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.

Significance Statement

Research on the antimicrobial immune defenses in fruit flies has led to key insights of innate immunity, particularly through studies of how the immune response is regulated in the insect humoral immune tissue, the fat body. However, there is a gap in our knowledge in understanding immunity against another major natural enemy of fruit flies, the parasitic wasps. Here we have focused on the regulation of humoral immune effectors in this response, where we found that three signaling pathways, JAK/STAT, NF-κB, and GATA, combine in the regulation of this immune response in the fat body. This gene regulation mechanism represents a novel system, underlying how parasites can elicit an immune response distinct from antimicrobial defenses.

62 ## Introduction

63

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

73

74 Several humoral immune effectors have been identified in the context of humoral anti-
75 parasitoid immunity in *Drosophila*. Humoral immunity is mediated by the *Drosophila* fat
76 body, an insect-specific organ functionally similar to the mammalian liver, the metabolic hub
77 of the organism. The invertebrate counterparts of the mammalian complement factors, called
78 thioester-containing proteins (TEPs) [8], a group of serine proteases [9,10], and the fat body-
79 derived peptide edin [11], are some of the secreted immune effectors shown to be involved in
80 this response. We have previously also shown that a C-type lectin, lectin-24A, secreted by the
81 larval fat body, plays an important role in this immune response [12]. The induction of *lectin-*
82 *24A* expression following parasitism, controlled by the *cis*-regulatory upstream region of the
83 gene, is associated with higher resistance to parasitoid infections [12]. Lectin-24A localizes to
84 the surface of the parasitoid egg before hemocytes attach to it, suggesting that it may play a
85 role in the recognition of the egg and subsequent recruitment of hemocytes.

86

87 *Drosophila* humoral immunity against bacterial and fungal infections has been investigated
88 intensively in the past and is well characterized. Following recognition of pathogens, humoral
89 defenses are induced via the activation of the two hallmark NF-κB immune signaling
90 pathways, Toll and Imd (reviewed in [13] and [14]). These two pathways are known for their
91 roles in regulating the secretion of many immune-related genes by the fat body, most
92 famously antimicrobial peptides (AMPs). Dif and Dorsal are the two NF-κB family
93 transcription factors (TFs) in the Toll pathway, which is mostly responsible for defense
94 against Gram-positive bacteria, fungi and other pathogens. Relish is the transcription factor
95 involved in Imd signaling, which mostly controls the defense against gram-negative bacteria.
96 In some cases, a combination of the two pathways is responsible for the expression of
97 immune effectors in the fat body [15,16]. Other than its role in the induction of AMP
98 expression, the Toll pathway is also involved in regulating the proliferation of hemocytes in
99 *Drosophila* larvae [17,18]. Loss-of-function mutations in the Toll pathway components result
100 in reduced capacity to melanize parasitoids [13,17], whereas overactivation of the Toll
101 pathway leads to a spontaneous melanization phenotype reminiscent of the melanotic
102 encapsulation response against parasitoid wasps [13,17,19].
103
104 The Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway has
105 been shown to be involved in the cellular immune response through the regulation of
106 hemocyte differentiation following parasitoid infection [18]. Downregulation of JAK/STAT
107 signaling in the anterior lobes of the larval lymph gland plays an important role in regulating
108 the differentiation of lamellocytes, a specialist type of hemocyte for the encapsulation
109 response [20,21]. It was also found that the activation of JAK/STAT signaling in somatic
110 muscles by hemocyte-derived cytokines Upd2 and Upd3 releases energy stored in the
111 muscles as glycogen for use in the immune response and is key to the immune response

112 against wasp infection [22,23]. Similar to the Toll pathway, overactivation of JAK/STAT
113 signaling in the whole larva also results in an over-proliferation of hemocytes and
114 spontaneous melanization [18,24,25].
115
116 A third group of transcription factors mediating *Drosophila* immunity is the GATA family of
117 transcription factors, a conserved group of TFs with zinc finger domains that recognize
118 variations of the GATA motif ((A/T) GATA (A/G)) (reviewed in [26]). *Drosophila* has a total
119 of five GATA factors: Pannier, Serpent, Grain, dGATAd, and dGATAe. Serpent is essential
120 for the differentiation of fat body and hemocytes [27,28], and Pannier has been found to play
121 an important role in hemocyte differentiation and maturation [29]. Strikingly, many immunity
122 genes expressed by the fat body exhibit Rel-Serpent synergy, where closely linked NF-κB
123 and GATA binding sites are found in the upstream regulatory region [30,31]. While Serpent is
124 the principal GATA factor regulating the antimicrobial response in the *Drosophila* fat body,
125 other GATA factors were reported to regulate immune responses in other *Drosophila* tissues
126 [31].
127
128 The anti-parasitoid humoral response is mainly driven by wasp parasite-associated molecular
129 patterns (PAMPs) [32], but the signaling pathways involved in the response remain unknown.
130 Previous studies of the transcriptional response to parasitoid attack in *Drosophila* larvae have
131 identified a series of genes that are differentially expressed in response to wasp oviposition
132 [33–36]. These studies have implicated the three pathways outlined above in the response
133 [33,35]. In particular, Wertheim et al. found that transcription factor binding motifs (TFBMs)
134 for NF-κB, STAT, and GATA significantly associate with genes differentially expressed after
135 parasitism [33]. However, these observations were not followed up experimentally. Previous
136 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.

145 ## Results

146

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

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

161

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

168

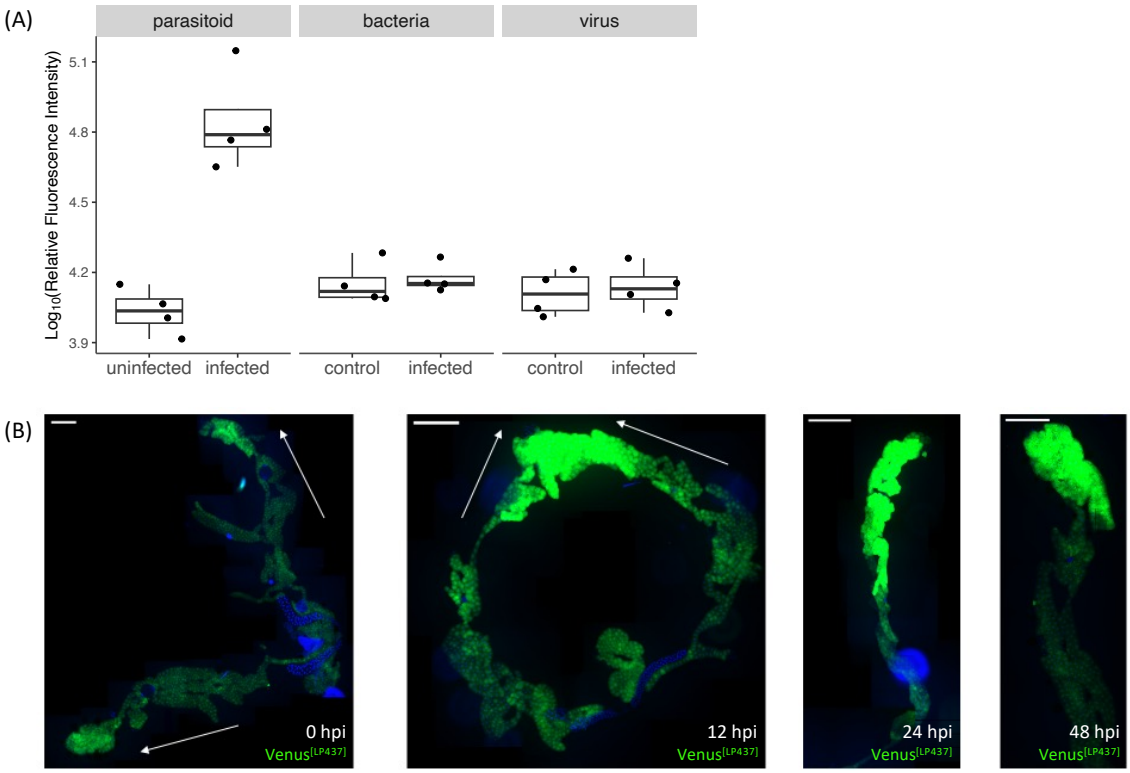


Figure 1. Expression of the *lectin-24A* reporter *Venus^{LP437}*. (A) Expression of *Venus^{LP437}* 24 hours post-infection (hpi) with different pathogens, left to right: *Leptopilina boulardi* parasitoid wasps, *Escherichia coli* bacteria, and *Drosophila* C virus. The relative fluorescence intensity 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

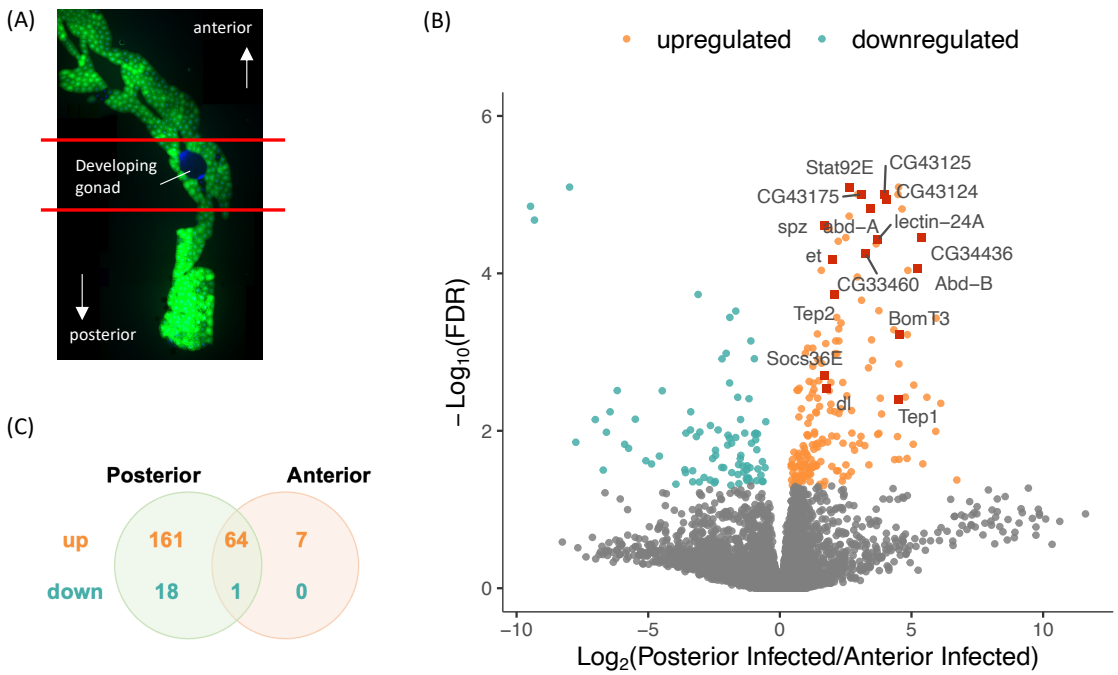
As we observed localized expression of *lectin-24A* in the posterior ends of the larval fat body, we investigated whether this localized response is seen in other immune-responsive genes. We conducted fat body RNA sequencing comparing gene expression changes in the anterior and posterior of the fat body 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).

214 Significantly downregulated genes in the larval fat body after parasitism suggest parasitoid
215 infection is causing shifts in metabolism and other aspects of immunity. *sug* and *daw*, genes
216 involved in sugar metabolism [44,45], are downregulated in the fat body after parasitoid
217 infection. We also found certain immune factors to be downregulated with parasitism,
218 including *Lectin-galC1* and the lyszyme *CG8492*, both involved in antimicrobial response
219 [46,47]. *RhoBTB*, a Rho-related BTB domain-containing protein involved in anti-parasitoid
220 response is downregulated in the posterior fat body after infection [48].

221



222

223 **Figure 2. RNA sequencing of larval fat body sections.** (A) Dissection of male larval fat
224 bodies along the representative red lines into anterior and posterior sections, where the
225 developing gonads were removed, for RNA sequencing. (B) Differentially expressed genes,
226 with absolute $\log_2$FC above 1 and False Discovery Rate (FDR) below 0.05, between anterior
227 and posterior larval fat body sections under infected condition. (C) Venn diagram showing
228 overlap of significantly differentially expressed genes with absolute $\log_2$FC above 1 after
229 infection in anterior and posterior fat body sections.
230

231

232 **Key immune signaling pathway components are differentially expressed in the fat body**

233 **after parasitism**

234 There are also interesting patterns of localized immune signaling gene expressions: key
235 components of the NF-κB and JAK/STAT pathways show up as top differentially expressed
236 genes between posterior and anterior sections in both control and infected conditions (Figure
237 2B and Supplementary Figure 2). GO enrichment of significantly upregulated genes in the
238 posterior of the fat body also showed enrichment of terms related to "receptor signaling
239 pathway via JAK-STAT", "immune system process", and "defense response" (Supplementary
240 Figure 3).
241
242 Selecting specific components from major immune signaling pathways including
243 transcription factors, we analyzed their expression patterns in anterior/posterior and
244 uninfected/infected conditions (Figure 3). It is striking to see that different transcription
245 factors show differential patterns of expression. The NF-κB transcription factor *dl* (*dorsal*) is
246 induced specifically in the posterior of the fat body in response to infection, with *Rel* (*Relish*)
247 showing a non-significant trend in the same direction. *Dif* shows similar levels of expression
248 across the four conditions (Figure 3). The gene encoding the Toll receptor *Tl* is also
249 upregulated with infection specifically in the posterior (Figure 3). While the Toll ligand, *spz*,
250 is more highly expressed in the posterior regardless of infection status (Figure 3).
251
252 The five known GATA transcription factors are encoded by *pnr* (*pannier*), *srp* (*serpent*), *grn*
253 *(grain), GATAd*, and *GATAe*. While the other four GATA transcription factors showed similar
254 expression between the fat body sections and infection statuses, *pnr* is upregulated with
255 infection in both the anterior and posterior (Figure 3).
256
257 The JAK/STAT pathway transcription factor *Stat92E* shows an entirely spatial differential
258 expression not affected by infection state, where it is constitutively upregulated in the

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

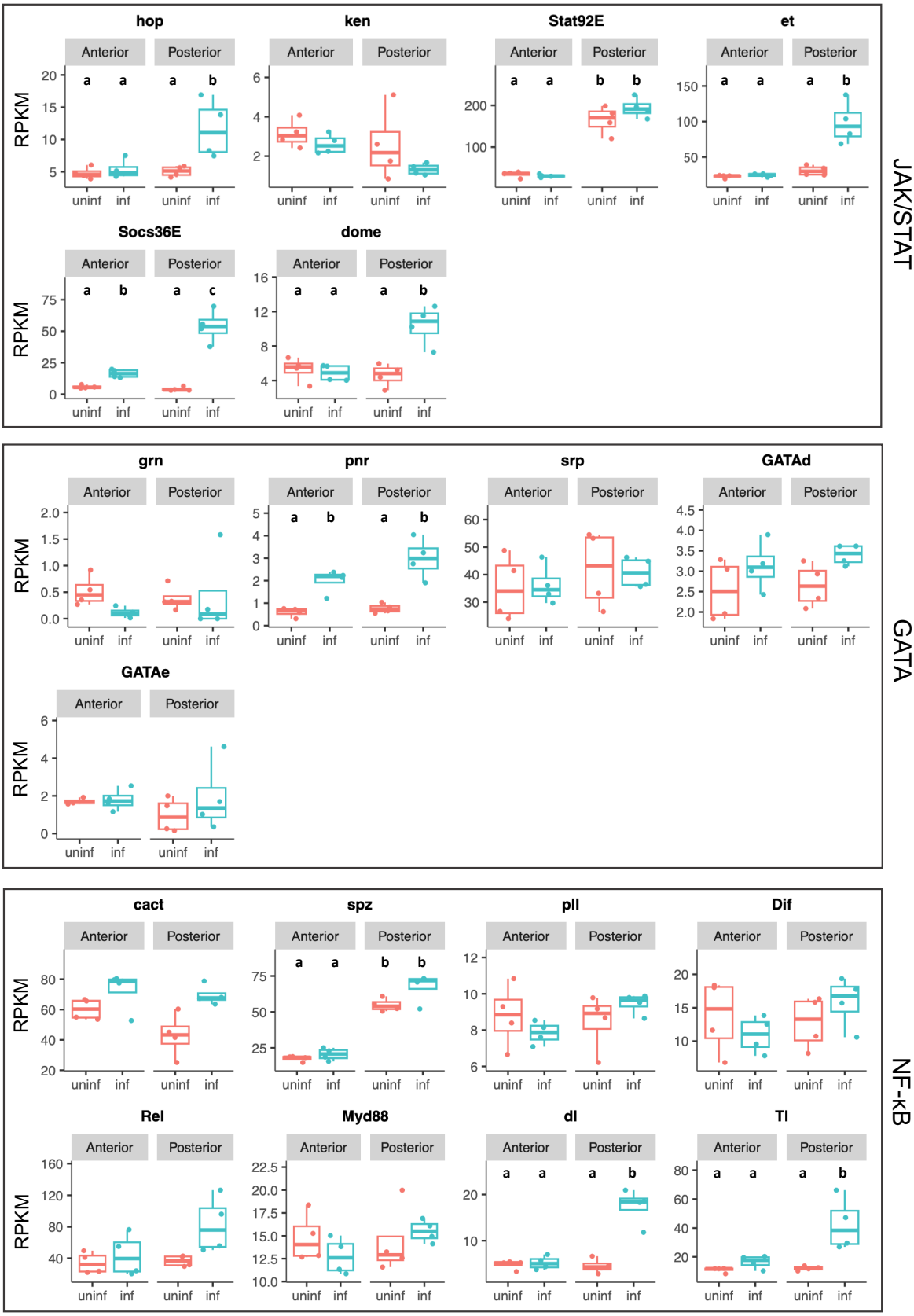


Figure 3. Gene expression patterns of components from major immune pathways in anterior and posterior sections with and without infection. Expression shown as Reads Per Kilobase of transcript per Million mapped reads (RPKM). Labels denote significant

273 (genome-wide false discovery rate < 0.05) differential expression with absolute $\log_2$FC of at
274 least 1 between treatment groups. Only significant differences are shown.
275
276

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

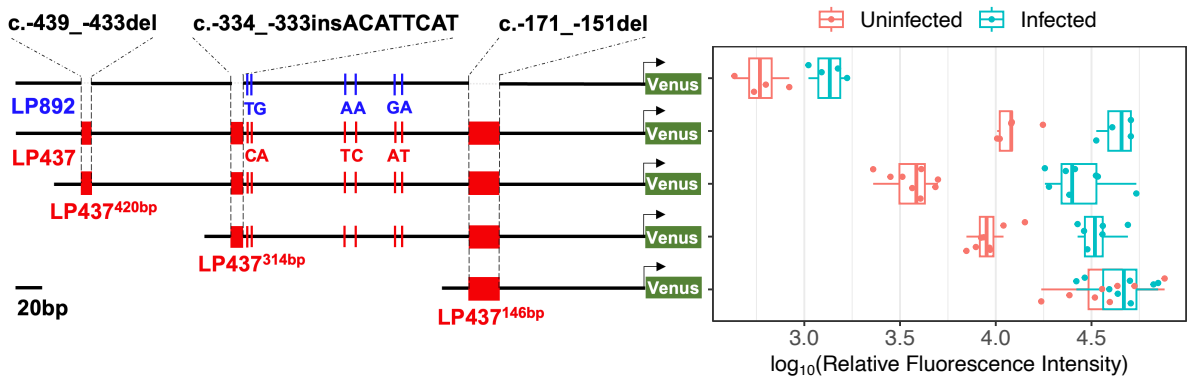
279 We have limited understanding of the regulation of the anti-parasitoid immune response in *D.*
280 *melanogaster*, particularly the early signaling that activates humoral immunity in the larval
281 fat body. We investigated the upstream region of the *lectin-24A* gene to further define the
282 specific sequences that underlie its induction with parasitization, and to understand the
283 transcription factors involved in the regulation of its expression. We created truncated
284 versions of the $Venus^{LP437}$ reporter construct where we deleted the sequence from the 5' end
285 (Figure 4A). While we saw little effect from the first two deletions to 420 bp and 314 bp
286 upstream of the gene, we found that, when we truncated the sequence to be 146 bp upstream
287 of the TSS, there were significant changes to the expression of the reporter where it becomes
288 expressed in other tissues such as the gut with and without parasitization (Supplementary
289 Figure 4). Therefore, the region 314 bp upstream of the gene is required for an inducible
290 response of the *lectin-24A* gene that is restricted to the fat body tissue.
291

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

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

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

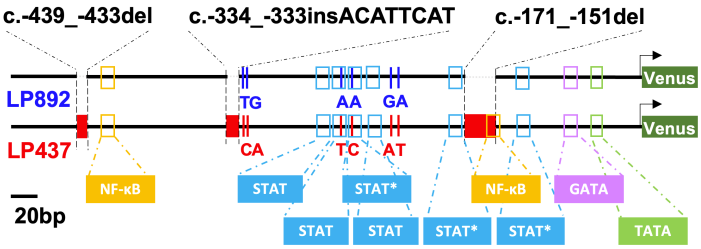
(A)



(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)

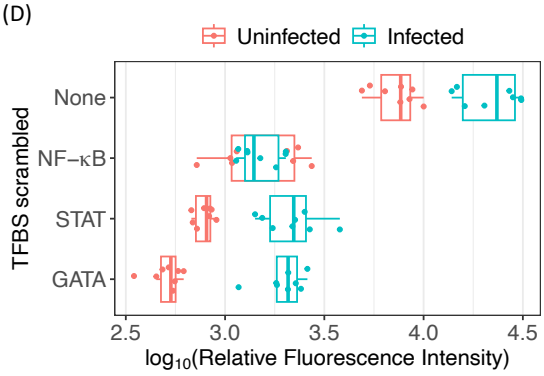


Figure 4. Transcription factor binding motifs (TFBMs) upstream of *lectin-24A*. (A) Truncated *lectin-24A* reporter Venus expression compared to the full-length reporter constructs with the susceptible DGRP-892 (LP892; blue) and resistant DGRP-437 (LP437; red) alleles. (B) Yeast one-hybrid screen results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

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

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

339 scrambling of the TFBMs for the GATA and STAT transcription factors resulted only in the
340 reduction of the basal expression while the induction with infection is still present (Figure
341 4D). These results suggest that all three of these pathways may bind to the upstream region of
342 *lectin-24A* and be involved in the regulation of expression.
343
344 We previously found that the *cis*-regulatory sequences of the resistant and susceptible alleles
345 of *lectin-24A* differed by three insertion-deletion polymorphisms (indels) of 7 bp, 8 bp, and
346 21 bp in length, and six single nucleotide polymorphisms (SNPs; Figure 4A, top two rows)
347 [12]. The 21 bp indel was found to be primarily responsible for the differential regulation of
348 *lectin-24A* expression between the two alleles [12]. Based on the positions of the TFBMs in
349 the region, we found that the NF-κB binding site is disrupted by the 21 bp deletion in the
350 susceptible DGRP-892 *lectin-24A* allele (Figure 4C). As scrambling this NF-κB binding site
351 abolishes the basal expression and induction of the reporter, we conclude that the loss of this
352 NF-κB binding site explains the differential expression we see from natural polymorphisms
353 in the *lectin-24A* upstream region. This in turn controls susceptibility to infection in natural
354 populations [12].
355
356
357 **The JAK/STAT pathway plays a crucial role in the regulation of *lectin-24A***
358 The JAK/STAT signaling pathway is an evolutionarily conserved pathway with a variety of
359 functions in hematopoiesis, immunity, and development [55]. The canonical JAK/STAT
360 signaling starts with ligand binding, resulting in receptor dimerization bringing two non-
361 receptor tyrosine kinases (JAKs) together to trans-phosphorylate one another [56,57].
362 Activated JAKs then phosphorylate the receptor which in turn phosphorylates the STAT
363 transcription factors. This leads to the normally cytosolic STAT molecule translocating to the

364 nucleus to activate gene expression. The *Drosophila* JAK/STAT pathway only has one JAK
365 (*hop*) [58], one active type I cytokine receptor (*dome*) [59], and one STAT (*Stat92E*) [51,60].
366 To better characterize the role of the JAK/STAT pathway in the regulation of *lectin-24A*, we
367 knocked down the expression of the transcription factor Stat92E in larvae expressing the
368 *lectin-24A* reporter using the ubiquitously expressed *da-GAL4* to drive the expression of a
369 hairpin RNAi construct. At 24 hpi, *Stat92E* knockdown abolished the induction of the
370 reporter, suggesting that the JAK/STAT pathway is involved in the regulation of the gene
371 (Figure 5A).
372
373 In our RNAseq analysis, we also found that the JAK/STAT pathway negative regulator *et*
374 showed specific induction in the posterior of the fat body. et is structurally similar to the
375 JAK/STAT receptor dome, and it can function as a negative regulator by forming inactive
376 heterodimers with dome [21,61]. It was previously found that et is required for the
377 downregulation of JAK/STAT activity in hematopoietic progenitors for lamellocyte
378 differentiation after wasp infection [21]. To further understand the role of JAK/STAT
379 signaling in *lectin-24A* regulation, we analyzed the expression of the reporter in a genetic
380 background where *et* is mutated [21]. We found that with a non-functional mutant *et*, the
381 reporter shows constitutively upregulated expression in the larval fat body (Figure 5B). In
382 addition, to confirm this result, we used a dominant negative allele of Domeless
383 (DomeΔCYT, cytoplasmic domain deleted), driven in the fat body by *Cg-GAL4* or
384 ubiquitously by *da-GAL4*, to decrease JAK/STAT activity. We observed a general lowering of
385 reporter expression both for basal and infected conditions (Figure 5C).
386
387 The *Drosophila* JAK, *hop*, showed a similar expression pattern as *et* in our RNAseq analysis,
388 being upregulated after infection in the posterior fat body. We therefore studied the

expression of the reporter in a genetic background with a constitutively active form of *hop* (*hop*^{Tum}). As predicted, the reporter showed elevated basal expression in the *hop*^{Tum} background (Figure 5D). When we then examined *lectin-24A* reporter expression in a background where we abolished *hop* in somatic cells using CRISPR/Cas9 and a guide RNA targeting the gene (somatic knock out), we found a reduction in the upregulation of the reporter after wasp infection (Figure 5E). Taken together, our results indicate clearly that the JAK/STAT pathway has a critical role in the induction of *lectin-24A* following parasitization.

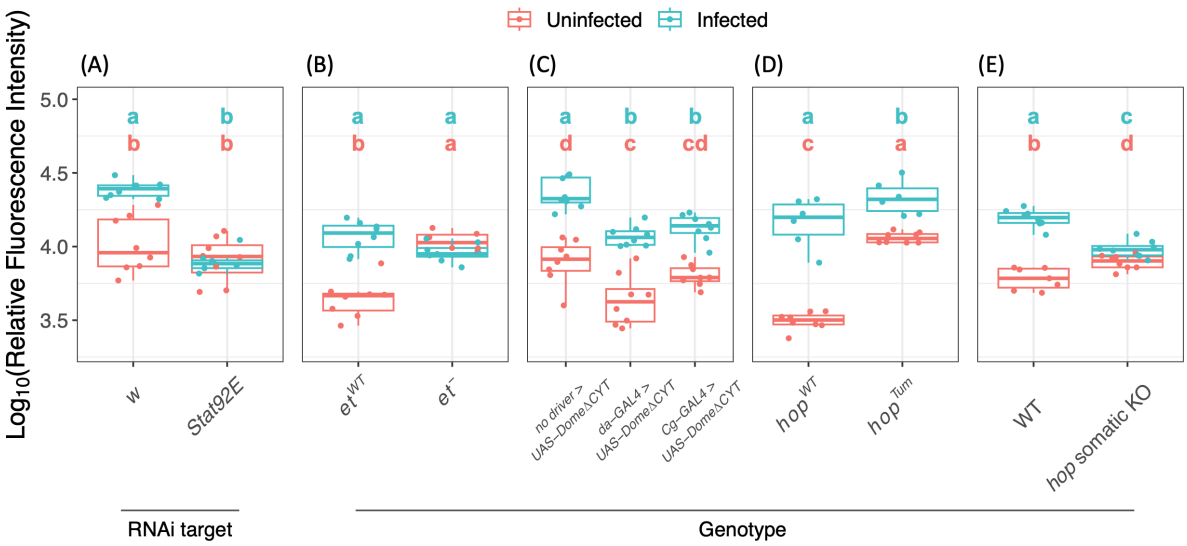


Figure 5. The role of the JAK/STAT pathway in the regulation of *lectin-24A*. In all panels the *Venus*^{LP437} reporter expression was measured. (A) Larvae with knockdown of *Stat92E* using RNAi, compared to the knockdown of the *white* gene as a control. (B) Larvae carrying a mutant or wildtype (WT)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

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

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

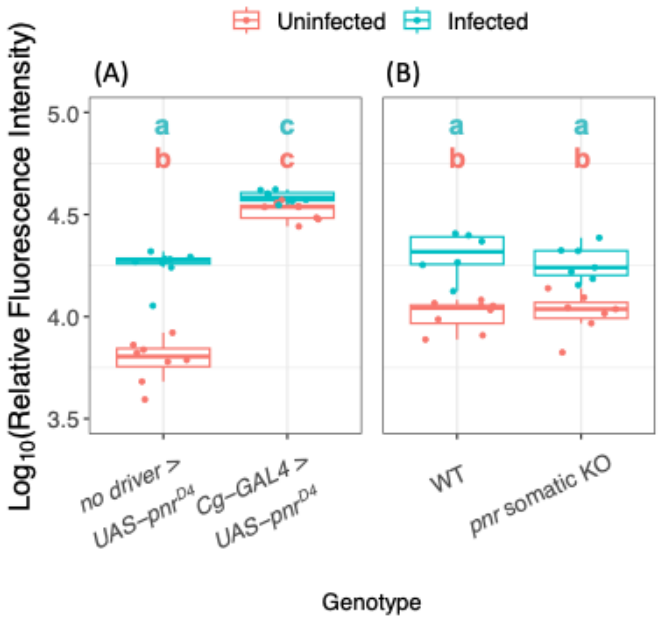
427

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

434

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

442 high expression, even in the absence of parasitization (Figure 6A). We then analyzed the
443 expression of *lectin-24A* Venus reporter in a genetic background where we knocked out *pnr*
444 expression using somatic mutagenesis with CRISPR/Cas9. However, knock out of *pnr*
445 expression in the whole larvae did not result in significant changes to the $Venus^{LP437}$ reporter
446 expression, which might reflect GATA factors may have redundant roles in regulating *lectin-*
447 *24A* (Figure 6B). Thus, our results indicate that Pnr is involved in the induction of *lectin-24A*
448 following parasitoid infection, but it may not be the only or the main GATA factor playing
449 this role.



450

451

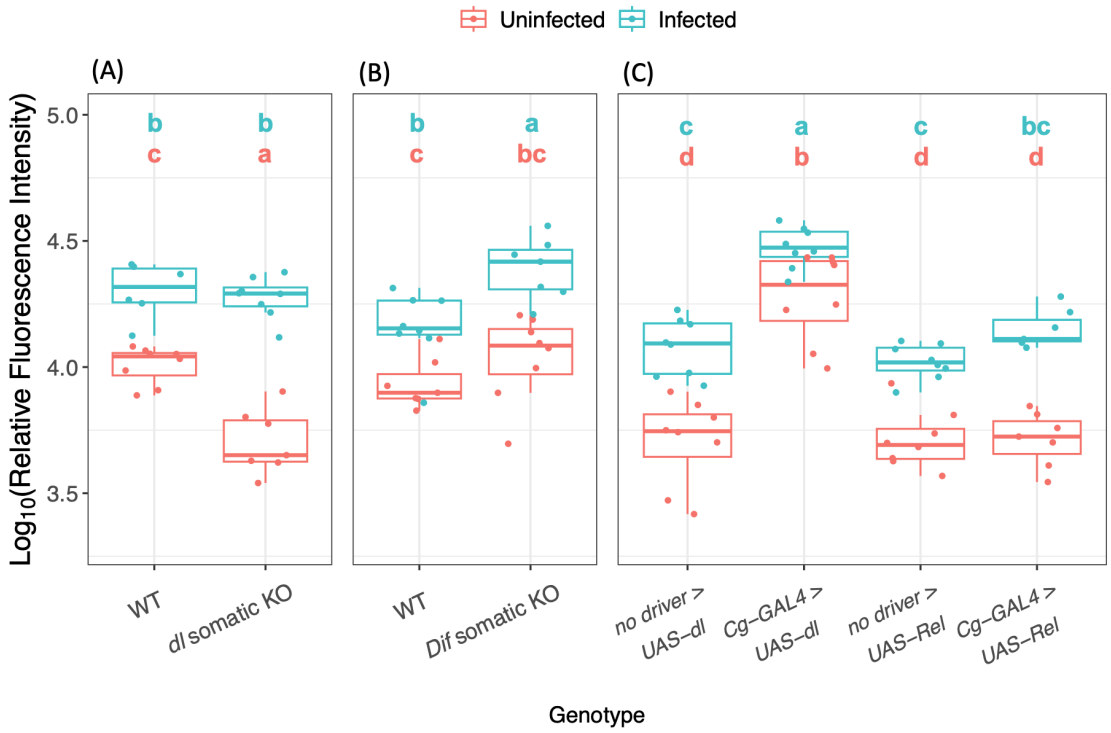
452 **Figure 6. The role of the GATA transcription factor, Pannier, in the regulation of *lectin-***
453 ***24A*.** The $Venus^{LP437}$ reporter expression was measured. (A) Larvae expressing the dominant
454 positive form of Pnr (Pnr^{D4}) under UAS control driven by the fat body and hemocyte-specific
455 *Cg-GAL4* driver or no driver. (B) Larvae expressing *Act-Cas9* and guide RNA targeting *pnr*
456 compared to control larvae. Each point represents a pool of 10 larvae. The relative
457 fluorescence intensity represents the fluorescence intensity normalized with the total protein
458 quantity of each sample. Tukey's test for each panel, $P < 0.05$ between groups.
459

460

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

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

477



478

479 **Figure 7. The role of NF-κB transcription factors in the regulation of *lectin-24A*.** The
480 *Venus*^{LP437} reporter expression was measured. (A) Larvae expressing *Act-Cas9* and guide
481 RNA targeting *dl* compared to control larvae. The same WT control samples were used here
482 as in Figure 6B. (B) Larvae expressing *Act-Cas9* and guide RNA targeting *Dif* compared to
483 control larvae. (C) Larvae expressing *dl* or *Rel* under UAS control driven by the fat body and
484 hemocyte-specific *Cg-GAL4* driver or no driver. F_1 heterozygote larvae were collected. As the
485 parental line expressing *UAS-dl* carried a balancer chromosome that could not be identified at
486 the larval stage in F_1 progeny, larvae collected from the *UAS-dl* crosses were a mix of *Cg-*
487 *GAL4/UAS-dl* and *Cg-GAL4/+*. Each point represents a pool of 10 larvae. The relative
488 fluorescence intensity represents the fluorescence intensity normalized with the total protein
489 quantity of each sample. Tukey's test for each panel, $P < 0.05$ between groups.
490

491

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

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

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

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

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

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

498 enriched in the upstream sequence of genes upregulated with infection in the posterior of the
499 fat body, relative to the full set of genes detected. We found that GATA binding motifs are
500 generally enriched around two-fold in the sequences upstream of upregulated genes. For
501 instance, the Pannier binding motif (Supplementary Table 1) is found an average of 0.60
502 times per kilobase (kb) of sequence 5' of TSSs for upregulated genes, whereas it is only
503 found an average of 0.33 times per kb of upstream sequences in the full set of genes (Welch's
504 *t*-test, t = 3.49, d.f. = 277.9, *p*-value = 5.65×10^{-4}). A similar case is observed in the genes
505 specifically upregulated in the posterior of the fat body after infection (Figure 2B), showing
506 even higher enrichment with a mean of 0.66 times per kb in these genes (Welch's *t*-test, t =
507 3.51, d.f. = 186.1, *p*-value = 5.72×10^{-4}).
508
509 When we searched for STAT motifs in the upstream sequences, we found significant
510 enrichment of the "TTC$(N)_4$AA" motif in the posterior-specific upregulated genes with a
511 mean of 8.46 instances per kb compared to 7.77 in the background (Welch's *t*-test, t = 2.10,
512 d.f. = 188.84, *p*-value = 0.037). Although the same motif also appears to be enriched in the
513 parasitism-induced gene set, with 8.27 occurrences per kb, it falls just below the significance
514 threshold (Welch's *t*-test, t = 1.91, d.f. = 284.98, *p*-value = 0.058). On the other hand, the
515 "TTC$(N)_5$AA" motif only shows significant enrichment in the parasitism-induced gene set,
516 with 6.29 occurrences per kb compared to 5.83 in the background set (Welch's *t*-test, t = 1.98,
517 d.f. = 284.47, *p*-value = 0.048).
518
519 Moreover, previous studies have described a specific organization of TFBMs in the upstream
520 region of fat body-specific immune genes, where NF-κB and GATA binding sites have been
521 found to be in close association, with less than 50 bp in-between them and within 200-300 bp
522 upstream of the TSSs [30,31]. We specifically searched for NF-κB sites within 50 bp of a

523 GATA site and checked for enrichment. Although, we see that NF-κB motifs linked to GATA
524 motifs are also more highly enriched in the upstream region of parasitism-induced genes and
525 posterior-specific anti-parasitoid genes, with a mean of 0.52 and 0.53 instances per kb,
526 respectively, compared to 0.39 in the background set, the differences were not significant.
527 Overall, the TFBMs of the GATA, NF-κB, and STAT transcription factors all appear to be
528 enriched in the 5' sequences of parasitism-induced genes in the *Drosophila* larval fat body,
529 suggesting that these transcription factors and the signaling pathways they belong to are
530 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).

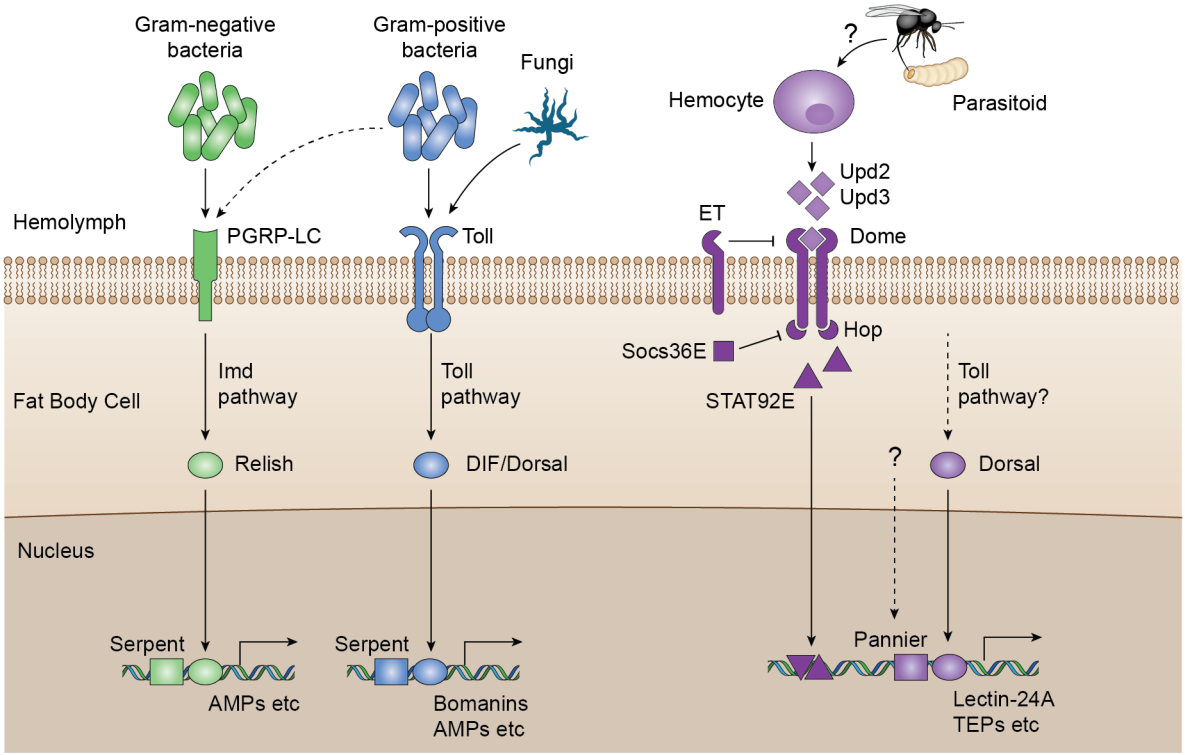


Figure 8. Schematic overview of *Drosophila* humoral defenses to microbial pathogens and parasitoid. Antimicrobial responses in the fat body are regulated by the NF-κB pathways, Toll and Imd. The Imd pathway is mostly activated by Gram-negative bacteria through the PGRP-LC receptor, whereas the Toll 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

550 closely linked GATA and κB binding sites in the upstream promoter region. The *Drosophila*
551 humoral anti-parasitoid response is regulated by a combination of inputs from JAK/STAT,
552 GATA and NF-κB pathways. After recognition of parasitism, hemocytes produce the
553 cytokines, Upd2 and Upd3, which bind to the receptor Dome to activate JAK/STAT pathway
554 signaling in the fat body. Dome dimerization after ligand binding leads to trans-
555 phosphorylation of Hop, which then activates the transcription factor STAT92E. STAT92E
556 binds the upstream region of anti-parasitoid genes including *lectin-24A*. ET and SOCS36E
557 are negative regulators of the JAK/STAT pathway that suppress this signaling. STAT, GATA,
558 and NF-κB TFBMs are found to be enriched in the upstream sequences of parasitism-induced
559 genes. The GATA transcription factor Pannier and the NF-κB transcription factor Dorsal also
560 participate in the regulation of anti-parasitoid genes in the larval fat body by binding to their
561 respective binding sites in the promoter region.
562
563

564 The transcriptional response induced by a parasitoid attack differs from that activated by
565 microbial infections [33]. We found that *lectin-24A* is a gene specifically induced by
566 parasitoid infection, providing a tool to understand broadly how wasp-responsive genes are
567 regulated. Based on our current results, it seems that this specificity is potentially conferred
568 by the coordinated inputs from different immune-signaling pathways: NF-κB, the GATA
569 factor Pannier, and JAK/STAT. This regulation differs from the well-characterized
570 antimicrobial defense, which mainly requires the two NF-κB pathways, Toll and Imd, with
571 additional inputs from the GATA transcription factor Serpent [30,74–78].
572

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

582 clustered together [33]. Genes in this cluster showed significant over-representation of NF-
583 κB, GATA, and STAT regulatory motifs [33], supporting out conclusion that the anti-
584 parasitoid humoral immune response mediated by the fat body is regulated by the concerted
585 action of these pathways. Further support comes from analyses of TEP expression in flies
586 where signaling pathways have been perturbed. TEPs are expressed mainly by the *Drosophila*
587 fat body and induced in response to various immune challenges [79]. The loss of the four
588 secreted TEPs, TEP1-4, results in reduced capacity to successfully encapsulate wasps [8].
589 *Tep1* shows constitutive expression in the absence of infection in the constitutively active
590 hop^{Tum} mutant [79]. Similarly, in a *Toll* gain-of-function mutant background, *Tep1* also
591 displayed constitutive activation [79]. However, in this case, the Toll pathway effects were
592 recognized to be indirect, and secreted TEPs were found to function upstream of Toll and
593 promote Toll activation [8,79].
594
595 It is still unknown how parasitoids are recognized by the *Drosophila* host. As hemocytes are
596 responsible for the secretion of Upd cytokines that activate the JAK/STAT pathway in other
597 tissues, they are likely to play a key role in the recognition of parasitoid attacks [22]. Our
598 study shows a significant role of the JAK/STAT pathway in *lectin-24A* regulation.
599 Interestingly, we see that JAK/STAT pathway negative regulators are strongly induced
600 alongside the activation of the pathway itself (Figure 3) [21,61,80,81]. As we only
601 investigated one time point (24 hpi) in our experiments, this result suggests potential negative
602 feedback loops in the shutdown of the pathway that were not captured. In mammals, negative
603 regulators of the pathway are known to be induced after infection and JAK/STAT activation,
604 playing a key role in regulating the cytokine response and preventing damaging inflammation
605 [82,83]. This is also the case in *Drosophila*, where expression of the negative regulator
606 SOCS36E is activated by JAK/STAT signaling, creating feedback inhibition [81,84,85].

607

608 In the *lectin-24A* upstream region, we predicted a GATA transcription factor binding site that
609 contains the consensus binding motifs from four of the *Drosophila* GATA transcription
610 factors (Figure 4 & Supplementary Table 1). We specifically focused on Pannier because it is
611 the only GATA transcription factor showing differential expression in the larval fat body
612 following parasitoid attack (Figure 3). Our results indicate that Pannier is involved in
613 regulating *lectin-24A* and likely the broader humoral anti-parasitoid response. However, we
614 have yet to investigate whether upregulation of Pannier expression is a primary signal of
615 infection or a consequence of activating other pathways such as JAK/STAT. Furthermore,
616 whether the other *Drosophila* GATA transcription factors also contribute to regulation
617 remains to be further studied. In particular, Serpent, which is known to be involved in the
618 regulation of immune genes in the larval fat body, is also predicted to bind upstream of *lectin-*
619 *24A* and might also have a role in the regulation of immunity against parasitoids [30,31,86].
620

621 Based on our RNAseq data, components of the Toll pathway showed significant differential
622 expression in the larval fat body following parasitism (Figure 3). Particularly, of the NF-κB
623 transcription factors, *dl* showed upregulation specifically in the posterior fat body with
624 infection (Figure 3). We also found that a Dif/dl binding motif in the *lectin-24A* upstream
625 region had a crucial role in the expression of the $Venus^{LP437}$ reporter (Figure 4D). Dif and
626 dorsal, the two transcription factors in the *Drosophila* NF-κB Toll pathway, are known to
627 regulate the defense to Gram-positive bacteria and fungi. In the antimicrobial response, Dif
628 and dl have different roles depending on developmental stage. In adults, Dif alone is required
629 for the humoral immune response, whereas in larvae, it shows some redundancy with dl
630 [76,87]. While there is evidence that in Dif and dl double mutants, expression of either
631 transcription factors is sufficient to rescue the expression of AMPs such as *Drosomycin* [87],

632 some other reports show that induction of certain AMPs specifically require Dif and cannot
633 be rescued with only dl [77]. Furthermore, Dif and dl, as well as Rel, form homodimers and
634 heterodimers during DNA binding, meaning that permutations of different dimers expand the
635 regulatory possibilities from these NF-κB proteins [88,89]. Since NF-kB transcription factors
636 activate transcription by translocating into the cell nucleus, our observation that the
637 expression of *Dif* does not increase after infection does not preclude it playing a role in
638 regulating the anti-parasitoid humoral response. Nevertheless, as *dl* but not *Dif* mutants
639 reduced expression of the $Venus^{LP437}$ reporter, dl is likely playing the key role in regulation of
640 this immune response.
641
642 In this study, we investigated individually how each of these pathways contributed to the
643 regulation of *lectin-24A* expression, but it remains unclear how they work together. It was
644 previously found that NF-κB and GATA regulatory synergy plays a key role in many
645 immunity-related fat body-specific genes that are activated in response to microbial
646 infections [30,31]. In particular, a fixed organization of NF-κB and GATA binding sites in the
647 5' regulatory region within 1 kb of predicted transcription start sites (TSSs) was defined,
648 where both sites are located in the proximal region within 200 or 300 bp from the TSS [30].
649 The sites were found to be closely linked, within 50 bp from each other and in the same
650 relative orientation [30]. The NF-κB and GATA binding sites we identified in the *lectin-24A*
651 upstream region fit this criterion exactly (Figure 4C). According to the previous study, each
652 individual NF-κB and GATA site has an essential role in the optimal expression of the genes
653 [30]. Furthermore, their results suggest that the GATA site is responsible for localizing the
654 expression to the fat body, and the NF-κB site is responsible for the induction of the gene in
655 response to infection [30]. Our analysis based on scrambling the binding sites in the *lectin-*
656 *24A* reporter construct are also in line with this model, with the addition that STAT binding is

657 also involved in regulation in a similar way as GATA (Figure 4D). However, notably, Pannier
658 is strongly upregulated by parasitoid infection and this upregulation does not differ between
659 anterior and posterior of the larval fat body (Figure 3). This result suggests that Pannier is not
660 just playing a role to localize *lectin-24A* expression to the fat body, but is also involved in the
661 induction of its expression. Nonetheless, when we manipulated specific genes in each of
662 these pathways the effects on *lectin-24A* expression become less straightforward, suggesting
663 more complex models of coordinated regulation and potential indirect effects from
664 knockdown or knockout in the whole larva.
665
666 Immune systems evolve rapidly as hosts adapt to an ever-changing array of parasites and
667 pathogens. We have previously shown that a polymorphic deletion upstream of Lectin-24A
668 has led to reduced parasitoid resistance in wild populations [12], and here we provide an
669 explanation of this by showing that it abolishes an NF-κB binding motif. Furthermore, a
670 group of four single nucleotide polymorphisms cause a smaller reduction in Lectin-24A
671 expression [12]. Notably, one of these is within a STAT binding motif, with the low
672 expression allele predicted to bind STAT92E with lower affinity (TTC$(N)_4$AA in the high
673 expression DGRP-437 line becomes TTA$(N)_4$AA in the low expression DGRP-892 line).
674 Therefore, the cis-regulatory elements that respond to immune signaling pathways may be
675 hotspots of host-parasite coevolution.
676
677

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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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