Published in final edited form as:

Science. 2011 June 3; 332(6034): 1210–1213. doi:10.1126/science.1199643.

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

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Abstract

A blood cell type termed crystal cell in *Drosophila* functions in clotting and wound healing and requires Notch for specification and maintenance. We report that crystal cells express elevated levels of Sima protein orthologous to mammalian hypoxia-inducible factor–α (Hif-α) even under conditions of normal oxygen availability. In these platelet-like crystal cells, Sima activates full-length Notch receptor signaling via a noncanonical, ligand-independent mechanism that promotes hemocyte survival during both normal hematopoietic development and hypoxic stress. This interaction initiates in early endosomes, is independent of Hif-β (Tango in *Drosophila*), and does not activate hypoxia response targets. Studies in vertebrate myeloid cells have shown a similar up-regulation of Hif-α protein in well-oxygenated environments. This study provides a mechanistic paradigm for Hif-α/Notch interaction that may be conserved in mammals.

The *Drosophila* lymph gland (hematopoietic organ) gives rise to myeloid blood cells: plasmatocytes, crystal cells, and lamellocytes (1). A majority of these cells are macrophages; a small fraction become crystal cells that function during wound healing (2, 3). Hemolectin (Hml) is the earliest marker expressed as cells initiate differentiation. The Runt-domain protein Lozenge (Lz) (4, 5) is essential for commitment to crystal-cell fate (6), and its expression initiates in these Hml^+ precursors, which then discontinue Hml expression (6). Mature crystal cells express prophenol oxidase (ProPO), essential for melanization (5), whereas macrophages remain Hml^+.

Sima is the *Drosophila* ortholog of hypoxia-inducible factor–α [Hif-α (7)], the key mediator of responses to hypoxia (8). Sima is stably expressed in mature crystal cells even under

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Supporting Online Material
www.sciencemag.org/cgi/content/full/332/6034/1210/DC1
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normal oxygen tension (Fig. 1A). Overexpression of *sima* in the lymph gland causes dramatic expansion of crystal cells (Fig. 1B and fig. S1A), whereas single-copy loss of *sima* reduces their number (Fig. 1C and fig. S1A). These phenotypes are similar to phenotypes resulting from *Notch* gain and loss of function (5) (Fig. 1, D and E, and fig. S1A). Furthermore, altering Sima can change Notch reporter [*12xSu(H)lacZ*] expression (Fig. 1, F to H, and fig. S1B), and a single mutant allele of *sima* suppresses the excess crystal-cell phenotype caused by $Notch^{activated}$ (N^{act}) (Fig. 1I and fig. S1A). Thus, Sima and Notch signaling appear to function in the same pathway in this tissue.

We examined the temporal requirement of Sima during crystal-cell development. Hph (hydroxy prolyl hydroxylase), which marks Sima for degradation (9), or *sima* RNA interference ($sima^{RNAi}$) was expressed in Hml^{+} precursors by using *Hml-gal4* as the driver that is switched off once a cell becomes Lz^{+} (10). Neither has any effect on crystal-cell number (Fig. 2, A, C, and E, and fig. S1C). However, expression of Hph or $sima^{RNAi}$ later in Lz^{+} crystal-cell precursors causes a significant reduction in crystal-cell number (Fig. 2, B and D to F, and fig. S1C, $P < 0.0001$). This loss is associated with bursting (compare Fig. 2, D′ with B′) of these cells, visualized by membrane green fluorescent protein (GFP) expression, a phenomenon (3) important for crystal cell–mediated blood clotting because of release of enzymes (3).

Crystal cell–fate specification requires canonical Notch signaling (6, 11). Expressing $Notch^{RNAi}$ in early differentiating Hml^{+} cells causes loss of crystal cells (Fig. 2, G and I). Additionally, late loss of Notch from already-specified crystal-cell precursors by either expressing N^{RNAi} or $Ofut^{RNAi}$ [modification of Notch by Ofut is required for proper Notch function (12)] causes a bursting phenotype (Fig. 2, H to J) as seen with loss of *Sima* (Fig. 2D ′). Thus, Notch function is required continuously: first in specifying the Lz^{+} precursor and then in expansion and maintenance of crystal cells.

High endogenous Notch expression in crystal cells (Fig. 2K) is further increased upon Sima overexpression (Fig. 2L), without change in *Notch* RNA (fig. S2, A to C). The majority of Notch protein in crystal cells is seen in intracellular vesicles (Fig. 2, M and N). In live trafficking assays (13), surface-labeled Notch internalized from the plasma membrane is rapidly degraded in all cells (Fig. 2, O and P) except in mature crystal cells, where Notch persists in Hepatocyte growth factor regulated tyrosine kinase substrate (Hrs)–positive early endocytic vesicles (Fig. 2, P and Q) and co-localizes with Sima (fig. S2, D to D‴).

Canonical Notch signaling requires the interaction of Notch with its membrane-bound ligand on an adjacent signaling cell (14, 15). Crystal cell–fate specification requires ligand-Serrate (Ser) (6, 11), but at late stages Ser is expressed in a restricted number of cells (6), not adjacent to a Ser-positive cell. This issue is even more acute for individualized circulating crystal cells, not in direct contact with a neighbor, that continue to require Notch function for their maintenance (Fig. 2J). The circulating crystal cells are of embryonic origin (16) and are not derived from the larval lymph gland (17). The other Notch ligand, Delta, lacks any obvious role in crystal-cell development (6, 11) (fig. S2E). Removal of Ser early prevents crystal cell–fate specification (Fig. 3, A and B), but unlike Notch and Sima, removing Ser after crystal cell–fate specification does not affect crystal-cell number or morphology (Fig.

3, A and C), suggesting that Sima-mediated Notch activation occurs independent of its ligand.

We addressed the question of ligand independence by using additional genetic criteria. Over-expression of Fng, a glycosyl transferase that inhibits Ser-Notch signaling (18) by using *Hml-gal4*, dramatically reduces crystal-cell number (Fig. 3, D and F), whereas late expression using *lz-gal4* has no effect (Fig. 3, D, E, and G). *Mib1* (19) and *neuralized* (20), which encode E3 ubiquitin ligases necessary for ligand endocytosis and promotion of ligand-dependent Notch signaling, have no role in crystal-cell development (fig. S2, F to I). We conclude that in Lz^+ cells, Notch activation is Ser-independent but Sima-dependent for its intracellular stabilization.

Full-length Notch (N^{fl}) can accumulate in endocytic vesicles in a ligand-independent manner (13, 21). This is also seen in hemocytes expressing Sima (fig. S3A), and N^{fl} is sufficient to increase crystal-cell number (Fig. 3I). This increase is not suppressed by *mib1* (Fig. 3J), but Rab5 co-expression, expected to enhance turnover of endocytic N^{fl} (22), causes strong suppression of Sima excess crystal-cell phenotype (fig. S3, B to D), confirming the involvement of endocytic vesicles in N^{fl}/Sima interaction. Although ligand-independent, the signaling entity is still cleaved Notch because expressing a dominant-negative form of presenilin (Psn^{D447A}, Fig. 3K) necessary for S3 cleavage of Notch (23) or feeding larvae with a γ-secretase inhibitor, DAPT {N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester} (fig. S3E), causes bursting of crystal cells. Also, expressing a dominant-negative form of Su(H) in Lz^+ cells causes bursting of crystal cells (fig. S2J).

In the context of hypoxia, Hif-α (Sima) functions as a heterodimer with Hif-β [Tango in *Drosophila* (24)]. However, *tgo* mutants or tgo^{RNAi} do not cause reduction but increase in crystal cells (Fig. 4, A to C). Crystal cells do not express the Sima/Tgo heterodimer-dependent hypoxia reporter (24) in wild-type (fig. S3F) and in Sima overexpression (fig. S3G) backgrounds. We hypothesize that in this context Tgo functions as a cytosolic sink to sequester Sima and prevents it from interacting with Notch.

Although normoxic Sima/Notch function does not require Tgo, the system responds to hypoxia by coopting the same developmental strategy. Conditions that stabilize Sima from degradation, such as blocking Hph (Fig. 4D) or exposing larvae to hypoxic stress (Fig. 4E), are sufficient to mediate an expansion of crystal cells. Under these conditions, the hypoxia-stabilized Sima further enhances Notch signaling independent of Tgo, causing an expansion of crystal cells.

Stimuli reported to stabilize Hif-α under normoxia include nitric oxide (NO) (25), reactive oxygen species (ROS) (26), and cations (27) that inhibit Hph (27) or stabilize Sima (28). Mature crystal cells express high levels of nitric oxide synthase1 (NOS1) (Fig. 4F), and expressing $NOS1^{RNAi}$ causes bursting of crystal cells (Fig. 4G). Additionally, feeding larvae NO inhibitor [L-NAME (NG-nitro-L-arginine methyl ester)] diminishes *Notch* reporter expression (Fig. 4, H compared to I). Lastly, clones expressing $NOS1^{RNAi}$ have low Sima protein and do not form crystal cells (fig. S3, H and H′). Thus, maturing crystal cells express

NOS1, which raises NO level, stabilizing Sima, which then functions with Notch to promote crystal cell maintenance and survival (fig. S4).

Earlier biochemical studies have established direct binding of Notch to Hif-α (29). Here, we describe a hypoxia and Notch ligand-independent developmental role for such an interaction. Normally a cell requiring continuous Notch signal needs to be in constant contact with a ligand-bearing cell, and a circulating cell will be unable to maintain active Notch signal. Crystal cells circumvent this need through noncanonical Notch activation via stabilization of N^{fl} receptor in the endocytic pathway mediated by Hif-α, even in the absence of ligand binding. Once stabilized, cleavage by presinilin creates an active signaling moiety, likely to be a N^{act}/Hif-α/Su(H) complex essential for crystal-cell maintenance. The Sima/Notch-dependent phase is a two-step process: the first is an expansion of Hml^{-} Lz^{+} crystal-cell precursors, followed by maintenance of Lz^{+} $ProPO^{+}$ mature crystal cells (see fig. S4 for a summary of all the data presented here). Circulating crystal cells in the larvae originate from the embryonic mesoderm (16) and not the lymph gland (17), and they show a similar Sima-mediated Notch requirement for their maintenance. Circulating Tcells also require Notch function to respond to pathogens (30) and express elevated levels of Hif-α (31). The source of ligand is the antigen-presenting cell, but additional alternate mechanisms are worth investigating.

Sima stabilization is important both for normal development and during response to hypoxia. A similar dual use of a signaling scenario is seen with ROS (32). At low levels, ROS functions as a signaling molecule in the stemlike progenitors, and scavenging ROS retards their differentiation. Under oxidative stress, the sensitized progenitors differentiate rapidly (32). Thus, the *Drosophila* myeloid system makes dual use of the same ROS/c-Jun N-terminal kinase and Sima/Notch signaling pathways for development and stress responses. Interestingly, vertebrate myeloid cells also maintain high Hif-α in normoxic environments to maintain their cellular energy pools and ability to mount an inflammatory response (33). Because *Drosophila* hemocytes are functionally most similar to mammalian myeloid cells, the concepts presented here are worthy of further investigation in mammalian systems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank H.Bellen, C. Frei, S. Artavanis-Tsakonas, C. Delidakis, M. Guo, M. Regulski, B. Shilo, G. Struhl, P. Wappner, National Institute of Genetics, Vienna Drosophila RNAi Center, Flybase, and Bloomington for reagents and D. Walker, G. Weinmaster, and C. Evans for helpful discussion. Supported by NIH grant R01HL067395.

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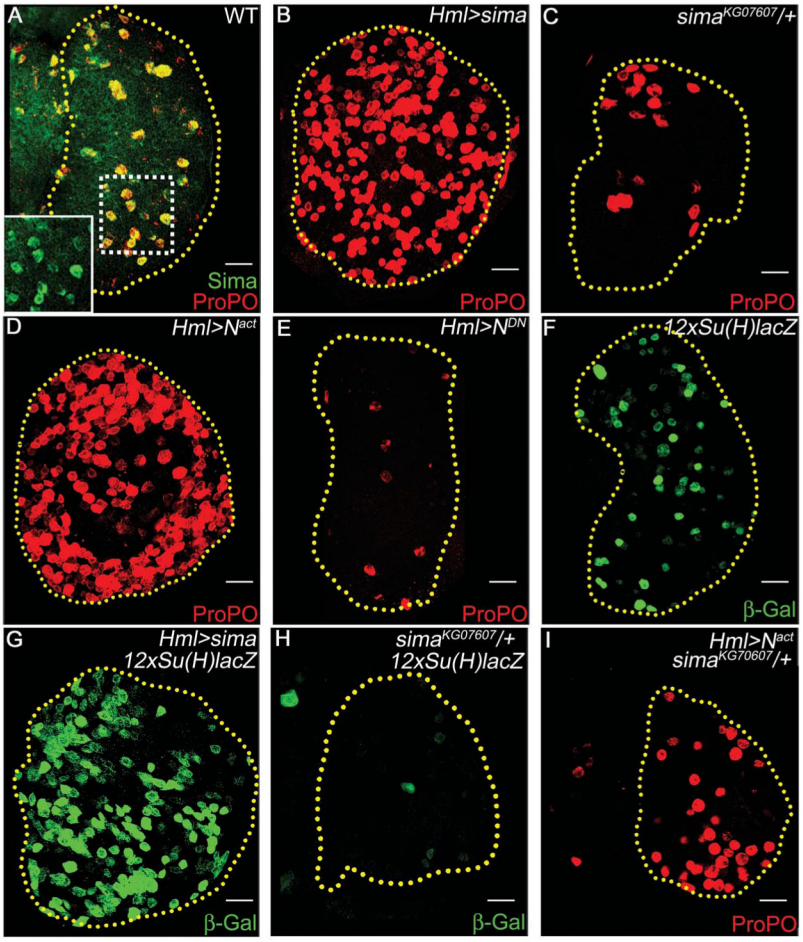

Fig. 1.

Sima functions with Notch during crystal-cell development. Crystal cells marked with ProPO (red). Scale bars indicate 20 μm. (**A**) Wild-type lymph gland, crystal cells show elevated levels of Sima (green; yellow because of ProPO co-localization). (Inset) Magnified view of crystal cells expressing Sima (green). (**B**) Overexpression or (**C**) single-copy loss of *sima* causes crystal-cell expansion or reduction, respectively. (**D**) Overexpression of activated Notch (N^{act}) that functions as a gain of function Notch or (**E**) dominant-negative Notch (N^{DN}) that functions as a loss of function Notch causes crystal-cell expansion and reduction, respectively. (**F**) Wild-type *Notch* reporter activity [*12xSu(H)-lacZ*, green] (**G**) increases with *sima* gain of function and (**H**) decreases with single-copy loss of *sima*. β-Gal, β-galactosidase. (**I**) Single-copy loss of *sima* suppresses N^{act}-driven crystal-cell expansion. Compare with (D).

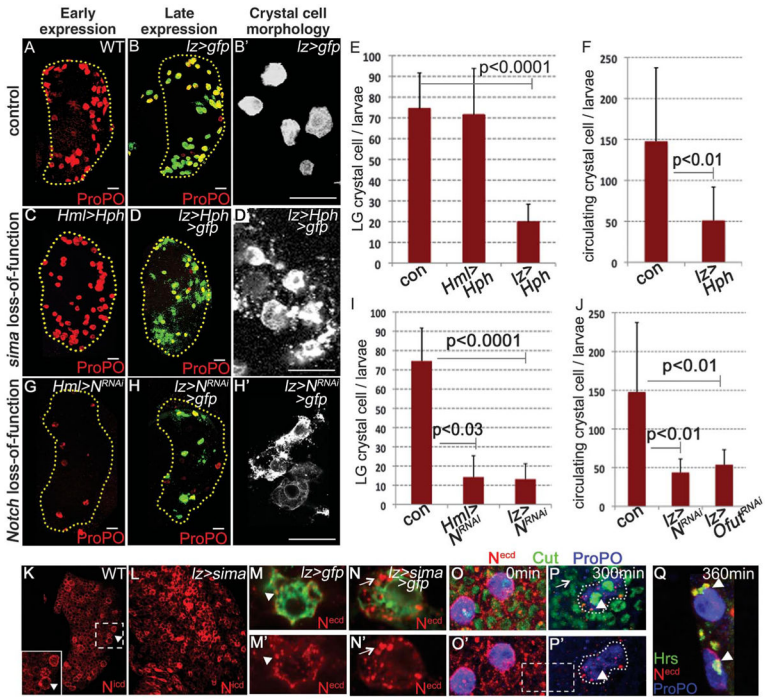

Fig. 2.

Sima stabilizes Notch in mature crystal cells, which is necessary for their maintenance and survival. Crystal cells marked with ProPO (red). Scale bars, 20 μm. (**A**) Wild-type (WT) lymph gland. (**B**) Control lymph glands expressing membrane GFP in crystal cells. Magnified in (**B′**). Overexpression of *Hph* (**C**) early does not affect crystal cells [compare with (A)]; (**D**) late expression in crystal cells causes their reduction and (**D′**) rupturing [compared with (B′)]. Quantification of (**E**) lymph gland ($n = 8$) and (**F**) circulating crystal-cell ($n = 8$) data. Error bars indicate standard deviation. Expressing N^{RNAi} (**G**) early and (**H**) late causes reduction in crystal cells [compared with (A) and (B)]. Loss of Notch late from crystal cells causes their rupturing [(**H′**) similar to (D′) compared with (B′)]. Quantification of (**I**) lymph gland ($n = 12$) data and (**J**) circulating crystal-cell data expressing N^{RNAi} ($n = 8$) and $Ofut^{RNAi}$ ($n = 8$). (**K**) Wild-type lymph glands with elevated Notch protein [Notch intracellular domain (N^{icd}), red] in crystal cells (arrowheads, magnified in inset). (**L**) *sima* overexpression causes further Notch (N^{icd}, red) accumulation. Antibody against the extracellular domain of Notch (N^{ecd}, red) antibody detects (**M** and **M′**) Notch in vesicles (arrowheads) in crystal cells from control lymph glands expressing GFP and (**N** and **N′**) overexpressing *sima* further increases Notch (N^{ecd}, red) accumulation in vesicles (arrows, compare with M and M′). (**O** to **Q**) Live endocytic trafficking assay: Notch antibody-recognizing epitope on N^{ecd} (red) in wild-type lymph glands marked with nuclear (Cut, green) and crystal-cell (ProPO, blue) markers. (O and O′) Notch protein is detected on all membranes at 0 min. (P) and (P′) At 300 min, endocytosed Notch is degraded from surrounding cells [compare N^{ecd} levels in the dashed areas in (O′) and (P′)] except in crystal cells (arrowheads) (Q) that retain Notch (red) in Hrs-positive (green, arrowheads) early endosomes. N^{ecd} (red) and Hrs (green) co-localize (yellow).

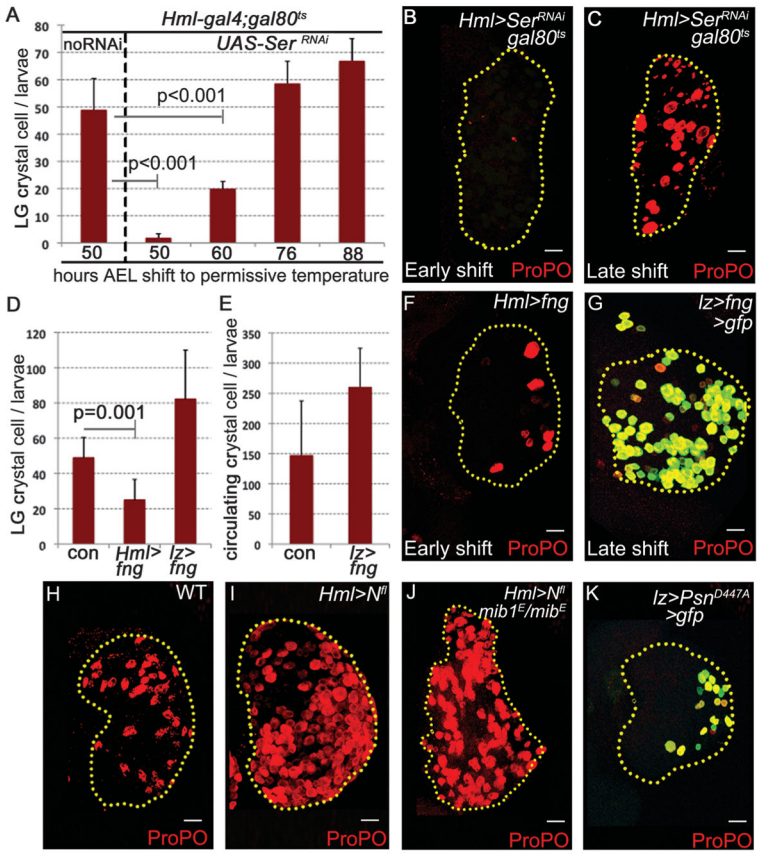

Fig. 3.

Sima promotes ligand-independent stabilization of full-length Notch protein for crystal-cell maintenance. (**A** to **C**) Ser^{RNAi} in signaling cells affects crystal cell [(A) and (B)] early [*n* = 5, 50, and 60 hours after egg laying (AEL)] but not [(A) and (C)] late (*n* = 5, 76 and 88 hours AEL). Crystal cells marked with ProPO (red). Scale bars, 20 μm. (**D** to **F**) *fng* overexpression [(D and (F)] early in signaling cells (*n* = 7) causes crystal-cell reduction but not [(D), (E), and (G)] late (*n* = 4). (**H**) Wild-type lymph gland. (**I**) Overexpressing N^{fl} increases crystal cells [compare with (H)]. (**J**) Expressing N^{fl} in *mib1* background shows no reduction in crystal cells [compare with (I) and (H)]. (**K**) Expressing dominant-negative presenilin (Psn^{D447A}) in crystal cells causes reduction.

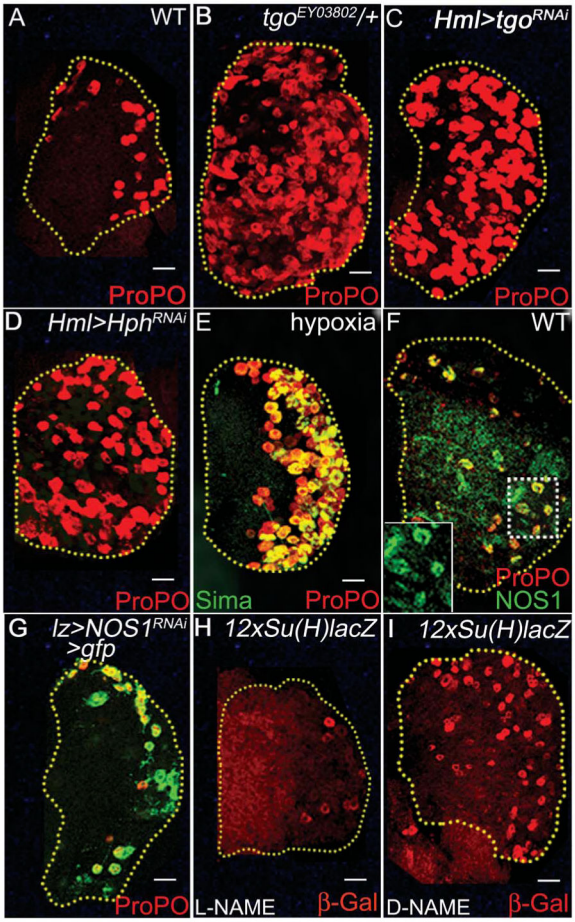

Fig. 4.

Sima function in mature crystal cells is independent of Tgo. ProPO (red) marks crystal cells (red). Scale bars, 20 μm. (**A**) Wild-type lymph gland. (**B**) Single-copy loss of *tgo*^{EY03802} or (**C**) expressing *tgo*^{RNAi} causes an increase in crystal cells. (**D**) Expressing *Hph*^{RNAi} or (**E**) exposing second instar wild-type larvae to 5% hypoxic stress increases Sima (green) stabilization and crystal-cell expansion. (**F**) Crystal cells from third instar WT lymph glands show elevated NOS1 (green, yellow because of overlap with ProPO; see inset). (**G**) *NOS1*^{RNAi} in crystal cells causes bursting [compare with (A)]. (**H** and **I**) Feeding larvae with (**H**) L-NAME (NO inhibitor) shows reduction in *Notch* reporter activity (red), whereas (**I**) D-NAME (inactive isomer) has no effect.