Mammalian African trypanosome VSG coat enhances tsetse's vector competence

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Tsetse flies are biological vectors of African trypanosomes, the protozoan parasites responsible for causing human and animal trypanosomiases across sub-Saharan Africa. Currently, no vaccines are available for disease prevention due to antigenic variation of the Variant Surface Glycoproteins (VSG) that coat parasites while they reside within mammalian hosts. As a result, interference with parasite development in the tsetse vector is being explored to reduce disease transmission. A major bottleneck to infection occurs as parasites attempt to colonize tsetse's midgut. One critical factor influencing this bottleneck is the fly's peritrophic matrix (PM), a semipermeable, chitinous barrier that lines the midgut. The mechanisms that enable trypanosomes to cross this barrier are currently unknown. Here, we determined that as parasites enter the tsetse's gut, VSG molecules released from trypanosomes are internalized by cells of the cardia—the tissue responsible for producing the PM. VSG internalization results in decreased expression of a tsetse microRNA (*mir-275*) and interferes with the Wnt-signaling pathway and the Iroquois/IRX transcription factor family. This interference reduces the function of the PM barrier and promotes parasite colonization of the gut early in the infection process. Manipulation of the insect midgut homeostasis by the mammalian parasite coat proteins is a novel function and indicates that VSG serves a dual role in trypanosome biology—that of facilitating transmission through its mammalian host and insect vector. We detail critical steps in the course of trypanosome infection establishment that can serve as novel targets to reduce the tsetse's vector competence and disease transmission.

tsetse | trypanosome | VSG | peritrophic matrix | vector competence

Insects are involved in transmission of fatal diseases to humans that collectively account for over 1 million deaths per year. Among these diseases are malaria, trypanosomiasis, and leishmaniasis, caused by the protozoan parasites *Plasmodium*, *Trypanosoma*, and *Leishmania*, respectively. Mosquitoes, tsetse flies, and sand flies, respectively, are required for transmission of these pathogens to their mammalian hosts. Disease control in humans is challenging due to lack of efficacious vaccines and emergence of drug resistance in parasites. Hence, reduction of the vector insect populations is an alternative approach to effectively curb disease. Current vector control methods rely on the use of insecticides, traps/targets, habitat modification, and release of sterile males—all of which decrease vector densities to interrupt disease transmission. However, the high cost of these methods, their reliance on community participation for sustainable implementation, and the widespread emergence of resistance in insects to the available insecticides threaten the efficacy of these approaches. An in-depth understanding of the molecular mechanisms that underlie vector–parasite associations can lead to the generation of alternative methods, such as modified insects that block parasite development, and thus prevent disease transmission.

Insects naturally possess multiple barriers that can prevent establishment of parasites in the gut early during the infection process. The first barrier that protozoan parasites encounter in the gut is the peritrophic matrix (PM), a thick semipermeable chitinous sheath that separates luminal contents from epithelial cells (1). For successful colonization of the gut, parasites must bypass the PM and translocate into the vector's ectoperitrophic space (area between the PM and gut epithelia). *Plasmodium* (2) and *Leishmania* (3) both produce chitinase enzymes that help degrade their vector host's PM during the course of blood digestion. African trypanosome genomes do not encode any protein(s) with putative chitinase function. Thus, the mechanism(s) by which trypanosomes traverse tsetse's PM remain controversial. Once the parasites invade the ectoperitrophic space of the gut, they are recognized by various immune-signaling pathways and molecules, which collectively result in their elimination in their respective insect vectors (4–6). In the susceptible tsetse where trypanosomes survive, parasites replicate and permanently colonize the ectoperitrophic space of the gut. These parasites subsequently undergo multiple stages of differentiation and eventually move to the salivary glands or mouthparts of the fly and mature into mammalian infective forms that cause disease in the next mammalian host (7). Here, we investigated the molecular mechanisms that influence trypanosome establishment dynamics in the adult tsetse gut early during the infection process.

Significance

Insects are responsible for transmitting protozoan parasites that cause fatal diseases in humans. While the underlying mechanisms by which parasites evade mammalian immune responses have been extensively studied, less is known about how parasites promote their survival in the insect vectors. An in-depth understanding of the molecular mechanisms that underlie vector–parasite associations can lead to the generation of novel transmission-blocking tools. Here, we show an adaptive coevolutionary process that enables the African trypanosomes to overcome the tsetse peritrophic matrix, a major barrier to infection outcome in the gut. Disrupting this manipulative process could prevent establishment of infections in the vector and reduce transmission.

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See Commentary on page 6821.

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Results and Discussion

Exposure to Trypanosomes Induces Expression of Functions Associated with Cell Signaling and Cell Remodeling. Experimental infection of newly eclosed tsetse adults (teneral) results in high infection prevalence (between ~25 and 70%) as flies at this stage present immature immune responses and an underdeveloped PM (8). Thus, teneral adults have been used as an efficient system to study trypanosome infection processes. Contrary to tenerals, mature adults that have acquired several blood meals display high resistance to trypanosomes (prevalence around 1%) (8, 9). Despite the high level of resistance in mature adults compared with tenerals, susceptible mature adults can still support parasite maturation and contribute to disease transmission (9). In natural settings, trypanosome infection prevalence in field-captured flies is also very low, but flies are commonly found to harbor coinfections with multiple trypanosome species (10, 11). Field flies also show an increase in infection prevalence as a function of fly age (12), suggesting that mature adults can acquire new infections and are epidemiologically relevant for disease transmission. Here, we used mature adults for transcriptome analyses in an effort to simulate parasite transmission dynamics under natural conditions. We provided 8-d-old flies bloodstream-form parasites in their fourth blood meal and then used high-throughput RNA-sequencing (RNA-seq) to monitor gut gene expression at 48 and 72 h post acquisition (hpa) of parasite-infected blood. For comparison, we performed identical experiments with control flies at 48 and 72 h post normal blood (hpb), respectively (overviews of the transcriptional analysis are shown in *SI Appendix*, Figs. S1 and S2). These temporal time points were selected based on prior studies, which showed induction of immune-related gene expression with impact on parasite infection outcome at 72 hpa in mature adults (13, 14).

Although minimal changes in gene expression were observed at 48 hpa, a significant number of tsetse genes exhibited differential expression at 72 hpa; 27% (1810) and 22% (1,507) of detectable transcripts were up- and down-regulated, respectively (Datasets S1 and S2). To obtain a global snapshot of tsetse responses that could influence parasite infection outcomes, differentially expressed genes were subjected to gene ontology analysis (Blast2GO). The majority of up-regulated gene products were associated with cell signaling (DNA/RNA binding, protein phosphorylation, and initiation of transcription) and cell-remodeling functions (cytoskeleton and actin binding) (*SI Appendix*, Fig. S3; Dataset S3). We also observed up-regulated immune-related genes, including several associated with the immune deficiency (Imd) (*Kenny* and *Imd*) and Toll (*cactus*, *dorsal/dif*, *DEAF-1*) signaling pathways (Fig. 1*A*). The Imd pathway produced antimicrobial peptides that have been shown to be important trypanolytic effectors (14, 16–18). The induction of gut immune-signaling pathways likely results from recognition of parasite-associated molecular patterns by the gut epithelia. Immune molecules produced by the gut epithelia, such as reactive oxygen species (19) or cardia-produced inducible nitric oxide (iNOS) (20), may further lead to systemic immune induction in other organs (21). Our previous study that investigated fat body responses in adult flies noted strong induction of the Imd pathway and one of its effector products (antimicrobial peptide *Attacin-A*) 72 h after exposure to trypanosomes (14). A systemic double-stranded RNA-based RNAi approach targeting the Imd pathway rendered tsetse highly susceptible to parasite infections (14). Thus, host recognition of parasites around 72 hpa initiates a cascade of responses that prevents parasite establishment in the majority of the individuals. Interestingly, neither tsetse PGRP-LB nor tsetse-EP, both of which restrict parasite infections (22, 23), were differentially expressed. This finding suggests that endogenous levels of these effector molecules already present in the gut at the time of parasite acquisition may interfere with parasite colonization or survival.

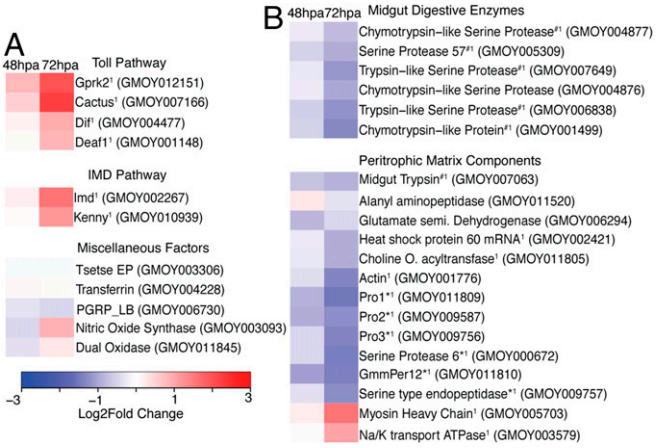

Fig. 1. Tsetse gut responses to trypanosome challenge. Transcriptome analysis of guts from 8-d-old adults after exposure to *T. b. rhodesiense* bloodstream parasites. (*A* and *B*) Heat maps denoting relative transcript abundance of immune-related genes and genes associated with digestion and PM structural integrity. Tsetse gene IDs are indicated in parentheses. Heat maps denoting relative transcript abundance of immune-related genes and genes associated with digestion and PM structural integrity at 48 and 72 hpa. Fold-change values are represented as a fraction of the average normalized gene expression levels from age-matched trypanosome-challenged versus -unchallenged flies. A number 1 by the gene ID indicates differential expression (FDR value < 0.05) in the 72-hpa dataset by EdgeR. $n = 2$ or 3. In *A* and *B*, # or * indicates preferential expression in the midgut or cardia organ, respectively. "Peritrophic Matrix Components" designations are based on proteomic analysis (15).

Trypanosome Exposure Reduces Expression of PM-Associated Proteins and Modifies Gut Physiology. We noted transcripts linked to three broad categories that were reduced upon trypanosome challenge: energy generation/mitochondrial activity, translation processes, and metabolism (*SI Appendix*, Fig. S3; Dataset S4). The most abundant transcripts that were down-regulated included several that encode major gut enzymes (trypsins and chymotrypsins) and molecules associated with formation of the PM barrier (Fig. 1*B*). Tsetse produce a PM that is continuously synthesized by cells in the fly's cardia organ (also known as proventriculus) (24). In particular, we noted a reduction in cardia-specific transcripts (*pro1*, *pro2*, *pro3*, and *per12*) that encode the major PM-associated Peritrophins. Peritrophins are structural PM proteins that bind chitin fibers and influence elasticity or porosity of the PM (15, 25). In addition to Peritrophins, we also surveyed for changes in expression of additional PM constituents that were identified through proteomic analysis of PM dissected from teneral adult guts (15) and noted a reduction in *serine protease 6*, *serine endopeptidase*, and *hsp60*. PM integrity is a critical mediator of tsetse's susceptibility to trypanosome infection. Experimental reduction of PM integrity in adult flies (via RNAi-mediated knockdown of *chitin synthase*, *pro1*, and *pro2*) increased parasite infection prevalence by ~50-fold (18). Thus, trypanosome-mediated inhibition of PM functions soon after acquisition could reduce the efficacy of the first immune barrier and facilitate parasite translocation from the fly's gut lumen to the ectoperitrophic space where parasites reside in infected flies.

Trypanosome Exposure Reduces *mir-275*, Which Interferes with Tsetse PM Integrity. microRNAs (miRNAs) modulate gene expression at the posttranscriptional level and are increasingly recognized as key regulators of important insect physiologies (26–28). To investigate whether trypanosome exposure modulates expression of tsetse miRNAs, we deep-sequenced small RNAs from the same gut samples used for 72-hpa and 72-hpb transcriptional analysis (*SI Appendix*, Fig. S4, Dataset S5, and Fig. 2*A*). We noted several miRNAs that were differentially expressed upon parasite acquisition (Gmo-miR-184–3p, Gmo-miR-275–3p, Gmo-miR-263b-5p,

Gmo-miR-1000–5p, and two novel predicted miRNAs). Out of these miRNAs, *mir-275* is linked to gut function and blood digestion processes in the mosquito *Aedes aegypti* (29) although its molecular targets remain unknown. Here, we focused on *mir-275*, which is reduced upon parasite challenge in tsetse and is expressed over 30-fold higher in tsetse's cardia compared with midgut when analyzed at 24 hpb (Fig. 2*B*). Thus, we speculated that *mir-275* could regulate cardia-specific processes in tsetse.

PM Synthesis Involves Wnt-Signaling Pathway and Iroquois/IRX Family of Transcription Factors. For functional inferences, we provided two distinct groups of flies with a blood meal containing either mir-275–specific antisense oligonucleotide (antagomir, ant-275) or a randomly scrambled "missense" antagomir (ant-ms), respectively. We found that ant-275 treatment significantly reduced *mir-275* expression in the cardia 24 hpb (Fig. 2*C*). We next measured midgut weight as an indicator of digestion efficiency (30). We found that midgut weights from ant-275 individuals significantly increased 24 hpb, suggesting that *mir-275* influences digestive processes and/or fluid excretion (diuresis) (Fig. 2*D*). Cardia tissue from ant-275 individuals also displayed a reduction in *pro1–3* transcript abundance when analyzed 24 hpb (Fig. 2*E*). To investigate *mir-275*–regulated pathways that could influence PM synthesis, we compared midgut and cardia-specific RNAseq libraries and identified Hedgehog (*hh*) and Wingless (*wg*), as well as the Iroquois/IRX gene family of transcription factors (*caupolican*, *araucan*, and *mirror*) as cardia-specific regulatory molecules (*SI Appendix*, Fig. S5). We further observed that expression of *wg*, *caupolican*, *araucan*, and *mirror* was significantly reduced in the cardia of ant-275–treated individuals where *mir-275* is preferentially expressed (Fig. 2*E*). Wg belongs to the highly conserved Wnt family of proteins that are tied to regulating the Wnt-signaling pathway. This pathway is a critical mediator of embryonic development, cell proliferation, and cell-to-cell communication in eukaryotes (31) and is involved in secretory processes in the adult mosquito fat body (32). The Iroquois/IRX family of transcription factors regulates many processes during embryonic and larval development in *Drosophila* (33). We next used gene-specific siRNAs to knock down *wg* and *mirror*, the latter identified as a downstream target of Wg regulation during the development of *Drosophila*'s dorsoventral axis eye disk (34). We noted decreased expression of *caupolican*, *mirror*, and *pro1–3* in the cardia of siWg-treated individuals (Fig. 2*F*). siMir-treated individuals also displayed a similar pattern of decreased *caupolican* and *pro1–3* expression (Fig. 2*F*). Collectively, our results indicate that *mir-275* modulates tsetse's Wnt pathway and the Iroquois/IRX family of transcription factors and that these pathways ultimately regulate PM production.

Components of the Mammalian Parasites Are Responsible for Altering Tsetse Gut Physiology. The transcriptional modifications that we observed in PM-associated genes (Fig. 1*B*) could be mediated either by the mammalian bloodstream-form parasites that are in the infectious blood or by the procyclic forms into which they differentiate into within hours after entering the gut. We addressed this question by feeding different groups of tsetse a blood meal supplemented with heat-killed extracts prepared from bloodstream-form *Trypanosoma brucei brucei*, *T. b. rhodesiense*, and *T. congolense* or from procyclic forms of the same *T. b. brucei* and *T. b. rhodesiense* parasites, respectively. To determine the physiological effect of these treatments, we subsequently used a highly sensitive microbial infection assay that has been used as an indicator of PM functional integrity in tsetse and *Drosophila* (18, 35). All extract-treated flies were provided a blood meal inoculated with entomopathogenic *Serratia marcescens*. Tsetse that present an intact PM fail to immunologically detect this microbe, which thus proliferates rapidly in the gut lumen and translocates into the hemolymph and kills the fly by sepsis (18). Conversely, when PM structure is experimentally compromised, the fly's immune system can detect the presence of *Serratia* in the gut early in the infection process and express robust antimicrobial immunity that limits pathogen replication and increases host survival (18). Thus, tsetse's survival upon acquisition of *Serratia* reflects the functional integrity of the PM. We observed that a significantly higher percentage of tsetse survived after infection with *Serratia* when they were previously provided blood-meal supplements with extracts from bloodstream form parasites. No difference in survival rate was observed when flies were provided procyclic form parasite extracts (Fig. 3 *A–C*). These results indicate that component(s) of the mammalian bloodstream form trypanosomes cause the functionally compromised PM phenotype.

Mammalian Parasite VSG Coat Proteins Mediate the Cardia Gene Expression Modifications and Altered Gut Physiology. We next set out to determine the molecular component(s) of the bloodstream trypanosomes that manipulate vector functions. One major difference between mammalian and insect-stage parasites relates to their surface coat proteins. Two forms of bloodstream trypanosomes,

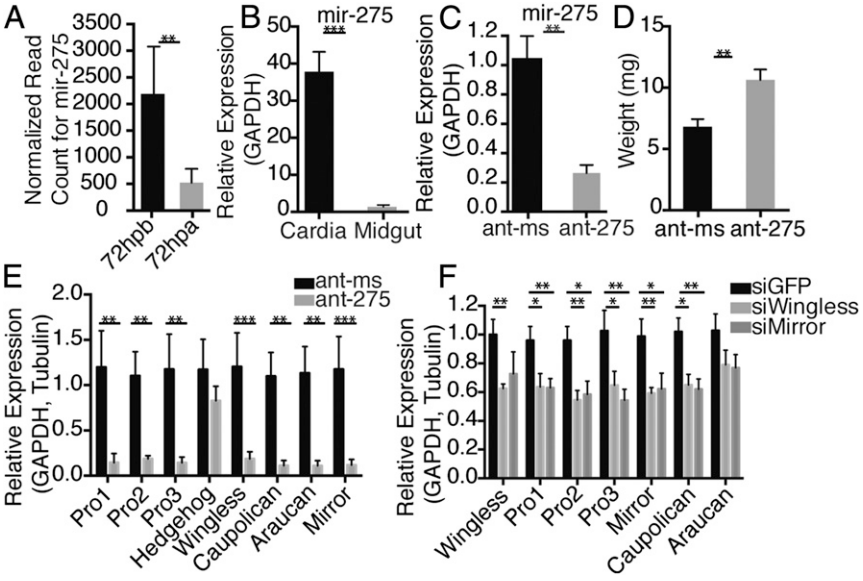

Fig. 2. Mir-275 affects the Wnt-signaling pathway. (*A*) Normalized read counts for *mir-275* expression from 72-hpb and 72-hpa individuals. (*B*) Relative expression of *mir-275* between cardia and midgut at 24 hpb. (*C*) Relative *mir-275* expression in ant-275– and ant-ms–treated tsetse (control). Ant-ms encodes a scrambled derivative of *mir-275*. (*D*) Relative midgut weight of tsetse (n = 13) treated with either ant-275 or ant-ms measured 24 h post per os antagomir inoculation. (*E*) Relative gene expression of PM-associated *peritrophins* (*pro1–3*), Iroquois/IRX family transcription factors (*caupolican*, *araucan*, *mirror*), *hedgehog* (*hh*) and *wingless* (*wg*) in cardia 24 h post ant-275, or ant-ms per os dietary provisioning. (*F*) Relative gene expression of *peritrophins*, Iroquois transcription factors, and *wg* in cardia 24 h post siGFP, siWingless, or siMirror per os dietary provisioning. Biological replicates n = 5–6. Midgut weight experiments were performed in duplicate. Results are shown as the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.0001$. Student's t-test (midgut weight comparison) and REST software Pair Wise Fixed Reallocation Randomization Test (relative gene expression) were used for statistical analyses. All primers used in the study are detailed in *SI Appendix*, Table S1.

termed "slender" and "stumpy," are present in the vertebrate blood (reviewed in ref. 36). Slender forms proliferate and cause the devastating effects of disease. Stumpy forms are developmentally arrested and are responsible for continuing the disease cycle in the insect vector. Both forms are covered with about 5×10^6 dimers per cell of identical glycosylphosphatidylinositol (GPI)-anchored VSG protein, which displays periodic variation and protects parasites from antibody-mediated lysis (37). When tsetse feed on infectious blood, both forms of the bloodstream parasite are taken up into the gut. Slender forms, which are unable to survive in the gut environment, are readily lysed, thus releasing their abundant VSG coat and other cellular components into the gut lumen. In contrast, stumpy forms, which are preadapted for survival in tsetse, receive an unknown signal and differentiate to insect-stage procyclic forms (reviewed in ref. 38). Stumpy parasites release their VSG coat via a bimodal process that involves GPI hydrolysis by a parasite GPI phospholipase C and endoproteolytic cleavage (39, 40). Next, these parasites synthesize a new surface coat composed of GPI-anchored Procyclins (41), which are necessary for efficient colonization of the tsetse midgut (42). The process of VSG release and stumpy cell differentiation to procyclic cells is thought to be completed within hours of parasite acquisition in the gut (43).

Large amounts of VSG release, both through lysis of slender parasites and GPI-PLC–mediated shedding from stumpy parasites, led us to hypothesize that this molecule could be responsible for the gut modifications that we noted upon parasite exposure. To experimentally determine VSG-mediated effects on tsetse gut physiology, we fed soluble Glycosyl-Inositol-Phosphate VSG (sVSG) purified from infected rat blood at a dose equivalent to the number of bloodstream form parasites that we provided to flies for transcriptomics analysis (Fig. 4*A*). We observed a relative increase in midgut weight 24 h post dietary supplementation of sVSG, similar to our earlier results from the ant-275 treatment group (Fig. 4*B*). Midguts from flies that received sVSG dietary supplementation also had higher levels of hemoglobin relative to flies provisioned with BSA, indicating that sVSG treatment interferes with normal digestive processes (*SI Appendix*, Fig. S6). We performed our *Serratia* survival assay with flies provisioned with sVSG and found that comparatively more sVSG-treated individuals survived *Serratia* infection (Fig. 4*C*). This bacterium was less proliferative in the midgut of sVSG-treated flies (Fig. 4*D*), likely as a result of the earlier antibacterial responses elicited by the host epithelia due to compromised PM (18). Despite the initial negative effects of sVSG on digestive processes and PM integrity, flies that were provisioned a single treatment of sVSG did not exhibit long-term survival defects.

We also performed immunohistochemistry and detected sVSG within cells of the cardia 24 h after per os exposure of flies to live bloodstream parasites as well as to purified sVSG (Fig. 4*E*). We found that sVSG exposure reduced the expression of *mir-275* (Fig. 4*F*), as well as the major PM-associated *peritrophins* (*pro1–3*), iNOS, the Iroquois/IRX family of protein-encoding genes, and the extracellular ligand *wg* when analyzed 72 h post exposure (Fig. 4*G*). These findings suggest that the sVSG component of the mammalian trypanosome coat impairs transcriptional activities in tsetse's cardia and collectively results in the synthesis of a functionally compromised PM.

VSG Supplementation Increases Tsetse's Vector Competence. Our prior RNAi-based studies, in which we eliminated tsetse's PM, resulted in higher parasite susceptibility in mature adults that normally exhibit strong resistance to infection (18). To test whether VSG-mediated PM interference would increase tsetse's parasite susceptibility, we performed two different infection experiments. Designed similarly to our *Serratia* experiments described earlier, we fed sVSG to mature flies to compromise the PM and then provided these individuals bloodstream form trypanosomes in their subsequent blood meal. When we measured midgut infection prevalence 2 wk later, we observed that significantly more flies in the sVSG treatment group were infected compared with controls (BSA) ($P < 0.02$; Fig. 4*H*). However, this increase in infection prevalence occurred only when mature flies were fed a dose of sVSG (10 μg/mL) higher than the physiological dose used for transcriptional profiling experiments (1 μg/mL). Obtaining a large-enough sample size with our laboratory colony to demonstrate an increase in infection prevalence in adult flies using the physiological dose of sVSG that we defined can be challenging due to the innate resistance that mature flies display. Hence, we next used newly eclosed teneral flies, which display comparatively higher susceptibility to infection with trypanosomes (8). Teneral flies have been extensively used as a model to study parasite dynamics in tsetse and are also susceptible to infection with laboratory-cultured procyclic forms. We provided teneral adults a blood meal containing procyclic trypanosomes supplemented with either sVSG or BSA and then measured midgut infection prevalence. As procyclic parasites lack the VSG coat, this experimental approach allowed us to measure the exclusive effect of sVSG on parasite infection outcome. We observed significantly more midgut infections when flies were provisioned parasites with VSG relative to the BSA controls ($P < 0.001$; Fig. 4*I*). These results collectively indicate that VSG-mediated alteration of tsetse gut physiology directly promotes parasite infection establishment.

Conclusions. Tsetse present multiple immune barriers that function in an effort to thwart parasite infections. Here we show that mammalian trypanosomes manipulate tsetse's defenses early in the infection process to promote their survival in tsetse gut. At the core of this feud are the prominent VSG antigens covering the surface of the mammalian trypanosomes. The VSG coat is most notable for its role in antigenic variation that enables parasite survival in the mammalian host. We describe an unprecedented adaptive function for VSG that promotes parasite colonization of the tsetse gut. We propose a

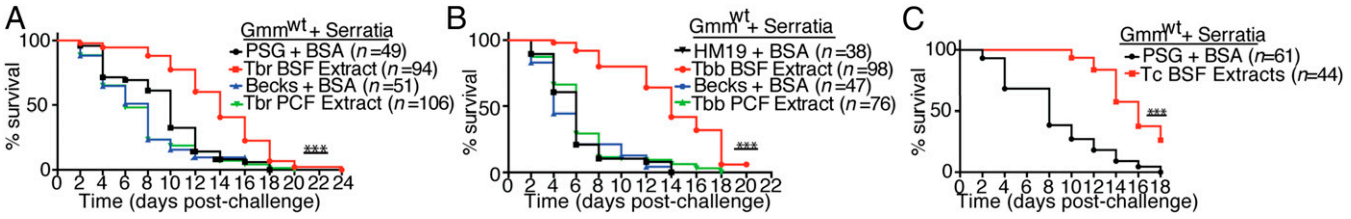

Fig. 3. Components of BSF trypanosome modulate the functional integrity of tsetse's PM. Tsetse survival curves after sequential per os provisioning with heat-killed bloodstream (BSF) or procyclic (PCF) form *T. b. rhodesiense* (*Tbr*), *T. b. brucei* 427 (*Tbb*), and *T. congolense* Trans Mara (*Tc*) extracts followed by live *Serratia*. (*A*) Fly diets supplemented with heat-killed BSF *Tbr*, heat-killed PCF *Tbr*, PSG (Phosphate Saline Glucose) buffer with BSA, or PCF culture media (Becks) with BSA before receiving *Serratia*. (*B*) Fly diets supplemented with heat-killed BSF *Tbb*, heat-killed PCF *Tbb*, and BSF culture media (HM19) with BSA or Becks with BSA. (*C*) Fly diets supplemented with heat-killed BSF *Tc* or PSG with BSA. ***$P < 0.0001$, log-rank (Mantel–Cox) test. Statistical comparisons are between the respective control and experimental group (PSG/HM19 BSA vs. BSF and Becks BSA vs. PCF). The *n* corresponds to the number of flies used per group across two independent biological experiments (displayed in *SI Appendix*, Fig. S8).

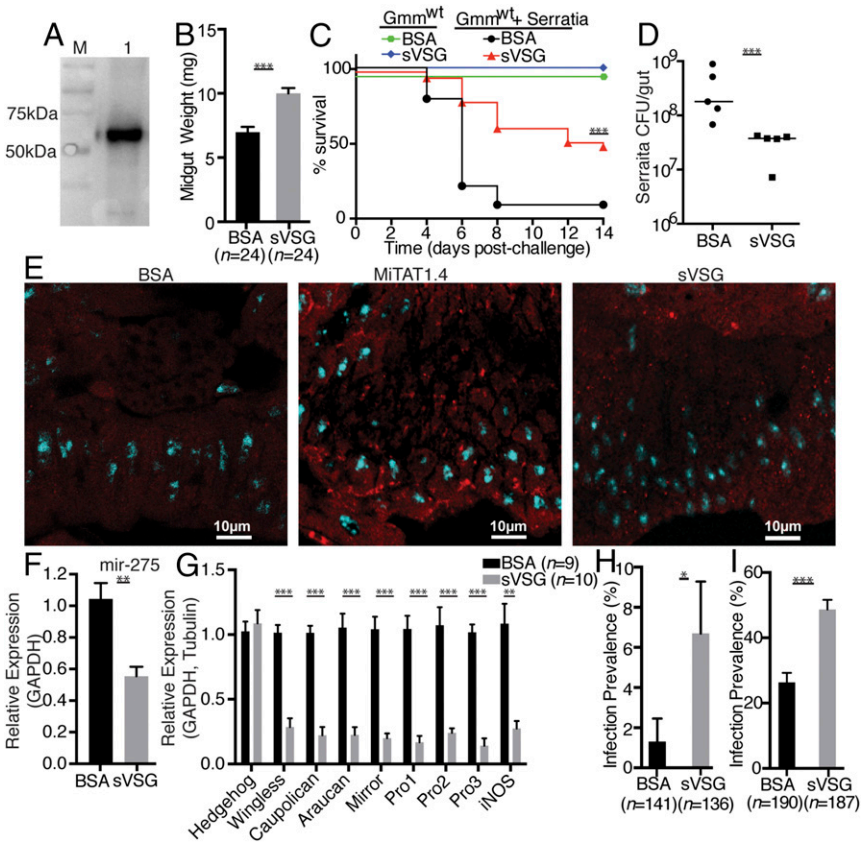

Fig. 4. Trypanosome VSG modulates tsetse gut physiology. (*A*) Western blot analysis of purified sVSG (VSG117 MITat1.4) using anti-VSG polyclonal antibodies (M, Marker; 1 VSG). (*B*) Midgut weights measured 24 hpb from flies that received 1 μg/mL BSA or 1 μg/mL sVSG supplemented blood meals as 8-d-old adults, respectively. (BSA average = 6.89 mg and VSG average = 10.0 mg.) (*C*) Tsetse survival curve after sequential per os provisioning with either BSA (1 μg/mL) or sVSG (1 μg/mL) followed by live *Serratia* in the next blood meal. (*D*) *Serratia* densities determined from tsetse midgut previously exposed to either BSA or sVSG. Each point indicates one fly midgut, and the bar represents the group average. Experiment was performed only once. (*E*) Immunohistochemistry of 5-μm-thick sectioned cardia 24 h after provisioning in the blood meal BSA (*Left*) and live *T. b. brucei* (strain Lister 427) purified from rat blood (*Middle*) and purified sVSG (*Right*). Full images are displayed in *SI Appendix*, Fig. S7. (*F* and *G*) Relative expression of *mir-275*, *peritrophins*, Iroquois/IRX family transcription factors, *iNOS*, at 72 hpb of either BSA or sVSG. Data for midgut weight, *Serratia* survival assay, and gene expression were pooled from two independent biological experiments. Experiments are displayed as the mean ± SEM. $*P < 0.05$, $**P < 0.01$, $***P < 0.0001$ between the two independent experiments. (*H*) Infection prevalence in flies that were provisioned with either 10 μg/mL BSA (1.31%) or 10 μg/mL VSG (6.69%) as 8-d-old adults before receiving BSF *Tbr* parasites in the next blood meal, respectively. Results are displayed as the mean ± SEM between three independent experiments. (*I*) Infection prevalence in teneral flies co-fed PCF *Tbr* parasites with either 1 μg/mL BSA (26.33%) or 1 μg/mL VSG (48.67%), respectively. Results are displayed as the mean ± SEM between three independent experiments. The *n* corresponds to the total number of flies used in the experiment. Independent biological experiments for survival, gene expression, and individual infection experiments are displayed *SI Appendix*, Fig. S8.

model in which sVSG, either passively or actively internalized by cardia cells, leads to a cascade of events that involve the inhibition of a vector miRNA (*mir-275*), the subsequent interference with the Wnt-signaling pathway and the actions of the Iroquois/IRX family of transcription factors. Collectively, this process interferes with the synthesis and function of the PM and provides trypanosomes a transient advantage to bypass this barrier. In the sand fly–*Leishmania* system, a vector-produced chitinase enzyme degrades the PM late in the blood digestion process, thereby also facilitating the parasite transmission process (44). Currently, no studies in tsetse have been performed to characterize the functional role of vector chitinase(s) in PM formation/development or in parasite transmission. Additionally, the molecular mechanism(s) that underlie VSG-mediated reduction of PM structural integrity remains to be determined. Interestingly, our transcriptome Gene Ontology analysis highlights that a number of genes involved in ribosomal and protein synthesis are reduced, thus suggesting that trypanosomes may interfere with a global process of cardia function(s) including PM production. Despite this interference, the majority of flies go on to eliminate gut infections through immune responses, as evidenced by extremely low parasite infection prevalence in laboratory and field populations. However, in nature the few susceptible flies can still facilitate the natural disease transmission cycle. How parasites evade the next set of immune barriers to establish a permanent gut infection in susceptible flies is unknown. In the mosquito–*Plasmodium* system, direct evidence indicates that a *Plasmodium falciparum* protein (*Pfs47*) can disrupt and block *Anopheles gambie* parasite recognition system, thus allowing parasites to bypass immunological detection (45). A similar coevolutionary manipulative system may also be occurring to enable procyclic trypanosome to permanently establish in the tsetse midgut.

In mice, sVSG mediates the induction of the major inflammatory cytokine TNF-α and sVSGs with different galactose side chains have been shown to have varying effects on host physiology (46). We noted that exposure of tsetse to different trypanosome species, each expressing a different VSG coat, resulted in PM-reducing phenotype. However, it remains to be seen whether antigenically distinct VSGs can lead to fine-tuned differences in vector gut physiology that might impact parasite infection establishment and disease transmission traits. Discovering the vector-parasite dialogue that shapes parasitism early in the infection process is of biological interest. Furthermore, this information can help strengthen the antiparasite defenses of insect vectors and aid in the development of parasite-resistant insects that can provide alternative control strategies to reduce disease.

Materials and Methods

Biological Materials. Bloodstream forms (BSF) and procyclic forms (PCF) of *T. b. brucei* strain 427 (VSG 221). BSF and PCF forms of *T. b. rhodesiense* (YTat 1.1), *T. b. brucei* (*Lister 427* MiTAT 1.4), and *T. congolense* (Trans Mara strain variant antigenic type TC 13) were prepared as described in *SI Materials and Methods*. Wild-type female *Glossina morsitans morsitans* (Gmm^{WT}) were maintained at the Yale University insectary.

mRNA and small RNA Library Constructions. RNA-sequencing libraries were prepared by Yale University Center of Genome Analysis. The experimental design and RNA preparation are described *SI Materials and Methods*. Small RNA analysis was performed using the same 72-hpa and 72-hbp biological replicates obtained above for RNA-seq analysis and is described in *SI Materials and Methods*.

Data Processing. The bioinformatic pipeline used to determine differentially expressed mRNA genes and microRNAs is described in *SI Materials and Methods*.

Antagomir and siRNA Treatments. Antagomirs for *mir-275* and *ant-ms* were synthesized at the Yale University Keck Center (Oligo Synthesis Resource) as

described in ref. 29. siRNAs targeting *mirror* and *wingless* were generated using the IDT siRNA design tool (https://www.idtdna.com). Details on gene expression and quantitative PCR analysis are described in *SI Materials and Methods*.

***Serratia* Feeding and Density Determination.** *Serratia marcescens* strain Db11 inoculation and microscopic dissection of the whole gut and subsequent plating of its contents on LB plates 4 d postbacterial challenge are as described (18). The parasite extract preparation and experimental design are provided in *SI Materials and Methods*.

sVSG Feeding and Immunohistochemistry. sVSG 117 was purified from *T. b. brucei* (strain Lister 427) grown in rats as described (47). Description of sVSG purification and our experimental design are provided in *SI Materials and Methods*.

Immunohistochemistry. For immunohistochemistry, dissected cardia were fixed for at least 24 h at 4 °C in 4% (wt/vol) paraformaldehyde in PBS and then processed for immunostaining. Detailed description on tissue sectioning and preparation can be found in *SI Materials and Methods*.

Infection Experiments. Groups of 8-d-old adults received a blood meal containing either BSA (10 μg/mL) or sVSG (10 μg/mL). All flies received bloodstream form *T. b. rhodesiense* (5×10^6/mL) in the next blood meal provided 72 h later. For teneral infections, newly emerged adult females received either BSA (1 μg/mL) or sVSG (1μg/mL) in their first blood meal supplemented with procyclic form *T. b. rhodesiense* (1×10^6/mL). Thereafter, flies were maintained on a normal blood diet for 12 d at which time flies were blindly dissected and midgut infection status was microscopically determined.

Statistical Reporting. REST software was used for pairwise gene expression analysis, including an internal multiple tests correction. A Shapiro–Wilk test was first performed to assess the normal distribution of the weight data. The Student's *t*-test was used for midgut weights pairwise comparison. A log-rank (Mantel–Cox) test was used to compare survival distribution. Infection experiment data were statistically analyzed using R software (version 3.2.2) by a generalized linear model (GLM) from the binomial family (link logit; equivalent to a logistic regression). Full details of the linear model are included in *SI Materials and Methods*.

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