An evolutionary conserved function of the JAK-STAT pathway in anti-dengue defense

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Here, we show that the major mosquito vector for dengue virus uses the JAK-STAT pathway to control virus infection. Dengue virus infection in *Aedes aegypti* mosquitoes activates the JAK-STAT immune signaling pathway. The mosquito's susceptibility to dengue virus infection increases when the JAK-STAT pathway is suppressed through RNAi depletion of its receptor Domeless (Dome) and the Janus kinase (Hop), whereas mosquitoes become more resistant to the virus when the negative regulator of the JAK-STAT pathway, PIAS, is silenced. The JAK-STAT pathway exerts its anti-dengue activity presumably through one or several STAT-regulated effectors. We have identified, and partially characterized, two JAK-STAT pathway-regulated and infection-responsive dengue virus restriction factors (DVRFs) that contain putative STAT-binding sites in their promoter regions. Our data suggest that the JAK-STAT pathway is part of the *A. aegypti* mosquito's anti-dengue defense and may act independently of the Toll pathway and the RNAi-mediated antiviral defenses.

Aedes aegypti | dengue fever | innate immunity | resistance | transcriptome

Dengue fever has become the most important arthropod-borne viral disease and thereby a major public health concern worldwide (1). The dengue virus (DENV) is an enveloped, positive-stranded RNA virus of the Flaviviridae family that comprises four closely related but antigenically distinct serotypes (DENV-1 to -4). Dengue virus causes both dengue and dengue hemorrhagic fever in human populations and the *Aedes aegypti* mosquito is its principal vector, while virus transmission can also be supported by the closely related secondary vector species *Aedes albopictus* (2). The dengue virus will first infect the mosquito midgut upon ingestion of infected blood, then replicate and bud off to infect other tissues such as the trachea, fat body and the salivary glands from where they can be transmitted to a new host upon a second blood meal (3).

Mosquitoes have, similar to other insects, an effective innate immune system that is capable of defending against a variety of invaders (4–6). The insect innate immune responses are largely orchestrated by three major signaling pathways, the Toll, the Immune Deficiency (Imd) and the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathways (7–11). The Toll pathway is mainly activated by Gram-positive bacteria, fungi (6, 12) and virus (13), and largely controls the expression of AMPs, whereas the Imd pathway is mainly Gram-negative bacteria responsive and controls the synthesis of several AMPs (6, 12). In contrast to the well-characterized Toll and Imd immune pathways, relatively little is known about the transcriptional responses induced by the JAK-STAT pathway, although this pathway has been implicated in insect immunity (8, 14). Activation of the canonical *Drosophila melanogaster* JAK-STAT pathway (Fig. 1A), is initiated by the extracellular binding of the unpaired ligand Upd to the transmembrane receptor Domeless (Dome), a distant homolog of the vertebrate type I cytokine receptors (15), which will undergo a conformational transformation leading to the self-phosphorylation of the associated

JAKs (Hop). The activated Hop will in turn phosphorylate Dome, resulting in the formation of docking sites for the cytoplasmic STATs. The recruitment of STATs by the Dome/Hop complex will induce STATs phosphorylation and dimerization that ultimately leads to its translocation into the nucleus where it will activate transcription of specific target genes. Two negative regulators, PIAS (protein inhibitor of activated STAT) and SOCS (suppressor of cytokine signaling), have been shown to suppress the JAK-STAT pathway in *D. melanogaster* (16). The completion of the *A. aegypti* genome sequence (17) and its comparative genomic analysis to *D. melanogaster* and *Anopheles gambiae* have identified orthologs for the core JAK-STAT pathway components (Dome, Hop and STAT) in these three species (18). We have further identified orthologs for PIAS (SUMO) and SOCS and used comparative genomics to confirm the proximity and evolutionary conservation of the JAK-STAT pathway between insects and mammals (Fig. 1B and Fig. S1).

The JAK-STAT pathway was originally identified as a cytokine signaling pathway in mammals (19–23), and shown to be a key player in antiviral (24, 25) including anti-dengue defenses (26). The JAK-STAT pathway has also been implicated in the control of the *Drosophila* C virus (DCV), suggesting a conserved function of the JAK-STAT signaling pathway in antiviral defenses in humans and insects (8). Furthermore, we have shown that two putative JAK-STAT pathway genes, *dome* and *SOCS*, are induced upon dengue virus infection (13). This finding suggested involvement of this pathway in the mosquito's anti-dengue defenses, along with the Toll pathway (13) and the RNAi-mediated defenses (27). Here, we show that the JAK-STAT immune signaling pathway is a key player in the *A. aegypti* anti-dengue defenses, and we present two putative JAK-STAT pathway-regulated dengue virus restriction factors (DVRF1 and -2).

Results

JAK-STAT Pathway Controls Dengue Virus Infection in *A. aegypti*. Our previous studies have shown that the Toll pathway is activated by, and controls, dengue virus infection in the *A. aegypti* mosquito vector (13). The dengue virus infection responsive nature of the JAK-STAT pathway genes, *dome* and *SOCS*, also suggested the implication of this pathway in the mosquito's anti-dengue defense. To investigate this hypothesis, we have used a combination of reverse genetic and transcriptomic analyses. We rendered mosquitoes more resistant or more susceptible to DENV-2 through RNAi-mediated gene silencing of the JAK-STAT pathway negative regulator PIAS or positive regulators Hop and Dome, respectively, as a measure of altered virus loads in the

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Fig. 1. The canonical JAK-STAT signaling cascade. (A) Core JAK-STAT pathway factors identified in *Drosophila* and their putative roles in the cascade. (B) Evolutionary conservation of the JAK-STAT immune signaling in insects and humans. Insect orthologs are indicated with solid black circles at the branch node. Genes that are transcriptionally regulated by the insect STAT are indicated with a star. The pathway core intracellular machinery is highly conserved in human and insects. The upstream ligands and effectors are less conserved, which may suggest a host-pathogen-specific response. Accession numbers are presented in Fig. S1.

midgut tissue at 3 and 7 days post infectious blood meal (pibm) (Fig. 2). The RNAi-mediated silencing of these negative and positive regulators simulates the transient activation or repression, respectively, of the pathway. PIAS gene silencing (77% silencing efficiency) reduced DENV-2 infection by ≈ 2 ($P = 0.44$) and 5-fold ($P < 0.05$) at 3 and 7 days after pibm, respectively (Fig. 2A and B). Conversely, both Hop gene silencing (60% silencing efficiency) and Dome gene silencing (69% silencing efficiency) resulted in an increase of virus load at 3 and 7 days pibm, respectively; Hop depletion led to a 2.3-fold ($P = 0.07$) increase in infection at 3 days pibm (Fig. 2A) and Dome depletion resulted in a 3-fold increase of virus loads ($P < 0.05$) at 7 days pibm (Fig. 2B) compared with their respective *GFP* dsRNA-treated control mosquitoes. Although nonsignificant when compared with their respective *GFP* dsRNA-treated controls, the

effect of Hop and PIAS gene silencing on DENV-2 infection becomes statistically significant if compared with each other ($P < 0.01$) and follow the same trend demonstrated by repression or activation of the JAK-STAT pathway at 7 days pibm. Our data show that the pathway is acting against the dengue virus in the mosquito midgut as early as 3 days after virus ingestion.

Dissecting the JAK-STAT Pathway-Regulated Transcriptome and Its Activation in Response to Dengue Virus Infection. The implication of the JAK-STAT pathway in limiting DENV-2 infection suggests that this immune pathway regulates transcription of one or several dengue virus restriction factors. The JAK-STAT pathway-regulated transcriptome in mosquitoes and other organisms has not been characterized at a genome-wide level as yet. To identify candidate JAK-STAT pathway-regulated anti-dengue effector genes, we determined the *A. aegypti* putative JAK-STAT pathway-regulated transcriptome by comparing the transcript abundance between *PIAS* dsRNA-treated and *GFP* dsRNA-treated control mosquitoes, at 4 days after dsRNA injection, using high-density oligonucleotide microarrays. Similar approaches have been fruitfully used to determine the Toll and Imd pathway regulated transcriptomes in *A. aegypti* and *A. gambiae* mosquitoes (13, 28). The depletion of *PIAS* resulted in the up-regulation of 63 genes and down-regulation of 66 genes, belonging to diverse functional groups (Fig. 3A and Table S1). Only 14% of the putative JAK-STAT-regulated genes could be assigned putative immune functions based on sequence homology, whereas as much as 22% of the regulated genes were of unknown function. Interestingly, a putative Toll receptor ligand gene, *spätzle 6* (*spz6*, AEEL012164), was up-regulated, and five anti-microbial peptide genes (four Cecropin A-like genes: AEEL000611, AEEL000627, AEEL015515, and AEEL000598 and one Defensin 1-like gene: AEEL003832) were down-regulated. This may indicate a competition of resources between the JAK-STAT pathway and the other immune pathways.

To assess the extent of JAK-STAT pathway activation by DENV-2 infection, at the transcriptomic level, we compared the

Fig. 2. The JAK-STAT pathway modulates mosquito susceptibility to dengue infection. Dengue titers in the midguts of *PIAS* dsRNA-, *hop* dsRNA-, *dome* dsRNA-, and *GFP* dsRNA-injected mosquitoes were determined by plaque assays in C6/36 cells at 3 (A) or 7 (B) days after feeding on DENV-2-infected blood. The significance of gene silencing effect on DENV-2 loads in experimental samples, compared with dsGFP-treated controls, was determined by Student's *t* test. *, $P < 0.05$. Standard deviation bars for four biological replicates are shown.

Fig. 3. The JAK-STAT pathway-regulated transcriptome and its activation upon dengue virus infection. (A) Functional classification of the JAK-STAT pathway-regulated transcriptome according to refs. 17 and 18. The graph shows the functional class distributions of genes that are regulated upon PIAS gene silencing (see Table S1 for complete gene expression dataset). Up- and down-regulated genes are indicated by + and −, respectively. Functional group abbreviations are: IMM, immunity; R/S/M, redox, stress and mitochondrion; CSR, chemosensory reception; DIG, blood and sugar food digestive; C/S, cytoskeletal and structural; TRP, transport; R/T/T, replication, transcription, and translation; MET, metabolism; DIV, diverse functions; UNK, unknown functions. (B) Venn diagram representing unique and shared gene regulation in PIAS- and Cactus-depleted mosquitoes or dengue infected carcasses (DENV CAR). The overlapping regions represent genes that are concomitantly regulated in two or three experimental conditions. The direction of gene regulation is represented by upward-pointing and downward-pointing arrows. (C) Hierarchical cluster analysis of the genes that were regulated in at least two of the three experimental conditions.

putative JAK-STAT pathway regulated transcriptome to the DENV-2 infection-responsive midgut and carcass transcriptomes at 10 days pibm (13). We also coclustered the putative Toll pathway regulated transcriptome (13) to assess potential overlaps in gene regulation between the two anti-dengue immune pathways. This analysis revealed 18 genes that were transcriptionally induced by both PIAS gene-silenced and DENV-2-infected mosquitoes (Fig. 3B). Interestingly, none of these genes were induced in Cactus-depleted (Toll pathway-activated) mosquitoes, suggesting that the JAK-STAT and the Toll immune pathways modulate DENV-2 infection through different factors and mechanisms. However, the concomitant down-regulation of 36 genes by both pathways corroborates certain relationships that may mainly involve developmental processes (7, 8, 29–31).

Hierarchical clustering of genes that were differentially expressed in at least two of the three experiments (*PIAS* silencing, *cactus* silencing, and DENV-2 infection responses in the carcass tissue) generated six distinct expression clusters (Fig. 3C and Table S2). All 18 JAK-STAT pathway induced and DENV-2 infection responsive genes were grouped in the same cluster (Fig. 3C, Cluster VI, and Table S2), further supporting the relationship between the JAK-STAT pathway and anti-dengue immunity. Expression cluster VI comprises six genes of putatively known functions: two t-box transcription factors, *tbx6* (AAEL004174) and *tbx20* (AAEL014429); a zinc finger protein (*ZFP*, AAEL009508); a *cadherin* (AAEL000700); a forkhead box protein (*foxn2*, AAEL009900); and a host cell factor C1 (*HCF1*, AAEL008905) that belongs to a gene family that has been linked to host-virus interactions (32–35). The majority (12 of 18) of genes in cluster VI have no known function. Expression cluster V (Fig. 3C, Cluster V, and Table S2) is mostly populated by known immune genes (18 of 19) that are induced by *cactus* silencing (Toll pathway) but not by *PIAS* silencing. One Toll pathway-induced Cecropin gene (*cecA*-like) (AAEL015515) was suppressed by JAK-STAT activation, further suggesting that the JAK-STAT and the Toll pathway are acting independently against DENV-2.

Screening and Characterization of PIAS-Suppressed (Putative JAK-STAT-Induced) Dengue Virus Restriction Factors (DVRFs). To identify putative anti-dengue factors among the 18 genes in cluster VI that were induced by both PIAS gene silencing (putative JAK-STAT-induced) and DENV-2 infection, we considered their expression patterns along with sequence characteristics that could be indicative of antiviral proteins. We selected the *ZFP*, *foxn2*, *HCF1*, and two additional genes that coded for hypothetical proteins, *Q1HR00* [AAEL008492, putative secreted protein, (36)] and *AAEL000896*. The selection of *AAEL000896* and *Q1HR00* was purely based on protein features such as a predicted signal peptide (Table S3).

We used an established RNAi-mediated gene silencing approach, in conjunction with DENV-2 infection assays, to test the putative implication of the five selected gene candidates in anti-dengue defense (Fig. 4). This screen confirmed two of these genes, *QIHR00* (33% silencing efficiency) and *AAEL000896* (50% silencing efficiency) as putative JAK-STAT pathway-regulated dengue virus restriction factors *DVRF1* and *DVRF2*, respectively. RNAi-mediated depletion of *DVRF1* resulted in a 2.5-fold increase of DENV-2 infection ($P < 0.05$), and silencing of *DVRF2* resulted in 2.2-fold increased infection in midgut tissue ($P < 0.05$) when compared with *GFP* dsRNA-treated control mosquitoes. The lack of effects on virus infection for the other candidate genes does not exclude their implication in the anti-dengue defense because they could be acting in different tissues and/or at different infection stages.

To validate the JAK-STAT pathway regulation of DVRF1 and DVRF2, we investigated the effect of PIAS gene silencing on their transcript abundance. PIAS depletion resulted in 1.7- and 1.8-fold induction of *DVRF1* and *DVRF2*, respectively. To confirm the relationship between the JAK-STAT pathway and the anti-dengue effectors, we also performed double gene-silencing assays, in which the effect of PIAS depletion on the DVRFs gene expression was reversed through simultaneous Dome depletion (Fig. S4 and Table S4). To confirm the DENV-2 infection-responsive nature of the DVRFs, we compared the relative

Fig. 4. RNAi screen for anti-dengue effectors. Five JAK-STAT-regulated dengue virus-responsive genes were selected for this screen. Dengue titers in the midguts of *Q1HR00* dsRNA-, *ZFP* dsRNA-, *HCF1* dsRNA-, *AEEL000896* dsRNA-, *foxn2* dsRNA-, and *GFP* dsRNA-injected mosquitoes were determined by plaque assays in C6/36 cells at 7 days after feeding on DENV-2-infected blood. The significance of gene silencing effect on DENV-2 loads in experimental samples, compared with controls, was determined by Student's *t* test. *, *P* < 0.05. Standard deviation bars for four biological replicates are shown.

mRNA abundance of these two genes in the whole body of uninfected and DENV-2-infected adult females at 24 h pibm. Dengue virus infection induced *DVRF1* by a 3-fold and *DVRF2* by a 3.6-fold (Fig. 5B and Table S4). The activation of the JAK-STAT pathway at this early stage of dengue virus infection was also confirmed by measuring the relative expression of the JAK-STAT pathway reporter gene *SOCS* with qRT-PCR. At 24 h pibm, *SOCS* was 3.4-fold up-regulated in response to DENV-2 infection (Fig. 5B and Table S4).

To test whether the JAK-STAT pathway is a general anti-dengue defense system in *A. aegypti* or whether it is specific for the Rockefeller strain, we also evaluated the activation of these genes in the whole body of the recently colonized *A. aegypti* Costa Rica strain (CRICA) upon infection with Vero cell (mammalian)-derived dengue virus (mDENV-2) at 24 h pibm. We used the mammalian cell-derived virus to better simulate a

Fig. 5. Validation of *DVRF1* and *DVRF2* JAK-STAT pathway-regulated transcription and dengue virus-responsive activation. (A) Bars represent *DVRF* gene expression compared with respective GFP dsRNA-treated controls under single or double gene silencing of *PIAS* and *dome*. (B) *DVRFs* and *SOCS* gene expression at 24 h after DENV-2 infection, compared with uninfected blood-fed controls. (C) *DVRFs* and *SOCS* gene expression in *A. aegypti* Costa Rica strain at 24 h pibm with Vero cell-derived DENV-2, compared to uninfected blood-fed controls. Standard deviation bars for three biological replicates are shown.

Fig. 6. Molecular analysis of *DVRF1* and *DVRF2*. (A) Putative STAT-binding sites in the *DVRFs* promoter regions. The 1 kb upstream DNA sequences were analyzed by using the MatInspector program from Genomatix (<http://www.genomatix.de/products/MatInspector/MatInspector1.html>). (B) Signal peptide prediction was performed with SignalP 3.0.

field condition when mosquitoes feed on human-derived virus and thereby test whether the potentially different glycosylation pattern of the virion surface could influence the immune response. *DVRF2* and *SOCS* were 2- and 1.7-fold induced, respectively, in the CRICA/mDENV-2 combination (Fig. 5C and Table S4), suggesting that the JAK-STAT pathway is activated in different *A. aegypti* strains when infected with mammalian cell-derived virus. The lack of *DVRF1* induction is likely to indicate variations in function and regulation patterns for this gene between different mosquito strains.

At least one STAT-binding site, TTCN₂₋₄GAA, was identified within the 1-kb upstream genome region of both *DVRFs* (Fig. 6A and Table S5) (37). *DVRF1* encodes a putative 120-aa transmembrane protein, suggesting a putative receptor function. The 210-aa predicted polypeptide encoded by *DVRF2* is a putative secreted protein that contains putative antifreezing 1 (AF1) and allergen C (All.C) domains (Fig. 6B and Table S6) and shares 64% and 53% identity with the *A. gambiae* cuticular proteins 1 (CPF1, AGAP010900) and 2 (CPF2, AGAP010901), respectively.

Discussion

The evolutionary conserved JAK-STAT pathway mediates mammalian cytokine signaling and is involved in antiviral defense in mammals and flies (8, 19–25). Here, we show that the *A. aegypti* JAK-STAT pathway is involved in anti-dengue defense.

The many unknown genes in the *PIAS* depletion-regulated and DENV-2 infection-responsive transcriptomes is a reflection of the largely understudied nature of the JAK-STAT pathway and its unrelatedness to the better characterized Toll and Imd pathways. The large proportion on non-immunity-related genes suggests implication of JAK-STAT in other biological processes such as development (30, 38–42). In *Drosophila*, both Toll and JAK-STAT have been linked to immunity and development (7, 8, 29–31). Interestingly, none of the immune genes that were coregulated by DENV-2 infection and the Toll pathway (13) were regulated by *PIAS* depletion, further suggesting that the Toll and the JAK-STAT pathways represent two independent anti-dengue defense systems. Although the JAK-STAT pathway activation upon bacterial infection has been previously reported (14, 38, 43, 44), RNAi-mediated suppression or activation of the *A. aegypti* JAK-STAT pathway resulted only in a marginal but nonsignificant effect on mosquito survival upon *Escherichia coli* infection (Fig. S2A). No proliferation of opportunistic bacteria in the mosquito hemolymph and midgut could be detected when the pathway was silenced (Fig. S2B and C), corroborating earlier observations of Dostert et al. (8). These findings may suggest a more specialized role of the JAK-STAT pathway in antiviral defense. Future studies will address potential relationships be-

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