1 **Loss of self-tolerance leads to altered gene expression and IMD pathway activation in**

2 ***Drosophila melanogaster***

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ABSTRACT

Immune self-tolerance is the ability of a host's immune system to recognize and avoid triggering immune responses against self-tissue. This allows the host to avoid self-directed immune damage while still responding appropriately to pathogen infection. A breakdown of self-tolerance can lead to an autoimmune state in which immune cells target healthy self-tissue, leading to inflammation and tissue damage. In order to better understand the basic biology of autoimmunity and the role of the innate immune system in maintaining self-tolerance, we have recently characterized the *Drosophila melanogaster* *tuSz* autoimmune mutant. This mutant strain can serve as a model of innate immune mediated self-tolerance, and here we identify transcripts that are deregulated in flies experiencing a loss of self-tolerance. We found that these changes include the ectopic activation of pro-inflammatory signaling through the Relish/NFκB transcription factor, alterations in transcripts encoding proteins predicted to mediate organismal metabolism, and a downregulation of transcripts linked to developmental processes. This study can provide insight into the transcriptional and physiological changes underlying self-tolerance and autoimmunity.

INTRODUCTION

The maintenance of self-tolerance, the process in which self-tissues are identified and self-directed immune mechanisms are repressed, is an important characteristic of the immune system that protects an organism from pathogen infection (Medzhitov and Janeway 2002; Romagnani 2006). The breakdown of the immune system's ability to discriminate healthy self-tissue from non-self pathogens can lead to damage of a host's own tissues by the immune system. Multiple studies have revealed the importance of self-tolerance and failure of this system has been implicated in a variety of autoimmune disorders and other immune mediated diseases. Although much of the research uncovering mechanisms of immune recognition and

self-tolerance has focused on adaptive immune mechanisms, a growing body of work has demonstrated the importance of innate immune mechanisms in the suppression of autoimmunity (Waldner 2009; Toubi and Vadasz 2019).

Innate immune responses are evolutionarily conserved and serve as a first line of defense against infection (Hoffmann *et al.* 1999). The innate immune system relies upon germ-line encoded receptors, signaling components and other molecules for the recognition and destruction of pathogens. When activated, these receptors induce intracellular signaling that results in the activation of genes involved in host defense. The inappropriate activation and targeting of these innate immune effectors against self-tissue can contribute to autoimmune pathology (Toubi and Vadasz 2019). However, mechanisms underlying innate immune mediated self-tolerance and the genes associated with the maintenance of this system remain largely unexplored.

The genetic model organism *Drosophila melanogaster* is a valuable model for understanding conserved innate immune mechanisms (Lemaitre and Hoffmann 2007). Flies are host to a wide range of pathogens including bacteria, fungi, viruses, and parasites and use both humoral and cell mediated immune responses to combat these pathogens (Brennan and Anderson 2004). In *Drosophila*, hemocytes (immune cells) known as plasmatocytes are found in circulation, and following stimulation, surveil the hemolymph (body cavity) for the presence of invading pathogens and tissue damage. These macrophage-like plasmatocytes are responsible for the phagocytosis of microbial pathogens and are involved in the encapsulation response against macroparasites including parasitoid wasp eggs (Mortimer *et al.* 2012; Honti *et al.* 2014; Parsons and Foley 2016). Another immune cell type, the lamellocytes, are generally not present in a healthy fly larva, but are induced following parasitoid wasp infection and are required for melanotic encapsulation of parasitoid eggs and other foreign objects (Mortimer *et al.* 2012; Honti *et al.* 2014; Anderl *et al.* 2016).

Drosophila hemocytes also play a role in self-recognition and maintaining self-tolerance. We have recently characterized the *Drosophila tuSz¹* temperature sensitive mutant strain which mounts a self-directed encapsulation response at the restrictive temperature (Mortimer *et al.* 2021). We found that the loss of self-tolerance in *tuSz¹* is due to two genetic changes. The first is a gain of function mutation in *hopscotch (hop)*, the *Drosophila* orthologue of Janus Kinase (JAK). The JAK-STAT signaling pathway plays a key role in immune priming, and the *hop^{Sz}* allele leads to hemocyte activation and the ectopic production of lamellocytes in naïve larvae (Mortimer *et al.* 2021). These hemocyte phenotypes are also seen in the *hop^{Tum}* gain of function allele (Hanratty and Dearolf 1993; Luo *et al.* 1995), but interestingly, even though *hop^{Tum}* and *tuSz¹* mutants both have hemocyte activation and excess lamellocyte production, *hop^{Tum}* mutants do not display an autoimmune phenotype (Mortimer *et al.* 2021). This would suggest that JAK-STAT mediated immune activation alone is insufficient for the loss of self-tolerance, and that loss of self-tolerance and immune priming are genetically separable. The second mutation in the *tuSz¹* strain is a conditional loss of function mutation in the *GCS1* gene. At the restrictive temperature, the *GCS1^{Sz}* allele leads to disruption of protein N-glycosylation of extracellular matrix proteins (Mortimer *et al.* 2021). Together these findings define a system in which *Drosophila* self-tolerance is maintained by the ability of primed hemocytes to sense a self-signal that is encoded in the N-glycosylation of extracellular matrix proteins, and the loss of this self-recognition leads to the melanotic encapsulation of self-tissues. Thus, hemocytes can mediate both the activation of an immune response and the maintenance of self-tolerance thereby regulating immune homeostasis.

Here, we utilize the *Drosophila tuSz¹* and *hop^{Tum}* mutant strains to identify key transcriptional changes underlying immune priming, self-tolerance, and autoimmunity. Differentially expressed transcripts often reveal disease-induced changes (Porcu *et al.* 2021), so our analysis of transcript abundance changes in these mutants may allow us to identify

processes that occur in autoimmune reactions following the loss of self-tolerance. Our results further suggest that the pro-inflammatory Immune deficiency (Imd) signaling pathway may be dysregulated in the *tuSz*¹ loss of self-tolerance phenotype.

MATERIALS AND METHODS

Insect Stocks

The *D. melanogaster* strains *w*¹¹¹⁸ (BDSC: 5905), *tuSz*¹ (BDSC: 5834), *PBac{GFP.FPTB-Rel}VK00037 (Rel-GFP)* (BDSC: 81268), *hop*^{Tum} (BDSC: 8492) were obtained from the Bloomington Drosophila Stock Center (BDSC). All fly stocks were maintained on standard *Drosophila* medium on a 12-hour light-dark cycle. The study also uses the parasitoid wasp *Leptopilina boulardi* (strain Lb17) which is maintained on the *Canton S D. melanogaster* strain.

RNA Isolation

Total RNA was extracted from late third instar *w*¹¹¹⁸, *tuSz*¹ and *hop*^{Tum} larvae raised at 28°. In total, three biological replicates were prepared with each biological replicate consisting of 20 pooled larvae. RNA was extracted from whole larvae using Trizol (Thermo Fisher Scientific) followed by QIAGEN RNeasy Micro clean-up (QIAGEN; both according to manufacturer's instructions). RNA concentration was measured using a nanodrop and the integrity was verified using agarose gel electrophoresis. The RNA samples were sent to Novogene (Sacramento, CA) for RNA sequencing (150bp paired end reads, Illumina).

Differential Gene Expression Analysis

Sequence data were obtained in the form of FASTQ reads and analyzed using the Galaxy web platform (<https://usegalaxy.org/>) (Afgan *et al.* 2018). Initial quality control was

performed using FastQC (version 0.11.8) (Andrews 2010) to trim reads below a quality score threshold of 30. Reads were then mapped to the *D. melanogaster* reference genome dm6 using HISAT2 (version 2.1.0) (Kim *et al.* 2015). The resulting BAM format files were analyzed using featureCounts (version 1.6.3) (Liao *et al.* 2014) to generate read counts for each gene in the reference genome annotation. The counts generated by featureCounts were subsequently used to determine the differentially expressed genes using DESeq2 (version 2.11.40.6) (Love *et al.* 2014). The results were filtered and significant differentially expressed genes (DEGs) were identified after applying significance cut-offs of a false discovery rate (FDR) < 0.05 and log₂ fold change ≥ 1 (for upregulated transcripts) or ≤ -1 (for downregulated transcripts).

Functional Annotation

The Gene Ontology (GO) Resource (<http://geneontology.org/>) and PANTHER classification system (Ashburner *et al.* 2000; Mi *et al.* 2013; Gene Ontology Consortium 2021) were used for GO term analysis of each list of DEGs. The FDR threshold to determine term enrichment was 0.05.

Statistical Analysis

All statistical analyses described in the following sections were done in the R statistical computing environment (R Core Team 2021) as detailed. Graphs were produced using the ggplot2 and ggvenn R packages (Wickham 2009; Yan 2021).

Quantitative PCR (qPCR)

qPCR was used to confirm the differential expression of selected genes of interest. Primers for individual genes were designed according to the FlyPrimerBank (Hu *et al.* 2013) and obtained from Integrated DNA Technologies (Table S1). Ten larvae from each genotype raised at 28° were collected and total RNA was extracted using Trizol (Thermo Fisher Scientific). RNA

concentration was measured using a Nanodrop and samples were normalized to 2.5 µg for reverse transcription into cDNA using SuperScript IV (Thermo Fisher Scientific). cDNA samples were diluted 1:100 in nuclease-free water and 1 µl was used as a template for qPCR experiments. qPCR was performed using the PowerUp SYBR Green Supermix (Thermo Fisher Scientific). Samples were run as technical triplicates and a total of three biological replicates were performed for each primer in each genotype.

For comparison across genotypes, transcript levels for the genes of interest were normalized to the *Arp1* housekeeping control from the same sample. Within each biological replicate, the ΔC_t value was calculated by subtracting the C_t value of *Arp1* from the C_t value of each experimental primer set in each genotype. Differences in transcript ΔC_t values were tested across biological replicates using mixed linear models, with genotype as a fixed effect and biological replicate as a random effect using the lmerTest package (Kuznetsova *et al.* 2017) in R. Relative quantification was calculated as $2^{-\Delta\Delta C_t}$ (where $\Delta\Delta C_t$ is the difference between the *tuSz¹* ΔC_t values and *w¹¹¹⁸* control ΔC_t values for each gene of interest).

Rel Localization

To determine Rel subcellular localization in hemocytes in control and mutant larvae, *w¹¹¹⁸* and *tuSz¹* unmated females were crossed to *Rel-GFP* males and the crosses were raised at 28°C. From these crosses we selected *w¹¹¹⁸/Y; Rel-GFP/+* control larvae and *tuSz¹/Y; Rel-GFP/+* mutant larvae. Five male third instar larvae from each genotype were bled in PBS and hemocytes were stained using DAPI (1:500; Invitrogen) to mark nuclei and mounted in Vectashield mounting medium. To determine Rel localization in hemocytes of parasitoid infected larvae, late second instar *w¹¹¹⁸/Y; Rel-GFP/+* larvae were placed on 35mm Petri dishes filled with Drosophila medium only (uninfected control) or together (infected) with *L. boulardi* wasps at 25°C. Larvae were dissected at 72-hours post infection (hpi) in PBS and hemocytes were

stained using DAPI mounted in Vectashield for imaging. To determine the *Rel-GFP* expression in fat body tissue, anterior and posterior regions of fat bodies from w^{1118}/Y ; *Rel-GFP/+* and *tuSz¹/Y*; *Rel-GFP/+* larvae raised at 28° were dissected in PBS and mounted in Vectashield mounting medium. Cells and tissues were imaged using a Leica SP8 confocal microscope in the ISU Confocal Microscopy Facility to assay Rel localization.

The relative proportion of Rel-GFP signal coming from the nucleus vs cytoplasm (N/C ratio) was calculated using Fiji (Schindelin *et al.* 2012). Image analysis was performed as described in (Noursadeghi *et al.* 2008). Specifically, the DAPI stain was used to mask the nucleus allowing for mean cytoplasmic and nuclear fluorescence intensity values to be independently determined. The N/C ratio was then calculated based on the ratio of mean nuclear intensity to mean cytoplasmic intensity. Cells with an N/C ratio greater than 1.5 were considered to have nuclear Rel-GFP localization and cells with an N/C ratio less than 1.5 were considered to have diffuse Rel-GFP (Rel-GFP exclusion from the nucleus was not observed). The proportion of cells with nuclear Rel-GFP localization in the *tuSz¹* genetic background was compared to w^{1118} using Fisher's exact test in R.

RESULTS AND DISCUSSION

Identification of differentially expressed genes in *tuSz¹* and *hop^{Tum}*

To gain a better understanding of the changes involved in JAK-STAT mediated immune priming and the consequences of the loss of self-tolerance, we performed RNA sequencing on w^{1118} , *tuSz¹* and *hop^{Tum}* larvae raised at 28° to assess differential transcript abundance. The resulting sequence reads were mapped to the *D. melanogaster* genome (Table S2) to quantify transcript levels. We separately compared transcript levels in each mutant genotype to the w^{1118} control genotype (Tables S3-S6). Both *tuSz¹* and *hop^{Tum}* have gain of function mutations in *hop*, so any genes that are differentially expressed in both genotypes are likely to be regulated by the

JAK-STAT pathway. We found 832 shared genes that were differentially expressed in both *tuSz¹* and *hop^{Tum}* mutants compared to wildtype, with 384 genes that were upregulated, and 438 genes that were downregulated (Figure 1). Because the JAK-STAT pathway plays a key role in immune activation (Agaisse and Perrimon 2004), these identified genes might be relevant to the priming of the immune response.

To begin characterizing these putative immune activation genes, we conducted Gene Ontology (GO) analysis to identify the biological processes and molecular functions that are enriched among the common set of differentially expressed genes (Table 1). Analysis of genes that were upregulated in both *tuSz¹* and *hop^{Tum}* identified GO terms that have been linked to host immunity including defense response to other organism (GO:0098542) and response to external stimulus (GO:0009605). General immune response genes identified in these categories include five members of the antimicrobial peptide (AMP)-like *Bomanin* gene family (*BomBc1*, *BomS1*, *BomS2*, *BomS4*, *BomT1*) (Clemmons *et al.* 2015) and the Cecropin family AMP gene *CecC* (Tryselius *et al.* 1992), along with several immune receptors (*NimB2*, *NimC1*, *NimC4*) (Kurucz *et al.* 2007; Somogyi *et al.* 2010), the prophenoloxidase family members *PPO1* and *PPO3* (Nam *et al.* 2008; Dudzic *et al.* 2015), and the *induced by infection (IBIN)* gene (Valanne *et al.* 2019). We also found differential expression of important immune related signaling pathways such as the JAK-STAT signaling pathway components *dome*, *et* and *upd3* (Myllymäki and Rämét 2014), the Toll pathway genes *nec* and *SPE* (Kambris *et al.* 2006), and *Rac2* and *Rap1* which are linked to hemocyte function (Williams *et al.* 2005; Huelsmann *et al.* 2006). The *tuSz¹* and *hop^{Tum}* mutations both lead to the ectopic production of lamellocytes, and accordingly, we see lamellocyte related genes such as *ItgaPS4*, *ItgaPS5*, and *drip* upregulated in these genotypes (Stofanko *et al.* 2010; Tattikota *et al.* 2020). The proteolysis (GO:0006508) GO term is also enriched among genes upregulated in both *tuSz¹* and *hop^{Tum}*. Notably, 29 members of the S1A serine protease family are significantly upregulated in both *tuSz¹* and *hop^{Tum}*, including

CG10764 and *CG4793* which have recently been shown to be involved in the cellular immune response to parasitoid infection (Kr *et al.* 2021).

The induction of host immunity is associated with life history tradeoffs, primarily in the redistribution of energy resources from development or reproduction to the production of an immune response (Zuk and Stoehr 2002). We found that genes involved with cellular lipid metabolism (GO:0044255) are enriched among the transcripts upregulated in *tuSz¹* and *hop^{Tum}*, and conversely that genes linked to development, including regulation of multicellular organismal development (GO:2000026), tissue morphogenesis (GO:0048729), and cell fate commitment (GO:0045165) are among those transcripts downregulated in both *tuSz¹* and *hop^{Tum}* (Table 1). The upregulation of transcripts involved in metabolism and downregulation of transcripts linked to developmental processes possibly reflects the immune response-development tradeoff, and suggests that this tradeoff may be regulated in part by JAK-STAT signaling or downstream processes. Further study of these genes may help to provide an understanding of the energy trade-offs and other physiological changes that occur during immune responses.

Loss of self-tolerance in *tuSz¹* mutants results in differential gene expression

Despite the similarities between the *tuSz¹* and *hop^{Tum}* mutants, *tuSz¹* specifically displays a loss of self-tolerance phenotype (Mortimer *et al.* 2021), so we wanted to investigate transcripts that were specifically differentially expressed in *tuSz¹* to identify genes that might be involved in innate immune self-tolerance and autoimmune mechanisms. Out of 1154 transcripts that were differentially expressed in *tuSz¹* but not *hop^{Tum}* (referred to as *tuSz¹* specific DEGs), we found 670 transcripts that were significantly upregulated and 484 transcripts that were significantly downregulated (Figure 1).

In contrast to the list of upregulated transcripts in common to *tuSz¹* and *hop^{Tum}*, GO Term analysis of these *tuSz¹* specific DEGs (Table 2) failed to find enrichment of any GO categories associated with positive regulation of host immunity. Indeed, one of the most highly enriched terms was the negative regulation of antimicrobial peptide biosynthetic process (GO:0002806). This term was linked to peptidoglycan recognition protein (PGRP) encoding genes, and we found that six PGRP gene transcripts are upregulated in *tuSz¹* mutants. There are 13 members of this family in the *Drosophila* genome (Table 3), which primarily play roles in the regulation of the Toll and Imd immune signaling pathways (Kurata 2014). There are five PGRP genes that encode negative regulators of Imd signaling, and we found that all five of these are specifically upregulated in *tuSz¹* mutants, with the sixth altered PGRP playing an unknown role (Table 3). The finding that whereas positive regulators of immunity were identified as upregulated genes in both *tuSz¹* and *hop^{Tum}*, the enrichment of genes involved in negative regulation of the Imd pathway specifically in *tuSz¹* mutants suggests a differential role of immune mechanisms in immune priming and the autoimmune response to a loss of self-tolerance. We additionally found 26 genes that map to the chitin-based cuticle development (GO: 0040003) term among the *tuSz¹* specific DEGs, including members of the *Lcp*, *Acp*, *Twdl*, *Cpr*, and *Ccp* gene families. Expression of cuticle genes has been linked to infection in *D. melanogaster* and other insects, although a role in host immunity has yet to be identified (Yadav *et al.* 2017; Hou *et al.* 2021; Pantha *et al.* 2021).

Many of the additional enriched GO categories among the *tuSz¹* specific DEGs are associated with stress responses and metabolism, perhaps providing insight into physiological changes in *tuSz¹* mutant larvae due to the occurrence of an autoimmune response (Table 2). We found an enrichment of oxidoreductase activity (GO: 0016491) genes, including the glutathione transferase (GST) encoding genes *GstD2*, *GstD6*, *GstD7*, *GstD8*, *GstE1*, *GstE3*, *GstE5*, and *GstE7* (Low *et al.* 2007). We also found that 18 cytochrome P450 (CYP450)

encoding genes are upregulated in *tuSz*¹ mutants. CYP450s are involved in a wide range of cellular activities including roles in hormone signaling and detoxification of xenobiotics (Bergé *et al.* 1998; Chung *et al.* 2009). The specific upregulation of these stress response genes suggests that loss of self-tolerance may lead to the production of an internal oxidizing environment.

As mentioned previously, metabolic changes often accompany immune responses, and we found that two general regulators of host metabolism, the *Adipokinetic hormone receptor* (*AkhR*) (Bharucha *et al.* 2008) and *Linking immunity and metabolism* (*Lime*) (Mihajlovic *et al.* 2019), are upregulated in *tuSz*¹ mutants. *Lime* encodes a transcription factor that plays a key role in metabolic changes following infection. Accordingly, we found that genes associated with a wide range of metabolic activities including generation of precursor metabolites and energy (GO:0006091), carbohydrate derivative metabolic process (GO:1901135), alpha-amino acid metabolic process (GO:1901605) and lipid metabolic process (GO:0006629) were enriched among those upregulated in *tuSz*¹ larvae (Table 2). Further analysis of these genes and the mechanisms they mediate will help to identify the metabolic pathways involved in the response to a loss of self-tolerance.

Along with upregulated transcripts, transcripts that are downregulated in these genotypes may also provide insight into the cellular activities that are altered. We found that genes associated with tissue morphogenesis (GO:0048729) and the establishment of tissue polarity (GO:0007164) are enriched among the downregulated *tuSz*¹ specific DEGs (Table 2). The loss of cellular polarity and disrupted tissue organization has also been observed in both autoimmune mouse models and patients with autoimmune conditions such as inflammatory bowel disease (Ahmad *et al.* 2017; Klunder *et al.* 2017; Guo and Shen 2021). This may suggest that tissue structural maintenance is a key self-tolerance checkpoint, in agreement with previous findings in *Drosophila* (Rizki and Rizki 1974; Kim and Choe 2014; Mortimer *et al.* 2021).

Relish/NFκB is activated in immune tissues in *tuSz¹* mutants

Based on the specific upregulation of PGRP genes that act as negative regulators of the Imd pathway (Table 3) (Bischoff *et al.* 2006; Zaidman-Rémy *et al.* 2006; Maillet *et al.* 2008), we hypothesized that Imd signaling may be dysregulated in the *tuSz¹* loss of self-tolerance phenotype. While the role of the Imd pathway in mediating the *Drosophila* humoral immune response to Gram-negative bacteria has been well established (Lemaitre *et al.* 1995), the mechanism by which it may participate in the response to loss of self-tolerance is unknown. The *tuSz¹* autoimmune mutant may therefore provide an effective model to begin exploring the role of the Imd pathway in self-tolerance and autoimmune reactions.

To verify the elevated expression of PGRP genes in *tuSz¹* larvae, we assayed the expression of *PGRP-SC1a* and *PGRP-SC2* in *tuSz¹* and *w¹¹¹⁸* by qPCR. As expected, both *PGRP-SC1a* and *PGRP-SC2* were significantly upregulated in *tuSz¹* compared to *w¹¹¹⁸* (*PGRP-SC1a*: 11.3-fold upregulated, $p < 2.2 \times 10^{-16}$; *PGRP-SC2*: 1.3-fold upregulated, $p = 0.0094$) (Figure 2A-B). To further evaluate the ability of qPCR to detect altered expression in *tuSz¹* mutants, we also assayed transcript levels of *scrib* (a cell polarity gene) and observed the expected downregulation in *tuSz¹* larvae compared to *w¹¹¹⁸* (1.7-fold downregulated, $p = 1.4 \times 10^{-8}$) (Figure 2C).

The Imd pathway mainly responds to infection via differential expression of target genes (Kleino and Silverman 2014). A previous study has identified genes whose expression is modified by constitutive Imd pathway activation (Davoodi *et al.* 2019). We found that 58 of the 324 genes identified in this study also showed differential expression in *tuSz¹* larvae (Table S7), including three of the identified PGRP genes (*PGRP-LB*, *PGRP-LF*, *PGRP-SC2*), and other immune genes such as *AttA*, *Rala*, *Gbp1* and *GILT2*. These findings provide support for the hypothesis that Imd signaling is dysregulated in *tuSz¹* mutants.

Gene expression downstream of Imd is largely mediated by the activation of *Relish* (*Rel*), a *D. melanogaster* member of the NF- κ B family of transcriptional activators (Hetru and Hoffmann 2009). *Rel* activation is regulated by signal-dependent translocation into the nucleus. Whereas full-length *Rel* protein is autoinhibited and remains in the cytoplasm, Imd activation results in cleavage of *Rel* and the production of an active N-terminal fragment, which translocates to the nucleus to drive gene expression (Stöven *et al.* 2000). To determine whether *Rel* is activated in *tuSz¹* mutants, we used a *Rel-GFP* fusion protein to track the subcellular localization within the main immune tissues, the hemocytes and fat body. This reporter has GFP fused to the N-terminal domain of *Relish*, and GFP localization in the nucleus versus cytoplasm gives a relative readout of pathway activity.

Rel-GFP expression was observed in plasmatocytes in *tuSz¹/Y; Rel-GFP/+* and *w¹¹¹⁸/Y; Rel-GFP/+* larvae (Figure 3A-B). We found that *Rel-GFP* was significantly more likely to be concentrated in the nucleus in plasmatocytes from *tuSz¹/Y; Rel-GFP/+* larvae compared to *w¹¹¹⁸/Y; Rel-GFP/+* control larvae (Fisher's exact test: $p = 1.23 \times 10^{-5}$; odds ratio = 9.02) (Figure 3D), suggesting that Imd/*Rel* activation is induced in *tuSz¹* mutants. In addition, *Rel-GFP* expression and nuclear localization were observed in lamellocytes from *tuSz¹/Y; Rel-GFP/+* larvae (Figure 3C), suggesting that the Imd pathway is also active in this hemocyte type in the *tuSz¹* mutant background.

To determine if this *Rel* activation is common to cellular immune responses in *Drosophila* or if it may be specific to loss of self-tolerance reactions, we assayed *Rel-GFP* expression and localization in the immune cells of *w¹¹¹⁸/Y; Rel-GFP/+* larvae during the response to parasitoid wasp infection. *w¹¹¹⁸/Y; Rel-GFP/+* larvae were infected by *Leptopilina boulardi* and hemocytes were isolated and imaged 72hpi. We observed minimal *Rel-GFP* expression in both plasmatocytes (Figure 4A-B) and lamellocytes (Figure 4C) in these infected larvae. Circulating non-hemocyte cells (marked by an asterisk* in Figure 4B-C) showed strong

Rel-GFP expression and acted as an imaging control. These results suggest that *Rel* and the Imd pathway are not likely to be involved in the cellular immune response to parasitoid infection. In agreement with this finding, a previous study has shown that *Rel* mutants have a normal encapsulation response against parasitoid wasp infection (Hedengren *et al.* 1999).

To test whether *Rel* activation is specific to hemocytes or seen in other immune tissues in *tuSz¹* mutants, we imaged fat body tissue dissected from *tuSz¹/Y; Rel-GFP/+* and *w¹¹¹⁸/Y; Rel-GFP/+* larvae raised at 28°. We observed minimal *Rel-GFP* expression throughout the fat body of control larvae (Figure 5A). The *tuSz¹* self-encapsulation phenotype is specific to the posterior fat body tissue in mutant larvae, so we imaged both anterior and posterior fat body regions. We found that similar to control, *Rel-GFP* showed minimal expression in anterior fat body tissue from *tuSz¹/Y; Rel-GFP/+* larvae (Figure 5B). However, *Rel-GFP* expression was elevated in posterior fat body tissue, including at the sites of self-encapsulation, in *tuSz¹/Y; Rel-GFP/+* larvae (Figure 5C).

Rel/NFκB transcriptional activity is associated with pro-inflammatory signaling in *Drosophila* and other species (Libert *et al.* 2006; Lawrence 2009; Ganesan *et al.* 2011; Myllymäki *et al.* 2014). The observed *Rel* activation suggests that inflammation is a feature of loss of self-tolerance responses in *Drosophila*, a mechanism that is conserved in human autoimmune disease (Zhang *et al.* 2017; Furman *et al.* 2019). Interestingly, *tuSz¹* mutants appear to attempt to block pro-inflammatory *Rel* activity by inducing transcription of negative regulators of the Imd pathway. A similar mechanism is seen in the establishment of immune tolerance; immune tolerance can be defined as an attempt by the host to mitigate the effects of infection rather than eliminating the infecting pathogen (Ayres and Schneider 2012), and is often accompanied by a downregulation of pro-inflammatory signaling (Sears *et al.* 2011; Vale *et al.* 2014). Indeed, a recent study has revealed that the downregulation of Imd signaling plays an

important role in establishing immune tolerance in *Drosophila* (Prakash *et al.* 2021), highlighting this possible relationship.

The constitutive activation of NF- κ B is associated with various chronic inflammatory conditions, autoimmune diseases, and cancer (Baker *et al.* 2011; Zhang *et al.* 2017; Sun 2017). Studies using *in vivo* mouse models and human patients with autoimmune disorders have demonstrated that NF- κ B signaling is implicated in the pathogenesis of a number of autoimmune diseases, such as rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis, and systemic lupus erythematosus (Atreya *et al.* 2008; Zubair and Frieri 2013; Miraghazadeh and Cook 2018; Zhou *et al.* 2020; Ilchovska and Barrow 2021). The involvement of aberrant NF- κ B activity in disease has attracted focus in the therapeutic targeting of this signaling pathway (Viatour *et al.* 2005; Lin *et al.* 2010). Thus, our model may serve as a suitable system to further understand the role of NF- κ B in autoimmunity and allow for investigation into its full potential as a therapeutic target.

noncoding RNAs are differentially expressed in *hop*^{Tum} and *tuSz*¹ mutants

Similar to a recent study looking at the transcriptional response to aging and viral infection in *Drosophila* (Sheffield *et al.* 2021), we found widespread changes in the expression of predicted noncoding RNAs (ncRNAs) in *tuSz*¹ and *hop*^{Tum} larvae (Figure 6A-B). ncRNAs are a class of RNA molecules that are transcribed from the genome but are not translated into proteins. Major classes of ncRNAs include ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), small ncRNAs, and long ncRNAs (Cech and Steitz 2014). While the roles of rRNAs and tRNAs are well characterized, multiple studies have found that small and long ncRNAs participate in a range of biological processes such as genome imprinting, organismal development, and disease pathogenesis (Wang and Chang 2011; Anastasiadou *et al.* 2018; Fasolo *et al.* 2019), as well as immune regulation (Atianand *et al.* 2017; Yuan *et al.* 2018).

ncRNAs are categorized according to their transcript size, with small ncRNAs being <200 nucleotides (nt). These small ncRNA species include microRNAs (miRNAs), small nuclear RNAs (snRNAs), and small nucleolar RNAs (snoRNAs), and only a limited number of small RNAs are differentially expressed in *tuSz¹* and *hop^{Tum}* larvae (Table 4). On the other hand, a large number of long ncRNAs (lncRNAs; >200 nt) show differential expression in these mutants (Figure 6C-D). Amongst the lncRNAs upregulated in both *tuSz¹* and *hop^{Tum}*, *Induced by infection (IBIN)* is the only lncRNA that has been studied in the context of *Drosophila* immunity, although it has been proposed that *IBIN* encodes a short peptide that confers its function (Valanne *et al.* 2019; Ebrahim *et al.* 2021). The significant upregulation of lncRNAs in both genotypes highlights the potential importance of lncRNAs in mediating immune priming and autoimmune responses.

CONCLUSION

Immune self-tolerance is essential for maintaining immune homeostasis, and breakdown of self-tolerance mechanisms results in autoimmunity and other immune related diseases. Although great progress has been made in understanding self-tolerance in the context of adaptive immunity, innate immune mechanisms regulating self-tolerance and its role in autoimmunity remain less well characterized. In this study we have identified genes that are differentially expressed following loss of self-tolerance in the *Drosophila melanogaster tuSz¹* mutant. These results provide insight into the biological processes that take place in *tuSz¹* mutant larvae and may contribute toward a better understanding of the events that take place in loss of self-tolerance autoimmune reactions.

DATA AVAILABILITY

RNAseq data will be made available through the NCBI Gene Expression Omnibus (submission pending). The authors are happy to make these data available during the review process, and we will update with the NCBI GEO accession number when available.

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FIGURE LEGENDS

Figure 1. Venn diagrams illustrating the numbers of transcripts that are (A) upregulated and (B) downregulated in the indicated genotypes in comparison with w^{1118} control larvae.

Figure 2. Mean relative quantification $\pm$ standard error of *PGRP-SC1a* (A), *PGRP-SC2* (B) and *scrib* (C) transcript levels in *tuSz¹* mutant larvae compared to w^{1118} controls. Transcripts were quantified by qPCR, * indicates $p < 0.05$ relative to w^{1118} control.

Figure 3. Localization of the Rel-GFP fusion protein in hemocytes. Hemocytes were dissected from w^{1118}/Y ; *Rel-GFP/+* and *tuSz¹/Y*; *Rel-GFP/+* larvae raised at 28°C, and stained with propidium iodide (PI). Shown are representative images of hemocytes: brightfield (A-C), GFP to visualize Rel-GFP localization (A'-C') and PI to mark nuclei (A''-C''). Rel-GFP shows enriched nuclear localization in plasmatocytes (B-B'') and lamellocytes (C-C'') in *tuSz¹/Y*; *Rel-GFP/+*, but not in plasmatocytes (A-A'') in w^{1118}/Y ; *Rel-GFP/+*. Note that w^{1118}/Y ; *Rel-GFP/+* larvae do not

produce lamellocytes. (D) Bar graph showing the proportion of hemocytes with a predominantly nuclear Rel-GFP localization (see Materials and Methods for analysis details). Complete genotypes *tuSz¹/Y; Rel-GFP/+* and *w¹¹¹⁸/Y; Rel-GFP/+* are simplified to *tuSz¹* and *w¹¹¹⁸* for clarity. * indicates $p < 0.05$ relative to *w¹¹¹⁸/Y; Rel-GFP/+* control.

Figure 4. Expression of the Rel-GFP fusion protein in hemocytes from parasitoid infected *w¹¹¹⁸/Y; Rel-GFP/+* larvae. Brightfield (A-C) and GFP (A'-C') images. The Rel-GFP fusion protein is barely detectable in plasmatocytes (A, B) and lamellocytes (C). Strong Rel-GFP expression is seen in an unknown non-hemocyte cell type (indicated by * in B and C).

Figure 5. Expression of the Rel-GFP fusion protein in the fat body dissected from *w¹¹¹⁸/Y; Rel-GFP/+* and *tuSz¹/Y; Rel-GFP/+* larvae raised at 28°C. Minimal Rel-GFP expression is observed in fat body tissue dissected from *w¹¹¹⁸/Y; Rel-GFP/+* (A-A') or anterior fat body tissue dissected from *tuSz¹/Y; Rel-GFP/+* (B-B') larvae. Increased Rel-GFP expression is observed in posterior fat body tissue dissected from *tuSz¹/Y; Rel-GFP/+* (C-C') larvae. The posterior fat body is the site of the loss of self-tolerance in *tuSz¹* mutant larvae, and the characteristic self-encapsulation phenotype is evident in (C).

Figure 6. Venn diagrams illustrating the numbers of differentially expressed ncRNA transcripts that are (A) upregulated and (B) downregulated, and the numbers of differentially expressed lncRNA transcripts that are (C) upregulated and (D) downregulated in the indicated genotypes in comparison with *w¹¹¹⁸* control larvae.

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A

Upregulated genes

B

Downregulated genes

A **B** **C**

D

Proportion of Hemocytes with
Nuclear Localization

A

Upregulated ncRNAs

B

Downregulated ncRNAs

C

Upregulated lncRNAs

D

Downregulated lncRNAs

Table 1. Enriched Gene Ontology (GO) Terms among DEG in common to both *tuSz*¹ and *hop*^{Tum}

GO Term	Fold enrichment	FDR
<i>Upregulated genes</i>		
Defense response to other organism (GO:0098542)	2.58	0.014
Response to external stimulus (GO:0009605)	2.11	2.6 x10 ⁻⁴
Proteolysis (GO:0006508)	2.87	1.2x10 ⁻⁹
Cellular lipid metabolic process (GO:0044255)	2.43	0.022
<i>Downregulated genes</i>		
Developmental process (GO:0032502)	1.59	4.7x10 ⁻⁴
Regulation of multicellular organismal development (GO:2000026)	2.24	0.015
Tissue morphogenesis (GO:0048729)	2.15	0.029
Cell fate commitment (GO:0045165)	2.99	0.005

Table 2. Enriched GO Terms among *tuSz*¹ specific DEGs

GO Term	Fold enrichment	FDR
<i>Upregulated genes</i>		
Negative regulation of antimicrobial peptide biosynthetic process (GO:0002806)	12.42	0.031
Chitin-based cuticle development (GO: 0040003)	2.89	4.9x10 ⁻⁶
Oxidoreductase activity (GO: 0016491)	2.80	5.4x10 ⁻¹³
Generation of precursor metabolites and energy (GO:0006091)	2.32	0.023
Carbohydrate derivative metabolic process (GO:1901135)	2.14	5.0x10 ⁻⁵
Alpha-amino acid metabolic process (GO:1901605)	3.29	0.006
Lipid metabolic process (GO:0006629)	1.98	0.003
<i>Downregulated genes</i>		

Tissue morphogenesis (GO:0048729)	2.43	2.3x10 ⁻⁴
Establishment of tissue polarity (GO:0007164)	4.17	0.049
Appendage development (GO:0048736)	2.63	0.004
Central nervous system development (GO:0007417)	2.53	0.042
Cell differentiation (GO:0030154)	1.53	0.043
Pattern specification process (GO:0007389)	2.09	0.035
Regulation of cell communication (GO:0010646)	1.83	0.025
Positive regulation of stem cell proliferation (GO:2000648)	9.6	0.003
Metamorphosis (GO:0007552)	2.42	0.002

Table 3. Expression of PGRP family genes in *tuSz*¹ and *hop*^{Tum} compared to *w*¹¹¹⁸ control larvae.

Gene	Pathway	Function	<i>tuSz</i> ¹ log ₂ FC	<i>hop</i> ^{Tum} log ₂ FC
<i>PGRP-LA</i>	IMD	Positive Regulator	-0.38	-0.30
<i>PGRP-LB</i>	IMD	Negative Regulator	1.83*	-0.18
<i>PGRP-LC</i>	IMD	Positive Regulator	-1.12	-0.63
<i>PGRP-LD</i>	Unknown	Unknown	-2.77*	-0.81
<i>PGRP-LE</i>	IMD	Positive Regulator	-.018	-0.75
<i>PGRP-LF</i>	IMD	Negative Regulator	1.37*	0.32
<i>PGRP-SA</i>	Toll	Positive Regulator	1.67	0.99
<i>PGRP-SB1</i>	IMD	Immune Effector	-0.92	-1.95
<i>PGRP-SB2</i>	IMD	Immune Effector	Not detected	Not detected
<i>PGRP-SC1a</i>	IMD	Negative Regulator	3.46*	1.70
<i>PGRP-SC1b</i>	IMD	Negative Regulator	3.38*	1.51
<i>PGRP-SC2</i>	IMD	Negative Regulator	1.23*	-0.19
<i>PGRP-SD</i>	Toll	Positive Regulator	0.66	-0.83

* indicates FDR < 0.05 compared to *w*¹¹¹⁸ control; log₂FC = log₂ Fold Change

Table 4. Number of differentially expressed ncRNAs of each class that are upregulated and downregulated in the indicated genotype in comparison with *w*¹¹¹⁸ control larvae.

	<i>tuSz¹</i>		<i>hop^{Tum}</i>		Both mutants	
ncRNA Class	Up	Down	Up	Down	Up	Down
lncRNAs	55	136	53	130	14	68
miRNAs	0	5	4	1	0	1
snRNAs	1	2	3	1	1	1
snoRNAs	2	2	4	1	2	1