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Characterization of the Rel2-regulated transcriptome and proteome of *Anopheles stephensi* identifies new anti-*Plasmodium* factors

Andrew Pike¹, Alekhya Vadlamani¹, Simone L. Sandiford¹, Anthony Gacita¹, and George Dimopoulos^{1,*}

Andrew Pike: apike@jhsph.edu; Alekhya Vadlamani: alvadlam@jhsph.edu; Simone L. Sandiford: ssandifo@jhsph.edu; Anthony Gacita: agacita1@jhu.edu; George Dimopoulos: gdimopou@jhsph.edu

¹W. Harry Feinstone Department of Molecular Microbiology and Immunology and the Johns Hopkins Malaria Research Institute, Bloomberg School of Public Health, Johns Hopkins University, 615 N. Wolfe Street, Baltimore, MD, USA

Abstract

Mosquitoes possess an innate immune system that is capable of limiting infection by a variety of pathogens, including the *Plasmodium* spp. parasites responsible for human malaria. The *Anopheles* immune deficiency (IMD) innate immune signaling pathway confers resistance to *Plasmodium falciparum*. While some previously identified *Anopheles* anti-*Plasmodium* effectors are regulated through signaling by Rel2, the transcription factor of the IMD pathway, many components of this defense system remain uncharacterized. To begin to better understand the regulation of immune effector proteins by the IMD pathway, we used oligonucleotide microarrays and iTRAQ to analyze differences in mRNA and protein expression, respectively, between transgenic *An. stephensi* mosquitoes exhibiting blood meal-inducible overexpression of an active recombinant Rel2 and their wild-type conspecifics. Numerous genes were differentially regulated at both the mRNA and protein levels following induction of Rel2. While multiple immune genes were up-regulated, a majority of the differentially expressed genes have no known immune function in mosquitoes. Selected up-regulated genes from multiple functional categories were tested for both anti-*Plasmodium* and anti-bacterial action using RNA interference (RNAi). Based on our experimental findings, we conclude that increased expression of the IMD immune pathway-controlled transcription factor Rel2 affects the expression of numerous genes with diverse functions, suggesting a broader physiological impact of immune activation and possible functional versatility of Rel2. Our study has also identified multiple novel genes implicated in anti-*Plasmodium* defense.

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*Corresponding Author: Bloomberg School of Public Health, Johns Hopkins University, 615 N. Wolfe street, Baltimore, MD 21205-2179, USA, Tel. +1 443 287 0128, Fax. +1 410 955 0105.

Competing Interests

The authors have declared that no competing interests exist.

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Keywords

Mosquitoes; transgenesis; Rel2; transcriptome; proteome; innate immunity; Plasmodium

1. Introduction

Mosquitoes are vectors for many important human pathogens, including viruses, filarial worms, and apicomplexan parasites. A number of *Plasmodium* spp. parasites, vectored exclusively by *Anopheles* spp. mosquitoes, cause human malaria. Because of difficulties in the distribution of anti-malarial chemotherapeutics and the rise of drug resistance in the parasite, vector control remains at the forefront of malaria control efforts. However, after decades of insecticide spraying, bed net distribution, and habitat remodeling, the disease remains established, so novel vector-control methods must be developed. Recently, methods have been developed to generate genetically modified mosquitoes (Ito *et al.*, 2002), and various strategies based on their release are being investigated for malaria control. Conversion of a natural mosquito population to a transgenic population that overexpresses anti-*Plasmodium* immune system activators or effector molecules could represent one such method, and multiple mosquito lines expressing such transgenes in different tissues have already been developed (Dong *et al.*, 2011; Dong *et al.*, 2012).

Mosquitoes possess an innate immune system that is capable of responding to, and controlling, infection by diverse pathogens, including bacteria, viruses, and apicomplexan parasites (Cirimotich *et al.*, 2009; Garver *et al.*, 2009). Two immune pathways, the Toll and immune deficiency (IMD) pathways, have been extensively studied in both *Drosophila* and mosquitoes. Both pathways recognize invading pathogens through the association of host pattern recognition receptors (PRR) with pathogen associated molecular patterns (PAMPs), leading to a signaling cascade, nuclear localization of transcription factors, and subsequent induction of the expression of numerous immune effector molecules and anti-microbial peptides. Invading pathogens are then killed by various mechanisms, such as phagocytosis and complement-like killing.

The nuclear translocation of the NF- κ B transcription factor Rel2 leads to an induction of immune gene expression that constitutes the IMD pathway-mediated immune response (Meister *et al.*, 2005). The IMD pathway has been shown to regulate the mosquito's resistance to *P. falciparum* infection (Garver *et al.*, 2009), and numerous mosquito lines with inducible overexpression of the constitutively active short form of Rel2 have been created (Dong *et al.*, 2011). One such line (henceforth referred to as the CP15 line) uses the carboxypeptidase gene promoter to limit Rel2 overexpression to the midgut following a blood meal, while another line (the VG1 line) overexpresses the same Rel2 transgene under the control of the vitellogenin gene promoter, leading to fat body-specific expression after a blood meal (Dong *et al.*, 2011). Both these lines exhibit a greatly reduced susceptibility to *Plasmodium* infection following an infected blood meal and may represent viable tools for future release as part of a malaria control program. However, immune pathways and their downstream transcription factors can regulate a large variety of both immunity and non-immunity related processes (Dong *et al.*, 2006; Xi *et al.*, 2008). Hence, the overexpression

of the Rel2 transcription factor affects the immune system, but it is also likely to regulate other physiological processes entailing genes of diverse functions.

A majority of studies on the insect immune system have relied on infection with a pathogen and observation of the insect's response to the insult (Imroze and Prasad, 2012; McKean *et al.*, 2008; Zerofsky *et al.*, 2005). Using transgenic mosquitoes that overexpress Rel2 in an inducible fashion provides a pathogen-independent system to study IMD pathway-regulated immune response and eliminates any confounding factors brought about by the presence of the infecting organism. Zou and colleagues used a Rel2-overexpressing *Aedes aegypti* to study the IMD pathway-regulated transcriptome (Zou *et al.*, 2013).

We used whole-genome oligonucleotide microarrays to study recombinant Rel2-induced changes in mRNA abundance, as well as isobaric tags for relative and absolute quantitation (iTRAQ) to study changes in protein abundance after Rel2 overexpression in transgenic mosquitoes. Measuring the expression levels of both mRNA and protein allowed us to look for correlations between transcript and protein abundance following up-regulation of a transcription factor. We then used RNA interference (RNAi) assays to investigate a subset of genes, both with and without known immune function, for anti-*Plasmodium* and anti-bacterial activity, leading to the identification of multiple novel anti-*Plasmodium* effectors.

2. Materials and Methods

2.1 Mosquito rearing

A. stephensi Liston strain wild-type, CP, and VG transgenic Rel2-overexpressing lines (Dong *et al.*, 2011) were maintained according to standard insectary procedures. In brief, larvae were reared at low densities in trays and fed a combination of ground fish flakes (Tetra) and cat food pellets (Purina). Upon emergence, adults were maintained on a 12 h/12 h light/dark cycle at 27° C with 80% humidity and constant access to a 10% solution of sucrose in water. In order to stimulate egg production, adults were fed on ketamine-anesthetized mice according to IACUC-approved protocols.

2.2 RNA extraction and microarrays

One-week-old adult female mosquitoes were given a human blood meal from water-jacketed membrane feeders maintained at 37° C. Mosquito tissues were dissected in sterile PBS as follows: midguts were collected at 6 and 12 h after blood feeding, while fat bodies were collected at 12 and 18 h after the blood meal. Total mosquito RNA from dissected tissues was extracted using RNeasy kits (Qiagen) according to the manufacturer's protocols and quantified on a NanoDrop spectrophotometer before quality assessment on an Agilent Bioanalyzer 2100. Probes were synthesized using 200 ng of RNA and the Low-Input RNA Labeling Kit (Agilent) according to the manufacturer's protocol. These probes were hybridized to a custom-designed Agilent microarray slide, which was scanned with an Axon GenePix 4200AL scanner at 2- μ m resolution. After scanning, statistical analysis was performed using the TIGR, MIDAS, and TMEV software packages (Dudoit *et al.*, 2003), following standard laboratory protocol (Dong *et al.*, 2006), and analysis was performed using a *t*-test, with a significance level of $\alpha=0.05$. Changes in gene expression were considered significant if the absolute value of the gene regulation was ≥ 0.75 on a $\log_2$

scale. For each treatment, three biological replicates and one pseudoreplicate were performed. The array was designed using Array Designer software (Premier Biosoft, www.premierbiosoft.com) and based on an early version of the *A. stephensi* transcriptome obtained from Dr. Jake Tu of Virginia Polytechnic Institute and State University, and putative function and gene ontology (GO) terms were assigned to transcript sequences based on homology to previously annotated *A. gambiae* genes discovered by a blastn search (Altschul *et al.*, 1990). The blast search was performed against gene set AgamP3.7, downloaded from vectorbase.org; for each gene, the most significant hit was used for annotation, with a maximum e-value of 0.0001 used as a cutoff. Any genes that did not have significant homology to any previously annotated *An. gambiae* genes were used for a blastn (Altschul *et al.*, 1990) search against the non-redundant nucleotide database from NCBI to assign putative function if similar genes or conserved sequences were identified in other species. While the gene with the highest blast homology between *An. stephensi* and *An. gambiae* may not represent a true orthologue, this is our best prediction given the early state of the annotation of the *An. stephensi* genome. Seven genes were analyzed by qRT-PCR to verify the results of the microarray (Figure S4).

2.3 Protein extraction and iTRAQ

One-week-old adult female mosquitoes from the WT, CP, and VG lines were given a human blood meal from membrane feeders at 37° C. Prior to the blood meal and 24 h afterward, mosquitoes were dissected in sterile PBS and their midguts and fat bodies collected. Three replicates of 10 midguts or fat bodies were resuspended in lysis buffer (10 mM HEPES, 0.1 mM MgCl₂, 0.1% Triton, and protease inhibitors [Roche]) and left on ice for 30 min. Tissues were homogenized, freeze-thawed twice, and centrifuged at 14,000 rpm for 30 min at 4°C. The supernatant fraction was collected and used as the total protein extract. Total protein concentration was measured by Bradford assay. Consistency among the three replicates was assessed by running 0.1 µg of total extract on a 4–12% Tris gel and silver staining. Replicates were then combined and used for iTRAQ analysis: 50 µg of protein was reduced using 2 µL of 50 mM TCEP for 1 h at 60° C, cooled to room temperature, and incubated with 1 µL of 100 mM MMTS for 15 min in the dark. Samples were then TCA-precipitated, and air-dried pellets were submitted to the Johns Hopkins School of Medicine Mass Spectrometry and Proteomics Facility for identification and relative quantitation using iTRAQ. The samples were trypsin-digested, labeled with 8plex iTRAQ reagents, fractionated by both strong cation exchange and reverse phase chromatography, and identified by mass spectrometry using a LTQ Orbitrap Velos mass spectrometer (Thermo Scientific) as described in detail (Guo *et al.*, 2007; Martin *et al.*, 2008; Pierce *et al.*, 2008; Ross *et al.*, 2004). MS/MS spectra were searched against a custom *A. stephensi* database generated from sequences provided by Dr. Jake Tu (Virginia Polytechnic Institute and State University) using Mascot (Matrix Science) and Proteome Discoverer (v1.2 Thermo Scientific) with the high peptide confidence filter. The resulting data were considered significant if the ratio of GM:WT protein levels was >0.75 on a log₂ scale. Sequences obtained from the mass spectrometer were compared to previously annotated *A. gambiae* gene sequences using blastp (Altschul *et al.*, 1990) in order to assign them putative functions and GO terms.

2.4 dsRNA-mediated gene silencing

Double-stranded RNAs (dsRNAs) targeting selected genes were synthesized from PCR products using the HiScribe T7 *in vitro* transcription kit (NEB). Adult female mosquitoes (3–4 days old) were anesthetized on ice and injected with 69 nl of 3 µg/µl dsRNA targeting a gene of interest or GFP as a control, then maintained under normal mosquito rearing conditions. At 3 days post-injection, groups of 10 mosquitoes were collected for silencing efficiency measurement using qRT-PCR. Primers used for the PCR amplification of oligos were designed using the Primer3 program (<http://frodo.wi.mit.edu>) and are listed in table S1. Silencing of the genes was verified by qRT-PCR 3 days post-injection (Figure S5).

2.5 Quantitative Real-Time PCR (qRT-PCR)

Total RNA used to verify gene knockdown was extracted from whole mosquitoes with their heads and legs removed, while total RNA used to verify the microarray results was collected from dissected midguts or fat bodies using a Qiagen RNEasy kit according to the manufacturer's instructions. qRT-PCR was carried out using Sybr Green PCR Master Mix (ABI) on an ABI StepOnePlus Real-Time PCR System with the ABI StepOne Software. Transcript abundance was normalized to mosquito ribosomal protein S7 gene levels and the -fold change of each gene was calculated using the $2^{-\Delta\Delta C_t}$ method. Primers were designed using the Primer3 program (<http://frodo.wi.mit.edu>) and are listed in table S1.

2.6 *P. falciparum* infections

P. falciparum challenges were performed according to a standard laboratory protocol: Three days after dsRNA injection, mosquitoes were fed on human blood containing NF54W strain *P. falciparum* gametocytes through a membrane feeder at 37° C. Unfed mosquitoes were discarded and mosquitoes were maintained as usual for 7 days, at which point their midguts were dissected and stained with 0.1% mercurochrome for oocyst enumeration by manual counting under a light microscope (Olympus).

2.7 Enumeration of midgut bacteria

The number of colony forming units (CFUs) of midgut bacteria for gene-silenced sugar or blood-fed mosquitoes was counted as previously described: Mosquitoes were surface-sterilized with ethanol and rinsed in PBS before their midguts were dissected into PBS. Midguts were then homogenized in PBS, and serial dilutions were plated onto LB agar plates and incubated at room temperature. Three days after plating, the number of colonies per plate was counted, and the total number of culturable bacteria per midgut was calculated. Samples were collected for sugar-fed mosquitoes 3 days after gene silencing, while other mosquitoes were provided a blood meal 3 days after gene silencing, with dissections being performed 24 h later.

2.8 Statistical Analyses

Statistical analyses were performed using the GraphPad Prism 5 software (Graphpad Software). The tests used are indicated in the Results section and figure captions.

3. Results and Discussion

3.1 Expression of active Rel2 leads to broad transcriptome and proteome changes

Using whole-genome microarrays, we compared the mRNA abundance of all predicted *An. stephensi* genes in transgenic Rel2-overexpressing (Dong *et al.*, 2011) and WT mosquito lines at two time points following a blood meal in order to identify the Rel2-driven changes in the midgut and fat body transcriptomes. Mosquitoes with Rel2 expression driven by the midgut-specific carboxypeptidase promoter (CP line) were assayed at 6 and 12 h post-blood meal (PBM), and samples with Rel2 expression driven by the fat body-specific vitellogenin promoter (VG line) at 12 and 18 h PBM. Selection of these time points was based on the recombinant Rel2 induction profile in the two tissues (Dong *et al.*, 2011). At 6 h PBM, there were 190 up-regulated and 94 down-regulated genes in the midguts of CP line mosquitoes (Fig 1A), and these totals increased to 645 up-regulated and 596 down-regulated genes at 12 h (Fig 1A). In VG line mosquitoes, there were 173 up-regulated and 152 down-regulated genes in the fat body at 12 h PBM and 203 up-regulated and 189 down-regulated genes at 18 h (Fig 1A). This magnitude of gene regulation is similar to that observed in the fat bodies of Rel2 overexpressing *Ae. aegypti* mosquitoes, which upregulated 123 and downregulated 176 genes following Rel2 activation (Zou *et al.*, 2011). A total of 71 transcripts were regulated by recombinant Rel2 activation in both the CP midgut and VG fat body, with 19 being regulated in the same direction at 12 h PBM (10 up-regulated in both tissues and 9 down-regulated in both tissues) and 20 being up-regulated in the midgut and down-regulated in the fat body; 32 displayed the opposite mRNA abundance pattern (Fig 1B). It is unlikely that a transcription factor would up-regulate and down-regulate the same gene in different tissues, so it is much more likely that the differences in expression pattern are related to interactions with other transcription factors, feedback loops, or mRNA or protein degradation rates. Because we were looking at global changes following Rel2 induction, we could not differentiate between an mRNA directly up-regulated by Rel2 and another that was down-regulated because of a silencing factor, or an mRNA with a long half-life compared to another that was degraded by a micro-RNA that was up-regulated by Rel2.

Predicted gene ontology (GO) categories were assigned to genes based on homology to previously annotated *An. gambiae* genes (vectorbase.org). In both tissues and at all time points, the predicted GO category with the greatest number of significantly regulated genes was the diverse functional category (a total of 508 differentially regulated genes at 12 h PBM) followed by the unknown category (328 total differentially regulated genes at 12 hours PBM). This is to be expected, since these two GO categories represent over half of the annotated genes in the *An. gambiae* genome (vectorbase.org), and therefore the predicted GO terms for our *An. stephensi* genes. The broad functional spectrum of genes assigned to the diverse category reflects the far-reaching effects of Rel2 activation. Because Rel2 is the major transcription factor of the IMD pathway, we conjectured that numerous genes assigned to the immune/apoptosis GO category would be up-regulated following Rel2 expression. There were 26 genes from this category that were up-regulated in the midgut of CP mosquitoes at 12 h PBM, representing 4% of the total up-regulated genes at that time point. There were also 16 down-regulated genes in this category at the same time. In the VG line fat body, there were 36 up-regulated immune genes at 18 h PBM, representing 17.7% of

up-regulated fat body genes at 18 h PBM, with 12 down-regulated at the same time. Highly up-regulated immune genes included both known anti-*Plasmodium* effectors, such as TEPI and LRIM1 (Blandin *et al.*, 2004; Povelones *et al.*, 2009), and genes that have not yet been associated with *Plasmodium* resistance but that have shared domains with known anti-*Plasmodium* effectors, such as multiple leucine-rich repeat and fibrinogen domain-encoding genes and two MD-like genes (Garver *et al.*, 2009; Povelones *et al.*, 2009; Riehle *et al.*, 2008). While a smaller proportion of immune genes were found to be differentially regulated in the *An. stephensi* genome following Rel2 expression than in *Ae. aegypti* (Zou *et al.*, 2011), many of the same functional classes of immune genes are represented in the upregulated group, including thio-ester proteins and leucine rich repeat containing proteins. Representatives from other GO categories, such as serine proteases in the Proteolysis/Digestion group, redox responsive genes in the Redox/Stress/Mitochondrial category, and many others, are likely to be relevant for reciprocal interactions between *Plasmodium* and the mosquito. Other up-regulated genes such as cytochrome P450s may play a role in the mosquito's resistance to insecticides (David *et al.*, 2013) and thereby affect the ability of these transgenic mosquitoes to survive and compete in the wild.

To determine changes in midgut and fat body proteomes following Rel2 activation in these tissues, we used iTRAQ to quantify the relative amounts of all proteins in Rel2-overexpressing transgenic mosquitoes relative to their wild-type conspecifics both before and 24 h after a blood meal. Filtering for only high confidence peptides, we identified 31,392 peptide spectrum matches (PSMs) corresponding to 8,574 peptides that mapped to 2,244 unique protein contigs previously annotated by the Jake Tu Lab (Virginia Polytechnic Institute and State University). These proteins were assigned putative names and functions through a BLASTp search against the *An. gambiae* genome, yielding 2041 unique proteins, 2024 of which had significant similarity (BLASTp e-value < 0.01) to *An. gambiae* genes. Prior to a blood meal, the transgenic mosquito midgut displayed significantly higher levels of 89 proteins and lower levels of 45 proteins. The transgenic mosquito fat body displayed higher levels of 204 proteins and lower levels of 31 proteins prior to the blood meal (Fig. 2A). One reason for the differential expression of these proteins in the absence of Rel2 induction may be that they represent genes with permanently altered expression patterns, either as a result of position effects of transgenesis or adaptation during the numerous generations since the insertion of the transgene. Alternatively, there may be leaky expression from the CP and VG promoters, as has been observed before for the VG promoter for some autogenous mosquito species (Provost-Javier *et al.*, 2010), though not in *Anopheles* spp. mosquitoes. The reason for this leakiness could be further explained by examining the expression profiles of multiple lines with different transgene insertion locations. However, due to the labor intensive maintenance of transgenic lines, only one line of each strain, possessing the most potent anti-*Plasmodium* activity, has been kept and therefore limiting the analysis to a single line for each tissue. However, since these lines were selected based on their most potent anti-*P. falciparum* activity they represent the most relevant lines for this study (Dong *et al.*, 2011). As expected, at 24 h PBM, there were many more proteins displaying significantly altered abundances. Specifically, after a blood meal there were 1,230 up-regulated and 64 down-regulated proteins in the midgut and 26 up-regulated and 185 down-regulated proteins in the fat body (Fig. 2A). Of these, only 22 were significantly

regulated in the same direction (10 up and 12 down) in both the midgut and the fat body, while 78 were significantly regulated in opposite directions (77 up in the midgut and down in the fat body, and 1 with the reverse) (Fig. 2B). As at the transcript level, the GO category with the greatest number of differentially regulated proteins assigned to it was the diverse category (493 total differentially regulated proteins at 24 h PBM), although all the GO categories were represented, especially in the list of proteins up-regulated in the midgut at 24 h PBM. In the midgut, there were 50 proteins, or 4% of the total up-regulated proteins, belonging to the immune GO category that were significantly up-regulated at 24 h PBM, but there were only 2 significantly down-regulated immune proteins, or 3.1%, at that time. In the fat body at 24 h PBM, there were only 2 significantly up-regulated immune proteins, but these proteins represent 7.7% of the total significantly up-regulated proteins, and there were 7 significantly down-regulated proteins representing 3.7%. As for the transcriptome, there was a functionally diverse set of up-regulated immune proteins, including some corresponding to genes found to be regulated in the microarray-based transcriptome analyses, such as *An. gambiae* MD2-like protein 6 (AgMDL6) and neuronal leucine-rich repeat protein 3 (NLRR3). Other significantly up-regulated proteins were not found to be significantly up-regulated in the same tissue at the transcript level, but the regulated transcriptome and proteome sets contained many genes belonging to the same families, such as leucine-rich repeat and fibrinogen domain-containing proteins. Interestingly, the levels of both Toll and Rel1, which are Toll pathway-associated genes, were significantly up-regulated at the protein level following Rel2 activation, possibly indicating an interplay between the IMD and Toll pathways.

In both the transcriptomic and proteomic analyses, we observed differential expression of genes belonging to various functional classes (Figs. 1 and 2). Here, we present a brief overview of the several classes of genes that we believe are relevant to the Rel2 pathway or provide interesting insights on the physiological influence of this pathway.

Immune-related genes—In total, 102 separate immune-related genes were up-regulated under at least one experimental condition, while 44 were down-regulated. These genes represent a large number of different families of immune genes, including 8 leucine-rich repeat containing proteins, 5 tep proteins, 14 CLIP domain serine proteases, and 7 serine protease inhibitors, as well as many anti-microbial peptides and other genes. Interestingly, in addition to IMD pathway-associated genes such as Rel2, a number of Toll pathway-associated genes, including Toll and Rel1, were also significantly up-regulated, indicating that there may be interaction between the two major immune pathways. Similarly, Hop and JNK, components of the Jak/Stat and JNK pathways, were also up-regulated. These data indicate that the various immune pathways do not act in isolation and may instead act together to attain a broader immune response. The JNK pathway has recently been shown to play a role in *Plasmodium* resistance (Garver *et al.*, 2013), and interaction between the JNK and IMD pathways may lead to greater *Plasmodium* immunity. Up-regulation of AMP genes such as defensin and cecropins likely plays a direct role in the control of bacteria and other pathogens (Meister *et al.*, 2005), and similar upregulation of AMP expression has been seen following Rel2 induction in *Ae. aegypti* (Zou *et al.*, 2011), while other genes such as the

serine proteases likely trigger cascades to amplify and diversify the response (An *et al.*, 2010).

Digestive—485 digestion-related genes were up-regulated across the experimental conditions, and 280 were down-regulated. Among these were genes involved in both protein and sugar digestion, as well as many Ras family proteins. Numerous proteolysis related genes were also found to be differentially regulated in the *Ae. aegypti* Rel2 regulated transcriptome (Zou *et al.*, 2011). Up-regulated protein digestion-related genes, such as 26S proteosome subunits and serine/threonine phosphatases, work as part of the system for digesting blood meals following ingestion and may aid in the control of bacterial proliferation in the midgut following a blood meal. Ras family proteins are GTPases involved in cell proliferation and signaling, some of which have been implicated in *Drosophila* immunity (Ye and Zhang, 2013). While evidence from *Drosophila* implicates the Ran subfamily of Ras genes in the phagocytosis of virus-infected cells, other Ras genes may play a role in phagocytosis of other infectious organisms. Alternatively, GTPases, such as Ras family proteins, and kinases may act in a similar fashion to serine proteases and related genes to amplify and diversify the signal from the IMD pathway.

Cell structure genes—There were also 82 up-regulated and 48 down-regulated genes in the Cell Structure GO category. These included genes vital for muscle function or cell motility, such as 7 up-regulated and 3 down-regulated actins and 13 up-regulated and 8 down-regulated myosins; genes used for cell division, such as 3 up-regulated tubulins; and numerous cuticular proteins, actin- and chitin-binding proteins, and various other proteins. Previous research has shown that the anti-*Plasmodium* immune response involves remodeling of the midgut epithelium (Han and Barillas-Mury, 2002), as does bacterial resistance in the *Drosophila* midgut (Buchon *et al.*, 2013). In *Drosophila*, infected cells are expelled into the midgut lumen and must be regenerated, and both processes require action by the cell structure and motility system. Other studies have also shown that remodeling of the cytoskeleton is necessary for successful *Plasmodium* infection and traversal of the midgut (Han and Barillas-Mury, 2002), so changes in the cytoskeleton by the immune system may be important for resistance to infection.

Redox genes—Finally, many redox and stress-related genes showed differential regulation. Components of the ROS system have been implicated in resistance to both bacteria (Oliveira *et al.*, 2011) and *Plasmodium*, and multiple NOS and mitochondrial carrier (Goncalves *et al.*, 2012) genes are up-regulated following IMD pathway induction. The up-regulation of ROS-related genes by the IMD pathway may be a link between the various ways of fighting off midgut infection by both *Plasmodium* and bacteria. By up-regulating these genes, the mosquito can attack pathogens with both ROS and AMPs at the same time, increasing the potential for clearance. Many components of the oxidative phosphorylation system, including various reductases and oxidases, were also up-regulated under at least one condition, indicating that the mosquito may increase ATP production in order to compensate for energy use by the immune system.

Genes with few representatives—In addition to the gene families with large numbers of representatives listed above, there were also gene families with very few differentially regulated representatives. For instance, odorant receptors and other sensory proteins were almost completely absent from the differentially regulated datasets, despite having many representatives in the genome (vectorbase.org). Thus, the mosquito IMD pathway likely does not greatly affect the ability of mosquitoes to sense their environment.

While we have identified a large number of transcripts and proteins that displayed altered expression in our genetically modified mosquitoes following up-regulation of Rel2, not all of these genes are likely to be controlled directly by Rel2, and there are many Rel2-regulated genes that were not discovered through our approach. We tested only a limited number of time points, and there may be short-lived transcripts and proteins that were degraded before we collected our samples. Similarly, some genes may take longer to transcribe and translate than others, and our time points may have been too early to observe the changes in expression. Other genes may require the binding of different transcription factors not present in this study in addition to Rel2, and thus they would not be differentially regulated following the up-regulation of Rel2 alone. Conversely, some genes having promoter sequences with low affinity for Rel2 may be differentially regulated in our transgenic mosquitoes, likely because of the overabundance of an active Rel2 form in the system. The differential regulation of many genes upon activation of recombinant Rel2 is also likely to represent a secondary effect and a general physiological response to immune activation. However, we believe that the time points we have chosen are well chosen to capture both the timing of Rel2 up-regulation in our mosquitoes and the physiologically relevant times for *P. falciparum* invasion of the midgut, in the under-studied mosquito vector *An. stephensi*.

3.2 Correlation between mosquito transcript and protein expression levels

Because we measured both transcript abundance and protein abundance, we were able to compare the expression of genes at the two levels in order to assess a correlation between transcript levels and protein abundance. Previous studies in other organisms have shown only weak concordance between transcripts and proteins (de Sousa Abreu *et al.*, 2009; Vogel and Marcotte, 2012), but such studies are usually restricted to unicellular organisms or cell lines, and this study provided an opportunity to expand this knowledge to multicellular eukaryotes.

When we looked at all the genes that displayed a significant difference at the protein abundance level at 24 h PBM and a significant difference at the transcript abundance level at one of the two time points, we saw no significant correlation between protein and transcript levels in the midgut when we used a linear model (Fig. S6 A). However, we saw a significant correlation for the fat body (fig. S6 B). Our assays generated data for 1,273 genes at both the transcript and protein levels in the midgut, 240 of which were significantly regulated in the same direction at both the protein and transcript levels, while 119 were significantly regulated in opposite directions. In the fat body, we obtained data for 1,538 genes at both the transcript and protein levels, with 33 significantly being regulated in the same direction at both protein and transcript levels and only 8 significantly regulated in

opposite directions. The lack of significant correlation between mRNA and protein levels in the midgut is likely due to the wide variety of genes that are differentially expressed in that tissue and the various post-transcriptional modifications and regulatory mechanisms involved. The lack of correlation is also indicative of the large proportion of genes that are not directly regulated by the Rel2 transcription factor. Furthermore, factors such as transcript and protein degradation rates, miRNAs, mRNA secondary structure, the presence of other transcription regulators, and the availability of ribosomes and amino acids to build proteins could all contribute to differences between the abundance of mRNA and proteins (Maier *et al.*, 2009; Vogel and Marcotte, 2012). For instance, if the mRNA of a particular transcript is quickly degraded but the protein is long-lived, or vice-versa, then an increase in the transcription of that gene will not necessarily lead to a measurable change in the gene expression at both levels. Similarly, other transcriptional and translational regulators, including transcription factors, miRNAs, and feedback loops, may promote or inhibit transcription and translation differentially, leading to discordance between mRNA and protein levels. Finally, it is possible that the time points at which we measured expression levels did not adequately capture the timing of gene expression. Our previous work indicates that Rel2 expression levels should be high at 12 h and remain elevated for many hours afterward in both the midgut and fat body, and protein levels would, if directly governed by transcription, follow soon after. An increase in transcripts, even if not immediately translated, could allow mosquitoes to increase the abundance of a protein quickly if other signals necessary for the translation of that gene were present. Similarly, differential degradation of transcripts and proteins may allow mosquitoes to remove unnecessary gene products, even if transcription is initiated by a promiscuous promoter. Other studies have also shown a lack of correlation between mRNA and protein levels (Gedeon and Bokes, 2012; You and Yin, 2000). Our results show that a lack of correlation between mRNA and protein expression levels is also prominent in mosquitoes, likely providing mosquitoes a fine level of control over the proteins expressed in their cells.

3.3 Identification of novel anti-*Plasmodium* immune genes

The analyses of transcript and protein abundance following Rel2 induction in transgenic mosquitoes allowed us to select a variety of genes to investigate further for involvement in the mosquito's immune defense. Because Rel2 is an IMD pathway-associated transcription factor, we expected a large number of significantly up-regulated immune genes. Similarly, because this immune pathway is responsible for the mosquito's resistance to *Plasmodium* infection (Garver *et al.*, 2009), we were interested in identifying IMD pathway-regulated genes that control resistance to *P. falciparum*. We began by selecting five of the immune genes with the greatest up-regulation at the protein level at 24 h PBM in the midgut. We chose to consider only the protein level for these genes because it takes approximately 24 h for *P. falciparum* to exit the midgut lumen, so proteins with increased expression at this time will be available to act against the parasite.

From the 20 most abundant immune proteins, we chose 5 that may have anti-*Plasmodium* activity for further testing based on the level of up-regulation and predicted functions based on sequence homology. Specifically, we chose a class B scavenger receptor containing a SCRBQ1 domain (SCRBQ1), bacteria response protein 1 (AGBP1), Neimann-Pick type C-2

(NPC2), alpha-2-macroglobulin receptor-associated protein (A2MRAP), and a leucine-rich transmembrane protein (LRTP). The selected genes were knocked down by RNAi prior to parasite exposure, and the resulting impact on *P. falciparum* infection of the mosquito midgut was assayed by oocyst counting (Fig. 3). RNAi-based depletion of SCRBQ1, AGBP1, and NPC2 had no significant effect on *Plasmodium* infection levels; however, depletion of A2MRAP and LRTP led to significant increases in the number of oocysts per midgut (Kruskal-Wallis test, $p < 0.0001$; Dunn's post-hoc test: A2MRAP, $p < 0.01$; LRTP, $p < 0.001$). While the median number of oocysts was decreased following both A2MRAP and LRTP knockdown, none of the dsRNA treatments had a significant effect on oocyst prevalence, even though previous studies have shown that Rel2 knockdown by RNAi leads to an increase in oocyst prevalence. This difference in result may stem from the fact that the genes we knocked down are only a part of the whole immune response to *Plasmodium* infection, and their knockdown may not be efficient enough to affect the prevalence, whereas the full complement of IMD pathway-regulated immune effectors together cause a greater effect.

SCRBQ1 (ASTE009112/AGAP010132) is homologous to the *Croquemort* (CRQ) gene in *D. melanogaster*, which is essential for efficient phagocytosis of apoptotic cells in *Drosophila* embryos (Franc *et al.*, 1996; Franc *et al.*, 1999), and may play a role in anti-*Plasmodium* defense through apoptosis or phagocytosis (Blandin and Levashina, 2007; Hurd *et al.*, 2006). AGBP1 (STE009712/AGAP008061) is one of a number of bacterial infection-responsive proteins that have been identified in *An. gambiae*, and is up-regulated following infection with *P. berghei* (Dong *et al.*, 2006). However, *P. berghei* infection is principally controlled by the Toll pathway (Garver *et al.*, 2009), so the relevance of this protein in defending against *P. falciparum* infection may be minimal. NPC2 (ASTE004995/AGAP002851) is a small, highly conserved, secreted protein that plays an important role in regulating sterol homeostasis in *Drosophila* (Ioannou, 2007). *Plasmodium* parasites are unable to synthesize their own sterols and must scavenge these molecules from their host (Bano *et al.*, 2007), so changes in the abundance of sterols may affect the ability of *Plasmodium* to infect mosquitoes. NPC2 also plays an important role in dengue resistance of *Ae. aegypti* mosquitoes and may play a role in a variety of infection systems (Jupatanakul *et al.*, 2013). A2MRAP (ASTE011001/AGAP003521) is a protein associated with the receptor for alpha-2-macroglobulins. Alpha-2-macroglobulin is an abundant protein that binds to a variety of ligands and is involved with the lectin-dependant cytolytic pathway in arthropods (Armstrong and Quigley, 1999). The diversity of ligands to which alpha-2-macroglobulin can bind and its importance for the lysis of cells indicate that it, and its receptors, could be important for the lysis of *Plasmodium* infected cells and help mosquitoes fight off the parasite. Finally, LRTP (ASTE008359/AGAP007061) bears structural similarity to other proteins containing leucine-rich repeats. Two such proteins, LRIM1 and APL1C, have been shown to be important for the anti-*Plasmodium* defense in *An. gambiae* (Dong *et al.*, 2006; Povelones *et al.*, 2011), suggesting that similar proteins may also act against *Plasmodium* in *An. stephensi*.

The lack of an increase in *Plasmodium* oocysts per midgut following knockdown of SCRBQ1 is likely a result of gene redundancy, with alternative proteins also controlling

apoptosis. CRQ is largely expressed in the *Drosophila* embryo and not in adults, and it is possible that SCRBP1 is similarly more active during mosquitoes' immature stages than in adults. Both AGBP1 and NPC2 are known to be immune modulators for other parasites and may simply display specificity for bacteria and viruses and not act against *Plasmodium*. Knockdown of both A2MRAP and LRTP led to significant increases in *Plasmodium* infection, indicating that these proteins are important in modulating *P. falciparum* infection in the mosquito.

3.4 Serine proteases affect mosquito anti-*Plasmodium* defenses

A large number of proteases and digestive enzymes were highly up-regulated at both the protein and transcript levels. Serine proteases and other proteolytic enzymes are often part of proteolytic cascades that can lead to the amplification of signals that control downstream effector mechanisms (An *et al.*, 2010). In mosquitoes, some serine proteases have been implicated in blood digestion (Yang and Davies, 1971), the anti-*Plasmodium* defense (Blumberg *et al.*, 2013; Volz *et al.*, 2005), signal transduction, and many other diverse functions. The TOLL immune pathway, for instance, is activated through a serine protease-dependent signaling cascade (Ligoxygakis *et al.*, 2002; Weber *et al.*, 2003). A number of serpins are involved in the prophenoloxidase (PPO) activation cascade, an important part of the innate immune system (Christophides *et al.*, 2002; Ligoxygakis *et al.*, 2003; Weber *et al.*, 2003), but the functions of many other serpins have yet to be elucidated. The presence of many proteolytic regulators, including serine proteases and their inhibitors, in the highly up-regulated gene group at both the transcript and protein levels indicates that serine proteases play a role in the IMD pathway-based immune response and the mosquito anti-*Plasmodium* defense. It may also help to explain the broad diversity of genes affected by increased expression of Rel2, since serine protease-dependent signaling cascades can both amplify and diversify the signal, causing changes in the regulation of many different genes.

Given the significant up-regulation of numerous proteases and related enzymes at both the protein and transcript levels, we decided to investigate a number of them further. Serine protease inhibitor 10 (SRPN10), Rel2-responsive serine protease 1 (R2RSP1), Rel2-responsive serine protease 2 (R2RSP2), serine protease precursor 1 (SEPRP1), trypsin precursor (TRYPP), angiotensin converting enzyme precursor (ACEP), and serine protease precursor 2 (SEPRP2) were all chosen because they showed at least a 2-fold induction at the transcript level at either 6 or 12 h PBM and at the protein level 24 h PBM in the midgut. Silencing of R2RSP2, ACEP, and SEPRP2 significantly increased oocyst loads, suggesting that these factors are *P. falciparum* antagonists (Kruskal-Wallis test, $p < 0.0001$; Dunn's post-hoc test for R2RSP2, $p < 0.01$, ACEP $p < 0.05$, SEPRP2 $p < 0.001$). However, as with the immune genes, none of the silenced genes had a significant effect on oocyst prevalence. Again, this may be due to the limited activity of only a few genes, when compared to the overall effects of the full IMD pathway-based response. Serine proteases act in diverse processes in mosquitoes and other dipterans, including activating signaling cascades (such as the Toll and PPO cascades) and regulating development. SRPN10 (ASTE007248/AGAP005246) is one of many serine protease inhibitors (serpins) found in mosquitoes. Studies in *An. gambiae* have shown that some isoforms of SRPN10 are up-regulated during the parasite's traversal of the midgut and may be involved in anti-*Plasmodium* defense

(Danielli *et al.*, 2005). R2RSP1 (ASTE006240/AGAP007142) and R2RSP2 (ASTE014104/AGAP007165) are both examples of trypsin-like serine proteases. SEPRP1 (ASTE009202/AGAP005065) and SEPRP2 (ASTE010540/AGAP005310) are both precursors to serine proteases and may be involved in many of the same functions as R2RSP1 and R2RSP2. TRYPP (ASTE010330/AGAP006709) is a precursor for a chymotrypsin which are proteolytic enzymes and form a subset of serine proteases that play a role in the digestion of mosquito blood meals (Yang and Davies, 1971). Serine proteases similar to those identified in this study cleave a variety of targets and may have many different functions, including the regulation of anti-*Plasmodium* activity. Finally, ACEP (ASTE004060/AGAP004563) is a precursor for angiotensin converting enzyme (ACE), a family of proteins found in the hemolymph of insects and cleave a broad range of substrates (Burnham *et al.*, 2005; Riordan, 2003). While various ACE-like proteins have been studied in *An. gambiae* and an immune function has been suggested, their potential effect on *Plasmodium* has not been investigated.

Two serpins, SRPN6 and SRPN7, have previously been shown to play roles in the anti-*Plasmodium* defense (Abraham *et al.*, 2005; Blumberg *et al.*, 2013), and we have identified a series of new serine proteases and precursors that also play a role in this defense. R2RSP2 is one of many mosquito serine proteases and may play a role in amplifying the immune signal. Other serine proteases have also been shown to be vital for the melanization response in mosquitoes (Christophides *et al.*, 2002), which can help to clear parasites; therefore, it is possible the R2RSP2 increases melanization, although we have not measured this specific immune action. While ACEs have not been shown to have a direct effect on *Plasmodium* infection in mosquitoes, it has been suggested that they can cleave immune-related substrates and thereby alter immune activity. Our results demonstrate that ACEP has anti-*Plasmodium* activity in *An. stephensi* mosquitoes. Further investigation is needed to determine whether this activity is a direct or indirect effect. Similarly, since SEPRP2 was identified as a precursor for an unknown serine protease, it is difficult to accurately predict all effects of altering the expression patterns of this gene, but, given the ability of serine proteases to act against *Plasmodium* discussed above, a precursor of any one of a number of serine proteases has the potential to be important for insect immunity.

3.5 Effect of anti-*Plasmodium* effector genes on midgut bacterial load

In order to determine whether the genes identified previously as having an anti-*Plasmodium* effect act as general immune factors or are *Plasmodium*-specific, we tested them for involvement in controlling the mosquito midgut microbiota. The mosquito midgut is colonized by a variety of bacteria that need to be tightly controlled to prevent overproliferation and damage to the insect host (Pumpuni *et al.*, 1996; Straif *et al.*, 1998). Many different bacterial strains can be present in the mosquito gut, and the community varies from mosquito to mosquito and species to species; however, Gram-negative bacteria are considered to make up the majority of the species (Straif *et al.*, 1998). Previous studies have implicated the IMD pathway as the main pathway involved in controlling the levels of bacteria in the mosquito midgut (Dong *et al.*, 2009). Similarly, other studies have shown that the midgut microbiota is necessary to stimulate and prime the mosquito immune system and prepare it for future challenge (Clayton *et al.*, 2012; Dong *et al.*, 2009). Some anti-

Plasmodium factors also act against the midgut microbiota, while others do not. Therefore, we tested our newly identified anti-*Plasmodium* effectors for an effect on the levels of bacteria in the mosquito midgut. The number of culturable bacteria per mosquito midgut was quantified by CFU assays following RNAi knockdown of potential novel anti-*Plasmodium* effector genes. Thus, we tested A2MRAP, LRTP, R2RSP2, ACEP, and SEPRP2 for antibacterial effect in both sugar-fed and blood-fed mosquitoes. In sugar-fed mosquitoes, one-way ANOVA showed a significant change in the log transformed number of colonies per midgut following knockdown of A2MRAP and LRTP (ANOVA $p < 0.05$); however, a Dunnett's multiple comparison test showed no difference between either A2MRAP or LRTP and the GFP control, indicating that neither gene significantly affects midgut bacterial load (Fig 5A). The ANOVA revealed no significant differences between GFP, R2RSP2, ACEP, and SEPRP2 in sugar-fed mosquitoes (Fig 5B). Following a blood meal, ANOVA revealed a highly significant difference in bacterial load between GFP, A2MRAP, and LRTP (ANOVA $p < 0.0001$), and a Dunnett's multiple comparison test showed a significant increase in bacterial load following knockdown of A2MRAP (Dunnett's $p < 0.001$) (Fig 5C). As with the sugar meal, there was no significant difference in bacterial loads after knockdown of GFP, R2RSP2, ACEP, or SEPRP2 after a blood meal (Fig 5D).

The fact that none of the five genes tested had any effect on culturable bacteria levels in the sugar-fed mosquito midgut may indicate that the midgut microbiota is somewhat stable at this point. The midgut microbiota is adapted to the midgut environment and may therefore be able to evade action by various mosquito immune effectors. Alternatively, while multiple immune genes are able to affect the midgut microbiota, the genes we tested may display specificity for *Plasmodium* parasites and therefore not affect bacteria to a considerable degree. Specificity in mosquito immune response is common, since even pathogens in the same genus, such as *P. falciparum* and *P. berghei*, elicit strikingly different immune responses (Garver *et al.*, 2009), and infection by the two is controlled by separate immune pathways. Thus, it is likely that highly divergent pathogens such as *Plasmodium* and bacteria would also be affected differently. Similarly, we saw no effect of AGBP1 on *Plasmodium* infection, although it has been shown to have an effect on *Staphylococcus aureus* infection in the mosquito (Dong *et al.*, 2006), adding credence to the theory of divergent immune action.

Following a blood meal, only A2MRAP had an effect on bacterial load. Blood meals have been shown in the past to have a large effect on the midgut microbiota of mosquitoes, leading to a large increase in the number of bacteria (Oliveira *et al.*, 2011). This disturbance and proliferation may allow more opportunity for anti-bacterial genes to take effect. Alternatively, the expansion in bacterial numbers following a blood meal may allow for dangerous levels of bacteria in the midgut that need to be controlled by the immune system in order to prevent damage to the mosquito. Alpha-2-macroglobulins have relatively broad binding specificities (Riordan, 2003), which may explain why this protein can act against divergent pathogens such as *Plasmodium* and bacteria. Alternatively, since A2MRAP is an alpha-2-macroglobulin receptor-associated protein, it may be able to bind to multiple

different alpha-2-macroglobulin receptors and therefore have a broad specificity based on the different combinations of alpha-2-macroglobulins and their receptors.

Our findings support the hypothesis that the mosquito's immune system is able to react in a specific manner to different pathogens, especially when they are distantly related. In addition, the fact that many more genes are up-regulated following Rel2 induction in the midgut than in the fat body implies that mosquito immune pathways exhibit tissue specificity and that the IMD pathway may be more important for immune defense in the midgut than in the fat body. The IMD pathway acts specifically against Gram-negative bacteria, which make up the majority of the mosquito midgut microbiota (Straif *et al.*, 1998), and this pathway has been shown to be the major pathway involved in controlling the midgut microbiota in both mosquitoes and *Drosophila*. The greater expression of IMD pathway-responsive genes in the midgut than in the fat body may allow better control and a faster response to perturbations in the midgut microbiota.

Conclusions

In this work, we have measured the changes in expression, at both the transcript and protein level, in the midguts and fat bodies of *P. falciparum*-resistant, genetically modified mosquitoes that are transiently overexpressing the IMD pathway-regulated transcription factor Rel2 (Dong *et al.*, 2011). Through a combination of full-genome microarray-based expression analyses and iTRAQ proteomic analyses, we were able to see that specific up-regulation of Rel2 leads to differential regulation of a large number of both immune-related and other genes, including general immune genes and a large number of serine proteases cascade-related genes. We measured the effect of knockdown of multiple immune genes including serine protease cascade-related genes on *P. falciparum* infection in mosquitoes, thereby identifying a number of novel genes implicated in anti-*Plasmodium* defense. The presence of multiple serine proteases and their observed effect on *Plasmodium* infection indicate that these genes may be responsible for expanding and amplifying the IMD pathway signal and support the importance of serine proteases in the mosquito's immune defense. Of the five newly identified anti-*Plasmodium* genes, only one, A2MRAP, had an effect on the mosquito microbiota, and only after a blood meal. Finally, by observing significant changes at both the transcript and protein levels, we were able to look for any correlation between transcript and protein levels. In the midguts of our GM mosquitoes, we observed no correlation between the two, while in the fat body we saw a significant correlation, although many fewer genes were included in the fat body analysis. The lack of a strong correlation between transcripts and proteins concurs with the results of other studies that have observed a similar lack of correlation, and it likely indicates a large role for post-transcriptional processing and control of translation, allowing mosquitoes to have a finer level of control over protein expression. Overall, our results indicate that Rel2 has a significant impact on both the mosquito transcriptome and proteome. The patterns of differential gene expression in the fat body were similar to those seen in transgenic *Ae. aegypti* overexpressing Rel2 under the VG promoter, indicating that these pathways may be well conserved across mosquito species (Zou *et al.*, 2011). Also, as expected, we found that a number of genes controlled by the IMD pathway have an effect on anti-*Plasmodium* defenses, while others have no known immune function. This result indicates that the IMD pathway, and Rel2

specifically, controls or affects the expression of many non-immune processes. By means of these effects, the mosquito's immune response may be influenced by many factors that have not previously been considered to be part of canonical immune pathways. Similarly, induction of the mosquito immune system may alter many non-immune processes that can have far-reaching implications for mosquito fecundity and fitness. Overall, these results indicate that mosquito immune pathways act on a global level to produce complex changes in gene expression that will require further investigation to unravel fully.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

IMD	Immune Deficiency
PRR	pattern recognition receptors
PAMPs	pathogen associated molecular patterns
CP15	carboxypeptidase gene promoter –driven Rel2 line 15
VG	vitellogenin gene promoter –driven Rel2 line
GO	gene ontology
RNAi	RNA interference
iTRAQ	isobaric tags for relative and absolute quantitation
dsRNA	double-stranded RNA
qRT-PCR	quantitative Real-Time PCR
CFUs	colony forming units
PBM	post-blood meal
PSMs	peptide spectrum matches
AgMDL6	<i>An. gambiae</i> MD2-like protein 6
NLRR3	neuronal leucine-rich repeat protein 3
SCRBQ1	scavenger receptor BQ1 domain

AGBP1	bacteria response protein 1
NPC2	Neimann-Pick type C-2
A2MRAP	alpha-2-macroglobulin receptor-associated protein
LRTP	leucine-rich transmembrane protein
CRQ	Croquemort
SRPN10	serine protease inhibitor 10
R2RSP1	Rel2-responsive serine protease 1
R2RSP2	Rel2-responsive serine protease 2
SEPRP1	serine protease precursor 1
TRYPP	trypsin precursor
ACEP	angiotensin converting enzyme precursor
SEPRP2	serine protease precursor 2
GFP	green fluorescent protein
SRPN10	serpin 10
R2RSP1	Rel2-responsive serine protease 1
R2RSP2	Rel2-responsive serine protease 2
SEPRP1	serine protease precursor 1
TRYPP	trypsin precursor
ACEP	angiotensin-converting enzyme precursor
SEPRP2	serine protease precursor 2

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Highlights

1. Transgenic over-expression of the IMD immune pathway-controlled transcription factor Rel2 affects the expression of numerous genes with diverse functions, suggesting a broader physiological role of this immune pathway.
2. Our study has identified multiple novel *A.stephensi* anti-*Plasmodium* effectors.

Figure 1. Global changes in transcript levels in transgenic *A. stephensi* following Rel2 induction

A) The total number of genes significantly up- or down-regulated that are predicted to be in each GO category. Genes were considered significantly differentially regulated if the -fold change was ≥ 0.75 on a $\log_2$ scale. B) Venn diagram comparing the total number of regulated transcripts between the midgut of CP line mosquitoes and fat body of VG line mosquitoes at 12 h PBM. Red arrows correspond to midgut samples, and green arrows correspond to fat body samples; the arrow direction indicates significant up- or down-regulation.

Figure 2. Global changes in protein levels in transgenic *A. stephensi* following Rel2 induction

A) The total number of proteins significantly up- or down-regulated that are predicted to be in each GO category. Genes were considered significantly differentially regulated if the ratio of transgenic to wild type was ≥ 0.75 on a $\log_2$ scale. B) Venn diagram comparing the total number of regulated proteins between the midgut of CP line mosquitoes and fat body of VG line mosquitoes at 24 h PBM. Red arrows correspond to midgut samples, and green arrows correspond to fat body samples; the arrow direction indicates significant up- or down-regulation.

Figure 3. *P. falciparum* infection intensity following RNAi knockdown of immune genes

The number of oocysts per midgut of wild-type *A. stephensi* following RNAi-mediated depletion of GFP, SCRQB1, bacterial response protein (AGBP1), Niemann-Pick type-C (NPC2), alpha-2-macroglobulin (A2MRAP), or leucine-rich transmembrane protein (LRTP). Depletion of both A2MRAP and LRTP led to a significant increase in the number of oocysts per mosquito midgut. Each circle represents a single midgut, and horizontal black bars represent the median of the sample. Significance was determined by a Kruskal-Wallis test followed by Dunn's post-hoc test to compare immune-depleted mosquitoes to GFP controls. Significance was assessed at $\alpha=0.05$. Supplementary data for this figure is given in table S2.

Figure 4. *P. falciparum* infection intensity following RNAi knockdown of protease genes

The number of oocysts per midgut of wild-type *A. stephensi* following RNAi-mediated depletion of: A) green fluorescent protein (GFP), serpin 10 (SRPN10), Rel2-responsive serine protease 1 (R2RSP1), Rel2-responsive serine protease 2 (R2RSP2), or serine protease precursor 1 (SEPRP1); and B) trypsin precursor (TRYPP), angiotensin-converting enzyme precursor (ACEP), or serine protease precursor 2 (SEPRP2). Silencing of R2RSP2, ACEP, and SEPRP2 all led to significant increases in the number of oocysts per midgut. Each circle represents a single midgut, and horizontal black bars represent the median of the sample. Significance was determined by a Kruskal-Wallis test followed by Dunn's post-hoc test to compare immune-depleted mosquitoes to GFP controls. Significance was assessed at $\alpha=0.05$. Supplementary data for this figure is given in table S2.

Figure 5. Influence of novel anti-*Plasmodium* genes on midgut microbiota

The number of colony forming units of culturable bacteria in the midguts of females following RNAi-mediated knockdown of genes shown above to have anti-*Plasmodium* effects. A) Sugar-fed mosquitoes following depletion of GFP, A2MRAP, and LRTP. B) Sugar-fed mosquitoes following depletion of GFP, R2RSP2, ACEP, and SEPRP2. C) Blood-fed mosquitoes following depletion of GFP, A2MRAP, and LRTP showed that A2MRAP depletion leads to a significant increase in CFUs per midgut. D) Blood-fed mosquitoes following depletion of GFP, R2RSP2, ACEP, and SEPRP2. Significance was determined by a one-way ANOVA, followed by Dunnett's multiple comparison test, with significance assessed at $\alpha=0.05$. Bars represent the mean of three biological replicates, and error bars represent the standard error of the mean.