCD8.ChRFP	27392	Reporter	BDSC	P{UAS-mCD8.ChRFP}3
UAS-myrRFP	7118	Reporter	BDSC	P{UAS-myr-mRFP}1
Act57B	31551	RNAi	BDSC	P{TRiP.JF01113}attP2
Alk	27518	RNAi	BDSC	P{TRiP.JF02668}attP2
Ama	33416	RNAi	BDSC	P{TRiP.HMS00297}attP2
Ama	22944	RNAi	VDRC	P{GD12733}v22944
Argk	41697	RNAi	BDSC	P{TRiP.HMS02262}attP2
Argk	31951	RNAi	BDSC	P{TRiP.JF02243}attP2
bib	27691	RNAi	BDSC	P{TRiP.JF02771}attP2
CadN	41982	RNAi	BDSC	P{TRiP.HMS02380}attP2
CadN	27503	RNAi	BDSC	P{TRiP.JF02653}attP2
CG11835	41725	RNAi	BDSC	P{TRiP.HMS02291}attP2
CG5953	57287	RNAi	BDSC	P{TRiP.HMJ21225}attP40
CG5953	57543	RNAi	BDSC	P{TRiP.HMC04860}attP40
CG9650	58323	RNAi	BDSC	P{TRiP.HMJ22453}attP40
CG9650	26713	RNAi	BDSC	P{TRiP.JF02253}attP2
chinmo	33638	RNAi	BDSC	P{TRiP.HMS00036}attP2
chinmo	31738	RNAi	BDSC	P{TRiP.HM04048}attP2
chinmo	62873	RNAi	BDSC	P{TRiP.HMC05346}attP40
chinmo	26777	RNAi	BDSC	P{TRiP.JF02341}attP2
Col4a1/Cg25C	44520	RNAi	BDSC	P{TRiP.HMC02910}attP2
Con	28967	RNAi	BDSC	P{TRiP.HM05178}attP2
E(spl)m3-HLH	55302	RNAi	BDSC	P{TRiP.HMC03989}attP2
E(spl)m3-HLH	25977	RNAi	BDSC	P{TRiP.JF01999}attP2
E(spl)m6-BFM	66950	RNAi	BDSC	P{TRiP.HMS05416}attP40
E(spl)m7-HLH	29327	RNAi	BDSC	P{TRiP.JF02489}attP2
E(spl)malpha-BFM	66949	RNAi	BDSC	P{TRiP.HMS05415}attP40
E(spl)mdelta-HLH	26203	RNAi	BDSC	P{TRiP.JF02101}attP2
E(spl)mdelta-HLH	65129	RNAi	BDSC	P{TRiP.HMC05943}attP2
edl	57552	RNAi	BDSC	P{TRiP.HMC04869}attP40

eEF1alpha2	64659	RNAi	BDSC	P{TRiP.HMC05694}attP40
EGFP	41559	RNAi	BDSC	P{VALIUM20-EGFP.shRNA.3}attP40
h	34326	RNAi	BDSC	P{TRiP.HMS01313}attP2
hoip	21752	RNAi	VDRC	P{GD11169}v21752
LamC	31621	RNAi	BDSC	P{TRiP.JF01406}attP2
mamo	26766	RNAi	BDSC	P{TRiP.JF02330}attP2
mamo	60111	RNAi	BDSC	P{TRiP.HMC05105}attP2
mamo	51770	RNAi	BDSC	P{TRiP.HMC03325}attP40
mCherry	35785	RNAi	BDSC	P{VALIUM20-mCherry}attP2
mid	50681	RNAi	BDSC	P{TRiP.HMC03082}attP2
nkd	67788	RNAi	BDSC	P{TRiP.HMS05702}attP40
nop5	55262	RNAi	BDSC	P{TRiP.HMC03949}attP40
Nrt	28742	RNAi	BDSC	P{TRiP.JF03170}attP2
side	50642	RNAi	BDSC	P{TRiP.HMC03042}attP2
sp1	35777	RNAi	BDSC	P{TRiP.HMS01526}attP2
SPARC	40885	RNAi	BDSC	P{TRiP.HMS02133}attP40
stg	34831	RNAi	BDSC	P{TRiP.HMS00146}attP2
stg	29556	RNAi	BDSC	P{TRiP.JF03235}attP2
Ten-a	29439	RNAi	BDSC	P{TRiP.JF03375}attP2
vkg	50895	RNAi	BDSC	P{TRiP.HMC02400}attP2
wb	29559	RNAi	BDSC	P{TRiP.JF03238}attP2
attP2	36303	wildtype/control	BDSC	P{CaryP}attP2

Appendix Table S3. List of fly stocks used in this work.

Ab/dye	host species	identifier	source	dilution
Anti-cut	mouse IgG1	2B10	DSHB	1/50
Anti-GFP (FITC)	goat	ab6662	abcam	1/500
Anti-beta-gal	mouse IgG1	40-1a	DSHB	1/200
Anti-Zfh1	rabbit		Ruth Lehmann (NYU Langone Medical Center, New York)	1/1000
Anti-Kettin	rat IgG1	MAC155	Babraham Institute	1/1000
Anti-mCherry 594	rat IgG2a	M11240	Life Technologies Corporation	1/750
Anti-beta-PS-integrin	mouse IgG2b	CF.6G11	DSHB	1/50
Anti-futsch	mouse IgG1	22C10	DSHB	1/30
Anti-Neurotactin	mouse IgG2a	BP 106	DSHB	1/100
Anti-Lamin C	mouse IgG1	LC28.26-s	DSHB	1/100
Anti-Fasciclin III	rabbit	7G10	DSHB	1/100
Anti-Hairy	mouse	1279	T. Orenic, UIC	1/4
Anti-wingless	mouse	4D4	DSHB	1/50
Anti-seven up	mouse	5B11	DSHB	1/20
Anti-Notch Intracellular domain (NICD)	mouse	C17.9C6	DSHB	1/100
Anti- Notch Extracellular domain (NECD)	mouse	458.2H	DSHB	1/100
Anti-Phospho-Histone H3	rabbit	06-570	Millipore	1/200
Anti-Cleaved Drosophila Dcp-1	rabbit	Asp216	Cell Signaling Technology	1/500
Phalloidin-TRITC		P1951	Sigma	1/1000
Phalloidin-Atto 488		49409	Sigma	1/1000
4',6-Diamidino-2-phenylindole dihydrochloride (DAPI)		D8417	Sigma	1/1000
Cy™3 AffiniPure Donkey Anti-Mouse IgG (H+L)	donkey	715-165-151	Jackson Immunoresearch Laboratories	1/300
Alexa Fluor® 647 AffiniPure Donkey Anti-Mouse IgG (H+L)	donkey	715-605-151	Jackson Immunoresearch Laboratories	1/300
Cy™5 AffiniPure Donkey Anti-Rabbit IgG (H+L)	donkey	711-175-152	Jackson Immunoresearch Laboratories	1/300
Cy3-AffiniPure Donkey Anti-Rabbit IgG (H+L)	donkey	711-165-152	Jackson Immunoresearch Laboratories	1/300
Alexa Fluor® 647 AffiniPure Donkey Anti-Rat IgG (H+L)	donkey	712-605-153	Jackson Immunoresearch Laboratories	1/300
Cy™3 AffiniPure Donkey Anti-Rat IgG (H+L)	donkey	712-165-153	Jackson Immunoresearch Laboratories	1/300

Appendix Table S4. List of antibodies and dyes used in this work.