A humoral immune response to parasitoid wasps in *Drosophila* is regulated by JAK/STAT, NF-κB and GATA

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Abstract

The two arms of innate immunity consist of the cell-mediated cellular defenses and the systemic humoral immune responses. *Drosophila* humoral immune defenses in the context of antimicrobial immunity, particularly the regulation and activation of antimicrobial peptide secretion from the fat body, have been studied extensively. How *Drosophila* regulates humoral immunity against another major natural enemy, the parasitoid wasp, is less well-characterized. In this study, we focused on a gene crucial in anti-parasitoid immunity, *lectin-24A*, which is specifically induced following parasitization. We found that a fluorescent reporter driven by the region upstream of *lectin-24A* showed localized posterior expression in the larval fat body, the *Drosophila* tissue mediating humoral immunity. Furthermore, with RNA sequencing of the anterior and posterior fat body sections, we found that components of JAK/STAT, GATA, and Toll pathways were regulated differentially in the anterior-posterior axis of the fat body and/or by infection. Predicted binding motifs for transcription factors in all three of these pathways were identified in the 444bp upstream region of the *lectin-24A* gene, where scrambling these motifs leads to reduced basal or induced expression of the fluorescent reporter. Investigating each of these pathways, we found that JAK/STAT, the GATA factor Pannier, and the NF-κB factor dorsal all modulate the expression of *lectin-24A*. The binding motifs associated with these transcription factors were also enriched in the upstream sequences of parasitism-induced genes in the fat body. Taken together, these results indicate that JAK/STAT, Pannier, and NF-κB signaling are involved in the regulation of *lectin-24A* and, more generally, *Drosophila* humoral anti-parasitoid immunity after infection.

Introduction

Parasitoid wasps are major natural parasites of insects. In *Drosophila melanogaster*, the most common parasitoids attack larvae. Females of species such as *Leptopilina boulardi* insert an egg via their ovipositor inside the *Drosophila* larva, where the parasitoid larva, once emerged, feeds on host tissue during development. Without a successful immune response, the *Drosophila* host is eventually killed at the pupal stage. However, parasitization induces an anti-parasitoid defense in the larva termed melanotic encapsulation that involves both cellular and humoral immune responses [1–3]. *Drosophila* immune cells (hemocytes) are recruited to the parasitoid egg and surround it in a multi-layered cellular capsule [4–6]. This capsule then becomes melanized, killing the parasitoid [7].

Several humoral immune effectors have been identified in the context of humoral anti-parasitoid immunity in *Drosophila*. Humoral immunity is mediated by the *Drosophila* fat body, an insect-specific organ functionally similar to the mammalian liver, the metabolic hub of the organism. The invertebrate counterparts of the mammalian complement factors, called thioester-containing proteins (TEPs) [8], a group of serine proteases [9,10], and the fat body-derived peptide edin [11], are some of the secreted immune effectors shown to be involved in this response. We have previously also shown that a C-type lectin, lectin-24A, secreted by the larval fat body, plays an important role in this immune response [12]. The induction of *lectin-24A* expression following parasitism, controlled by the *cis*-regulatory upstream region of the gene, is associated with higher resistance to parasitoid infections [12]. Lectin-24A localizes to the surface of the parasitoid egg before hemocytes attach to it, suggesting that it may play a role in the recognition of the egg and subsequent recruitment of hemocytes.

Drosophila humoral immunity against bacterial and fungal infections has been investigated intensively in the past and is well characterized. Following recognition of pathogens, humoral defenses are induced via the activation of the two hallmark NF-κB immune signaling pathways, Toll and Imd (reviewed in [13] and [14]). These two pathways are known for their roles in regulating the secretion of many immune-related genes by the fat body, most famously antimicrobial peptides (AMPs). Dif and Dorsal are the two NF-κB family transcription factors (TFs) in the Toll pathway, which is mostly responsible for defense against Gram-positive bacteria, fungi and other pathogens. Relish is the transcription factor involved in Imd signaling, which mostly controls the defense against gram-negative bacteria. In some cases, a combination of the two pathways is responsible for the expression of immune effectors in the fat body [15,16]. Other than its role in the induction of AMP expression, the Toll pathway is also involved in regulating the proliferation of hemocytes in *Drosophila* larvae [17,18]. Loss-of-function mutations in the Toll pathway components result in reduced capacity to melanize parasitoids [13,17], whereas overactivation of the Toll pathway leads to a spontaneous melanization phenotype reminiscent of the melanotic encapsulation response against parasitoid wasps [13,17,19].

The Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway has been shown to be involved in the cellular immune response through the regulation of hemocyte differentiation following parasitoid infection [18]. Downregulation of JAK/STAT signaling in the anterior lobes of the larval lymph gland plays an important role in regulating the differentiation of lamellocytes, a specialist type of hemocyte for the encapsulation response [20,21]. It was also found that the activation of JAK/STAT signaling in somatic muscles by hemocyte-derived cytokines Upd2 and Upd3 releases energy stored in the muscles as glycogen for use in the immune response and is key to the immune response

100 against wasp infection [22,23]. Similar to the Toll pathway, overactivation of JAK/STAT
101 signaling in the whole larva also results in an over-proliferation of hemocytes and
102 spontaneous melanization [18,24,25].
103
104 A third group of transcription factors mediating *Drosophila* immunity is the GATA family of
105 transcription factors, a conserved group of TFs with zinc finger domains that recognize
106 variations of the GATA motif ((A/T) GATA (A/G)) (reviewed in [26]). *Drosophila* has a total
107 of five GATA factors: Pannier, Serpent, Grain, dGATAd, and dGATAe. Serpent is essential
108 for the differentiation of fat body and hemocytes [27,28], and Pannier has been found to play
109 an important role in hemocyte differentiation and maturation [29]. Strikingly, many immunity
110 genes expressed by the fat body exhibit Rel-Serpent synergy, where closely linked NF-κB
111 and GATA binding sites are found in the upstream regulatory region [30,31]. While Serpent is
112 the principal GATA factor regulating the antimicrobial response in the *Drosophila* fat body,
113 other GATA factors were reported to regulate immune responses in other *Drosophila* tissues
114 [31].
115
116 The anti-parasitoid humoral response is mainly driven by wasp parasite-associated molecular
117 patterns (PAMPs) [32], but the signaling pathways involved in the response remain unknown.
118 Previous studies of the transcriptional response to parasitoid attack in *Drosophila* larvae have
119 identified a series of genes that are differentially expressed in response to wasp oviposition
120 [33–36]. These studies have implicated the three pathways outlined above in the response
121 [33,35]. In particular, Wertheim et al. found that transcription factor binding motifs (TFBMs)
122 for NF-κB, STAT, and GATA significantly associate with genes differentially expressed after
123 parasitism [33]. However, these observations were not followed up experimentally. Previous
124 investigations have also focused on transcriptional changes in the whole larvae, where

differential expression in different tissues can confound the interpretation of the results, or in hemocytes, where transcriptional changes reflect mainly hemocyte differentiation [37–40]. Based on these studies, here we investigated the regulation of *Drosophila* humoral anti-parasitoid immunity mediated by the larval fat body. We focused on the *lectin-24A* gene, a key humoral immune effector in this response [12], and studied its induction with parasitization to understand the regulation of anti-parasitoid humoral immune mechanisms more broadly.

133 ## Results

134

135 **The upstream cis-regulatory region of the *lectin-24A* gene underlies a specific response**
136 **to parasitoid wasp infection**

137 We have previously found that the expression of *lectin-24A* increases resistance to
138 parasitoids, and the Drosophila Genetic Reference Panel (DGRP) line 437 carries a resistant
139 allele of the gene whereas the DGRP-892 line carries a susceptible allele [12]. These two
140 alleles differed in their expression levels, where the resistant allele is expressed at a higher
141 level than the susceptible one in homeostasis and when the *lectin-24A* is upregulated after
142 infection [12]. To investigate if the proximal upstream region of the *lectin-24A* gene responds
143 specifically to parasitoid wasp infection, we infected *D. melanogaster* larvae expressing a
144 *lectin-24A* reporter driven by 444 bp of sequence upstream from the transcription start site
145 (TSS) of the resistant DGRP-437 allele ($Venus^{LP437}$) [12]. This upstream region was chosen
146 because it is the entire intergenic sequence between *lectin-24A* and its adjacent gene *Shaw*.
147 We infected larvae with virus, bacteria, or parasitoid wasps, and found that only parasitoid
148 wasp infection significantly induces the expression of the reporter (Figure 1A).

149

150 Consistent with previous reports of *lectin-24A* expression [41], when we observed infected
151 larvae under a fluorescent dissecting microscope the *lectin-24A* reporter $Venus^{LP437}$ construct
152 was expressed in the larval fat body. We also confirmed the expression by removing the larval
153 fat body through dissection (Figure 1B). Microscopy imaging of the dissected larval fat body
154 shows localization of expression after infection with parasitoid wasps, where the reporter is
155 consistently expressed at higher levels in the posterior ends (Figure 1B).

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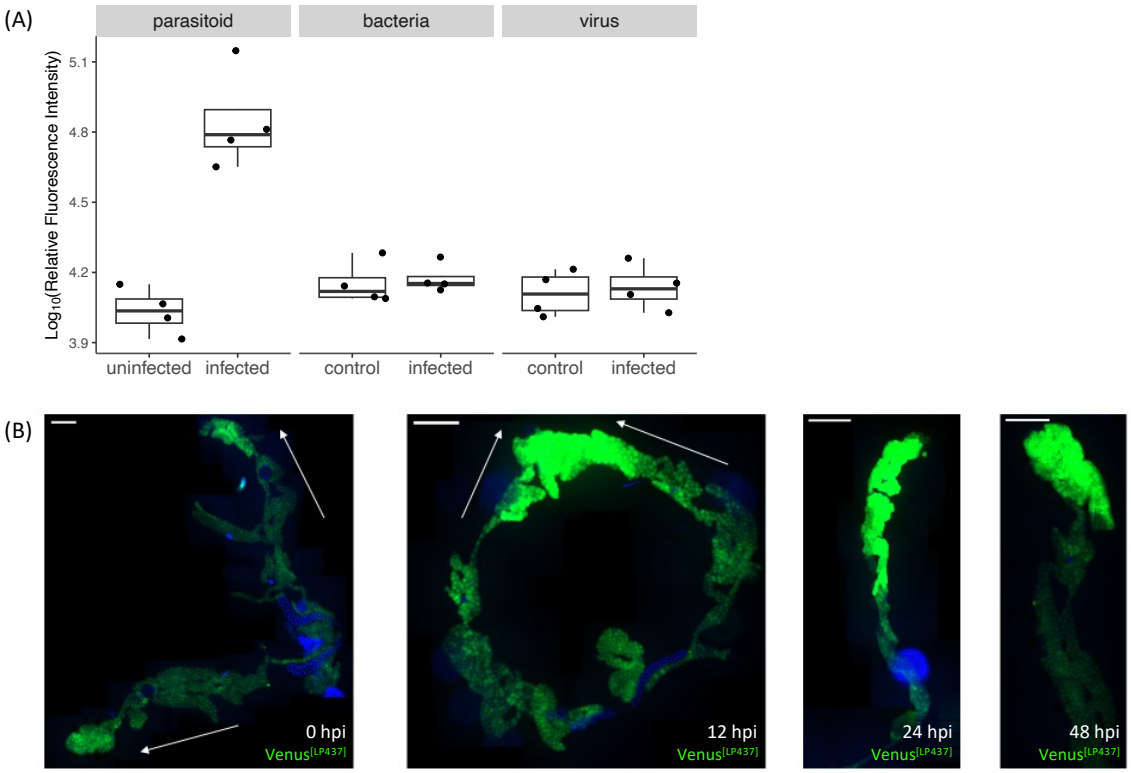


Figure 1. Expression of the *lectin-24A* reporter *Venus^{LP437}*. (A) Expression of *Venus^{LP437}* 24 hours post-infection (hpi) with different pathogens, left to right: *Leptopilina boulardi* parasitoid wasps, *Escherichia coli* bacteria, and *Drosophila* C virus. The relative fluorescence intensity represents the fluorescence intensity normalized with total protein quantity of each sample. Fluorescence was measured in homogenized larvae with a plate reader in a 96-well plate. Each point represents a pool of 15 larvae. (B) Expression pattern of the reporter *Venus^{LP437}* in the *Drosophila* larval fat body. Microscopy imaging of larval fat bodies dissected from uninfected and infected larvae, at 0, 12, 24, and 48 hpi. Fat bodies are shown with posterior pointing up unless indicated by the direction of white arrows. Green represents Venus expression, and blue represents Hoescht nuclear staining. Scale bars represent 500µm.

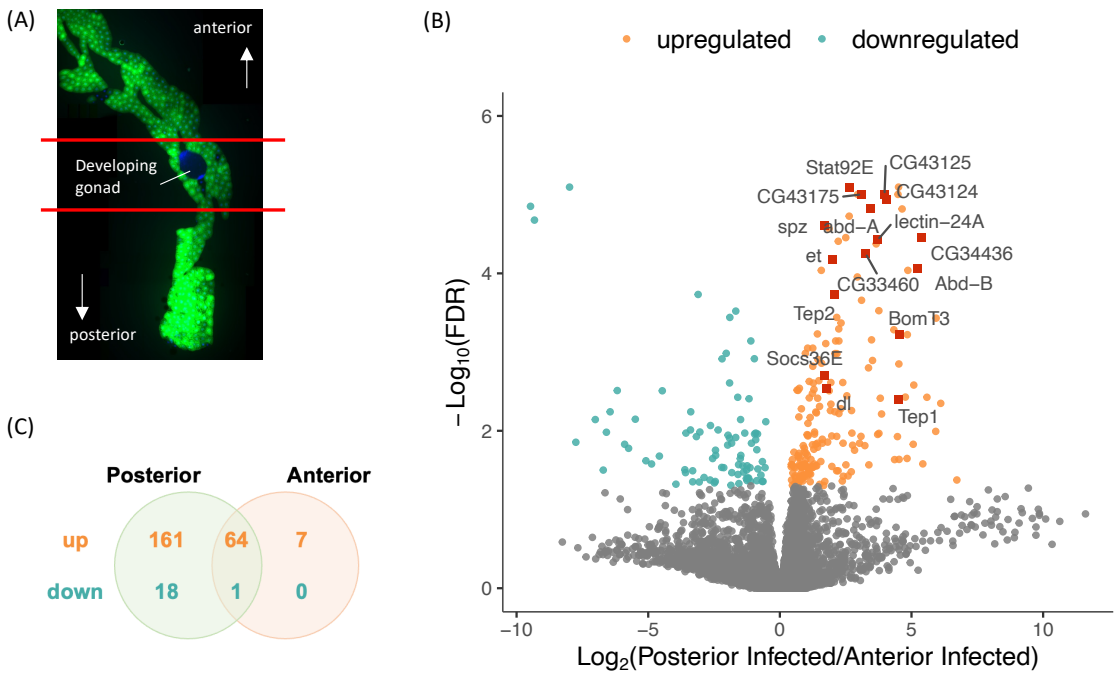
RNA sequencing reveals humoral immune response is localized to the posterior larval fat body

As we observed localized expression of *lectin-24A* in the posterior ends of the larval fat body, we investigated whether this localized response is seen in other immune-responsive genes. We conducted fat body RNA sequencing comparing gene expression changes in the anterior and posterior of the fat body in response to parasitoid infection. The fat bodies were separated into anterior and posterior sections according to the red line indicated in Figure 2A. After

filtering out lowly expressed genes, we detected a total of 10499 genes expressed in the fat body. Under infected condition, there is a total of 213 significantly differentially expressed genes with absolute $\log_2$FC of greater than 1, between the anterior and posterior sections. The expression of homeobox (Hox)-containing transcription factor genes, *abd-A* and *Abd-B*, are known for determining the anteroposterior body axis in *Drosophila*, including the fat body, and we see both genes differentially expressed between the anterior and posterior, indicating we have successfully separated the sections by dissection (Figure 2B) [42,43]. The results confirm our observation of localized $Venus^{LP437}$ expression in the posterior ends of the fat body upon wasp infection, as *lectin-24A* is among the top upregulated genes in the posterior with infection (Figure 2B).

Moreover, it seems that the posterior of the fat body showed generally higher immune responsiveness than the anterior to parasitoid infection. Following infection, 72 genes were significantly differentially expressed with an absolute $\log_2$FC of greater than 1 in the anterior, whereas in the posterior the number is 244 genes (Supplementary Figure 2). Most genes differentially expressed in the anterior after infection were also differentially expressed in the posterior, while there were 179 genes that were significantly differentially expressed in the posterior but not in the anterior after parasitization (Figure 2C). We also see that differentially expressed genes in the posterior are enriched for "extracellular" in Cellular Components Gene Ontology (GO) terms, suggesting that the posterior fat body is enriched for secreted proteins (Supplementary Figure 3). In particular, putative immune effectors, including the thioester-containing protein genes *Tep1* and *Tep2*, as well as a number of serine proteases (*CG34436*, *CG33460*, *CG43124*, *CG43125*, and *CG43175*), appear to be specifically upregulated in the posterior of the fat body with parasitization (Figure 2B).

202 Significantly downregulated genes in the larval fat body after parasitism suggest parasitoid
203 infection is causing shifts in metabolism and other aspects of immunity. *sug* and *daw*, genes
204 involved in sugar metabolism [44,45], are downregulated in the fat body after parasitoid
205 infection. We also found certain immune factors to be downregulated with parasitism,
206 including *Lectin-galC1* and the lysozyme *CG8492*, both involved in antimicrobial response
207 [46,47]. *RhoBTB*, a Rho-related BTB domain-containing protein involved in anti-parasitoid
208 response is downregulated in the posterior fat body after infection [48].
209



210

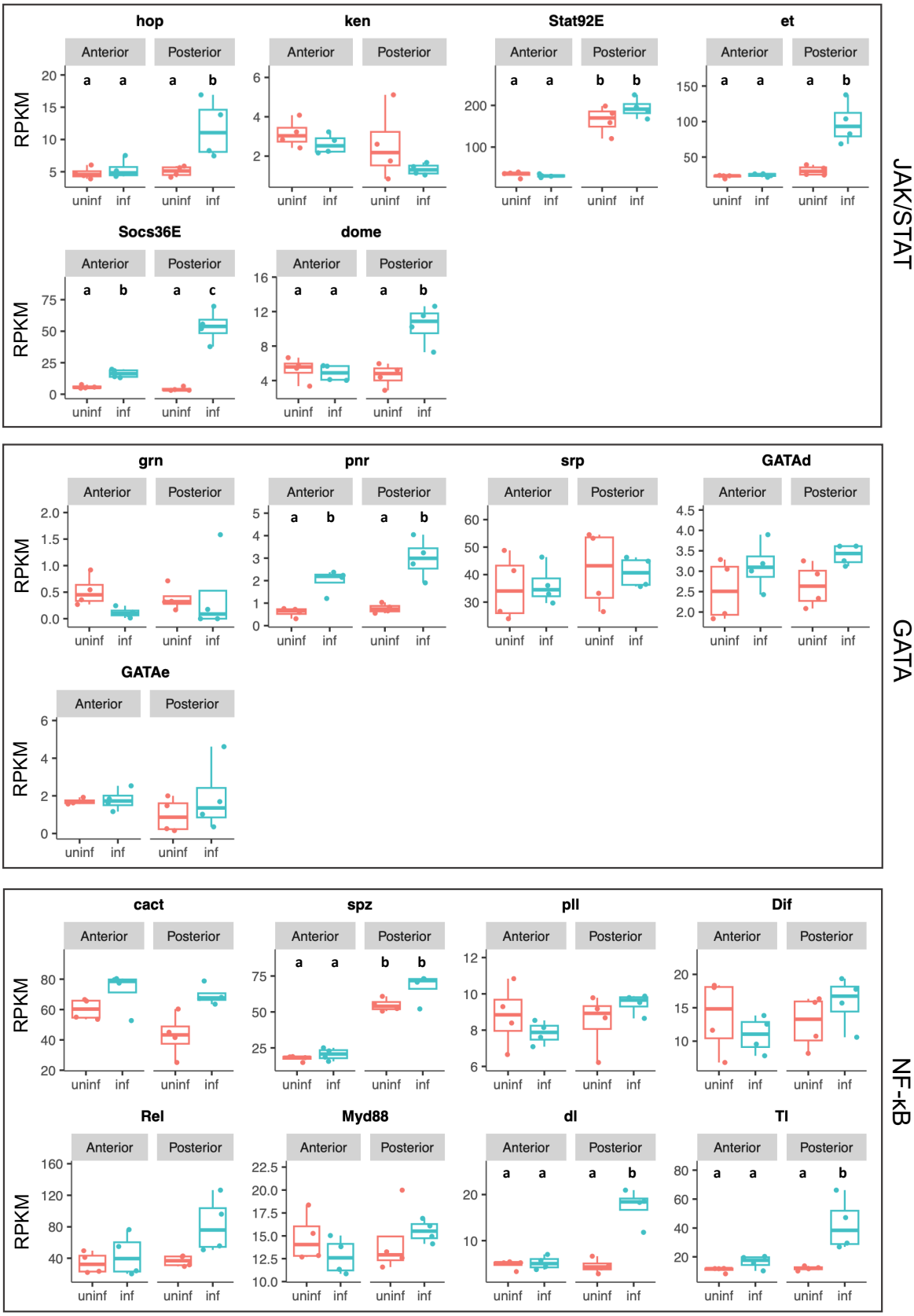
211 **Figure 2. RNA sequencing of larval fat body sections.** (A) Dissection of male larval fat
212 bodies along the representative red lines into anterior and posterior sections, where the
213 developing gonads were removed, for RNA sequencing. (B) Differentially expressed genes,
214 with absolute $\log_2$FC above 1 and False Discovery Rate (FDR) below 0.05, between anterior
215 and posterior larval fat body sections under infected condition. (C) Venn diagram showing
216 overlap of significantly differentially expressed genes with absolute $\log_2$FC above 1 after
217 infection in anterior and posterior fat body sections.
218

219

220 **Key immune signaling pathway components are differentially expressed in the fat body**
221 **after parasitism**

222 There are also interesting patterns of localized immune signaling gene expressions: key
223 components of the NF-κB and JAK/STAT pathways show up as top differentially expressed
224 genes between posterior and anterior sections in both control and infected conditions (Figure
225 2B and Supplementary Figure 2). GO enrichment of significantly upregulated genes in the
226 posterior of the fat body also showed enrichment of terms related to "receptor signaling
227 pathway via JAK-STAT", "immune system process", and "defense response" (Supplementary
228 Figure 3).
229
230 Selecting specific components from major immune signaling pathways including
231 transcription factors, we analyzed their expression patterns in anterior/posterior and
232 uninfected/infected conditions (Figure 3). It is striking to see that different transcription
233 factors show differential patterns of expression. The NF-κB transcription factor *dl* (*dorsal*) is
234 induced specifically in the posterior of the fat body in response to infection, with *Rel* (*Relish*)
235 showing a non-significant trend in the same direction. *Dif* shows similar levels of expression
236 across the four conditions (Figure 3). The gene encoding the Toll receptor *Tl* is also
237 upregulated with infection specifically in the posterior (Figure 3). While the Toll ligand, *spz*,
238 is more highly expressed in the posterior regardless of infection status (Figure 3).
239
240 The five known GATA transcription factors are encoded by *pnr* (*pannier*), *srp* (*serpent*), *grn*
241 *(grain), GATAd*, and *GATAe*. While the other four GATA transcription factors showed similar
242 expression between the fat body sections and infection statuses, *pnr* is upregulated with
243 infection in both the anterior and posterior (Figure 3).
244
245 The JAK/STAT pathway transcription factor *Stat92E* shows an entirely spatial differential
246 expression not affected by infection state, where it is constitutively upregulated in the

247 posterior (Figure 3). However, some other JAK/STAT pathway components have different
248 patterns of expression: *hop* and *dome* are induced specifically in the posterior with infection,
249 while the negative regulator *et* shows the same pattern of posterior induction (Figure 3).
250 Another negative regulator, *Socs36E*, shows induction with infection in both anterior and
251 posterior, but is more strongly induced in the posterior (Figure 3). Overall, we see patterns of
252 localized differential expression of immune pathway component genes in the *Drosophila*
253 larval fat body between the anterior and posterior sections in response to wasp parasitization.
254 The expression patterns of these genes in relation to the localized expression of *lectin-24A*
255 suggest potential roles of these pathways in the regulation of *lectin-24A* and the wider
256 humoral immunity against parasitoid infections.



257

258 **Figure 3. Gene expression patterns of components from major immune pathways in**
259 **anterior and posterior sections with and without infection.** Expression shown as Reads
260 Per Kilobase of transcript per Million mapped reads (RPKM). Labels denote significant

261 (genome-wide false discovery rate < 0.05) differential expression with absolute $\log_2$FC of at
262 least 1 between treatment groups. Only significant differences are shown.
263
264

265 **Truncation of the upstream promoter sequence of *lectin-24A* defines the minimal region**
266 **to maintain fat body specific induction of the gene**

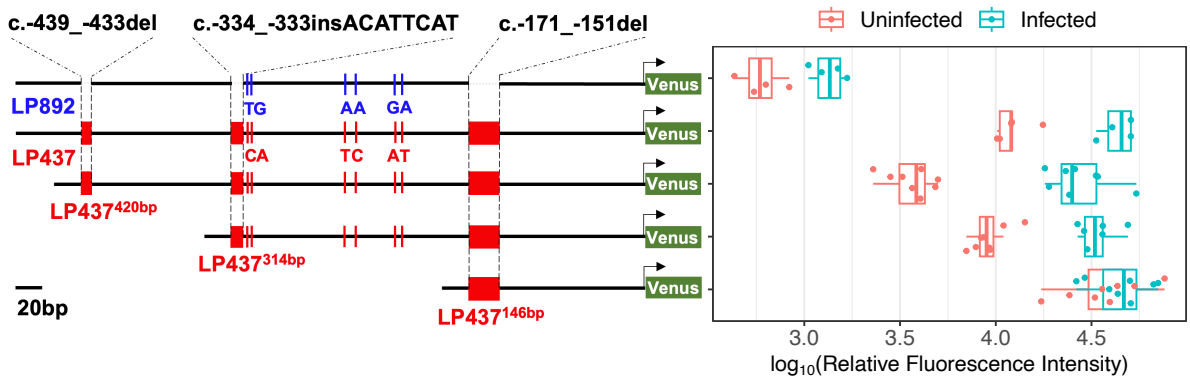
267 We have limited understanding of the regulation of the anti-parasitoid immune response in *D.*
268 *melanogaster*, particularly the early signaling that activates humoral immunity in the larval
269 fat body. We investigated the upstream region of the *lectin-24A* gene to further define the
270 specific sequences that underlie its induction with parasitization, and to understand the
271 transcription factors involved in the regulation of its expression. We created truncated
272 versions of the $Venus^{LP437}$ reporter construct where we deleted the sequence from the 5' end
273 (Figure 4A). While we saw little effect from the first two deletions to 420 bp and 314 bp
274 upstream of the gene, we found that, when we truncated the sequence to be 146 bp upstream
275 of the TSS, there were significant changes to the expression of the reporter where it becomes
276 expressed in other tissues such as the gut with and without parasitization (Supplementary
277 Figure 4). Therefore, the region 314 bp upstream of the gene is required for an inducible
278 response of the *lectin-24A* gene that is restricted to the fat body tissue.
279

280 **NF-κB, GATA, and STAT binding motifs are all involved in the regulation of *lectin-24A***

281 Our RNAseq results suggest that the NF-κB, GATA, and JAK/STAT pathways may be
282 involved in the regulation of the *lectin-24A* gene and humoral anti-parasitoid immunity in
283 general. We also screened for transcription factors binding to the upstream region of both the
284 resistant and susceptible alleles of *lectin-24A* in a Yeast one-hybrid (Y1H) screen. In
285 particular, we found that dorsal from the Toll pathway binds to both the resistant allele from
286 DGRP-437 and the susceptible allele from DGRP-892 (Figure 4B). We then predicted
287 TFBMs in the 444 bp upstream region according to the consensus binding motifs for

288 transcription factors in each of the immune pathways. One consensus motif was used for the
289 transcription factors Dif/dl, two motifs were used for STAT92E, and four motifs were used
290 for the different GATA transcription factors [49–52] (Figure 4C & Supplementary Table 1).
291 We predicted TFBMs for each of these transcription factors in this region (Figure 4C).
292 Furthermore, the GATA motif is predicted to bind all four of the GATA transcription factors
293 (Supplementary Table 1).

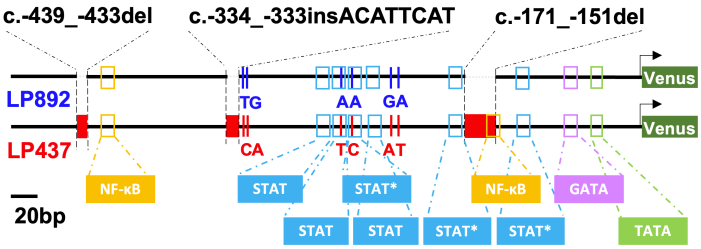
(A)



(B)

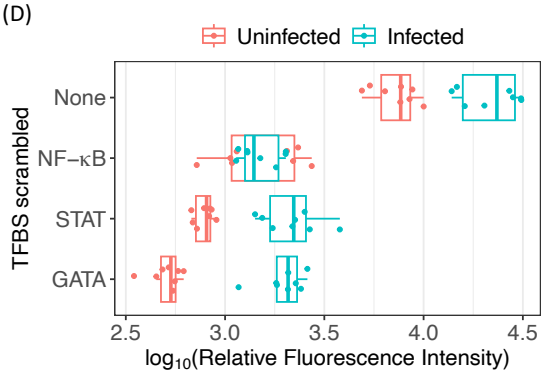
Allele	Gene	Expressed in larval fat body
Both	Rfx	No
	sd	Yes
	dl	Yes
DGRP-437	CTCF	No
	CG2202	Yes
	CG14965	No
DGRP-892	CG11902	No
	Dif ¦ dl	Yes

(C)



Pathway	Transcription factor	Motif consensus sequence
NF-κB	Dif/dl	GGGHHNNDVH
GATA	Pnr/Srp/GATAd/GATAe	WGATAAR
STAT	STAT92E	$TT(N)_5AA/TT(N)_6AA$

(D)



294

295 **Figure 4. Transcription factor binding motifs (TFBMs) upstream of *lectin-24A*.** (A)
296 Truncated *lectin-24A* reporter Venus expression compared to the full-length reporter
297 constructs with the susceptible DGRP-892 (LP892; blue) and resistant DGRP-437 (LP437;
298 red) alleles. (B) Yeast one-hybrid screen results showing proteins that bind to the sequence
299 444 bp upstream of the resistant DGRP-437 *lectin-24A* allele and/or the susceptible DGRP-
300 892 *lectin-24A* allele. Dif¦dl represents a heterodimer. (C) Predicted TFBMs of transcription
301 factors from the JAK/STAT, NF-κB, and GATA pathways upstream of *lectin-24A*. The STAT
302 consensus sequence shown corresponds to the conserved STAT binding site "TT(*n*)AA", with
303 5-6 variable *n* sites in the middle. (D) Expression of the reporter constructs with the 314 bp
304 upstream sequence of *lectin-24A* where predicted NF-κB, GATA, and three of the STAT
305 TFBMs are scrambled. The scrambled STAT sites are indicated with asterisks in (C). Each
306 point represents a pool of 10 larvae. The relative fluorescence intensity represents the
307 fluorescence intensity normalized with total protein quantity of each sample.
308

309

310 To further study the role of TFBMs in the regulation of *lectin-24A*, we created promoter
311 reporter constructs where motifs have been scrambled to abolish transcription factor binding.
312 Since the truncated version with 314 bp upstream of the TSS showed similar expression as
313 the full-length LP437 construct, we scrambled the TFBMs with this version of the reporter
314 (Figure 4A, $LP437^{314bp}$). The universally conserved STAT binding site is defined as
315 "TT(*n*)AA", with variable 4-6 *n* sites in the middle [53,54]. The *Drosophila* STAT binds
316 "TTC$(N)_3$GAA" sites with high affinity [50,51]. However, gene regulation can rely on STAT
317 binding motifs that have lower affinity [50]. This includes *Dome,* which is upregulated by
318 STAT binding "TTC$(N)_4$GAA" sites [50], and which we found to be upregulated in the
319 posterior fat body after infection (Figure 3). Here we scrambled three sites with the motif
320 "TTC$(N)_5$AA" in the *lectin-24A* upstream region, which are half-way between the universal
321 STAT motif and the 4*n Drosophila* STAT motif (Figure 4C, sites with asterisks &
322 Supplementary Table 2). We also scrambled motifs predicted to bind Dif/dl and GATA.
323

324 We found that the predicted TFBMs associated with Dif/dl, STAT92E, and GATA are all
325 involved in the regulation of the gene. Scrambling of the TFBM for Dif/dl resulted in the
326 abolishment of the upregulation of the reporter and reduction in the basal expression, whereas

327 scrambling of the TFBMs for the GATA and STAT transcription factors resulted only in the
328 reduction of the basal expression while the induction with infection is still present (Figure
329 4D). These results suggest that all three of these pathways may bind to the upstream region of
330 *lectin-24A* and be involved in the regulation of expression.
331
332 We previously found that the *cis*-regulatory sequences of the resistant and susceptible alleles
333 of *lectin-24A* differed by three insertion-deletion polymorphisms (indels) of 7 bp, 8 bp, and
334 21 bp in length, and six single nucleotide polymorphisms (SNPs; Figure 4A, top two rows)
335 [12]. The 21 bp indel was found to be primarily responsible for the differential regulation of
336 *lectin-24A* expression between the two alleles [12]. Based on the positions of the TFBMs in
337 the region, we found that the NF-κB binding site is disrupted by the 21 bp deletion in the
338 susceptible DGRP-892 *lectin-24A* allele (Figure 4C). As scrambling this NF-κB binding site
339 abolishes the basal expression and induction of the reporter, we conclude that the loss of this
340 NF-κB binding site explains the differential expression we see from natural polymorphisms
341 in the *lectin-24A* upstream region. This in turn controls susceptibility to infection in natural
342 populations [12].
343
344
345 **The JAK/STAT pathway plays a crucial role in the regulation of *lectin-24A***
346 The JAK/STAT signaling pathway is an evolutionarily conserved pathway with a variety of
347 functions in hematopoiesis, immunity, and development [55]. The canonical JAK/STAT
348 signaling starts with ligand binding, resulting in receptor dimerization bringing two non-
349 receptor tyrosine kinases (JAKs) together to trans-phosphorylate one another [56,57].
350 Activated JAKs then phosphorylate the receptor which in turn phosphorylates the STAT
351 transcription factors. This leads to the normally cytosolic STAT molecule translocating to the

352 nucleus to activate gene expression. The *Drosophila* JAK/STAT pathway only has one JAK
353 (*hop*) [58], one active type I cytokine receptor (*dome*) [59], and one STAT (*Stat92E*) [51,60].
354 To better characterize the role of the JAK/STAT pathway in the regulation of *lectin-24A*, we
355 knocked down the expression of the transcription factor Stat92E in larvae expressing the
356 *lectin-24A* reporter using the ubiquitously expressed *da-GAL4* to drive the expression of a
357 hairpin RNAi construct. At 24 hpi, *Stat92E* knockdown abolished the induction of the
358 reporter, suggesting that the JAK/STAT pathway is involved in the regulation of the gene
359 (Figure 5A).
360
361 In our RNAseq analysis, we also found that the JAK/STAT pathway negative regulator *et*
362 showed specific induction in the posterior of the fat body. et is structurally similar to the
363 JAK/STAT receptor dome, and it can function as a negative regulator by forming inactive
364 heterodimers with dome [21,61]. It was previously found that et is required for the
365 downregulation of JAK/STAT activity in hematopoietic progenitors for lamellocyte
366 differentiation after wasp infection [21]. To further understand the role of JAK/STAT
367 signaling in *lectin-24A* regulation, we analyzed the expression of the reporter in a genetic
368 background where *et* is mutated [21]. We found that with a non-functional mutant *et*, the
369 reporter shows constitutively upregulated expression in the larval fat body (Figure 5B). In
370 addition, to confirm this result, we used a dominant negative allele of Domeless
371 (DomeΔCYT, cytoplasmic domain deleted), driven in the fat body by *Cg-GAL4* or
372 ubiquitously by *da-GAL4*, to decrease JAK/STAT activity. We observed a general lowering of
373 reporter expression both for basal and infected conditions (Figure 5C).
374
375 The *Drosophila* JAK, *hop*, showed a similar expression pattern as *et* in our RNAseq analysis,
376 being upregulated after infection in the posterior fat body. We therefore studied the

377 expression of the reporter in a genetic background with a constitutively active form of *hop*

378 (*hop*^{Tum}). As predicted, the reporter showed elevated basal expression in the *hop*^{Tum}

379 background (Figure 5D). When we then examined *lectin-24A* reporter expression in a

380 background where we abolished *hop* in somatic cells using CRISPR/Cas9 and a guide RNA

381 targeting the gene (somatic knock out), we found a reduction in the upregulation of the

382 reporter after wasp infection (Figure 5E). Taken together, our results indicate clearly that the

383 JAK/STAT pathway has a critical role in the induction of *lectin-24A* following parasitization.

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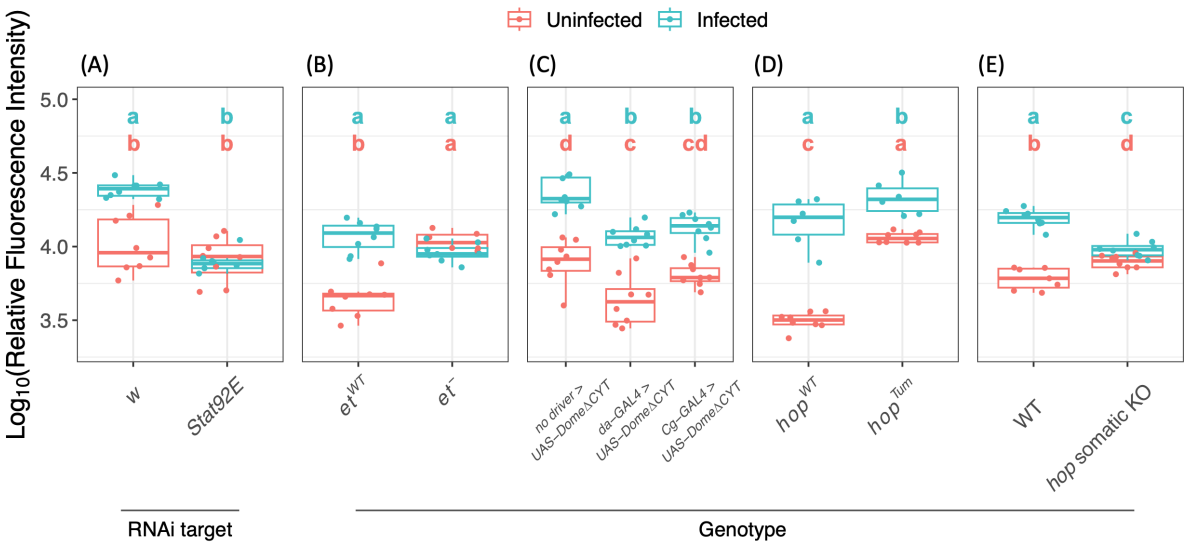

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386

387 **Figure 5. The role of the JAK/STAT pathway in the regulation of *lectin-24A*.** In all panels
388 the *Venus*^{LP437} reporter expression was measured. (A) Larvae with knockdown of *Stat92E*
389 using RNAi, compared to the knockdown of the *white* gene as a control. (B) Larvae carrying
390 a mutant or wildtype (WT) form of the JAK/STAT negative regulator *et*. (C) Larvae
391 expressing a dominant negative form of the JAK/STAT receptor Dome (DomeΔCYT) in the
392 fat body and hemocytes under the control of the *Cg-GAL4* driver, with the ubiquitous *da-*
393 *GAL4* driver, and with no driver. As the parental stock expressing *UAS-DomeΔCYT* also
394 expresses the JAK/STAT negative regulator *et* under UAS control on a balanced chromosome
395 II, a portion of the larvae also expresses *UAS-et*. Larvae expressing GAL4 drivers also
396 expressed *tub-GAL80*^{ts}. (D) Larvae expressing the constitutively active *hop* (*hop*^{Tum})
397 compared to larvae expressing the wild type (WT) *hop*. (E) Larvae expressing *Act-Cas9* and
398 guide RNA targeting *hop* compared to control larvae. Each point represents a pool of 10
399 larvae. The relative fluorescence intensity represents the fluorescence intensity normalized
400 with total protein quantity of each sample. All infected larvae were collected at 24 hpi and
401 uninfected age-matched larvae were collected for the controls. Tukey's test for each panel, P
402 < 0.05 between groups.
403
404
405

406 **GATA transcription factors modulate *lectin-24A* expression in the fat body**

407 GATA transcription factors comprise a family of DNA binding proteins named after the
408 consensus sequence (WGATAAR) they recognize. GATA factors are highly conserved across
409 invertebrates and vertebrates, where they play major roles in development. *Drosophila* and
410 mammalian GATA factors share functional similarities in the regulation of developmental
411 processes, such as hematopoietic development [26], blood cell differentiation [28,62], and
412 liver/fat body development [28,63,64]. *Drosophila* has a total of five GATA factors, of which
413 only three display the canonical two zinc finger domains found in all mammalian GATA
414 factors: Pannier (Pnr), Serpent (Srp) and Grain [65–67].

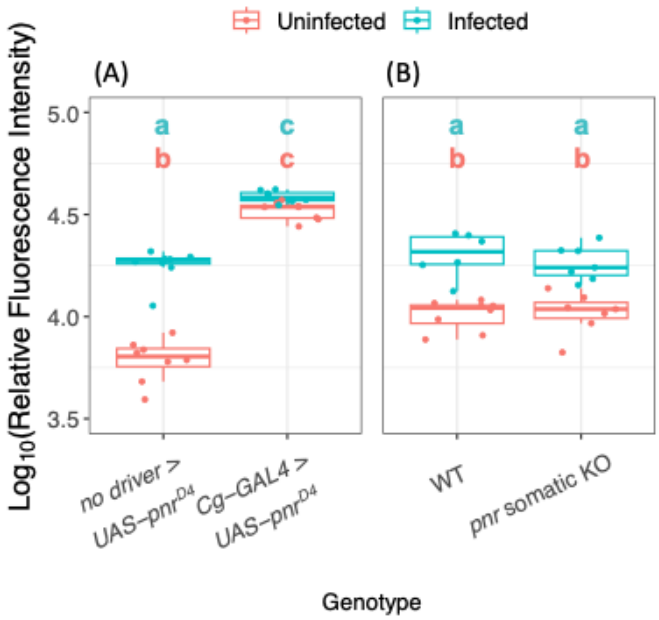
415

416 The DNA-binding activity of GATA factors is mainly mediated by the C-terminal zinc finger
417 domain, whereas the N-terminal zinc finger domain is required for interactions between the
418 GATA factor and its cofactor Friend of GATA (FOG) proteins [68]. It has previously been
419 shown that the FOG protein u-shaped (Ush) interacts with the N-terminal zinc finger of both
420 Pnr and Srp [65,68,69]. In particular, Pnr and Ush form heterodimers, where Ush antagonizes
421 the activator function of the Pnr transcription factor [65,69,70].

422

423 As we observed significant differential expression of *pnr* in our RNAseq analysis following
424 parasitization (Figure 3), we studied the effect of Pnr on *lectin-24A* expression. We utilized
425 the pnr^{D4} mutant, which belongs to a class of *pnr* mutants with point mutations in the N-
426 terminal zinc finger domain, resulting in very weak interactions with Ush [65,69]. Using the
427 fat body *Cg-GAL4* driver, we expressed the dominant pnr^{D4} in the fat body with the *UAS-*
428 pnr^{D4} construct (Figure 6A). As pnr^{D4} interacts poorly with the negative Pnr regulator Ush, it
429 functions as a dominant positive mutant. Strikingly, $Venus^{LP437}$ reporter showed constitutive

430 high expression, even in the absence of parasitization (Figure 6A). We then analyzed the
431 expression of *lectin-24A* Venus reporter in a genetic background where we knocked out *pnr*
432 expression using somatic mutagenesis with CRISPR/Cas9. However, knock out of *pnr*
433 expression in the whole larvae did not result in significant changes to the $Venus^{LP437}$ reporter
434 expression, which might reflect GATA factors may have redundant roles in regulating *lectin-*
435 *24A* (Figure 6B). Thus, our results indicate that Pnr is involved in the induction of *lectin-24A*
436 following parasitoid infection, but it may not be the only or the main GATA factor playing
437 this role.



438

439

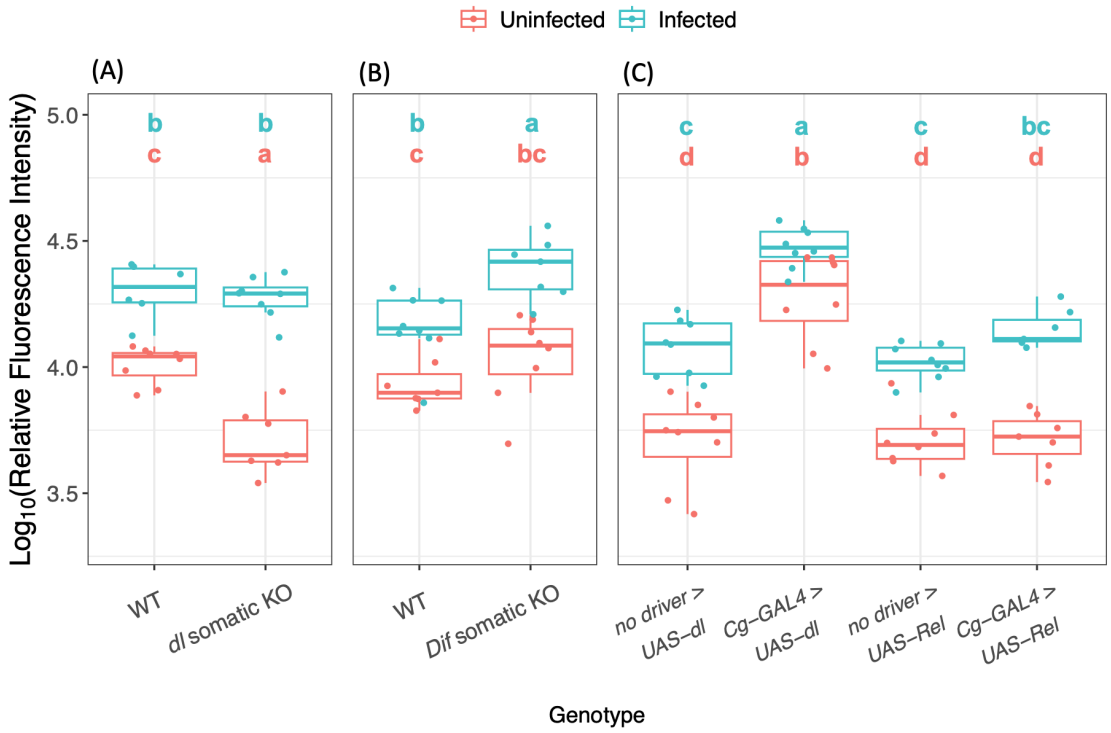
440 **Figure 6. The role of the GATA transcription factor, Pannier, in the regulation of *lectin-***
441 ***24A*.** The $Venus^{LP437}$ reporter expression was measured. (A) Larvae expressing the dominant
442 positive form of Pnr (Pnr^{D4}) under UAS control driven by the fat body and hemocyte-specific
443 *Cg-GAL4* driver or no driver. (B) Larvae expressing *Act-Cas9* and guide RNA targeting *pnr*
444 compared to control larvae. Each point represents a pool of 10 larvae. The relative
445 fluorescence intensity represents the fluorescence intensity normalized with the total protein
446 quantity of each sample. Tukey's test for each panel, $P < 0.05$ between groups.
447

448

449 **NF-κB transcription factors dl regulates humoral antiparasitoid immunity**

450 NF-κB transcription factors of the Toll and Imd pathways have major roles in the regulation
451 of antimicrobial humoral immunity for *Drosophila*. Our scrambling of TFBMs in the *lectin-*
452 *24A* upstream region suggests that the Toll pathway might also participate in regulating the
453 anti-parasitoid response in the larval fat body (Figure 4D). Of the three *Drosophila* NF-κB
454 transcription factors, Dif, dl, and Rel, we found that only dl becomes significantly
455 upregulated with parasitism specifically in the posterior of the larval fat body (Figure 3). To
456 further understand the role of NF-κB transcription factors in the regulation of *lectin-24A*, we
457 created somatic mutants of the NF-κB transcription factors Dif and dl. The effects of somatic
458 mutagenesis of these genes suggest that knocking out *dl* reduces the expression of $Venus^{LP437}$
459 in the uninfected state (Figure7A), while removing *Dif* expression seems to give the opposite
460 effect (Figure 7B). We then overexpressed dl and Rel in the larval fat body using the driver
461 *Cg-GAL4*, where we show that dl overexpression leads to a constitutively high expression of
462 the $Venus^{LP437}$ reporter with and without infection (Figure 7C). Overexpression of Rel did not
463 show significant effects on the reporter expression (Figure 7C). Altogether, we conclude that
464 dl is the NF-κB transcription factor regulating *lectin-24A* expression.

465



466

467 **Figure 7. The role of NF-κB transcription factors in the regulation of *lectin-24A*.** The
468 *Venus*^{LP437} reporter expression was measured. (A) Larvae expressing *Act-Cas9* and guide
469 RNA targeting *dl* compared to control larvae. The same WT control samples were used here
470 as in Figure 6B. (B) Larvae expressing *Act-Cas9* and guide RNA targeting *Dif* compared to
471 control larvae. (C) Larvae expressing *dl* or *Rel* under UAS control driven by the fat body and
472 hemocyte-specific *Cg-GAL4* driver or no driver. F_1 heterozygote larvae were collected. As the
473 parental line expressing *UAS-dl* carried a balancer chromosome that could not be identified at
474 the larval stage in F_1 progeny, larvae collected from the *UAS-dl* crosses were a mix of *Cg-*
475 *GAL4/UAS-dl* and *Cg-GAL4/+*. Each point represents a pool of 10 larvae. The relative
476 fluorescence intensity represents the fluorescence intensity normalized with the total protein
477 quantity of each sample. Tukey's test for each panel, $P < 0.05$ between groups.
478

479

480 **Transcription factor binding motifs found in the upstream region of *lectin-24A* are**

481 **generally enriched in the upstream sequences of parasitism-induced genes**

482 As we have found the TFBMs of GATA, NF-κB, and STAT are important in the regulation of

483 the *lectin-24A* gene, we were interested to find out if this generalizes to the other genes

484 upregulated with infection. We searched for TFBMs in the 300 bp upstream region of all the

485 genes detected in the larval fat body RNAseq (Figure 2). Then we assessed if a TFBM is

486 enriched in the upstream sequence of genes upregulated with infection in the posterior of the
487 fat body, relative to the full set of genes detected. We found that GATA binding motifs are
488 generally enriched around two-fold in the sequences upstream of upregulated genes. For
489 instance, the Pannier binding motif (Supplementary Table 1) is found an average of 0.60
490 times per kilobase (kb) of sequence 5' of TSSs for upregulated genes, whereas it is only
491 found an average of 0.33 times per kb of upstream sequences in the full set of genes (Welch's
492 *t*-test, t = 3.49, d.f. = 277.9, *p*-value = 5.65×10^{-4}). A similar case is observed in the genes
493 specifically upregulated in the posterior of the fat body after infection (Figure 2B), showing
494 even higher enrichment with a mean of 0.66 times per kb in these genes (Welch's *t*-test, t =
495 3.51, d.f. = 186.1, *p*-value = 5.72×10^{-4}).
496
497 When we searched for STAT motifs in the upstream sequences, we found significant
498 enrichment of the "TTC$(N)_4$AA" motif in the posterior-specific upregulated genes with a
499 mean of 8.46 instances per kb compared to 7.77 in the background (Welch's *t*-test, t = 2.10,
500 d.f. = 188.84, *p*-value = 0.037). Although the same motif also appears to be enriched in the
501 parasitism-induced gene set, with 8.27 occurrences per kb, it falls just below the significance
502 threshold (Welch's *t*-test, t = 1.91, d.f. = 284.98, *p*-value = 0.058). On the other hand, the
503 "TTC$(N)_5$AA" motif only shows significant enrichment in the parasitism-induced gene set,
504 with 6.29 occurrences per kb compared to 5.83 in the background set (Welch's *t*-test, t = 1.98,
505 d.f. = 284.47, *p*-value = 0.048).
506
507 Moreover, previous studies have described a specific organization of TFBMs in the upstream
508 region of fat body-specific immune genes, where NF-κB and GATA binding sites have been
509 found to be in close association, with less than 50 bp in-between them and within 200-300 bp
510 upstream of the TSSs [30,31]. We specifically searched for NF-κB sites within 50 bp of a

511 GATA site and checked for enrichment. Although, we see that NF-κB motifs linked to GATA
512 motifs are also more highly enriched in the upstream region of parasitism-induced genes and
513 posterior-specific anti-parasitoid genes, with a mean of 0.52 and 0.53 instances per kb,
514 respectively, compared to 0.39 in the background set, the differences were not significant.
515 Overall, the TFBMs of the GATA, NF-κB, and STAT transcription factors all appear to be
516 enriched in the 5' sequences of parasitism-induced genes in the *Drosophila* larval fat body,
517 suggesting that these transcription factors and the signaling pathways they belong to are
518 generally involved in the regulation of genes in the humoral anti-parasitoid response.

Discussion

The *Drosophila* anti-parasitoid immune response involves the concerted action of hemocytes and the fat body to encapsulate and melanize the wasp [4,63,64]. This complex and multifaceted response is distinct from responses to microbial or viral infection, and is specifically induced by a wasp PAMP [32]. Here we have demonstrated that the regulation of *Drosophila* anti-parasitoid humoral immunity, mediated by the larval fat body, involves complex inputs from multiple immune pathways (Figure 8).

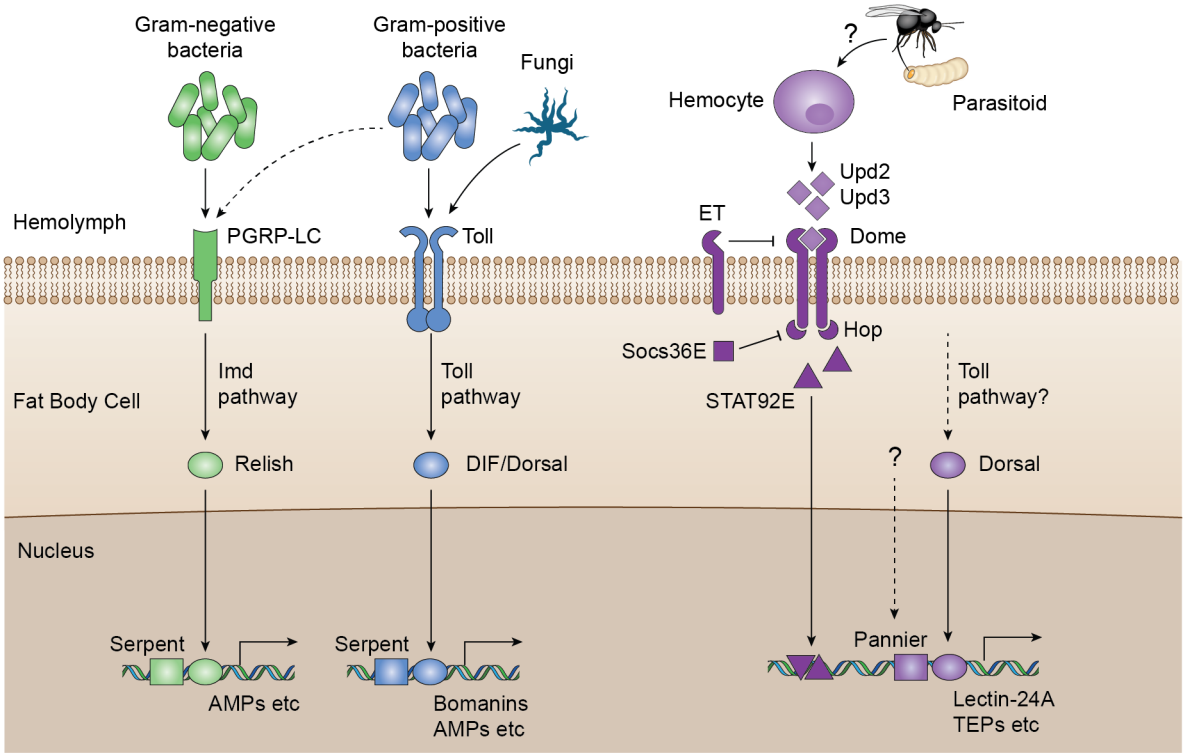


Figure 8. Schematic overview of *Drosophila* humoral defenses to microbial pathogens and parasitoid. Antimicrobial responses in the fat body are regulated by the NF-κB pathways, Toll and Imd. The Imd pathway is mostly activated by Gram-negative bacteria through the PGRP-LC receptor, whereas the Toll pathway is largely activated by fungi and Gram-positive bacteria through the Toll receptor. Activation of the Imd pathway leads to the NF-κB transcription factor Relish translocating into the nucleus after its cleavage and activation, and the transcription factors Dif and Dorsal are activated through Toll pathway signaling. These NF-κB transcription factors along with the GATA transcription factor Serpent induce the expression of genes encoding antimicrobial effectors in the fat body via

538 closely linked GATA and κB binding sites in the upstream promoter region. The *Drosophila*
539 humoral anti-parasitoid response is regulated by a combination of inputs from JAK/STAT,
540 GATA and NF-κB pathways. After recognition of parasitism, hemocytes produce the
541 cytokines, Upd2 and Upd3, which bind to the receptor Dome to activate JAK/STAT pathway
542 signaling in the fat body. Dome dimerization after ligand binding leads to trans-
543 phosphorylation of Hop, which then activates the transcription factor STAT92E. STAT92E
544 binds the upstream region of anti-parasitoid genes including *lectin-24A*. ET and SOCS36E
545 are negative regulators of the JAK/STAT pathway that suppress this signaling. STAT, GATA,
546 and NF-κB TFBMs are found to be enriched in the upstream sequences of parasitism-induced
547 genes. The GATA transcription factor Pannier and the NF-κB transcription factor Dorsal also
548 participate in the regulation of anti-parasitoid genes in the larval fat body by binding to their
549 respective binding sites in the promoter region.
550
551

552 The transcriptional response induced by a parasitoid attack differs from that activated by
553 microbial infections [33]. We found that *lectin-24A* is a gene specifically induced by
554 parasitoid infection, providing a tool to understand broadly how wasp-responsive genes are
555 regulated. Based on our current results, it seems that this specificity is potentially conferred
556 by the coordinated inputs from different immune-signaling pathways: NF-κB, the GATA
557 factor Pannier, and JAK/STAT. This regulation differs from the well-characterized
558 antimicrobial defense, which mainly requires the two NF-κB pathways, Toll and Imd, with
559 additional inputs from the GATA transcription factor Serpent [30,74–78].
560

561 It is likely that our results reflect mechanisms of regulation in the wider humoral anti-
562 parasitoid response, not just limited to *lectin-24A*. Our analysis of upstream regulatory motifs
563 found enrichment of NF-κB, GATA, and STAT binding sites in the upstream region of
564 differentially expressed genes in the larval fat body in response to wasp infection. This result
565 is consistent with a previous report of TFBMs significantly associated with anti-parasitoid
566 genes in the whole larvae [33]. In that study, Wertheim et al. defined clusters of differentially
567 expressed genes based on patterns of co-expression over nine time points in the 72 hours after
568 parasitization [33]. *lectin-24A* as well as the genes encoding three thioester-containing
569 proteins (TEPs), which are structurally similar to mammalian complement proteins, were

570 clustered together [33]. Genes in this cluster showed significant over-representation of NF-
571 κB, GATA, and STAT regulatory motifs [33], supporting out conclusion that the anti-
572 parasitoid humoral immune response mediated by the fat body is regulated by the concerted
573 action of these pathways. Further support comes from analyses of TEP expression in flies
574 where signaling pathways have been perturbed. TEPs are expressed mainly by the *Drosophila*
575 fat body and induced in response to various immune challenges [79]. The loss of the four
576 secreted TEPs, TEP1-4, results in reduced capacity to successfully encapsulate wasps [8].
577 *Tep1* shows constitutive expression in the absence of infection in the constitutively active
578 hop^{Tum} mutant [79]. Similarly, in a *Toll* gain-of-function mutant background, *Tep1* also
579 displayed constitutive activation [79]. However, in this case, the Toll pathway effects were
580 recognized to be indirect, and secreted TEPs were found to function upstream of Toll and
581 promote Toll activation [8,79].
582
583 It is still unknown how parasitoids are recognized by the *Drosophila* host. As hemocytes are
584 responsible for the secretion of Upd cytokines that activate the JAK/STAT pathway in other
585 tissues, they are likely to play a key role in the recognition of parasitoid attacks [22]. Our
586 study shows a significant role of the JAK/STAT pathway in *lectin-24A* regulation.
587 Interestingly, we see that JAK/STAT pathway negative regulators are strongly induced
588 alongside the activation of the pathway itself (Figure 3) [21,61,80,81]. As we only
589 investigated one time point (24 hpi) in our experiments, this result suggests potential negative
590 feedback loops in the shutdown of the pathway that were not captured. In mammals, negative
591 regulators of the pathway are known to be induced after infection and JAK/STAT activation,
592 playing a key role in regulating the cytokine response and preventing damaging inflammation
593 [82,83]. This is also the case in *Drosophila*, where expression of the negative regulator
594 SOCS36E is activated by JAK/STAT signaling, creating feedback inhibition [81,84,85].

595

596 In the *lectin-24A* upstream region, we predicted a GATA transcription factor binding site that
597 contains the consensus binding motifs from four of the *Drosophila* GATA transcription
598 factors (Figure 4 & Supplementary Table 1). We specifically focused on Pannier because it is
599 the only GATA transcription factor showing differential expression in the larval fat body
600 following parasitoid attack (Figure 3). Our results indicate that Pannier is involved in
601 regulating *lectin-24A* and likely the broader humoral anti-parasitoid response. However, we
602 have yet to investigate whether upregulation of Pannier expression is a primary signal of
603 infection or a consequence of activating other pathways such as JAK/STAT. Furthermore,
604 whether the other *Drosophila* GATA transcription factors also contribute to regulation
605 remains to be further studied. In particular, Serpent, which is known to be involved in the
606 regulation of immune genes in the larval fat body, is also predicted to bind upstream of *lectin-*
607 *24A* and might also have a role in the regulation of immunity against parasitoids [30,31,86].
608

609 Based on our RNAseq data, components of the Toll pathway showed significant differential
610 expression in the larval fat body following parasitism (Figure 3). Particularly, of the NF-κB
611 transcription factors, *dl* showed upregulation specifically in the posterior fat body with
612 infection (Figure 3). We also found that a Dif/dl binding motif in the *lectin-24A* upstream
613 region had a crucial role in the expression of the $Venus^{LP437}$ reporter (Figure 4D). Dif and
614 dorsal, the two transcription factors in the *Drosophila* NF-κB Toll pathway, are known to
615 regulate the defense to Gram-positive bacteria and fungi. In the antimicrobial response, Dif
616 and dl have different roles depending on developmental stage. In adults, Dif alone is required
617 for the humoral immune response, whereas in larvae, it shows some redundancy with dl
618 [76,87]. While there is evidence that in Dif and dl double mutants, expression of either
619 transcription factors is sufficient to rescue the expression of AMPs such as *Drosomycin* [87],

620 some other reports show that induction of certain AMPs specifically require Dif and cannot
621 be rescued with only dl [77]. Furthermore, Dif and dl, as well as Rel, form homodimers and
622 heterodimers during DNA binding, meaning that permutations of different dimers expand the
623 regulatory possibilities from these NF-κB proteins [88,89]. Since NF-kB transcription factors
624 activate transcription by translocating into the cell nucleus, our observation that the
625 expression of *Dif* does not increase after infection does not preclude it playing a role in
626 regulating the anti-parasitoid humoral response. Nevertheless, as *dl* but not *Dif* mutants
627 reduced expression of the $Venus^{LP437}$ reporter, dl is likely playing the key role in regulation of
628 this immune response.
629
630 In this study, we investigated individually how each of these pathways contributed to the
631 regulation of *lectin-24A* expression, but it remains unclear how they work together. It was
632 previously found that NF-κB and GATA regulatory synergy plays a key role in many
633 immunity-related fat body-specific genes that are activated in response to microbial
634 infections [30,31]. In particular, a fixed organization of NF-κB and GATA binding sites in the
635 5' regulatory region within 1 kb of predicted transcription start sites (TSSs) was defined,
636 where both sites are located in the proximal region within 200 or 300 bp from the TSS [30].
637 The sites were found to be closely linked, within 50 bp from each other and in the same
638 relative orientation [30]. The NF-κB and GATA binding sites we identified in the *lectin-24A*
639 upstream region fit this criterion exactly (Figure 4C). According to the previous study, each
640 individual NF-κB and GATA site has an essential role in the optimal expression of the genes
641 [30]. Furthermore, their results suggest that the GATA site is responsible for localizing the
642 expression to the fat body, and the NF-κB site is responsible for the induction of the gene in
643 response to infection [30]. Our analysis based on scrambling the binding sites in the *lectin-*
644 *24A* reporter construct are also in line with this model, with the addition that STAT binding is

645 also involved in regulation in a similar way as GATA (Figure 4D). However, notably, Pannier
646 is strongly upregulated by parasitoid infection and this upregulation does not differ between
647 anterior and posterior of the larval fat body (Figure 3). This result suggests that Pannier is not
648 just playing a role to localize *lectin-24A* expression to the fat body, but is also involved in the
649 induction of its expression. Nonetheless, when we manipulated specific genes in each of
650 these pathways the effects on *lectin-24A* expression become less straightforward, suggesting
651 more complex models of coordinated regulation and potential indirect effects from
652 knockdown or knockout in the whole larva.
653
654 Immune systems evolve rapidly as hosts adapt to an ever-changing array of parasites and
655 pathogens. We have previously shown that a polymorphic deletion upstream of Lectin-24A
656 has led to reduced parasitoid resistance in wild populations [12], and here we provide an
657 explanation of this by showing that it abolishes an NF-κB binding motif. Furthermore, a
658 group of four single nucleotide polymorphisms cause a smaller reduction in Lectin-24A
659 expression [12]. Notably, one of these is within a STAT binding motif, with the low
660 expression allele predicted to bind STAT92E with lower affinity (TTC$(N)_4$AA in the high
661 expression DGRP-437 line becomes TTA$(N)_4$AA in the low expression DGRP-892 line).
662 Therefore, the cis-regulatory elements that respond to immune signaling pathways may be
663 hotspots of host-parasite coevolution.
664
665

666 # Methods

667

668 **Lectin promoter reporter assay with bacterial, viral, or wasp infection**

669 Six vials of cornmeal fly food (per 1200 ml water: 13g agar, 105g dextrose, 105g maize, 23g
670 yeast, 35ml Nipagin 10% w/v) were prepared for overnight egg lay with the LP437 line,
671 where three vials were used for wasp infection and three vials used as uninfected controls.
672 Three hundred flies of the LP437 line, with approximately even distribution of males and
673 females, were prepared in a cage for egg lay onto a 90mm agar plate coated with a thin layer
674 of yeast paste. After an overnight egg lay in the cage onto an agar plate, the eggs were
675 transferred onto 50mm plates containing cornmeal fly food and some yeast. The eggs were
676 removed from the agar plate by washing them down with a phosphate-buffered saline (PBS)
677 solution (prepared from tablets Oxoid# BR0024G) and dislodging the eggs with a clean
678 plastic brush. The egg solution was transferred into a 15ml Falcon tube and fly eggs allowed
679 to sink to the bottom, where 500µl were then transferred using a cut pipette tip into an
680 Eppendorf tube. Using a cut 200µl pipette tip, 15µl of fly eggs were transferred onto each
681 50mm plate. Four plates were prepared. The vials and plates were all incubated at 25ºC and
682 70% humidity, and on the fourth day infections were carried out: three female parasitoid
683 wasps were added to each of the three vials for infection for three hours; bacterial and viral
684 infections were carried out according to the protocol below. At 24 hours post-infection (hpi),
685 larvae were collected from the fly food by adding 15% sugar solution to allow the larvae to
686 float to the top of the vial. Groups of fifteen larvae were collected and transferred to a piece
687 of filter paper to dry. They were then transferred to a PCR tube containing 5-10 1.0mm
688 zirconia beads (BioSpec Products # 11079110zx) with 100µl PBS (Gibco# 10010-015). This
689 procedure was repeated until we have four samples for each infected and uninfected
690 conditions with each pathogen.

691

692 **Bacterial and viral cultures for infection**

693 A LB-agar plate with no antibiotic added was prepared under sterile conditions, *E.coli* of the
694 OP50 strain was transferred to the plate using a sterile inoculation loop. The loop was then
695 wiped around the plate to produce single colonies. One colony was picked from the plate
696 following an overnight incubation at 37ºC. The colony was then transferred to 5ml of sterile
697 LB with no antibiotic. The culture was shaken overnight at 37ºC. We then recorded the initial
698 concentration of the *E. coli* culture at OD600 (600nm) by measuring absorbance with a
699 NanoDrop™. 1ml of the bacterial culture was transferred to a 1.5ml Eppendorf tube and spun
700 down at 10,000xg for 5 minutes to concentrate the culture. It was then diluted according to
701 the initial concentration to be slightly above 0.5 at OD600. This culture was then read again
702 on the nanodrop and diluted further to be exactly 0.5 at OD600. The DCV viral cultures were
703 prepared according to Bruner-Montero et al., and aliquots were thawed immediately before
704 use [90].

705

706 ***Drosophila* larval infection with virus and bacteria using tungsten wires**

707 We cut a short section of about 1-2 cm of Tungsten wire using scissors or a wire cutter. The
708 wires were mounted into nickel plated holders and adjusted to an appropriate length. The tips
709 of the Tungsten wires were sharpened by connecting them to a circuit with Sodium
710 Hydroxide (NaOH) solution and a battery pack. While sharpening, we checked the tips of the
711 Tungsten wires continuously to see if it needs to be sharpened further.

712 We transferred the prepared bacterial or viral culture to the caps cut off from 0.5ml Eppendorf
713 tubes and the caps were filled as much as possible. Control cultures were also prepared for
714 either bacteria or virus infections, which consisted of LB for bacteria and cell culture medium
715 for virus. *Drosophila* larvae were removed at around late second instar/early third instar from

716 the 50mm cornmeal plates, and washed in PBS. Twenty larvae were transferred at a time to a
717 piece of filter paper. The prepared Tungsten needle was dipped into the prepared culture and
718 used to prick the larva. This was repeated for each larva. We then gently brushed water
719 through the line of 20 larvae for easier removal from the filter paper and transferred the
720 larvae onto the fly food in a glass vial.
721
722 **RNA extraction, cDNA preparation, and quantitative polymerase chain reaction (qPCR)**
723 Fifteen *Drosophila* larvae were used for RNA extraction for each treatment using the total
724 RNA extraction protocol using the chloroform isopropanol TRIzol method following the
725 manufacturer's protocol (Invitrogen #15596026).
726 *DiptericinB* and *CecropinA* were targeted with qPCR to confirm the infection with *E.coli*.
727 *DptB* primers: fwd-"TTCTCGAGTGCCTGGGCTTA" and rev-
728 "ATTGGGAGCATATGCCAGTG", and *CecA*1 primers: fwd-
729 " TTGGCAAGAAAAATCGAACGC" and rev-"GCTTGTTGAGCGATTCCCAG". DCV
730 infection was detected using primers specifically targeting a region of the DCV genome: fwd-
731 "GACACTGCCTTTGATTAG" and rev- "CCCTCTGGGAACTAAATG".
732
733 **Quantitative Polymerase Chain Reaction (qPCR)**
734 One µl of RNA per sample was reverse-transcribed with Promega GoScript reverse
735 transcription system (Promega #A5001), using random hexamer primers. The complementary
736 DNA (cDNA) samples were then diluted 10-fold with nuclease free water for qPCR. qPCR
737 was performed on Applied Biosystems StepOnePlus system (ThermoFisher Scientific
738 #4376600) or using Applied Biosystems QuantStudio5 system (ThermoFisher Scientific
739 #A34322), with SensiFast SYBR Hi-ROX mix (Bioline #BIO-92020).
740

741 **Localized *lectin-24A* expression microscopy**

742 Larval fat bodies were dissected from 3rd instar *D. melanogaster* larvae. The whole fat body
743 was obtained by first removing the head of the larva then pulling back the cuticle to expose
744 the fat body and other inner tissues. The gut, salivary gland, and other tissues were then
745 removed and discarded. Each isolated fat body is transferred into an Eppendorf tube
746 containing ice-cold PBS. The fat bodies were incubated in 3.7% formaldehyde diluted in PBS
747 for 20 minutes with gentle agitation on an orbital shaker, with the tubes covered in foil. The
748 formaldehyde was then removed by pipetting and washed with ice-cold PBS for three times.
749 Then we again washed the fat bodies two times for 30 minutes each with 0.3% ice-cold PBT
750 with gentle agitation on an orbital shaker, with the tubes covered in foil. The fat bodies were
751 then washed for three times again with ice-cold PBS by pipetting. We then stained the nuclei
752 with 1μg/ml Hoescht staining solution diluted in PBS for 5 minutes, while making sure the
753 staining solution sufficiently cover the whole fat body. The fat bodies were washed again
754 with PBS for three times, then each transferred individually to a clean Eppendorf tube.
755 Immediately cover the fat body tissue with cold Vectashield mounting media (Vector
756 Laboratories # H-1000-10). Pipette tips with the ends cut were used to transfer each fat body
757 with minimal volume of mounting media on to a microscope slide. The fat body was then
758 spread out on the microscope slide using two nickel plated pin holders holding 0.1mm
759 diameter pins. We then slowly added a cover slip to the slide, making sure there were no
760 bubbles. The fat bodies were then imaged using a Leica upright fluorescence microscope
761 (Leica # DM6000M).

762

763 **Larval fat body posterior vs. anterior RNAseq dissection**

764 Larval fat bodies were dissected according to the protocol in the previous section "**Localized**
765 ***lectin-24A* expression microscopy protocol**". The anterior and posterior of the fat body were

766 separated along the representative line as shown in Figure 2A.

767

768 **Library preparation for RNA sequencing**

769 At 24 hpi, groups of 10 male larvae from the stock expressing the *lectin-24A* promoter
770 reporter were dissected, with the anterior and posterior larval fat body sections separated.
771 Age-matched uninfected fat bodies were also dissected and separated into anterior and
772 posterior sections. RNA was isolated from each sample of pooled fat body sections, where for
773 each sample the PBS supernatant was removed and 250µl of Tri-reagent (Ambion
774 #10296010) was added. The fat bodies were homogenized using TissueLyzer II (Qiagen
775 #85300) for 2 minutes at 30Hz. 62.5µl of chloroform was added to each sample and the tubes
776 were shaken for 15 seconds followed by 3 minutes of incubation at room temperature. The
777 samples were then centrifuged for 10 minutes at 12,000xg at 4ºC. 66µl of the upper aqueous
778 phase was transferred to a fresh Eppendorf tube and 156µl of propan-2-ol was added. The
779 tubes were then inverted 5 times to mix thoroughly. Following a 10-minute incubation at
780 room temperature, the samples were centrifuged at 12,000xg at 4ºC for 10 minutes. The
781 supernatant was removed by pipetting, watching out for small white pellets at the bottom of
782 the tubes, and 250µl of ice-cold 70% ethanol was added. The samples were centrifuged again
783 at 4ºC at 12,000xg for 2 minutes and the ethanol was removed. The RNA pellets at the
784 bottom of the tubes were allowed to air-dry very briefly and 20µl of nuclease-free water was
785 added. The RNA pellets were dissolved by incubating the tubes on a heat block adjusted to
786 45ºC for 5 minutes. RNA was quantified using a Qubit RNA HS assay kit (Thermofisher
787 #Q32852). RNAseq libraries were then prepared using a NEBNext Ultra II Directional RNA
788 Library Prep Kit for Illumina (New England Biolabs #E7760S) with NEBNext Multiplex
789 Oligos for Illumina (96 Unique Dual Index Primer Pairs) (New England Biolabs #E64460S)
790 and polyA enrichment module (NEB #E7490S). Up to 1µg of total RNA was used to make

791 each RNAseq library, where mRNA was fragmented at 94ºC for 15 minutes following polyA
792 enrichment, according to the protocol recommendations. The adaptor concentrations and
793 number of amplification cycles used were adjusted based on the amount of starting RNA. The
794 quantity in each prepared library sample was measured using Qubit DNA HS assay kit
795 (Thermofisher #Q32851). The library quality was assessed using a Bioanalyzer high
796 sensitivity kit on an Agilent 2100 Bioanalyzer (Agilent #5067-4626). The libraries were
797 sequenced at Source BioScience using Illumina NovaSeq 150bp paired-end reads.
798

799 **Statistical analyses of RNAseq data**

800 We used Trim Galore (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) for
801 trimming raw RNAseq reads, setting a Phred score of 20 for quality control. Reads with
802 fewer than 50 bases after trimming were removed. The reads were then mapped and counted
803 using STAR v2.6.0 [91] to *D. melanogaster* reference genome Dmel-r6.40. The R package
804 edgeR (RRID:SCR_012802) was used for differential expression analyses. Genes that had a
805 count per million (CPM) above ten in at least four libraries were kept, serving as a threshold
806 for identifying genes with detectable expression in our dataset. Dispersions were estimated
807 using the Cox-Reid profile-adjusted likelihood (CR) method in edgeR, and the expression
808 data were fitted with a negative binomial generalized linear model (GLM). Differential
809 expression of genes was then determined with a quasi-likelihood (QL) *F*-test, where a false
810 discovery rate (FDR) of 0.05 was set as the significance threshold.

811

812 **Gene Ontology enrichment analysis**

813 A Gene Ontology (GO) enrichment analysis was conducted using the R package goseq [92].
814 The list of genes detected in RNAseq analysis was used as a background set. Sets of

815 significantly upregulated and significantly downregulated genes with an FDR < 0.05 and up
816 or down-regulation of at least one logFC were then analyzed separately to determine GO
817 enrichment. The length bias inherent to RNAseq data were accounted for by calculating a
818 Probability Weighting Function (PWF) using goseq, that gives a probability that a gene will
819 be differentially expressed based on its length alone. A null distribution for GO category
820 membership was approximated with Wallenius distribution, and each GO category is then
821 tested for over and under representation amongst the set of differentially expressed genes.
822 Where there are more than 5 GO terms in any of the three major Gene Ontology branches
823 (Biological Processes, Cellular Components, and Molecular Functions), the list of GO terms
824 was summarized using REVIGO [93] (RRID:SCR_005825).
825
826 **Yeast-1-hybrid (Y1H)**
827 The Y1H analysis of the *lectin-24A* upstream region was carried out following the system
828 developed in [94].
829
830 **RNAi knockdown assay**

831 We used fly stocks from the Transgenic RNAi Project (TRiP), which consisted of flies that
832 utilize the GAL4/UAS driven system to silence a specific gene using short hairpin RNA and
833 RNA interference (RNAi). To perform the experiment, a fly stock that expresses a
834 *daughterless* driven GAL4 (*da*-Gal4), a temperature-sensitive tubulin driven GAL80 (*tub*-
835 Gal80^{ts}), and the *lectin-24A* promoter Venus reporter (LP437) was crossed to the RNAi-
836 expressing line targeting *Stat92E* (BDSC#33637). The cross generated F_1 progeny with the
837 genotype LP437/+; tub-Gal80ts/+; da-Gal4/UAS-TRiP. Egg lay was carried out at 25ºC
838 overnight, then the developing larvae were reared at 29ºC. On the 4th day, larvae were
839 infected for 3 hours with 3 female wasps in each vial, and larvae were collected 24 hpi for

840 protein extraction to analyze fluorescence expression of *Venus*^{LP437}, as stated in "**Lectin**
841 **promoter reporter assay**".

842

843 **Truncated *lectin-24A* promoter fluorescence reporter**

844 We created three truncated versions of the *lectin-24A* promoter fluorescence reporter, starting
845 20bp 5' of each of the three indels in the *lectin-24A* upstream region. The constructs were
846 made in the same way as the original *lectin-24A* fluorescence reporter construct [12]. We
847 digested pWallium20 backbone plasmid with the restriction enzymes HpaI and PstI (NEB #
848 R0105S and R0140T) followed by gel purification. The forward primers used to amplify each
849 promoter sequence are listed in the following table. The reverse primer used to amplify each
850 sequence was 5'-
851 ATTATAAGCTGCAATAAACAAGTTTTACTTGTACAGCTCGTCCATGCC-3'. The insert
852 sequence is amplified from the full-length *lectin-24A* promoter fluorescence reporter, and it
853 includes both the truncated promoter sequence as well as the Venus open reading frame. The
854 digested pWallium20 backbone and the amplified PCR fragment were assembled using a
855 HiFi assembly reaction (NEB #E5520), with an incubation time of 1 hour at 50 ^{o}C. The
856 transformation plasmids were then purified by miniprep (Qiagen # 27104). Plasmid
857 sequences were then verified by Sanger sequencing using the primers M13 Reverse 5'-
858 CAGGAAACAGCTATGAC-3' and the reverse primer to amplify the insert sequence. All
859 constructs were injected into *D.melanogaster* line attP2Ar5, harboring an attP landing site on
860 the X chromosome (BDSC#24480) by Bestgene Inc, USA, and transformants were selected
861 by eye color.

Primer name	**Primer sequence**
truncated LP7bp Fwd	GCTATACGAAGTTATCTGCAACCGATGAAATTTTCATGC
truncated LP8bp Fwd	GCTATACGAAGTTATCTGCATCTTTTGCTCTGACATTCAC
truncated LP21bp Fwd	GCTATACGAAGTTATCTGCAGCAACTATTTTTTA CTGAATAAATAAGG

862

863

864 **Scrambling TFBMs with site-directed mutagenesis (SDM)**

865 To generate *lectin-24A* promoter fluorescence reporter with scrambled transcription factor
866 binding sites, we used Q5 site-directed mutagenesis kit (NEB# E0554S). Primers were
867 designed according to the sites to be scrambled as shown in the table below, where the
868 lowercase letters represented changes made to the original promoter sequence. The truncated
869 LP8 plasmid was used for scrambling instead of the full-length *lectin-24A* fluorescence
870 promoter reporter.

871

Primer name	Primer sequence
Dif_dl_fwd	AAGGCTATCTttATCAAGTTTTTTAAGTAG
Dif_dl_rev	ATTTATTCAGTAAAAAATAGTTGC
GATA_fwd	ATCAAGTAATccccccGCGCCTATAGAAAC
GATA_rev	TGCTTTATTTTCTTCAGTCTTG
Stat_1_fwd	ATTTAATAAAggaCTTTTAATTCGTTTTGATAATATTTAAATATTTC
Stat_1_rev	AATTCTTTAAATATGAAATTTGAAAAGG
Stat_2_fwd	ATTTTTTACTtccTAAATAAGGCTATCTGGG
Stat_2_rev	AGTTGCTATGTATACAATATTG
Stat_3_fwd	TTTTTAAGTAtccACACAAGACTGAAGAAAATAAAG
Stat_3_rev	ACTTGATCCCAGATAGCC

872

873

874 **JAK/STAT pathway *et* mutant and *lectin-24A* reporter recombination**

875 The *et* mutant line is a kind gift from Michèle Crozatier [21]. The *lectin-24A* promoter
876 fluorescence reporter was recombined with the *et* mutant, as both the construct and the gene
877 are on the X chromosome. A line with attached-X chromosomes (genotype: C(1)DX, y w f/y
878 cv v shi[ts1] f mal/y[+]Y) from the Genetics Department at the University of Cambridge was
879 used to follow chromosomes with recombination events. Females from the *lectin-24A*
880 promoter fluorescence reporter line was first crossed with *et* mutant line males, then the

881 female offspring were crossed to *lectin-24A* promoter fluorescence reporter males. Individual
882 male offspring of this cross, which might carry a recombined X chromosome are crossed to
883 females of the attached-X line. As the X chromosomes of males are always inherited through
884 the male offspring when crossed to an attached-X female, any recombined X chromosomes in
885 the males are kept in males.
886 Male offspring from each of the cross above were first screened for fluorescence using an
887 epifluorescence microscope. Stocks that show Venus fluorescence were then screened for *et*
888 mutation by PCR. Genomic DNA was extracted from males of each stock. Two pairs of
889 primers from [21] were used to check *et* mutation. Fwd-1 & Rev-1 give an amplicon of
890 1186bp, and Fwd-2 & Rev-2 give an amplicon of 526bp. Stocks carrying the *et* mutation
891 would not give a PCR product from either of these primer pairs. Male larvae from stocks that
892 have successfully recombined were used in subsequent assays for *lectin-24A* promoter
893 fluorescence.

Primer name	**Primer sequence**
Fwd-1	TGGAGTTCAATGGAGCCGA
Fwd-2	CAGCATGCTCGCGATCTTCA
Rev-1	GGAGCAGCAATGTCGTCGGT
Rev-2	AAATGGCGGACATCCGGGTT

894

895

896 ***DomeΔCYT* assay**

897 The stock expressing *DomeΔCYT* under UAS control is a kind gift from Michèle Crozatier.
898 We crossed females expressing the larval fat body driver *Cg-GAL4* or the ubiquitous *da-*
899 *GAL4* driver, as well as *tub-GAL80*^{ts}, with males expressing *UAS-DomeΔCYT*. The stock also
900 carried *UAS-et* with a balancer chromosome that could not be identified in larvae, so a
901 portion of the F_1 progeny also expressed *UAS-et*. The control cross was with females
902 expressing only the *Venus*^{LP437} reporter with no driver. We carried out egg lay and allowed the

903 larvae to develop at 18ºC, until the 7th day after egg lay, when we moved the larvae to 29ºC 3
904 hours prior to infection with parasitoids. Sample collection was performed at 24 hpi. The
905 assays were then carried out as stated in "**Lectin promoter reporter assay**".
906
907 ***hop^{Tum}* assay**

908 Females expressing the *Venus^{LP437}* reporter were crossed with males of the *hop^{Tum}* genotype.
909 Larvae were allowed to develop at 25ºC. Since both the reporter and *hop* are located on the X
910 chromosome, only female F_1 heterozygotes were selected during sample collection at 24 hpi.
911 The assays were then carried out as stated in "**Lectin promoter reporter assay**".
912
913 **Dominant positive *Pnr* mutant expression in the *Drosophila* fat body**
914 We expressed the dominant positive mutant *Pnr^{D4}* under the control of UAS (BDSC#36546)
915 in the *Drosophila* larval fat body using the driver *Cg-GAL4*. The larvae were allowed to
916 develop at 25ºC until 3 hours prior to infection with parasitoids, then they were moved to
917 29ºC until sample collection at 24 hpi, as stated in "**Lectin promoter reporter assay**".
918
919 **Somatic mutagenesis of key immune pathway genes**

920 In order to create somatic knockouts of immune pathway genes, we created a line that
921 expresses the *lectin-24A* promoter reporter and *Act5c-Cas9*. We crossed virgins of this line to
922 males expressing guide RNAs (gRNAs) targeting each gene. The gRNA-expressing lines
923 used are listed below. Primers used for PCR and sequencing to confirm the somatic
924 mutagenesis are also listed. The assays were carried out as stated in "**Lectin promoter**
925 **reporter assay**".

gRNA target gene	BDSC line #	Fwd primer for sequencing	Rev primer for sequencing
Dif	84190	TGTTATTGCTCTGGGCCTGG	GCTGCTCTTGCCGTAAATCG
Tl	83044	TGTGTTGCCACTAGGTGTCG	TACACCGTCGCATCTGAACC
dl	81730	TTGGCCACGTAATGTTTCCG	AAGGCCTCCCAAACACTCTG
Rel	84191	CCGAACATCGGGGAAGTACC	CGACCAGCTGATGGGAATGT
hop	79787	AGGAGCATTCGCAGTAAGCC	CAATTCGGGCACACGGAAAC
pnr	81993	AAAATGGCTGCCAAGCAAGG	GGGGTCTACTCACTGCACTC

926

927 **Overexpression of NF-κB transcription factors dl and Rel**

928 The stocks for expressing *dl* (FlyORF Stock# F000638) and *Rel* (FlyORF Stock# F001780)
929 under UAS control were obtained from the Zurich ORFeome Project [95]. We crossed
930 females expressing the larval fat body driver *Cg-GAL4* and *tub-GAL80*^{ts}, with males
931 expressing *UAS-dl* or *UAS-Rel*. The control cross was with females expressing *Venus*^{LP437}
932 with no GAL4 driver. Egg lay and larval development were carried out at 25°C until 3 hours
933 prior to wasp infection on the 4th day after egg lay, when they were moved to 29°C, and
934 sample collection was done at 24 hpi, following "**Lectin promoter reporter assay**". The
935 UAS-dl stock carried a balancer that could not be identified at the sample collection stage in
936 larvae, so the larvae collected from the UAS-dl crosses were with a mix of *Cg-GAL4/UAS-dl*
937 and *Cg-GAL4/+* genotypes.

938

939 **Enrichment of TFBMs in the upstream region of parasitism-induced genes**

940 FIMO from the MEME suite version 5.5.5 was used to scan sequences for occurrences of
941 TFBMs [96,97]. Default parameters were used for GATA TFBMs, and a significance

threshold of 0.005 was used for NF-κB and STAT92E TFBMs. MEME format position weight matrices (PWMs) were generated from binding sites collected in flyfactorsurvey [98] for GATA sites, and from IUPAC motifs for NF-κB and STAT sites (Figure 4C).

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Data availability

The RNAseq data has been submitted to the NCBI Sequence Read Archive under the BioProject number PRJNA1021619, with BioSample accessions from SAMN37565424 to SAMN37565439. Processed data files and scripts to analyze data are available on Apollo, the University of Cambridge repository (https://doi.org/10.17863/CAM.108488).

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