SUMOylation of Jun fine-tunes the *Drosophila* gut immune response

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Running Title: Conjugation of Jun by SUMO regulates gut immunity

ABSTRACT

Post-translational modification by the small ubiquitin-like modifier, SUMO can modulate the activity of its conjugated proteins. The transcriptional regulator Jun, a member of the AP-1, complex is one such SUMO target. We find that Jra, the *Drosophila* Jun ortholog, is a regulator of the *Pseudomonas entomophila* induced gut immune gene regulatory network, modulating the expression of a few thousand genes, as measured by quantitative RNA sequencing. Decrease in Jra in gut enterocytes is protective, suggesting that reduction of Jra signaling favors the host over the pathogen. In Jra, lysines 29 and 190 are SUMO conjugation targets, with the Jra^{K29R+K190R} double mutant being SUMO conjugation resistant (SCR). Interestingly, a Jra^{SCR} fly line, generated by CRISPR/Cas9 based genome editing, is more sensitive to infection, with adults showing a weakened host response and increased proliferation of *Pseudomonas*. Transcriptome analysis of the guts of Jra^{SCR} and Jra^{WT} flies suggests that lack of SUMOylation of Jra significantly changes core elements of the immune gene regulatory network, that include antimicrobial agents, secreted ligands, feedback regulators, and transcription factors. SUMOylation attenuates Jra activity, with the master immune regulator Relish being an important transcriptional target. Our study implicates Jra as a major immune regulator, with dynamic SUMO conjugation/deconjugation modulating the kinetics of the gut transcriptional immune response.

Keywords

NFkB, *Pseudomonas*, Enterocytes, infection, SUMO, Jra, AP-1

INTRODUCTION

Proteins undergo post-translational modifications (PTM) by reversible and covalent attachment of a diverse set of molecules. PTMs can modulate the target protein's structure, stability, interactions, or location and alter its function. This modulation increases the complexity and diversity of a proteome. The effect of the PTM on the target protein is usually context-dependent, with the combination of different PTMs affecting function in a differential manner. PTMs come in all shapes and sizes with a wide variety of chemical groups. One common PTM is the conjugation of one protein to another. The best-known examples of such protein modifiers are Ubiquitin-like proteins (UBLs) (VAN DER VEEN AND PLOEGH 2012; VIERSTRA 2012; EIFLER AND VERTEGAAL 2015; CAPPADOCIA AND LIMA 2018), a large group of proteins exhibiting a Ubiquitin fold. These, along with Ubiquitin, include Nedd8, ISG15, SUMO, ATG8/12, and many others. These protein PTMs conjugate a wide variety of proteins, usually targeting lysine side chains.

The small ubiquitin-like modifier, SUMO (MELCHIOR 2000; MULLER *et al.* 2001; HAY 2005) can be reversibly and covalently conjugated to a target protein. For conjugation, the translated SUMO protein is made conjugation-competent by proteolytic cleavage of the C-terminal tail of SUMO, exposing a –GG dipeptide. The SUMO-GG is then picked up by the SUMO activating enzymes (SAEs) which transfer the SUMO to the SUMO conjugase Ubc9. Ubc9 interacts with the target protein and works to attach the C-terminal GG-COO- to a lysine side chain, on the target protein, via an isopeptide bond (FLOTHO AND MELCHIOR 2013; CELEN AND SAHIN 2020). The conjugation is reversible and requires deconjugating enzymes (HICKEY *et al.* 2012). Ubc9, during conjugation, can be assisted by one or multiple SUMO ligases, which are believed to provide specificity and enhance conjugation.

Our laboratory is interested in the role of SUMO conjugation of proteins during an innate immune response in flies (HANDU *et al.* 2015; HEGDE *et al.* 2020). A subset of proteins involved in immune regulatory networks are known to be SUMOylated both in vertebrates or invertebrates (HANNOUN *et al.* 2016; ADORISIO *et al.* 2017; HEGDE *et al.* 2020). Our approach to the problem was first to identify proteins that changed their SUMOylation status on infection. We used quantitative proteomics to identify a set of proteins whose SUMOylation state either increased or decreased with infection (HANDU *et al.* 2015). One SUMO target uncovered in our quantitative proteomics screen (HANDU *et al.* 2015) is the *Drosophila* c-Jun ortholog Jra (Flybase ID, FBgn0001291) - the Jun related antigen (PERKINS *et al.* 1988; SLUSS *et al.* 1996). Jra

is one of the transcriptional effectors of the JNK (Jun N-terminal kinase) signaling pathway, the other being Kayak (Kay, ortholog of c-Fos) (NOSELLI 1998; NOSELLI AND AGNÈS 1999). Jra function has been uncovered in multiple developmental contexts (BOHMANN *et al.* 1994; RIESGOESCOVAR AND HAFEN 1997; KOCKEL *et al.* 2001; RIOS-BARRERA AND RIESGO-ESCOVAR 2013), as well as in the adult animal (BITEAU *et al.* 2011; LA MARCA AND RICHARDSON 2020). The JNK kinase cascade has been broadly classified as a ‘stress’ pathway in organisms, and our current research focuses on its role in immune signaling.

In this study, we have explored roles for SUMO conjugation of Jra in the gut immune response. Using the loss of function genetics, in combination with quantitative RNA sequencing, we find that Jra is a major regulator of the genes involved in the gut immune response, triggered by oral *Pseudomonas entomophila* (*Pe*) infection. Our data suggest that Jra is part of an extensive immune Gene regulatory network (i-GRN) and is crucial for evoking a robust immune response and maintain gut immune homeostasis. We find that SUMO conjugates Jra at lysines 29 and 190, and a Jra^{K29R+190R} mutant is SUMO-conjugation resistant (SCR). The Jra^{SCR} double mutant line, generated by CRISPR Cas9 genome editing, is sensitive to *Pe* infection and carries a higher load of bacteria, suggesting that the absence of Jra SUMOylation weakens the ability of the host to resist infection. At the level of transcription, lack of Jra SUMOylation changes the kinetics of the immune response, delaying and weakening the activation of defense genes, an important example being the NFκB factor Relish (Rel).

Our study thus uncovers Jra as a major transcriptional regulator of the gut immune response. Increased Jra signaling appears to favor *Pseudomonas*, while SUMOylation of Jra favors the host, presumably by enhancing the host response to pathogens. Thus, we postulate that SUMOylation of Jra fine-tunes the host transcriptional response to infection.

RESULTS

Jra is a target for SUMO conjugation

We initiated our studies by validating our initial observation (HANDU *et al.* 2015) that Jra is SUMO conjugated (SUMOylated). Jra SUMOylation was validated using a previously described *in-bacto* approach (NIE *et al.* 2009). Here, Jra was co-expressed in bacteria with *Drosophila* SAE1, SAE2, Ubc9, and mature 6XHis-tagged SUMO (SUMO-GG) to facilitate target SUMOylation. Affinity purification using glutathione beads, followed by western blotting, was used to detect SUMOylated species migrating at a higher molecular weight than Jra, based on the degree of conjugation. A similar experiment with an immature/dead 6XHis-tagged SUMO (SUMO-ΔGG) served as a negative control. We consistently find two high molecular weight bands that correspond to 1X and 2X SUMO conjugated species in the Western blots (Fig. 1A), confirming that Jra is a target of SUMO conjugation. We also find a third higher molecular weight band corresponding to >3X SUMO conjugated species. To discover the lysine residues that are targets of SUMO machinery, we used the web-based tool, Joined Advanced SUMOylation Site and Sim Analyser (JASSA) (BEAUCLAIR *et al.* 2015) to predict the putative SUMO sites (Fig. 1B). SUMO is known to prefer lysine residues that follow the broad consensus SUMO motif (ψ KXE/D), where ψ is a hydrophobic residue. A total of seven sites (Fig. 1B) were targeted based both on JASSA predictions and the evolutionary conservation of SUMO sites. Of these, K214 was a strong consensus motif while K29 and K190 were part of Negatively charged amino acid-dependent SUMOylation (NSDM) site and Synergy Control (SC) – SUMO motifs, respectively (Fig. 1C). The three-dimensional structures of Jun available in the Protein Data Bank do not include the N-terminal and C-terminal regions, with secondary structure predictions (MITCHELL *et al.* 2019) suggesting that K29 and K190 are in unstructured regions. We replaced each of the predicted lysine residues, one at a time, with an arginine residue, whose side chain is resistant to SUMO conjugation (Fig. 1D & E). Our goal was to identify SUMO conjugation sites and generate a SUMO conjugation resistant mutant (SCR) of Jra. Of the seven site-directed mutants tested (Fig. 1C, D and E), only two, K190R and K29R, showed partial loss of SUMOylation. There were changes in the intensity of SUMOylated bands for the remaining single mutations, but their SUMOylation status was maintained (Fig. 1D & E). As a further step to generate a Jra SCR mutant, we cloned and tested the Jra double mutant, Jra^{K29R+K190R}. Interestingly, we found that the double mutant could not be SUMO conjugated, with all three SUMOylation bands missing (Fig. 1F).

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Figure 1: Site-directed lysine mutagenesis screen identifies Jra(K29) and Jra(K190) as acceptor sites for SUMOylation,

A. Anti-His western blot (upper panel) post Glutathione agarose affinity pulldown (PD) shows three SUMOylated bands for GST-Jra. GST-Jra^{WT} co-transformed with immature SUMO (SUMO-ΔGG) serves as a negative control for SUMO conjugation. Anti-GST WB (lower panel) post affinity PD indicates similar levels of expression of GST-Jra^{WT} for the experimental and control lysates.

B. Schematic of the primary sequence of *Drosophila* Jra. The conjugation sites to be tested (panel C) are mapped onto Jra. Secondary structure prediction indicates that the Jra N-terminus is unstructured (black line), while the C-terminus (residues 210-275) is helical. This is in agreement with structures deposited in the Protein Data Bank

(PDB:1FOS), which correspond only to the C-terminal leucine zipper (orange box), formed from c-Jun/c-Fos dimers.

C. Putative SUMO motifs predicted for Jra include the negative amino-acid dependant SUMO motif (NDSM; K29), synergy consensus SUMO motif (SCSM; K190), four consensus SUMO motifs (K214, K233, K239 and K248) and an inverted consensus motif (K218).

D. Anti-His western blot (upper panel) post PD showing the SUMOylated bands of Jra and its mutant variants. Jra^{K190R} shows partial loss of SUMOylation where the upper and lowermost SUMOylated bands disappear. Jra^{K214R} and Jra^{K218R} do not show any loss of bands and behave like the wildtype. Anti-GST western blot (lower panel) post PD showing equal expression of different variants of GST-Jra. The lanes expressing the SUMO(⊗GG) variant serve as a negative control for SUMO conjugation.

E. Anti-His western blot (upper panel) post PD showing the SUMOylated bands of Jra and its mutant variants. Jra^{K29R} shows partial loss of SUMOylation where the two bands on the top disappear. Jra^{K233R}, Jra^{K239R} and Jra^{K248R} do not show loss of higher molecular weight SUMO bands. Anti-GST western blot (lower panel) post PD showing expression of different variants of GST-Jra. The lanes expressing the SUMO-⊗GG variant serve as a negative control for SUMO conjugation.

F. Anti-His western blot (upper panel) of Jra post PD showing the three distinct SUMOylated Jra bands in Jra^{WT}. Partial loss of SUMOylation in Jra^{K29R} and Jra^{K190R}, as seen in panels D and E, is confirmed. Jra^{K29R+K190R} shows complete loss of SUMOylation. Anti-GST western blot (lower panel) post PD showing expression levels of unmodified Jra.

G. *In-vivo* demonstration of SUMOylation of Jra post-Ni-NTA affinity pull-down. Anti-Flag western blot in the upper panel represents the SUMOylated species of Jra and was seen as two distinct bands, marked by asterisks (*). Jra^{K190R} shows loss of the upper band, indicating partial loss of SUMOylation. Jra^{K29R} and Jra^{K29R+K190R} show complete loss of SUMOylation. Jra^{K218R} that was previously shown not to alter SUMOylation status in the bacterial assay, is at par with Jra^{WT}. The empty vector was used as a master negative control. Anti-His western blot (lower panel) represents unmodified Jra and serves as input post affinity PD.

Based on the molecular weight of the SUMOylated species in the Jra^{K29R} and Jra^{K190R} lanes, we suspect that there is a third SUMO site in Jra which is dependent on SUMOylation of Jra^{K29}. However, this third site is not populated either in the Jra^{K29R} single mutant or the Jra^{K29R+K190R} double mutant. Another possibility, di-SUMOylation at Jra^{K29R}, exists, but there is evidence against poly-SUMOylation in insects (URENA *et al.* 2016).

The Jra^{K29R+K190R} is our putative SCR mutant (Jra^{SCR}). To confirm the resistance of this mutant to SUMO conjugation *in-vivo*, we cloned Jra^{WT}, Jra^{K29R}, Jra^{K190R} and Jra^{K29R+K190R} with a 6XHis tag into an inducible pRM vector and transfected 529SU cells transiently with these constructs. 529SU is a stable S2 cell line (BHASKAR *et al.* 2002) that expresses FLAG-SUMO-GG, SAE1, SAE2 and Ubc9 using inducible metallothionein promoters. Expression of Jra variants was initiated by adding CuSO₄, which activates the metallothionein promoter. We have performed Ni-NTA affinity pulldown under denaturing conditions, followed by western blots probed with an anti-FLAG antibody and an anti-6XHis antibody (Fig. 1F). In S2 cells, Jra^{WT} showed two SUMO-conjugated bands, corresponding to 1X and 2X SUMOylated species. Jra^{K190R} retained one SUMO conjugation site, but interestingly both Jra^{K29R} and Jra^{K29R+K190R} did

not show any SUMO conjugation. Since all further experiments were to be conducted in the animal, and both these sites were high confidence SUMO conjugation sites, we chose to work further with the double mutant, $Jra^{K29R+K190R}$, and defined it as Jra^{SCR} .

Jra and SUMO play critical roles in host defense in the fly gut

After exploring published roles for *Jra*, we decided to explore *Jra* function in the gut, which is one of the primary immune organs in the fly (BUCHON *et al.* 2013; MIGUEL-ALIAGA *et al.* 2018). *Jra* and the other components of the JNK pathway are expressed in various cell types of the gut, with *Jra* upregulated in response to *Pe* infection (DUTTA *et al.* 2015)(Fig. S1). *Jra* transcripts are highest in the enterocytes (ECs, 4.3-fold), followed by the entero-endocrine cells (EEs, 2.7-fold), the interstitial stem cells (ISCs, 1.6-fold) and the enteroblasts (EBs, 1.2-fold) post *Pe* infection (Fig. S1). Flies orally fed with *Pe* rapidly succumb despite the induction of both local and systemic immune responses (VODOVAR *et al.* 2005; LIEHL *et al.* 2006). Hence, we used the *Pe* infection model to study the function of *Jra* in gut immunity. Since *Jra* null animals are embryonic lethal, we tested the ability of two well characterised null alleles of *Jra*, Jra^{IA109} and Jra^{76-19} , to resist *Pe* infection in a heterozygous combination with w^{1118} (Fig. 2A). Interestingly, the absence of one copy of *Jra* protected the animal against infection, significantly extending lifespan (Fig. 2A). In order to validate this result, we used a gut EC driver, $Myo31DF-Gal4^{ts}$ (also called $NP1-Gal4^{ts}$), to reduce *Jra* function by both RNA interference and by overexpressing a dominant-negative (Jra^{DN}) allele. In both experiments (Fig. 2B), reduction of *Jra* activity in the gut led to partial protection against the pathogen, leading to an extension of lifespan.

In contrast, reduction of *Jra* activity either in the whole animal (Fig. 2A) or in the gut (Fig. 2B) in the absence of infection does not appear to affect lifespan, suggesting that *Jra* is haplo-sufficient in this context. In flies, the *Jra* function is activated by a Jun kinase (JNK), Basket (Bsk) (RIESGO-ESCOVAR *et al.* 1996; SLUSS *et al.* 1996). Bsk modulates stem cell proliferation and impacts gut regeneration in *Drosophila* (BITEAU *et al.* 2008; JIANG *et al.* 2009; ZHOU *et al.* 2017). Also, Bsk synergizes with the Imd pathway to regulate epithelial cell shedding during gut infection (ZHAI *et al.* 2018). However, the role of Bsk in the regulation of the gut immune response is not well understood. To test this, Bsk function was attenuated by overexpressing a dominant-negative allele (Bsk^{DN}) in the ECs, and the flies were infected with *Pe* (Fig 2C). Interestingly, as in the case of *Jra* knockdown, reduction of Bsk activity also

increased lifespan to a similar extent, suggesting that Bsk functions to regulate Jra activation in the host response to infection (Fig. 2C).

Figure 2

Figure 2: Jra signalling and components of SUMO conjugation machinery have important host-defence roles in the *Drosophila* gut.

A. Survival curves of unchallenged (UC, dotted) and infected (I, closed) flies: *w¹¹¹⁸* (black), *Jra^{A109}/+* (purple) and *Jra⁷⁶⁻¹⁹/+* (blue). Both *Jra^{A109}/+* and *Jra⁷⁶⁻¹⁹/+* flies show enhanced survival upon infection. Log-rank test for trend was used to compare *Jra^{A109}/+* (I) to *w¹¹¹⁸* (I) and *Jra⁷⁶⁻¹⁹/+* (I) to *w¹¹¹⁸* (I). ****p<0.0001.

B. Survival curves of UC (dotted) and I (closed), flies after over-expression of *Jra^{RNAi}* (purple) and *Jra^{DN}* (blue). The *Gal4* control is represented in black. Loss of function of Jra showed enhanced survival upon infection and suggested a gut-specific role of Jra as a suppressor of the immune response. Log-rank test for trend was used to compare *>Jra^{RNAi}* (I) to *>w¹¹¹⁸* and *>Jra^{DN}* (I) to *>w¹¹¹⁸* (I). ****p<0.0001.

C. Survival curves of UC (dotted) and I (closed) flies, post-over-expression of *Bsk^{DN}* (purple) and *Kay^{DN}* (blue). The *Gal4* control is represented in black. Loss of function of Bsk (but not Kay) in the gut showed enhanced survival upon infection. Log-rank test for trend was used to compare *>Bsk^{DN}* (I) to *>w¹¹¹⁸* (I), *>Kay^{DN}* (I) to *>w¹¹¹⁸* (I) and *>Bsk^{DN}* (UC) to *>w¹¹¹⁸* (UC). ****p<0.0001.

D. Survival curves of unchallenged UC (dotted) and I (closed) flies: *>w¹¹¹⁸* (black) and *>SUMO^{RNAi}* (purple). Loss of SUMO in the gut enhances lifespan post-infection. Log-rank test for trend was used to compare *>SUMO^{RNAi}* (I) to *>w¹¹¹⁸* (I). ****p<0.0001.

E. Survival curves of UC (dotted) and I (closed) flies after perturbing different components of the SUMO cycle machinery: $>w^{1118}$ (black), $>Uba2^{RNAi}$ (green), $>Aos1^{RNAi}$ (pink) and $>Ubc9^{DN}$ (orange). Log rank test for trend was used to compare $>Uba2^{RNAi}$ (I) to $>w^{1118}$ (I), $>Aos1^{RNAi}$ (I) to $>w^{1118}$ (I) and $>Ubc9^{DN}$ to $>w^{1118}$ (I) and $>Aos1^{RNAi}$ (UC) to $>w^{1118}$ (UC). * $p=0.03$; *** $p=0.0008$ **** $p<0.0001$.

F. Scatter dot plot representing bacterial load as colony-forming units (CFUs) post-infection. The student's t-test was performed comparing w^{1118} and $Jra^{IA109}/+$ at respective time points. ** $p=0.0025$; * $p=0.0194$.

Enterocyte specific NP1-Gal4^{ts} was used to express the transgenes. Data related to the number of animals per experiment and replicates listed in Fig. S2A. Data not significant, not represented.

Surprisingly, loss of function of Bsk in the gut enhanced lifespan in non-infected conditions. This may suggest that Bsk is a kinase for additional proteins in the gut that regulate the fly's lifespan. Kayak (Kay) can also partner with Jra for transcriptional regulation, with the heterodimeric Jra/Kay complex orthologous to the Jun/Fos AP-1 complex (RIESGO-ESCOVAR AND HAFEN 1997; CIAPPONI AND BOHMANN 2002). In the fly gut, however, reducing the function of Kay did not appreciably change the lifespan of the adult fly (Fig. 2C) post-infection, possibly indicating a Kay-independent role for Jra in responding to *Pe* infection in the gut.

Since this study aims to understand the roles of Jra SUMOylation, we also tested the effect of the reduction of global SUMOylation in the gut. Knockdown of *Smt3/SUMO* in the gut using *NP1-Gal4^{ts}* was again protective under infective conditions (Fig. 2D). Further, knockdown of the enzymes that contribute to SUMO conjugation, namely *SAE1/Aos1*, *SAE2/Uba2*, was also protective, extending adult fly lifespan during *Pe* infection (Fig. 2D), as was the expression of a dominant-negative allele of the conjugating enzyme, *Lwr/Ubc9* (*Ubc9^{DN}*). Under non-infective conditions, loss of SUMO conjugation appeared to affect lifespan, with *Aos1* knockdown reducing lifespan significantly compared to *NP1-Gal4^{ts}>w¹¹¹⁸* (Fig. 1E).

The protective effect of *Jra* knockdown suggests that the *Pe* may be resistant to clearance by the host-defense response in the presence of *Jra*. We measured the colony-forming units (CFUs) in the gut of adult flies, 2 hours, 8 hours and 24 hours post-infection (hpi, Fig. 2F). The number of CFUs gradually decreased as the infection progressed in w^{1118} and $Jra^{IA109}/+$. However, there were fewer CFUs in the gut, by order of magnitude, in $Jra^{IA109}/+$ animals, when compared to controls at 8 and 24 hours post-infection, suggesting that absence of *Jra* signaling in the gut epithelium led to better clearance of gut microbes. Early clearance of the pathogen could also lead to decreased damage of the gut epithelium and a concomitant longer lifespan.

Infection with *Pe* damages the gut epithelium, triggering the ISCs to divide asymmetrically to replace the differentiated cells (CHAKRABARTI *et al.* 2012). Immunostaining with an anti-phospho-Histone 3 (pH3) antibody in the gut post-infection allows us to measure the extent of ISC proliferation. For this, *w¹¹¹⁸* and *Jra^{IA109}/+* flies were fed with *Pe* for 8 hours and dividing cells per midgut were counted (Fig. S2B). When compared to UC flies, there was a strong induction of mitoses upon infection. The number of dividing cells was similar between *w¹¹¹⁸* (I) and *Jra^{IA109}/+* (I), suggesting that *Jra* might not play a dominant role in regulating the intestinal stem cell proliferation upon an immune challenge in the current context.

The reduction of *Jra* activity during a *Pe* infection appears to be protective to the fly in the context of the gut. Although *Jra* transcripts increase in the gut epithelium in response to *Pe* infection, peaking at 12 hours (next section); Their absence reduces the CFUs and also enhances the survival of flies post-infection. Further, reduction of activity of *Bsk* is also protective, suggesting that *Bsk* and *Jra* function in a common Jun kinase (JNK) regulatory pathway in the gut. The gut thus appears to merit a detailed study on the roles for SUMO conjugation of *Jra*. Our data *prima facie* suggests that *Jra* acts as an important regulator of the host defense response in the gut epithelium.

Jra is a major regulator of the transcriptional response to Pe infection

To understand the effect of reduced *Jra* function in the clearance of *Pe* and extending lifespan in infective conditions, we measured the changes in the midgut transcriptome using 3'mRNA sequencing (Fig. S3A and described in Materials and Methods). To start with, we measured the kinetics of activation of *Jra* in the gut during the infection and also the extent of knockdown at each time point using quantitative real-time PCR (qRT-PCR; Fig. 3A). We find that *Jra* levels peak at 12 hpi in our experimental setup. In terms of efficiency of knockdown, the *Jra* levels were reduced by at least 60% during the time course of the infection (Fig. 3A).

Next, transcriptomes of the midguts were measured at 4, 12, and 24 hpi for both wild-type and after *Jra* knockdown. Principal component analysis (PCA) was performed to estimate the robustness of the dataset (Fig. S3B). We plotted the first two principal components and observed that the data sets were distinct as the infection progressed and were seen as separate clusters (Fig. S3B). We compared the transcriptional changes in *NP-Gal4^{ts} >Jra^{RNAi}* (>*Jra^{RNAi}* here onwards) to *NP-Gal4^{ts} >w¹¹¹⁸* (>*w¹¹¹⁸* here onwards), without an immune challenge (Unchallenged; UC). Upon knockdown, *Jra* is downregulated by 1.2 times on a log

2-fold change ($\log_2$ FC scale; Table S1). We also report that 292 genes were differentially expressed with a false discovery rate (FDR) <0.1 , amongst which 168 genes were upregulated and 124 genes were downregulated in $>Jra^{RNAi}$ as compared to $>w^{1118}$ (Fig. 3B and Table S1). The genes including components of immune signalling are putative direct (or indirect) transcriptional targets of Jra. The gut-specific AMPs *Drosomycin-like 2* (*Drsl2*), *Drsl3* and peptidoglycan recognition proteins *PGRP-SC1a* and *PGRP-SC1b* are upregulated (Fig. 3C), suggesting a role for Jra in regulating defense genes even in the absence of infection.

Next, we compared the transcriptome of $>w^{1118}$ and $>Jra^{RNAi}$ in response to infection (I). We normalised data from each infection time point to UC of that genotype and have separated the total genes that were differentially expressed in each pairwise comparison. We then looked at the number of genes differentially expressed at each timepoint in $>w^{1118}$ and $>Jra^{RNAi}$. The genes identified ranged from 1000-2000 at each time point. Of note, at 12 hpi, the number of significantly different genes in $>Jra^{RNAi}$, were numerically half of that in $>w^{1118}$, indicating a major perturbation in the immune transcriptome (Fig. 3D). We also compared the genes that were common to both the genotypes and genes that were unique to either of the genotypes during infection (FDR <0.1 ; Fig. S4A). A total of 3505 genes were differentially expressed in both the genotypes, with all timepoints taken together (Table S1). Gene ontology (GO) analysis on all the 3505 genes indicates enrichment of several key GO terms like metabolic process, response to stress, Toll and Imd signalling pathway (Fig. S4B).

Fig. 3E categorizes a representative set of genes. Analysing $\log_2$ FC values of individual genes (Table S1; Fig. 3E) at the three kinetic time points allows us to understand quantitative differences between the immune response for the two genotypes. At the level of the gene transcripts, the effect appears to be context-specific; most AMPs (Fig. 3E) are upregulated post-infection. In $>Jra^{RNAi}$ genes such as *AttA*, *AttB*, *DptA*, *DptB*, *CecA1*, *CecA2*, *Drsl4* show higher $\log_2$ FC values and appear to be activated strongly in the earliest time point, i.e 4 hpi, further confirmed by qRT-PCR (Fig. S5A and S5B) for *DiptA* and *AttD*. $\log_2$ FC values for the Imd pathway members, *Relish* (*Rel*), seem to be comparable between both the genotypes.

Figure 3: Jra is a major regulator of the *Pe* infected gut transcriptome.

A. Kinetics of activation of *Jra* transcripts, as measured by qRT-PCR in $>w^{1118}$ and $>Jra^{RNAi}$ without and with gut infection. * $p=0.0144$, **** $p<0.0001$ as determined by 2-way ANOVA with Bonferroni's post-hoc test for multiple comparisons. Data from three independent experiments. Means and SEMs represented.

B. Bar plot representation of a total number of significantly differentially expressed genes (FDR<0.1) obtained by comparing $>w^{1118}$ and $>Jra^{RNAi}$ under UC conditions post a 3' mRNA sequencing experiment.

C. Volcano plot showing the up or down-regulated genes, comparing $NP-Gal4^{ts} >w^{1118}$ and $NP-Gal4^{ts} >Jra^{RNAi}$. A few genes previously known to function in regulating immunity are highlighted.

D. Bar plot representation of the total number of significantly differentially expressed genes (FDR<0.1) obtained at different hpi after comparing to UC animals of the respective genotype.

E. Tabular representation of log₂FC values of transcripts for a few categories of defence genes regulated during the infection. Log₂FC values in the range 0.58 to -0.58, indicating no significant changes compared to UC, are marked in white.

F. Jra binds to the promoters of several genes (data plotted from modERN dataset ENCSR471GSA). Heatmap represents the normalised expression counts of genes for transcription factors, enriched in the RNA-seq data set, with Jra binding peaks on the promoters.

G. Schematic of Jra immune regulatory network representing the effect of Jra on a subset of transcription factors. The balance between activation and repression of genes by Jra is necessary to evoke a proper and robust immune response.

The same is true for the NFκB transcription factor *dorsal* (*dl*) and members of the JNK pathway, *Puckered* (*Puc*) and *Kay*. However, *poor Imd response upon knock-in* (*Pirk*), the negative regulator of the Imd pathway (KLEINO *et al.* 2008), had higher log₂FC values in *>Jra^{RNAi}* compared to *w¹¹¹⁸* at 12hpi. Also, the Jak/STAT pathway components *Upd2* and *Dome* show higher log₂FC values in *>Jra^{RNAi}* suggesting that the Jak/STAT pathway might be strongly activated in the guts of *>Jra^{RNAi}* flies. Interestingly, transcriptional regulators like *forkhead* (*fkh*), *Ets at 21C* (*Ets21C*), *chronologically inappropriate morphogenesis* (*chinmo*), *simganj* (*simj*), *Protein interacting with Ttk69 and Sin3A* (*Pits*), *hairy* (*h*), *Bicoid interacting protein 1* (*Bin1*), and *anterior open* (*aop*) show stronger levels of activation in *>Jra^{RNAi}* throughout the infection. This suggests that Jra could be a major player in a larger gut-specific i-GRN that is activated during an immune response.

Jra can influence the transcription of genes by directly binding to their promoters. We hypothesised that Jra could directly influence the transcription of a subset of the 3505 genes obtained in our experiment. To test this, we used the Jra ChIP-seq dataset available from the modERN consortium (KUDRON *et al.* 2018) and mapped the occupancy of Jra on the promoters of 3505 genes (Fig. S6A, Table S1). We identified a total of 778 genes with enriched peaks of Jra binding on the promoters (Fig. S6A) and considered them as direct transcriptional targets of Jra. We plotted the normalized counts of these 778 genes across all timepoint and two genotypes and observed clear differences in the expression of several genes in *>Jra^{RNAi}* as compared to *>w¹¹¹⁸* (Fig. S6B). We then performed GO analysis on this subset and confirmed that ‘gene-specific transcription’ is one of the most represented GO terms (Fig. S6C). Interestingly, there were 37 transcription factors (TFs) in this list, with each transcription factor having the ability to upregulate/downregulate its own set of target genes. Thus the GRN of Jra, along with its target TFs, would define the core Jra-dependent immune gene

regulatory network (Jra i-GRN). Hence, we had a closer look at the expression patterns of these TFs (Fig. 3F). Of the 37 TFs, a subset of them responds strongly to infection. These include *h*, *kay*, *Cyclic-AMP response element-binding protein A (CrebA)*, *Rel*, *Ets21C*, *cabut (cbt)*, *dorsal (dl)*, *daughterless (da)*, *fkh*, *aop*, *Activating transcription factor 3 (Atf3)*, *Kruppel homolog 1 (Kr-h1)*, *chinmo*, *Myocyte enhancer factor 2 (Mef2)*, *NK7.1*, and *Jra*. Reduction in *Jra* affected *Rel*, *Ets21c*, *Promyelocytic leukaemia zinc finger (Plzf)*, and *brother of odd with entrails limited (bowl)* consistently in all the time points and can be seen as reduced levels of transcripts suggesting positive regulation by *Jra*. Genes like *h*, *fkh*, *aop*, *atf3* and *CG8944* show increased transcript levels upon knockdown of *Jra*, suggesting negative regulation by *Jra*.

Our data from the transcriptome analysis suggests that loss of *Jra* in the gut both enhances and suppresses a set of genes (Fig. 3G). The kinetics of upregulation of the AMPs in *>Jra^{RNAi}* appears to be faster than *>w¹¹¹⁸*, and this could lead to early clearance of bacteria and thus increase the longevity of the infected animal. The *Jra* i-GRN appears to contain several key transcriptional factors with known gut-specific roles in maintaining homeostasis. These, among several others, include *Rel*, *Ets21C*, *fkh*, *aop*, and *atf3*, which are also confident targets of *Jra*. The influence of *Jra* on the transcription of these factors could be part of a larger network that is required to activate and suppress genes to evoke a robust immune response (Fig. 3G). Physiologically, the integrated result of the reduction of *Jra* function and subsequent kinetic modulation of the i-GRN appears to strengthen the host defences, to delay the final victory of the pathogen.

Generation of *Jra^{SCR}* by CRISPR/Cas9 genome editing

Having established the functional landscape of *Jra* function in the gut, we focused on understanding specific roles for SUMO conjugation of *Jra*. For this, we generated a *Jra* SCR animal using CRISPR/Cas9 technology. This technology has emerged as a powerful tool to edit the genome and generate point mutations within a gene (BASSETT *et al.* 2013; GRATZ *et al.* 2013; GRANT *et al.* 2019). A major benefit of the methodology is the directed nature of the process and the absence of modifications distal from the targeted locus, with the expression of the mutant allele driven by the native promoter. Our strategy involved the use of the Scarless system (GRATZ *et al.* 2014), where a mutant *Jra* genomic template was available after double-stranded break by Cas9 for homology dependant repair (HDR). The stages for generating a *Jra^{K29R+K190R}* were as follows. A guide RNA (gRNA) that targeted the third exon in the *Jra* locus was designed and cloned into the pBFv-U6.2B vector (Fig. 4A). A transgenic line

was generated by targeting the integration of this vector into the second chromosomal attP40 site by embryonic microinjection (Fig. 4A). The single guide-RNA line (*Jra^{sg2}*) was balanced and validated. The two SCR mutations that we planned to incorporate into the genomic locus of *Jra* were cloned in the pHD-Scarless-DsRed vector ((GRATZ *et al.* 2014); Fig. 4B). In the Scarless vector, the DsRed cassette is flanked by PBac inverted repeats (IR) that target a TTAA site, and the IRs, in turn, are flanked by the homology regions (HR) specific to the genomic region of interest (Fig. 4B). The DsRed is driven specifically in the eye by the P3 promoter and serves as a proxy for the insertion of the entire cassette into the genomic locus. We incorporated the desired K->R mutations in the 3'HR region and assembled two fragments of *Jra* and two vector fragments using Gibson assembly creating the pHD-Scarless-*Jra^{SCR}* vector (Fig. 4B), which was validated by DNA sequencing.

pHD-Scarless-*Jra^{SCR}* was injected into 600 embryos expressing Cas9 and *Jra^{sg2}* (Fig. 4C, D). The F1 animals that emerged were balanced with a 2nd chromosome balancer. Lines that were successfully balanced were screened for positive insertions of the pHD-Scarless-*Jra^{SCR}* by the presence of red fluorescence in the eye of the fly. HDR seemed to be efficient, with 82 lines showing integration. A small subset (20) of DsRed positive lines were crossed with a transposase line. The intent was to excise the DsRed cassette. The TTAA site that is duplicated upon insertion of the cassette is restored into a single genomic TTAA site leaving no 'scar'. Six out of the 20 lines showed a loss of DsRed fluorescence, and these flies were rebalanced and sequenced. Only two out of the six lines had K->R mutations at both positions (29th and 190th), and we have extensively characterised these lines in the further sections. Three lines harboured K->R mutation only on the 29th position and not the 190th position. One line did not harbour any K->R mutations, and this line was used as a CRISPR control for all the further experiments and referred to as *Jra^{WT}* (Fig. 4E). The *Jra* genomic region was sequenced to confirm that no new mutations were generated in the region. All the six lines were homozygous viable, isogenic and lost the balancers in a couple of generations.

Figure 4: CRISPR/Cas9 genome editing is used to generate the *Jra*^{SCR} line.

A. The guide RNA transgenic line, *Jra*^{sg2}, was created for generating a double-stranded break in the *Jra* locus. A *Jra* locus-specific gRNA fragment (5'-CCCGTTTCCGCTGCTGCG3') was designed and inserted into the pBFV-U6.2B vector.

B. Design and cloning strategy for generating the pHD-scarless-*Jra*^{SCR} vector. Homology regions (HR, in dark grey) from the *Jra* locus (bottom panel) were cloned into the 5' and 3' arms of the vector as shown in the Schematic. The majority of *Jra* coding region was part of the 3' arm. As part of the design, the gRNA (panel A) was targeted to the CDS which was a part of the 3rd exon. Details of cloning of the vector are listed in Materials and Methods. The construction of the pHD-Scarless-*Jra*^{SCR} was validated by sequencing.

C. Schematic of injection of pHD-Scarless-*Jra*^{SCR} and genetic crosses implemented to mutate the genomic locus of *Jra* and generate a *Jra*^{SCR} transgenic fly using CRISPR/Cas9 technology. *actin-Cas9* flies were crossed with the

stable Jra gRNA transgenic fly, *Jra^{sg2}*. The embryos from the cross were injected with pHD-Scarless-*Jra^{SCR}*. Flies that emerged were screened for the presence of fluorescent red eyes. DsRed positive flies, with the pHD-Scarless-*Jra^{SCR}* cassette integrated into the Jra locus, were balanced against the w⁻;CyO balancer. SCR mutations would presumably have been incorporated as part of the 3' HR arm. Next, excision of the dsRed cassette was carried out by crossing the flies to a line expressing pBac transposase. Lines in the next generation were screened for loss of DsRed. The lines were balanced, post excision and *Jra* locus sequenced to confirm the loss of DsRed and gain of SCR mutations (panel E).

D. Tabular representation of the efficiency of CRISPR/Cas9 mediated genome editing of *Jra*. 600 embryos of the Cas9 and gRNA cross were injected with pHD-Scarless-*Jra^{SCR}*. 530 embryos emerged into adults, of which 490 were females, and 40 were males. Only 82 of 490 females had red fluorescence in their eyes. 20 fly lines out of the 82 positives were randomly selected and crossed to flies expressing pBac transposase. 6 flies showed loss of red fluorescence in the eye, and all were sequenced. Only 2 of the 6 lines harboured both the desired lysine to arginine mutations.

G. Snapshot of the chromatogram of sequences showing K29R and K190R mutations in the genomic locus of *Jra*. L1 and L2 represent two independent lines of *Jra^{SCR}*, and a third line that did not harbour either of the mutations, was used as wildtype control.

SUMO conjugation of Jra attenuates the suppression of immune response by Jra

Earlier, we have shown that *Jra* is a major regulator of the gut i-GRN. Mechanistically, *Jra* directly or indirectly regulates key immune effector genes in the gut of *Drosophila* during infection. A comparison of the gut immune transcriptome between *Jra^{WT}* and *Jra^{SCR}* animals would lead to an understanding of the role of SUMOylation in regulating *Jra* function (Next Section). Before that, we looked for phenotypical differences in *Jra^{WT}* and *Jra^{SCR}* during an infection to attribute an allelic definition to *Jra^{SCR}*. *Jra^{SCR}* flies fed with *Pe* responded poorly to infection and succumbed early (Fig. 5A). This was true for both lines (L1, L2) that we generated. Interestingly, in terms of survival against infection, *Jra^{SCR}* flies were more sensitive, unlike the *Jra* hypomorph (compare Fig. 2A and Fig. 5A). To define the nature of the SCR allele, we crossed the *Jra^{WT}*, and *Jra^{SCR}* flies to *Jra^{IA109}* and performed lifespan experiments on the heterozygotes. Post feeding with *Pe*, *Jra^{IA109}/Jra^{WT}* flies lived longer than homozygous *Jra^{WT}* flies upon infection, consistent with the idea that loss of *Jra* was protective, enhancing the ability of the fly to fight the infection. *Jra^{IA109}/Jra^{SCR}* animals also survived longer when compared to homozygous *Jra^{SCR}* flies, but not when compared to *Jra^{IA109}/Jra^{WT}*.

To further characterize the allele, we generated overexpression (OE) lines for both *Jra^{WT}* and *Jra^{SCR}*. Wild-type and SCR sequences were cloned into the pUASp-attB vector and UAS-*Jra^{WT}* and UAS-*Jra^{SCR}* lines balanced on the third chromosome. Flies with one copy of *Jra^{WT}* and *Jra^{SCR}* survived better than homozygous *Jra^{SCR}* flies suggesting that a single copy of WT *Jra* is sufficient to rescue the lethality and restore homeostasis in the gut (Fig. 5C). Also,

OE of *Jra*^{WT} in the gut was sufficient to partially rescue the survival seen in *Jra*^{SCR} homozygous flies, while overexpression of *Jra*^{SCR} in the gut of homozygous *Jra*^{SCR} flies did not show any change in lifespan post-infection (Fig. 5C). Since the rescue with OE of *Jra*^{WT} in the enterocytes was only partial, we used *Jra* specific *Gal4* (*Jra-Gal4*) and looked at the rescue of *Jra*^{SCR} with OE of *Jra*^{WT}. Similar to what was previously observed, flies with a single copy of *Jra*^{WT} and *Jra*^{SCR} survived better than homozygous *Jra*^{SCR} flies. Interestingly, OE of *Jra*^{WT} with *Jra-Gal4* completely rescued the survival of homozygous *Jra*^{SCR}. OE of *Jra*^{SCR}, however, did not alter the survival of the homozygous *Jra*^{SCR} flies. This suggests that the expression of *Jra* needs to be tightly regulated to evoke a robust immune response and maintain homeostasis. Also, the role of other organs/tissues in contributing to the expression of *Jra* cannot be ruled out. Our data rule out *Jra*^{SCR} as a loss of function allele or a dominant-negative allele. Based on the behaviour of the allele and the equivalent data from hypomorphic alleles (Fig. 2A, B), *Jra*^{SCR} appears to be a hypermorphic allele with increased activity of *Jra*. This would also suggest that SUMO conjugation of *Jra* could reduce *Jra* activity, an observation previously reported in the mammalian ortholog, c-Jun (MULLER *et al.* 2000; BOSSIS *et al.* 2005).

Next, we tested if the sensitivity of *Jra*^{SCR} flies correlates with the ability of the pathogen to survive in the gut. For this, we performed the bacterial clearance assay on *Jra*^{WT} and *Jra*^{SCR} flies. We observed that *Jra*^{SCR} flies were weaker in their ability to clear *Pe* as there were more CFUs as compared to *Jra*^{WT} at 24 hpi (Fig. 5E). This suggested that the defence factors produced by the gut were weaker in *Jra*^{SCR} animals. We also tested for the ability of ISCs to proliferate post-infection in *Jra*^{WT} and *Jra*^{SCR} flies. In both cases, pH3⁺ cells increased, indicating a stem cell response, but the response appears to be equal in both genotypes (Fig. S7B). SUMOylation of *Jra* does not appear to have a role in regulating the division of ISCs during infection.

The function of *Jra*^{SCR} is thus distinct from *Jra*^{WT}. Compared to *Jra* hypomorphs, the effect is contrary, both in terms of lifespan and bacterial clearance. This further agrees with our assertion that *Jra*^{SCR} is a hypermorphic allele. Loss of SUMOylation of *Jra* increases the activity of *Jra* and thus reduces the ability of the fly to fight infection.

Figure 5

Figure 5: Survival curves post-infection suggest that *Jra*^{SCR} is a hypermorphic allele

A. Survival curves of unchallenged (UC, dashed lines) and orally infected (I, closed lines), CRISPR/Cas9 modified *Jra*^{SCR} flies, compared to *Jra*^{WT}. L1 (red) and L2 (blue) indicate two independent *Jra*^{SCR} lines used in the experiment. Log-rank test for trend was used to individually compare *Jra*^{SCR} L1 (I) and *Jra*^{SCR} L2 (I) to *Jra*^{WT} (I). ****p<0.0001.

B. Survival curves of UC (dotted) and I (closed), flies: *Jra*^{WT} (black), *Jra*^{SCR} (purple), *Jra*^{IA109}/*Jra*^{WT} (grey) and *Jra*^{IA109}/*Jra*^{SCR} (lavender). Log rank test for trend was used to compare *Jra*^{IA109}/*Jra*^{WT} (I) to *Jra*^{WT} (I), *Jra*^{IA109}/*Jra*^{SCR} (I) to *Jra*^{SCR} (I) and *Jra*^{IA109}/*Jra*^{SCR} (I) to *Jra*^{IA109}/*Jra*^{WT} (I). ****p<0.0001.

C. Survival curves of UC (dotted) and I (closed) flies: *Jra*^{SCR}, NP1-Gal4^{ts}/*Jra*^{SCR} (black), *Jra*^{SCR}, NP1-Gal4^{ts}/*Jra*^{WT} (grey), *Jra*^{SCR}, NP1-Gal4^{ts}/*Jra*^{SCR}; UAS-*Jra*^{WT}/+ (blue) and *Jra*^{SCR}, NP1-Gal4^{ts}/*Jra*^{SCR}; UAS-*Jra*^{SCR}/+ (purple). Log rank test for trend was used to compare *Jra*^{SCR}, NP1-Gal4^{ts}/*Jra*^{WT} (I), *Jra*^{SCR}, NP1-Gal4^{ts}/*Jra*^{SCR}; UAS-*Jra*^{WT}/+ (I) and *Jra*^{SCR}, NP1-Gal4^{ts}/*Jra*^{SCR}; UAS-*Jra*^{SCR}/+ to *Jra*^{SCR}, NP1-Gal4^{ts}/*Jra*^{SCR} (I). ****p<0.0001.

D. Survival curves of UC (dotted) and I (closed) flies: *Jra*^{SCR}, *Jra*-Gal4/+ (black), *Jra*^{SCR}/*Jra*^{WT}, *Jra*-Gal4/+ (grey), *Jra*^{SCR}, *Jra*-Gal4/UAS-*Jra*^{WT} (blue) and *Jra*^{SCR}, *Jra*-Gal4/UAS-*Jra*^{SCR} (purple). Log rank test for trend was used to compare *Jra*^{SCR}/*Jra*^{WT}, *Jra*-Gal4/+ (I), *Jra*^{SCR}, *Jra*-Gal4/UAS-*Jra*^{WT} (I) and *Jra*^{SCR}, *Jra*-Gal4/UAS-*Jra*^{SCR} to *Jra*^{SCR}, *Jra*-Gal4/+ (I). ****p<0.0001.

E. Scatter dot plot representing bacterial load as colony-forming units (CFUs) post oral feeding with *Pe* Student's t-test was performed comparing *Jra*^{WT} and *Jra*^{SCR} at respective time point post-infection. **p=0.0015.

Data related to the number of animals per experiment and replicates listed in Fig. S7A. Data not significant is not represented.

Jra^{SCR} suppresses the activation of defence genes

To gain further mechanistic insight into the role of *Jra^{SCR}*, we have performed a comparative 3'mRNA sequencing experiment with the guts isolated from *Jra^{SCR}* and *Jra^{WT}* at 4 and 12 hours post *Pe* infection. PCA indicates that both the genotypes form distinct clusters (Fig. S8A), with the progression of infection. *Jra* levels in *Jra^{WT}* and *Jra^{SCR}* flies were comparable during infection, indicating similar levels of activation (Fig. 6A). Comparing *Jra^{WT}* and *Jra^{SCR}* in UC animals, we find that the transcriptional differences were restricted to a very small number (87; FDR<0.1; Table S2) of genes (Fig. 6B). Genes that are known to regulate immune response were found to be both upregulated, (e.g., *Drosomycin-like 2 (Drsl2)*, *Attacin-D (AttD)* and *Charon*) and downregulated, (e.g. *Peptidoglycan recognition protein -SC1a (PGRP-SC1a)*, *PGRP-SC1b* and *Pirk*), in the *Jra^{SCR}* flies (Fig. 6C). Post *Pe* infection, we identified a total of 3134 (FDR<0.1; Table S2) genes to be differentially expressed at 4 hpi and 12 hpi, as normalised to UC in both the genotypes taken together. These genes are plotted in terms of number upregulated downregulated at each time point of the respective genotype (Fig. 6D). We also observed that the changes in genes that were differentially expressed were maximum at 12 hpi (Fig. 6D, Fig. S8B), an observation we previously made (Fig 3) suggesting that major changes in gene regulation occur at 12 hpi in our experimental setup. Gene ontology analysis reveals several key GO terms like metabolic process, gene expression, response to stress, MAPK signalling pathway etc., to be significantly enriched (Fig. S8C) in the genes that are differentially expressed. In order to relate this data to earlier data on *Jra* loss of function, we evaluated the same set of genes as in Fig. 3E. The log₂FC values of a majority of AMPs (*AttB*, *AttD*, *Cecropin A2 (CecA2)*, *Diptericin A (DptA)*, *Drsl3*, and *Drsl4*) were lower in *Jra^{SCR}* as compared to that of *Jra^{WT}*. The lower levels of AMPs could be one of the reasons for *Jra^{SCR}* flies to succumb early upon infection. Interestingly, the log₂FC values of the transcription factor *Rel* were considerably lower in *Jra^{SCR}* than *Jra^{WT}* in both the infection time points. Also, the log₂FC values of the negative regulator (of the Imd pathway), *Pirk*, appeared to be higher in *Jra^{SCR}* flies than *Jra^{WT}* flies indicating that in *Jra^{SCR}* flies, Imd signalling is dampened. Since *Rel* is a master immune regulator, we validated this result by performing a quantitative real-time PCR (qRT-PCR) on the guts of *Jra^{WT}* and *Jra^{SCR}* post-infection.

Figure 6: The modulation of the immune response by *Jra*^{SCR} is distinct from that of *Jra*^{WT}.

A. Kinetics of activation of *Jra* transcripts, as measured by qRT-PCR in *Jra*^{WT} and *Jra*^{SCR} without and with gut infection. Data from three independent experiments. Means and SEMs represented.

B. Bar plot representation of a total number of significantly differentially expressed genes (FDR<0.1) obtained by comparing *Jra*^{WT} and *Jra*^{SCR} under UC conditions post a 3' mRNA sequencing experiment.

C. Volcano plot showing the up or down-regulated genes, on comparing *Jra*^{WT} and *Jra*^{SCR}. A few genes previously known to function in regulating immunity are highlighted.

D. Bar plot representation of the total number of significantly differentially expressed genes (FDR<0.1) obtained at 4 and 12 hpi after comparing to UC animals for the respective genotype.

E. Tabular representation of log₂FC values of transcripts for a few categories of defence genes regulated during the infection, with a color code at the bottom of the table. Log₂FC values in the range 0.58 to -0.58, indicating no significant changes compared to UC, are marked in white.

F. Heatmap representing the normalised expression counts of genes for transcription factors, enriched in the RNA-seq data set, with Jra binding peaks on their promoters.

G. Cartoon representation of the effect of SUMOylation of Jra on *Rel* transcription during infection. In the left panel, Jra^{WT} can be conjugated to SUMO, and this feature helps in the normal activation of *Rel* during the immune response. Whereas, in the case of Jra^{SCR} (right panel), which cannot conjugate to SUMO, *Rel* expression is significantly weaker.

We found that the activation of *Rel* was suppressed in the guts of *Jra*^{SCR} (Fig. S9A), the same was true with the activation of *DptA* (Fig. S9B) and *AttD* (Fig. S9C), which are transcriptionally activated by Rel. In contrast, the log₂FC values of components of the JNK pathways were comparable in *Jra*^{WT} and *Jra*^{SCR}. A number of transcriptional regulators that showed higher log₂FC values in *>Jra*^{RNAi} (Fig. 3E) in comparison to *w*¹¹¹⁸ showed lower log₂FC values in *Jra*^{SCR} when compared to *Jra*^{WT}. Also, the levels of *Upd3* and *Dome* were lower in the *Jra*^{SCR} flies. Taken together, this suggests that at a molecular level, the genes necessary to negate *Pe* infection are either suppressed or insufficiently activated in *Jra*^{SCR}, leading to the better survival of the pathogen.

As before (Fig. 3), we focused on the genes that could be directly regulated by Jra. For this, we mapped the occupancy of Jra on the 3134 genes differentially expressed, as before. We identified 702 genes with enriched Jra binding peaks on the promoters (Fig. S10A). Plotting the normalised expression of the 702 genes indicated changes between both the genotypes as the infection progressed (Fig. S10B; Table S2) and as previously observed in *>w*¹¹¹⁸ and *>Jra*^{RNAi} dataset, the ‘gene-specific transcription’ GO term was one of the most represented categories (Fig. S10C). We evaluated gene function and identified 42 transcription factors in the list. We plotted the normalised expression counts between the two genotypes during infection to look for changes in the expression pattern of these transcription factors (Fig. 6F). Surprisingly, we observed that the mRNA levels in *Jra*^{SCR} animals for this subset of genes were lower as compared to *Jra*^{WT} as the infection progressed. This decrease was considerable in *kay*, *Rel*, *dl*, *aop*, *Kr-h1* *cbt* and *Ets21C* at 12 hpi (Fig. 6F) and modest for *h*, *pebbled* (*peb*), *chinmo*, *Jra*, *da*, *fkh* and *Atf3*.

The early lethality seen in *Jra*^{SCR} flies thus strongly correlates with the findings from the transcriptomics data. We observed that in *Jra*^{SCR}, several AMPs and key genes that

regulate the immune response were insufficiently activated, allowing the pathogen to thrive at the expense of the host. The Jra i-GRN previously described appears to be weakly activated in the *Jra^{SCR}* flies shifting the balance in favor of the pathogen. The mRNA levels of *Rel*, in particular, are significantly lowered in *Jra^{SCR}* (Fig S9), suggesting that SUMOylation of Jra is a necessary event for robust activation of *Rel* in the gut (Fig. 6G).

DISCUSSION

The Jun-kinase (JNK) pathway is a well-characterized signalling module used in a wide variety of cellular processes, including, but not limited to proliferation, immunity, apoptosis, and embryonic development. The module is complex with multiple receptors, signalling intermediates, and transcriptional effectors (reviewed by (EFERL AND WAGNER 2003; XIA AND KARIN 2004; DHANASEKARAN AND REDDY 2008; WAGNER AND NEBRED A 2009; ARTHUR AND LEY 2013)). A central theme of the pathway is a cascade of kinases that lead to the activation of a number of the effector transcription factor(s), including c-Jun. In *Drosophila*, the components of the JNK pathway have been well studied (IP AND DAVIS 1998; NOSELLI AND AGNÈS 1999; PEREZ-GARIJO *et al.* 2005; BITEAU *et al.* 2011; RAZZELL *et al.* 2011; RIOS-BARRERA AND RIESGO-ESCOVAR 2013; GALENZA AND FOLEY 2019; BAKER 2020) with Jra also implicated as a transcriptional effector of the systemic immune response in *Drosophila* (KIM *et al.* 2005; KIM *et al.* 2007)(Fig. S11). Current models for the role of Jra in immune signalling for gram-negative infections place it downstream of the PGRP-LC receptor. The pathway bifurcates downstream of Immune Deficient (Imd), with the MAP kinase kinase kinase Tak1 signalling to both Jra and Rel (Fig. S11). The pathway, which is well characterized in flies, involves the IKK complex Kenny/Ird5 and leads to phosphorylation and subsequent cleavage of Rel. The N-terminal 68 kD fragment of Rel translocates to the nucleus where it activates the host-defense response. In the parallel path, Tak1 signals to the Jun kinase-kinase Hep, which signals to the JNK Bsk, which in turn activates Jra by phosphorylation (Fig. S11). Jra can function to negatively regulate genes of the host immune response, with a specific example of the AMP *AttA*, where Jra competes with Rel, and shuts off *AttA* transcription (KIM *et al.* 2005; KIM *et al.* 2007). Both Jra and Rel are thus effectors of immune signalling (BOUTROS *et al.* 2002; SILVERMAN *et al.* 2003; KALLIO *et al.* 2005; DELANEY *et al.* 2006; BOND AND FOLEY 2009; MYLLYMAKI *et al.* 2014) and there may be considerable overlap between the gene-regulatory networks they activate.

Our interest in the functional consequences of SUMO conjugation in the immune response led us to Jra, which is a SUMO target. Also, the role of Jra has not been studied in the context of the gut immune response, making it an exciting candidate to explore. As a first step, we used loss of function Jra alleles, gut-specific Jra knockdowns, and infection with *Pe* to elucidate the role of Jra in regulating the gut immune response. Knockdown of either Jra or Bsk leads to an increase in lifespan post-infection, suggesting that JNK signalling is active in

the gut and that pathogen infectivity is facilitated by increased JNK/Jra signalling. Additionally, reduction of global SUMO conjugation in gut ECs was also protective, indicating that the availability of the SUMOylation machinery during infection was helpful to the pathogen. Using gut-specific transcriptomics, we report that the kinetics of activation of AMPs is faster in the guts of the flies with reduced function of Jra, leading to the rapid clearance of bacteria. We have also uncovered a Jra dependent immune gene regulatory network (Jra i-GRN), and this complex network involves several key transcription factors known to maintain gut homeostasis. Examples include *Rel* (BUCHON *et al.* 2009; BUCHON *et al.* 2014), *Fkh* (VARMA *et al.* 2014; BOLUKBASI *et al.* 2017), *Chinmo* (GRMAI *et al.* 2018), *Ets21C* (TOGGWEILER *et al.* 2016; MUNDORF *et al.* 2019), *Atf3* (RYNES *et al.* 2012; ZHOU *et al.* 2017), and *Aop* (ALIC *et al.* 2014; SLACK *et al.* 2015). Jra is required to activate or suppress the transcription of these factors to evoke a robust immune response in the gut against an invading pathogen (Fig. 3G).

The Jra SUMO conjugation resistant flies, generated by the CRISPR/Cas9 mediated genome editing, are sensitive to infection and succumb early. One contributory factor to this sensitivity is the persistence of bacteria in the *Jra^{SCR}* flies, suggesting a weaker host-defense response to the pathogen. In contrast, the loss of Jra function, validated using different hypomorphic alleles, led to a longer lifespan and comparatively fewer bacteria under infective conditions. This would indicate that the Jra-dependent GRN required to evoke a robust immune response in the gut is altered when Jra cannot be SUMOylated. An analysis of the differential gut transcriptional response to the pathogen in *Jra^{SCR}* and *Jra^{WT}* animals suggests that the differences lie in the ability of the wild type allele to regulate a subset of defense genes. We propose that the *Jra^{SCR}* allele is a hypermorph of Jra with increased Jra activity. Interestingly, this increase in activity leads to a lowered and delayed transcriptional response, which gives the pathogen a better foothold and improves its chances for growth and subsequent colonization in the host.

One interesting finding is that the NFkB factor *Rel* is insufficiently activated in *Jra^{SCR}* flies. In addition to this, we have identified other transcription factors of the Jra i-GRN to be either suppressed or insufficiently activated in *Jra^{SCR}* flies (Fig. 7A). It is important to note that the perturbation of secreted factors such as *upd2* and *upd3* could also influence inter-organ signalling (RAJAN AND PERRIMON 2012; CHAKRABARTI *et al.* 2016), indicating long-range, systemic effects for Jra SUMOylation. We hypothesize that the repression of key transcription factors

would influence the transcription of several other genes and compromise the fly's ability to fight infection (Fig. 7A). We propose that SUMOylation of Jra is a crucial step to fine-tune the immune response and maintain homeostasis in the gut of *Drosophila*.

Figure 7: Regulation of the gut immune response by SUMOylation of Jra

A. The invading pathogen is recognised by the cell surface receptor and the Imd/JNK pathways get activated. Under wildtype conditions where Jra can be SUMOylated (left panel), Jra is required to activate the transcription of *Rel*, *Ets21C* and repress the transcription of *fkh*, *aop* and *Atf3*. This is required to evoke a robust immune response and maintain gut immune homeostasis. In a scenario where Jra is resistant to SUMO conjugation, it suppresses the transcription of *Rel* and *Ets21C* along with *fkh*, *aop* and *Atf3*. This leads to insufficient activation of the Jra dependent immune GRN and finally leads to a dampened immune response.

B. Dynamics of SUMO conjugation/deconjugation of Jra on the chromatin indicates the fine-tuning of Jra activity by SUMOylation.

C. SUMOylation of Jra can alter the interactome of Jra and yield context specific regulation of Jra activity.

Images generated using BioRender.

How does SUMOylation reduce the activity of Jra in the context of transcriptional regulation? Traditionally, Bsk phosphorylates Jra on the N-terminus, leading to the activation of Jra (Sluss *et al.* 1996; Kockel *et al.* 2001), while the mode of deactivation of Jra has been elusive to date. We propose SUMOylation of Jra as a mechanism to attenuate the function of

Jra in the context of the gut immune response. We hypothesise that SUMO conjugation/deconjugation is a dynamic event that happens near the promoters of Jra target genes, and the two states are in equilibrium (Fig. 7B). Depending on the context, there is a shift in this equilibrium, which dictates the outcome of Jra activity. During infection, the transcript levels of Jra rise, leading to an increase in protein levels of Jra. More Jra protein would lead to more active Jra, which could be detrimental to the organism and beneficial to the pathogen. Hence, Jra is SUMOylated to reduce its activity and evoke a potent immune response.

One possibility is that SUMOylation of Jra causes Jra to exit the promoter leading to a decrease in overall transcription activity. This argument can be further strengthened by data from the mammalian ortholog, c-Jun, where a SUMO conjugation resistant variant shows higher transcriptional activity (MULLER *et al.* 2000; BOSSIS *et al.* 2005). Consistent with this, the c-Jun binding partner, c-Fos, also shows increased transcriptional activity upon abrogating SUMO conjugation (BOSSIS *et al.* 2005). The SUMOylated form of c-Fos is less abundant on the promoters of select target genes, where SUMOylation knocks c-Fos off the chromatin to disrupt its activity (TEMPE *et al.* 2014). As Jun and Fos are binding partners and perform similar functions, we strongly believe that the same could be happening with SUMOylation of Jra (Fig. 7B). Also, there is evidence that SUMO conjugation negatively regulates the function of a set of transcription factors (BHASKAR *et al.* 2002; MILES *et al.* 2008; OUYANG AND GILL 2009; BOULANGER *et al.* 2021). Another possible mechanism could be a change in interactors, particularly the transcriptional apparatus, that could alter the outcome of Jra activity upon SUMOylation (Fig. 7C). However, we do not rule out the possibility of both these mechanisms acting parallelly in a highly context-specific manner on individual genes.

The levels of SUMOylation of any protein at a given time is very low. To test out either of the proposed hypotheses in the current context, one needs to capture the SUMOylated species in abundant amounts in the gut epithelium. With current tools, it becomes challenging to perform ChIP experiments or mass-spectrometry based assays to enrich the SUMOylated species and understand the context-specific role of SUMO conjugation of Jra.

In summary, our study establishes Jra's role as a major regulator of the gut immune transcriptome. The Jra and Rel GRN's have substantial overlap with Jra activity regulating defense genes activated by Rel. SUMO conjugation of Jra modulates the immune response, and our study suggests that dampening of the transcriptional regulatory networks is a major feature.

MATERIALS AND METHODS

Fly husbandry. Flies were reared on standard cornmeal agar at 25°C in a 12-hour light-dark cycle. Only 6-8 day old females were used in all the experiments in this study. All *NP1-Gal4^{ts}* crosses were maintained at 21°C to inactivate the Gal4. 3 days post eclosion, flies were shifted to 29°C for 3 days (to activate the Gal4) until bacterial feeding. The flies were shifted to 29°C from the beginning of the infection till the end of the experiment. Table 1 lists the lines used in our studies.

Cloning of gRNA and *Jra^{SCR}* for CRISPR/Cas9 mediated genome editing: The gRNA was designed using <http://targetfinder.flycrispr.neuro.brown.edu/>. The gRNA sequence with no off-targets and optimal for our experimental design was chosen and cloned into the pBFv-U6.2B vector. The 2.5 kb genomic locus of *jra* was amplified from a single *w¹¹¹⁸* fly. This was used to generate 3'HR and 5'HR fragments of *jra*. The SCR mutations were incorporated in the 3'HR region. The pHD-scarless-DsRed vector was amplified as two separate fragments using specific primers. The two fragments of *jra* along with the two fragments of the vector were ligated using Gibson assembly (NEB) and sequenced. The 3'HR region cloned into the vector was resistant to the gRNA due to the modification of a few degenerate nucleotides and the PAM sequence. For screening of *Jra^{SCR}* after the excision of DsRed cassette, the genomic DNA was extracted by homogenising single flies in 50ul of a buffer containing 10mM TRIS, 1mM EDTA and 20uM Proteinase K. The homogenate was incubated at 37°C for 30 min followed by 85°C for 5 min. 2.5kb of the genomic region of *Jra* was amplified and sequenced for each line. The *Jra^{SCR}* and *Jra^{WT}* fly lines were regularly sequenced to confirm the desired genotype. All the primers used are listed in table S3.

Table 1: Genotypes and source of *Drosophila* lines used for our experiments.

Line/Genotype	Source	Description
<i>Jra^{WT}</i>	This study	<i>Jra</i> locus modified using CRISPR/Cas9. No mutations incorporated into the genome
<i>Jra^{SCR} L1</i>	This study	<i>Jra</i> locus modified using CRISPR/Cas9. K29R+K190R mutations are incorporated into the genome. L1 = line 1. Extensively used in this study
<i>Jra^{SCR} L2</i>	This study	<i>Jra</i> locus modified using CRISPR/Cas9. K29R+K190R mutations are incorporated into the genome. L2 = line 2.
<i>Jra^{SCR}; Jra-Gal4</i>	This study	<i>Jra^{SCR}</i> balanced with <i>Jra-Gal4</i>
<i>Jra^{SCR}, NP1-Gal4^{ts}/CyO</i>	This study	<i>Jra^{SCR}</i> recombined with <i>NP1-Gal4^{ts}</i>
<i>UAS-Jra^{WT}</i>	This study	pUASp-attB- <i>Jra^{WT}</i> inserted into attp40 site

<i>UAS-Jra^{SCR}</i>	This study	pUASp-attB- <i>Jra^{SCR}</i> inserted into attp40 site
<i>Jra^{sg2}</i>	This study	gRNA targetting exon 3 of <i>jra</i> genomic locus
<i>Jra^{sg2}</i>	This study	gRNA targetting exon 3 of <i>jra</i> genomic locus
<i>Myo81F-Gal4, UAS-GFP, tub-gal80^{ts} (NP1-Gal4^{ts})</i>	Sveta Chakrabarti	Enterocyte specific Gal4 line (<i>NP1-Gal4^{ts}</i>)
<i>y v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF01184}attP2</i>	BDSC:31595	<i>UAS-Jra^{RNAi}</i>
<i>y[1] w[1118]; P{w[+mC]=Jbz}10</i>	BDSC:7218	<i>UAS-Jra^{DN}</i>
<i>w[1118] P{w[+mC]=UAS-bsk.DN}2</i>	BDSC:6409	<i>UAS-Bsk^{DN}</i>
<i>w[1118]; P{w[+mC]=UAS-Fra.Fbz}5</i>	BDSC:7214	<i>UAS-Kay^{DN}</i>
<i>cn[1] Jra[IA109] bw[1] speck[1]/CyO</i>	BDSC:3273	Jra null
<i>Adh[fn23] pr[1] cn[1] Jra[76-19]/CyO</i>	BDSC:9880	Jra null
<i>y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HM05055}attP2 /TM3, Sb[1]</i>	BDSC:28569	<i>UAS-Uba2^{RNAi}</i>
<i>y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HM05183}attP2 /TM3, Sb[1]</i>	BDSC:28972	<i>UAS-Aos1^{RNAi}</i>
<i>y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMS01540}attP2</i>	BDSC:36125	<i>UAS-SUMO^{RNAi}</i>
<i>UAS-lwr^{DN}</i>	Shubha Govind (HUANG <i>et al.</i> 2005)	<i>UAS-Ubc9^{DN}</i>
<i>w1118; P{GMR61B05-GAL4}attP2</i>	BDSC:46459	<i>Jra-Gal4</i>
<i>y[1] M{Act5C-Cas9.P.RFP-}ZH-2A w[1118] DNAlig4[169]</i>	BDSC:58492	<i>Actin5c</i> Cas9
<i>w[1118]</i>	BDSC:3605	<i>w¹¹¹⁸</i>

Cloning and generation of constructs for overexpression: Jra (FBpp0087498) was amplified from the *Drosophila* gold collection library (https://www.fruitfly.org/EST/gold_collection.shtml) using specific primers (Table S3). These amplicons were independently cloned into the pGEX-4T1 vector for bacterial SUMOylation assay and pRM-HA3 vector for transfection into S2 cells using a modified Seamless Ligation Cloning Extract (SLiCE) protocol (ZHANG *et al.* 2012). The site-directed mutagenesis approach with specific primers was used to modify all the lysine residues to arginine residues to abrogate SUMO conjugation. pUASp-AttB vector was procured from the *Drosophila* Genomics Resource Centre (DGRC, #1358) and was used for targeted insertions of WT/SCR constructs into the attP2 site on the 3rd chromosome. All the clones were confirmed by sequencing and used for downstream experiments.

Bacterial SUMOylation assay: This is a modified in-vitro SUMOylation assay that was previously described (Nie *et al.* 2009). The quartet vector comprising of the *Drosophila* SUMO machinery components was co-transformed with GST tagged Jra. The bacterial culture was induced with 1mM of Isopropyl β -D-1-thiogalactopyranoside (IPTG) for 6 hours at 25°C. 10ml of the bacterial culture was harvested in 1ml 50mM *tris* aminomethane (TRIS) buffer containing 150mM NaCl, 1mM Dithiothreitol (DTT), 1ug/ml lysozyme, and 1mM phenylmethylsulfonyl fluoride (PMSF). The cells were lysed using a VibraCell™ probe sonicator with 2/3sec ON/OFF cycle for 2 min at 60% amplitude.

S2 cell culture and transfections: S2 cells that were stably transfected with Flag-SUMO (referred to as 529SU cells) were a kind gift from Prof. Albert Courey. The cells were grown and maintained in Gibco® Schneider's *Drosophila* Medium (Thermo Fischer Scientific, #21720024) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Thermo Fischer Scientific, #10082147) at 23°C. 1ug of plasmid was transfected per 1ml of cells using TransIT®-Insect Transfection Reagent (Mirus, #6100) as per the manufacturer's protocol. The cells were induced with 0.5M CuSO₄ and harvested after 48h of induction.

Pulldown, Immunoprecipitation and Western blotting: Glutathione affinity PD for the bacterial SUMOylation assay was performed using Pierce™ Glutathione Agarose beads (Thermo Fischer Scientific, #16101). Post lysis, the bacterial lysate was incubated with the equilibrated beads for 12-14h at 4°C. The beads were then washed 3 times with the lysis buffer with 0.1% Triton-X100, and bound protein was eluted using 1X laemmli buffer. Ni-NTA superflow resin (Qiagen, #30430) was used to pull down His-Jra from 529SU cells as per the manufacturer's protocol. To maintain stringent denaturing conditions, 8M Urea was used throughout the experiment in all the buffers. 20mM N-ethylmaleimide was also used to inactivate the SENPs. Pierce™ BCA Protein Assay Kit (Thermo Fischer Scientific, #23225) was used to estimate the protein concentration before performing the Western blotting. 1-2mg of total protein for PD and IP experiments and 50ug of total protein for inputs. The following antibodies were used as primary antibodies for the WB. Anti-His (SCBT, #sc-8036; used in 1:2000), anti-GST (SCBT, #459; used in 1:5000) and anti-Flag (Sigma-Aldrich, #F7425; used in 1:2000). Peroxidase conjugated anti-mouse (Jackson Immunoresearch, #115-035-003; used in 1:10000) and peroxidase-conjugated anti-rabbit (Jackson Immunoresearch, #111-035-003; used in 1:10000) were used as secondary antibodies. All the blots were developed using Immobilon Western Chemiluminescent HRP Substrate (Sigma-Aldrich, #WBKLS). Anti-GST WB

and anti-6XHis WB in Fig1 were run on duplicate gels with the same samples. Anti-Flag WB and anti-6XHis WB in Fig 1F were run on duplicate gels with the same samples.

Bacterial culture, gut infection, fly survival assays and bacterial clearance assays:

Pseudomonas entomophila (Pe) was used in this study to evoke a robust immune response in the fly gut. The pathogen was always selected for rifampicin (100ug/ml) resistance and hydrolysis of casein on a milk agar plate before the infection experiments. The bacterial pellet obtained from an overnight grown culture was resuspended in a 5% sucrose solution, so the final OD₆₀₀ was ~200. For all the infection experiments, flies were first starved for 2 hours without food and water. Post starvation, the flies were transferred to a fly vial (at 20-30 flies/vial) containing Whatman filter disk that was dipped in the concentrated bacterial solution and were allowed to feed for a certain amount of time specific to the experiment. The flies were transferred to a vial containing standard food post-feeding, and the dead flies were counted every 24 hours. Bacterial feeding was carried out for 24 hours for all the survival experiments except when Jra^{SCR} flies were used, where feeding time was 6 hours. For assessing the bacterial load, flies were fed on Pe for 2 hours and transferred to vials containing standard food. Before plating, the flies were rinsed in 70% ethanol and left to dry. Whole flies were crushed in sterile PBS, and the lysate was plated on a bacterial agar plate containing rifampicin. The bacterial plates were imaged, and colonies were counted in Fiji (ImageJ) post an intensity cutoff for each plate.

Gut dissections, immunostaining and microscopy: The guts were precleared by growing the flies on 5% sugar agar overnight for all the immunofluorescence experiments. The next day, flies were fed with Pe for 8 hours after 2 hours of starvation. Post feeding, the guts were dissected in ice-cold 1X PBS and fixed in a solution containing 1X PBS, 0.1% TritonX100 and 4% paraformaldehyde for 20 minutes. The guts were then incubated at room temperature for an hour in a blocking solution that contained 1X PBS, 0.5% bovine serum albumin, 0.3% TritonX100 and 3% normal goat serum. This was followed by incubation with 1:200 anti-phosphoHistone3 (pH3) antibody (Cell Signalling Technology, #9701L) diluted in blocking solution at 4°C overnight. Anti-rabbit alexa568 (Thermo Fisher Scientific, #A-11011) was used as a secondary antibody in 1:1000 dilution. DAPI was used to mark the nuclei, and the samples were mounted in Slowfade gold antifade mountant (Thermo Fisher Scientific, #36940). The mounted samples were imaged using a 20X oil immersion objective on a Leica SP8 confocal

microscope. pH3 positive cells per gut were counted using ImageJ after specifying a threshold and size cutoff (4-20 μm^2).

qRT-PCR: 1 μg of extracted RNA was used to generate cDNA using a high-capacity cDNA reverse transcriptase kit (Thermo Fisher Scientific, #4374966) using the manufacturer's protocol. The cDNA was diluted in a 1:5 ratio for all the experiments. qRT-PCR was performed using gene-specific primers (Table S3) with Kapa SyBr Fast (Sigma-Aldrich, #KK4618) in an Applied Biosystem ViiA 7 real-time PCR system.

RNA isolation, 3'mRNA library preparation and sequencing: Total RNA was extracted from 50-60 midguts/experiment using RNeasy Plus Universal Kits (Qiagen, #73404) according to the manufacturer's protocol. 3' mRNA specific libraries from three independent biological replicates were amplified using QuantSeq 3' mRNA-Seq Library Prep Kit FWD (Lexogen, #015.96) using the manufacturer's instructions. The concentrations of the libraries were estimated using Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific, #Q32851). Quality assessment and library size estimation of the individual libraries was done using an HS DNA kit (Agilent, #5067-4626) in a Bioanalyzer 2100. The libraries were pooled in equimolar ratio, and single-end 75bp reads were sequenced on the Illumina HiSeq 2500 platform.

Read mapping, counts generation and differential expression analysis: On average, 4-5 million reads were uniquely mapped per sample. Sequencing quality was assessed using FastQC v0.11.5. Post QC, the reads were mapped to the *Drosophila* genome (dm6) using STAR aligner v.2.5.2a (DOBIN *et al.* 2013). Gene expression levels were measured using the counts generated by HTSeq-count v 0.6.0 (ANDERS *et al.* 2015). To obtain differential expression of the genes, the biological conditions were compared pairwise using DESeq2 (LOVE *et al.* 2014). The above steps were carried out on the bluebee platform (<https://lexogen.bluebee.com/quantseq>).

ChIP-seq data analysis: Jra specific ChIP-seq dataset (ENCSR471GSA) against an input dataset (ENCSR908EFA) was obtained from the model organism Encyclopedia of Regulatory Networks (modERN) consortium (<https://epic.gs.washington.edu/modERN/>) (KUDRON *et al.* 2018). Input normalised Bigwig files were used, and the occupancy of Jra was plotted on the gene body (+/- 2kb of the mRNA) of all the significantly differentially expressed genes from the 2 RNA sequencing datasets using deeptools 3.5.0 (RAMIREZ *et al.* 2016).

Bioinformatic analysis: The FDR cutoff < 0.1 was considered for the entire analysis. Normalised counts were generated using the CPM function of edgeR (ROBINSON *et al.* 2010).

The PCA was performed using a custom R script. GO enrichment analysis of the significantly differentially expressed gene set was performed using gProfiler (<https://biit.cs.ut.ee/gprofiler/gost>) (REIMAND *et al.* 2011) and panther database (<http://pantherdb.org/>). GO:0003700 'DNA-binding transcription factor activity' term was used to fetch the transcription factors used in the analysis.

Statistics: All experiments were performed in three biological replicates unless otherwise specified. Adequate sample size was chosen for each experiment and appropriate statistical test was performed using GraphPad Prism 8.0.

Data Deposition: The RNA sequencing data will be deposited in the Gene-Expression Omnibus database on acceptance of the Manuscript.

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CONTRIBUTIONS

AS and GR conceptualized the project and designed the experiments. AS performed all the experiments. AS and GR analysed the data and wrote the manuscript. GR supervised the project and acquired funding.

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Figure S1: Expression of JNK components in different cell types of the adult gut.

A. Relative mRNA expression of JNK pathway components in different cell types of the adult gut. ISC, Interstitial stem cells; EB, Enteroblast; EC, Enterocytes; EE, Enteroendocrine cells.

B. Fold change of *Bsk*, *Jra* and *Kay* post oral infection with *P.e*.

Both Graphs plotted from data available at <http://flygutseq.buchonlab.com/>

A

Figure 2	No of Flies used for the experiment	# experiments
A	w^{1118} (UC) – 84; w^{1118} (I) – 154; $Jra^{IA109/+}$ (UC) – 83; $Jra^{IA109/+}$ (I) – 161; $Jra^{76-19/+}$ (UC) – 87; $Jra^{76-19/+}$ (I) – 141.	3
B	$>w^{1118}$ (C) – 103; $>w^{1118}$ (I) – 141; $>Jra^{RNAi}$ (C) – 91; $>Jra^{RNAi}$ (I) – 141; $>Jra^{DN}$ (C) – 103; $>Jra^{DN}$ (I) – 159.	3
C	$>w^{1118}$ (UC) – 66; $>w^{1118}$ (I) – 77; $>Bsk^{DN}$ (UC) – 79; $>Bsk^{DN}$ (I) – 89; $>Kay^{DN}$ (UC) – 102; $>Kay^{DN}$ (I) – 89.	2
D	$>w^{1118}$ (UC) – 86; $>w^{1118}$ (I) – 128; $>SUMO^{RNAi}$ (UC) – 91; $>SUMO^{RNAi}$ (I) – 141.	3
E	$>w^{1118}$ (C) – 20; $>w^{1118}$ (I) – 51; $>Uba2^{RNAi}$ (C) – 18; $>Uba2^{RNAi}$ (I) – 50; $>Aos^{RNAi}$ (C) – 17; $>Aos^{RNAi}$ (I) – 47; $>Ubc9^{DN}$ (C) – 17; $>Ubc9^{DN}$ (I) – 24.	1
F	w^{1118} (2 hpi) – 20; $Jra^{IA109/+}$ (2 hpi) – 20; w^{1118} (8 hpi) – 17; $Jra^{IA109/+}$ (8 hpi) – 19; w^{1118} (24 hpi) – 20; $Jra^{IA109/+}$ (24 hpi) – 19.	2

B

Figure S2: Jra regulates the gut immune response.

A. Tabular representation of the total number of flies used for experiments and number of independent experiments performed in Fig 2.

B. Quantitation of the number of pH3⁺ cells per midgut of UC flies and flies orally fed with *Pe* ****p<0.0001; ***p=0.0011 as determined by 2-way ANOVA with Tukey's post-hoc test. Number of gut used; w^{1118} (UC) – 16; w^{1118} (I) – 17; $Jra^{IA109/+}$ (UC) – 14; $Jra^{IA109/+}$ (I) – 17. Means and SEMs represented.

Figure S3: Gut transcriptomics of >*w*¹¹¹⁸ and >*Jra*^{RNAi}.

A. Schematic of 3' mRNA sequencing experiment. Flies were initially starved (without food and water) for 2h. Flies were then transferred onto a filter paper soaked in concentrated culture of *P.e* and 5% sucrose solution. 2h post feeding, flies were transferred to normal fly food and guts were dissected at 4, 12 and 24 hours post infection (hpi). Gut tissue was homogenized in trizol and RNA was extracted for 3'mRNA sequencing.

B. Principal component analysis (PCA) plot showing the first two principal components of the entire data set.

A

B

Figure S4: Gut transcriptomics of *>w¹¹¹⁸* and *>Jra^{RNAi}*.

A. Venn diagram of significantly differentially expressed genes (FDR < 0.1) upregulated and downregulated in *>w¹¹¹⁸* and *>Jra^{RNAi}*. The intersection shown in a shade of green represent genes that are significantly differentially expressed in *>w¹¹¹⁸* and *>Jra^{RNAi}*.

B. Gene ontology (GO) enrichment analysis of the significantly differentially expressed genes in *>w¹¹¹⁸* and *>Jra^{RNAi}* at 4, 12 and 24 hpi. Some key GO terms are highlighted and described

Figure S5: *>Jra^{RNAi}* shows increased expression of AMPs during infection.

qRT-PCR data showing the activation of *DptA* (A) and *AttD* (B) transcripts during gut infection in *>w¹¹¹⁸* and *>Jra^{RNAi}*. **p<0.0091 as determined by 2-way ANOVA with Bonferroni's post-hoc test for multiple comparisons. Data from three independent experiments. Means and SEMs represented.

Suppl. Figure 6

Figure S6: Mapping Jra occupancy on the promoters of differentially expressed genes in $>w^{1118}$ and $>Jra^{RNAi}$.

A. Heatmap showing occupancy of Jra on the gene body of the differentially expressed genes in $>w^{1118}$ and $>Jra^{RNAi}$. Cluster 1 represents a set of genes with enriched binding. Heatmap plotted with data extracted from Jra ChIP-seq dataset (ENCSR471GSA) post input (ENCSR908EFA) normalization

B. Heatmap representing the normalised expression counts of genes with enriched Jra (Cluster 1) binding on the promoters.

C. GO terms (Panther-Protein class) of genes with enriched Jra binding on the promoters.

A

Figure 5	No of Flies used in the experiment	# experiments
A	<i>Jra</i> ^{WT} (UC) – 142; <i>Jra</i> ^{WT} (I) – 154; <i>Jra</i> ^{SCR} L1 (UC) – 103; <i>Jra</i> ^{SCR} L1 (I) – 154; <i>Jra</i> ^{SCR} L2 (UC) – 111; <i>Jra</i> ^{SCR} L2 (I) – 127.	3
B	<i>Jra</i> ^{WT} (UC) – 51; <i>Jra</i> ^{WT} (I) – 99; <i>Jra</i> ^{SCR} (UC) – 49; <i>Jra</i> ^{SCR} (I) – 74; <i>Jra</i> ^{IA109} / <i>Jra</i> ^{WT} (UC) – 41; <i>Jra</i> ^{IA109} / <i>Jra</i> ^{WT} (I) – 95; <i>Jra</i> ^{IA109} / <i>Jra</i> ^{SCR} (UC) – 46; <i>Jra</i> ^{IA109} / <i>Jra</i> ^{SCR} (I) – 92.	2
C	<i>Jra</i> ^{SCR} , <i>NP1-Gal4</i> ^{ts} / <i>Jra</i> ^{SCR} (UC) – 84; <i>Jra</i> ^{SCR} , <i>NP1-Gal4</i> ^{ts} / <i>Jra</i> ^{SCR} (I) – 168; <i>Jra</i> ^{SCR} , <i>NP1-Gal4</i> ^{ts} / <i>Jra</i> ^{WT} (UC) – 89; <i>Jra</i> ^{SCR} , <i>NP1-Gal4</i> ^{ts} / <i>Jra</i> ^{WT} (I) – 185; <i>Jra</i> ^{SCR} , <i>NP1-Gal4</i> ^{ts} / <i>Jra</i> ^{SCR} ; <i>UAS-Jra</i> ^{WT} /+ (UC) – 80; <i>Jra</i> ^{SCR} , <i>NP1-Gal4</i> ^{ts} / <i>Jra</i> ^{SCR} ; <i>UAS-Jra</i> ^{WT} /+ (I) – 162; <i>Jra</i> ^{SCR} , <i>NP1-Gal4</i> ^{ts} / <i>Jra</i> ^{SCR} ; <i>UAS-Jra</i> ^{SCR} /+ (UC) – 82; <i>Jra</i> ^{SCR} , <i>NP1-Gal4</i> ^{ts} / <i>Jra</i> ^{SCR} ; <i>UAS-Jra</i> ^{SCR} /+ (I) – 163.	3
D	<i>Jra</i> ^{SCR} ; <i>Jra-Gal4</i> /+ (UC) – 142; <i>Jra</i> ^{SCR} ; <i>Jra-Gal4</i> /+ (I) – 155; <i>Jra</i> ^{SCR} / <i>Jra</i> ^{WT} ; <i>Jra-Gal4</i> /+ (UC) – 117; <i>Jra</i> ^{SCR} / <i>Jra</i> ^{WT} ; <i>Jra-Gal4</i> /+ (I) – 153; <i>Jra</i> ^{SCR} ; <i>Jra-Gal4</i> / <i>UAS-Jra</i> ^{WT} (UC) – 136; <i>Jra</i> ^{SCR} ; <i>Jra-Gal4</i> / <i>UAS-Jra</i> ^{WT} (I) – 155; <i>Jra</i> ^{SCR} ; <i>Jra-Gal4</i> / <i>UAS-Jra</i> ^{SCR} (UC) – 132; <i>Jra</i> ^{SCR} ; <i>Jra-Gal4</i> / <i>UAS-Jra</i> ^{SCR} (I) – 150.	3

B

Figure S7: *Jra*^{SCR} dampens the gut immune response.

A. Tabular representation of the total number of flies used for experiments and number of independent experiments performed in Fig 5.

B. Quantitation of number of pH3⁺ cells per midgut of UC flies and flies orally fed with *P.e.* **:p=0.0071; ***p=0.0005 as determined by 2-way ANOVA with Tukey's post-hoc test. Number of gut used; *Jra*^{WT} (UC) – 20; *Jra*^{WT} (I) – 23; *Jra*^{SCR} (UC) – 24; *Jra*^{SCR} (I) – 24. Means and SEMs represented.

Suppl. Figure 8

Figure S8: Gut transcriptomics of *Jra*^{WT} and *Jra*^{SCR}.

A. Principal component analysis (PCA) plot showing the first two principal components of the entire data set.

B. Venn diagram of significantly differentially expressed genes (FDR<0.1) upregulated and downregulated in *Jra*^{WT} and *Jra*^{SCR}. The intersection shown in a shade of green represent genes that are common in *Jra*^{WT} and *Jra*^{SCR}.

C. Gene ontology (GO) enrichment analysis of the significantly differentially expressed genes from the entire data set. Some key GO terms are highlighted and described

Figure S9: *Jra*^{SCR} shows insufficient activation of key immune effectors.

qRT-PCR data showing the activation of *Rel* (A), *DptA* (B) and *AttD* (C) transcripts during gut infection in *Jra*^{WT} and *Jra*^{SCR}. *p=0.0182 (*DptA*-12hpi), *p=0.0365 (*AttD*-4hpi), **p=0.0027, and ****p<0.0001 as determined by 2-way ANOVA with Bonferroni's post-hoc test for multiple comparisons. Data from three independent experiments. Means and SEMs represented.

Suppl. Figure 10

Figure S10: Mapping *Jra* occupancy on the promoters of differentially expressed genes in *Jra*^{WT} and *Jra*^{SCR}.

A. Heatmap showing occupancy of *Jra* on the gene body of the differentially expressed genes in *Jra*^{WT} and *Jra*^{SCR}. Cluster 1 represents a set of genes with enriched binding. Heatmap plotted with data extracted from *Jra* ChIP-seq dataset (ENCSR471GSA) post input (ENCSR908EFA) normalization

B. Heatmap representing the normalised expression counts of genes with enriched *Jra* binding (cluster 1) on the promoters.

C. GO terms (Panther-Protein class) of genes with enriched *Jra* binding on the promoters.

Figure S11: The Imd/JNK pathway in the *Drosophila* immune response.

Upon binding of peptidoglycan (PGN) molecules to the peptidoglycan recognising proteins (PGRPs) presents on the surface of the cell, the Imd pathway gets activated. The adaptor molecule, Imd is cleaved and recruits the TAB2/Tak1 complex. This phosphorylates and activates the IKK complex comprising Key and Ird5. The IKK phosphorylates and activates Rel. Active Rel is cleaved by Dredd and translocates into the nucleus to activate the transcription of antimicrobial peptides (AMPs) and other immune factors. Tak1 parallelly phosphorylates and activates the JNKK, Hemipterous (Hep). Hep activates the JNK, Basket (Bsk) and this in turn activates the AP-1 complex consisting of the dimer Jra and Kay. Jra interacts with HDAC1, STAT92E and Dsp1 to antagonise the activation of AttA by Rel.