



Figures and figure supplements

Embryonic hematopoiesis modulates the inflammatory response and larval hematopoiesis in *Drosophila*

Wael Bazzi et al

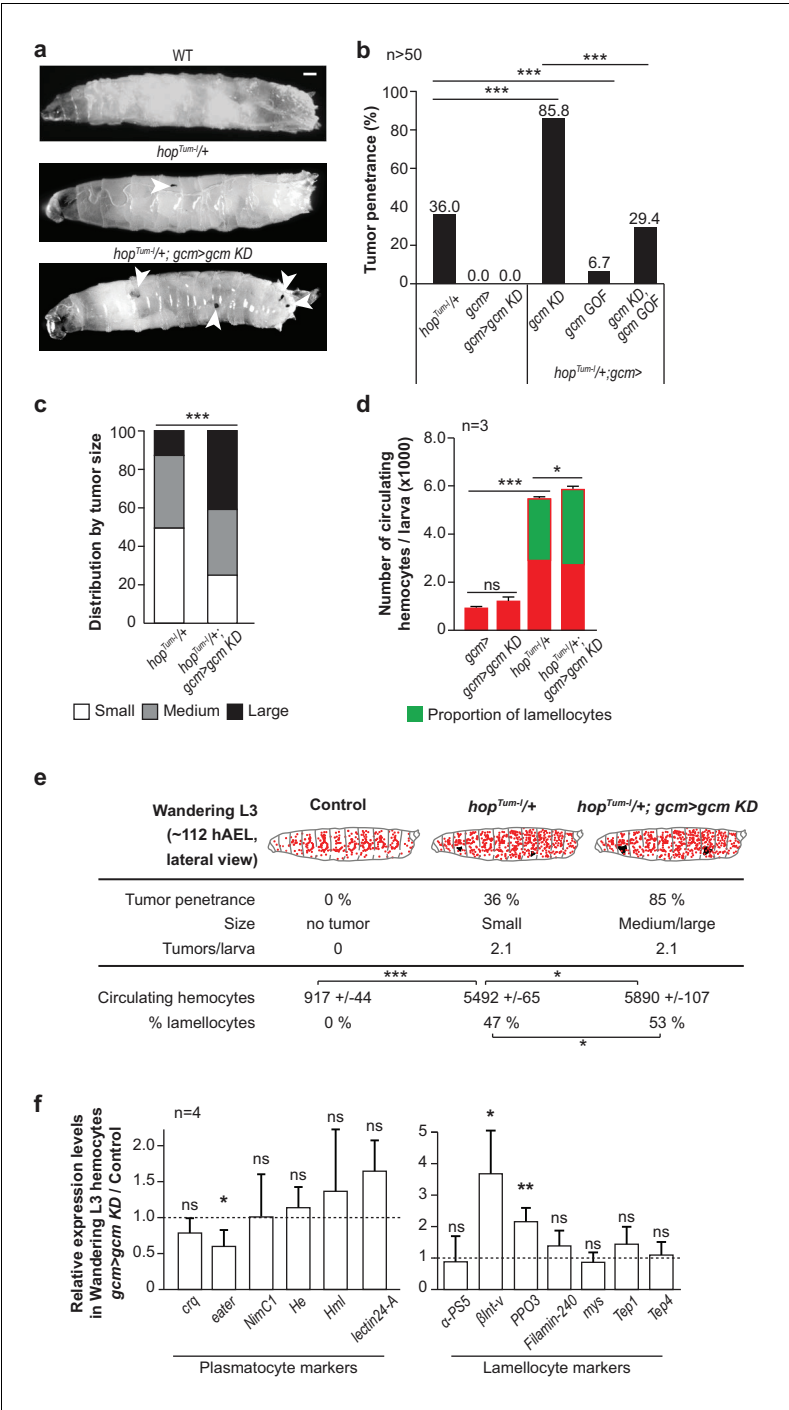


Figure 1. Gcm hinders Jak/Stat-mediated melanotic tumor formation. (a) Third instar larvae of the indicated genotypes (Note that all the detailed genotypes are in the Supplementary Methods, Fly strains and genetics section). White arrowheads indicate the melanotic tumors. (b) Penetrance of melanotic tumors (50). (c) Phenotype expressivity assessed as tumor size (*n* > 40). (d) Total number of circulating hemocytes and lamellocyte contribution (*n* = 3, using 10 larvae/replicate). (e) Hemocytes (red) and tumors (black) in third instar larvae of the mentioned genotypes. Assessment in terms of tumor penetrance, size and number of tumors/larva, number of circulating hemocytes and percentage of lamellocytes (*n* = 3, using 10 larvae/replicate). In all figures, **p*<0.005, ***p*<0.01; ****p*<0.001, ns: not significant; scale bar: 50 μ m unless otherwise specified. All the P values shown in this and in the following figures are in **Figure 1—source data 1**. (f) Relative expression levels of plasmatocyte and lamellocyte markers in larval hemocytes *gcm>gcm KD* compared to *gcm>*, measured by qPCR. The data are

Figure 1 continued on next page

Figure 1 continued

normalized to two housekeeping genes and then the ratio ($gcm > gcm\ KD/gcm$) is plotted ($n = 4$). The error bars represent the sum of the s.e.m. of $gcm > gcm\ KD$ and $gcm > .$

DOI: <https://doi.org/10.7554/eLife.34890.002>

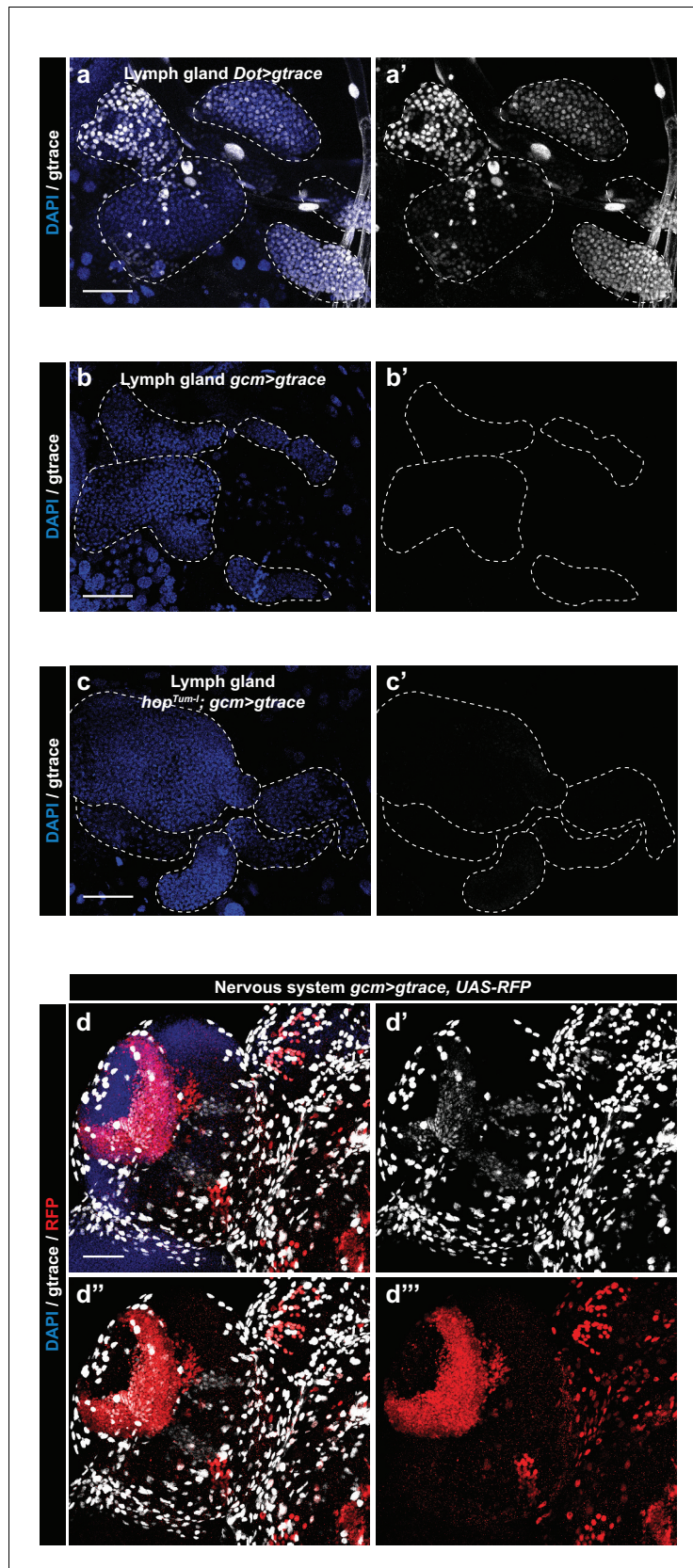


Figure 1—figure supplement 1. *Gcm* is not expressed in the second hematopoietic wave. Control lineage tracing in the lymph gland of Wandering L3 using the lymph gland specific *Dot>gtrace* line (Kimbrell et al., 2002) (a,a'). Figure 1—figure supplement 1 continued on next page

Figure 1—figure supplement 1 continued

Lineage tracing in Wandering L3 using *gcmgtrace* (**b,b'**) and *hop^{Tum-I};gcm>gtrace* lines (**c,c'**). The *gtrace* construct allows the constitutive expression of GFP as soon as the driver (here *Dot>* or *gcm>*) is expressed in the cell. Thus, the GFP signal indicates cells that have expressed the driver during development and/or are still expressing it (Evans et al., 2009). DAPI in blue and *gtrace* in white, maximum Z-projections. Note the expression of *Dot* in all the cells of the lymph gland (**a,a'**) and the absence of *Gcm* expression (**b,b'**) even upon constitutive Jak/Stat activation (**c,c'**). (**d–d'''**) Control lineage tracing showing *Gcm* expression in the larval nervous system. In this case, the *gcmgtrace,UAS-RFP* construct makes it possible to specifically identify the cells currently expressing *Gcm* as RFP positive. *Gcm* is expressed in many cell lineages (glia for *gtrace* signal and lamina neurons (Chotard et al., 2005) for RFP+ *gtrace* signals).

DOI: <https://doi.org/10.7554/eLife.34890.003>

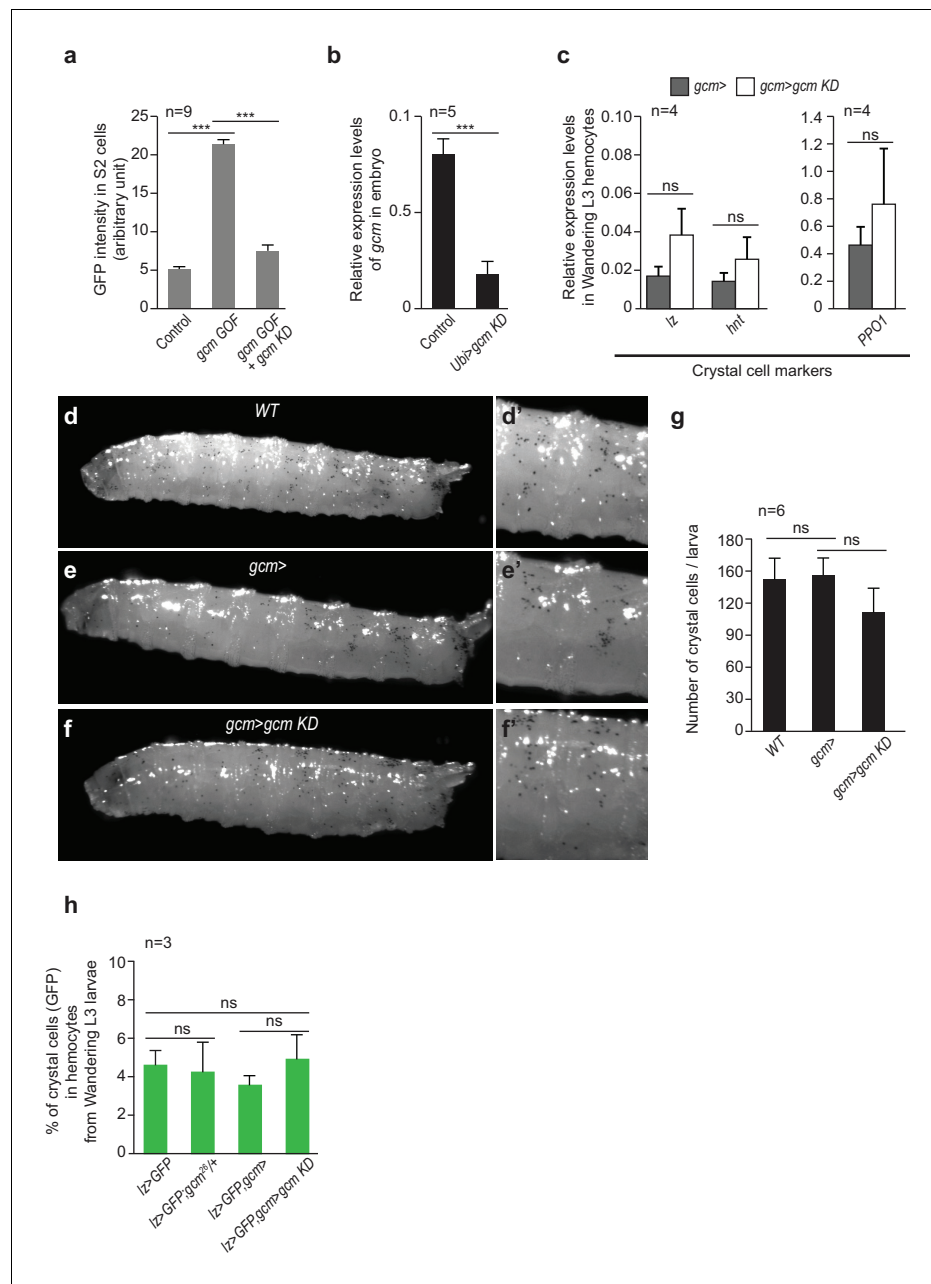


Figure 1—figure supplement 3. Crystal cell phenotype in *gcm* KD larvae. (a) *gcm* RNAi efficiency in S2 cells detected by GFP intensity in *gcm* GOF + *gcm* KD transfected S2 cells as compared to controls (n = 9). GFP signal measured upon transfection with *pPac-gal4*, *pUAS-gcm* and *repo-GFP* plasmids (column 2) or *pPac-gal4*, *pUAS-gcm*, *pUAS-gcmRNAi* and *repo-GFP* plasmids (column three) respectively. *repo-GFP* represents the reporter for Gcm activity (Laneve et al., 2013). (b) *gcm* RNAi efficiency in embryos. The levels of *gcm* were measured by qPCR on embryos (overnight lay) of the following genotypes: *UAS-gcmRNAi/+* (Control) and *Ubi>gcm* KD (*Ubi>* is a driver ubiquitously expressed). The levels are represented relative to the two housekeeping genes *Gapdh1* and *Act5c*. (c) Relative expression levels of crystal cell markers in hemocytes from *gcm>* and *gcmgcm* KD Wandering L3 measured by qPCR and normalized to two housekeeping genes (n = 4). (d–f) Crystal cells visualized in Wandering L3 after heat treatment at 70°C for 10 min. (g) Average number of crystal cells/larva in WT, *gcm>* and *gcmgcm* KD animals (n = 6). (h) Average percentage of crystal cells in circulating and sessile compartments in the mentioned genotypes, using the *lz>GFP* driver specific to crystal cells (Waltzer et al., 2003; Osman et al., 2009) (n = 3).

DOI: <https://doi.org/10.7554/eLife.34890.005>

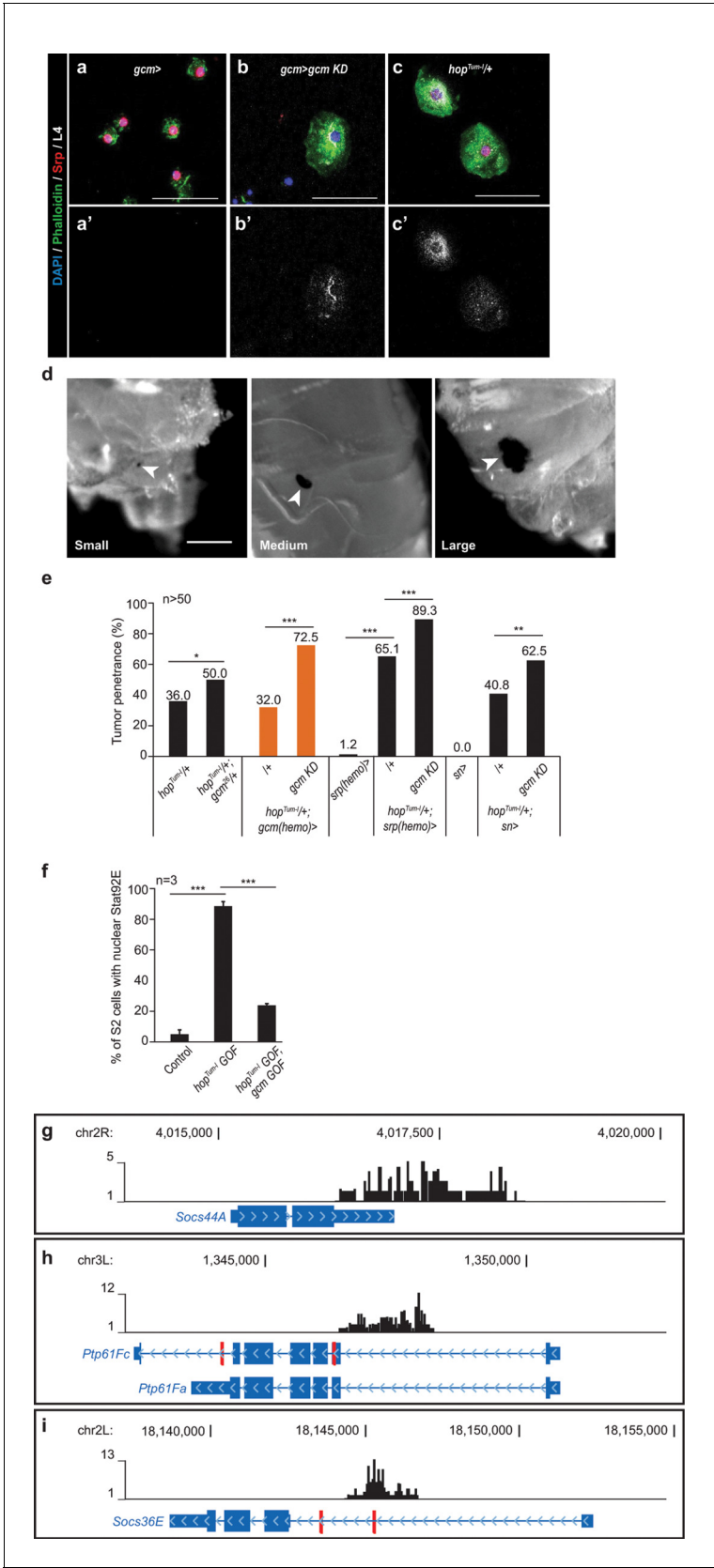


Figure 1—figure supplement 4. Gcm inhibits Jak/Stat-mediated melanotic tumor formation. (a–c) Immunolabelling of hemocytes from Wandering L3 of the mentioned genotypes (DAPI in blue, Phalloidin in green, Srp in red, lamellocyte marker L4 in white), (n > 3). Strong Phalloidin labelling is specific Figure 1—figure supplement 4 continued on next page

Figure 1—figure supplement 4 continued

to lamellocytes (**Avet-Rochex et al., 2010; Stofanko et al., 2010**). In all fluorescent confocal images, maximum Z-projections are presented. (d) Melanotic tumors of different size (arrowheads). See Materials and methods for quantitative assessment. (e) Tumor penetrance in *hop^{Tum-I/+};gcm26/+* larvae (column 2), *hop^{Tum-I/+};gcm(hemo)Gal4/+* (column 3), *hop^{Tum-I/+};srp(hemo)Gal4/+* (column 6) and *hop^{Tum-I/+};snGal4/+* (column 9) or upon *gcm* KD (columns 4, 7 and 10) as compared to controls ($n > 50$). (f) Percentage of S2 cells presenting nuclear Stat92E labelling after transfection with *pPac-gal4* and *pUAS-RFP* (Control), or *pPac-gal4*, *pUAS-RFP* and *pUAS- hop^{Tum-I}* (*hop^{Tum-I}* GOF), or *pPac-gal4*, *pUAS-RFP*, *pUAS- hop^{Tum-I}* and *pPac-gcm* (*hop^{Tum-I}* GOF, *gcm* GOF). The quantification was done on at least 20 cells in triplicates. The labelling is displayed in **Figure 2a–c**. (g–i) Loci containing DamID peaks (black), Gcm binding sites (GBSs, in red), blue arrows within the loci indicate the direction of transcription, histograms above the locus show a region of 1 kb on each side of a DamID peak scoring a $FDR < 0.001$, genomic coordinates of the loci are indicated above the histograms: *Socs44A* (g), *Ptp61Fa* (cytoplasmic isoform) and *Ptp61Fc* (nuclear isoform) obtained upon alternative splicing at the 3' carboxyl terminal of *Ptp61F* (h) and *Socs36E* (i).

DOI: <https://doi.org/10.7554/eLife.34890.006>

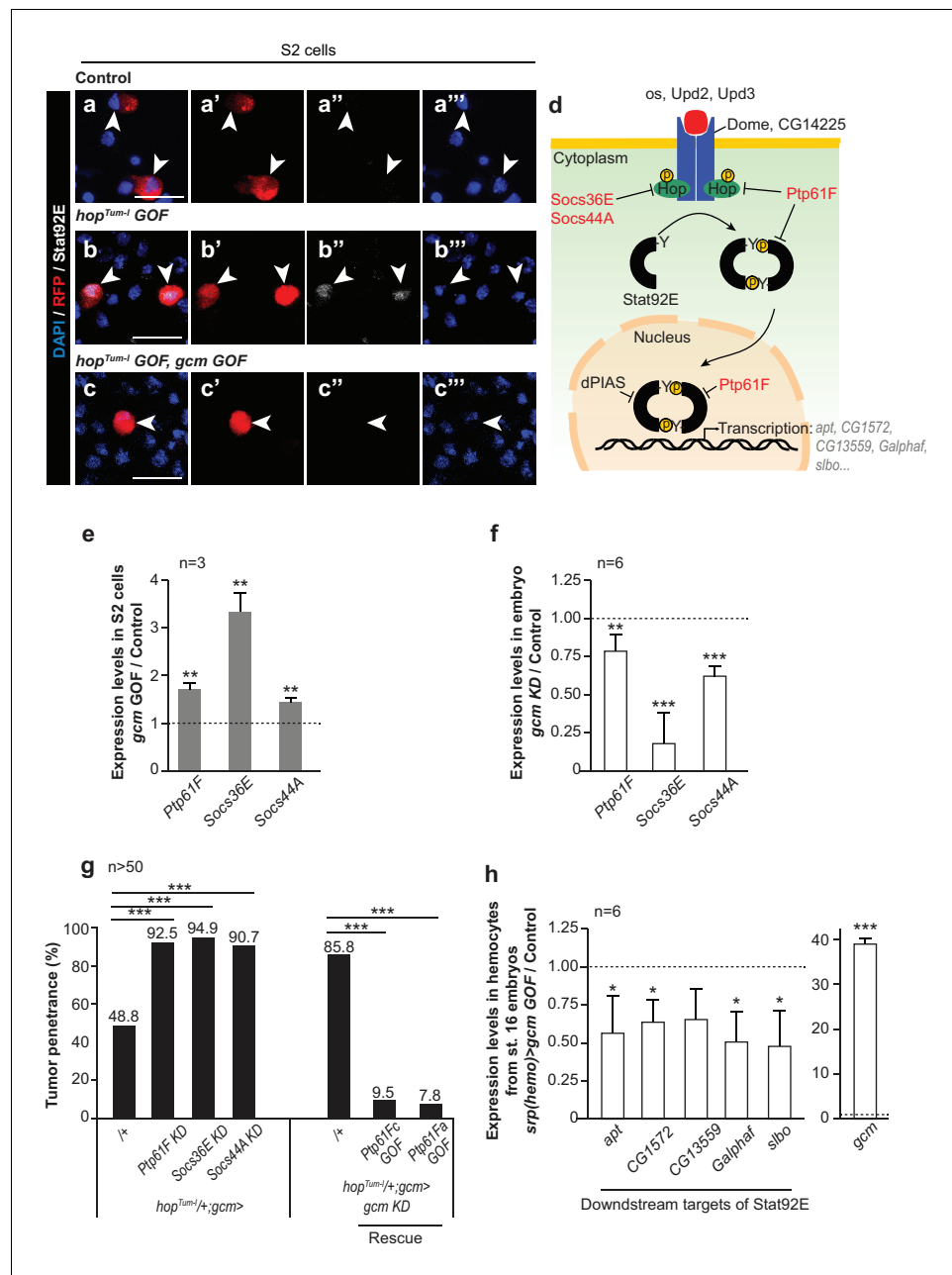


Figure 2. Gcm induces the expression of Jak/Stat inhibitors and hinders Jak/Stat-mediated melanotic tumor formation. (a–c) Immunolabelling of S2 cells transfected with *pPac-gal4* and *pUAS-RFP* (Control, (a), or *pPac-gal4*, *pUAS-RFP* and *pUAS- hop^{Tum-I}* (*hop^{Tum-I} GOF*, (b), or *pPac-gal4*, *pUAS-RFP*, *pUAS- hop^{Tum-I}* and *pPac-gcm* (*hop^{Tum-I} GOF, gcm GOF*, (c) and labelled for DAPI (blue), RFP (red) and Stat92E (gray). (a,b,c) show the merge of the three channels, (a',b',c') show RFP alone, (a'',b'',c'') show Stat92E alone and (a''',b''',c''') show DAPI. Arrowheads indicate transfected S2 cells. Scale bar: 20 μ m. The percentage of cells presenting nuclear Stat92E is displayed in **Figure 1—figure supplement 4f**. (d) Jak/Stat pathway: inhibitors of the pathway that are regulated by Gcm are in red. (e,f) Relative expression levels of Jak/Stat inhibitors in S2 cells transfected with a *pPac-gcm* expression plasmid (three independent assays) (e) and in embryo *gcm> gcm KD* (f). In both cases, the data are relative to the levels in controls as described in **Figure 1f**. (g) Penetrance of melanotic tumors ($n > 50$). (h) Relative expression levels of Jak/Stat downstream targets (*apt* (Starz-Gaiano et al., 2008), *CG1572* (Bina et al., 2010), *CG13559* (Bina et al., 2010), *Galphaf* (*Ga73b*) (Bina et al., 2010; Bausek and Zeidler, 2014) and *slbo* (Silver and Montell, 2001)) in hemocytes from stage 16 embryo *srp(hemo)> gcm GOF*. The data are relative to the levels in controls as described in **Figure 1f**.

DOI: <https://doi.org/10.7554/eLife.34890.008>

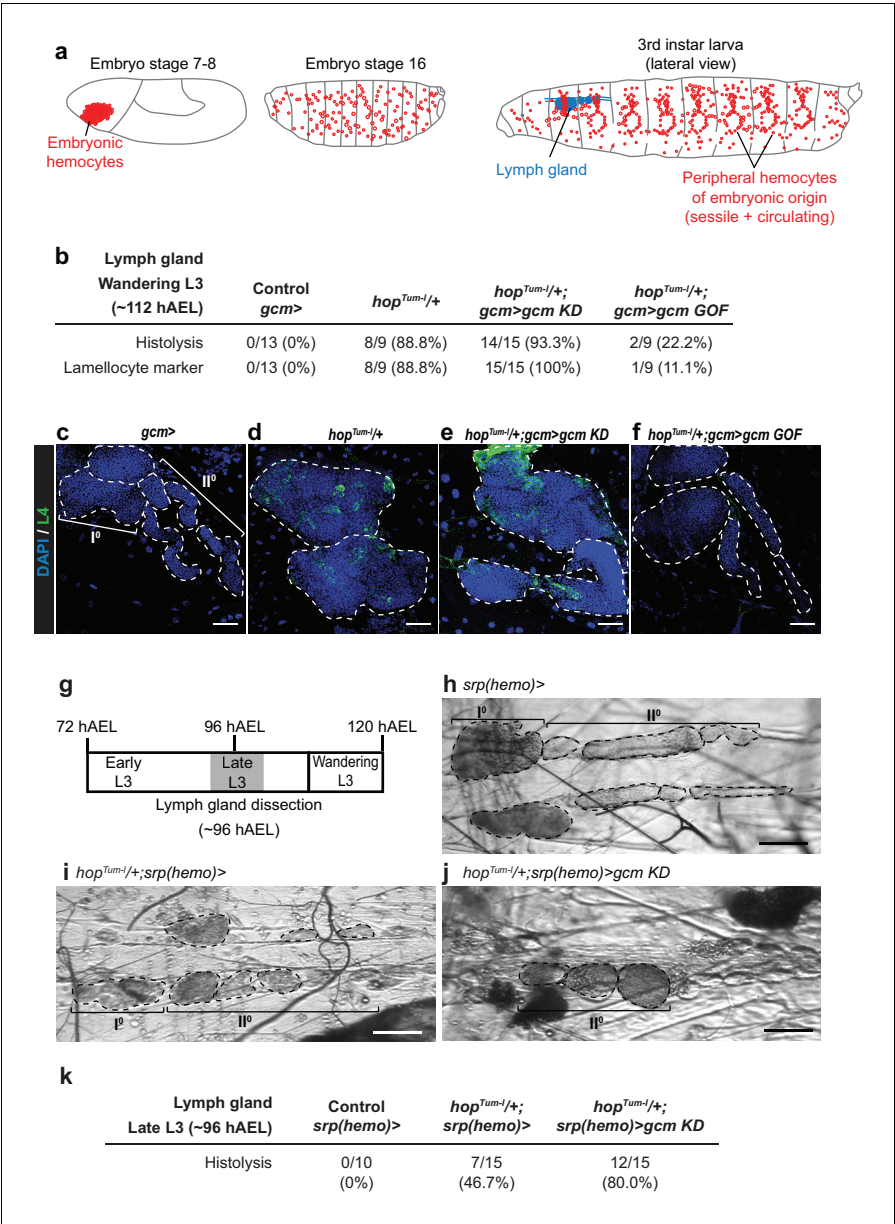


Figure 3. Embryonic hemocytes signal to the lymph gland. (a) Embryonic hemocytes (red) in early and late embryos as well as in a third instar larva. The lymph gland (blue) histolyzes at the larva to pupa transition. (b) Number and percentage of lymph glands showing histolysis and lamellocyte labelling (L4 marker, green) at wandering L3 stage, DAPI is in blue. (c–f) Lymph glands are indicated by hatched lines. (c) Control lymph gland (*gcm>*): I° and II° indicate primary and secondary lobes, respectively. (d,e) show hypertrophic glands, lack of lobes and L4 expression. (f) rescue of the phenotype. (g–j) Bright-field images of lymph glands of the indicated genotypes (h–j) from Late L3 larva (~96 hAEL) as indicated in (g). I° and II° indicate primary and secondary lobes, respectively. Note that the lymph glands are partially histolyzed in *hop^{Tum-1/+}; srp(hemo)>* (i) and *hop^{Tum-1/+}; srp(hemo)> gcm KD* (j) animals. (k) Number and percentage of lymph glands showing histolysis at Late L3 stage (~96 hAEL).

DOI: <https://doi.org/10.7554/eLife.34890.009>

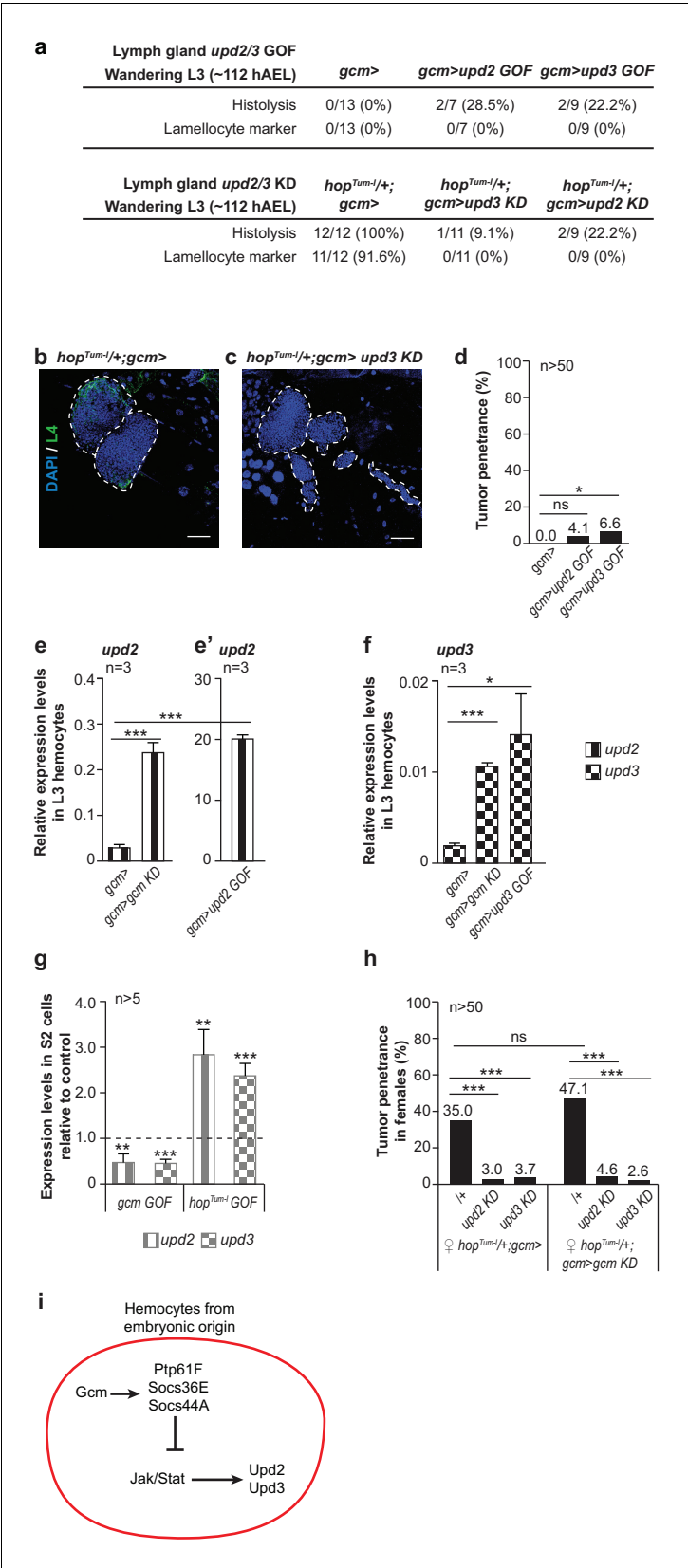


Figure 4. Embryonic hemocytes signal through Upd2 and Upd3. (a) Lymph gland phenotypes. (b,c) Lymph gland immunolabelling as in **Figure 3c–f**. (d) Tumor penetrance. (e–f) Relative expression levels of *upd2* and *upd3* in **Figure 4** continued on next page

Figure 4 continued

hemocytes from wandering L3 larvae of the mentioned genotypes. *upd2* and *upd3* expression levels increase in *gcmgcm* KD (e), first two columns from the left in e) and even further in *gcm > upd2/3* GOF third instar larval hemocytes (e'), column three in (f). Note the different scale between (e) and (e'). (g) *upd2* and *upd3* expression levels in S2 cells upon transfection with *pPac-gcm* or *pUAS-hop^{Tum-I}* expression vectors, compared to control levels shown by the dashed line (transfection of an empty expression vector) ($n > 5$). (h) Tumor penetrance in third instar female larvae. (i) Schematic representation of Gcm contribution to Upd2 and Upd3 production from embryonic hemocytes.

DOI: <https://doi.org/10.7554/eLife.34890.010>

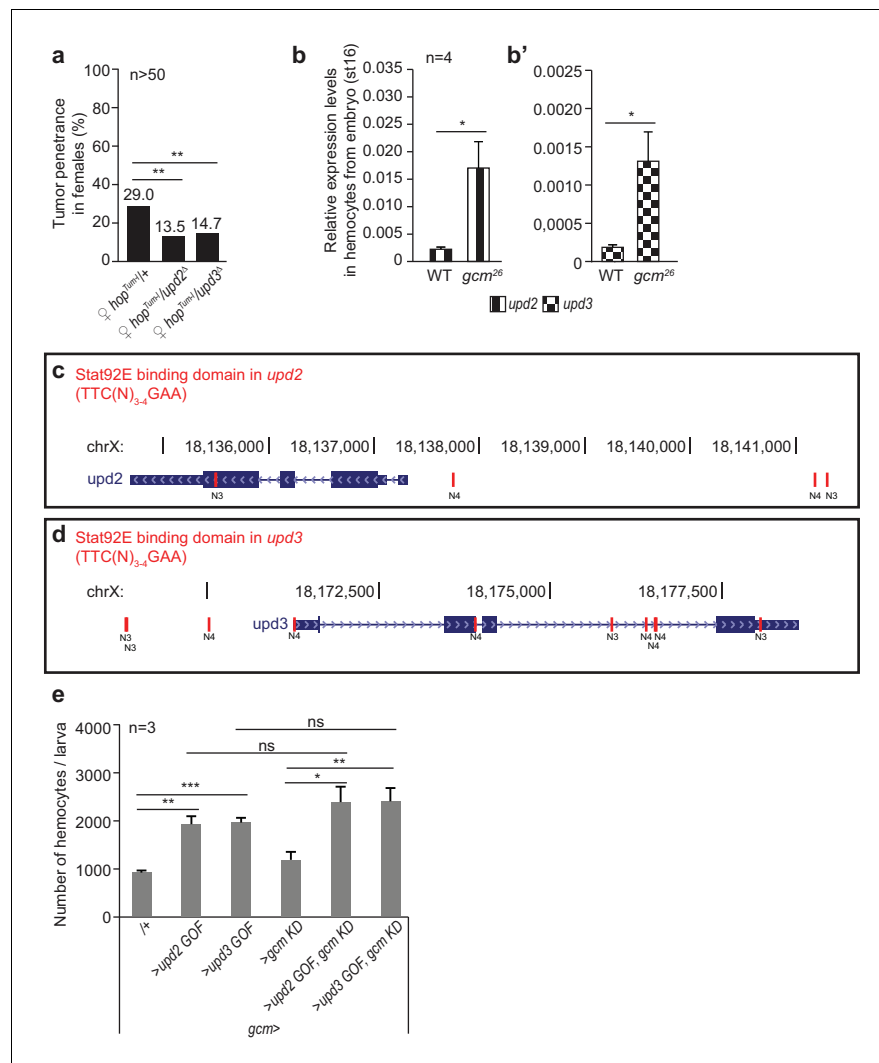


Figure 4—figure supplement 1. Interaction between Jak/Stat pathway, Gcm and Upd2/Upd3 cytokines. (a) Tumor penetrance in double heterozygous female larvae *hop^{Tum-I}/upd2^Δ* and *hop^{Tum-I}/upd3^Δ*. (b,b') *upd2* and *upd3* expression levels in hemocytes sorted from stage 16 control embryos (*srp(hemo)Gal4/UAS-RFP*) or *gcm26* embryos (*srp(hemo)Gal4, gcm26/UAS-RFP,gcm26*). Note that the levels of *upd2* and *upd3* are at the limit of the detection range in WT hemocytes and their levels increase strongly in *gcm26* hemocytes. (c,d) Canonical Stat92E binding sites (TTC(N)₃₋₄GAA) (Yan et al., 1996) at *upd2* and *upd3* loci (in red), symbols as in Figure 1—figure supplement 1. (e) Total number of circulating hemocytes in the indicated genotypes (n = 3).

DOI: <https://doi.org/10.7554/eLife.34890.011>

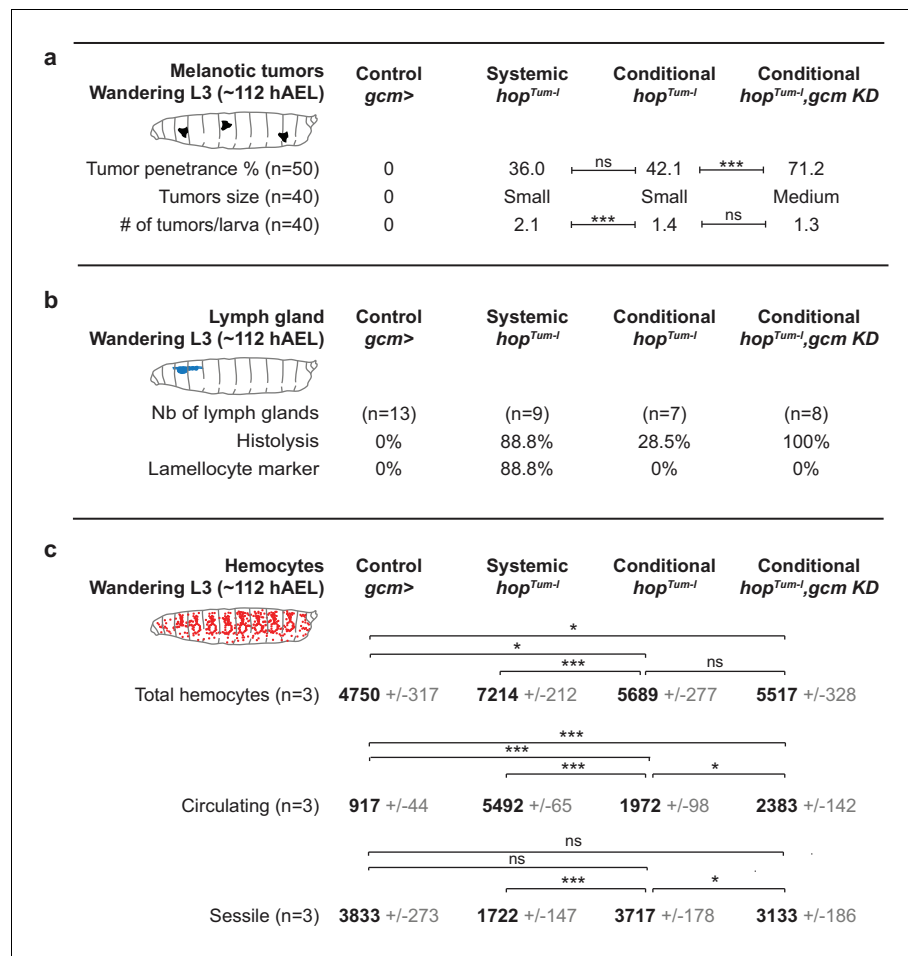


Figure 5. Inflammatory response upon systemic and conditional Jak/Stat activation. (a) Tumor penetrance, phenotype expressivity assessed as number of tumors/larva and tumor size of control animals (*gcm>*), systemic *hop^{Tum-I}*, conditional *hop^{Tum-I}* (*gcm> UAS-hop^{Tum-I}*) and conditional *hop^{Tum-I},gcm KD* (*gcm(hemo)> gcm KD,UAS-hop^{Tum-I}*). The distribution of the tumor size is detailed in **Figure 5—figure supplement 1b**. (b) Precocious lymph gland histolysis and lamellocyte labelling, note the 100% histolysis in conditional *hop^{Tum-I},gcm KD*. (c) Total number of hemocytes (circulating +sessile), number of circulating hemocytes and number of sessile hemocytes in systemic and conditional *hop^{Tum-I}* mutations as compared to controls. n = 3, the numbers in gray indicate s.e.m. DOI: <https://doi.org/10.7554/eLife.34890.012>

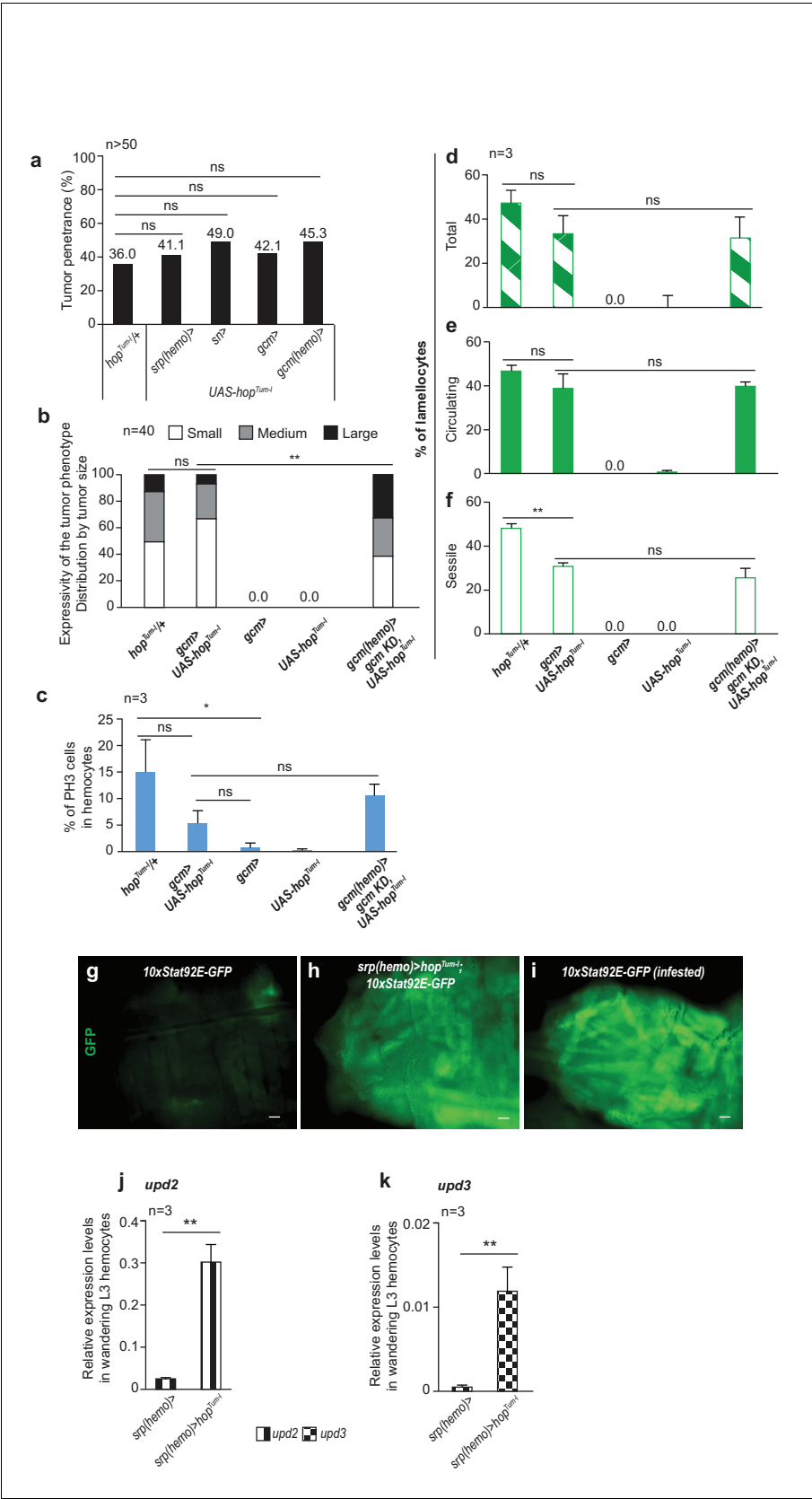


Figure 5—figure supplement 1. Phenotypes induced by conditional activation of the Jak/Stat pathway in the embryonic hemocytes. (a) Tumor penetrance in conditional *hop^{Tum-I}* animals (*UAS-hop^{Tum-I}*) using *srp(hemo)>* ,
Figure 5—figure supplement 1 continued on next page

Figure 5—figure supplement 1 continued

sn>, *gcm>* and *gcm(hemo)>* drivers as compared to *hop^{Tum-1}/+* ($n > 50$). (b) Tumor expressivity assessed as tumor size ($n = 40$). (c) Fraction of PH3 positive (dividing) cells in circulating and sessile compartment in the systemic and conditional *hop^{Tum-1}* mutations as compared to controls ($n = 3$). (d–f) Percentage of lamellocytes in whole hemocyte population (circulating + sessile, Total) (d), circulating (e) and sessile (f) compartments in the mentioned genotypes ($n = 3$). (g–i) 10XStat92E-GFP reporter intensity in somatic muscles. (j–k) Relative expression levels of *upd2* (j) and *upd3* (k) in hemocytes from wandering L3 hemocytes *srp(hemo)>* and *srp(hemo)> hop^{Tum-1}*, $n = 3$.

DOI: <https://doi.org/10.7554/eLife.34890.013>

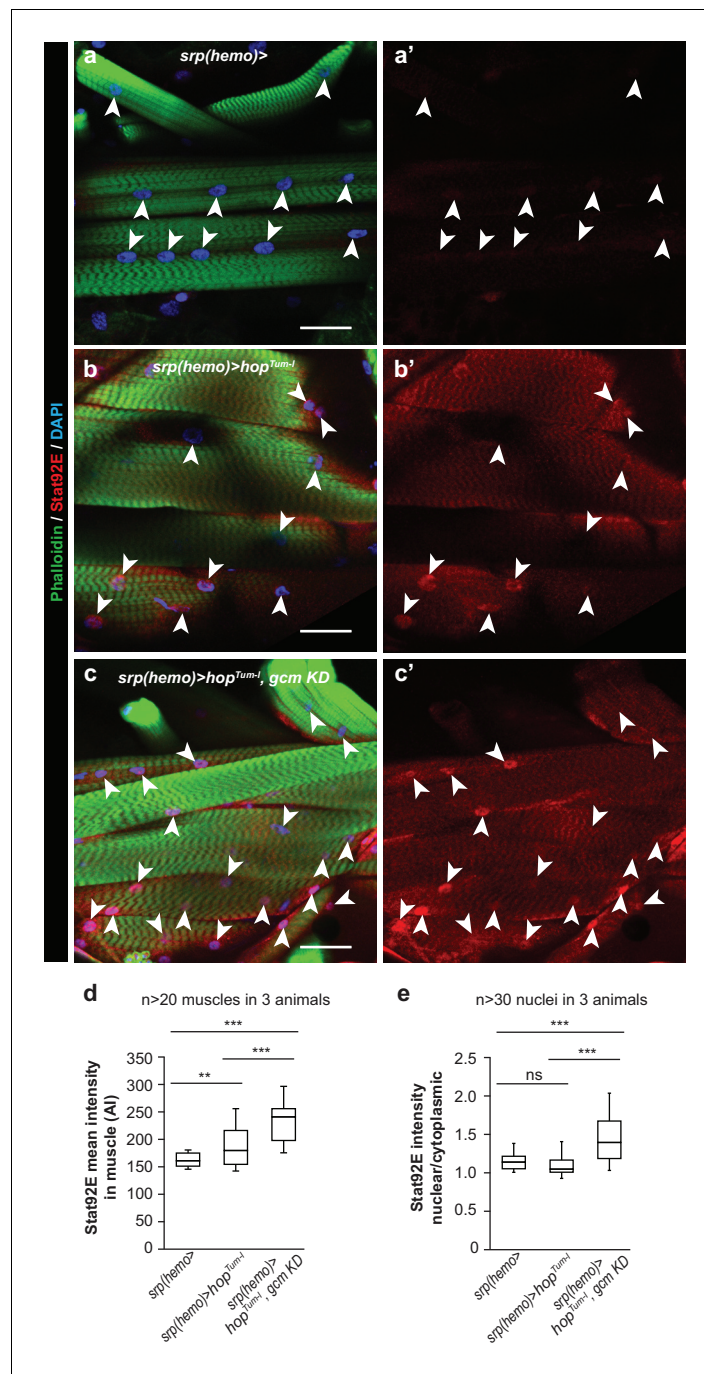


Figure 6. Jak/Stat activation in the embryonic hemocytes activates the Jak/Stat pathway in Wandering L3 muscles. (a–c) Muscle immunolabelling in Wandering larvae of the indicated genotypes. The Phalloidin labelling (in green) highlights the striated muscle fibers rich in actin filament, Stat92E is in red and DAPI in blue. (a,b,c) show the merge of the three channels, (a',b',c') show Stat92E alone. Scale bar: 50 μ m. Arrowheads indicate nuclei in the muscles. (d,e) Box-plots representing the distribution of Stat92E intensity (d) and the ratio nuclear/cytoplasmic Stat92E (e) in Wandering L3 muscles of the indicated genotypes. Stat92E intensity was quantified in more than 20 muscles in three animals and the ratio nuclear/cytoplasmic Stat92E was measured in more than 30 nuclei in three animals for each genotype. From bottom to top, the boxes represent the 5% percentile, the first quartile, the median, the third quartile and the 95% percentile. P-values were measured using student test after variance analysis.

DOI: <https://doi.org/10.7554/eLife.34890.014>

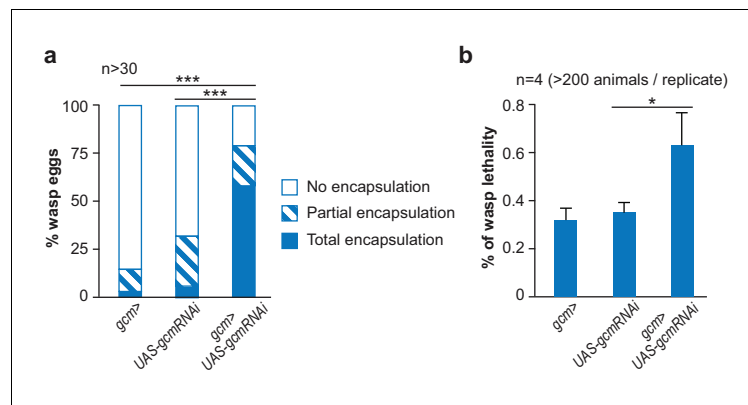


Figure 7. The inflammatory response upon wasp infestation is exacerbated in *gcm* *KD* animals. **(a)** Histogram representing the percentage of total, partial and no wasp egg encapsulation ($n > 30$). **(b)** Lethality of the parasitic wasp after infestation of *Drosophila* larvae ($n = 4$) (> 200 animals).

DOI: <https://doi.org/10.7554/eLife.34890.015>

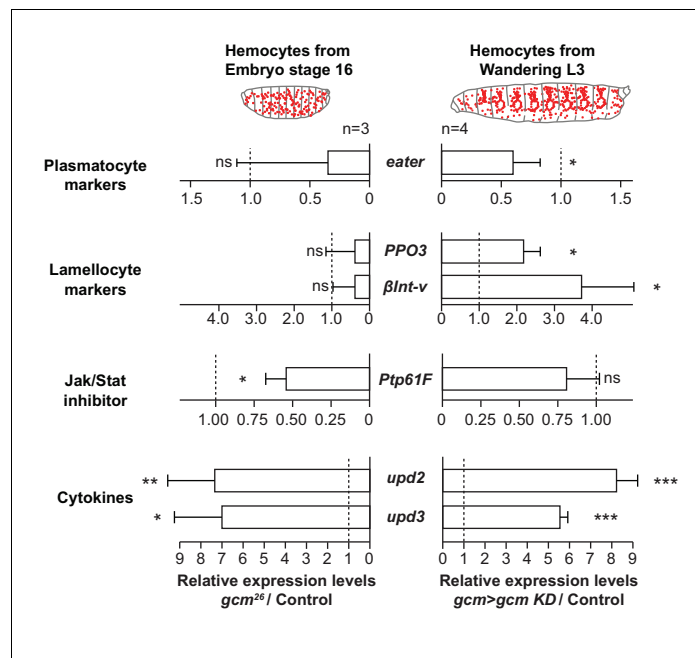


Figure 8. *Gcm* impacts the development and the inflammatory response of hemocytes. Relative expression levels of plasmotocyte and lamellocyte markers, Jak/Stat inhibitor and cytokines in hemocytes from stage 16 mutant embryos (*srp(hemo)> RFP,gcm* [Cattenoz et al., 2016]) or from third instar mutant larvae (*gcm> gcm KD*). The levels represented are relative to the levels in controls (*srp(hemo)> RFP* for the embryos and *gcm>* for the larvae) after normalization against housekeeping genes. The data are displayed as described in **Figure 1f**.

DOI: <https://doi.org/10.7554/eLife.34890.016>

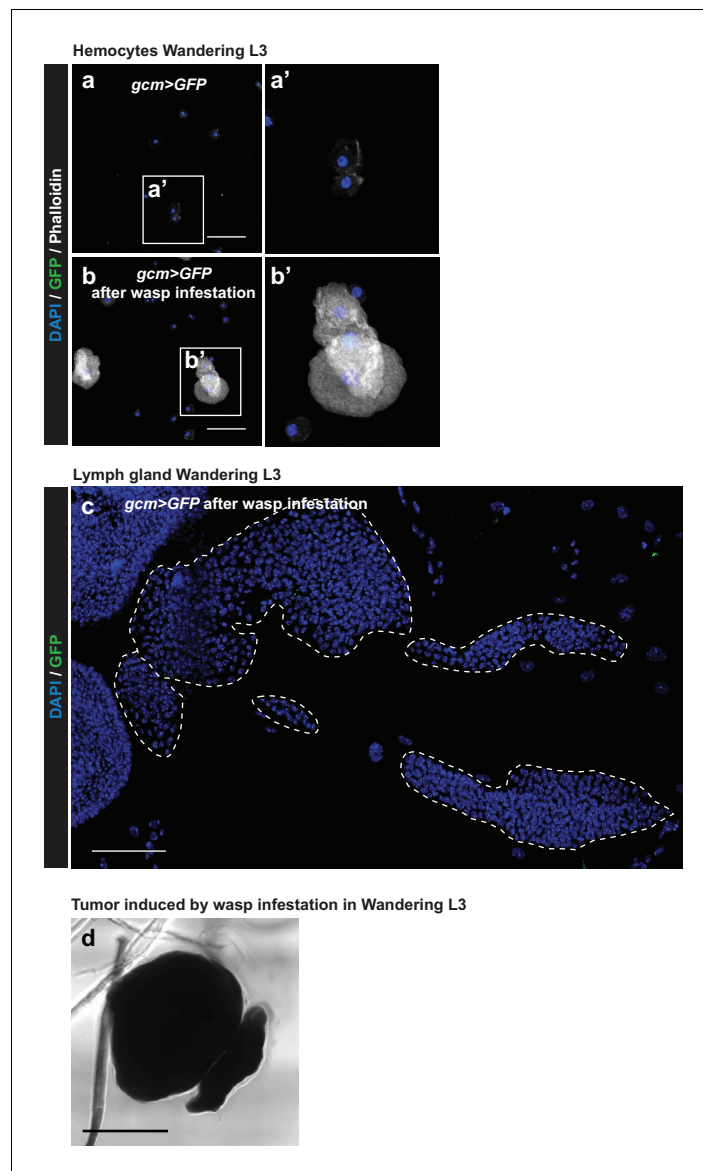


Figure 8—figure supplement 1. *Gcm* is not induced in circulating hemocytes nor in lymph glands of 3rd instar larvae upon wasp infestation. Immunolabelling of hemocytes from third instar larvae *gcm> GFP* without (a) or after wasp infestation (b). (DAPI in blue, GFP in green and Phalloidin in gray). (c) Immunolabelling of the lymph gland from third instar larva *gcm> GFP* after wasp infestation. (DAPI in blue, GFP in green). (d) Bright-field image of the melanised tumor induced by wasp infestation in *gcm> gtr* LacZ Wandering L3 shown in **Figure 9e**. Scale bar: 100 μ m.

DOI: <https://doi.org/10.7554/eLife.34890.017>

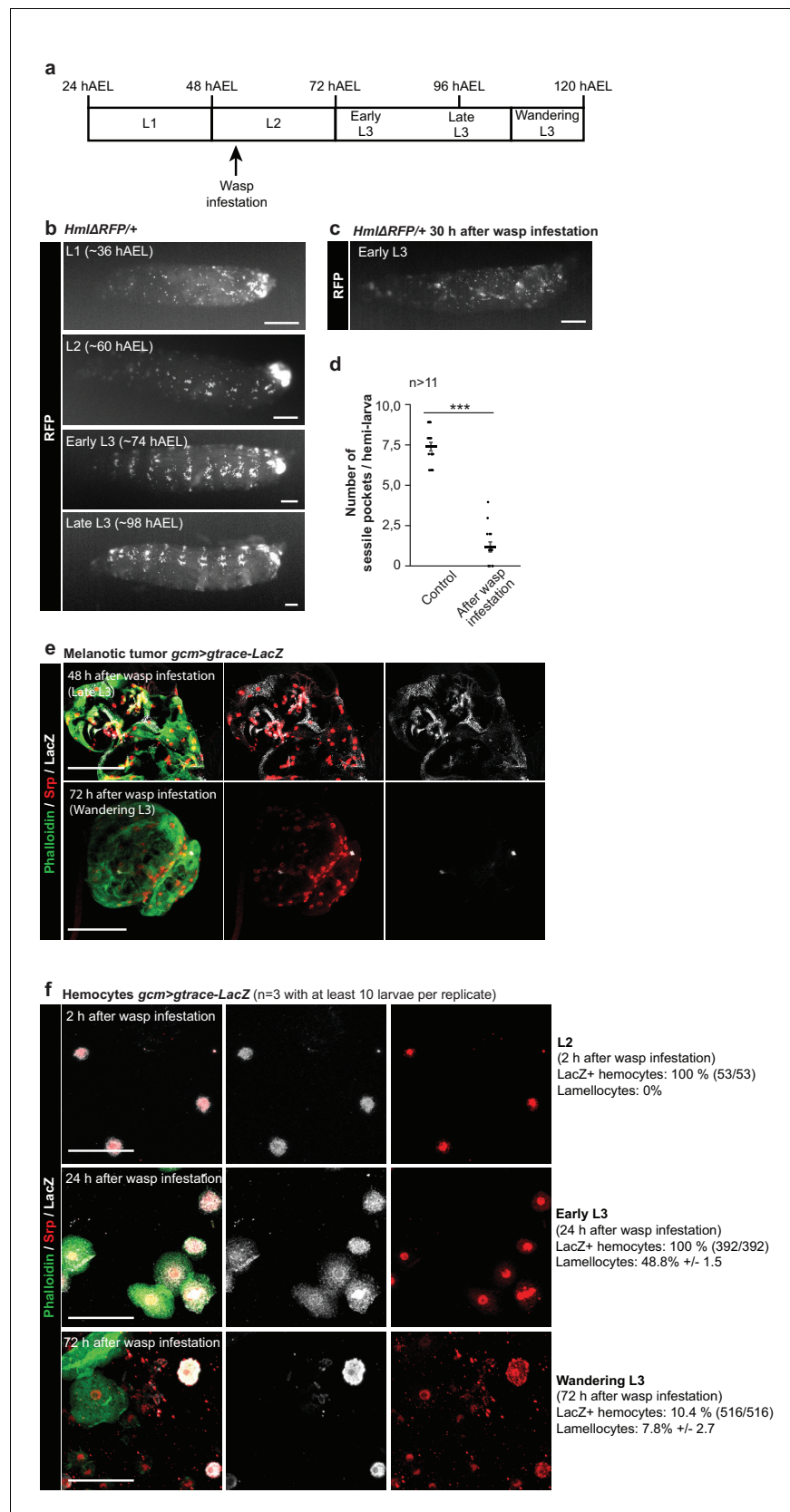


Figure 9. Impact of the wasp infestation on the hemocytes. (a) Schematic of the correspondence between the timeline after egg laying (AEL) and the larval developmental stage. The wasp infestation was carried out at the L2

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Figure 9 continued

stage. (b–c) RFP signals from *HmlΔRFP*/+ larvae at L1, L2, Early L3 and Late L3 stages (b) and Early L3 after wasp infestation (c). The *HmlΔRFP* transgene induces the expression of RFP in hemocytes starting from L1 (Makhijani et al., 2011). The larvae display an accumulation of hemocytes in the sessile pockets from the L2 stage onward. Upon wasp infestation, the sessile hemocytes are released into circulation. Scale bar: 200 μm. (d) Number of sessile pockets per Late L3 larva (one lateral view per larva = hemi larva) in normal conditions and after wasp infestation. Each hemi-larva is represented by a dot, the bar represents the average and the error-bars s.e.m., $n > 11$, p-value measured using student test after variance analysis. (e) Representative examples of melanotic tumors at different stages. Immunolabelling of tumors from *gcm > gtrace LacZ* larvae, 48 hr and 72 hr after wasp infestation. Phalloidin (green), Srp (red) and LacZ (white). Left panels show the merged channels, mid panels show LacZ and Srp labelling and the right panels show LacZ labelling alone. Scale bar: 100 μm. (f) Immunolabelling of *gcm > gtrace LacZ* larval hemocytes 2 hr, 24 hr and 72 hr after wasp infestation. Phalloidin (green), Srp (red) and LacZ (white). The left panels show the merged channels, the mid panels show LacZ labelling alone and the right panels show Srp labelling alone. Lamellocytes can be recognized by the strong expression of Phalloidin and by their very large size. Scale bar: 50 μm.

DOI: <https://doi.org/10.7554/eLife.34890.018>

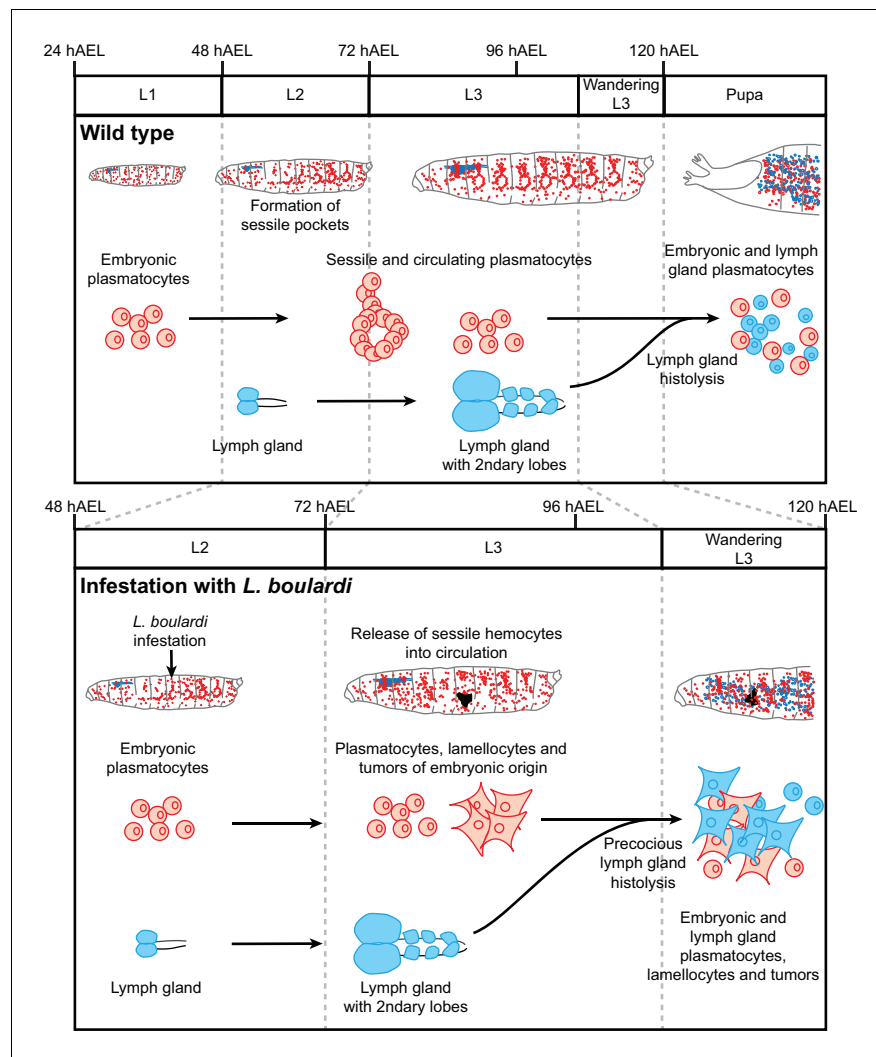


Figure 10. Wasp infestation recruits first the embryonic hemocytes and then the LG hemocytes. Timeline summarizing the sequence of events occurring in the immune system of the larva in normal conditions (top panel) and upon wasp infestation (lower panel). In normal conditions, only embryonic hemocytes (in red) are present in the hemolymph until the pupal stage. They start to colonize the sessile pockets by L2 and continue to do so during L3. The lymph gland (in blue) histolyzes at the beginning of pupal stage to release plasmatocytes into circulation. Upon wasp infestation (occurring at L2 stage), the sessile hemocytes are mobilized, embryonic plasmatocytes differentiate into lamellocytes (rhomboid shaped cells) and tumors start forming at Late L3 upon aggregation of the embryonic hemocytes. The lymph gland histolyzes precociously, to release plasmatocytes and lamellocytes that contribute to tumor formation in Wandering L3. Plasmatocytes and lamellocytes originating from the embryo are indicated in red, the ones from the lymph gland in blue. Lamellocytes originating from the embryo are indicated in red, the ones from the lymph gland are in blue.

DOI: <https://doi.org/10.7554/eLife.34890.019>

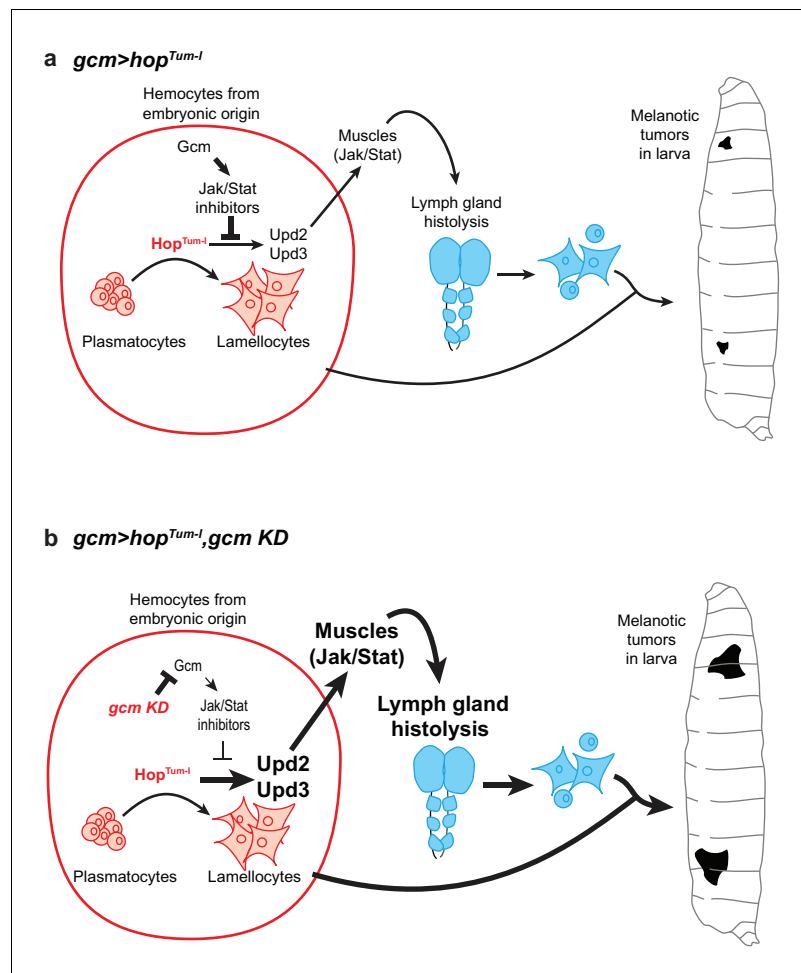


Figure 11. Schematic model on the molecular cascade leading to tumor formation upon Jak/Stat activation in the embryonic hemocytes. (a) *gcm>hop^{Tum-1}* activates the Jak/Stat pathway exclusively in the hemocytes at embryonic stage. This leads to the production of the Upd2/Upd3 pro-inflammatory cytokines in those cells and to the activation of the Jak/Stat pathway in the larval muscles. This ultimately results in the formation of melanotic tumors and in lymph gland histolysis. When *gcm>hop^{Tum-1}* is combined with *gcm KD* (b), the levels of the inhibitors of the Jak/Stat pathway controlled by Gcm (i.e. Ptp61F, Socs36E and Socs44A) decrease in the embryonic hemocytes. This leads to a stronger activation of the Jak/Stat pathway in embryonic hemocytes, which enhances the production of Upd2/Upd3 and the activation of the Jak/Stat pathway in the muscles. As a consequence, *gcm>hop^{Tum-1}, gcm KD* animals display stronger phenotypes in terms of lymph gland histolysis and melanotic tumors.

DOI: <https://doi.org/10.7554/eLife.34890.020>