Supplementary Files
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Cell adhesion-mediated actomyosin assembly regulates the activity of Cubitus interruptus for hematopoietic progenitor maintenance in *Drosophila*

Shiv Kumar Sharma[1], Saikat Ghosh[1,3], Aarathy RavisundarJose Geetha[1,4], Sudip Mandal[2] and Lolitika Mandal[1]

[1]Developmental Genetics Laboratory

[2]Molecular Cell and Developmental Biology Laboratory

Department of Biological Sciences, Indian Institute of Science Education and Research (IISER).

Mohali, Knowledge City, Sector 81, Manauli P.O. 140306, India

Current Address

[3]Eunice Kennedy Shriver National Institute of Child Health and Human development

(NICHD)National Institute of Health (NIH) Bethesda, MD 20892-3758

[4]Max F.Perutz Laboratories (MFPL), Universityof Vienna,1030, Vienna, Austria Cell and

Developmental Biology Laboratory

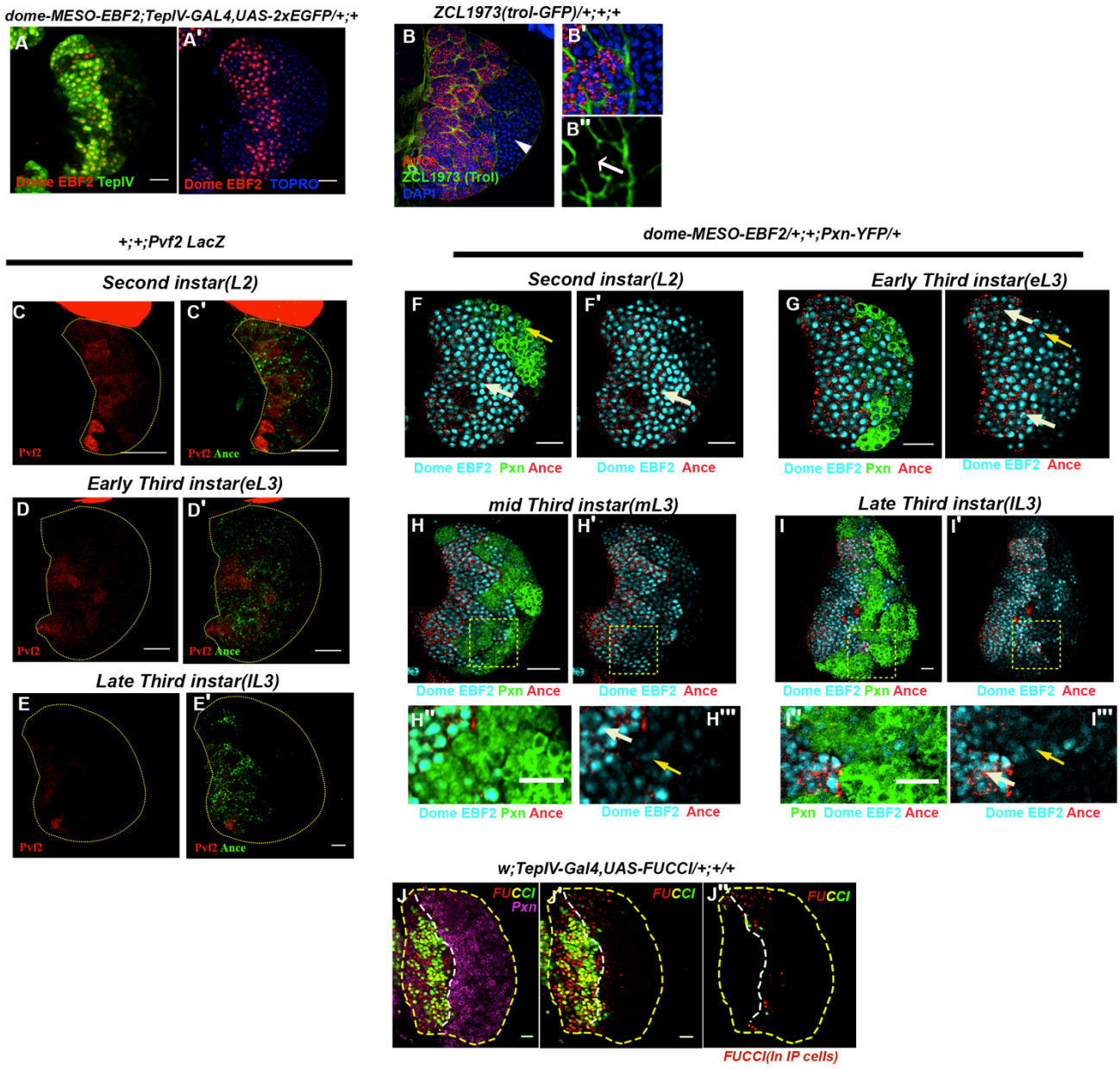


Figure S1(related to Fig.1)

***Drosophila* hematopoietic progenitors are heterogeneous.**

The genotypes are mentioned on the top of the relevant panels. Scale bar: 20 μ m

Supplementary Figure S1

(A-A') The bona fide hemocyte progenitor specific Gal4 drivers: Dome (red) and TepIV (green) expresses in overlapping domain.

(B-B') The progenitors of late third-instar larval lymph gland visualized by Trol-GFP expression are positive for Ance. Trol is one of the extracellular matrix (ECM) protein present in the lymph gland. In the lymph gland, the ECM forms thin basement membrane around individual or small groups of blood progenitors (arrow in B''). The undifferentiated progenitors are enclosed in many, closely spaced trol positive membranes whereas differentiated hemocytes have very few of them (arrowhead in B).

(C-E') Pvf2 (red) expresses in the niche/PSC and the innermost core of the lymph gland progenitors (Ance, green marks the progenitors) of the developing lymph gland.

(F-I''') Co-labeling of Pxn, Dome and, Ance throughout development reveals that a subset of Dome expressing hemocyte progenitors are positive for Ance (white arrows). This subset constitutes the core progenitors of the MZ and due to their position they never overlap with Pxn expression. Pxn expresses in the peripheral MZ progenitors (yellow arrow).

(J-J'') The intermediate progenitors Tep IV+ and Pxn + are cycling as revealed by FUCCI labeling (red: indicating S phase).

Related to Figure1

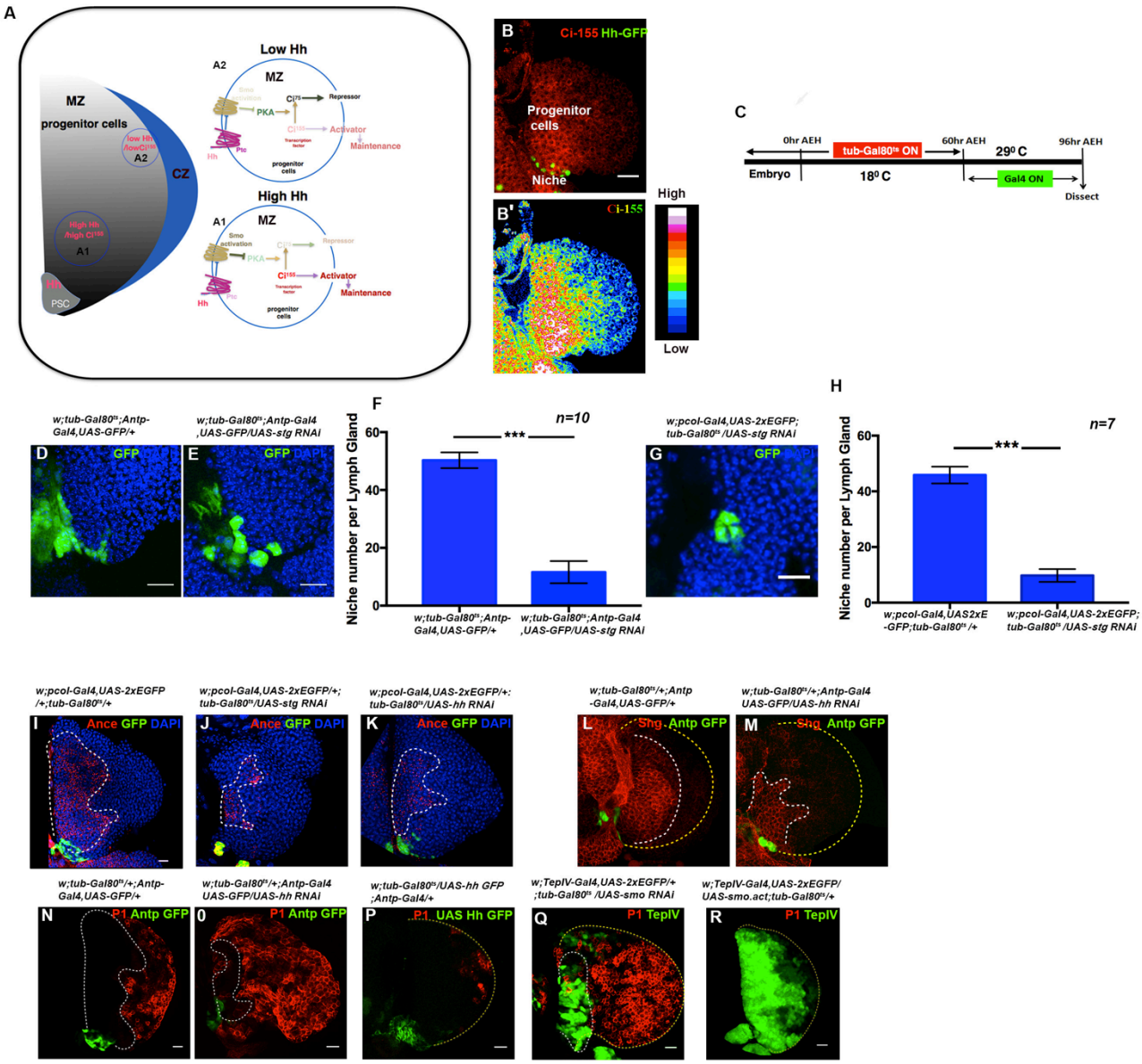


Figure S2(related to Fig.2)

Requirement of Hh signalling in the developing lymph gland

The genotypes are mentioned on the top of the relevant panels. Scale bar: 20 μm

(A-A2) Hh signaling in the lymph gland.

The source of Hedgehog is the hematopoietic niche of the lymph gland. Reception of Hh signaling requires the activity of two transmembrane proteins Patched (the receptor) and

Smoothened (transducer of Hh signaling). In the absence of Hh/very low Hh, Ptc inhibits signaling by Smo, thereby prevents the activation of the intracellular effectors. Binding of Hh with Ptc immediately triggers their internalization, thereby allowing Smo to accumulate and signal the downstream effectors. The signaling thus initiated culminates in activation of Cubitus interuptus. The full-length protein accumulated acts as transcriptional activator of Hh target genes. Interestingly, Ci-155 expression in the mature lymph gland corresponds with the above description. Hemocyte precursors cells near the source of Hh are higher in Ci-155 **(A1** and **B-B')** while those that are farther away have down-regulated it **(A2** and **B-B').**

(C) Scheme illustrating the timeline adopted for all our studies.

The synchronized larvae collected from the crosses were kept at 18^{o}C to keep the GAL80 repression on till 7days AEL (equivalent to 60hrs @25^{o}C). Following this, the vials were shifted to 29^{o}C to downregulate the desired gene function.

(D-H) Down-regulation of String in the niche either by *Antp-Gal4* **(D-F)** or *pcol-Gal4* **(G-H)** affects the cell number**.** P-value for *tub-GAL80^{ts};Antp-Gal4,UAS-GFP/UAS-stg RNAi:*4.349x10^{-7}, *pcol-Gal4,UAS-GFP;tub-GAL80^{ts}/UAS-stg RNAi:7.163x10^{-6}*.

(I-O) Loss of Hh signaling from the niche affects progenitor number. Compare to control, loss of Hh from the niche post 60 hrs AEL by *pcol-Gal4* causes a decline in progenitor number (visualized by Ance) compared to control **(I-K)**. Similar observations are seen upon using *AntpGal4* as the driver or Shg as another independent marker for progenitors **(L-M).**

(N-O) An increase in differentiation seen upon loss of Hh signaling from the niche (P1 red) compared to control.

(P) Overexpression of Hh in the niche results in expansion of progenitors and concomitant loss of differentiated cells (P1).

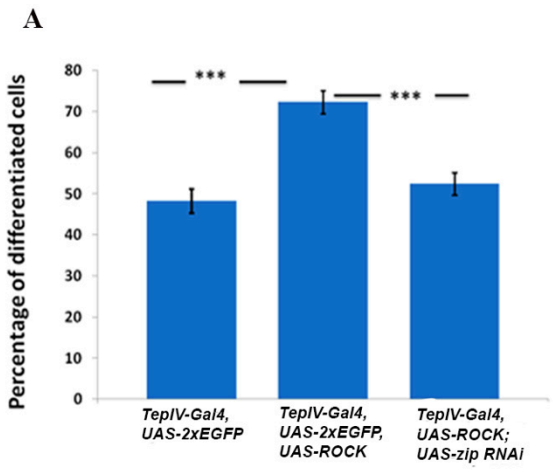
(I-J) Downregulation of Smo in the progenitors (green) affects their maintenance while activation of Smo sustains their maintenance at the cost of differentiation (P1, red).

The yellow dotted line mark whole of the lymph gland in L, M, P, and Q, while white dotted line marks the progenitors in I, J, K, L, M, N, O, and Q.

Error Bar S.D

Related to Figure2

Figure S3(related to Fig.3)



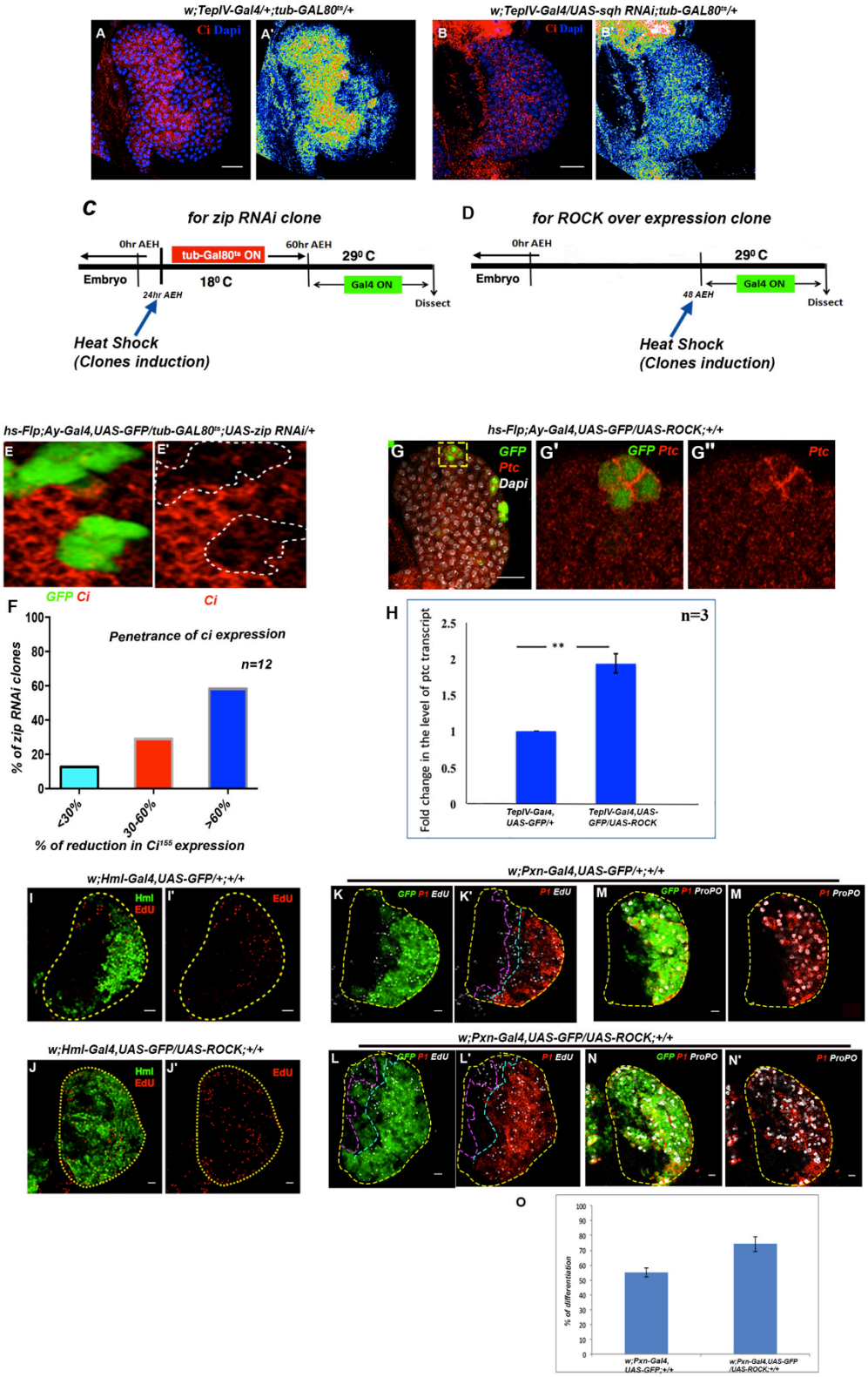
Hemocyte Progenitors maintenance require the activity of Actomyosin network

(A) Quantitative Analyses of P1 Compared to control *P value for TepIV-Gal4,UAS-2xEGFP/UAS-ROCK; tub-GAL80^{ts} = 5.865x10^{-12}. While compared to TepIV-Gal4,UAS-2xEGFP/UAS-ROCK; tub-GAL80^{ts}, the P-value for TepIV-Gal4 /UAS-ROCK; tub-GAL80^{ts}/ UAS-zipRNAi= 5.891x10^{-11}*

Error Bar S.D

Related to Figure 3

Figure S4(related to Fig.4)



The actomyosin network regulates progenitor maintenance through Ci activity

The genotypes are mentioned on the top of the relevant panels.

Scale bar: 20 μ m

(A-B') Ci-155 expression in the progenitors decreased upon sqh loss (*TepIV-Gal4/UAS-sqh RNAi*; tubgal80^{ts} compared to control (*TepIV-Gal4*). All images analyzed are from 72 hrs AEH.

(C-D) Schemes are representing the timeline of clonal induction for both *zipRNAi* (C) and ROCK overexpression (D).

(E-E') Ci-155 expression level declines in *hsFLP;ay-GAL4/tub-GAL80^{ts}; UAS-zip RNAi* clones.

(F) Penetrance of phenotype (loss of Ci-155 expression) in *hsFLP;ay-GAL4/tub-GAL80^{ts}; UAS-zip RNAi* clones.

(G-G'') Ptc expression is upregulated in *hsFLP; ay-GAL4/UAS-ROCK* clones.

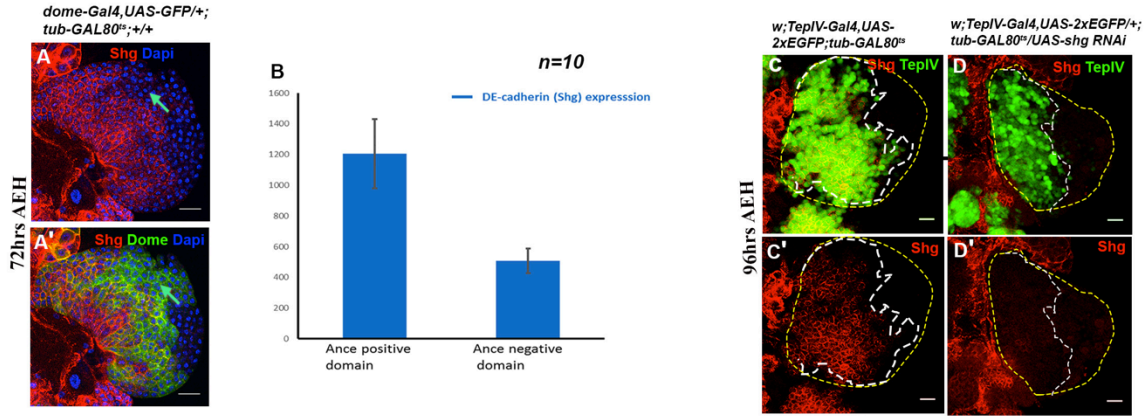
(H) A two-fold increase in *ptc* transcript level seen upon overexpression of ROCK in the progenitors. P-value : 6.832622X 10^{-3}

(I-J') Overexpression of ROCK leads to increase in Hml population (visualized by EdU incorporation) and disturbed zonation in the lymph gland.

(K-N') Overexpression of ROCK leads to increase proliferation (EdU) of IP and disturbed zonation in the lymph gland without affecting terminal differentiation (assayed by P1: Plasmatocytes and ProPO: Crystal Cells). Quantified in **O**.

Related to Figure 4

Figure S5 (related to Fig.5)



DE-cadherin expression in the developing hemocyte progenitors

The genotypes are mentioned on the top of the relevant panels.

(A-B) The inner core of Dome (green) expressing progenitors are rich in Shg (red). Dome positive progenitors in the periphery of the MZ have down-regulated Shg expression (green arrow in **A'**).

(B) Quantitative data illustrating the DE-cadherin/Shg expression level in Ance positive and Ance negative progenitors. P value: 2.187×10^{-3}.

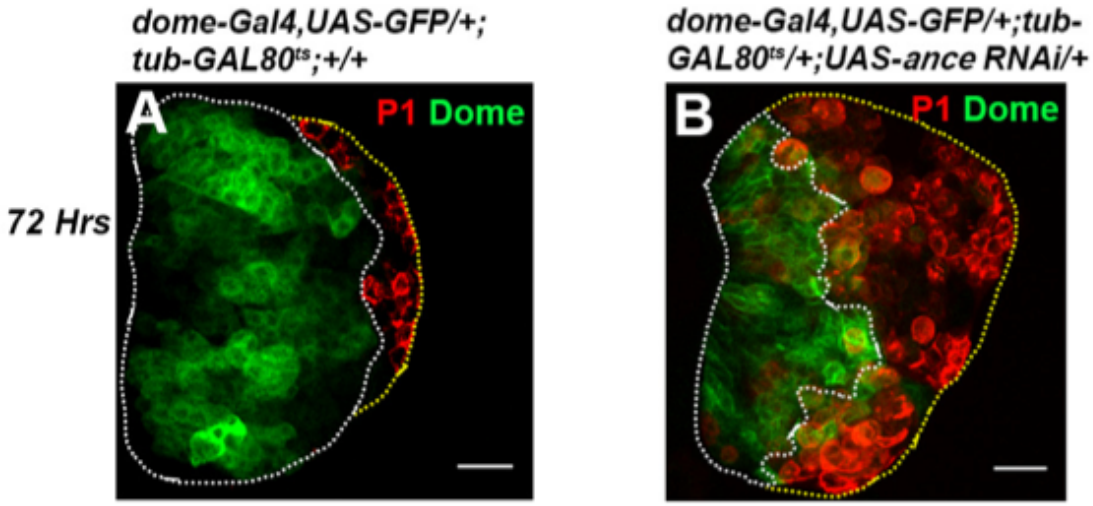
All images analyzed are from 72 hrs AEH.

(C-D') Validation of both Shg antibody expression and the *UAS-shgRNAi* construct in the lymph gland.

Error Bars: SD

*Related to Figure **5***

Figure S6(related to Fig.6)



Supplementary Figure 6

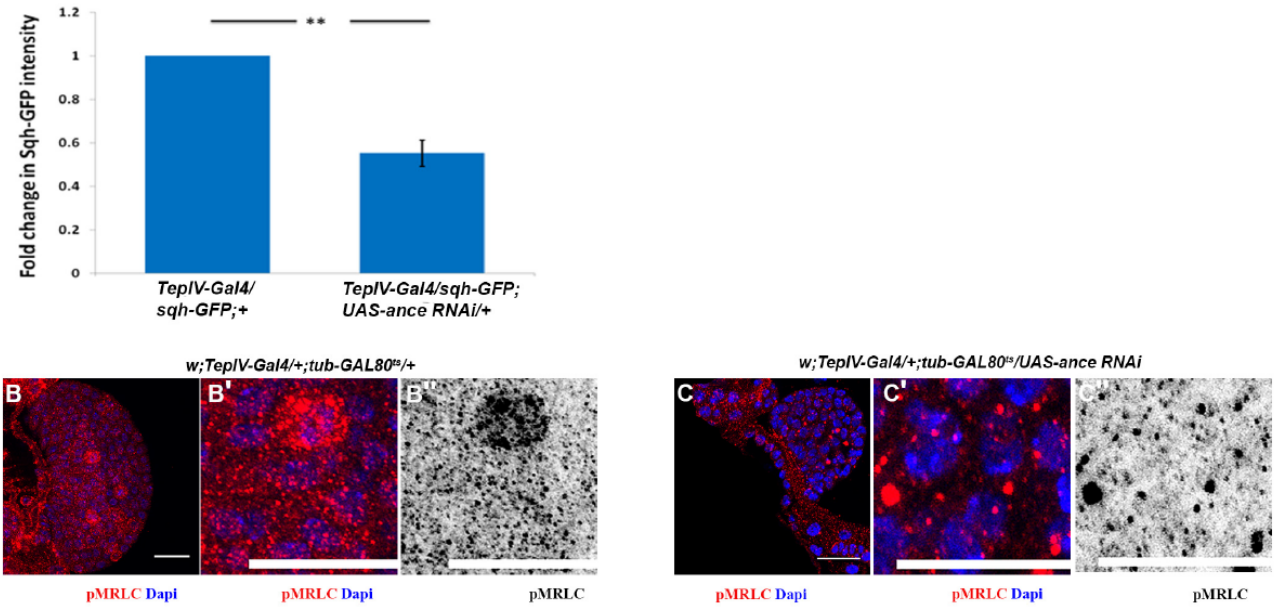
Ance is essential for progenitor maintenance

The genotypes are mentioned on the top of the relevant panels.

(A-B) Ance down-regulation from progenitors affects progenitor number and maintenance leading to their differentiation. Increased differentiation can be detected even at 72 hrs AEH.

Related to Figure ***6***

Figure S7(related to Fig.7)



Supplementary Figure 7

DE-Cadherin and the expression of actomyosin components declines upon down-regulation of Ance.

The genotypes are mentioned on the top of the relevant panels.

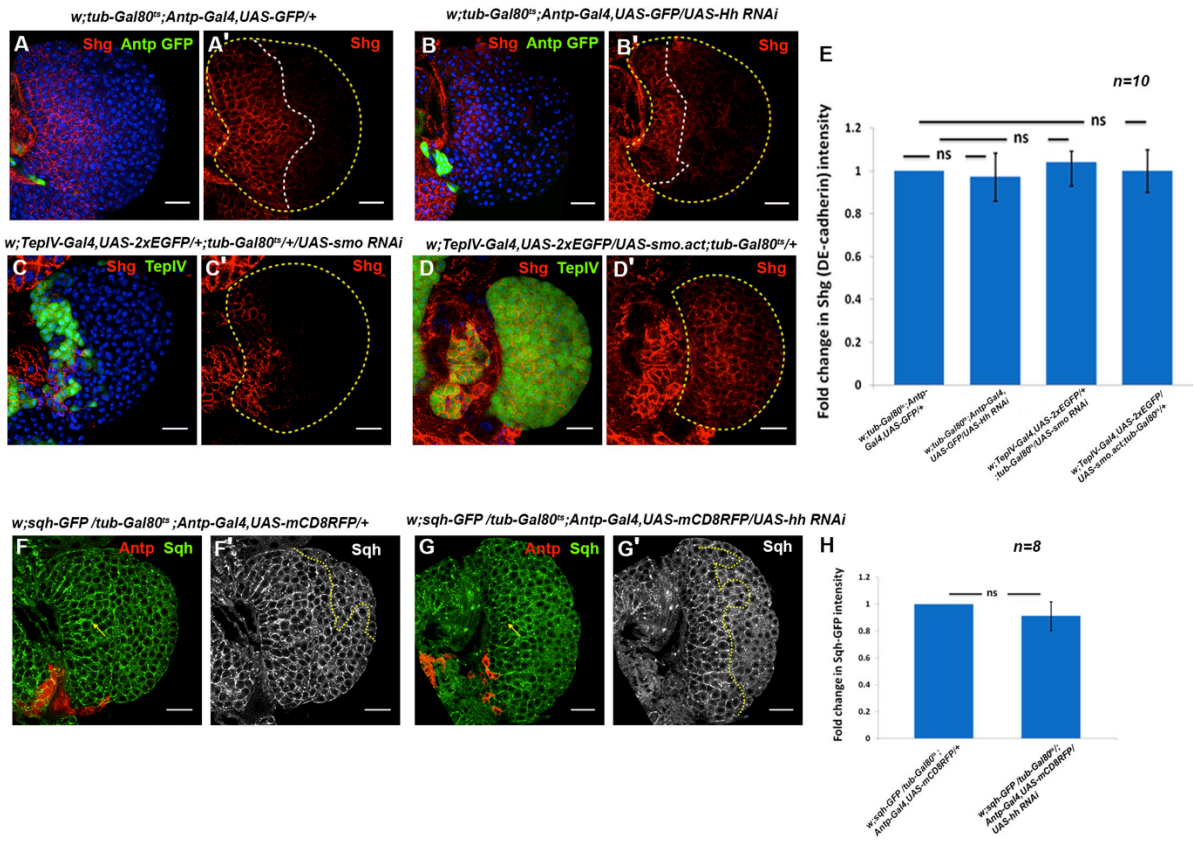
(A) Quantification of sqhGFP expression reveals a substantial decline upon loss of Ance from the progenitors *(TepIV-GAL4;tub-GAL80^{ts}/UAS-anceRNAi)*

P-value:6.03×10^{-3}.

(B-C'') Upon down regulation of Ance *(TepIV-GAL4;tub-GAL80^{ts}/UAS-anceRNAi)* there is a decrease in pMRLC (red and grey) expression in the progenitors compared to control.

Error Bar S.D

Related to Figure 7



Supplementary Figure 8

Supplementary Figure 8

Downregulation of Hh signalling does not affect the expression of Shg or actomyosin components.

The genotypes are mentioned on the top of the relevant panels.

(A-B') Although the Shg domain significantly decreases, the level of Shg expression is not affected upon Hh downregulation from the niche (*tub-GAL80^{ts}, Antp-Gal4.UAS-GFP/UAS-hh RNAi*).

(C-C') Lack of Smo in the progenitors (*TepIV-Gal4, UAS-2xEGFP;tub-GAL80^{ts}/UAS-smoRNAi*) affect their number, but the level of Shg remains comparable to control lymph gland progenitors.

(D-D') Overexpression of Smo in the progenitors (*TepIV-Gal4, UAS-2xEGFP / UAS-smo.act;tub-GAL80^{ts}*) enhances their number, but the level of Shg expression is equivalent to control.

(E) Quantitative analyses of the above results illustrating that Shg expression level in the progenitors is not dependent on Hh signaling.

P values: (*tub-GAL80^{ts}, AntpGal4-UASGFP/UAS-hh RNAi*): 0.71x10^{-1}

TepIV-Gal4, UAS-2xEGFP;tub-GAL80^{ts}/UAS-smoRNAi: 6.82 x10^{-1}

(*TepIV-Gal4, UAS-2xEGFP/ UAS-smo.act; tub-GAL80^{ts}*): 9.79 x10^{-1}

(F-G') The loss of Hh signaling from the niche (*tub-GAL80^{ts}, Antp-Gal4-UASRFP/ UAS-hhRNAi*) affects the progenitor number but does not alter the level of sqh expression in the residual progenitors.

(H) Quantitative analyses of the above results illustrating that level of SqhGFP expression in the progenitors is independent of Hh signaling.

P-value: (*tub-GAL80^{ts}, Antp-Gal4.UASRFP/UAS-hhRNAi*): 2.838x10^{-1}.

Age of the larvae: 72hr AEH

The yellow dotted line mark whole of the lymph gland in A', B', C', and D' while white dotted line marks the progenitor domain in A' and B'. However, yellow dotted line mark progenitor domain in F' and G'.

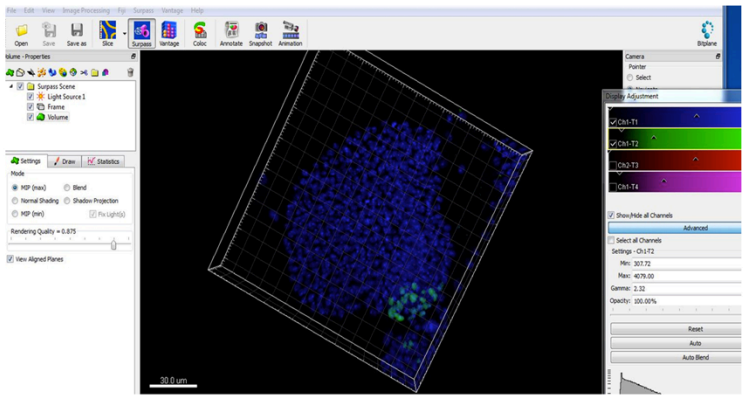
Error Bars: SD

Related to Figure 7

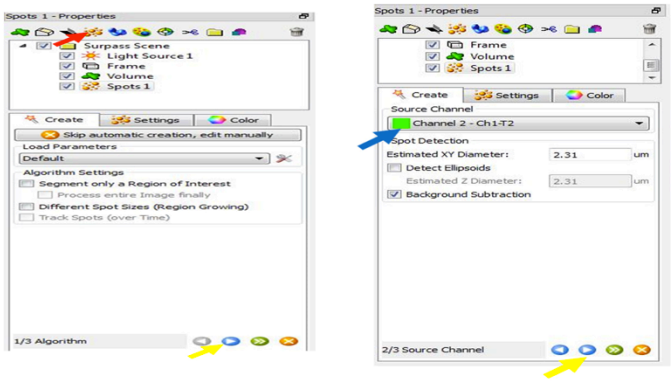
Supplementary Figure Method 1 and Method 2

Following method was used to count the Hh positive niche cells-

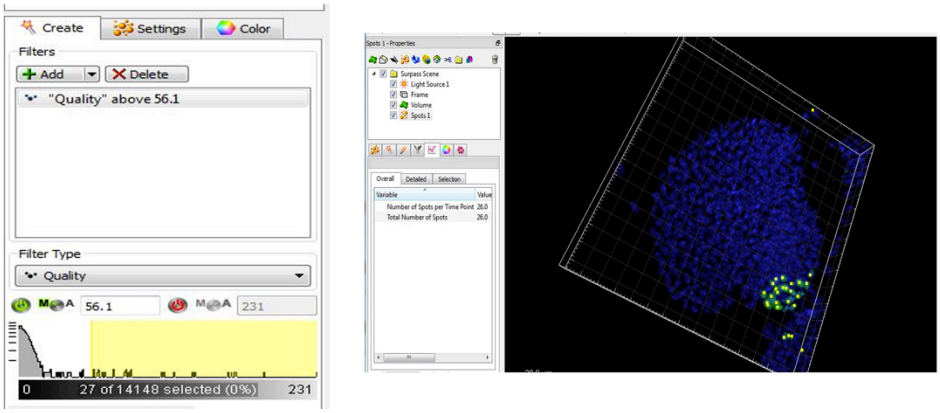
(a) Open the lymph gland image in Imaris as in this example, we have marked niche with Hh (green) and nucleus with Dapi(Blue).



(b) To count the Hh positive niche cell number, we have used spot function in imaris. choose the the **spot function (red arrow)** and start the algorithm **(click on next, indicate via yellow arrow).**Set the green channel (HH positive) in create tab and set the estimated XY diameter.Than click on the next (yellow arrow).

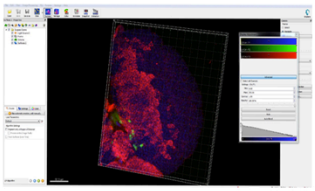


(c() It will autiomatically detect the Hh positive cells as a spot indicate in yellow dots and we can acquired the quantitated data from statistics tab

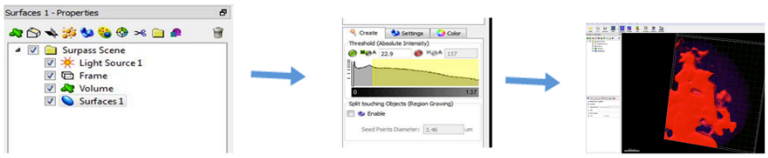


Following method was used to count the progenitor cells-

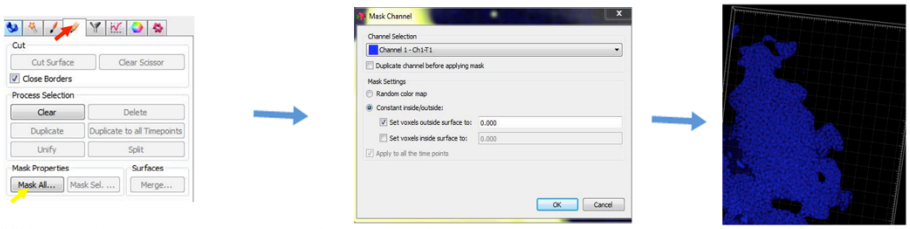
(a) Open the lymph gland image in Imaris as in this example, we have marked progenitor cells with Ance (red) and nucleus with Dapi(Blue).



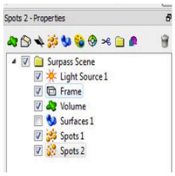
(b) To count the Ance positive progenitor cell number, we have used surface tool in imaris.For doing this, we first need to create surface on the red channel (Ance positive).



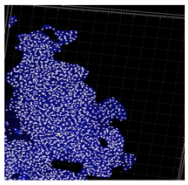
(c) Next, we masked the DAPI channel in surface1 (Ance positive domain). Then click on the the **Edit tab(red arrow) of** surface 1 and choose **Mask All (yellow arrow).** In a new window that apperars, we have to c the channel that we want to mask. In mask channel selection, we choose Dapi and to clear all the non-progenitor cell's nucleus,we choose **set voxels outside surface** to 0.



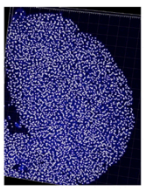
(d) To analyse the percentage of Ance positive cells, we then counted the total nucleus (DAPI) and Ance positive nuc using spot function identical to the one adopted for counting Hh positive cells .



Ance positive nucleus



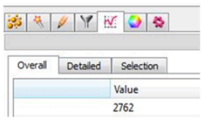
Total nucleus

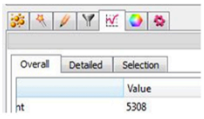


Percentage of Ance positive cells $= \frac{\text{Ance positive nucleus}}{\text{Total nucleus}} \times 100$

$= \frac{2762}{5308} \times 100$

$= 52.05$





Details of Fly Stocks and Reagents used:

Stock name	Detail Genotype	Reference
dome-Gal4,UAS-GFP	*dome-Gal4,UAS-mCD8GFP/ FM7; +/+; +/+*	(Mandal et al.,2007)
dome-Gal4,UAS-GFP; tub-GAL80^{ts}	*dome-Gal4,UAS-mCD8GFP/ FM7; tub-GAL80^{ts} / tub-GAl80^{ts} ;+/+*	(Mandal et al.,2007)
dome Gal4,UAS YFP	*dome Gal4,UAS YFP / FM7;+/+;+/+*	(Sinenko et al., 2009)
ZCL1973 (Trol GFP)	ZCL1973(X);+;+	(Jung et al.,2005)
TepIV-Gal4 (DGRC # 105442)	*yw,TepIV-Gal4* (NP7379)	(DGRC # 105442)
antp Gal4	*yw,+;antp-Gal4,UAS GFP/TM2*	(Mandal et al.,2007)
UAS FUCCI (BL 55121)	*w; P{w[+mC]=UASGFP. E2f1.1-230}32 P{w[+mc]=UASmRFP. NLS.CycB.1-266}19/cyo,P{ry[+t7.2]=en1} wg[en11];MKRS/TM6B, Tb[1]*	(Dey et al., 2016)
Pxn-Gal4,UAS GFP	*w;Pxn-Gal4,UAS-GFP/ Pxn-Gal4,UAS-GFP*	(Nelson et al., 1994)
Ance^{34Eb2}	*w;Ance^{34Eb-2} Adh^{D} pr^{1} cn^{1}/CyO,$^{AdhnB;+/+}$*	(Hurst et al., 2003)
Df(2L)b88f32 (BL3897)	*Df(2L)b88f32/In(2L)Cy In(2R)Cy, Cy^{1} dpy^{lvl} b^{1} pr^{1} Bl^{1} L^{4} sp^{2}*	(Hurst et al., 2003)
UAS-Ance RNAi (BL36749)	*y^{1}sc*v^{1};+/+;P{TRiP.HMS03009} attP2/P{TRiP.HMS03009}attP2*	Verified by immunostaining(current study)
Pvf2 LacZ	*w;+/+;Pvf2 LacZ/TM3Sb*	(Choi N H., et al 2008)
UAS-2xEGFP (BL6874)	*w;UAS-2xEGFP/UAS-2xEGFP; +/+*	(BL6874)
UAS-Hh RNAi (BL 32489)	*y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00492}attP 2/TM3, Sb[1]*	(Kuzhandaivel A et al.,2014) (Liu Z et al.,2015) (Pankaj Sahai-Hernandez et al 2013)
UAS-String RNAi (BL 34831)	*y[1] sc[*] v[1]; P{y[+t7.7 v[+t1.8]=TRiP.HMS00146}attP2*	(Spéder P et al.,2018)
UAS-Sqh RNAi (GD 7916)	*w[1118]; P{GD1695}v7916*	(Aranjuez et al., 2016) (Majumder et al., 2012) (Ku HY et al., 2017)
UAS-Zip RNAi (BL 36727)	*y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS01618}attP2*	(Nie et al., 2014)
UAS ROCK (BL 6668)	*y[1] w[*]; P{w[+mC]=UASRok. CAT}7.1*	(Rauskolb et al., 2014)

sqh[AX3]; sqh-GFP.RLC}2 (BL 57144)	*y[1] w[*] cv[1] sqh[AX3]; P{w[+mC]=sqh-GFP.RLC}2*	(Barros et al., 2003) (Katsuhiko et al., 2015)
UAS-Shg RNAi (BL 32904)	*y1 sc* v1; P{TRiP.HMS00693} attP2*	(Katsuhiko et al., 2015) (Cai D et al., 2015) (Kim M J et al., 2014)
UAS ci.HA (BL 32569)	*w*; P (UAS-ci.HA.m1-4)2;hhIJ3,P(Gal4 prd.Gal4/TM2*	(Chen Y et al., 2000)
hh4fF-GFP	*hhF4f GFP;+;+*	(Tokusumi et al., 2010)
UAS-Hh GFP	*w; UAS-Hh.EGFP;+*	(Vyas N et.al, 2008)
UAS-Smo RNAi (BL 27037)	*y1 v1; P{TRiP.JF02363}attP2*	(Tian A et., 2015) (Liu Z et., 2015)
UAS-Smo-act (BL44621)	*y1 w*; P{UAS-FLAG smo. act}2*	(Tian A et., 2015) (Liu Z et., 2015)
Pxn-GFP (DGRC 115452)	*w[1118]; PBac{802.P.SVS-2}Pxn[CPTI003897]*	DGRC 115452
tub-GAL80^{ts2} (2) BL 7019	*w*; P{tubP-GAL80^{ts}}20; TM2/TM6B, Tb1*	BL 7019
tub-GAl80t^{s2} (3) (BL7017)	w*; P{tubP-GAL80^{ts}}2/TM2*	BL 7017
dome-MESO-EBF2	*dome-MESO-EBF2.dome-MESO-EBF2;+;+*	(CJ Evans et., 2014) (Dey et al., 2016)
hs Flp (BL8862)	*P{ry(+7.2)=hsFLP}22*	(Bosch J A et.al., 2016)
Ay-Gal4(BL 3953)	*y1 w*; P(AyGAL4)25*	(Bosch J A et.al., 2016)
pCol85-Gal4	*P{GAL4}col85*	(Krzemień et al., 2007)
Hml-GAL4.Δ	*P{Hml-GAL4.Δ}3*	(Sinenko et al., 2004)

To knockdown the gene in temporal and spatial fashion, following GAL4 lines were recombined with tub-GAL80^{ts}:-

1. *dome-Gal4,UAS-GFP/FM7;+/+;+/+*

dome-Gal4,UAS-GFP/FM7;+/+;+/+; tub-GAL80^{ts20}/ tub-GAL80^{ts20}; +/+

2. *w/w; TepIV-Gal4, UAS-GFP/TepIV-Gal4,UAS GFP; +/+*

w/w; TepIV-Gal4, UAS-GFP/TepIV-Gal4,UAS GFP; +/+; tub-GAL80^{ts10}/ tub-GAL80^{t10}; +/+

3. *w/w; TepIV Gal4/TepIV Gal4; +/+*

w/w; TepIV Gal4/ TepIV Gal4; tub-GAL80^{ts20}/ tub-GAL80^{ts20}; +/+

4. *w/w; +/+; antp-Gal4,UAS-mCD8 RFP/TM6,Tb^{1}*

w/w; tub-GAL80^{ts20}/ tub-GAL80^{ts20}; antp-Gal4,UAS-mCD8 RFP/TM6,Tb^{1}

To generate clones Ay-Gal4 line were recombined with hs Flp:-

5. *hsflp/ hsflp; Ay-Gal4,UAS-GFP/ CyO; +*

RRID of Antibodies used in this study:

The Primary antibodies used in this study :

Rabbit anti-Ance (1:500, RRID:AB_2569408)

Mouse anti-P1 (1:50, RRID:AB_2568423)

Mouse anti-Patched (Ptc APA1, 1:30, RRID: AB_528441)

Rat anti-DE Cadherin (DCAD2, 1:100, RRID: AB_528120)

Rat anti-Ci (1:5, 2A1, RRID:AB_2109711)

Rabbit pMRLC (1:50, Cell Signaling RRID:AB_330248)

Mouse anti-β galactosidase (1:100, Promega RRID:AB_430877).

The RRID for Secondary antibodies used in our study

anti-mouse FITC (RRID:AB_2338601

anti-rabbit FITC (RRID:AB_2337978)

anti-mouse Cy3 (RRID:AB_2338692)

anti-rabbit Cy3 (RRID:AB_2307443)

anti-rat Alexa Fluor 647 (RRID:AB_2340693)

anti-mouse Alexa Fluor 647 (RRID:AB_2338902)

DAPI- RRID: AB_2629482