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Supporting Online Material for

Interaction Between Notch and Hif-α in Development and Survival of *Drosophila* Blood Cells

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Materials and methods

Drosophila Stocks and Genetics

The following fly stocks were used: *Hml$^{\Delta}$-gal4, UAS-2xEGFP* and *Hml$^{\Delta}$-gal4, UAS-2xEGFP*; *tubGal80^{ts10}* (S. Sinenko), *lz-gal4, UAS-mCD8::GFP* and *Hand-gal,Hml-gal4, UAS-2xEGFP, UAS-FLP; A5C-FRT-STOP-FRT-GAL4 (HHLT,* C. Evans), *UAS-sima* and *sima^{KG07607}*(P. Wappner), *UAS-Hph* (C. Frei), *UAS-N^{act}*, *UAS-N^{DN}*, *UAS-N^{fl}* and *12x Su(H)-lacZ* (S. Artavanis-Tsakonas), *y w,hs-FLP122; mib1^{EY09780} FRT82B neur1/TM6B* and *y w; mib1^{EY09780} FRT82B Ubi-GFP/TM6B* (C. Delidakis), *UAS-Psn^{D447A}* (M. Guo), *UAS-Su(H)^{DN}* (R. Chavourkar), *mib1^{3}, mib1^{EY097890}* (G. Struhl), *UAS-Dl, UAS-fng*, *w^{1118}*, *w; P{tubP-GAL80^{ts}}10; TM2/TM6B, Tb, UAS-N^{RNAi}*, *UAS-Rab5*, *tgo^{EY03802}* stocks from the Bloomington stock center. The RNAi stocks: *UAS-Dl^{RNAi}, UAS-Ser^{RNAi}, UAS-Ofut^{RNAi}, UAS-tgo^{RNAi}, UAS-sima^{RNAi}*, and *UAS-Hph^{RNAi}* were obtained from Vienna Drosophila RNAi Center (Austria), *UAS-NOS1^{RNAi}* was obtained from National Institute of Genetics-fly stock center (Japan).

To express the following transgenes: *UAS-sima*, *UAS-sima^{RNAi}*, *UAS-Hph, UAS-Hph^{RNAi}*, *UAS-tgo^{RNAi}*, *UAS-N^{act}*, *UAS-N^{DN}, UAS-N^{RNAi}, UAS-N^{fl}*, *UAS-Dl^{RNAi}, UAS-Ser^{RNAi}*, *UAS-fng*, and *UAS-Rab5* prior to or at the stage of crystal-cell commitment, but not later once the cell has become Lz^{+}, we used *Hml$^{\Delta}$-gal4, UAS-2xEGFP* as the driver. As a control to compare crystal-cell number and morphology, for these crosses we used lymph glands expressing *Hml$^{\Delta}$-gal4, UAS-2xEGFP/+*. The representative figures from these lymph

glands do not show the GFP (green channel) as it has been omitted for clarity.

For experiments to express *UAS-Hph, UAS-N*^{RNAi}, *UAS-Ofut*^{RNAi}, *UAS-fng, UAS-sima*^{RNAi}, *UAS-NOS1*^{RNAi}, *UAS-Psn*^{D447A} and *UAS-Su(H)*^{DN} specifically in the Lz^+ crystal-cell specified population we used *lz-gal4, UAS-mCD8::GFP* as the driver. As a control we used *lz-gal4, UAS-mCD8::GFP/+*.

Temporal analyses of Ser and Dl requirement during crystal-cell development was carried out by expressing *UAS-Ser*^{RNAi} or *UAS-Dl*^{RNAi} in the signaling cells at various time points from 50-88h AEL under the control of *Hml*$^{\Delta}$*-gal4, UAS-2xEGFP* inhibited by the expression of Gal80^{ts} (genotypes: *Hml*$^{\Delta}$*-gal4, UAS-2xEGFP*; *tubGal80*^{ts10}*; UAS-Ser*^{RNAI} and *Hml*$^{\Delta}$*-gal4, UAS-2xEGFP*; *tubGal80*^{ts10}*; UAS-Dl*^{RNAi}*)*. This inhibition was eliminated at selected time points (50, 60, 76 and 88h AEL) by shifting the crosses to permissive temperature (29°C) to express *Ser*^{RNAi} or *Dl*^{RNAi}, and were grown until wandering 3rd instar following which they were dissected and analyzed for crystal-cell development.

All the crosses were set up and maintained at 25°C, except crosses with Gal80^{ts} where they were set up and maintained at 18°C until the selected time point, after which the larvae were shifted to 29°C and grown until dissection.

For clonal analysis of *NOS1*^{RNAi} in the lymph gland, we generated "flip-out" clones expressing *UAS-NOS1*^{RNAi} using *Hand-gal, Hml-gal4, UAS-2xEGFP, UAS-FLP; A5C-FRT-STOP-FRT-GAL4 (HHLT).*

Crosses involving reporter analyses with *12xSu(H)-LacZ* or *HRE-LacZ*, the reporters were analyzed in single copy, except for NO feeding assay where *12xSu(H)-LacZ* reporter was kept homozygous.

Immunohistochemistry

Lymph glands were stained as previously described (*1, 2*) using the following antibodies: rat anti-Sima (P. Wappner and B. Shilo), rabbit anti-ProPO (H. Müller), mouse anti-βgal (Promega), mouse anti-Notch^{ecd} and Notch^{icd} (Developmental Studies Hybridoma Bank, Iowa), guinea pig anti-Hrs (H. Bellen) and mouse anti-NOS1 (M. Regulski). Cy3, Cy5 and FITC conjugated secondary antibodies were from Jackson Laboratory. Images were captured using a BioRad Radiance 2000 Confocal microscope with LaserSharp 2000 acquisition software.

***In Situ* hybridization**

For *in situ* hybridization sense and anti-sense probes for Notch were prepared using direct PCR products amplified from genomic DNA with T7-containing primers. These were used as templates for *in vitro* transcription using the DIG labeling kit (Roche). The color reaction was developed in NBT/BCIP solution (Roche). Lymph glands from wild-type and animals over-expressing Sima or Notch were prepared and stained in parallel and the color reaction was developed for the same time. The discs were dissected and mounted in

70% glycerol and photographed using a Zeiss Axioskop2 MOT microscope.

Endocytic Assay

Endocytic assay for Notch was performed as described in (*3*). Briefly, wild-type live lymph glands were dissected in fresh phosphate buffered saline, transferred for 30min into Scheneiders medium containing antibody against Notch extracellular domain. The antibody was then chased for 0, 30, 60, 90, 240, 300 and 360 min respectively. The lymph glands were fixed in 4% formaldehyde, followed by the normal antibody staining protocol.

NO and DAPT feeding experiment

For NO feeding experiments, mid 2nd instar larvae (genotype: *12xSu(H)-LacZ*) were transferred from standard fly food to food containing 500mM NOS inhibitor nitro-L-arginine methyl ester (L-NAME) or the inactive isomer *N*-nitro-D-arginine-methylester (D-NAME, Sigma) into a yeast plus food coloring mix. Wandering 3rd instar larvae were then dissected and further assessed for *Notch* reporter levels.

For DAPT feeding, late 2nd to early 3rd instar larvae (genotype: *lz-gal4, UAS-mCD8::GFP*) were transferred to standard fly food containing 500μM DAPT (Sigma), inhibitor of γ-secretase. Wandering 3rd instar larvae were then dissected and stained to assess crystal-cell development.

Hypoxic Stress

Embryos from WT larvae (genotype: w^{1118}) were collected for 2h under normoxic conditions. These embryos were allowed to hatch and grow until early 2nd instar larval life. Following this, the 2nd instar larvae were transferred to a 5% hypoxia chamber where they were grown until wandering 3rd instars. These 3rd instar larvae were then collected followed by dissection and immunostaining to assay for crystal-cell phenotype.

Statistics

Statistical analysis of data sets was undertaken using either Microsoft Excel (mean and standard deviation (SD) measurements) and u-tests (see http://faculty.vassar.edu.html).

Additional Figure legend information and genotypes used

In all the graphs control is abbreviated as con, lymph gland is abbreviated as LG and larvae were examined at late wandering 3rd instar.

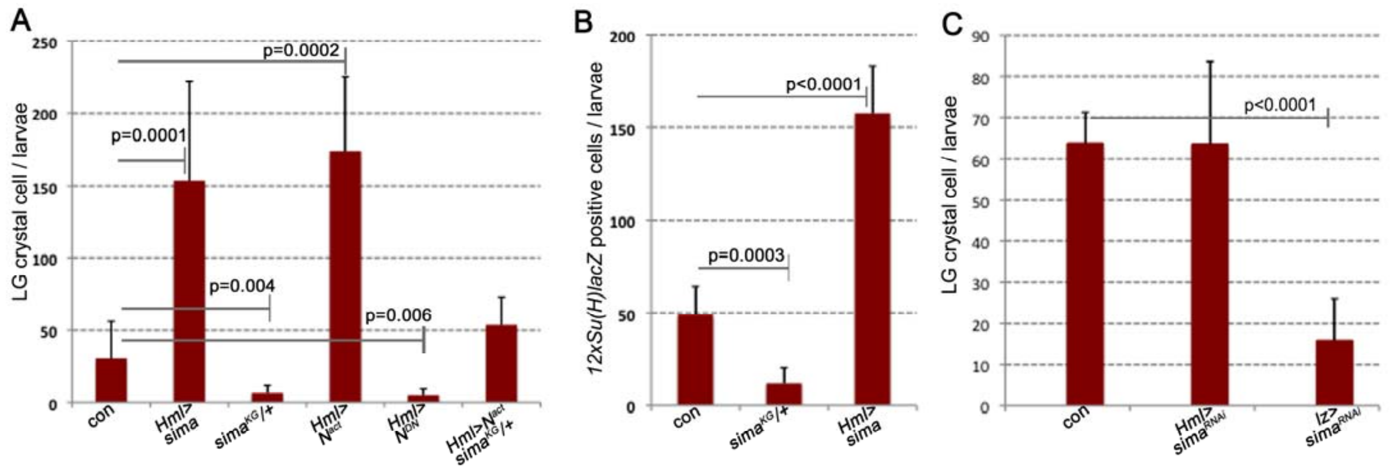
Figure 1. (**A**) w^{1118}, (**B**) *Hml$^{\Delta}$-gal4, UAS-2xEGFP; UAS-sima*, (**C**) *sima^{KG07607}/+*, (**D**) *Hml$^{\Delta}$-gal4, UAS-2xEGFP; UAS-N^{act}*, (**E**) *Hml$^{\Delta}$-gal4, UAS-2xEGFP; UAS-N^{DN}*, (**F**) *12xSu(H)-lacZ/+*, (**G**) *Hml$^{\Delta}$-gal4/12xSu(H)-lacZ; UAS-sima*, (**H**) *Hml$^{\Delta}$-gal4/12xSu(H)-lacZ; sima^{KG07607}/+* and (**I**) *Hml$^{\Delta}$-gal4/UAS-N^{act}; sima^{KG07607}/+*.

Figure2. (**A**) w^{1118}, (**B, B'**) *lz-gal4, UAS-mCD8::GFP*, (**C**) Hml^{Δ}*-gal4, UAS-2xEGFP; UAS-Hph,* (**D-E**) *lz-gal4, UAS-mCD8::GFP; UAS-Hph,* (**F**) *lz-gal4, UAS-mCD8::GFP; UAS-Hph*, (**G, I**) Hml^{Δ}*-gal4, UAS-2xEGFP*; *UAS-*N^{RNAi} , (**H, I**) *lz-gal4, UAS-mCD8::GFP; UAS-*N^{RNAi}, (**J**) *lz-gal4, UAS-mCD8::GFP; UAS-*N^{RNAi} and *lz-gal4, UAS-mCD8::GFP; UAS-*$Ofut^{RNAi}$, (**K**) w^{1118}, (**L**) *lz-gal4, UAS-mCD8::GFP; UAS-sima*, (**M, M'**) *lz-gal4, UAS-mCD8::GFP,* (**N, N'**) *lz-gal4, UAS-mCD8::GFP; UAS-sima* and (**O**) w^{1118} .

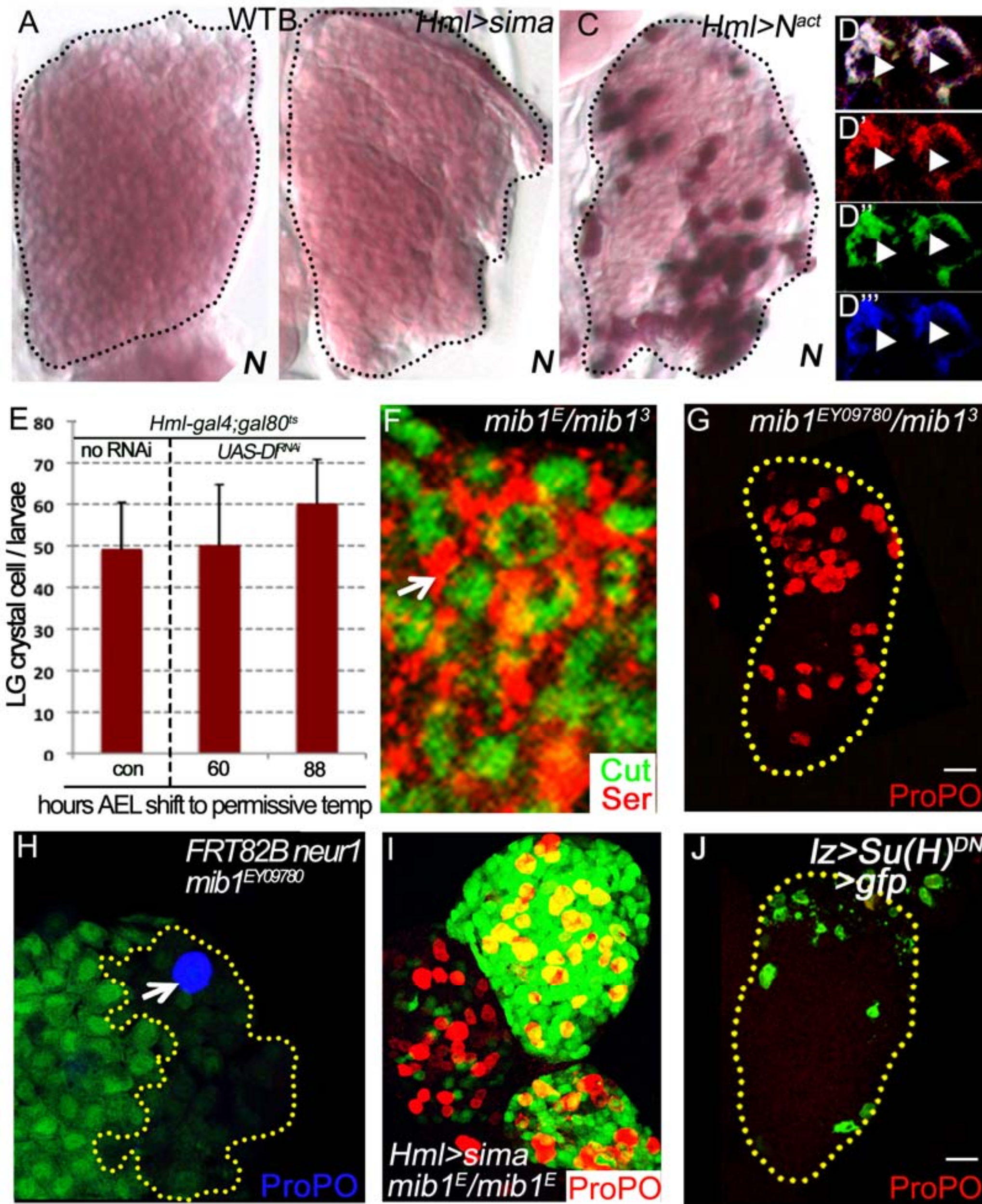
Figure 3. (**A-C**) Hml^{Δ}*-gal4, UAS-2xEGFP*; *tubGal80*^{ts10}*; UAS-*Ser^{RNAi}, (**D** and **F**) Hml^{Δ}*-gal4, UAS-2xEGFP*; *UAS-fng* and (**D**, **E** and **G**) *lz-gal4, UAS-mCD8::GFP; UAS-fng*, (**H**) w^{1118}, (**I**) Hml^{Δ}*-gal4, UAS-2xEGFP; UAS-*N^{fl} , (**J**) Hml^{Δ}*-gal4, UAS-2xEGFP; UAS-*N^{fl}*; mib1*^{EY09780}*/mib1*^{EY09780}, (**K**) *lz-gal4, UAS-mCD8::GFP; UAS-*Psn^{D447A} .

Figure 4. (**A**) w^{1118}, (**B**) $tgo^{EY03802}$/+, (**C**) Hml^{Δ}*-gal4, UAS-2xEGFP/UAS-*tgo^{RNAi}, (**D**) Hml^{Δ}*-gal4, UAS-2xEGFP/UAS-*Hph^{RNAi}, (**E** and **F**) w^{1118}, (**G**) *lz-gal4, UAS-mCD8::GFP; UAS-*$NOS1^{RNAi}$, (**H** and **I**) *12x Su(H)-lacZ.*

Supplementary figure 1



Supplementary figure 2



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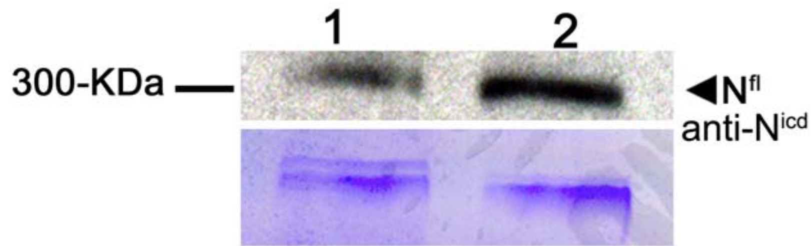
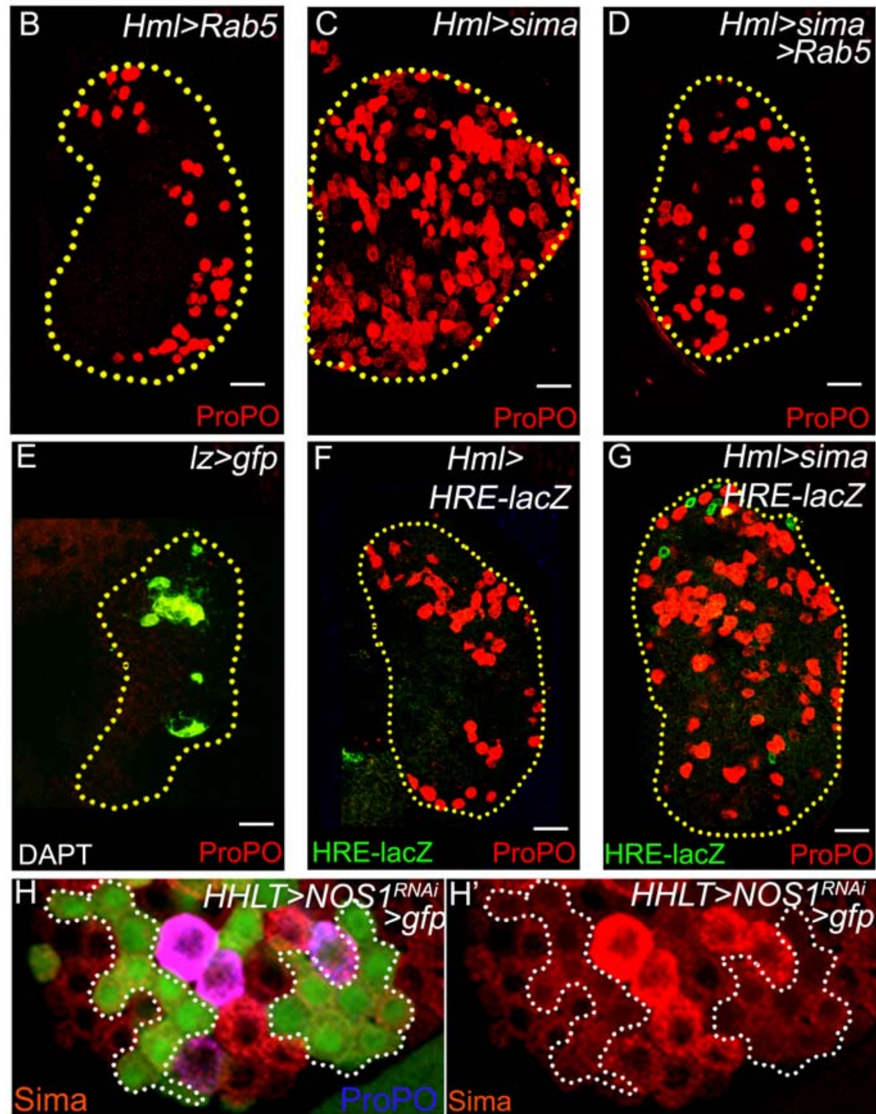
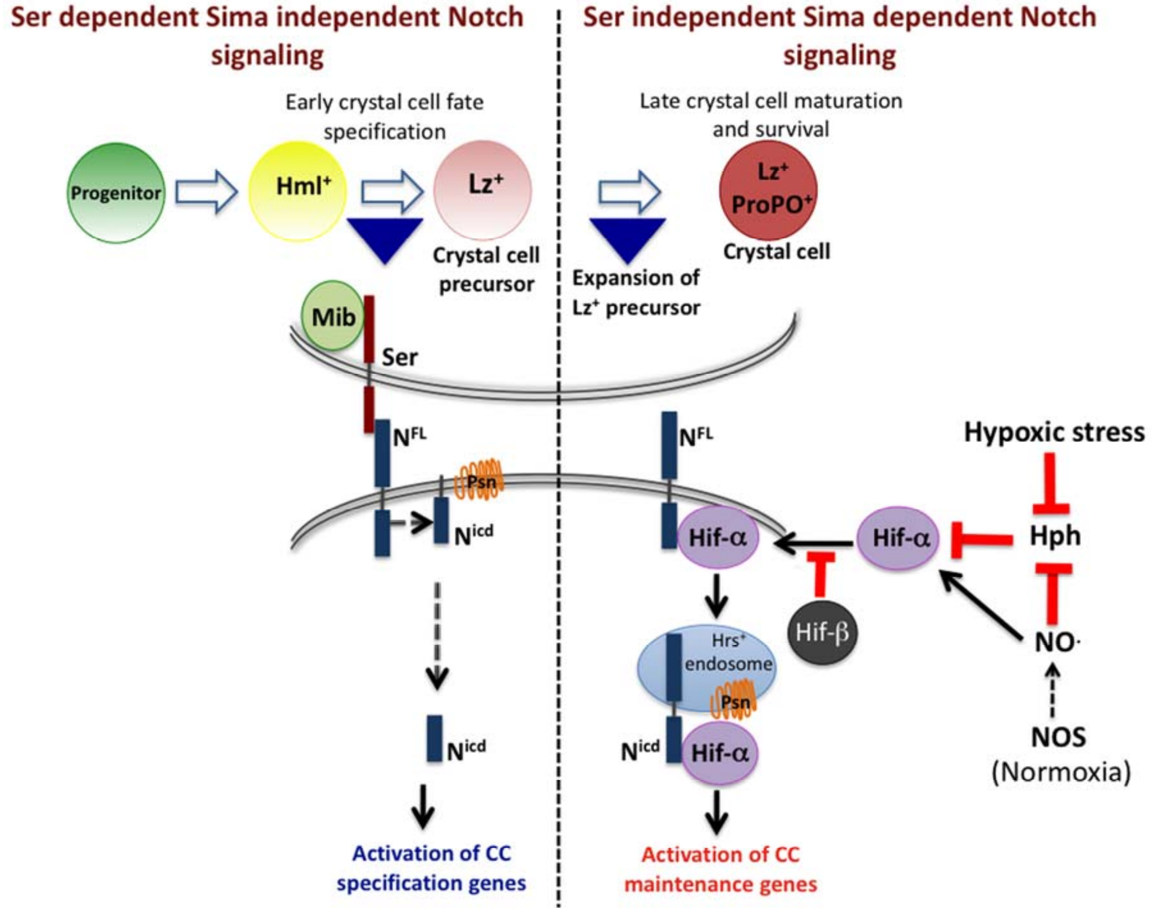




Supplementary figure 4



Supplementary figure legends

Supplementary figure 1. Sima function required late in crystal-cells but not early.

(**A**) Quantification of data in Fig.1A-E and I (n= 5).

(**B**) Quantification of *Notch* reporter data in Fig.1F-H (n= 5).

(**C**) Quantification of crystal-cell counts from lymph glands expressing $sima^{RNAi}$ early and late. Expression of $sima^{RNAi}$ early (genotype: *Hml*$^{\Delta}$*-gal4, UAS-2xEGFP; UAS-* $sima^{RNAi}$, n= 7) does not affect crystal-cell development, while late expression of $sima^{RNAi}$ in crystal-cells (genotype: *lz-gal4, UAS-mCD8::GFP; UAS-* $sima^{RNAi}$, n= 14) causes their rupturing and a subsequent reduction in their numbers.

Supplementary figure 2. Notch function late in crystal-cells is ligand-independent.

(**A-C**) Sima does not stabilize *Notch* transcriptionally. (**A**) In wild-type (WT) lymph glands *Notch* RNA is ubiquitously expressed. (**B**) Lymph gland over-expressing *sima* (genotype: *Hml*$^{\Delta}$*-gal4, UAS-2xEGFP; UAS-sima*) do not show any increase in *Notch* RNA levels. (**C**) Lymph glands over-expressing N^{act} (genotype: *Hml*$^{\Delta}$*-gal4, UAS-2xEGFP; UAS-*N^{act}) used as a positive control.

(**D-D’’’**) Crystal-cells (arrowheads) retaining N^{ecd} (red, **D’**) in Hrs positive (green, **D’’**) endosomes contain Sima (blue, **D’’’**). The vesicles appear white (**D**) due to colocalization of N^{ecd} (red), Hrs (green) and Sima (blue).

(**E**) Sima function in crystal-cells (ProPO: red) is independent of Notch ligands. Sequentially removing Dl from the lymph gland by expressing Dl^{RNAi} (genotype: Hml^{Δ}-*gal4, UAS-2xEGFP*; $tubGal80^{ts10}$*; UAS-*Dl^{RNAi}) during early (n= 6, 50 and 60 hours AEL) and late (n= 5, 76 and 88 hours AEL) stages of crystal-cell development does not show any effect in crystal-cell number (control abbreviated as con).

(**F**) $mib1^{EY09780}/mib1^{3}$ (abbreviated as $mib1^{E}/mib1^{3}$) mutant lymph gland stained with anti-Ser antibody (red), shows retention of Ser protein on the membrane (arrow), as it fails to get transendocytosed (cell nuclei marked with anti-Cut antibody in green).

(**G**) $mib1^{EY09780}/ mib1^{3}$ mutant lymph glands have normal crystal-cells (ProPO: red).

(**H**) *neur1* mutant clones in a *mib1* mutant background in the lymph gland have crystal-cells (marked ProPO in blue (arrow), genotype: *y,w, hs-FLP122;* $mib1^{EY09780}$ *FRT82B neur1/* $mib1^{EY09780}$ *FRT82B Ubi-GFP*).

(**I**) Over-expression of Sima causes an increase in crystal-cell (ProPO: red) number that is not suppressed in $mib1^{EY09780}$ mutant background (genotype: Hml^{Δ}-*gal4, UAS-2xEGFP/ UAS-sima;* $mib1^{EY09780}/mib1^{EY09780}$, abbreviated as *Hml>sima;* $mib1^{E}/mib1^{E}$).

(**J**) Expressing dominant negative Su(H) in crystal-cells causes them to burst (*lz-gal4, UAS-mCD8::GFP; UAS- $Su(H)^{DN}$*).

Supplementary figure 3. Sima stabilizes full-length Notch protein.

(**A**) Western blot for Notch protein in bleeds from (1) control (genotype: *Hml^{Δ}-gal4, UAS-2xEGFP*), and samples over-expressing *sima* (2) *Hml>sima* (genotype: *Hml^{Δ}-gal4, UAS-2xEGFP /UAS-sima*) using an antibody against Notch intracellular domain (N^{icd}) detects elevated levels of full-length Notch protein (300 kDa). Lower panel shows Coomassie stained lanes as loading control.

(**B**) Expressing Rab5 on its own in Hml^{+} cells has no effect on crystal-cells (ProPO: red, genotype: *Hml^{Δ}-gal4, UAS-2xEGFP; UAS-Rab5*).

(**C**) *sima* over-expression (*Hml^{Δ}-gal4, UAS-2xEGFP; UAS-sima*), crystal-cell (ProPO: red) expansion phenotype is suppressed by (**D**) co-expressing Rab5 (*Hml^{Δ}-gal4, UAS-2xEGFP; UAS-sima; UAS-Rab5*).

(**E**) Late 2nd to early 3rd instar larve were transferred to standard fly food containing 500μM DAPT. Reduction in crystal-cell number (ProPO: red) and bursting of these cells (genotype: *lz-gal4, UAS-mCD8::GFP*) was observed.

(**F**) The hypoxia reporter (*HRE-lacZ*: green) is not expressed in crystal-cells (ProPO: red, genotype: *Hml^{Δ}-gal4, UAS-2xEGFP; HRE-lacZ*). (**G**) Ectopic expression of Sima causes

an increases in crystal-cells (red), but not of the hypoxia reporter (green, genotype: *Hml*$^{\Delta}$-*gal4, UAS-2xEGFP/UAS-sima; HRE-lacZ*).

(**H** and **H'**) Clones expressing *NOS1*^{RNAi} (green, outlined in white dotted lines) have lower levels of Sima (red) and no crystal-cells (blue) when compared to the surrounding non-green wild-type tissue (genotype: *Hand-gal, Hml-gal4, UAS-2xEGFP, UAS-FLP; A5C-FRT-STOP-FRT-GAL4/UAS-NOS1*^{RNAi} abbreviated as *HHLT>NOS1*^{RNAi}).

Supplementary figure 4. Non-canonical mechanism of Notch activation mediated by Sima in crystal-cell

Crystal-cell (CC) fate specification is marked by the expression of Lz in a small subset of early differentiating Hml^{+} hemocytes arising from hematopoietic progenitors. This specification step requires activation of canonical Notch signal dependent on ligand-Ser. Upon Lz expression the precursors discontinue Hml expression, proliferate and mature to form crystal-cells expressing Lz and ProPO that are then maintained. Notch activation continues to be required in these stages of crystal-cell development as well. However, this function of Notch is independent of Notch ligands. The maturing crystal-cells express elevated levels of NOS1, the key enzyme responsible for generating Nitric Oxide (NO). NO mediates stabilization of Hif-α even under normoxic conditions, either directly or indirectly through inhibition of Hydroxy prolyl hydroxylase (Hph). Hif-α stabilization in the maturing crystal-cells causes ligand-independent, activation of full-length Notch (N^{fl}) receptor signaling by stabilization of the N^{fl} receptor in early Hrs^{+} endocytic

vesicles. Once stabilized, cleavage by Presenilin (Psn) releases an active signaling moiety likely to be a N^{act}/Hif-α /Su(H) complex essential for activation of crystal-cell maturation and survival genes. This Hif-α/Notch function is independent of Hif-β and does not lead to activation of canonical hypoxia target genes. However, upon hypoxic stress, which causes further stabilization of Hif-α, allows more Hif-α/Notch interaction and causes further expansion of crystal-cell number.

References

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