

1 **Blood cells of adult *Drosophila* do not expand, but control survival after bacterial infection**
2 **by induction of *Drosocin* around their reservoir at the respiratory epithelia**

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24 **Summary**

25 *Drosophila melanogaster* has been an excellent model for innate immunity, but the role and regulation
26 of adult blood cells and organismal immunity have remained incompletely understood. Here we
27 address these questions in a comprehensive investigation of the blood cell system in adult *Drosophila*.
28 As a central finding, we reveal the largest reservoir of blood cells (hemocytes) at the respiratory
29 epithelia (tracheal air sacs) and fat body of the thorax and head. We show that most hemocytes of adult
30 *Drosophila* are phagocytic macrophages (plasmatocytes), derived by more than 60% from the
31 embryonic lineage that parallels vertebrate tissue macrophages. Surprisingly, in contrast to hemocytes
32 at the larval stage, we find no capacity of the adult blood cell system to expand. Instead, we
33 demonstrate its central role in relaying an innate immune response to tissues surrounding the blood cell
34 reservoir: Hemocytes, through Imd signaling and the Jak/Stat pathway ligand Upd3, act as sentinels of
35 bacterial infection that induce expression of the antimicrobial peptide gene *Drosocin* in the respiratory
36 epithelia and colocalizing domains of the fat body. We demonstrate that endogenous *Drosocin*
37 expression in these tissues promotes animal survival after bacterial infection. Our work identifies the
38 first molecular step in a new relay of organismal immunity, establishing adult *Drosophila* as model to
39 dissect mechanisms of inter-organ immunity.

40

41 **Keywords**

42 *Drosophila melanogaster*; hemocyte; macrophage; innate immunity; local humoral immune response;
43 antimicrobial peptide; *Drosocin*; NFκB; *imd*; Jak/Stat; *upd3*; respiratory epithelia; tracheal air sacs; fat
44 body; hematopoiesis

45

46 **Introduction**

47 *Drosophila melanogaster* has greatly promoted our understanding of innate immunity and blood cell
48 development, but the capacity of the adult animal as a model remains a matter of debate. Most studies
49 reported a lack of new blood cell production (Lanot et al., 2001; Mackenzie et al., 2011; Woodcock et
50 al., 2015) and immunosenescence (Felix et al., 2012; Mackenzie et al., 2011), while a recent
51 publication claimed continued hematopoietic activity in adult *Drosophila* (Ghosh et al., 2015).

52 *Drosophila* blood cells, or hemocytes, emerge from two lineages that persist into adult life, showing
53 parallels with the two myeloid systems in vertebrates (Gold and Brückner, 2014, 2015; Holz et al.,
54 2003). First, hemocytes originating in the embryo parallel vertebrate tissue macrophages, as they
55 quickly differentiate into plasmatocytes (macrophage-like cells), and subsequently proliferate
56 extensively, mainly in the hematopoietic pockets (HPs) of the larva (Gold and Brückner, 2014, 2015;
57 Makhijani et al., 2011; Makhijani and Brückner, 2012). At least some embryonic-lineage
58 plasmatocytes can further differentiate into other blood cell types such as crystal cells and, under
59 immune challenge, lamellocytes (Bretscher et al., 2015; Gold and Brückner, 2015; Leitao and Sucena,
60 2015; Makhijani et al., 2011; Markus et al., 2009). Second, hemocytes originating in the lymph gland
61 (LG) also give rise to plasmatocytes, crystal cells and lamellocytes, yet in the lymph gland they are
62 predominantly generated via differentiation from blood cell progenitors (prohemocytes) (Banerjee et
63 al., 2019; Gold and Brückner, 2015; Jung et al., 2005; Letourneau et al., 2016). At the beginning of
64 metamorphosis, hemocytes from both the hematopoietic pockets and the lymph gland enter the open
65 circulatory system, leading to intermixing of the two blood cell lineages. (Gold and Brückner, 2015;
66 Grigorian et al., 2011; Lanot et al., 2001; Makhijani et al., 2011). The subsequent fate and capacity of
67 the adult blood cells is an ongoing matter of debate. To clarify this question, we dedicated the first part
68 of our study to comprehensively investigate the hematopoietic capacity of the blood cell system in
69 adult *Drosophila*.

70 For the second part of the study, we focused on the role of the adult blood cell pool in the humoral
71 immune response, identifying a new system of organismal innate immunity in *Drosophila*. Historically,
72 *Drosophila* has been instrumental in the discovery of innate immunity and Toll like receptor (TLR)
73 signaling (Lemaitre and Hoffmann, 2007). Toll- and the related Immune Deficiency (Imd) signaling,
74 are evolutionary conserved NF κ B family pathways that have been studied in detail regarding their
75 upstream activation by pathogens and other inputs, and downstream signal transduction components
76 and mechanisms (Lemaitre and Hoffmann, 2007). Targets include antimicrobial peptides (AMPs),
77 which have been investigated for their transcriptional gene regulation and functional properties
78 (Lemaitre and Hoffmann, 2007; Zasloff, 2002). TLR signaling has been well established also in
79 vertebrate systems for its roles in infection and inflammation (Beutler, 2009; Kopp and Medzhitov,
80 1999; Takeda and Akira, 2005). However, despite the detailed knowledge of TLR signaling and innate
81 immunity at the cellular and molecular level, it has been far less understood how multiple tissues or
82 organs communicate with each other to elicit local innate immune responses.

83 Addressing these two major questions, our study comprehensively illuminates basic principles of the
84 blood cell system in adult *Drosophila* and its role in multi-tissue organismal immunity. We identify an
85 extensive, previously unknown blood cell reservoir at the respiratory epithelia and fat body, investigate
86 its dynamics, and probe for various signs of hematopoiesis. We demonstrate a key role of adult blood
87 cells as sentinels of bacterial infection that trigger a humoral response in their reservoir, i.e. the
88 respiratory epithelia and colocalizing domains of the fat body. This response culminates in the
89 expression of the AMP gene *Drosocin*, which we show is significant for animal survival after bacterial
90 infection. We identified a requirement for Imd signaling and Upd3 expression in hemocytes as a first
91 molecular step in this relay of organismal immunity, laying the foundation for the use of adult
92 *Drosophila* to dissect additional mechanisms of multi-tissue innate immunity in the future.

93

94 **Results**

95 ***The respiratory epithelia are the largest reservoir of blood cells in adult Drosophila***

96 To investigate the blood cell system in adult *Drosophila*, we started out by examining the anatomical
97 sites of hemocytes. We visualized blood cells by fluorescent labeling with macrophage (plasmatocyte)-
98 specific *Hemolectin HmlΔ-GAL4* (Sinenko and Mathey-Prevot, 2004) driving *UAS-GFP*, or direct
99 reporters *HmlΔ-DsRed* or *HmlΔDsRednls* (Makhijani et al., 2011). To gain an unbiased overview of
100 hemocyte locations throughout the animal, we took a cryosectioning approach. In addition, we imaged
101 hemocytes through the cuticle of whole flies. Hemocytes are largely resident (sessile), and show
102 consistent enrichment in specific areas. Surprisingly, we found that the largest pool of hemocytes
103 colonizes the respiratory epithelia, in particular the extensive air sacs of the thorax and head (Fig. 1A-
104 E). This location was identified in our cryosections by comparison with anatomical features of the
105 respiratory epithelia (Manning and Krasnow, 1993; Whitten, 1957), and the unique blue
106 autofluorescence of the respiratory epithelia when exposed to UV light (Kim et al., 2012). Localization
107 of hemocytes with the respiratory epithelia was further confirmed by colabeling with the tracheal
108 driver *btl-GAL4* expressing *UAS-GFP* (Guha and Kornberg, 2005) (Fig. 1D, Suppl.Fig. 1C). In intact
109 flies, hemocyte localization at the respiratory epithelia is visible around the eyes and posterior head,
110 and in the thorax laterally, and dorsally near the wing hinges and scutellum (Suppl. Fig. 1A,C,D).

111 Consistent with previous reports (Dionne et al., 2003; Elrod-Erickson et al., 2000; Ghosh et al., 2015),
112 we also saw a smaller fraction of hemocytes surrounding the heart (Suppl.Fig. 1A,B,D), accumulating
113 in clusters at the ostia (Suppl. Fig.1B), which are the intake valves of the heart toward the open
114 circulatory system. Quantification of hemocytes released from flies split into two parts, at the boundary
115 of the thorax and abdomen, confirmed that the majority of hemocytes in adult *Drosophila* is located in
116 the head and thorax (see Fig. 3F). Overall we conclude that, in adult *Drosophila*, the respiratory

117 epithelia of the head and thorax provide the major reservoir of blood cells, which is distinct from
118 smaller clusters of hemocytes at the ostia of the heart.

119 ***Hemocytes relocate during maturation of adult *Drosophila****

120 Next we investigated the developmental timing of hemocyte localization to the respiratory system and
121 heart. Newly eclosed *Drosophila* expressing a fluorescent plasmatocyte reporter show a diffuse glow
122 in live imaging, which over the following 5-7 days develops into a more defined hemocyte pattern (Fig.
123 2B-C); in these mature adults, hemocytes then remain rather stationary over time (Suppl. Fig2A-C and
124 (Woodcock et al., 2015)). While the visual change of hemocytes in young adults may suggest an
125 increase in total hemocyte numbers, we actually found a different process to be underlying this
126 phenomenon. Specifically, we discovered a major redistribution of hemocytes within the first days of
127 adult life. Dissection and lipid dye staining of newly eclosed adults illustrates that hemocytes are
128 attached to dissociated larval fat body cells (Fig. 2D) (Nelliot et al., 2006), forming a large mass
129 throughout the abdomen and other parts of the fly (Fig. 2E). Around 5-7 days into adult life, cytolysis
130 of the larval fat body cells is completed, allowing hemocytes to relocate to resident sites at the
131 respiratory system and heart (Fig. 2F). Therefore, counting local hemocytes, e.g. in the heart area,
132 gives the false impression of an increase in blood cells during the first days of adult life (Fig. 2E-F
133 dashed box). An impression of increasing blood cell numbers is also given when counting
134 fluorescently labeled hemocytes that can be visually recognized through the external cuticle (Fig. 2G).
135 In contrast, when assessing hemocytes in cryosections (Fig. 2E-F), or quantifying total hemocytes from
136 dissected adult flies, we discovered a continuous decline in hemocytes over various time points, even
137 during the first week of adult life (Fig. 2H and (Woodcock et al., 2015)).

138 In summary, we find no evidence for a significant increase in total hemocyte numbers during adult
139 maturation. Instead, we observe a dramatic redistribution of existing hemocytes during the first week

140 of adult life; once larval fat body cells have cytolysed, hemocytes are free to move toward the
141 periphery to their final destinations, with the majority of hemocytes colonizing the respiratory epithelia.

142 ***Hemocytes do not expand after septic injury***

143 Next we investigated whether bacterial infection could have an effect on hemocytes of the respiratory
144 system and the heart (Fig. 3B, C). Injection of adult flies with gram-negative *Escherichia coli* (*E.coli*),
145 *Enterobacter cloacae* (*E. cloacae*), *Erwinia carotovora carotovora 15* (*Ecc15*), or gram-positive
146 *Micrococcus luteus* (*M. luteus*) resulted in increased hemocyte numbers in external counts 6 days post
147 infection (Fig. 3D). Surprisingly, we discovered that absolute hemocyte numbers, which were
148 quantified by blood cell release ex vivo, did not increase upon infection (Fig. 3E). Asking whether
149 hemocytes may redistribute upon infection, we assessed ex vivo blood cell counts from flies split into
150 2 parts consisting of head plus thorax, versus abdomen (Fig. 3F). Similar as in maturation, bacterial
151 immune challenge did not affect absolute hemocyte numbers in the two sections; likewise, there was
152 no effect by the site of injection in the thorax or abdomen (Fig. 3F). Tracking down what could be the
153 basis for the seemingly increased hemocyte appearance after infection, we found a rather unexpected
154 explanation. Using qPCR analysis, we saw that bacterial infection leads to transcriptional upregulation
155 of macrophage-specific *Hml* and other commonly used marker genes such as *croquemort* (*crq*) (Fig.
156 3G), a phenomenon that actually has been described before (De Gregorio et al., 2001; Franc et al.,
157 1999). This suggests that increased *Hml*Δ reporter fluorescence after bacterial infection likely accounts
158 for elevated GFP expression, increasing the number of hemocytes that can be visually recognized
159 through the cuticle, while absolute hemocyte counts are not raised.

160 To further address the role of the respiratory hemocyte reservoirs in infection, we examined the
161 dynamics of particle accumulation, injecting fluorescently labeled microbeads or *E. coli* bioparticles
162 (Molecular Probes/Invitrogen). Independent of the site of injection in the thorax or abdomen,
163 microbeads or bioparticles quickly accumulate along the respiratory epithelia of the thorax and head

164 (Fig. 3H-Q), and at the ostia of the heart (Fig. 3H,I), matching typical sites of hemocyte residence.
165 Comparable localization of bioparticles was observed under hemocyte ablation conditions (Suppl. Fig.
166 3B), suggesting that this accumulation pattern is independent of hemocytes or their phagocytic activity.
167 Hemocytes at the respiratory epithelia and other locations are capable of phagocytosis, as we
168 confirmed by ingestion of injected bioparticles marked with regular label (Fig. 3S) or pHrodo, a pH
169 dependent dye highly fluorescent in acidic compartments such as lysosomes following phagocytic
170 ingestion (Suppl. Fig. 3C). Fractions of pHrodo positive hemocytes were similar when released from
171 head, thorax or abdomen of split flies (Fig. 3R, T-V), suggesting comparable phagocytic activity and
172 similar ratios of hemocyte- and particle accumulation in all body parts.

173 In summary, we find major accumulation of foreign particles in the hemocyte reservoirs lining the
174 respiratory epithelia of *Drosophila*. This localization is independent of the presence of phagocytes,
175 suggesting passive transport through the hemolymph and physical retention in these areas. Bacterial
176 infection does not substantially change the overall localization of hemocytes, and does not cause
177 significant increase in the total number of hemocytes per animal. Instead, we find a substantial increase
178 in the expression of macrophage markers, which likely accounts for the impression of increased
179 hemocyte numbers as they appear through the cuticle.

180 ***The majority of adult hemocytes derive from the embryonic lineage***

181 Next we sought to determine the ontogenesis of the adult blood cell system. Blood cells in adult
182 *Drosophila* are known to derive from two lineages (Holz et al., 2003), the embryonic lineage that
183 parallels tissue macrophages, and the lymph gland lineages that parallels progenitor-based blood cell
184 production (Fig. 4A) (Banerjee et al., 2019; Gold and Brückner, 2014, 2015). To address their relative
185 contributions to the adult animal, we used a flipout-*lacZ* lineage tracing approach (Makhijani et al.,
186 2011; Weigmann and Cohen, 1999). In this approach, spatially and temporally controlled Flp
187 recombinase induces reconstitution and permanent expression of a *lacZ* reporter transgene, which can

188 be followed and compared relative to other cell markers. First, we used the method to mark early
189 embryonic hemocytes (under control of *srpHemo-Gal4*) and examined animals at the pupal and adult
190 stage (Fig. 4B-E). Indeed, this approach showed many *lacZ* positive hemocytes in the pupa (Fig.
191 4D,D') and in the adult, including hemocytes attached to larval fat body cells in newly eclosed flies
192 (Fig. 4E). We obtained similar results when we labeled differentiated embryonic-lineage plasmatocytes
193 in the young larva using *HmlΔ-GAL4* (Fig. 4F-I). The method also allowed to quantify the contribution
194 of embryonic-lineage hemocytes to the adult blood cell pool. Comparing the fraction of *lacZ* positive
195 cells among plasmatocytes in the adult, relative to those in the 3rd instar larva, we estimate that more
196 than 60% of adult macrophages originate from the embryonic lineage (Fig. 4I). This is surprising,
197 given the previously prevailing view that the majority of adult hemocytes would derive from the lymph
198 gland (Lanot et al., 2001). Based on our findings we expect that less than 40% of adult hemocytes
199 derive from the lymph gland lineage, although we can only infer this contribution as the relatively
200 weak expression of early lymph gland GAL4 drivers left our lineage tracing attempts of the lymph
201 gland unsuccessful.

202 Taken together, we conclude that the majority of adult blood cells derive from the embryonic
203 hemocyte lineage that proliferates in the larva and resembles tissue macrophages in vertebrates (Gold
204 and Brückner, 2014, 2015; Makhijani et al., 2011; Makhijani and Brückner, 2012).

205 ***Adult Drosophila show no signs of new hemocyte production***

206 Given that adult hemocytes originate from embryonic-lineage hemocytes, which retain the ability to
207 proliferate as macrophages in the larva (Makhijani et al., 2011), and the lymph gland hemocyte lineage,
208 which may comprise undifferentiated progenitors with putative proliferation potential that arise in the
209 posterior lobes (Grigorian et al., 2011; Jung et al., 2005), we wanted to investigate the proliferative
210 capacity and differentiation status of adult hemocytes. First we used in vivo EdU (5-ethynyl-2'-
211 deoxyuridine) incorporation into newly synthesized DNA to detect proliferation of macrophages and

212 their putative progenitors. Labeling all EdU-incorporating cells over a continuous time period of two
213 weeks and examining the entire pool of adult hemocytes *ex vivo*, we surprisingly did not find any EdU
214 positive hemocytes (Fig. 5A). Fragments of other tissues that are side products of the hemocyte release
215 method harbored EdU positive cells, serving as positive control (Fig. 5A). To explore the possibility of
216 hemocyte proliferation upon immune challenge, we examined adults following natural infection
217 (feeding) with the gram-negative bacterium *Serratia marcescens* (*S. marcescens*), or septic injury
218 using gram-negative *E. coli*, *Ecc15*, or gram-positive *M. luteus*. However, even under conditions of
219 infection we did not detect any EdU positive hemocytes (Fig. 5A). This suggests that neither
220 differentiated macrophages, nor any potentially unlabeled progenitors giving rise to macrophages,
221 proliferate under the tested conditions. Some hemocytes carry EdU positive cellular inclusions, which
222 however result from phagocytosis of other EdU-incorporating polyploid or proliferative cells, rather
223 than from hemocyte proliferation *per se* (Suppl.Fig. 4G-I).

224 We employed numerous other methods to detect proliferation of plasmatocytes or their progenitors.
225 Using a hemocyte-specific two-color Fucci cell cycle indicator line, we found no Fucci S/G2/M green-
226 positive hemocytes in adult flies (Fig. 5C-F'), in contrast to proliferating larval hemocytes that served
227 as positive control (Fig. 5B-B'). Likewise, hemocyte MARCM (Mosaic analysis with a repressable cell
228 marker) (Lee and Luo, 1999; Makhijani et al., 2011), which labels all dividing cells that eventually
229 would give rise to hemocytes, resulted only in minimal numbers of MARCM positive hemocytes; rare
230 events detected could simply be due to the high background labeling seen with this assay under all
231 induction conditions (Suppl.Fig. 4B, C) and in other systems (von Trotha et al., 2009). Lastly,
232 PermaTwin labeling, a MARCM variant designed to detect all dividing cell progeny (Fernandez-
233 Hernandez et al., 2013), did not produce any labeled hemocytes over induction periods of up to 3
234 weeks, as we determined by external imaging of intact flies and hemocyte releases. As expected, the
235 method generated many positive cells in control tissues such as the gut (Suppl.Fig. 4D, E).

236 We also examined adult hemocytes for putative hemocyte progenitors. We focused on Srp, a GATA
237 factor required for prohemocyte specification in the embryo (Rehorn et al., 1996), which was recently
238 proposed as progenitor marker in the adult fly (Ghosh et al., 2015). We examined Srp-positive
239 hemocytes in the adult using a Srp antibody or *srp-GAL4* driver, both of which labeled very similar,
240 overlapping populations of hemocytes in young and more mature adults (Suppl.Fig. 4F). Surprisingly,
241 we found that most Srp-positive hemocytes in the adult show hallmarks of phagocytosis, including
242 internal phagocytic vesicles and the ability to phagocytose experimentally injected fluorescent
243 microbeads (Fig. 5I-M). In maturing adults between day 3 and 11 after eclosion, an increasing number
244 of Srp-positive hemocytes also gained expression of the plasmatocyte marker *HmlΔDsRednls* (Fig. 5G,
245 H). However, even single positive Srp-only hemocytes are largely capable of ingesting injected beads
246 (Fig. 5J, L), similar to *Hml* positive or double positive cells (Fig. 5K,M).

247 We conclude that, in the adult fly, Srp is not a marker for hemocyte progenitors. Overall, we find no
248 indication that the adult blood cell system has significant hematopoietic capacity. Instead, we observe
249 mainly active macrophages and a continuous decline of blood cell numbers in adult animals. Our
250 model proposes that the adult blood cell pool results from hemocyte expansion in the larva, and over
251 time diminishes without any significant new blood cell production (Fig. 5N).

252 ***Hemocytes, through cell-autonomous Imd signaling, are required for an infection-induced Drosocin*** 253 ***response***

254 After proving that the adult blood cell population does not expand upon infection, we investigated
255 whether the anatomical arrangement of hemocytes at the respiratory epithelia has consequences for
256 humoral immunity. Comparing hemocyte-ablated and control animals, we examined the transcriptional
257 induction of several antimicrobial peptide (AMP) genes following bacterial infection. Hemocyte
258 ablation was achieved by expression of the proapoptotic genes *reaper* (*rpr*) and *head involution defect*
259 (*hid*) (Arefin et al., 2015; Bergmann et al., 1998; Charroux and Royet, 2009; Defaye et al., 2009;

260 White et al., 1996) and confirmed through live imaging of larvae and adults and quantification of
261 released hemocytes (Suppl.Fig. 5A,B). *E. coli* was selected for these infections, owing to its common
262 use as model for gram-negative infection in similar *Drosophila* studies (Ghosh et al., 2015; Lemaitre
263 and Hoffmann, 2007). Following septic injury in the absence of hemocytes, *Drosomycin* showed little
264 change, and *Cecropin A1* showed increased expression within the first hours of infection (Fig. 6A,B).
265 However, expression of three AMPs was consistently reduced under hemocyte-ablated conditions, in
266 particular *Drosocin* and, to a lesser extent, *Attacin A* and *Diptericin*, suggesting that their induction
267 depends on hemocytes (Fig. 6C-E).

268 Focusing on *Drosocin* expression as a paradigm, we asked whether TLR immune signaling in
269 hemocytes is required for the infection-induced *Drosocin* response. We examined requirement for the
270 NF κ B-related Imd signaling pathway for detection and response to gram-negative bacteria by RNAi
271 silencing of pathway components in hemocytes. Indeed, constitutive *imd* knockdown in hemocytes
272 dramatically reduced *Drosocin* expression following gram-negative infection at various time points
273 post infection (Suppl.Fig. 5C,D). Similar results were obtained when we silenced *imd* only transiently
274 before infection (Fig. 6F), ruling out that a general shift in the immune status (Arefin et al., 2015)
275 would cause the observed phenotype. Likewise, hemocyte-specific knockdown of the upstream
276 peptidoglycan receptor *PGRP-LC* substantially reduced *Drosocin* induction (Fig. 6G), suggesting that
277 recognition of bacterial cell wall components such as DAP-type peptidoglycans is required to trigger
278 the response (Kaneko et al., 2006). Overexpression of *imd*, which leads to pathway activation (Georgel
279 et al., 2001), mildly enhanced *Drosocin* expression after sterile and septic injury (Fig. 6H, Suppl.Fig.
280 5E) consistent with the Imd pathway in hemocytes being required, albeit not sufficient, for the
281 response.

282 We conclude that, in response to gram-negative infection, hemocytes are essential players in the
283 induction of humoral immunity and the expression of *Drosocin*. Signaling through the PGRP-LC/Imd
284 pathway in hemocytes is required, albeit not sufficient, for the *Drosocin* response.

285 ***Hemocytes are sentinels of infection that induce Drosocin expression in the respiratory epithelia***
286 ***and fat body***

287 Next we examined the sites of *Drosocin* expression in the adult fly. Using a *Drosocin-GFP* transgenic
288 reporter (Tzou et al., 2000), we found a unique expression pattern in the thorax and head of infected
289 flies that strikingly matched locations of the hemocyte reservoir at the respiratory epithelia (Fig. 7A-C).
290 This *Drosocin-GFP* pattern was independent of the site of bacterial injection in the thorax or abdomen
291 of the animal (Suppl.Fig. 6A,B). We confirmed the localized *Drosocin* expression by qPCR analysis
292 on infected flies split into two parts, one containing head and thorax, and the other consisting of
293 abdomen only (Fig. 7D). Dissection revealed that *Drosocin-GFP* is expressed in the respiratory
294 epithelia and in adjacent domains of the fat body, which covers the respiratory epithelia and occupies
295 the space toward the cuticle of the fly (Fig. 7F-F'', see also Fig. 7J-K'''). This restriction to specific fat
296 body domains was particularly obvious when comparing the *Drosocin-GFP* pattern to the overall
297 expression of a fat body driver (Fig. 7E). No apparent expression of *Drosocin-GFP* was detected in
298 hemocytes in vivo or ex vivo (Fig. 7B,F and data not shown).

299 In order to further confirm the contribution of each tissue to the overall *Drosocin* response, we
300 performed tissue specific RNAi knockdowns of *Drosocin* and analyzed the levels of total *Drosocin*
301 expression by qPCR of whole flies. Silencing of *Drosocin* in the respiratory system shows partial
302 contribution to the total *Drosocin* expression (Fig. 7H). Silencing of *Drosocin* in fat body
303 demonstrated that the major contribution comes from this tissue (Fig. 7I), consistent with the observed
304 high levels of *Drosocin-GFP* expression in fat body, and its established role as major site of AMP
305 expression. In contrast, *Drosocin* silencing in hemocytes caused little to no reduction in overall

306 *Drosocin* levels, again confirming that hemocytes themselves are not a significant source of total
307 *Drosocin* expression in the fly (Fig. 7G). Overall *Drosocin* knockdown efficiency was close to 100%
308 using a ubiquitous driver (Suppl.Fig. 6D).

309 Considering that hemocytes are required for *Drosocin* induction, yet *Drosocin* is predominantly
310 expressed in domains of the fat body and the respiratory epithelia, we hypothesized that hemocytes
311 might be required as sentinels of infection that signal through some molecular mechanism to the
312 *Drosocin*-expressing tissues. Cryosections of whole adult animals and head dissections show that
313 hemocytes tightly colocalize with, and are layered in between, the respiratory epithelia and fat body,
314 suggesting an interface that facilitates signaling communications among the tissues (Fig. 7J-K''',
315 Suppl.Fig. 6C,C'). Hypothesizing communication via a secreted factor, we tested potential candidate
316 genes of secreted signaling molecules by RNAi and overexpression. One gene that stood out was
317 *unpaired 3 (upd3)* (Agaïsse et al., 2003), a ligand of the Jak/Stat pathway in *Drosophila*. Hemocyte
318 specific knockdown of *upd3* strongly reduced *Drosocin* expression after gram-negative infection (Fig.
319 7L), suggesting that Upd3 is a required hemocyte produced signal in the communication to the
320 respiratory epithelia and fat body. Conversely, *upd3* overexpression mildly enhanced the response
321 under conditions of sterile and septic injury (Fig. 7M), resembling the effect of *imd* overexpression
322 (see Fig. 6H) and suggesting that *upd3* in hemocytes is required albeit not sufficient.

323 Since Upd3 is a ligand of the Jak/Stat pathway, we also probed for the requirement of pathway
324 components in the putatively receiving tissues. Indeed, RNAi silencing of the pathway components
325 *hopscotch (hop)* (*Drosophila* Jak) or *Stat92E* in the respiratory system partially reduced the overall
326 *Drosocin* response (Fig. 7N, O). Likewise, silencing of *hop* or *Stat92E* in the fat body also led to a
327 partial reduction in total *Drosocin* expression (Fig. 7P,Q). This suggests that Jak/Stat signaling in both
328 the respiratory epithelia and fat body are required to respond to hemocyte-expressed Upd3 and to
329 contribute jointly to overall *Drosocin* expression, consistent with our earlier findings. Unexpectedly,

330 overexpression of activated Jak *hop^{TumL}* in fat body, or activated *Stat92E* (combined *Stat92E*;
331 *Stat92E^{ΔNΔC}*) in the respiratory system or fat body, reduced *Drosocin* levels after infection (Suppl. Fig.
332 6E-G). Crosses of *UAS-hop^{TumL}* with the tracheal driver *bil-GAL4* were largely lethal and gave rise to
333 only a minimal number of escapers, despite the use of *tub-GAL80^{ts}* to avoid expression during
334 development. The unexpected effects of Jak/Stat overexpression might be due to some unexpected
335 activation of a negative feedback loop or other complex signaling changes.

336 Taken together, our findings suggest that hemocytes act as sentinels of bacterial infection, which signal
337 to neighboring cells of the respiratory epithelia and fat body. Hemocyte-expressed *upd3* and Jak/Stat
338 signaling in the respiratory epithelia and fat body are required, albeit not sufficient, in this process. In
339 response, both fat body and respiratory epithelia concertedly contribute to the expression of *Drosocin*.

340 ***Drosocin* expression in the respiratory epithelia and fat body promote animal survival after infection**

341 Lastly, we asked whether *Drosocin* expression in the respiratory epithelia and fat body is crucial for
342 animal survival after infection. First we determined whether ubiquitous RNAi silencing of *Drosocin*
343 affects animal survival after immune challenge. Indeed we found that survival after bacterial infection
344 with gram-negative *E. coli* or *E. cloacae* was significantly reduced, an effect seen with two
345 independent *Drosocin* RNAi lines (Fig. 8A,D). *Drosocin* knockdown also affected survival after injury
346 (PBS injection) (Fig. 8B). Ubiquitous knockdown of *Drosocin* did not affect the induction of other
347 AMPs following bacterial infection (Suppl.Fig. 7A-D), strengthening the idea that endogenous
348 *Drosocin* has direct antibacterial role/s, and does not act as signal or mediator that would sustain the
349 expression of other AMPs following bacterial infection.

350 Next we probed the role of *Drosocin* expression specifically in the respiratory system or fat body.
351 Indeed, *Drosocin* silencing in the respiratory system or fat body significantly reduced survival after *E.*
352 *coli* infection (Fig. 8H,K), an effect that was again seen with two independent *Drosocin* RNAi lines.
353 *Drosocin* expression in the respiratory system and fat body may also contribute to survival after injury,

354 as *Drosocin* knockdown in either of the two tissues showed a partially penetrant effect on survival after
355 PBS injection (Fig. 8I,L). As expected, silencing of *Drosocin* in hemocytes had no significant effect on
356 survival following gram-negative infection or injury (Fig. 8E,F).

357 Taken together, we conclude that endogenous *Drosocin* expression in the respiratory system and fat
358 body of adult *Drosophila* is effective in promoting animal survival after gram-negative infection. This
359 protective role of localized *Drosocin* expression is directly linked to the reservoirs of blood cells in
360 adult *Drosophila*. Our findings highlight the role of hemocytes as sentinels of infection that relay the
361 innate immune response to colocalizing domains of the fat body and the respiratory epithelia. By
362 identifying Imd signaling and upd3 expression in hemocytes as the first molecular steps in this relay,
363 our work establishes a new *Drosophila* model to study multi-tissue organismal immunity.

364

365 **Discussion**

366 Adult *Drosophila* has been a powerful system to dissect molecular mechanisms underlying innate
367 immunity, from pattern recognition, TLR- and cytokine- related signaling cascades, to humoral
368 effectors such as antimicrobial peptides (Imler and Bulet, 2005; Lemaitre and Hoffmann, 2007; Morin-
369 Poulard et al., 2013; Royet and Dziarski, 2007) However, major questions have remained regarding the
370 hematopoietic capacity of the *Drosophila* adult blood cell system, and the role of blood cells in
371 organismal immunity. Addressing these questions, we discovered a central role for a new blood cell
372 reservoir at the respiratory epithelia and fat body of adult *Drosophila*. The reservoir serves as major
373 receptacle of blood cells and foreign particles, and in addition executes a local humoral immune
374 response of *Drosocin* expression that promotes animal survival after bacterial infection. Both functions
375 are tied together by hemocytes acting as sentinels of infection, that signal through the Imd pathway and
376 Upd3 to induce the *Drosocin* in the tissues of their surrounding reservoir, i.e. the respiratory epithelia
377 and colocalizing domains of the fat body.

378 In the past, studies on *Drosophila* and other insects typically focused on the adult heart as the site of
379 hemocyte accumulation. Historic insect literature described clusters of hemocytes at the ostia of the
380 heart as “immune organ” (Gupta, 2009), which were confirmed as locations where hemocytes and
381 bacteria accumulate (King and Hillyer, 2012). More recently, a model of adult blood cell production at
382 the heart was proposed (Ghosh et al., 2015). Some studies described functions of hemocytes in other
383 locations, such as at the ovaries or along the gut of adult flies (Ayyaz et al., 2015; Van De Bor et al.,
384 2015). In our study we took a more global approach. Surveying the whole animal by cryosectioning
385 afforded us to identify the largest reservoir of hemocytes in adult *Drosophila*, which is surrounding the
386 respiratory epithelia of the thorax and head. The respiratory hemocyte reservoir is lined by fat body, a
387 major immune tissue, and is directly connected with the open circulatory system (Fig. 7R). Our
388 evidence from microbead- and bioparticle injections and developmental studies suggest that hemocytes
389 and particles are delivered to these areas by the streaming hemolymph, even though the detailed
390 anatomy of the open circulatory system remains to be mapped in more detail. Hemocytes may get
391 physically caught in these locations, or in addition may use a more active adhesion mechanism., The
392 intimate relationship of hemocytes with the respiratory epithelia, hemolymph, and adjacent fat body
393 may serve dual interconnected roles, (1) guarding the respiratory epithelia as a barrier to the
394 environment with respect to the roles of hemocytes in both phagocytosis and the induction of humoral
395 immunity, and (2) facilitating gas exchange of hemocytes and nearby immune tissues, which in turn
396 may again benefit defense functions. The former may be particularly advantageous in the defense
397 against fungal pathogens, such as the entomopathogenic fungus *B. bassiana*, which invades
398 *Drosophila* via the tracheal system as primary route of infection (Clarkson and Charnley, 1996;
399 Lemaitre et al., 1997). Regarding the latter, a study in caterpillars described the association of
400 hemocytes with trachea, proposing a function for the respiratory system to supply hemocytes with
401 oxygen (Locke, 1997).

402 *Drosophila* adult blood cells derive from two lineages: one that originates in the embryo and resembles
403 vertebrate tissue macrophages, and another that produces blood cells in the lymph gland through a
404 progenitor-based mechanism (Banerjee et al., 2019; Gold and Brückner, 2014, 2015; Holz et al., 2003)
405 Based on flipout-*lacZ* lineage tracing we estimate that more than 60% of adult hemocytes derive from
406 the embryonic lineage, inferring that the rest derive from the lymph gland. This is surprising,
407 considering older reports that proposed the majority of adult hemocytes would derive from the lymph
408 gland (Lanot et al., 2001), Our findings places more importance on the *Drosophila* embryonic lineage
409 of hemocytes and suggest additional parallels with tissue macrophages in vertebrates, which persist
410 into adulthood and form a separate myeloid system independent of the progenitor-derived monocyte
411 lineage (Davies et al., 2013; Gold and Brückner, 2014, 2015; Perdiguero et al., 2014; Sieweke and
412 Allen, 2013). Future research will show whether the relative contribution of the two hemocyte lineages
413 to the adult blood cell pool will be the same or different under conditions of stress and immune
414 challenges.

415 Given that embryonic-lineage plasmatocytes are highly proliferative in the hematopoietic pockets of
416 the *Drosophila* larva, and lymph gland hemocyte progenitors and some lymph gland plasmatocytes
417 proliferate during larval development (Evans et al., 2003; Jung et al., 2005; Letourneau et al., 2016),
418 the absence of hemocyte proliferation in the adult is surprising. However, no significant new
419 plasmatocyte production, neither from direct proliferation nor a proliferating progenitor, was detected
420 using a variety of approaches including 2-week in vivo EdU incorporation, Fucci, PermaTwin- and
421 hemocyte MARCM analyses, all under standard laboratory conditions and bacterial infection.

422 Two additional lines of evidence support a lack of hematopoietic activity in adult *Drosophila*. First,
423 quantifications of total hemocytes ex vivo demonstrate a continuous decline of blood cells in the
424 animal over time, even under conditions of immune challenge. Second, we find no evidence that *Srp* in
425 adult *Drosophila* is a progenitor marker, as Ghosh et al. postulated in support of their adult

426 hematopoiesis model (Ghosh et al., 2015). Instead, we demonstrate that the vast majority of Srp-
427 expressing hemocytes in the adult are active phagocytes, therefore lacking hallmarks of
428 undifferentiated progenitors. This contrasts the above model (Ghosh et al., 2015), and reveals
429 differences to embryonic development, where Srp is required for the specification of undifferentiated
430 prohemocytes (Rehorn et al., 1996)

431 As an advantage to our study, we globally examined *Drosophila* adults for new blood cell production,
432 taking into account all hemocyte populations of the animal. This allowed us to clarify a process that we
433 believe formed another pillar in the model claiming blood cell production at the heart of adult
434 *Drosophila* (Ghosh et al., 2015). During maturation of the adult animal, hemocytes relocate to the
435 respiratory epithelia and heart upon completion of cytolysis of larval fat body cells (Nelliot et al.,
436 2006). This recruitment increases local hemocytes visible at the heart and in areas underlying the
437 cuticle, but does not increase the absolute number of blood cells. However, when focusing on this area
438 in isolation, the false impression of an expansion in the blood cell population could arise. Similarly, we
439 do not discern a significant increase in total blood cells following bacterial infection, although
440 increased numbers of fluorescently labeled hemocytes become visible through the cuticle. We attribute
441 this to the infection-induced upregulation of hemocyte-specific genes such as *Hml* and *crq* and their
442 respective enhancers in e.g. *Hml* Δ -*GALA*, driving expression of *UAS-GFP*. Enhanced hemocyte
443 expression of these genes post infection was also described previously (De Gregorio et al., 2001; Franc
444 et al., 1999).

445 Taken together, our broad evidence speaks to a lack of significant hematopoietic capacity of the blood
446 cell system in adult *Drosophila*. Our findings are in agreement with other studies that have reported a
447 lack of hemocyte proliferation in adult *Drosophila* (Lanot et al., 2001; Mackenzie et al., 2011;
448 Woodcock et al., 2015), and functional immunosenescence in ageing flies (Felix et al., 2012;
449 Mackenzie et al., 2011). Of course we cannot exclude the possibility that some other specific immune

450 challenge or stress might exist that would be potent enough to trigger proliferation- or differentiation-
451 based blood cell production in adult *Drosophila*. Likewise, although the majority of Srp positive
452 hemocytes are active macrophages, we cannot exclude that adult *Drosophila* may possess small
453 numbers of proliferation-competent progenitors that may have persisted e.g. from the lymph gland
454 posterior lobes (Grigorian et al., 2011; Jung et al., 2005); such cells might give rise to new
455 differentiated hemocytes, although according to our data they would remain insignificant in number.

456 Taking into account the relatively short life span and short reproductive phase of *Drosophila*, the adult
457 fly may be sufficiently equipped with the pool of hemocytes that is produced in the embryo and larva.
458 In fact, hemocytes may not be essential for the immediate survival of adult flies (see below). We
459 propose a model that places emphasis on larval development as the sensitive phase for the expansion
460 and regulation of the adult blood cell pool (Gold and Brückner, 2015) (Fig. 5N). In the larva, both the
461 embryonic-lineage hemocytes in the hematopoietic pockets and blood cells deriving from the lymph
462 gland integrate signals from a variety of internal and external stimuli to adapt to existing life conditions
463 (Gold and Brückner, 2014, 2015; Shim, 2015).

464 *Drosophila* ablated of hemocytes, and mutants devoid of hemocytes, survive to adulthood (Arefin et al.,
465 2015; Braun et al., 1998; Charroux and Royet, 2009; Defaye et al., 2009). However, hemocyte-
466 depleted animals have been known to be more prone to, and succumb more rapidly to, infection
467 (Arefin et al., 2015; Basset et al., 2000; Braun et al., 1998; Charroux and Royet, 2009; Defaye et al.,
468 2009). Our work reveals a role for hemocytes in a local humoral immune response of the fat body and
469 respiratory epithelia. Previous studies on hemocyte-ablated flies have reported increases in *Defensin*
470 and *IMI* expression (Charroux and Royet, 2009; Defaye et al., 2009). Likewise, we find that hemocyte
471 ablation enhances initial *Cecropin A1* expression after infection. This may indicate a negative
472 regulatory role of hemocytes on these and possibly other AMP genes. It may also be directly or
473 indirectly linked to the altered inflammatory status of hemocyte-ablated flies, which show increased

474 Toll and decreased Imd signaling (Arefin et al., 2015). In contrast, we find a positive role for
475 hemocytes in the induction of *Attacin A*, *Diptericin*, and *Drosocin*. Expression of *Drosocin* is
476 particularly intriguing, as its pattern of expression matches the tissues that form the hemocyte reservoir,
477 i.e. the respiratory epithelia and fat body domains of the head and thorax. The concept of hemocytes
478 promoting AMP expression in other tissues is well established (Agaisse et al., 2003; Basset et al.,
479 2000; Chakrabarti et al., 2016; Yang et al., 2015). A role for AMP expression in surface epithelia that
480 interface with the environment was reported by Ferrandon et al. (Ferrandon et al., 1998), and *Drosocin*
481 expression was described in embryonic and larval trachea and the abdominal tracheal trunks of adult
482 *Drosophila*, albeit not in the respiratory epithelia (Akhouayri et al., 2011; Tan et al., 2014; Tzou et al.,
483 2000). *Attacin A* is also expressed in the larval trachea (Akhouayri et al., 2011), warranting future
484 investigation into its expression in the adult respiratory epithelia and putative regulatory links with
485 hemocytes. In adult *Drosophila*, hemocytes tightly localize between the respiratory epithelia and a
486 layer of adult fat body tissue that occupies the space toward to the cuticle exoskeleton, forming an
487 anatomical and functional triad of hemocytes, respiratory epithelia and fat body, in which hemocytes
488 act as sentinels of infection.

489 We propose that this close anatomical relationship facilitates rapid local signaling by (1) detection of
490 gram-negative bacteria through PGRP-LC/Imd signaling in hemocytes and (2) communication of
491 hemocytes with the fat body and respiratory epithelia through Upd3/Jak/Stat signaling, culminating in
492 the induction of *Drosocin* expression in these tissues to protect the animal following bacterial infection
493 (Fig. 7R). Consistent with previous knowledge that *Drosocin* expression is lost in *imd* mutant
494 backgrounds (Lemaitre PNAS 1995; Rahel et al. JBC 2004), we find that hemocyte-autonomous Imd
495 signaling is required, albeit not sufficient, to trigger the infection-induced *Drosocin* response. Likewise,
496 the Imd pathway upstream receptor *PGRP-LC* is required in hemocytes, suggesting that DAP-type
497 peptidoglycan recognition and initiation of Imd signaling are a critical step in triggering the *Drosocin*

498 response. Our data suggest roles for hemocyte-expressed *upd3*, and corresponding Jak/Stat signaling in
499 cells of the fat body and respiratory system, all of which are required albeit not sufficient.
500 Overexpression of activated *Stat92E* (combined *Stat92E*; *Stat92E*^{ΔNΔC}) or the activated Jak *Hop*^{*TumL*}
501 paradoxically suppress *Drosocin* expression. Crosses of *UAS-hop*^{*TumL*} with the tracheal driver *btl-GAL4*
502 were largely lethal despite our use of *tub-GAL80^{ts}*, which might indicate leaky expression of the *UAS-*
503 *hop*^{*TumL*} transgene. Overall, we can only speculate that the unexpected effects of Jak/Stat
504 overexpression might be due to activation of some negative feedback loop or other complex signaling
505 changes.

506 Several reports provide precedent for a role of hemocyte-expressed Upd3 in the induction of immune
507 responses in other target tissues. Following septic injury, upregulation of *upd3* in hemocytes triggers
508 induction of stress peptide genes of the *turandot* family including *totA* in fat body (Agaisse et al.,
509 2003; Brun et al., 2006). Similarly, in response to injury, hemocyte-produced Upd3 induces Jak/Stat
510 signaling in the fat body and gut (Chakrabarti et al., 2016). Further, under lipid-rich diet, *upd3* is
511 induced in hemocytes, causing impaired glucose homeostasis and reduced lifespan in adult *Drosophila*
512 (Woodcock et al., 2015). Lastly, in the larva, hemocyte-derived Upd2 and -3 activate Jak/Stat signaling
513 in muscle, which are required for the immune response against parasitic wasps (Yang et al., 2015).
514 However, in the *Drosocin* response around the reservoir of hemocytes, our data predict that additional
515 signal/s and/or signaling pathway/s are needed to initiate *Drosocin* expression and potentially restrict
516 its expression to defined fat body domains of the head and thorax. Additional events may rely on
517 diverse mechanisms. They could include signaling through Toll or other signaling pathways in
518 hemocytes and/or other tissues including the respiratory epithelia and fat body. Likewise, other types
519 of signals may be required or permissive for the *Drosocin* response in the adult fly, such as reactive
520 oxygen species (ROS) or nitric oxide (NO) which were reported to play roles in the relay of innate
521 immune responses to infection and stress (Caceres et al., 2011; Di Cara et al., 2018; Eleftherianos et al.,

522 2014; Myers et al., 2018; Wu et al., 2012), or non-peptide hormones including ecdysone, which
523 confers competence in the embryonic tracheal *Drosocin* response to bacterial infection, and enhances
524 humoral immunity under conditions of dehydration (Tan et al., 2014; Zheng et al., 2018). Lastly, there
525 could be e.g. a requirement for additional processing to make bacterial ligands accessible for receptors
526 in other tissues, as has been reported for Psidin, a lysosomal protein required in blood cells for
527 degradation of engulfed bacteria and expression of *Defensin* in the fat body (Brennan et al., 2007),
528 although this mechanism may not be universal in all systems (Nehme et al., 2007).

529 Our work reveals an active role of endogenous *Drosocin* expression in survival after bacterial infection.
530 Since the cloning of *Drosocin* and its classification as an inducible antibacterial peptide (Bulet et al.,
531 1993), *Drosocin* has been studied for its transcriptional regulation (Charlet et al., 1996), illustrating its
532 induction under a variety of bacterial and other immune challenges (Akbar et al., 2011; Akhouayri et
533 al., 2011; Becker et al., 2010; Clark et al., 2013; Fernando et al., 2014; Gendrin et al., 2013; Lemaitre
534 et al., 2012; Yagi et al., 2013). *Drosocin* structure and antimicrobial function have been studied in vitro
535 (Ahn et al., 2011; Bikker et al., 2006; McManus et al., 1999; Otvos et al., 2000b), and by
536 overexpression from transgenes in *Drosophila* (Loch et al., 2017; Tzou et al., 2002; Vonkavaara et al.,
537 2013) and in heterologous vertebrate systems (Otvos et al., 2000a). Consistent with our findings, a
538 recent study that examined new CRISPR-based *Drosocin* null mutants reached similar conclusions
539 regarding the requirement of endogenous *Drosocin* expression for animal survival following *E.*
540 *cloacae* infection (Hanson et al., 2019). However, this study did not address anatomical features of
541 *Drosocin* expression, nor its unique path of induction. In addition to *Drosocin*'s role in animal survival
542 after bacterial infection, our data suggest contribution of *Drosocin* to animal survival after injury (PBS
543 injection); however, due to the possibility of inadvertent contamination of the injection site we cannot
544 exclude that this effect may also involve aspects of infection. A role for endogenous *Drosocin* levels in
545 the antimicrobial response is strongly supported by independent data in the literature. Specifically, the

546 minimum inhibitory concentration (MIC) of Drosocin against *E. coli* and *E. cloacae* was determined to
547 be well within the range or below the endogenous concentration of Drosocin in the *Drosophila*
548 hemolymph (MIC is 1 μ M or 2 μ M for the glycosylated forms, and 8 or 10 μ M for the unglycosylated
549 form, respectively (Bulet et al., 1996), compared to 40 μ M Drosocin in the *Drosophila* hemolymph
550 ((Uttenweiler-Joseph et al., 1998)).

551 In conclusion, our work recognizes adult *Drosophila* as a promising model to study organismal
552 immunity centering around the reservoir of blood cells, which involves immune signaling in the triad
553 of hemocytes, respiratory epithelia and fat body. At the evolutionary level, this model shows parallels
554 with vertebrate immune cells of the lung and innate immune responses to bacterial infection (Byrne et
555 al., 2015; Divangahi et al., 2015; Opitz et al., 2010). The *Drosophila* model opens countless new
556 avenues for exciting future research, for example to investigate additional molecular and cellular
557 mechanisms in the immune signaling relay, the role and regulation of the system in the defense against
558 pathogens that invade the trachea as natural route of infection, the anatomical and functional features
559 of the respiratory epithelia and other parts of the hemocyte reservoir, features of the open circulatory
560 system and its particle streaming dynamics, effects of the blood cell reservoir on hemocyte activity, the
561 impact of other tissues and systemic factors on the immune response, the use of the same axis by gram-
562 positive or non-bacterial pathogens, and the induction of other AMPs and immune effector genes in the
563 same axis of regulation.

564

565 **Experimental Procedures**

566

567 ***Drosophila* strains and fly husbandry; *HmlΔFucci* transgenic lines; Flipout-*LacZ* lineage tracing;**

568 **Fucci analysis and quantification of hemocytes; Hemocyte MARCM; PermaTwin MARCM;**

569 **Quantification of AMP expression by qPCR**

570 Please see Supplemental Experimental Procedures.

571

572 **Cryosectioning, head dissections**

573 For cryosectioning, adult whole flies were embedded in OCT (Tissue-Tek) and snap frozen on dry ice

574 with 95% ethanol. Cryosections of 55 μ M thickness were obtained using a cryostat (Leica CM3050 S

575 or Leica CM1950). Tissue sections were placed on charged glass slides and fixed with 4% PFA in

576 2xPBS with 2x Complete protease inhibitor (Roche) for 10 min, followed by 2% PFA for 10 min, and

577 permeabilized with 0.2% Triton-X100 for 15 min. Dissections of the respiratory epithelia (air sacs) of

578 the head were done using forceps and a dissection well filled with PBS. Heads were detached from the

579 rest of the body. By holding heads at the proboscis, the cuticle of the head was opened through the

580 antennae first, progressively working toward the posterior, trying to avoid damage of the respiratory

581 epithelia. Once the brains with the overlying respiratory air sacs were exposed, they were fixed with

582 4% PFA/PBS for 10 minutes, washed in PBS and processed for imaging. In both cryosections and

583 dissections, hemocyte attachment to the respiratory epithelia is quite fragile. Accordingly, some

584 hemocyte loss occurs in many preparations.

585 **Immunohistochemistry and other staining methods**

586 To analyze hemocytes ex vivo, hemocytes of single flies were released in 100 μ l of Schneider's

587 medium (Gibco, Millipore) or PBS supplemented with Complete 2x (Roche) and proteinase inhibitor

588 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride AEBSF (Sigma); flies were dissected and
589 hemocytes were scraped from all inside areas of the fly (head, thorax and abdomen).

590 For immunohistochemistry, antibodies used were goat anti-GFP (Rockland Immunochemicals, 1:2000),
591 rabbit anti-βGal (Thermo, 1:1000), rabbit anti-DsRed (Rockland Immunochemicals, 1:1000), mouse
592 P1 (P1a+P1b) (Kurucz et al., 2007) (kind gift of I. Ando, 1:10), anti-Crq (Franc et al., 1996) (kind gift
593 of C. Kocks, 1:1000), anti-Srp (kind gift of A. Giangrande, 1:1000) and secondary antibodies
594 conjugated to Alexa dyes (Molecular Probes, 1:500), fluorescently labeled phalloidin (Molecular
595 Probes), DAPI (Sigma), DRAQ5 (ThermoFisher).

596 Fat body cells were labeled using OilRedO (37%) dissolved in triethyl phosphate (6ml triethyl
597 phosphate and 4 ml water) for 30 min, followed by three to four washes with distilled water. Other
598 stainings used fluorescent LipidTOX dyes (LifeTech), diluted in PBS.

599 **Edu incorporation assays**

600 To assess cell proliferation in adult hemocytes, adult F1 progeny of *yw* or *HmlΔ-GAL4, UAS-GFP x*
601 *CantonS* were maintained for 14 days on fly food supplemented with a stock of 0.5 mg/ml EdU
602 (Invitrogen) to a final concentration of 0.4mM EdU. Animals were immune challenged with bacterial
603 infections both before or during EdU feedings. Various ages of adult animals (starting labeling at 0-1
604 week after eclosion) and crosses with other control lines (Oregon R, w1118 etc.) were examined.
605 Hemocytes were released in multi-well dishes ex vivo and Click-IT EdU detection was performed
606 according to the manufacturer's instructions (Invitrogen).

607 **Microbead and bioparticle injections**

608 For microbead injections, adult females aged 3 or 11 days after eclosure were injected with a
609 suspension of fluorescent beads (FluoSpheres carboxylate-modified 0.2 μm; Life Technologies); 50-69
610 nl of a 1:10 dilution in PBS of was injected as described previously (Makhijani et al., 2011). Flies were

611 incubated at 25°C for 30min-1h, followed by imaging, hemocyte releases or embedding for
612 cryosectioning. For quantification of bead-positive hemocytes, images were taken with a Leica
613 DMI4000B microscope followed by manual counting.

614 For bioparticle injections, adult females aged 6 to 11 days after eclosure were injected with fluorescent
615 bioparticles (*E. coli* K-12 Strain Bioparticles TexasRed Conjugate; Invitrogen) or pH sensitive
616 fluorescent bioparticles (pHrodo *E. coli* Bioparticles Conjugate for Phagocytosis; Invitrogen). Each fly
617 was injected with 27.6-32.2 nl of bioparticles (1mg/100µl) and then incubated at 25° C for 4 hours
618 before analysis. For ex vivo hemocyte analysis of differential body sections, flies were split into head,
619 thorax, abdomen using a scalpel, individually placed into wells of 20 µl of PBS, and hemocytes were
620 released from each section by poking and crushing. Hemocytes were imaged as described under
621 Microscopy.

622 **Microscopy**

623 Standard fluorescence images were obtained on a Leica DMI4000 microscope. A Leica M205FA
624 stereomicroscope with motorized stage and Leica LAS Montage module was used to image hemocytes
625 labeled by fluorescent reporters in live CO₂-anesthetized flies or whole mount stainings.
626 Immunostained adult cryosections and dissected preparations were imaged using a Leica SP5 confocal
627 microscope.

628 **Hemocyte quantification from adult flies**

629 To determine hemocyte numbers by visualization of fluorescent reporter expressing hemocytes
630 through the cuticle, the number of GFP labeled hemocytes in thorax, three legs and head of one side of
631 the animal was counted from montage images (described above) for 1, 7, 19, 24, 37 and 63 days old
632 adults. For each time point, 4-10 males and 4-10 females were used to determine the mean value of
633 number of hemocytes and standard deviation. For hemocyte quantification in the dorsal
634 thorax/abdominal area in (Fig. 5C-F'), see 'Fucci analysis and quantification of hemocytes'. To

635 determine total hemocyte numbers by release, single flies were CO₂-anesthetized, and dissected in a
636 glass well slide containing defined amount of 70µl of Schneider's *Drosophila* medium (Millipore).
637 Under a fluorescence stereomicroscope, the head was removed and the ventral side of the head was
638 carefully torn between the eyes, and subsequently pinched with forceps until all of the hemocytes were
639 released. The empty head was then removed from the well. Next, the abdomen was torn open and,
640 starting from the posterior end, squeezed and poked until the majority of hemocytes were released. The
641 thorax was squeezed/crushed multiple times to release the remaining hemocytes. The carcass was
642 transferred out of the well. 10µl of this hemocyte suspension was pipetted into a hemocytometer, and
643 cells were counted under a fluorescence microscope. Cell numbers from four 1mm² squares were
644 averaged, and the total number of hemocytes per fly was calculated. A total of 10 females plus 2 males
645 per time point were quantified which were processed in groups of 4 animals. Average number of
646 hemocytes/fly from 12 animals and standard deviation were calculated for each time point.

647 **Bacterial infection**

648 Animals for hemocyte MARCM, PermaTwin MARCM or EdU based proliferation experiments and
649 qPCR to detect AMP expression were immune challenged by either injection or feeding of bacterial
650 strains as outlined below.

651 For injections, bacterial cultures were grown overnight in LB broth with suitable antibiotics. The
652 following morning, a 1:20 culture dilution was incubated at 37°C for 3 hours, OD measured
653 (NanoDrop 2000c spectrophotometer, Thermo Scientific) while typically <1, and the culture was spun
654 down and the resulting pellet resuspended in PBS to achieve specific calculated ODs as summarized
655 below. Bacterial strains (kind gifts from Bruno Lemaitre laboratory) and ODs were as follows;
656 *Escherichia coli* OD 3 or 6 (see figure legends); *Micrococcus luteus* OD 1.5 or 2; *Erwinia carotovora*
657 *carotovora 15* (Lemaitre et al., 1997) OD 1.5; *Enterobacter cloacae* (β12, Jean Lambert(Lemaitre et
658 al., 1997)) OD 2 or 4 (see figure legends). Bacterial suspension or control (sterile PBS) was injected

659 into the thorax or abdomen of anesthetized females aged 5-7 days using a Nanoject II injector
660 (Drummond Scientific Inc.) fitted with a pulled glass capillary, using volumes of 9.2nl to 27.6nl as
661 indicated; experiments with *btl-GAL4* used 1-2 days old flies. Infected adults were incubated at 29°C,
662 or as indicated. F1 crosses of transgenes regulated by the temperature-sensitive *tub-GAL80^{ts}* (McGuire
663 et al., 2003) were initially maintained at 18°C, and then induced by shifting animals to 29°C, starting
664 24 hours before injection for crosses with *HmlA-GAL4, UAS-GFP; tub-GAL80^{ts}*, or 48 hours before
665 injection for crosses with *btl-GAL4, UAS-GFP, tub-GAL80^{ts}*. Immune challenge by feeding of *S.*
666 *marcescens* was performed as described previously (Nehme et al., 2007). *S. marcescens* grown
667 overnight from a single colony inoculated into 5 ml LB broth with 50ug/ml ampicillin was diluted to
668 OD600 of 0.1 in 10 ml of fly food. At least 10 adults per cohort were used.

669 **qRT-PCR**

670 Adult flies were frozen at -80°C after incubation at 29°C following injections (see figure legends for
671 specific incubation times). Frozen flies were homogenized in TRIzol (Invitrogen), and RNA was
672 isolated using Direct-zol RNA MiniPrep kit, following the manufacturer's instructions (Zymo). 1 µg of
673 RNA was reverse transcribed using iScript cDNA synthesis kit (BioRad) and the resulting cDNA was
674 diluted 1/10 for the qPCR. qPCR was run in a BioRad CFX96 Touch Real-Time PCR System, or ABI
675 Viia7 Real-Time PCR System using the BioRad iTaq SYBR Green Supermix. Primers used for qPCR
676 are summarized in Supplemental Table 1.

677

678 **Author Contributions**

679 This study was made possible by the combined talent of all authors over the course of more than ten
680 years. PSB expertly planned, performed and statistically analyzed the majority of qPCR experiments,
681 added the aspect of Upd3/Jak/Stat signaling, contributed some survival data, and majorly revised
682 qPCR figures. KM expertly planned and generated complex *Drosophila* genotypes and planned,

683 performed and analyzed lineage tracing, MARCM, EdU, hemocyte quantification and marker
684 experiments, and provided key information on the *Drosophila* respiratory epithelia. LH creatively
685 developed new ways to visualize *Drosophila* adult respiratory epithelia, and planned, performed and
686 analyzed adult fly dissections, confocal microscopy, microbead and bioparticle localization,
687 phagocytosis assays, qPCR and survival experiments. KG planned, performed and analyzed
688 experiments related to tissue colocalizations, microbead localization, confocal imaging, EdU
689 incorporation, reporter and marker analyses, qPCR experiments, and contributed to the writing of the
690 manuscript. RB planned, performed and analyzed whole and split-fly hemocyte counts and –qPCRs,
691 and other qPCR and initial survival experiments. BA developed and performed fly cryosectioning,
692 and performed EdU, lineage tracing, dye and marker experiments. KK performed and analyzed
693 survival experiments. SC performed and analyzed experiments related to bioparticle injections and
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990

991

992 **Figure Legends**

993 **Figure 1. The respiratory epithelia provide the largest reservoir of hemocytes in adult *Drosophila*.**

994 (A-C) Cryosections of adult *Drosophila*, *HmlΔ-DsRednls* hemocytes red, phalloidin green, respiratory
995 epithelia (air sacs) blue. (A) Longitudinal section, anterior up; (B) cross section of head, dorsal up; (C)
996 cross section of thorax, dorsal up. (D) Adult *Drosophila*, genotype *HmlΔ-DsRed/+; btl-GAL4, UAS-*
997 *GFP/+*; hemocytes red, tracheal marker green; respiratory epithelia (air sacs) blue; dissection of head,
998 anterior up; size bar 250μm. (E) Schematics of respiratory epithelia (tracheal air sacs) of the thorax and
999 head and other parts of the tracheal system in blue), hemocytes in red. Dashed lines indicate sections
1000 and dissected area shown in A-D. Note that model omits heart area, which is not visible in longitudinal
1001 section in (A). For full model including heart area, see Suppl. Fig. 1.

1002 **Figure 2. Developmental changes of hemocytes.**

1003 (A-C) Lateral view of adult *Drosophila*, (A) Model, hemocytes red, (B,C) *HmlΔ-GAL4, UAS-GFP*
1004 (hemocytes, red pseudo color), (B) day 1 post eclosure, (C) day 6 post eclosure. (D) Larval fat body
1005 cells (Oil Red O pseudo-colored in green) with associated hemocytes (*HmlΔ-GAL4, UAS-GFP* pseudo-
1006 colored in red), dissection of abdomen. (E, F) cross sections of anterior abdomen, *HmlΔ-DsRednls*
1007 (hemocytes red), LipidTox (green), DAPI (blue); dashed box marks heart region; (E) day 1 post
1008 eclosure; (F) day 6 post eclosure. (G) External hemocyte quantification, time course; fluorescently

1009 labeled hemocytes that can be visually recognized through the cuticle were counted (see Methods). (H)
1010 Total hemocyte counts per animal, time course; all hemocytes were released by scraping and counted
1011 ex vivo.

1012 **Figure 3. Infection-induced changes of hemocytes and accumulation of particles at the**
1013 **respiratory epithelia.**

1014 (A-C) Lateral view of adult *Drosophila*; (A) Model, hemocytes red, (B, C) *HmlΔ-GAL4, UAS-GFP*
1015 (hemocytes red pseudo color), (B) no infection control, (C) *Ecc15* injection. (D) External hemocyte
1016 quantification, hemocyte number of the dorsal thorax and anterior abdomen, controls and injected flies
1017 as indicated; p values of paired 2-tailed t test are shown, *, **, ***, or **** corresponding to $p \leq 0.05$,
1018 0.01, 0.001, or 0.0001. (E) Total hemocyte counts, control and injected flies as indicated, all
1019 hemocytes were released by scraping. Average and standard deviation; 2-tailed t test shows no
1020 statistically significant difference (NS). (F) Total hemocyte counts of flies split in two parts, head and
1021 thorax versus abdomen; control and injected flies as indicated. Flies were injected into thorax or
1022 abdomen at 5 days post eclosion and assayed at 12h and 6d post injection. Average and standard
1023 deviation are shown. (G) qPCR expression levels of *Hml* and *Crq* from whole flies, +/- infection, 48h
1024 post infection. 8 day old adults were injected with *E.coli* at OD 2. (H-M) Injection of fluorescent
1025 microbeads (green pseudocolor), *HmlΔ-GAL4, UAS-GFP* (hemocytes, red pseudocolor), respiratory
1026 epithelia (air sacs, blue). (H, J, L) Injection in thorax, (H) external view, (J) head dissection, (L) thorax
1027 cross section. (I, K, M) Injection in abdomen, (I) external view, (K) head dissection, (M) thorax cross
1028 section. (N-Q) Injection of fluorescent *E. coli* bioparticles (green), *HmlΔ-DsRed* for head dissections or
1029 *HmlΔ-DsRednls* for cryosections (hemocytes, red), respiratory epithelia (air sacs, blue). (N, P)
1030 Injection in thorax, (N) head dissection, (P) thorax cross section. (O, Q) Injection in abdomen, (O)
1031 head dissection, (Q) thorax cross section. (R) Injection of pHrodo *E. coli* bioparticles into *HmlΔ-*
1032 *DsRed* adults; percentages of hemocytes that phagocytosed pHrodo bioparticles from dissected head,

1033 thorax and abdomen 4 hours after injection. Mean with standard deviation and one-way ANOVA
1034 significance show no statistically significant difference (NS). (S) Dissected respiratory epithelia of the
1035 head from adults *HmlΔ-DsRed* (hemocytes, red) injected with pHrodo *E. coli* bioparticles (green) 4
1036 hours after injection. (T-V) Examples of hemocytes with incorporated pHrodo bioparticles isolated
1037 from head, thorax, abdomen, corresponding to (R).

1038 **Figure 4. Contribution of the two hemocyte lineages to the adult blood cell pool.**

1039 (A) Timeline of the two blood cell lineages in *Drosophila*, i.e. the embryonic and lymph gland lineage,
1040 with the major sites of hematopoiesis in the larva (hematopoietic pockets and lymph gland). Both
1041 lineages persist into the adult but the relative contribution has remained unclear. (B-E) *flipout-lacZ*
1042 lineage tracing using embryonically expressed *srpHemo-GAL4*. (B) Experimental genotype *tub-*
1043 *GAL80^{ts} / srpHemo-GAL4, UAS-srcEGFP; UAS-Flp/ act>stop>nuc-lacZ*. (C) Timeline of induction,
1044 hemocytes of the embryo were labeled in a 6h time window of Flp expression (grey box); blue bars
1045 mark time points of samples shown in (D-E). (D-D') *lacZ/βGal* positive hemocytes in the pupa, x-gal
1046 staining (blue); (E) *lacZ/βGal* positive hemocytes in the dissected abdomen of an adult, x-gal staining
1047 (blue); note that adult staining appears stronger due to decreased permeability of whole mount pupae,
1048 individual variation of lineage tracing, and occasional labeling of larval fat body cells. (F-I) *flipout-*
1049 *lacZ* lineage tracing of embryonic-lineage hemocytes in the larva, using *HmlΔ-GAL4*. (F) Experimental
1050 genotype *UAS-Flp; HmlΔ-GAL4, UAS-GFP/ tub-GAL80^{ts}; act>stop>lacZ/ +*. (G) timeline, induction
1051 at 0-48h AEL (grey box). Note that at this stage lymph gland hemocytes do not express *HmlΔ-GAL4*
1052 and therefore remain unlabeled; *HmlΔ-GAL4* is expressed in embryonic-lineage hemocytes from late
1053 embryonic stage onward. Blue bars mark time points of samples examined and quantified (see H, I);
1054 (H) Thorax cross section of adult fly, genotype as in (F), *lacZ/βGal* positive hemocytes green (anti-
1055 β Gal), air sacs and DAPI in blue. (I) Fraction of *lacZ/βGal* positive hemocytes relative to all Crq
1056 positive hemocytes in late 3rd instar larvae and in the adult; relative to each other, these numbers

1057 suggest contribution of the embryonic lineage to more than 60% of the adult blood cell pool (dashed
1058 line).

1059 **Figure 5. Adult hemocytes do not expand; *Srp* marks active phagocytes in adult *Drosophila*.**

1060 (A) In vivo EdU incorporation. Adult flies were kept on EdU containing food continuously for 2 weeks,
1061 in the absence or presence of immune challenges as indicated (see Methods). Bar chart shows
1062 percentage of EdU positive cells among hemocytes or control tissue; average and standard deviation.
1063 (B-F') 2-color Fucci analysis of hemocytes in larvae (positive control) and adult animals, control
1064 (uninfected), sterile injury (PBS), and infection (*M. luteus*, *E. coli*) as indicated; genotype is
1065 $w^{1118}; Hml\Delta FucciOrange^{G1}; Hml\Delta FucciGreen^{G2/S/M}$; (B-B') embryonic-lineage hemocytes released
1066 from larvae, note presence of green cells; (C-F') imaging of Fucci hemocytes in adult flies, dorsal
1067 views of thorax and anterior abdomen, anterior is up; note that no green hemocytes corresponding to
1068 expression of $Hml\Delta FucciGreen^{G2/S/M}$ can be found. (G-I) *Srp* labels phagocytic plasmatocytes in the
1069 adult fly. (G) *Srp* and *Hml* positive hemocytes of young (3 days) and mature (11 days) adults. Note the
1070 trend of hemocytes shifting from *srp* single positive to *srp Hml* double positive with increasing
1071 maturation. (H) Thorax cross section of adult fly showing *srp* and *Hml* (double-) positive hemocytes,
1072 genotype is $Hml\Delta Dsrednls/UAS-CD4-GFP; +/srpD-GAL4$; respiratory epithelia (air sacs, blue). (I)
1073 $Srp-Gal4, UAS-lifect-GFP$ positive plasmatocytes (green) with red phagocytic vesicles, released ex
1074 vivo from adult fly; DAPI (blue). (J-M) In vivo phagocytosis assay, based on fluorescent blue bead
1075 injection into living adult animals, followed by ex vivo examination of bead incorporation into
1076 hemocytes. (J) Quantification of hemocytes that incorporated blue beads in vivo, comparing *srp* and
1077 *Hml* single- and double positive hemocytes of young (3 days) and mature (11 days) adults. Note that
1078 *srp* single positive hemocytes show an increasing fraction of phagocytic cells upon animal maturation,
1079 and overall compare to the fraction of phagocytic hemocytes among the *srp Hml* double positives. (K,
1080 L, M) examples of labeled hemocytes as indicated. Genotype is $Hml\Delta Dsred/ UAS-stinger; +/srpD-$

1081 *GAL4*. (N) Model, hemocyte production takes place during the larval stage (mainly in the
1082 hematopoietic pockets and the lymph gland), while in the adult hemocyte numbers decline over time.

1083 **Figure 6. Hemocytes and Imd signaling are required for the induction of antimicrobial peptide**
1084 **genes including *Drosocin*.**

1085 (A-E) Expression of AMPs in hemocyte-ablated flies and controls. 5 day-old adult *Drosophila*
1086 untreated, injected with sterile PBS, or with *E.coli* in PBS (OD 6), 9.2 nl; genotypes are *HmlΔ-GAL4*,
1087 *UAS-GFP/+* (control) or *w; HmlΔ-GAL4, UAS-GFP/ UAS-rpr; UAS-hid/+* (hemocyte ablation); flies
1088 were harvested at 6h and 24h post injection. Each chart displays mean and standard error of the mean
1089 (SEM) of samples from a representative biological replicate experiment, using pools of 10 females per
1090 condition, and triplicate qPCR runs. Values of all charts are displayed relative to the RNA level
1091 induced by the sterile PBS injections in control flies. (A) *Drosomyacin*; (B) *Cecropin A1*; (C)
1092 *Diptericin*; (D) *Attacin A*; (E) *Drosocin*. (F-H) Expression of *Drosocin* in adult flies upon manipulation
1093 of Imd pathway activity. 5 day-old adult *Drosophila* untreated, injected with sterile PBS, or with *E.coli*
1094 in PBS (OD 6), 9.2 nl; flies were harvested at 6h post injection. Each chart displays the mean and
1095 confidence interval (CI) of samples from 3 averaged biological replicate experiments, using pools of
1096 10 females per condition, and triplicate qPCR runs for each sample. Values of all charts are displayed
1097 relative to the average RNA level induced by the sterile PBS injections in control flies. Two-way
1098 ANOVA with Sidak's multiple comparison test was performed, *, **, ***, or **** corresponding to
1099 $p \leq 0.05$, 0.01, 0.001, or 0.0001, respectively (Prism). Transgenes were inducibly expressed in
1100 hemocytes 24 hours before injections. (F) *Drosocin* RNA levels of control (*HmlΔ-GAL4, UAS-GFP/+*;
1101 *tub-GAL80^{ts}/+*) versus *HmlΔ-GAL4, UAS-GFP/+*; *tub-GAL80^{ts}/UAS-imd RNAi*; (G) control versus
1102 *HmlΔ-GAL4, UAS-GFP/+*; *tub-GAL80^{ts}/UAS-PGRP-LC RNAi*; (H) control versus *HmlΔ-GAL4, UAS-*
1103 *GFP/UAS-imd*; *tub-GAL80^{ts}/+*.

1104 **Figure 7. The *Drosocin* response is localized to the reservoir of hemocytes at the respiratory**
1105 **epithelia and colocalizing fat body domains, and requires Upd3 signaling from hemocytes.**

1106 (A-C) *Drosocin-GFP* expression is restricted to the head and thorax. (A) *Dro-GFP* uninfected control;
1107 (B) *Dro-GFP* infected; (C) model of *Dro-GFP* expression (green), hemocytes (red), tracheal system
1108 (blue). (D) *Drosocin* qPCR of head/thorax versus abdomen tissue. Flies were left untreated, or injected
1109 with sterile PBS, or *E.coli* in PBS (OD 6), 9.2 nl, and harvested at 6 and 24h post infection. Two-way
1110 ANOVA with Sidak's multiple comparison test was performed for head/thorax versus abdomen,
1111 *, **, ***, or **** corresponding to $p \leq 0.05$, 0.01, 0.001, or 0.0001, respectively. (E) Location of fat
1112 body throughout the animal marked by *ppl-GAL4, UAS-GFP* (green). (F-F'') Dissected heads of
1113 genotype *Drosocin-GFP/HmlΔ-DsRed* (*Drosocin-GFP* green, hemocytes red), respiratory epithelia (air
1114 sacs, blue). Note *Drosocin-GFP* expression is high in fat body and moderate in respiratory epithelia
1115 (arrowhead). (G-I) Tissue specific RNAi knockdown of *Drosocin*; overall *Drosocin* mRNA levels
1116 were quantified by qPCR. 6-7 day-old adult females were left untreated, injected with PBS, or *E.coli*
1117 in PBS (OD 6), 9.2 nl, and harvested 6 and 12h post infection. Each chart displays mean and SEM of
1118 samples from a representative biological replicate experiment, using pools of 10 females per condition,
1119 and triplicate qPCR runs. Values of all charts are displayed relative to the RNA level induced by the
1120 sterile PBS injections in control flies. (G) *Drosocin* RNAi silencing in hemocytes; (H) in respiratory
1121 system; (I) in fat body. (J-K'') Anatomy of fat body tissue lining the respiratory epithelia and
1122 hemocytes; *HmlΔ-DsRednls* (hemocytes, red), fat body (LipidTOX, large, distinct green cells),
1123 respiratory epithelia (air sacs, blue) (J) Sagittal section of adult *Drosophila*. (J') Closeup of region
1124 indicated in (J). (K'-K'') Closeup of regions indicated in (K). Note that hemocytes are layered
1125 between tracheal tissue and fat body. (L-Q) Expression of *Drosocin* in adult flies upon silencing or
1126 overexpression of *upd3* and silencing of genes of the Jak/Stat pathway. 5 day-old adult *Drosophila*
1127 untreated, injected with sterile PBS, or *E.coli* in PBS (OD 6), 9.2 nl; flies were harvested at 6h post

1128 injection. Each chart displays the mean and CI of samples from 3 averaged biological replicate
1129 experiments, using pools of 10 females per condition, and triplicate qPCR runs for each sample.
1130 Values of all charts are displayed relative to the average RNA level induced by the sterile PBS
1131 injections in control flies. Two-way ANOVA with Sidak's multiple comparison test was performed,
1132 *,**,***,or **** corresponding to $p \leq 0.05$, 0.01, 0.001, or 0.0001, respectively. (L, M) *Drosocin* qPCR
1133 of whole flies, inducible transgene expression in hemocytes, (L) Genotypes are control (*HmlΔ-
1134 GAL4,UAS-GFP/+; tub-GAL80^{ts} /+*) versus *HmlΔ-GAL4,UAS-GFP/+; tub-GAL80^{ts} /UAS-upd3* RNAi.
1135 (M) Control versus *HmlΔ-GAL4,UAS-GFP/ UAS-upd3; tub-GAL80^{ts} /+*. (N, O) *Drosocin* qPCR of
1136 whole flies, inducible transgene expression in tracheal system. (N) Genotypes are control (*btl-GAL4,
1137 tub-GAL80^{ts}, UAS-GFP / +*) versus *btl-GAL4, tub-GAL80^{ts}, UAS-GFP / UAS-hop* RNAi; (O)
1138 Genotypes are control versus *btl-GAL4, tub-GAL80^{ts}, UAS-GFP / UAS-Stat92E* RNAi. (P, Q) *Drosocin*
1139 qPCR of whole flies, transgene expression in fat body. (P) Genotypes are control (*ppl-GAL4, UAS-
1140 GFP / +*) versus *ppl-GAL4, UAS-GFP /+; UAS-hop* RNAi/+; (Q) Genotypes are control versus *ppl-
1141 GAL4, UAS-GFP/+; UAS-Stat92E* RNAi/+ . (R) Model of communication between hemocytes, fat body
1142 and respiratory epithelia, in which hemocytes act as sentinels of infection. Gram-negative bacteria that
1143 accumulate together with hemocytes in the reservoir between respiratory epithelia and fat body trigger
1144 activation of the Imd pathway through the peptidoglycan recognition protein PGRP-LC in hemocytes.
1145 Imd signaling drives induction of *upd3* expression in hemocytes, leading to Upd3 secretion. Upd3
1146 activates Jak/Stat signaling in adjacent domains of the fat body and the respiratory epithelia,
1147 contributing directly or indirectly to the induction of *Drosocin* expression.

1148 **Figure 8. *Drosocin* silencing in respiratory epithelia and fat body decreases animal survival after**
1149 **infection.**

1150 (A-D) Survival assays of adult female F1 progeny resulting from crosses of GAL4 drivers with the
1151 following transgenic lines were analyzed: *UAS-Drosocin RNAi* lines (D1, D2) or controls (γ^w (YW)),

1152 *w¹¹¹⁸* (W1118)). Mutant strains *Rel^{E20}* (R) and *spz^{rm7}* (S) served as controls. Figure displays one out of
1153 3 comparable biological replicate experiments; in each experiment, for each genotype and condition 40
1154 to 60 females were assessed; p- values log-rank (Mantel-Cox) test, *, **, ***, or **** corresponding to
1155 $p \leq 0.05$, 0.01, 0.001, or 0.0001, respectively; upper symbol corresponds to comparison *w1118* versus
1156 *Dro* RNAi D1; lower symbol corresponds to comparison of crosses of *yw* versus *Dro* RNAi D2. 5 day
1157 old female flies were treated as follows and then incubated at 29°C. (A-D) *Ubi-GAL4* crosses, (A) *E.*
1158 *coli* (OD6, 9.2 nl); (B) sterile PBS injection (9.2nl); (C) uninjected control; (D) *E. cloacae* (OD4,
1159 9.2nl). Note that *Drosocin* knockdown caused significantly reduced survival after gram-negative
1160 infection. After PBS injection, survival was significantly reduced for D1 in 2 out of 3 replicate
1161 experiments, for D2 in 3 out of 3 replicate experiments. (E-G) *HmlΔ-GAL4* crosses, (E) *E. coli* (OD6,
1162 9.2 nl); (F) sterile PBS injection (9.2nl); (G) uninjected control. (H-J) *btl-GAL4* crosses, (H) *E. coli*
1163 (OD6, 9.2 nl); (I) sterile PBS injection (9.2nl); (J) uninjected control. (K-M) *ppl-GAL4* crosses, (K) *E.*
1164 *coli* (OD6, 9.2 nl); (L) sterile PBS injection (9.2nl); (M) uninjected control. Note that ubiquitous
1165 *Drosocin* knockdown caused significantly reduced survival after gram-negative infection (A, D). After
1166 PBS injection, survival was significantly reduced (B), for D1 in 2 out of 3 replicate experiments, for
1167 D2 in 3 out of 3 replicate experiments. *Drosocin* knockdown in tracheal system or fat body caused
1168 significantly reduced survival after gram-negative infection (H, K). *Drosocin* knockdown in the
1169 tracheal system or fat body showed a partially penetrant effect on survival after PBS injection (I, L); in
1170 tracheal system knockdown, survival was significantly reduced for D1 in 0 out of 3 replicate
1171 experiments, for D2 in 2 out of 3 replicate experiments. For fat body knockdown, survival was
1172 significantly reduced for D1 in 1 out of 3 replicate experiments, for D2 in 1 out of 3 replicate
1173 experiments. As expected, survival is not affected when *Drosocin* is silenced in hemocytes (E, F, G).

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1176 **Supplemental Information**

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1178 **Supplemental Experimental Procedures**

1179 ***Drosophila* strains and fly husbandry**

1180 *Canton S*, *w¹¹¹⁸*, or *yw* were used as control strains to match the background of experiment crosses.
1181 Transgenic lines and mutants used were *HmlΔ-GAL4* (Sinenko and Mathey-Prevot, 2004), *HmlΔ-*
1182 *DsRed* (Makhijani et al 2011), *HmlΔ-DsRednls* (Makhijani et al 2011), *srpHemo-GAL4* (Brückner et al.
1183 2004), *srpGal4,UAS-lifeact-GFP* (from B. Stramer), *UAS-CD4-GFP; srpD-GAL4* (from M. Meister),
1184 *btl-GAL4,UAS-GFP* (Rao et al., 2015) (from P. Rao), *ppl-GAL4* (Bloomington 58768), *Ubi-GAL4/Cyo*
1185 (Bloomington 32551). *UAS-GFP* (Song et al., 2007), *UAS-CD8GFP* (from L. Kockel), *UAS-srcEGFP*
1186 (from E. Spana), *UAS-Drosocin RNAi* (VDRC 42503, GD line on chr. 2, here labeled ‘D1’), *UAS-*
1187 *Drosocin-RNAi* (VDRC 105251, KK line line on chr. 2, here labeled ‘D2’), *UAS-imd RNAi/TM3, sb¹*
1188 (Bloomington 38933), *UAS-PGRP-LC RNAi* (Bloomington 33383), *UAS-upd3 RNAi* (Bloomington
1189 28575), *UAS-hop RNAi* (Bloomington 31319), *UAS-Stat92E RNAi* (Bloomington 31317) (all
1190 Bloomington TRiP RNAi lines correspond to
1191 *y[1] v[1]; P{y[+t.7.7]v[+t.1.8]=TRiP.insertion}attP2*), *UAS-imd* (Georgel et al., 2001) (from N.
1192 Buchon), *UAS-upd3* (from E. Bach), *UAS-hop^{TumL}* (Harrison et al., 1995) (from E. Bach), *UAS-3HA-*
1193 *Stat92E; UAS-3HA-Stat92E^{ΔNΔC}* (Ekas et al., 2010) (from E. Bach), *tub-GAL80^{ts}* (McGuire et al., 2003)
1194 (Bloomington 7018), *UAS-hid;UAS-rpr* (Zhou et al., 1995),
1195 *w¹¹¹⁸;HmlΔFucciOrange^{G1};HmlΔFucciGreen^{G2/S/M}* (K. J Woodcock and F. Geissmann, see below);
1196 *Drosocin-GFP* (Tzou et al., 2000) (from B. Lemaitre), *Rel^{E20}* (Leulier et al., 2000) (from B. Lemaitre),
1197 *spz^{rm7}* (De Gregorio et al., 2002) (from B. Lemaitre). Lines for hemocyte MARCM *hsflp,UAS-GFP;*
1198 *HmlΔ-GAL4; FRT82B, tub-GAL80*, and *HmlΔ-GAL4, UAS-GFP; FRT82B* (Makhijani et al 2011)
1199 (experiment), and *HmlΔ-GAL4, UAS-GFP* (control). Lines for PermaTwin MARCM *w; FRT40A,*

1200 *UAS-CD8-GFP, UAS-CD2-Mir/Cyo; actGAL4, UAS-flp/TM6B* and *w; FRT40A, UAS-CD2-RFP, UAS-*
1201 *GFP-Mir/Cyo; tub-GAL80^{ts} /TM6B* (Fernandez-Hernandez et al., 2013). Lines for lacZ lineage tracing:
1202 *tub-GAL80^{ts}; UAS-Flp* and *srpHemo-GAL4, UAS-srcEGFP; Act>stop>nuc-lacZ* (Makhijani et al.,
1203 2011), or *HmlΔ-GAL4, UAS-GFP* and *UAS-Flp; tub-GAL80^{ts} /CyO wee p; act>stop>lacZnls* (kind
1204 gift from P. Rao, Kornberg lab). Recombinant chromosomes and combinations of transgenes were
1205 generated by standard genetic techniques. Unless stated otherwise, all genetic crosses were kept at
1206 25°C. Flies were raised on a standard dextrose cornmeal diet supplemented with dry yeast.

1207 **HmlΔFucci transgenic lines**

1208 To generate *HmlΔFucci-G1-Orange* and *HmlΔFucciGreenS/G2/M-Green* constructs, Fucci cloning
1209 vectors (MBL laboratories, AM-V9014 and AM-V9001) were both digested with HindIII and BamHI
1210 in order to excise the insert Fucci fluorescence genes; *Fucci-G1 Orange* and *Fucci-S/G2/M Green*. A
1211 previously cloned *p-Red H-Stinger-HmlΔ-DsRed* plasmid (Clark et al., 2011) was digested with
1212 BamHI and SpeI in order to excise *DsRed* whilst leaving the *HmlΔ* promoter in place. T4 DNA
1213 polymerase was used to blunt the *p-Red H-Stinger-HmlΔ* vector and the *Fucci* DNA inserts. Vector and
1214 *Fucci* insert DNA were then digested again with BamHI for a sticky-blunt ligation (Takara Bio Inc -
1215 TaKaRa DNA Ligation Kit LONG). Cloning was done according to standard procedures. The separate
1216 *HmlΔFucci-G1-Orange* and *HmlΔFucciGreenS/G2/M-Green* constructs were injected into *w¹¹¹⁸*
1217 *Drosophila* embryos to generate transgenic lines (BestGene Inc.). Plasmid maps and more detailed
1218 cloning information are available upon request.

1219 **Flipout-LacZ lineage tracing**

1220 Flipout-lacZ lineage tracing was essentially done as described by (Weigmann and Cohen, 1999).
1221 Lineage tracing of embryonic hemocytes was described previously in (Makhijani et al., 2011). F1
1222 progeny of the following cross were used *tub-GAL80^{ts}; UAS-Flp x srpHemo-GAL4, UAS-srcEGFP;*
1223 *Act>stop>nuc-lacZ*. To obtain specific lacZ labeling of embryonic hemocytes, expression of *srpHemo-*

1224 *GAL4* through *tub-GAL80^{ts}* was controlled by shifting 5 hour egg collections from 18°C to the
1225 permissive temperature of 31°C for 6 hours and then back to 18° C until pupal or adult stage. The no
1226 heat shock control was continuously maintained at of 18°C. For lineage tracing of embryonic-lineage
1227 hemocytes from the larva, F1 progeny of the following cross were used *HmlΔ-GAL4*, *UAS-GFP* *x*
1228 *UAS-Flp*; *tub-GAL80^{ts}* / *CyO wee p*; *act>stop>lacZnls*. Heat shock at 29°C was administered from 0-
1229 48h AEL, and animals were then maintained at 18°C for the remainder of their life. This time window
1230 was chosen to avoid labeling of lymph gland-lineage hemocytes that express the *HmlΔ-GAL4* driver
1231 from a later point during larval development. βGal positive hemocytes, relative to all Crq positive
1232 plasmatocytes, were determined by immunostaining of ex vivo released hemocytes at two points, (1)
1233 from 3rd instar larva before mobilization of differentiated lymph gland hemocytes takes place, and (2)
1234 from adult animals at 12 days post eclosure. The relative fraction of labeled hemocytes in the adult
1235 compared to the larva was calculated from the average and standard deviation from 16 adult females.

1236 **Fucci analysis and quantification of hemocytes**

1237 Fucci analysis, imaging and external hemocyte counts for Fig. 3D and 5C-F' were performed using a
1238 Leica SP5 confocal microscope. Flies were affixed dorsally to glass cover slips with superglue
1239 (Loctite) Sf435746 and imaged in vivo using 20x Dry NA 0.5 or 40x Oil NA 1.25 objectives. Cohorts
1240 of 15-21 flies per condition were imaged, and images were processed using Fiji and Imaris software.
1241 Cell counts were performed using the MATLAB spot detection function in Imaris, calculating the
1242 mean±SEM, and performing an unpaired t test to calculate statistical significance. For bacterial
1243 infections related to Fucci analyses and hemocyte counts, individual *E. coli*, *M. luteus*, or *E. cloacae*
1244 cultures were grown at 37°C overnight. The following morning, cultures were centrifuged at 4°C at
1245 1600rpm for 10 minutes. Bacterial pellets were re-suspended in PBS to a final OD600 of 1. Injection
1246 was performed using a PicospritzerR III (Parker Hannifin), and the injection volume was calibrated to

1247 50nl by injecting a drop into a plot of oil. Adult males 7 days post eclosion were used for injections of
1248 bacteria, sterile PBS, and non-injected controls.

1249 **Hemocyte MARCM**

1250 We developed Hemocyte MARCM as a derivation of the previously described MARCM (Lee and Luo,
1251 1999), a labeling method based on mitotic recombination. The ubiquitous *tub-GAL4* driver was
1252 replaced with *HmlΔ-GAL4* to allow specific GFP labeling of all dividing cells that would ultimately
1253 show Hml+ plasmatocyte characteristics (Makhijani et al., 2011). Genotypes of F1 progeny were as
1254 follows: Hemocyte MARCM: *hsflp,UAS-CD8GFP/ +; HmlΔ-GAL4/ HmlΔ-GAL4, UAS-GFP;*
1255 *FRT82B, tub-GAL80/ FRT82B*. Control: *hsflp, UAS-CD8GFP/ +; HmlΔ-GAL4/ HmlΔ-GAL4, UAS-*
1256 *GFP; FRT82B, tub-GAL80/ +*. Various heat shock and incubation schemes were tested to optimize
1257 Hemocyte MARCM labeling, using 1-2 week old adult progeny. Heatshock was induced at 37°C for
1258 1h 3 times/day for 3 days; 30°C continuously for 4 days; 32°C continuously for 4 days. Following heat
1259 shock schemes, flies were maintained at room temperature and observed under a fluorescence
1260 stereomicroscope for GFP labeled hemocytes. The control cross lacks one of the FRT82B chromosome
1261 and therefore does not allow Flp-induced recombination to occur. Progeny from control crosses was
1262 also exposed to heat shock, allowing to observe non-specific GFP labeling due to heat shock
1263 conditions and imbalance of GAL4 and GAL80, and/or occasional random recombination events.
1264 Groups of 12-18 animals (equal mix of males and females) were examined per condition. For immune
1265 challenges, 4-6 days old flies were infected with a mixture of *E. coli* and *M. luteus*, by pricking flies in
1266 the thorax with a needle dipped in a bacterial pellet (mix of equal OD600 from overnight cultures of *E.*
1267 *coli* and *M. luteus*).

1268 **PermaTwin MARCM**

1269 For PermaTwin MARCM, genotypes were essentially as described in (Fernandez-Hernandez et al.,
1270 2013). F1 progeny of the following cross were used: *w; FRT40A, UAS-CD8-GFP, UAS-CD2-Mir/Cyo;*

1271 *actGAL4, UAS-flp/TM6B* x *w; FRT40A, UAS-CD2-RFP, UAS-GFP-Mir/Cyo; tub-GAL80^{ts}/TM6B*.
1272 Flies were raised at 18°C until adulthood to repress *flp* expression. Adult progeny of different ages (3-5
1273 days and 2-3 weeks, n=12/age and induction condition) were examined. Animals were shifted to 29°C
1274 for varying time windows of 4 days, 8 days, 2 weeks, 3 weeks, respectively, and observed by live
1275 imaging for appearance of GFP and RFP labeled hemocytes; control tissues with labeled clones were
1276 dissected and observed in parallel. Adults were also dissected in PBS, to examine for hemocyte
1277 populations that might be attached to internal tissues. Negative control flies were maintained at 18°C
1278 continuously. In order to test the effect of immune challenge on hemocyte proliferation in adults, flies
1279 of the above age and genotypes were infected with either *E. coli* (OD 3) or *M.luteus* (OD 1.5 or 2)
1280 and observed 2-8 days post-infection.

1281 **Quantification of AMP expression by qPCR**

1282 For qPCR analysis, total RNA was harvested from 10 pooled adult female *Drosophila* for each
1283 experimental condition. For qPCR on split fly samples, 8 pooled females for each condition were snap
1284 frozen in LN2, and for each fly the head/thorax and abdomen were separated using a scalpel on a metal
1285 block on dry ice. Frozen body parts or whole flies were pestle-homogenized in 500 µl Trizol reagent
1286 (Millipore), and RNA was purified using a Direct-zol RNA Miniprep kit (Zymo Research). RNA was
1287 quantified and quality checked with a NanoDrop 2000c spectrophotometer (Thermo Scientific). 1 µl of
1288 total RNA was used in a 20 µl iScript cDNA Synthesis (BioRad) reverse transcription reaction. qPCR
1289 was carried out on a CFX96 c1000 Thermal Cycler (BioRad) using IQ SYBR Green Super Mix
1290 (BioRad) for *Drosocin*, *Drosomycin*, *Diptericin*, *Cecropin A1*, and *Attacin A*. Relative mRNA
1291 expression levels were normalized to that of *RpL32*; for primers see Supplemental Table 1. Results are
1292 presented as the mean and standard deviation of at least three (in rare cases two) biological repeats.

1293

1294

1295 **Supplemental Table 1.** Primer sequences.

Target	Forward	Reverse
Drosocin	CCATCGTTTTCTGCT	CTTGAGTCAGGTGATCC
Drosomycin	CGTGAGAACCTTTTCCAATAT	TCCCAGGACCACCAGCAT
Diptericin	GCTGCGCAATCGCTTCTACT	TGGTGGAGTGGGCTTCATG
Cecropin A1	GAACCTTCTACAACATCTTCGT	TCCCAGTCCCTGGATT
Attacin A	CCCGGAGTGAAGGATG	GTTGCTGTGCGTCAAG
RpL32	GACGCTTCAAGGGACAGTATC	AAACGCGGTTCTGCATGAG
Drosocin - 2	CGTTTTTGCCATGGGTGT	TTGAGTCAGGTGATCCTCGAT
RpL32 - 2	GTTCGATCCGTAACCGATGT	ATGCTAAGCTGTGACACAAATG

1296

1297

1298 **Supplemental Figures**

1299 **Supplemental Figure 1. The respiratory reservoir of hemocytes, and hemocyte clusters at the**
1300 **ostia of the heart.**

1301 (A) *Drosophila* adult, live imaging *HmlΔ-GAL4, UAS-GFP* (hemocytes red pseudocolor to match
1302 model), anterior up. (B) Cross section through the anterior abdomen, *HmlΔ-DsRednls* (hemocytes, red),
1303 phalloidin blue, dorsal up. (C) Thorax of adult *Drosophila*, live imaging of intact adult *HmlΔ-*
1304 *DsRednls/btl-GAL4, UAS-GFP* (hemocytes red, tracheal system green); arrowhead points to scutellum
1305 where hemocytes accumulate along the respiratory epithelia. (D) Schematics of heart (grey),
1306 respiratory epithelia of the thorax and head and other parts of the tracheal system (blue); associated
1307 hemocytes (red). Dashed lines indicate section shown in B, and area shown in C, arrowhead points to
1308 corresponding area in (C).

1309 **Supplemental Figure 2. Hemocytes are stationary; increased expression of *Hml* and *Crq* post**
1310 **infection.**

1311 (A-C) Time course of resident hemocyte pattern, live imaging of representative animal, genotype is
1312 *Hml* Δ -*GAL4*, *UAS-GFP*; day 5, 7, 10 post eclosure.

1313 **Supplemental Figure 3. Bioparticle accumulation at the respiratory epithelia.**

1314 (A-C) Confocal imaging of respiratory epithelia, hemocytes, and fluorescent bioparticles in dissected
1315 head preparations. (A) Control genotype *Hml-GAL4*, *UAS-GFP*+, no bioparticle injection; hemocytes
1316 green, respiratory epithelia blue. (B) Hemocyte ablation genotype *Hml-GAL4*, *UAS-GFP/UAS-rpr*;
1317 *UAS-hid*+, *E. coli* (K-12 strain) bioparticles, TexasRed, injected (1mg/100ml PBS; 32.2nl);
1318 hemocytes green (absent), bioparticles red, respiratory epithelia blue. (C) Control genotype *Hml-GAL4*,
1319 *UAS-GFP*+, pHrodo red *E. coli* bioparticles injected (1mg/100 μ L PBS; 32.2nl); hemocytes green,
1320 bioparticles red, respiratory epithelia blue.

1321 **Supplemental Figure. 4. No significant new production of hemocytes in adult *Drosophila*.**

1322 (A-C) MARCM clonal labeling to detect any dividing cells that eventually give rise to hemocytes. (A)
1323 genotypes of F1 progeny used; (B) titration of conditions for MARCM induction; (C) at 30°C 4 days
1324 continuous induction, experiment animals show at best an average of 6 labeled hemocytes over
1325 background; this number is not enhanced upon bacterial infection. (D, E) PermaTwin 2-color clonal
1326 analysis to search for dividing cells that would give rise to hemocytes; genotype of F1 progeny used is
1327 *w*¹¹¹⁸; *FRT40A*, *UAS-CD8-GFP*, *UAS-CD2-Mir/ FRT40A*, *UAS-CD2-RFP*, *UAS-GFP-Mir*; *actGAL4*,
1328 *UAS-flp/ tub-GAL80^{ts}*. (D) Adult fly after 5 days of heat shock induction and infection with *M. luteus*;
1329 arrowhead points to thorax and head, which show no sign of hemocyte labeling. Representative images
1330 were taken from 3 independent biological repeats (n=8/repeat) of various time points and infection
1331 conditions. (E) positive control, dissected gut showing PermaTwin clones. (F) Comparison of labeling
1332 efficiency of *srpGal4*, *UAS-lifeact-GFP* and anti-Srp antibody staining, day 3 and 11 animals were

1333 examined. (G-I) Examples of hemocytes with EdU positive inclusions. Careful examination and
1334 counterstaining with DAPI revealed that all EdU labeled elements correspond to phagocytic vesicles
1335 (arrowheads); hemocyte nuclei always remained EdU negative (open arrowhead).

1336 **Supplemental Figure 5. AMP gene expression depends on hemocytes; Imd signaling in**
1337 **hemocytes.**

1338 (A, B) Live images of hemocyte-ablated animals and controls, hemocytes in green; genotypes are
1339 *HmlΔ-GAL4,UAS-GFP/+* (control, animal on left side) and *w; HmlΔ-GAL4,UAS-GFP/ UAS-rpr; UAS-*
1340 *hid/+* (hemocyte ablation, animal on right side). (A) Larvae; (B) adults, lateral view. (C-D) *Drosocin*
1341 qPCR of whole flies, *imd* RNAi silencing in hemocytes. Genotypes are *HmlΔ-GAL4, UAS-GFP/+;*
1342 *UAS-imd RNAi/+* (experiment) and *HmlΔ-GAL4, UAS-GFP/+* (control). 5-6 day old adult females
1343 were left untreated (control) or injected with PBS or *E.coli* in PBS (OD 3), 27.6nl. Flies were analyzed
1344 at 24h (C) and 48h (D) post infection. Each chart displays mean and SEM of samples from a
1345 representative biological replicate experiment, using pools of 10 females per condition, and triplicate
1346 qPCR runs. (E) *Drosocin* qPCR of whole flies, *Imd* overexpression in hemocytes, genotypes are
1347 control *HmlΔ-GAL4, UAS-GFP/+* versus *HmlΔ-GAL4, UAS-GFP/ UAS-imd*; 5 day old adult females
1348 were left untreated (control) or injected with PBS or of *E.coli* in PBS (OD 3), 27.6nl. Each chart
1349 displays mean and SEM of samples from a representative biological replicate experiment, using pools
1350 of 10 females per condition, and triplicate qPCR runs.

1351 **Supplemental Figure 6. *Drosocin* expression in respiratory epithelia and fat body, and the role of**
1352 **Jak/Stat signaling.**

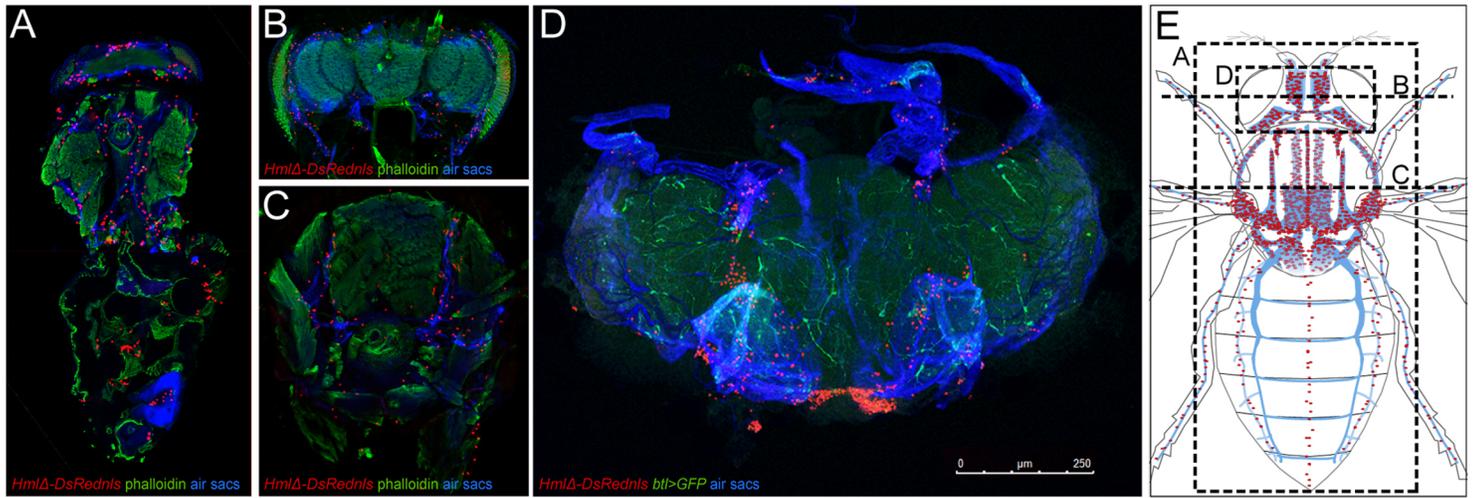
1353 (A-B) *Drosocin-GFP* expression is found in head and thorax, independent of the site of infection. (A)
1354 *Drosocin-GFP* 2 days post thorax injection (*E. coli*), respiratory epithelia (air sacs, blue); (B)
1355 *Drosocin-GFP* 2 days post abdomen injection (*E. coli*), respiratory epithelia (air sacs, blue). (C-C')
1356 Anatomy of fat body tissue lining the respiratory epithelia and hemocytes; *HmlΔ-DsRednls* (hemocytes,

1357 red), fat body (LipidTOX, green), respiratory epithelia (air sacs, blue) (C) Head cross section. (C')
1358 Closeup of region indicated in (C). Note that hemocytes are layered between respiratory epithelia and
1359 fat body. (D) *Drosocin* qPCR of whole flies, ubiquitous RNAi knockdown of *Drosocin*; genotypes are
1360 *Ubi-GAL4/UAS-Drosocin* RNAi line D1 (GD) or *Ubi-GAL4/UAS-Drosocin* RNAi line D2 (KK) or
1361 control *Ubi-GAL4/+* (cross with *yw* or *w¹¹¹⁸*). 5 day-old females were left untreated or injected with
1362 PBS or *E. coli* in PBS (OD 6), 9.2nl, and harvested at 6, 12, 24 h post infection. Chart displays mean
1363 and SEM of samples from a representative biological replicate experiment, using pools of 10 females
1364 per condition, and triplicate qPCR runs. *Drosocin* kd efficiency is at 6h=98.2%, 12h=98.8%,
1365 24h=98.9%. (E-G) qPCR of *Drosocin* in whole adult flies upon overexpression (OE) of Jak/Stat
1366 signaling components in the tracheal system or fat body. *Drosophila* untreated, injected with PBS, or
1367 with of *E.coli* in PBS (OD 6), 9.2 nl; flies were harvested at 6h post injection. Each chart displays the
1368 mean and CI of samples from 3 averaged biological replicate experiments, using pools of 10 females
1369 per condition, and triplicate qPCR runs for each sample. Values of all charts are displayed relative to
1370 the average RNA level induced by sterile PBS injections in control flies. Two-way ANOVA with
1371 Sidak's multiple comparison test was performed, *,**,***,or **** corresponding to $p \leq 0.05$, 0.01,
1372 0.001, or 0.0001, respectively (Prism). (E) *Drosocin* qPCR, genotype is control *btl-GAL4, tub-GAL80^{ts},*
1373 *UAS-GFP/+* versus *UAS-3HA-Stat92E /+; UAS-3HA-Stat92E^{ΔNΔC}/ btl-GAL4, tub-GAL80^{ts}, UAS-GFP*
1374 (G-H) *Drosocin* qPCR, (G) genotype is control (*ppl-GAL4, UAS-GFP/+*) versus *ppl-GAL4, UAS-*
1375 *GFP/ UAS-hop^{TumL}*; (H) genotype is control versus *UAS-3HA-Stat92E/ ppl-GAL4, UAS-GFP; UAS-*
1376 *3HA-Stat92E^{ΔNΔC}./+.*

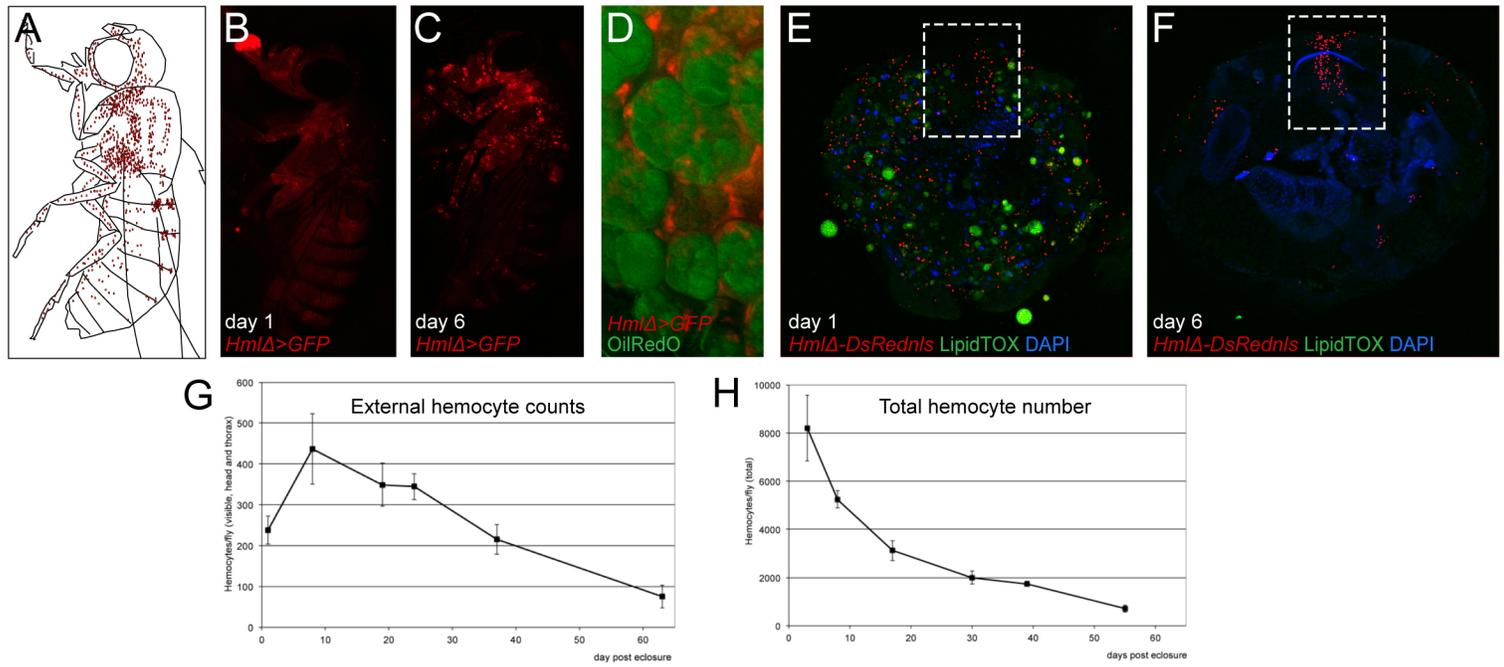
1377 **Supplemental Figure 7. Endogenous *Drosocin* promotes survival after infection; *Drosocin* is not**
1378 **required for induction of other AMPs after infection.**

1379 (A-D) Expression of AMP genes in the background of ubiquitous *Drosocin* silencing. Genotypes are
1380 *Ubi-Gal4/UAS-Drosocin* RNAi D1 and *Ubi-Gal4/UAS-Drosocin* RNAi D2, and control *Ubi-GAL4/+*

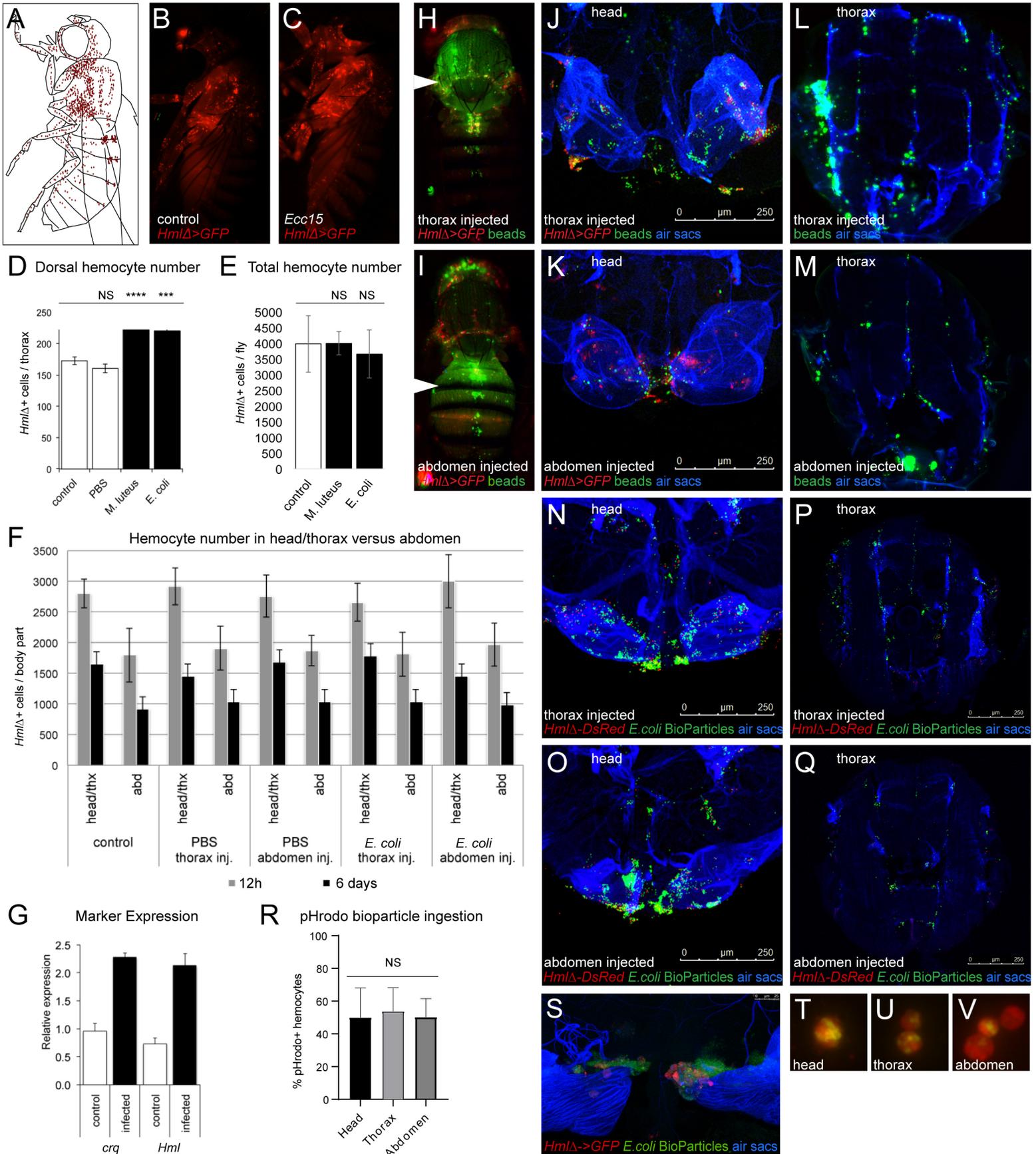
1381 cross with *w¹¹¹⁸*. Conditions are uninjured controls, injection of PBS and injection of *E. coli* in PBS
1382 (OD 6), 9.2 nl, with time points 6h and 12h post injection as indicated. Each chart displays the log₂
1383 mean and SEM of samples derived from pools of 10 females per condition, and triplicate qPCR runs.
1384 AMP genes quantified by qPCR for expression are (A) *Drosocin*; (B) *Attacin A*; (C) *Cecropin A1*; (D)
1385 *Diptericin*. The log₂ mean is displayed to illustrate subtle expression changes below baseline. Note
1386 that *Drosocin* knockdown does not affect the induction of other AMPs following gram-negative
1387 infection, although it may mildly affect expression levels of other AMPs in uninjected condition, and
1388 under sterile injury (PBS injection).



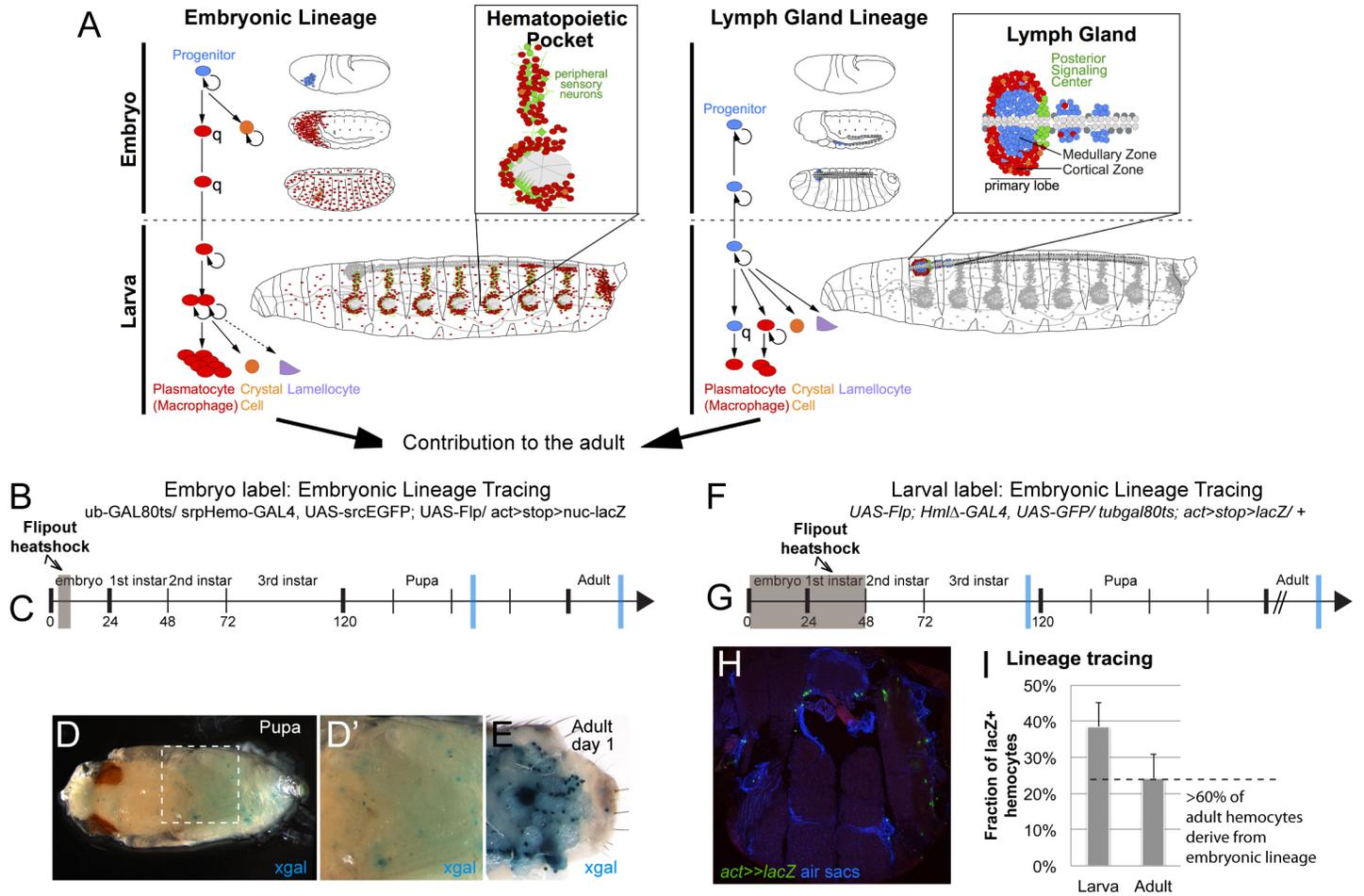
Sanchez Bosch et al. Figure 1



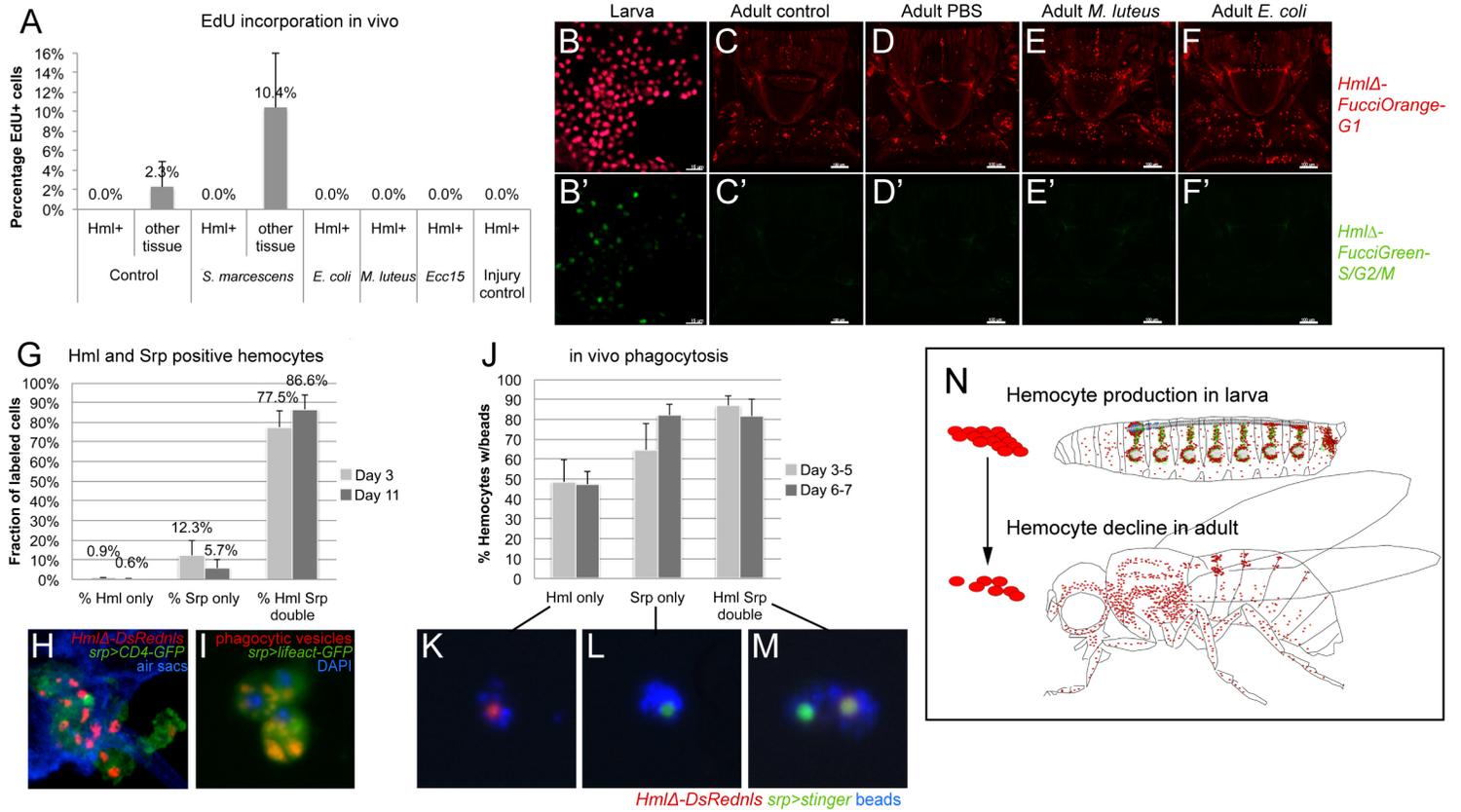
Sanchez Bosch et al. Figure 2



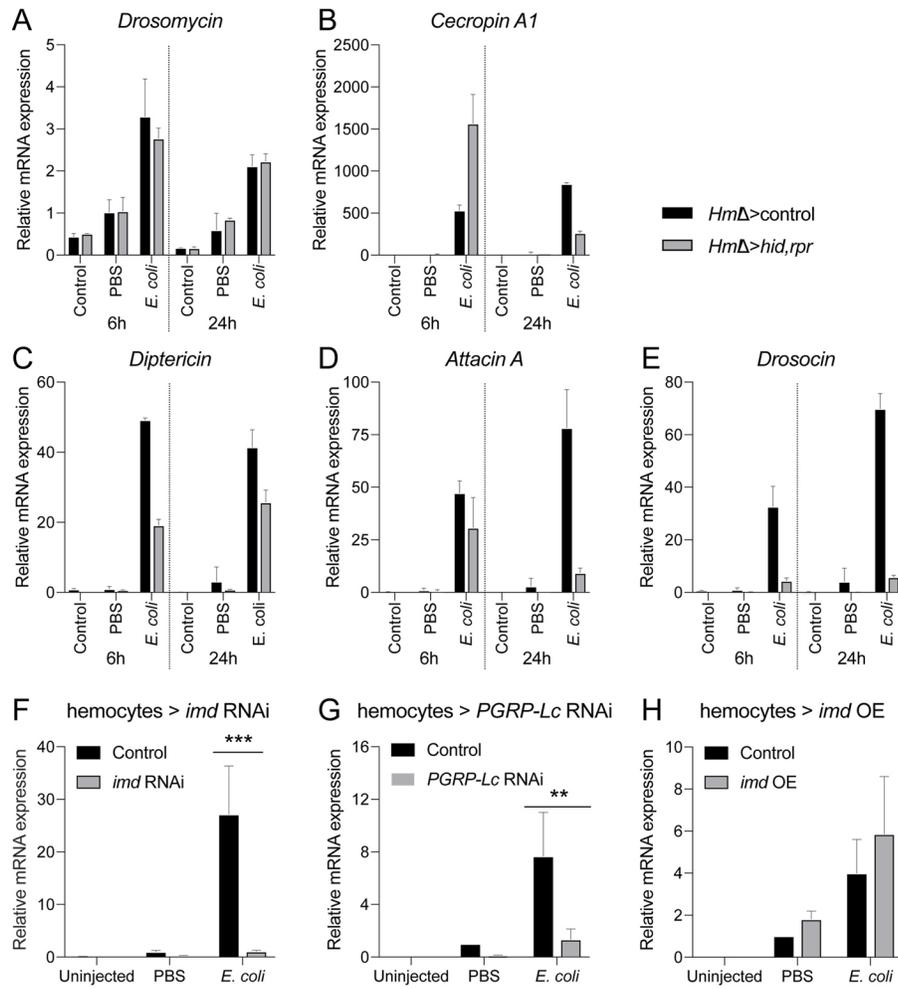
Sanchez Bosch et al. Figure 3



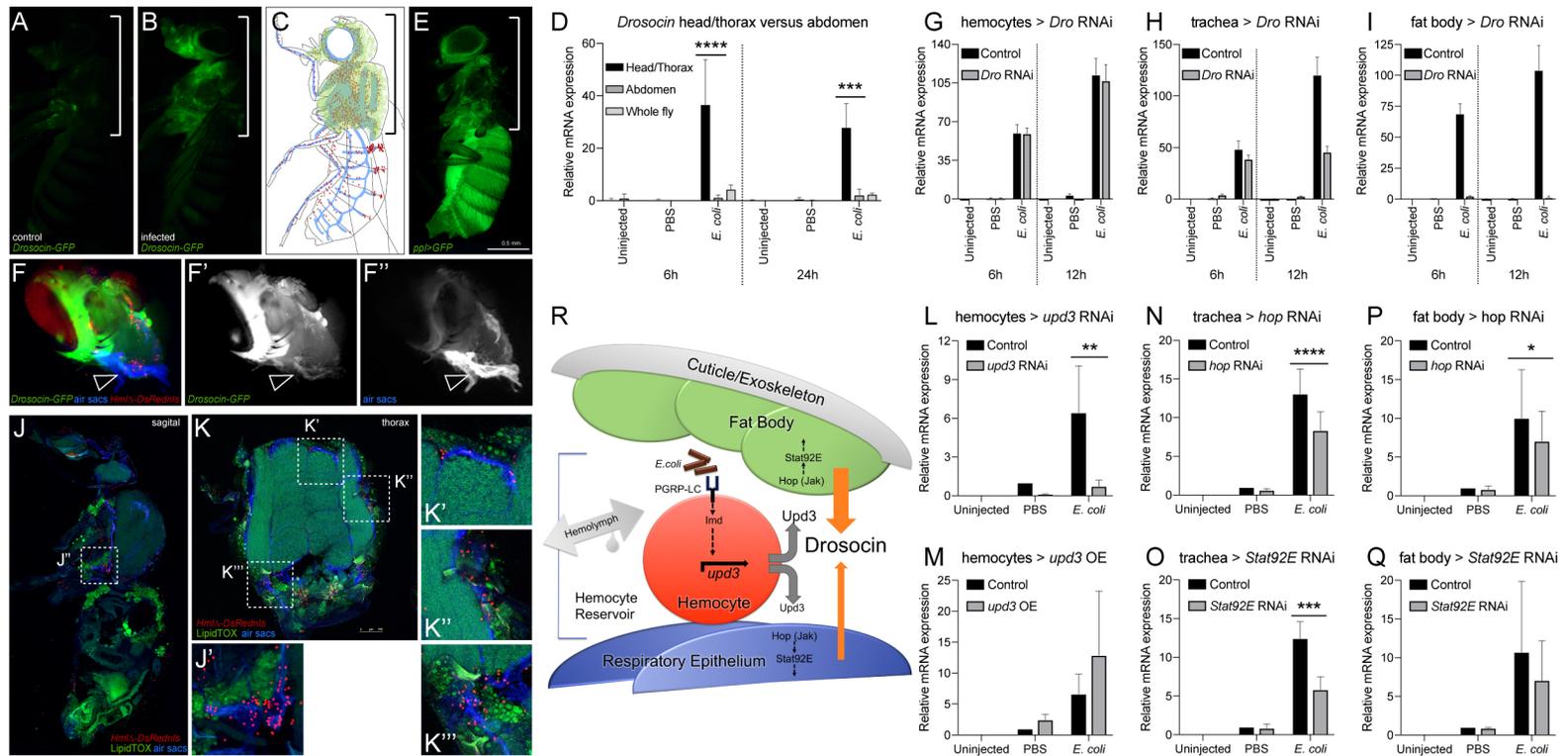
Sanchez Bosch et al. Figure 4



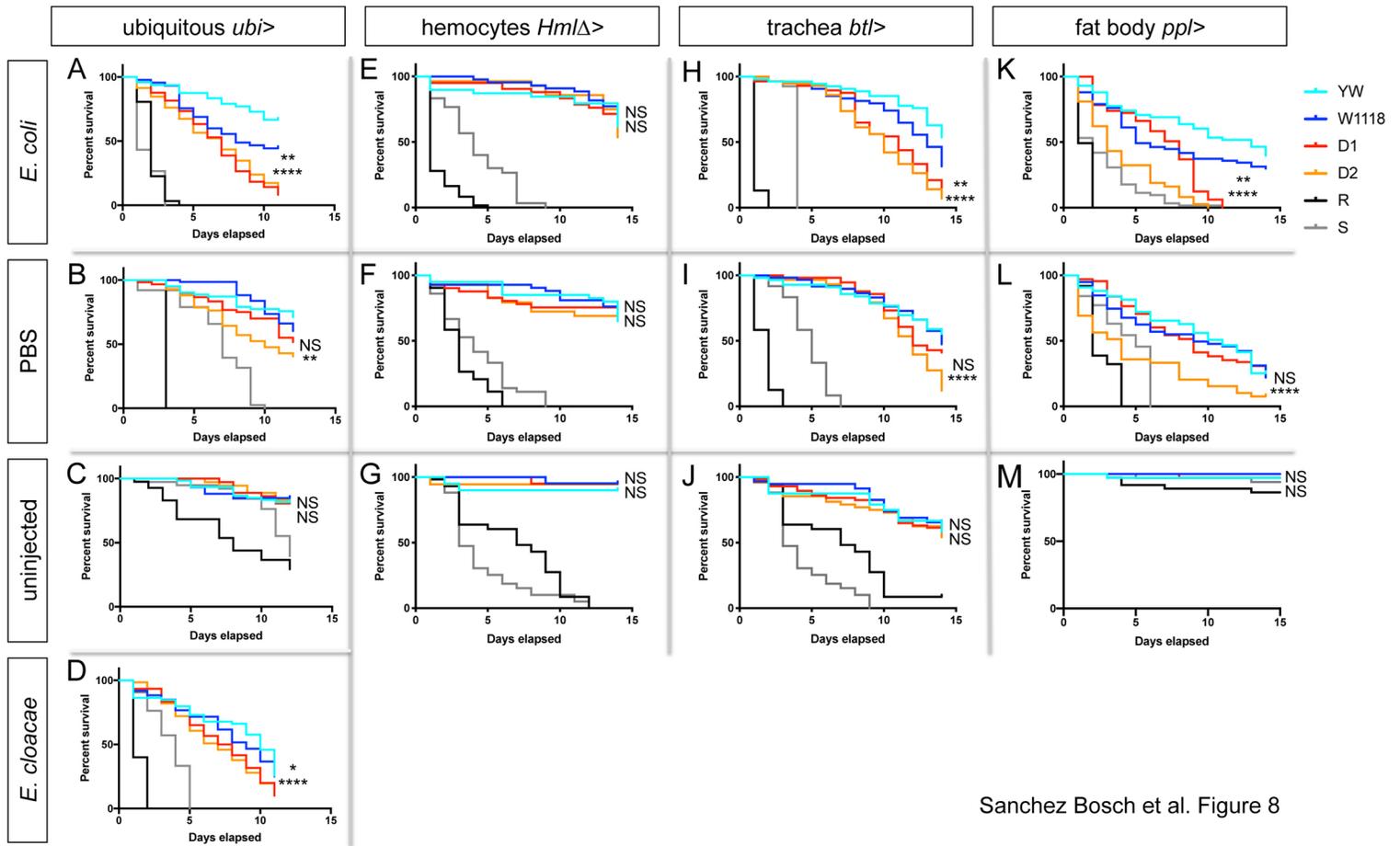
Sanchez Bosch et al. Figure 5



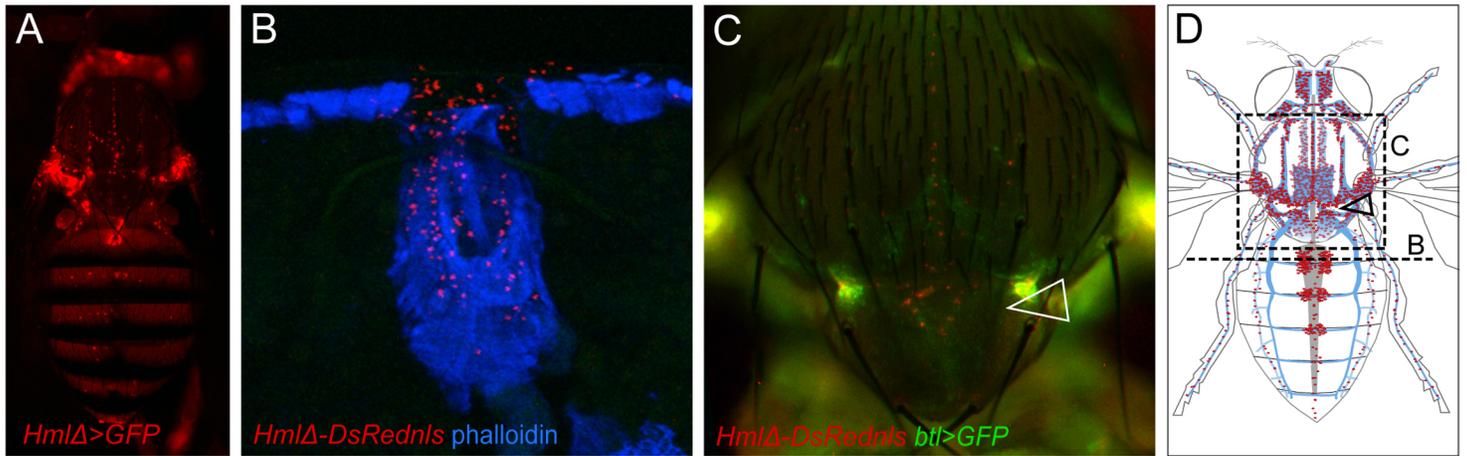
Sanchez Bosch et al. Figure 6



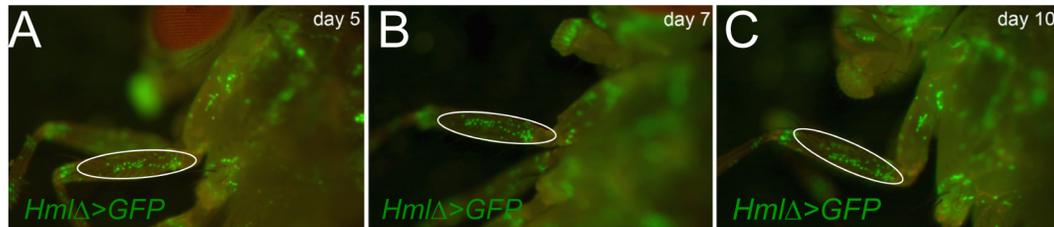
Sanchez Bosch et al. Figure 7



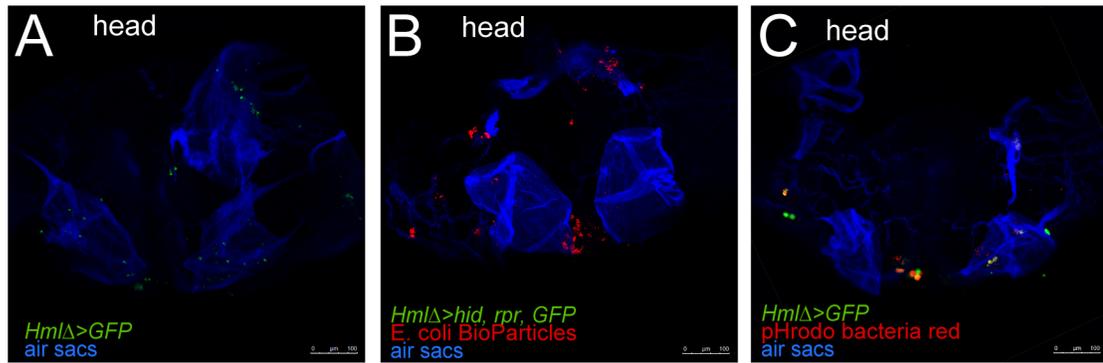
Sanchez Bosch et al. Figure 8



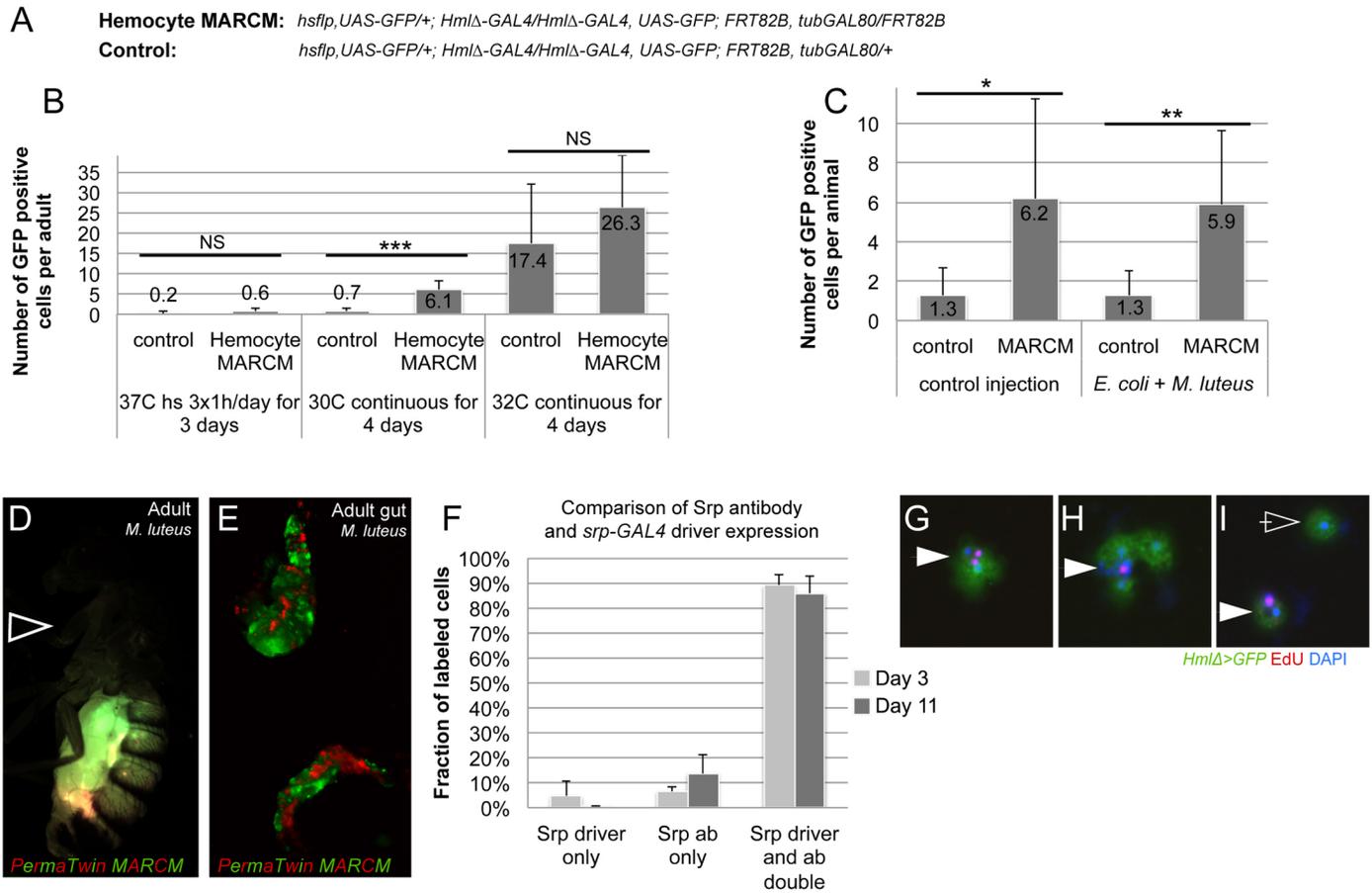
Sanchez Bosch et al. Supplemental Figure 1



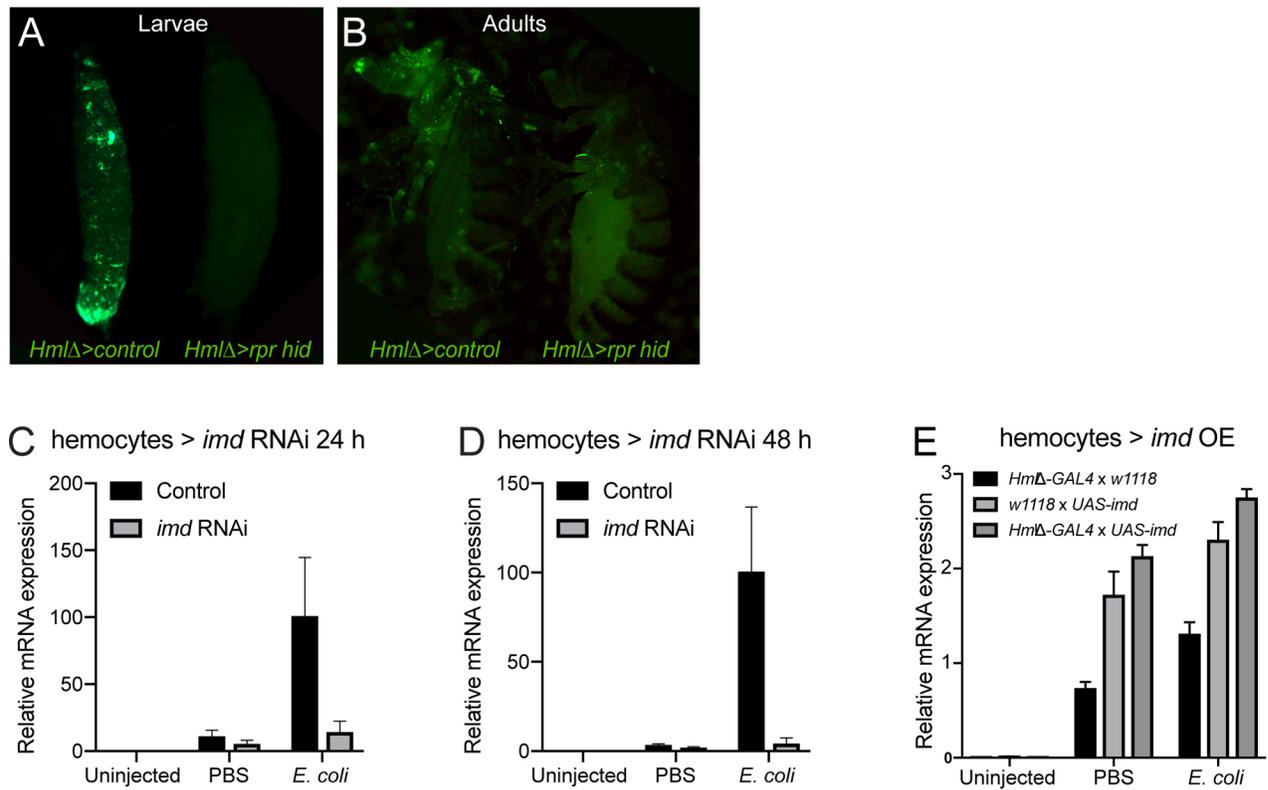
Sanchez Bosch et al. Supplemental Figure 2



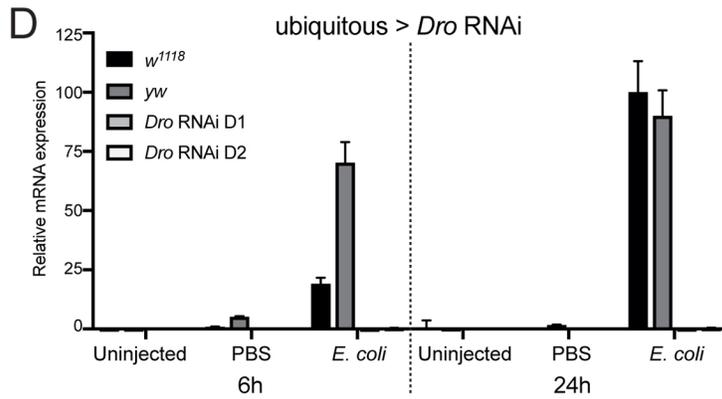
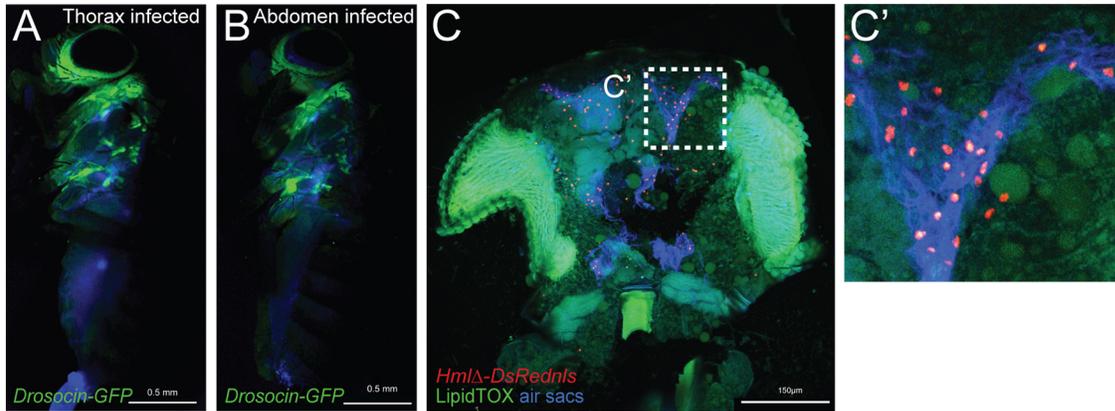
Sanchez Bosch et al. Supplemental Figure 3



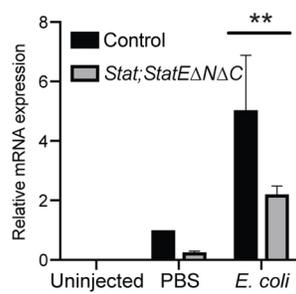
Sanchez Bosch et al. Supplemental Figure 4



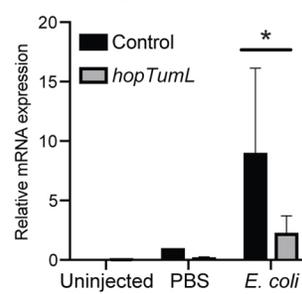
Sanchez Bosch et al. Supplemental Figure 5



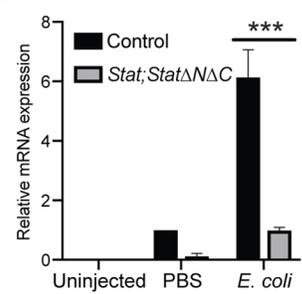
E trachea > *Stat;StatΔNΔC* OE



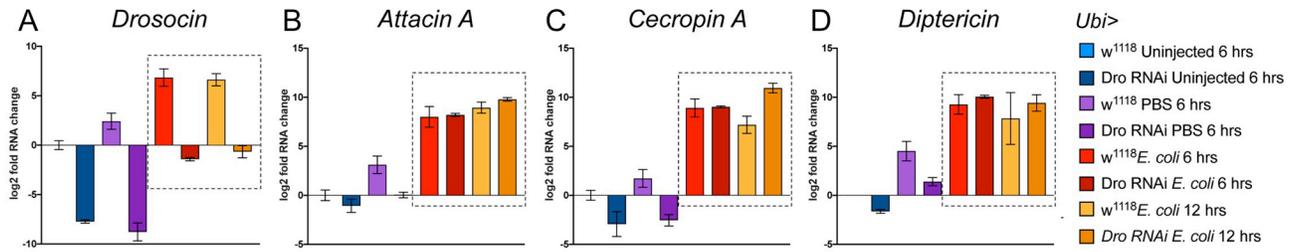
F fat body > *hopTumL* OE



G fat body > *Stat;StatΔNΔC* OE



Sanchez Bosch Supplemental Figure 6



Sanchez Bosch et al. Supplemental Figure 7