Paratransgenic manipulation of tsetse miR275 alters the physiological homeostasis of the fly's midgut environment

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Abstract

Tsetse flies are vectors of parasitic African trypanosomes (*Trypanosoma* spp.). Current disease control methods include fly-repelling pesticides, trapping flies, and chemotherapeutic treatment of infected people. Inhibiting tsetse’s ability to transmit trypanosomes by strengthening the fly’s natural barriers can serve as an alternative approach to reduce disease. The peritrophic matrix (PM) is a chitinous and proteinaceous barrier that lines tsetse’s midgut. It protects the epithelial cells from the gut lumen content such as food and invading trypanosomes, which have to overcome this physical barrier to establish an infection. Bloodstream form trypanosomes shed variant surface glycoproteins (VSG) into tsetse’s gut lumen early during the infection establishment. The VSG molecules are internalized by the fly’s PM-producing cardia, which results in a reduction in tsetse *miR*275 expression and a sequential molecular cascade that compromises the PM integrity. In the present study, we investigated the role(s) of *miR*275 in tsetse’s midgut physiology and trypanosome infection processes by developing a paratransgenic expression system. We used tsetse’s facultative bacterial endosymbiont *Sodalis glossinidius* to express tandem antagomir-275 repeats (or *miR*275 sponge) that constitutively reduce *miR*275 transcript abundance. This paratransgenic system successfully knocked down *miR*275 levels in the fly’s midgut, which consequently obstructed blood digestion and modulated infection outcomes with an entomopathogenic bacteria and with trypanosomes. RNA sequencing of cardia and midgut tissues from the paratransgenic tsetse confirmed that *miR*275 regulates processes related to the expression of PM-associated proteins and digestive enzymes as well as genes that encode abundant secretory proteins. Our
study demonstrates that paratransgenesis can be employed to study microRNA-regulated pathways in arthropods housing symbiotic bacteria.
Author Summary

Tsetse flies transmit African trypanosomes, which are the parasites that cause sleeping sickness in human in sub-Saharan Africa. When tsetse ingests a blood meal containing trypanosomes, the expression level of a microRNA (miR275) decreases in the fly’s gut. This process results in a series of events that interrupt the physiological homeostasis of the gut environment. To further understand the function of miR275 in tsetse fly, we genetically modified a tsetse’s native bacterial symbiont, reintroduced the genetically modified bacterium back into the fly, and successfully knocked down the miR275 expression in tsetse’s midgut. These ‘paratransgenic’ flies (which house genetically modified bacteria) presented impaired digestive processes and were highly susceptible to infection with trypanosomes. Lastly, we discovered that miR275 regulates tsetse secretory pathways. Our novel paratransgenic expression system can be applied to study the function of other microRNAs and how they regulate disease transmission in tsetse and other insect systems.
1. Introduction

Tsetse flies (*Glossina* spp.) are obligate vectors of pathogenic African trypanosomes (*Trypanosoma* spp.) throughout 37 countries in sub-Saharan Africa (1). These protozoan parasites cause human and animal African trypanosomiases (HAT and AAT, respectively), both of which are fatal if left untreated (2, 3). Current disease control methods include vector control to reduce population size and chemotherapeutic treatment of infected people and domesticated animals (4). A more complete molecular understanding of tsetse-trypanosome interactions will facilitate the development of novel control strategies, such as reducing or eliminating the fly’s capacity to transmit trypanosomes.

The tsetse-specific stages of the trypanosome life cycle begin when the fly ingests a bloodmeal that contains mammalian stage bloodstream form (BSF) parasites. Upon ingestion by tsetse, BSF parasites differentiate into insect adapted procyclic forms (PCF) in the lumen of the fly’s midgut (5, 6). PCF parasites then bypass the fly’s peritrophic matrix (PM) barrier in the anterior midgut and replicate within the ectoperitrophic space (ES, the region between the PM and the midgut epithelia) (7-9). As part of their development from BSF to PCF parasites, the BSF trypanosomes shed their abundant surface coat antigens, known as variant surface glycoprotein (VSG) into the fly’s midgut lumen. Free VSG is transiently internalized by cells of tsetse’s PM-producing cardia (also known as proventriculus) (10, 11). This process reduces the expression of genes that encode PM associated proteins and digestive enzymes, and modulates the expression of several microRNAs, including a drastic reduction in the expression of tsetse *microRNA 275 (miR275)* (11).
miRNAs are small (~23 nucleotides) non-coding RNAs that regulate many important physiological processes. miRNAs often suppress gene expression by guiding the Argonaute (AGO) protein to bind with its target mRNA, which induces the miRNA induced silencing complex (miRISC) and leads to post-transcriptional repression or degradation of the target mRNA (12-14). miRNAs can also upregulate gene expression by inducing translational activation (15, 16). When the expression of miR275 was experimentally reduced in tsetse’s cardia and midgut through the provisioning of synthetic anti-miR275 antagonirs (antagomir-275) or VSG purified from BSF trypanosomes, formation of the fly’s PM was impaired. This process disrupted blood meal digestion and enhanced the ability of trypanosomes to establish an infection in the fly’s midgut (11). In the mosquito Aedes aegypti, miR275 similarly influences midgut blood digestion and fluid excretion by regulating the expression of its target gene SERCA (sarco/endoplasmic reticulum Ca2+ adenosine triphosphatase) (17, 18) but the mRNA target of miR275 in tsetse remains unknown.

Tsetse flies house a consortium of symbiotic microbes that mediate numerous aspects of their host’s physiology (19, 20). One of these is the facultative endosymbiotic bacterium Sodalis glossinidius, which resides extra- and intracellularly within multiple tsetse tissues, including the midgut, salivary glands, and reproductive organs (21). Sodalis can be cultivated and genetically modified in vitro, and recolonized into tsetse’s gut via a blood meal (22, 23). Reintroducing recombinant Sodalis (recSodalis) does not elicit immune responses that would induce any fitness cost (23, 24). Per os provisioned recSodalis remains only in the gut (23). ‘Paratransgenic’ tsetse flies that house recSodalis have been successfully used to deliver anti-trypanosomal nanobodies (25-27). Paratransgenesis has also been used to deliver dsRNA for
gene silencing in kissing bugs (28, 29) and in the malaria mosquito *Anopheles gambiae* (30, 31).

However, paratransgenic expression of small RNA antagomirs to knockdown miRNA expression has not been reported to date. Herein we engineered *Sodalis* to paratransgenically express three tandem antagomir-275 repeats (3xant-*miR275*) in tsetse’s cardia and midgut environments, and then used this experimental system to investigate the mechanism(s) by which *miR275* regulates the physiological homeostasis of the fly’s gut environment. We found that paratransgenic flies presented multiple phenotypes that are associated with the production of a structurally compromised PM barrier and/or disrupted gut homeostasis. Our novel paratransgenenic expression system can be applied to further study functions of microRNAs that are involved in the tsetse-trypanosome interaction, thus advancing our understanding of parasite-deployed strategies to manipulates its host physiology. Additionally, this method could be broadly applied to other arthropod systems where a host interacts with microbes (especially with non-model systems where host genetic manipulation can be difficult), which could be particularly useful to study pathogen-host interactions in the field of vector biology.
2. Materials and methods

2.1 Tsetse fly and bacterial cultures

Tsetse flies (*Glossina morsitans morsitans*) were reared in the Yale University insectary at 25°C and 70% relative humidity (RH), and received defibrinated bovine blood every 48 h via an artificial blood feeding system. Wild-type *Sodalis glossinidius morsitans* were isolated from surface-sterilized *Gmm* pupae and plated on Difco™ Brain Heart Infusion Agar (BD Biosciences) plates that were supplemented with 10% bovine blood (BBHI). Clonal *Sodalis* populations were subsequently maintained *in vitro* in Bacto™ Brain Heart Infusion (BHI) medium (BD biosciences) at 26°C, 10% CO₂.

2.2. Generation of rec*Sodalis* strains

To generate rec*Sodalis*, two constructs (Fig. 1A) were made using a modified pgRNA-bacteria plasmid (NEB, Addgene plasmid # 44251). This plasmid, which encodes an ampicillin resistance cassette, was originally designed to express short guide RNAs for CRISPR application and is thus well suited for expressing small RNAs (32). An additional endonuclease cut site Sbfi was built into the original pgRNA plasmid backbone so as to include an RNA terminator sequence in the modified plasmid. Two pairs of two complementary single-stranded oligonucleotides (oligos) that encode either three copies of the *miR*275 antagomir (3xant-*miR*275) or the scrambled *miR*275 control (Scr-275) were synthesized at Yale Keck Oligo Synthesis Resource (Table 1). The two complementary single-stranded oligos, each of which encode Spel and Sbfi restriction endonuclease cut sites, were annealed at 95°C for 5 min, cooled to room temperature for 30 min and stored at -20°C for future use. Both pgRNA and the double stranded miRNA-encoding
oligos were subjected to restriction endonuclease treatment by SpeI and SbfI at 37°C for 2 h. The oligos were then ligated into pgRNA using T4 DNA ligase (NEB), and the constructs were propagated in *E. coli* DH5α cells. All purified plasmid constructs were sequenced at Yale’s Keck Sequencing Laboratory to confirm their structure.

**Table 1. Oligonucleotide sequences.** Capitalized letters represent restriction endonuclease cut sites. Red = antagomir-275.

| Name   | Strand | Sequence                                                                 |
|--------|--------|--------------------------------------------------------------------------|
| 3xant- | F      | CTAGTcgcgcgtacttcaggtacctgaaatcgcgcgcgtacttcaggtacctgaatcgcgcgcgtacttcag |
| miR275 | R      | gtacctgacCTGCAGGtcaacttgaaaaaagtggcaccgagtgcgggtcttttttga                 |
|        |        | AGCTtcaaaaaagcaccgacctgcgtgctttttcaagttgaCCTGCAGGtcaggtacctgaagtagcgccg |
|        |        | cgcggatttcaggtacctgaagtagcgccgcgcgggattcaggtacctgaagtagcgccgcgcggA        |
| Scr-275| F      | CTAGTaccggcttagtaagagggttagtttagctacgtcttcccattttgctcaatggcataggatgtcgttctggg |
|        |        | cgtgtcgggcacctcggagaagattttaaCCTGCA                                      |
|        | R      | GGttaatctcttgccaggtccggacacgcaccgacacgtcatctaatagcatgccatggcataggtgcatgggtg |
|        |        | ctaactagccctttactaagccgggtA                                               |

The purified DNA plasmids were electroporated into wild-type *S. glossinidius morsitans* (*Sgm*WT*) as described previously (33). Two *recSodalis* strains were used in this study: 1) *Sgm*3xant-*miR275*, which encodes 3xant-*miR275*, and 2) the *miR275* scrambled control (*Sgm*Scr-275) (Table 1). In brief, 25 mL of log-phase *Sodalis* cells (*OD*$_{600}$= 0.3~0.5; SmartSpec Plus spectrophotometer; Bio-Rad, Hercules, CA) were washed consecutively in 25 mL, 1 mL and 1 mL 10% sterile pre-chilled glycerol. After the three washes, the *Sodalis* cell pellets were resuspended in 50 μL...
sterile 10% glycerol. Each 50 μL of cell mixture was mixed with 1 or 2 μL (~100 ng) of plasmid DNA and subjected to electroporation (voltage, 1.9 kV; capacitance, 25 μF; resistance, 200 Ω). After electroporation, the recSodalis cells were immediately placed in 5 mL BHI medium for overnight recovery at 26°C, 10% CO₂. The recovered cells were then plated on BHI plates supplemented with 10% bovine blood, and transformants were selected with ampicillin (50 μg/mL). After a 1-week incubation, transformants were selected for PCR and sequencing. After the sequence was confirmed, a single recSodalis colony was grown in BHI medium for future experiments.

2.3 Establishment of paratransgenic tsetse flies

To generate paratransgenic tsetse flies, two groups of teneral female flies (newly emerged unfed adults) were given two consecutive blood meals (separated by 1 day) containing either Sgm³xant-miR275 or Sgm²Scr-275 (10⁶ CFU/mL each in the first two blood meals) and ampicillin (50 μg/mL). After a third blood meal (no recSodalis, no ampicillin), 8-day old paratransgenic flies were used in the experiments described below. All plasmid constructs, as well as recSodalis strains and paratransgenic tsetse lines, are summarized in Table 1.

2.4 Gentamicin exclusion assay and quantification of recSodalis

Gentamicin is unable to cross the eukaryotic cell wall and hence only kills extracellular bacteria (34). Cardia and midgut tissues were dissected from 8-day old paratransgenic and incubated in sterile 0.85% NaCl supplemented with 100 μg/mL gentamicin. Controls were incubated in the sterile NaCl in the absence of gentamicin. Tissues were agitated on a shaking platform at room
temperature for 1 h and washed 4 times in 500 μl sterile 0.85% NaCl. After the 4th wash, tissues were rigorously homogenized in sterile 0.85% NaCl. 50 μl of lysate from each treatment was plated onto BHI Agar plates supplemented with 10% blood and 50 μg/mL ampicillin. After 7 days of incubation at 26°C, 10% CO₂, colonies on each plate were counted as described in (23). Multiple colonies were randomly selected for colony PCR (with primers targeting the inserted section of the pgRNA plasmid) and subjected to sequencing to confirm they housed the correct plasmid construct.

2.5 Dual luciferase reporter assay

To clone the 3xant–miR-275 into psiCheck-2 (Promega), two complementary single-stranded oligos that encode 3xant–miR-275 and XhoI and NotI restriction endonuclease cut sites were synthesized at Yale Keck Oligo Synthesis Resource (Table 1). The complementary oligos were annealed at 95°C for 4 min and cooled to room temperature for 30 min. The psiCheck-2 vector and the doubled stranded miRNA-encoding oligos were subjected to XhoI and NotI treatment at 37°C for 2 h followed by inactivation at 65°C. The oligos were then ligated into the double digested psiCheck-2 plasmid using T4 DNA ligase (NEB) at room temperature for 2 h, and the constructs were propagated in E. coli DH5α cells. All purified plasmid constructs were sequenced at Yale’s Keck Sequencing Laboratory to confirm their structure. The psiCheck-2 vector containing the 3xant–miR275 sequence is hereafter referred to as psiCheck-2<sub>3xant-miR275</sub>.

For transfection, Drosophila S2 cells (Invitrogen) were maintained at 28°C in Schneider Drosophila medium supplemented with 10% heat inactivated FBS. We co-transfected 100 ng of psiCheck-2<sub>3xant-miR275</sub> and the synthetic tsetse miR275miScript miRNA mimic at 100 nM (Qiagen)
or with AllStars Negative Control (Qiagen) into S2 cell lines using Attractene Transfection reagent following the manufacturer’s protocol (Qiagen). A “no miRNA” treatment with only psiCheck-2 plasmid and transfection reagent was also conducted. Dual luciferase reporter assays were performed 48 h post transfection using the Dual Luciferase Reporter Assay System following the manufacturer’s protocol (Promega). The renilla (primary reporter) luciferase signal was normalized to the firefly (internal control) luciferase signal. Each treatment was conducted triplicate.

2.6 Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was used to quantify the expression levels of miR275, non-coding small nuclear RNA (snRNA) U6, and saliva-associated genes in our paratransgenic flies (described in section 2.3 above). Tsetse cardia, midgut and salivary glands were microscopically dissected 24-48 h after the third blood meal. Total RNA was extracted from pools of 5 cardia, 5 midgut or 10 salivary glands (as one biological replicate) using Trizol reagent (35). RNA was cleaned and purified using an RNA Clean and Concentrator Kit with in-column DNase treatment (Zymo Research). RNA quality and quantity was quantified using a NanoDrop 2000c (Thermo Scientific). A small portion of the RNA was then reverse transcribed into cDNA using the miScript II RT kit (Qiagen 218160) followed by qPCR. For each sample, two technical replicates were used. Relative expression (RE) was measured as RE= 2^{-ddCT}, and normalization was performed using U6 gene expression as a reference. Primers for amplifying miR275, saliva-associated genes and the reference gene are listed in Table S1.
qPCR was performed on a CFX96 PCR detection system (Bio-Rad, Hercules, CA) under the following conditions: 8 min at 95°C; 40 cycles of 15 s at 95 °C, 30 s at 57 °C or 55 °C, 30 s at 72 °C; 1 min at 95 °C; 1 min at 55 °C and 30 s from 55 °C to 95 °C. Each reaction consisted of 10 μl: 5 μl of iTaq™ Universal SYBR® Green Supermix (Bio-Rad), 1 μl cDNA, 2 μl primer pair mix (10 μM) and 2 μl nuclease-free H₂O.

2.7 Tsetse whole gut weight measurements

Individual guts from 8-day old paratransgenic flies (n=20 per group) were dissected 24 h after their last blood meal and weighed with a digital scale as an indicator for blood digestion.

2.8 Serratia infection assay

8-day old paratransgenic individuals were fed a blood meal containing 10³ CFU/mL S. marcescens strain Db11. Thereafter, all flies were maintained on normal blood and their mortality was recorded every other day for 14 days. Details of the Serratia infection assay are provided in (7, 10, 11).

2.9 Trypanosome infection prevalence

The 8-day old paratransgenic flies were challenged per os with a blood meal containing 10⁷ CFU/mL Trypanosoma brucei brucei strain 503 supplemented with 0.9 mg/mL of cysteine. Thereafter, the flies were maintained on normal blood meals for two weeks. Their guts were dissected and microscopically examined to determine trypanosome infection status.
2.10 mRNA library construction and RNA sequencing

Two groups of paratransgenic flies (\textit{Gmm}^{xant-miR275} vs. \textit{Gmm}^{Scr-275}) were generated as described in Section 2.3. All flies were dissected 36 h after the third blood meal; 10 individual cardia or 5 individual midgut were pooled as one biological replicate and stored in -80°C prior to RNA extraction, a total 3 biological replicates per treatment were used. Total RNA was extracted using Trizol reagent according to the manufacturer’s protocol (Invitrogen), followed by RNA Clean and Concentrator Kit and in-column DNase treatment (Zymo Research). RNA quality and quantity were quantified using a bioanalyzer. All 6 mRNA libraries were prepared and sequenced (pair-ended) at Yale Center for Genome Analysis (YCGA) using Illumina NovaSeq system.

2.11 RNA-seq data processing

RNA-seq raw reads were uploaded to FastQC (v. 0.11.9, www.bioinformatics.babraham.ac.uk/projects/) for quality check, and then trimmed and filtered to remove ambiguous nucleotides and low-quality sequences. The reads were mapped to \textit{Glossina morsitans morsitans} reference genome (36) using HISAT2 v2.1.0 with default parameters (37, 38). We then used the function ‘htseq-count’ in HTSeq v0.11.2 (39) to count the number of reads mapped to the genes annotated in the reference genome (version GmorY1.9 at Vectorbase.org) with option “-s reverse”. Reads that were uniquely aligned to \textit{Gmm} transcripts were used to calculate differential gene expression using \textit{EdgeR} package in R software (40). Significance was determined using \textit{EdgeR} General linear models, corrected with a False Discovery Rate (FDR) at p < 0.05. The differentially expressed (DE) genes were uploaded to
VectorBase (http://beta.vectorbase.org) for gene ontology (GO) enrichment analysis using the built-in web tool GO Enrichment analysis. REVIGO was used to remove the redundant GO terms (41).

2.12 Replicates and statistics

Biological replicates were obtained from samples derived from distinctly repeated experiments. Details about sample sizes and statistical tests used for data analyses in this study are indicated in the corresponding figure legends.
3. Results

3.1. Successfully developed the paratransgenic expression system

To knock down expression of tsetse miR275, we designed two expression constructs that encode 1) 3xant-miR275 to knockdown miR275, and 2) a scrambled miRNA sequence (Scr-275) that served as the control. Individual clonal populations of wild-type Sodalis (SgmWT) were transformed with one of the plasmids and are henceforth designated Sgm3xant-miR275 and SgmScr-275 (Fig. 1A). We then colonized individual groups of newly eclosed (teneral) adult tsetse per os with either Sgm3xant-miR275 or SgmScr-275, thus generating paratransgenic tsetse cohorts designated Gmm3xant-miR275 (treatment) and GmmScr-275 (control), respectively. During the development of the paratransgenic lines, we supplemented the first two bloodmeals with ampicillin to suppress the SgmWT population, which provided the antibiotic-resistant recSodalis populations a selective advantage over the indigenous antibiotic susceptible WT cells.

We performed gentamicin exclusion assays to confirm that the recSodalis successfully invaded tsetse cardia and midgut cells. Gentamicin cannot penetrate eukaryotic cell membranes, and thus treatment with this antibiotic effectively eliminates the extracellular bacteria but leaves the intracellular population intact (34). We incubated separate cardia and midgut tissues dissected from 8-day old paratransgenic flies in either gentamicin (treatment) or PBS (control). Tissues were subsequently rinsed, homogenized, and plated on BBHI plates supplemented with ampicillin. We recovered 214 (± 54.0) and 9.7x10⁵ (± 9.6x10⁴) gentamicin-resistant CFU from the cardia and midgut tissues, respectively (Fig. 1B). Sequencing of the transformation plasmid from several bacterial clones confirmed their identity as either Sgm3xant-miR275 or SgmScr-275. These findings indicate that recSodalis was successfully internalized by tsetse.
cardia and midgut cells where they were protected from the antibacterial effects of gentamicin. Additionally, significantly more recSodalis cells were present within midgut cells than cells of the cardia organ. We similarly quantified the Sgm\textsuperscript{3xant-miR275} and Sgm\textsuperscript{Scr-275} present in the no gentamicin control groups (cardia, 684 ± 90, \(p = 0.002\); midgut, 2.0\times10^6 ± 1.1\times10^5, \(p < 0.0001\)) (Fig. 1B), and found that 31% and 49% of recSodalis present in the gut were intracellular within cardia and midgut tissues, respectively. These data also indicated that our recSodalis successfully reside within tsetse’s gut at a density similar to that of indigenous Sgm\textsuperscript{WT} in age-matched flies (23). Thus, we demonstrated that recSodalis successfully colonized tsetse’s gut where they reside within cells that comprise the fly’s cardia and midgut tissues.

To test the binding efficacy of the antagomirs expressed by 3xant-miR275 to tsetse’s mature miR275, we performed a dual luciferase reporter assay. We cloned the 3xant-miR275 construct into the multiple cloning site located in the 3’-UTR region of the reporter gene (renilla) in the psiCheck-2 vector (psiCheck-2\textsuperscript{3xant-miR275}). When miR275 binds to the sponge construct cloned in the 3’UTR region of the reporter gene (which initiates the RNA interference (RNAi) process), we expect the renilla transcript to be degraded, and the renilla Luciferase signal to be decreased. The psiCheck-2 vector also contains a firefly reporter in the expression cassette that is designed to be an intra-plasmid transfection normalization reporter. Thus, the Renilla luciferase signal is normalized to the firefly signal to standardize between different biological samples. We measured luciferase activity in three different experiments: 1) psiCheck-2\textsuperscript{3xant-miR275} + synthetic miR275 mimic, 2) psiCheck-2\textsuperscript{3xant-miR275} + synthetic AllStars Negative Control, and 3) psiCheck-2\textsuperscript{3xant-miR275} alone, and we found that the relative luciferase activity (renilla/firefly) was significantly suppressed in experiment 1 compared to experiments 2 and 3.
In other words, in the presence of synthetic miR275 mimic, the luciferase activity was significantly repressed, which indicated that our sponge construct was successful when tested in vitro using an insect cell line. This outcome demonstrated that the miR275 effectively binds to the miR275 sponge and initiates the RNAi process with its associated mRNA.

To confirm the knockdown effect of miR275 levels in vivo, we used qPCR to quantify the relative expression of miR275 in Gmm3xant-miR275 (treatment) and Gmm5scr-275 (control) individuals. Using multiple biological samples (each of which contained 5 dissected tissues pooled per sample) to reduce variability, we confirmed that the expression level of miR275 was significantly reduced in the midgut of the treatment group compared to that of the control group (p < 0.05; Fig. 1D). However, our qPCR results did not consistently reveal a significant reduction of miR275 levels in the cardia organ of treatment versus control paratransgenic tsetse (Fig. 1E).

3.2 Gmm3xant-miR275 gut physiological homeostasis is compromised

We demonstrated that recSodalis successfully invaded tsetse cardia and midgut tissues, and that miR275 was knocked down in the midgut of Gmm3xant-miR275. We next sought to determine if midgut physiologies, such as blood meal digestion and PM functional integrity, were impaired in Gmm3xant-miR275 flies in a manner similar to what was observed when tsetse miR275 (11) and mosquito Ae. aegypti miR275 (17) were depleted through the use of synthetic miR275 antagonomers. We compared the weight of midguts from 14 individual 8-day old Gmm3xant-miR275 and Gmm5scr-275 flies 24 h after their last blood meal. We observed that guts from Gmm3xant-miR275
individuals weighed significantly more (8.37 ± 0.64 mg) than did those from $Gmm^{Scr-275}$ controls (4.03 ± 0.56 mg) ($p < 0.001$; Fig. 2A), thus indicating that blood digestion and/or excretory processes (diuresis) were greatly disrupted in $Gmm^{3xant-miR275}$.

We next employed a highly sensitive *Serratia* infection assay to test whether PM structural integrity was compromised in paratransgenic $Gmm^{3xant-miR275}$ compared to $Gmm^{Scr-275}$ flies. We observed that 22% of $Gmm^{3xant-miR275}$ individuals survived for 19 days following *per os* challenge with *Serratia*. Comparatively, 0% of $Gmm^{Scr-275}$ control flies survived this challenge ($p < 0.0001$; Fig. 2B). These data indicate that paratransgenic-mediated repression of $miR275$ expression impairs tsetse’s gut physiology and results in the production of a functionally compromised PM barrier, similar to what we had observed using synthetic antagonirs provided *per os* in a single bloodmeal (11).

Trypanosome infection establishment success in tsetse’s midgut inversely correlates with the structural integrity of the fly’s PM (7, 42). We next evaluated trypanosome infection outcomes in the midgut of $Gmm^{3xant-miR275}$ relative to $Gmm^{Scr-275}$ control individuals to further confirm that paratransgenic expression of $miR275$ sponges interferes with the efficacy of tsetse’s PM structure. We provided 8-day old adult paratransgenic flies a blood meal containing cysteine, which inhibits trypanolytic antioxidants present in the tsetse’s midgut (10, 43), and $10^7$ *T. b. brucei*/mL of blood. Thereafter, the flies were maintained on normal blood meals for two weeks and subsequently dissected and microscopically examined to determine their midgut infection status. We found that significantly more $Gmm^{3xant-miR275}$ individuals (49%) hosted trypanosome infections in their gut than did their $Gmm^{Scr-275}$ counterparts (11%) ($p < 0.0001$; Fig. 2C). The higher parasite infection prevalence we observed in $Gmm^{3xant-miR275}$
individuals further signifies that the functional integrity of tsetse’s PM is significantly compromised when \textit{miR275} sponges are paratransgenically expressed in the fly’s midgut.

3.3 Global gene expression profiling in paratransgenic cardia and midgut

Our paratransgenic expression system has confirmed prior phenotypes that we observed following \textit{per os} administration of synthetic antagomir-275, including a significant reduction of \textit{miR275} expression in the midgut and modified phenotypes associated with compromised gut physiological homeostasis such as dysfunctional digestive processes and compromised PM functional integrity. Additionally, we observed higher trypanosome infection prevalence in the midgut of \textit{Gmm}^{3x\text{ant-miR275}} compared to \textit{Gmm}^{Scr-275} flies. To obtain a broader understanding of the molecular mechanisms and pathways that are regulated by \textit{miR275}, we performed global transcriptomic profiling in cardia and midgut tissues that were harvested from paratransgenic \textit{Gmm}^{3x\text{ant-miR275}} relative to \textit{Gmm}^{Scr-275} controls. All flies were age matched and inoculated \textit{per os} with their respective rec\textit{Sodalis} strains in their 1\textsuperscript{st} and 2\textsuperscript{nd} blood meals. For both comparisons each biological replicate (\(n=3\)) contained pooled midguts (\(n=5\)) or cardia (\(n=10\)) tissues from 8-day old adults 36 h after their third blood meal. A total of 12 mRNA libraries were sequenced, and the total reads and uniquely mapped reads from each are summarized in Table S2. We generated multi-dimensional scaling (MDS) plots to understand the overall gene expression differences between the biological replicates and treatment groups. We found that all three replicates within each treatment group clustered closely together as did all control group replicates (Fig. 3A-B). When comparing gene expression differences in the cardia, we found that 265 genes (out of a total of 6101) were differentially expressed (DE; FDR< 0.05), with 99 (1.6%)
and 166 (2.7%) up- and down-regulated in \textit{Gmm}^{3\text{xant-miR275}} relative to that of \textit{Gmm}^{5\text{Scr-275}} control individuals, respectively (Fig. 3A). When comparing gene expression differences in midgut samples, we found that 283 genes (out of a total of 5540) were DE (FDR < 0.05), with 116 (2.1%) and 167 (3.0%) up- and down-regulated in the midgut of \textit{Gmm}^{3\text{xant-miR275}} relative to \textit{Gmm}^{5\text{Scr-275}} individuals, respectively (Fig. 3B).

3.4 Gene Ontology (GO) enrichment analysis in the paratransgenic cardia and midgut

We next applied GO enrichment analyses to acquire broad insights into the functional contributions of the DE genes we identified. In the 99 up-regulated transcripts of \textit{Gmm}^{3\text{xant-miR275}} cardia relative to controls, enriched GO terms included chitin binding in the molecular function category, whereas in the 166 down-regulated transcripts, enriched GO terms included iron binding, heme binding, adenosine deaminase activity, and hydrolase and peptidase activity (Fig. 4A; Table S3). In the 116 upregulated transcripts of \textit{Gmm}^{3\text{xant-miR275}} midguts relative to controls, enriched GO terms included catalytic activity, oxidase activity and peptidase activity in the molecular function category, while in the downregulated transcripts, enriched GO terms included ribosome and cellular component biogenesis in biological processes (Fig. 4B; Table S3).

3.5 Analysis of DE genes in the cardia from \textit{Gmm}^{3\text{xant-miR275}} vs. \textit{Gmm}^{5\text{Scr-275}} control

Given that our phenotypic analysis indicated that \textit{miR275} is involved in blood digestion and PM barrier function (Fig. 2), we first evaluated the DE genes whose products are likely associated with these functions. Among the genes whose putative products have been identified as PM structural proteins through proteomics analysis of the PM (44), we found that tsetse EP, midgut
trypsin (GMOY007063) and choline acyltransferase were significantly down-regulated, while serine type endopeptidase (GMOY009757), pro1 and GmmPer12 were up-regulated in Gmm\textsuperscript{3xant-miR275} relative to Gmm\textsuperscript{Scr-275} controls (Fig. 5A; Table S4). Among the secreted products localized to the PM, we found several digestive enzymes, serine proteases (Sp), trypsin and peptidases for which transcript abundance was significantly reduced in the treatment group (Fig. 5A; Table S4). The reduction in the production of these gene products may account for the impaired blood digestion we noted in Gmm\textsuperscript{3xant-miR275} individuals. The down-regulation of several genes whose products are associated with the PM, such as tsetse EP, midgut trypsin, Sp (GMOY006839), Sp15, and choline acyltransferase, were also noted from trypanosome-infected flies where PM functions were also compromised (10). Tsetse EP protein is localized to the midgut, PM, and hemolymph (45, 46). The gene that encodes this protein is immune responsive, as its expression level was upregulated in response to bacterial challenge (45). Furthermore, when tsetse EP was depleted via RNAi, trypanosome infection prevalence significantly increased (46).

Interestingly, the expression of chitinase (GMOY005519) and chitin binding protein (GMOY011054) was significantly upregulated in the cardia of Gmm\textsuperscript{3xant-miR275} individuals. Different from other arthropod vectors, such as mosquitoes and sandflies, adult tsetse flies have type II PM, which is continuously secreted by cells located within the cardia. The PM is composed of a lattice of chitin fibrils cross linked by glycoproteins (Peritrophins) that contain chitin binding domains (CBD) (47). Chitin is an extracellular polysaccharide that can be enzymatically hydrolyzed by chitinases (48). Prior studies on trypanosome-infected cardia (10) and midguts (49) also indicated upregulated expression of chitinases, which likely resulted in...
compromised PM integrity. The reduction in PM associated gene expression, and the
upregulation of the putative chitin degrading products, may contribute to the loss of PM
integrity observed in paratransgenic Gmm\(^{3x\text{ant-miR275}}\).

With respect to blood digestion processes, we detected 10 transcripts involved in heme
binding and detoxification processes that were downregulated in Gmm\(^{3x\text{ant-miR275}}\) compared to
controls (Fig. 5B; Table S4). Among these putative products were cytochrome (CYP) P450
enzymes, which belong to a superfamily involved in insect metabolism, detoxification and
insecticide resistance in many different species (50), as well as several CYPs regulated by
Plasmodium (51) and trypanosome (52) infections. Heme in the blood can induce oxidative
damage to insect tissues (53) and the presence of heme binding proteins in Ae. aegypti PM
suggest the structure exhibits a detoxification role (54).

Among the transcripts encoding transporters and/or transmembrane channel proteins
that would be involved in secreting, trafficking and absorbing digestive products, we detected
12 that were downregulated and 10 that were upregulated in Gmm\(^{3x\text{ant-miR275}}\) relative to
controls (Fig. 5C; Table S3). These up and down-regulated genes encode functions that involve
transporting nutrients such as sugar and amino acids (e.g., major facilitator super family sugar
transporter, glucose transporter, Slif and minidiscs), ions and water (e.g., Na/phosphate
cotransporter, calcium channel, Kir family member, magnesium transporter, and aquaporin),
and organic compounds (e.g. folate transporter). Annexin and Innexin are both upregulated in
Gmm\(^{3x\text{ant-miR275}}\). Annexin belongs to a large calcium dependent membrane binding protein
family and the functions range from receptors of proteases in the gut epithelium to inhibitors of
blood coagulation (55). Plasmodium ookinetes use annexin for protection or to facilitate their
development in the mosquito gut (56). Annexin is upregulated in trypanosome-infected salivary

glands (SG) (52). Innexin proteins form gap junction channels and play critical roles in cell-to-
cell communication in a variety of physiology activities (57). Innexin 2 is a target gene of the

*Wingless* signaling pathway in the proventricular cells in *Drosophila* (58). One innexin was DE

upon trypanosome infection in tsetse, *Glossina fuscipes fuscipes* (59).

We also noted 19 abundant and significantly downregulated transcripts encoding

secreted proteins in *Gmm*[^xant-mir275] cardia (Fig. 5D; Table S4), including Adenosine deaminase-

related growth factor 3 (*Adgf3*; FC= 4.94x10^-6 and FDR= 1.00x10^-152), salivary gland protein 3

(*SGP3*; FC= 6.24x10^-5 and FDR= 1.64x10^-122), antigen-5 precursor (*Ag5*; FC= 1.21x10^-3 and FDR=

2.86x10^-103), *Tsal1* protein precursor (FC= 2.21x10^-4 and FDR= 1.26x10^-61), 5′-nucleotidase

(*5′Nuc*; FC= 1.18x10^-4 and FDR= 1.10x10^-47), *Adgf2* (FC= 2.25x10^-5 and FDR= 2.49x10^-36) and one

of the two *Tsal2* protein precursors (GMOY012361) (FC= 5.81x10^-5 and FDR= 1.86x10^-34) (Table

S4). All of these 19 genes are preferentially expressed in SG tissue and downregulated in

trypanosome-infected SGs (52, 60, 61). Interestingly, our previous study with parasite-infected

cardia also indicated that 9 of these genes [*Adgf3, Ag5, Tsal1, Tsal2* (GMOY012360), *SGP1, Tsetse thrombin inhibitor (TTI), salivary secreted protein (GMOY012067) and two secreted

proteins (GMOY003214 and GMOY007077)] are expressed in the cardia, and 4 of them [*Ag5, *Tsal2, TTI and one of the secreted proteins (GMOY007077)] are significantly impacted by

trypanosome infection (10). Moreover, our earlier transcriptomic analysis of trypanosome-

challenged tsetse guts (48 h post provisioning of a parasite containing bloodmeal) has revealed

that the expression of sixteen of these genes [*Tsal1, TTI, SGP1, GRP2, 5′ Nuc, both Tsal2s, *Adgf1, Adgf2, Adgf3, Adgf5, salivary secreted protein and two secreted peptides (GMOY003214
and GMOY012286)] are significantly reduced relative to unchallenged controls (11). All of these SG preferential gene products were previously detected in tsetse saliva and thought to be essential for the fly’s ability to successfully blood feed (62). Adgf, TTI and 5’Nuc are associated with anticoagulant functions in tsetse’s saliva and gut (59, 60, 63-65), while Ag5 is a major allergen involved in hypersensitivity reactions in the mammalian host (66).

Lastly, six DE genes in Gmm3xant-miR275 flies encoded products associated with embryogenesis and imaginal cell proliferation. Among these genes, forkhead and wing blister (Wb) were downregulated, while imaginal disc growth factor (Idgf), GMOY004790 (homologous to integrin in Md), wingless (Wg), and Wnt6 were upregulated (Table S4). Idgf is involved in extracellular matrix formation in insects and participates in critical physiological activities such as larval and adult molting and wing development (67). The wingless pathway is an intracellular signaling network; Wg signaling in Drosophila involves embryonic epidermis and wing imaginal disc (68). Interestingly, Wg expression was reduced when tsetse miR275 was knocked down using the synthetic antagonir treatment (11), contrary to our data presented here using the constitutive silencing approach, which shows higher levels of Wg.

3.6 Analysis of DE genes in the midgut from Gmm3xant-miR275 vs. control GmmScr-275

Similar to our analysis with the cardia, we first analyzed DE genes that are associated with PM components and digestive enzymes in Gmm3xant-miR275 midgut transcriptomes. Among previously identified PM products (44), we found 7 that were upregulated in Gmm3xant-miR275 midguts, including pro2, pro3, Sp6, choline acetyltransferase, chitin deacetylase, midgut trypsin (GMOY007063), and a serine type endopeptidase (GMOY9757) (Table S5). In addition, we also
identified several digestive enzymes, including trypsin, proteases and peptidases that were upregulated in \textit{Gmm}^{3xant-miR275} midguts relative to the controls (Fig. 6A; Table S5). Pro3, Sp6 and serine type endopeptidase (GMOY009757) were upregulated in response to \textit{T. brucei gambiense} (\textit{Tbg}) infection (49). Higher levels of Chitin deacetylase, a hydrolytic enzyme that catalyzes the acetamido group in the N-acetylglucosamine units of chitin (69), could contribute to a compromised PM, similar to what we report for \textit{chitinase} expression in the paratransgenic cardias above. The increased midgut weight we observed in \textit{Gmm}^{3xant-miR275} flies could reflect a dysfunctional gut enzyme production and/or altered enzyme transport in response to the compromised PM integrity.

Among the twenty genes encoding transporters and/or transmembrane channel proteins DE in the midgut (Fig. 6B; Table S5), two (GMOY012503 and GMOY010388) were also identified DE in the cardia of \textit{Gmm}^{3xant-miR275}. In addition to transporters, we noted 7 DE genes, including down regulated members of \textit{CYP} p450, ubiquitin ligase and up regulated nitric-oxidase synthase (\textit{NOS}) that are associated with heme binding and oxidative response (Fig. 6C; Table S5). The ubiquitin ligase and a heme binding protein (GMOY001150) were also down regulated in the cardia of \textit{Gmm}^{3xant-miR275}. Ubiquitin ligase and \textit{CYP} p450, which are associated with insecticide resistance and metabolism of natural or xenobiotic products in many insect species (70), have been linked to toxin metabolism following a blood meal in \textit{An. gambiae} (71). \textit{CYP} p450-4g1 is also DE (FC>2) in response to \textit{Tbg} infections in the \textit{Gmm} midgut (49). \textit{NOS} is responsible for producing cellular nitric oxide, which is trypanocidal (72). \textit{NOS} expression is down regulated in trypanosome-infected SGs (52) and cardia (10, 73), and VSG-treated cardia as well (11).
Among the SG preferential genes that are dramatically reduced in Gmm\(^{3\text{xant-miR275}}\) cardia, we detected five that were expressed in the midgut: salivary C-type lectin (GMOY000466), Ag5, secreted peptides (GMOY007065 and GMOY007077) and TTI. However, only the salivary C-type lectin was DE in the midgut and upregulated in Gmm\(^{3\text{xant-miR275}}\) relative to controls.

3.7 The paratransgenic knockdown effect is gut tissue specific

We observed the significant downregulation of 19 SG preferential genes in the cardia transcriptome from Gmm\(^{3\text{xant-miR275}}\) versus Gmm\(^{5\text{Scr-275}}\) flies. Because per os provisioned recSodalis is restricted in the gut tissue not in the hemolymph (23), we tested whether miR275 is expressed in the SG (Fig. 7A). We anticipated that the miR275 knockdown effects would be restricted to the gut and not impact gene expression levels in other organs. To confirm this, we investigated whether paratransgenic knockdown of miR275 in tsetse’s cardia induces a systemic response that results in the knockdown of these genes in the fly’s SGs. We first dissected the SG organ from Gmm\(^{3\text{xant-miR275}}\) paratransgenic flies and tested the miR275 expression levels. We subsequently monitored the expression of Adgf3 (GMOY012374), Adgf5 (GMOY012375) and SGP1 (GMOY012268), which are abundantly expressed in tsetse’s SGs (52, 60, 61) and downregulated in Gmm\(^{3\text{xant-miR275}}\) cardia. We found that none of the three SG-preferential genes were significantly reduced in the SG of Gmm\(^{3\text{xant-miR275}}\) individuals despite being significantly down-regulated in the cardia (Fig. 7B-D). These results indicate that the effect of the paratransgenic knockdown is restricted to tsetse’s gut tissues where recSodalis reside, and does not impact gene expression at the systemic level.
4. Discussion

We developed a paratransgenic expression system using tsetse’s endosymbiont *Sodalis* to experimentally modify *miR275* transcript abundance in tsetse’s gut and to investigate the resulting physiological impact. Specifically, we engineered *Sodalis* to express *miR275* sponges (3 tandem antagomir-275 repeats), and demonstrated that the rec*Sodalis* successfully colonize tsetse’s cardia and midgut where they invade resident epithelial cells. We then demonstrated that the *miR275* sponges successfully bind *miR275*, which results in posttranslational knockdown *in vitro*. We detected a significant reduction of *miR275* levels in the midgut of paratransgenic tsetse expressing *miR275* sponges, although we could not reproducibly demonstrate its reduction in the cardia organ. The paratransgenic flies displayed several robust phenotypes that are similar to those of *miR275* depletion via synthetic antagomir-275, including altered blood meal digestion, compromised PM functional integrity, and susceptibility to parasite infection, all of which reflect impaired physiological homeostasis within the gut environment. Our transcriptomic studies further identified new molecular pathways heretofore unknown to be regulated by tsetse *miR275*, including the regulation of abundant secretory proteins functioning in vasoconstriction, platelet aggregation, coagulation, and inflammation or hemostasis. Our study is the first to use paratransgenesis as a strategy to constitutively modify the expression of a microRNA in midgut tissue where the endosymbionts reside. It is efficient, cost effective, and minimally invasive compared to feeding and/or injecting synthetic antagomirs, and as such, this approach serves as an efficacious alternative to investigate microRNA related functions in the tsetse fly gut. This strategy can similarly be employed in any
arthropod that houses genetically modifiable commensal gut symbionts that reside within host cells.

Several experimental approaches are available to modify miRNA expression in vivo. Chemically synthesized, cholesterol bound antisense oligonucleotides (antagomirs) are currently most commonly used. These single stranded oligos bind their complementary endogenous miRNA, thus preventing it from interacting with its target mRNA, which inhibits downstream protein production (74). While synthetic antagomirs interact exclusively with their complimentary miRNA, they must be administered repeatedly and often in large doses for long-term effect, their uptake by cells can be inefficient, and they are difficult to target to specific tissues (75). Transgenic expression of miRNA sponges is another widely used method, which can provide effective and specific inhibition of miRNA seed families (the conserved sequences among miRNAs) (76). This method, which involves the insertion of multiple, tandem antagomirs into the germline, has been successfully used to constitutively deplete miRNA abundance in mosquitoes in a tissue specific manner via the use of tissue specific promoters (18, 77-79). Because all embryonic and larval development occurs within the uterus of female tsetse (80), the generation of transgenic fly lines using traditional germline modification approaches has not been possible. To overcome this impediment, we developed the paratransgenic expression system described herein to constitutively express miR275 sponges in tsetse’s gut.

We consistently observed three phenotypes that are associated with modified tsetse midgut physiological homeostasis in our Gmm$^{3xant-miR275}$ flies compared to Gmm$^{Scr-275}$ controls. These phenotypes all correlate with the presentation of a structurally compromised PM, and they are similar to the phenotypes that we observed previously when synthetic antagomir-275
was administrated to tsetse. Specifically, we observed that Gmm$^{3xant-miR275}$ flies presented
significantly heavier gut weights, significantly higher survival rates upon challenge with an
entomopathogen, and significantly stronger vector competence, as compared to Gmm$^{Scr-275}$
controls. Increased midgut weight is indicative of impaired blood meal digestion and/or
excretion, and this phenotype was similarly observed following treatment of Ae. aegypti (17)
and tsetse (11) with synthetic miR275 antagonim. In hematophagous insects, the PM mediates
blood digestion by regulating the flux of digestive enzymes from their site of production in the
midgut epithelium into the blood bolus-containing gut lumen (81, 82). Our study also
demonstrated that significantly more Gmm$^{3xant-miR275}$ flies survive in the presence of
entomopathogenic Serratia than do Gmm$^{Scr-275}$ control flies, further indicating that PM
functional integrity is compromised in the former group of flies. Serratia marcescens strain
Db11 is an entomopathogenic bacterium (83) that can kill tsetse when provided in the
bloodmeal. Specifically, flies with an intact PM fail to immunologically detect Serratia, which
allows the bacterium to rapidly proliferate in the gut lumen, translocate into the hemolymph
and eventually to kill the tsetse and other insects (7, 10, 11, 83-86). Conversely, when PM
structural integrity is compromised, the bacterium is quickly detected by tsetse’s midgut
epithelium and eliminated by the fly’s robust antimicrobial immune response. The Serratia
infection assay thus serves as a highly sensitive indicator of tsetse’s PM structural integrity (7).
Lastly, we observed a higher trypanosome infection prevalence in Gmm$^{3xant-miR275}$ flies
compared to Gmm$^{Scr-275}$ controls. This outcome is similar to what observed in flies exposed to
anti-PM RNAi (dsRNA targeting pro1, pro2 and chitin synthase) (7) as well as in flies that were
provisioned a blood meal containing a purified trypanosome coat protein (sVSG), which
interferes with PM related gene expression in the cardia through the reduction of miR275 (11).

Taken together, our results confirm that interference with miR275 expression in the cardia and midgut of Gmm\textsuperscript{3xant-miR275} flies results in the modified gut environment we noted in this study. Herein we repeatedly observed phenotypes that correspond with a depletion of miR275 expression in tsetse’s cardia. However, despite these findings, we were unable to quantify a significant reduction in expression of the microRNA in tsetse’s cardia (although we could in the fly’s midgut). This outcome may be accounted for by one or several reasons. First, the concentration of paratransgenically expressed miR275 relative to the concentration of the binding sites may have reduced the inhibitory effect of the miRNA sponges (75). Prior investigations demonstrated that tsetse miR275 is highly abundant in the cardia compared to the midgut tissues (11). Thus, our depletion effect could have been diluted in the cardia organ where miR275 are highly abundant. This outcome is further exacerbated by the conspicuously low number of rec\textit{Sodalis} that colonized cells of tsetse’s cardia in comparison to the midgut. More experiments are required to optimize the uptake of rec\textit{Sodalis} by cells of tsetse’s cardia organ. Moreover, qRT-PCR can be an inaccurate method for quantifying the abundance of functional miRNAs, especially in the organ where the miRNAs are highly abundant such as tsetse’s cardia. The procedure measures the total amount of miRNAs and doesn’t distinguish between functional miRNAs and non-functional ones. Thus, qRT-PCR can quantify the amount of extracellular miRNA released from Trizol-lysed cells, and this represents a physiologically irrelevant population of miRNAs (87). Combined with the robust phenotypic changes and differential expression of blood digestion and PM related genes, we believe that our paratransgenic knockdown was successful at the functional level.
Our transcriptomic analyses of cardias and midguts from paratransgenic tsetse revealed several interesting insights into the broader functions of miR275 that are related to trypanosome infection. First, with regard to the genes that are associated with PM and digestion, midgut GO enrichment analysis indicated that downregulated genes in Gmm3xant-miR275 flies included an enriched population of transcripts that encode proteins involved in ribosome biogenesis and cellular component biogenesis. This suggests that protein synthesis is obstructed in the midguts of Gmm3xant-miR275 flies, which could reflect the compromised PM structure and disrupted digestion we observed in these fly’s guts. GO enrichment analysis of upregulated cardia specific genes indicated that genes in Gmm3xant-miR275 flies included a group of enriched transcripts that encode proteins involved in chitin metabolism and chitin binding processes. Chitinase produced by parasites degrades the sand fly and mosquito PM, which promotes Leishmania (88) and Plasmodium (89) transmission, respectively. The genome of African trypanosomes does not encode a chitinase gene. However, chitinase is a proteinaceous component of tsetse’s PM, and infection with trypanosomes induces chitinase expression in the fly’s cardia (10, 44, 90) and gut (11). These findings suggest that parasites may facilitate their transmission through the fly by transiently upregulating cardia/gut chitinase expression, thus degrading PM chitin fibrils and reducing the structure’s ability to serve as a barrier. We also observed that several genes encoding digestive enzymes were downregulated in the cardia of the Gmm3xant-miR275. Similarly, miR275 and digestive enzyme-encoding genes (e.g., those encoding trypsin and trypsin-like proteins) were down-regulated in tsetse’s cardia following trypanosome exposure (10, 11). In Ae. aegypti, gut-specific depletion of miR275 results in reduced expression of its target gene SERCA, as well as reduced digestive enzyme secretion,
disrupted gut microbiota homeostasis and compromised gut actin cytoskeleton integrity.

Notably, under these circumstances, protein levels of late trypsin, a late-phase digestive protease in female mosquitoes, are significantly reduced (18). This outcome likely accounts for the altered midgut phenotypes observed in miR275 knockdown mosquitoes. However, tsetse SERCA does not contain orthologous miR275 binding site motifs, and SERCA levels are not differentially expressed in Gmm<sup>3xant-miR275</sup> compared to Gmm<sup>Scr-275</sup> flies. These characteristics suggest that the target of tsetse miR275 may not be SERCA, and a currently unknown pathway(s) regulates the secretion of the above-mentioned proteins in tsetse’s gut.

Notably, in this study the expression of some PM-associated genes was the opposite of what was observed previously when tsetse miR275 was knocked down via synthetic antagomir-275 (11). In the previous study, expression of pro1-3 were significantly downregulated in the cardia samples after the per os provisioning of either synthetic antagomir-275 or sVSG. Conversely, in this study, pro1 in the cardia, and pro2 and pro3 in the midgut were significantly upregulated in Gmm<sup>3xant-miR275</sup> compared to Gmm<sup>Scr-275</sup> flies. However, in a different study of trypanosome-infected tsetse cardia pro1 is no significant different and pro2-3 are downregulated, and the downregulation effect of pro genes by provisioning sVSG in the cardia is transient (10). This finding suggests that the pro genes regulation might be different based on parasite infection status. The observed disparity in pro gene expression by miR275 can be explained by the possibility that the synthetic antagomir produces a one-time reduction in miR275 expression that causes a different physiological response in the fly compared to that when miR275 is constitutively suppressed in paratransgenic flies. However, further
investigation is required to acquire a more complete understanding of the miR275 regulatory network and physical fitness.

Our transcriptomic results consistently showed miR275 functions in reducing the expression of secretory enzymes and similarly impairing secretory and digestive pathways. Nineteen saliva-associated proteins were among the putative secretory products that were dramatically reduced in the cardia of the Gmm3xant-miR275 individuals. Interestingly, seventeen of these genes were reduced in trypanosome-infected salivary glands (52, 60, 61), but it remains to be seen if this reduction is also mediated by lower miR275 levels in infected salivary glands. Previous transcriptomic analyses found that sixteen of these genes were reduced in trypanosome-challenged guts (11). Nine of the saliva protein-encoding transcripts were detected in tsetse’s cardia, but only four were differentially expressed upon trypanosome infection (10). In addition to being major constituents of saliva, Adgf, TTI and 5’Nuc are expressed in tsetse’s cardia and gut tissues, suggesting that these molecules may also play a role in digestive processes (59, 60, 63-65). The reduction of these saliva-associated anticoagulants in infected flies causes increased probing and biting behaviors, which in turn increases the transmission potential of the parasite to multiple hosts (60). The significant reduction in expression of genes in the Adgf family was also very interesting. Adgf is a secreted enzyme that converts extracellular adenosine into inosine by deamination and is important in anti-inflammation, tissue damage and resistance to bacterial infection in Drosophila (91-93). A Adgf is expressed by immune cells to regulate the metabolic switch during bacterial infection in Drosophila, and the downregulation of Adgf increases extracellular adenosine and enhances resistance to bacterial infection (91). The loss of Adgf can induces intestinal stem cell
proliferation in *Drosophila* (93). As evidenced by reduced *Adgf* gene expressions in trypanosome-challenged tsetse guts, the downregulation of *Adgf* genes might be triggered by initial infection of trypanosomes to release anti-inflammatory response and/or to repair any damaged tissues. Interestingly, Matetovici *et al* (2016) noted significantly reduced expression of genes that encode saliva-associated products in the SGs of flies that house trypanosomes in their midgut but not yet in their SGs. This finding is suggestive of a molecular dialogue between the organs, and a possible anticipatory response of the SG environment prior to the parasites infecting the tissue, which may be mediated by *miR275* levels in these tissues. Given that these genes encode secreted proteins, their strong reduction in paratransgenic tsetse further supports the role of *miR275* in trypanosome infection, possibly through regulation of secretory pathways.

Arthropod-borne diseases impose a debilitating global public health burden. Due to the lack of effective vaccines capable of preventing the majority of these diseases, and the increasing resistance of vector arthropods to pesticides, alternative approaches for disease control are urgently needed. Paratransgenic systems have been applied in efforts to reduce vector competence in mosquitoes (30, 31, 94, 95), kissing bugs (96, 97), sand flies (98) and tsetse flies (25-27, 99). This technology has many benefits, including the absence of a reliance on inefficient germline modification procedures (88), and the fact that modified symbionts exert no fitness cost on their insect hosts (24) and can potentially spread through wild vector populations via vertical transmission (100). Additionally, paratransgenically expressed microRNAs costs significantly less than do their synthetically produced counterparts. Our study is the first to use this system to explore the function of an arthropod vector microRNA in
relation to disease transmission processes. This system can be easily applied to study the
function of other tsetse miRNAs and for future research aimed at experimentally interfering
with the physiological homeostasis of tsetse’s midgut environment with the intent of
interrupting trypanosome transmission through the fly. This study also expanded our
knowledge of the relationship between tsetse *miR275* and the regulation of key physiological
processes such as blood digestion, PM integrity, and gut environment homeostasis in tsetse.
Our transcriptomic data revealed functions regulated by *miR275* affecting tsetse’s secretory
pathways. These findings provide a foundation for discovering the target of tsetse *miR275* in
future studies.
Acknowledgements

This work was made possible with funding to Serap Aksoy from NIH/NIAID (R01AI139525), the Li Foundation and Ambrose Monell Foundation.
Reference cited

1. Simarro PP, Cecchi G, Franco JR, Paone M, Diarra A, Ruiz-Postigo JA, et al. Estimating and mapping the population at risk of sleeping sickness. PLoS Neglected Tropical Diseases. 2012;6(10):e1859.

2. Aksoy S, Gibson WC, Lehane MJ. Interactions between tsetse and trypanosomes with implications for the control of trypanosomiasis. Advances in Parasitology. 2003;53:1-83.

3. Beschin A, Van Den Abbeele J, De Baetselier P, Pays E. African trypanosome control in the insect vector and mammalian host. Trends Parasitol. 2014;30(11):538-47.

4. Sutherland CS, Stone CM, Steinmann P, Tanner M, Tediosi F. Seeing beyond 2020: an economic evaluation of contemporary and emerging strategies for elimination of Trypanosoma brucei gambiense. The Lancet Global Health. 2017;5(1):e69-79.

5. Abbeele JVD, Claes Y, Bockstaele Dv, D DLR, Coosemans M. Trypanosoma brucei spp. development in the tsetse fly: characterization of the post-mesocyclic stages in the foregut and proboscis. Parasitology. 1999;118(Pt 5):469-78.

6. Gibson W, Bailey M. The development of Trypanosoma brucei within the tsetse fly midgut observed using green fluorescent trypanosomes. Kinetoplastid Biology and Disease. 2003;2(1).

7. Weiss BL, Savage AF, Griffith BC, Wu Y, Aksoy S. The peritrophic matrix mediates differential infection outcomes in the tsetse fly gut following challenge with commensal, pathogenic and parasitic microbes. Journal of Immunology. 2014;193(2):773-82.

8. Weiss BL, Wang J, Maltz MA, Wu Y, Aksoy S. Trypanosome infection establishment in the tsetse fly gut is influenced by microbiome-regulated host immune barriers. PLoS Pathogens. 2013;9(4):e1003318.

9. Rose C, Casas-Sanchez A, Dyer NA, Solorzano C, Beckett AJ, Middlehurst B, et al. Trypanosoma brucei colonizes the tsetse gut via an immature peritrophic matrix in the proventriculus. Nat Microbiol. 2020;5(7):909-16.

10. Vigneron A, Aksoy E, Weiss BL, Bing X, Zhao X, Awuoché EO, et al. A fine-tuned vector-parasite dialogue in tsetse's cardia determines peritrophic matrix integrity and trypanosome transmission success. PLoS Pathogens. 2018;14(4):e1006972.

11. Aksoy E, Vigneron A, Bing X, Zhao X, O'Neil M, Wu Y-n, et al. Mammalian African trypanosome VSG coat enhances tsetse's vector competence. PNAS. 2016;113(25):6961-6.

12. Bartel DP. MicroRNAs: Target Recognition and Regulatory Functions. Cell. 2009;136(2):215-33.

13. Fabian MR, Sonenberg N, Filipowicz W. Regulation of mRNA translation and stability by microRNAs. Annu Rev Biochem. 2010;79:351-79.

14. Hussain M, Walker T, O'Neil SL, Asgari S. Blood meal induced microRNA regulates development and immune associated genes in the Dengue mosquito vector, Aedes aegypti. Insect Biochemistry and Molecular Biology. 2013;43(2):146-52.

15. Dharap A, Pokrywa C, Murali S, Pandi G, Vemuganti R. MicroRNA miR-324-3p Induces Promoter-Mediated Expression of RelA Gene. PLOS ONE. 2013;8(11):e79467.

16. Truesdell SS, Mortensen RD, Seo M, Schroeder JC, Lee JH, LeTeRougue O, et al. MicroRNA-mediated mRNA Translation Activation in Quiescent Cells and Oocytes Involves Recruitment of a Nuclear microRNP. Scientific Reports. 2012;2(1):842.
17. Bryant B, Macdonald W, Raikhel AS. microRNA miR-275 is indispensable for blood digestion and egg development in the mosquito Aedes aegypti. PNAS. 2010;107(52):22391–8.

18. Zhao B, Lucas KJ, Saha TT, Ha J, Ling L, Kokoza VA, et al. MicroRNA-275 targets sarco/endoplasmic reticulum Ca2+ adenosine triphosphatase (SERCA) to control key functions in the mosquito gut. PLoS Genetics. 2017;13(8):e1006943.

19. Wang J, Weiss BL, Aksoy S. Tsetse fly microbiota: form and function. Frontiers in Cellular and Infection Microbiology. 2013;3(69).

20. Doudoumis V, Blow F, Saridaki A, Augustinos A, Dyer NA, Goodhead I, et al. Challenging the Wigglesworthia, Sodalis, Wolbachia symbiosis dogma in tsetse flies: Spiroplasma is present in both laboratory and natural populations. Scientific Reports. 2017;7(1):4699.

21. Balmand S, Lohs C, Aksoy S, Heddi A. Tissue distribution and transmission routes for the tsetse fly endosymbionts. Journal of Invertebrate Pathology. 2013;112(Supplement 1):S116-S22.

22. Weiss BL, Wu Y, Schwank JJ, Tolwinski NS, Aksoy S. An insect symbiosis is influenced by bacterium-specific polymorphisms in outer-membrane protein A. Proceedings of the National Academy of Sciences. 2008;105(39):15088-93.

23. Maltz MA, Weiss BL, O'Neill M, Wu Y, Aksoy S. OmpA-mediated biofilm formation is essential for the commensal bacterium Sodalis glossinidius to colonize the tsetse fly gut. Applied and Environmental Microbiology. 2012;78(21):7760-8.

24. Weiss BL, Mouchotte R, Rio RVM, Wu Y-n, Wu Z, Heddi A, et al. Interspecific Transfer of Bacterial Endosymbionts between Tsetse Fly Species: Infection Establishment and Effect on Host Fitness. Applied and Environmental Microbiology. 2006;72(11):7013-21.

25. De Vooght L, Caljon G, Stijlemans B, Baetselier PD, Coosemans M, Abbeele JVD. Expression and extracellular release of a functional anti-trypanosome Nanobody® in Sodalis glossinidius, a bacterial symbiont of the tsetse fly. Microbial Cell Factories. 2012;11:23.

26. De Vooght L, Caljon G, Ridder KD, Abbeele JVD. Delivery of a functional anti-trypanosome Nanobody in different tsetse fly tissues via a bacterial symbiont, Sodalis glossinidius. Microbial Cell Factories. 2014;13:156.

27. De Vooght L, Keer SV, Abbeele JVD. Towards improving tsetse fly paratransgenesis: stable colonization of Glossina morsitans morsitans with genetically modified Sodalis. BMC Microbiology. 2018;18(Supplemental 1):31-8.

28. Taracena ML, Oliveira PL, Almendares O, Umaña C, Lowengerger C, Dotson EM, et al. Genetically modifying the insect gut microbiota to control Chagas disease vectors through systemic RNAi. PLoS neglected tropical diseases. 2015;9(2):e0003358-e.

29. Whitten MMA, Facey PD, Sol RD, Fernández-Martínez LT, Evans MC, Mitchell JJ, et al. Symbiont-mediated RNA interference in insects. Proceedings of the Royal Society B: Biological Sciences. 2016;283(1825):20160042.

30. Ren X, Hoiczky E, Rasgon JL. Viral paratransgenesis in the malaria vector Anopheles gambiae. Plos pathogens. 2008;4(8):e1000135.

31. Asgari M, Ilbeigikhamehnejad M, Rismani E, Dinparast Djadid N, Raz A. Molecular characterization of RNase III protein of Asaia sp. for developing a robust RNAi-based paratransgensis tool to affect the sexual life-cycle of Plasmodium or Anopheles fitness. Parasites & Vectors. 2020;13(1):42.
32. Qi LS, Larson MH, Gilbert LA, Doudna JA, Weissman JS, Arkin AP, et al. Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. Cell. 2013;152(5):1173-83.

33. Hrusa G, Farmer W, Weiss BL, Applebaum T, Roma JS, Szeto L, et al. TonB-Dependent Heme Iron Acquisition in the Tsetse Fly Symbiont Sodalis glossinidius. Applied and Environmental Microbiology. 2015;81(8):2900-9.

34. A. Elsinghorst E. Measurement of invasion by gentamicin resistance. Methods in Enzymology. 2361994. p. 405-20.

35. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Analytical Biochemistry. 1987;162(1):156-9.

36. Initiative IGG. Genome sequence of the tsetse fly (Glossina morsitans): Vector of African trypanosomiasis. Science. 2014;344(6182):380-6.

37. Kim D, Langmead B, Salzberg SL. HISAT: a fast spliced aligner with low memory requirements. Nature Methods. 2015;12(4):357-60.

38. Kim D, Paggi JM, Park C, Bennett C, Salzberg SL. Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. Nature Biotechnology. 2019;37(8):907-15.

39. Anders S, Pyl PT, Huber W. HTSeq—a Python framework to work with high-throughput sequencing data. Bioinformatics. 2014;31(2):166-9.

40. Chen Y, McCarthy D, Ritchie M, Robinson M, Smyth G. edgeR: differential expression analysis of digital gene expression data User’s Guide 2019 [Available from: http://www.bioconductor.org/packages/release/bioc/vignettes/edgeR/inst/doc/edgeRUserGuide.pdf.

41. Supek F, Bosnjak M, Skunca N, Smuc T. REVIGO summarizes and visualizes long lists of gene ontology terms. PLoS One. 2011;6(7):e21800.

42. Haines LR. Examining the tsetse teneral phenomenon and permissiveness to trypanosome infection. Front Cell Infect Microbiol. 2013;3:84.

43. MacleoD ET, MAUDLIN I, Darby AC, Welburn SC. Antioxidants promote establishment of trypanosome infections in tsetse. Parasitology. 2007;134(6):827-31.

44. Rose C, Belmonte R, Armstrong SD, Molynieux G, Haines LR, Lehane MJ, et al. An Investigation into the Protein Composition of the Teneral Glossina morsitans morsitans Peritrophic Matrix. PLoS Neglected Tropical Diseases. 2014;8(4):e2691.

45. Haines LR, Jackson AM, Lehane MJ, Thomas JM, Yamaguchi AY, Haddow JD, et al. Increased expression of unusual EP repeat-containing proteins in the midgut of the tsetse fly (Glossina) after bacterial challenge. Insect Biochemistry and Molecular Biology. 2005;35(5):413-23.

46. Haines LR, Lehane SM, Pearson TW, Lehane MJ. Tsetse EP Protein Protects the Fly Midgut from Trypanosome Establishment. PLoS pathogens. 2010;6(3):e1000793.

47. Hegedus D, Erlandson M, Gillott C, Toprak U. New insights into peritrophic matrix synthesis, architecture, and function. Annual Review of Entomology. 2009;54:285-302.

48. Merzendorfer H, Zimoch L. Chitin metabolism in insects: structure, function and regulation of chitin synthases and chitinases. Journal of Experimental Biology. 2003;206(24):4393.
49. Hamidou Soumana I, Tchicaya B, Rialle S, Parrinello H, Geiger A. Comparative Genomics of Glossina palpalis gambiensis and G. morsitans morsitans to Reveal Gene Orthologs Involved in Infection by Trypanosoma brucei gambiense. Frontiers in microbiology. 2017;8:540-.

50. Feyereisen R. 8 - Insect CYP Genes and P450 Enzymes. In: Gilbert LI, editor. Insect Molecular Biology and Biochemistry. San Diego: Academic Press; 2012. p. 236-316.

51. Félix RC, Müller P, Ribeiro V, Ranson H, Silveira H. Plasmodium infection alters Anopheles gambiae detoxification gene expression. BMC Genomics. 2010;11:312.

52. Matetovici I, Caljon G, Abbeele JVD. Tsetse fly tolerance to T. brucei infection: transcriptome analysis of trypanosome- associated changes in the tsetse fly salivary gland. BMC Genomics. 2016;17.

53. Sterkel M, Oliveira JHM, Bottino-Rojas V, Paiva-Silva GO, Oliveira PL. The Dose Makes the Poison: Nutritional Overload Determines the Life Traits of Blood-Feeding Arthropods. Trends in Parasitology. 2017;33(8):633-44.

54. Pascoa V, Oliveira PL, Dansa-Petretski M, Silva JR, Alvarenga PH, Jacobs-Lorena M, et al. Aedes aegypti peritrophic matrix and its interaction with heme during blood digestion. Insect Biochem Mol Biol. 2002;32(5):517-23.

55. Gerke V, Moss SE. Annexins: From Structure to Function. Physiological Reviews. 2002;82(2):331-71.

56. Kotsyfakis M, Ehret-Sabatier L, Siden-Kiamos I, Mendoza J, Sinden RE, Louis C. Plasmodium berghei ookinetes bind to Anopheles gambiae and Drosophila melanogaster annexins. Molecular Microbiology. 2005;57(1):171-9.

57. Güiza J, Barría I, Sáez JC, Vega JL. Innexins: Expression, Regulation, and Functions. Frontiers in physiology. 2018;9:1414-.

58. Bauer R, Lehmann C, Fuss B, Eckardt F, Hoch M. The Drosophila gap junction channel gene innexin 2 controls foregut development in response to Wingless signalling. J Cell Sci. 2002;115(Pt 9):1859-67.

59. Li S, Aksoy S. A family of genes with growth factor and adenosine deaminase similarity are preferentially expressed in the salivary glands of Glossina m. morsitans. Gene. 2000;252(1-2):83-93.

60. Van Den Abbeele J, Caljon G, De Ridder K, De Baetselier P, Coosemans M. Trypanosoma brucei modifies the tsetse salivary composition, altering the fly feeding behavior that favors parasite transmission. PLoS Pathog. 2010;6(6):e1000926.

61. Telleria EL, Benoit JB, Zhao X, Savage AF, Regmi S, Alves e Silva TL, et al. Insights into the trypanosome-host interactions revealed through transcriptomic analysis of parasitized tsetse fly salivary glands. PLoS Negl Trop Dis. 2014;8(4):e2649.

62. Alves-Silva J, Ribeiro JM, Abbeele JVD, Attardo G, Hao Z, Haines LR, et al. An insight into the sialome of Glossina morsitans morsitans. BMC Genomics. 2010;11.

63. Caljon G, De Ridder K, De Baetselier P, Coosemans M, Van Den Abbeele J. Identification of a tsetse fly salivary protein with dual inhibitory action on human platelet aggregation. PLoS One. 2010;5(3):e9671.

64. Cappello M, Bergum PW, Vlasuk GP, Furmidge BA, Pritchard DI, Aksoy S. Isolation and Characterization of the Tsetse Thrombin Inhibitor: A Potent Antithrombotic Peptide from the Saliva of Glossina morsitans morsitans. The American Journal of Tropical Medicine and Hygiene. 1996;54(5):475-80.
65. Cappello M, Li S, Chen X, Li CB, Harrison L, Narashimhan S, et al. Tsetse thrombin inhibitor: bloodmeal-induced expression of an anticoagulant in salivary glands and gut tissue of Glossina morsitans morsitans. Proceedings of the National Academy of Sciences of the United States of America. 1998;95(24):14290-5.

66. Caljon G, Broos K, De Goeyse I, De Ridder K, Sternberg JM, Coosemans M, et al. Identification of a functional Antigen5-related allergen in the saliva of a blood feeding insect, the tsetse fly. Insect Biochemistry and Molecular Biology. 2009;39(5):332-41.

67. Zhao Y, Li Z, Gu X, Su Y, Liu L. Imaginal Disc Growth Factor 6 (Idgf6) Is Involved in Larval and Adult Wing Development in Bactrocera correcta (Bezzi) (Diptera: Tephritidae). Front Genet. 2020;11:451-.

68. Swarup S, Verheyen EM. Wnt/Wingless signaling in Drosophila. Cold Spring Harb Perspect Biol. 2012;4(6):a007930.

69. Cohen E. Chapter 2 - Chitin Biochemistry: Synthesis, Hydrolysis and Inhibition. In: Casas J, Simpson SJ, editors. Advances in Insect Physiology. 38: Academic Press; 2010. p. 5-74.

70. Feyereisen R. Evolution of insect P450. Biochemical Society Transactions. 2006;34(6):1252-5.

71. Ribeiro JMC. A catalogue of Anopheles gambiae transcripts significantly more or less expressed following a blood meal. Insect Biochemistry and Molecular Biology. 2003;33(9):865-82.

72. Bogdan C. Nitric oxide and the immune response. Nat Immunol. 2001;2(10):907-16.

73. Hao Z, Kasumba I, Aksoy S. Proventriculus (cardia) plays a crucial role in immunity in tsetse fly (Diptera: Glossinidae). Insect Biochem Mol Biol. 2003;33(11):1155-64.

74. Horwich MD, Zamore PD. Design and delivery of antisense oligonucleotides to block microRNA function in cultured Drosophila and human cells. Nature Protocols. 2008;3:1537-49.

75. Ebert MS, Sharp PA. MicroRNA sponges: Progress and possibilities. RNA. 2010;16(11):2043-50.

76. Ebert MS, Neilson JR, Sharp PA. MicroRNA sponges: competitive inhibitors of small RNAs in mammalian cells. Nature methods. 2007;4(9):721-6.

77. Lucas KJ, Roy S, Ha J, Gervaise AL, Kokoza VA, Raikhel AS. MicroRNA-8 targets the Wingless signaling pathway in the female mosquito fat body to regulate reproductive processes. PNAS. 2015;112(5):1440-5.

78. Zhang X, Aksoy E, Girke T, Raikhel AS, Karginov FV. Transcriptome-wide microRNA and target dynamics in the fat body during the gonadotrophic cycle of Aedes aegypti. PNAS. 2017;114(10):E1895–E903.

79. Dong S, Fu X, Dong Y, Simões ML, Zhu J, Dimopoulos G. Broad spectrum immunomodulatory effects of Anopheles gambiae microRNAs and their use for transgenic suppression of Plasmodium. PLOS Pathogens. 2020;16(4):e1008453.

80. Benoit JB, Attardo GM, Baumann AA, Michalkova V, Aksoy S. Adenotrophic viviparity in tsetse flies: potential for population control and as an insect model for lactation. Annual review of entomology. 2015;60:351-71.

81. Kato N, Mueller CR, Fuchs JF, Mcelroy K, Wessely V, Higgs S, et al. Evaluation of the function of a type I peritrophic matrix as a physical barrier for midgut epithelium invasion by mosquito-borne pathogens in Aedes aegypti. Vector Borne and Zoonotic Diseases. 2008;8(5):701-12.
82. Baia-da-Silva DC, Alvarez LCS, Lizcano OV, Costa FTM, Lopes SCP, Orfanó AS, et al. The role of the peritrophic matrix and red blood cell concentration in Plasmodium vivax infection of Anopheles aquasalis. Parasites & Vectors. 2018;11(1):148.

83. Nehme NT, Liégeois S, Kele B, Giammarinaro P, Pradel E, Hoffmann JA, et al. A model of bacterial intestinal infections in Drosophila melanogaster. PLoS Pathog. 2007;3(11):e173.

84. Weiss BL, Maltz MA, Vigneron A, Wu Y, Walter KS, O’Neil MB, et al. Colonization of the tsetse fly midgut with commensal Kosakonia cowanii Zambiae inhibits trypanosome infection establishment. PLoS Pathogens. 2019;15(2):e1007470.

85. Muhammad A, Habineza P, Ji T, Hou Y, Shi Z. Intestinal Microbiota Confer Protection by Priming the Immune System of Red Palm Weevil Rhyynchophorus ferrugineus Olivier (Coleoptera: Dryophthoridae). Frontiers in Physiology. 2019;10(1303).

86. Kuraishi T, Binggeli O, Opota O, Buchon N, Lemaître B. Genetic evidence for a protective role of the peritrophic matrix against intestinal bacterial infection in <em>Drosophila</em> melanogaster</em>. Proceedings of the National Academy of Sciences. 2011;108(38):15966-71.

87. Thomson DW, Bracken CP, Szubert JM, Goodall GJ. On measuring miRNAs after transient transfection of mimics or antisense inhibitors. PLoS One. 2013;8(1):e55214.

88. Coutinho-Abreu IV, Sharma NK, Robles-Murguia M, Ramalho-Ortigao M. Targeting the Midgut Secreted PpChit1 Reduces Leishmania major Development in its Natural Vector, the Sand Fly Phlebotomus papatasi. PLOS Neglected Tropical Diseases. 2010;4(11):e901.

89. Li F, Patra KP, Vinetz JM. An Anti-Chitinase Malaria Transmission–Blocking Single-Chain Antibody as an Effector Molecule for Creating a Plasmodium falciparum–Refractory Mosquito. The Journal of Infectious Diseases. 2005;192(5):878-87.

90. Dyer NA, Rose C, Ejeh NO, Acosta-Serrano A. Flying tryps: survival and maturation of trypanosomes in tsetse flies. Trends in Parasitology. 2013;29(4):188-96.

91. Bajgar A, Dolezal T. Extracellular adenosine modulates host-pathogen interactions through regulation of systemic metabolism during immune response in Drosophila. PLOS Pathogens. 2018;14(4):e1007022.

92. Novakova M, Dolezal T. Expression of Drosophila Adenosine Deaminase in Immune Cells during Inflammatory Response. PLOS ONE. 2011;6(3):e17741.

93. Xu C, Franklin BJ, Tang H-W, Regimbald-Dumas Y, Hu Y, Ramos J, et al. An in vivo RNAi screen uncovers the role of AdoR signaling and adenosine deaminase in controlling intestinal stem cell activity. Proceedings of the National Academy of Sciences. 2019;117:464 - 71.

94. Wang S, Jacobs-Lorena M. Chapter 13 - Paratransgenesis Applications: Fighting Malaria With Engineered Mosquito Symbiotic Bacteria. In: Wikle SK, Aksoy S, Dimopoulos G, editors. Arthropod Vector: Controller of Disease Transmission, Volume 1: Academic Press; 2017. p. 219-34.
975 97. Beard CB, Cordon-Rosales C, Durvasula RV. Bacterial symbionts of the Triatominae and their potential use in control of Chagas disease transmission. Annual Review of Entomology. 2002;47:123-41.

978 98. Hurwitz I, Hillesland H, Fieck A, Das P, Durvasula R. The paratransgenic sand fly: A platform for control of Leishmania transmission. Parasites & Vectors. 2011;4.

980 99. Aksoy S, Weiss B, Attardo G. Paratransgenesis applied for control of tsetse transmitted sleeping sickness. Adv Exp Med Biol. 2008;627:35-48.

982 100. Cheng Q, Aksoy S. Tissue tropism, transmission and expression of foreign genes in vivo in midgut symbionts of tsetse flies. Insect Molecular Biology. 1999;8(1):125-32.
Figure 1. The successful development of paratransgenic expression system.

(A) recSodalis plasmid construct. Three tandem antagomir-275 repeats (3xant-miR275, in green) that are complementary to the tsetse miR275 mature sequence were cloned into plasmid pgRNA. Each repeat is separated by a 3-nucleotide linker sequence. 3xant-miR275, and a similarly engineered construct that encodes a scrambled antagomir-275 (Scr-275), were electroporated into SodalisWT to generate strains designated Sgm3xant-miR275 and SgmScr-275, respectively.

(B) Quantification of Sgm3xant-miR275 within cells of tsetse’s cardia (black) and midgut (grey) via gentamicin exclusion assay. Each dot represents one tsetse organ (n=5). A student’s t-test was used to determine statistical significance.

(C) Dual luciferase reporter assay. Each dot represents the average of normalized luciferase signal (Renilla/Firefly ratio) ± SEM of each experiment. The 3xant-miR275 construct was cloned into the psiCheck-2 plasmid containing two luciferase reporter genes, Renilla (reporter) and Firefly (internal control). The luciferase activity is measured by the Renilla signal normalized to the Firefly signal. Three different experiments were performed to test the binding efficacy between 3xant-miR275 and 1) synthetic miR275 mimic, 2) synthetic AllStars Negative Control, and 3) psiCheck plasmid without adding any miRNA. Three biological replicates (with 3 technical replicates each) per experiment were used. Bonferroni’s multiple comparison tests were used to determine statistical significance.

(D) miR275 expression level in the midgut of paratransgenic Gmm3xant-miR275 versus GmmScr-275 flies. Each dot represents 5 individual midguts. A student’s t-test was used to determine statistical significance.
miR275 expression in the cardia of Gmm$^{3xant-miR275}$ versus Gmm$^{Scr-275}$ flies. Each dot represents 5 individual cardia. A student’s t-test was used for statistical analysis.

Figure 2. Gut physiological homeostasis is compromised in Gmm$^{3xant-miR275}$.

(A) Tsetse gut weights. The gut weights were measured 24 h after the last blood meal. Each dot represents an individual fly gut. Mann-Whitney test was used for statistical analysis.

(B) Serratia infection assay. A total of 4 biological replicates ($n=25$ flies per replicate) were used. Gehan-Breslow-Wilcoxon test was used to determine statistical significance.

(C) Trypanosome midgut infection prevalence. Four biological replicates ($n=20$ flies per replicate) were used. Generalized linear model (GLM) with binomial distribution was used to determine statistical significance.

Figure 3. Overviews of transcriptome profiles in Gmm$^{3xant-miR275}$ compared to Gmm$^{Scr-275}$ flies.

(A) cardia and (B) midgut transcriptome profile overview. Left panel: MDS plots display the overall gene expression patterns among the samples and between the treatments.

Right panel: Venn diagrams show the number of downregulated (blue), upregulated (red) and not significantly different (white) genes in (A) cardia and (B) midgut. Genes were considered DE if they exhibited an FDR value <0.05.

Figure 4. GO enrichment analysis of the paratransgenic flies Gmm$^{3xant-miR275}$ vs. Gmm$^{Scr-275}$.

(A) cardia and (B) midgut tissues GO enrichment analyses. Three GO term categories were used: biological process (yellow), cellular component (green), and molecular function (pink).
The GO terms were considered significant (Bonferroni score < 0.05) using VectorBase built-in GO enrichment analysis web tool. Redundant GO terms were removed by REVIGO (0.5). The number of genes in our dataset/ the total number of genes that are associated to each individual GO term, are marked within parentheses next to each GO term description.

Figure 5. Heat map representation of DE genes in different functional groups (A-D) in paratransgenic cardia Gmm³xant-miR275 vs. Gmm⁵Scr-275. (A) PM and digestion associated, (B) heme binding and detoxification, (C) transporter associated, and (D) saliva associated. Heat maps were generated by plotting the read counts in treatment (3xant-miR275) and control (Scr-275) samples. Colors display normalized gene expression values from low (blue) to high (red). * indicates the unknown gene product’s orthologue in Drosophila melanogaster (Dm) and/or Musca domestica (Md).

Figure 6. Heat map representation of DE genes in different functional groups (A-C) in the midgut of Gmm³xant-miR275 and Gmm⁵Scr-275 flies. (A) PM and digestion associated, (B) transporter associated, and (C) heme binding and oxidative response associated. Heat maps were generated by plotting the read counts in treatment (3xant-miR275) and control (Scr-275) samples. Colors display normalized gene expression values from low (blue) to high (red). * indicates the unknown gene product’s orthologue in Drosophila melanogaster (Dm) and/or Musca domestica (Md).

Figure 7. The paratransgenic system is gut tissue specific.
(A) miR275, (B) Adgf3, (C) Adgf5 and (D) SGP1 expression levels in the salivary glands (SGs) of Gmm3xant-miR275 versus GmmScr-275 flies. Each dot represents 10 individual SGs. Student’s t-test was used to determine statistical significance.

Supporting information

Table S1. qPCR primer list.
Table S2. Summary of reads mapping.
Table S3. Dataset. GO enrichment analysis.
Table S4. Dataset. Raw data and DE analysis of cardia transcriptome.
Table S5. Dataset. Raw data and DE analysis of midgut transcriptome.
A

Down in 3xant-miR275

- adenosine metabolic process (3/4)
- adenosine catabolic process (3/4)
- aromatic compound catabolic process (8/84)
- drug catabolic process (4/13)
- purine-containing compound catabolic process (3/7)
- extracellular region (10/153)
- integral component of membrane (51/2510)
- intrinsic component of membrane (51/2514)
- membrane (54/2757)
- membrane part (51/2620)
- iron ion binding (10/89)
- heme binding (10/93)
- tetrapyrrrole binding (10/94)
- oxidoreductase activity (10/96)
- deaminase activity (4/16)
- catalytic activity (59/2985)
- adenosine deaminase activity (3/7)
- hydrolase activity (32/1279)
- gamma-glycylamyl-peptidase activity (2/2)

B

Down in 3xant-miR275

- ribosome biogenesis (13/64)
  - rRNA metabolic process (6/36)
  - cellular component biogenesis (13/220)
- nucleolus (7/37)

Up in 3xant-miR275

- (6/52) aminoglycan metabolic process
- (5/46) chitin metabolic process
- (5/48) glycosamine-containing compound metabolic process
- (5/49) amino sugar metabolic process
- (10/153) extracellular region
- (7/53) chitin binding
- (3/9) oxidoreductase activity
- (2/2) D-amino acid metabolic process
- (51/2985) catalytic activity
- (2/2) D-amino-acid oxidase activity
- (13/384) peptidase activity, acting on L-amino acid peptides
- (13/396) peptidase activity
### A. PM & Digestion Associated Genes

- **GMOY010995** *Zinc carboxypeptidase in Md*
- **GMOY003486** Aminopeptidase P
- **GMOY007063** Midgut trypsin
- **GMOY010142** *Brachyurin in Md*
- **GMOY010555** *Putative serine protease in Md*
- **GMOY010103** Aspartyl protease
- **GMOY011805** Choline acetyltransferase
- **GMOY000672** Serine protease 6
- **GMOY001499** Chymotrypsin-like protein
- **GMOY009531** *Carboxypeptidase in Md*
- **GMOY001946** Putative aminopeptidase
- **GMOY010996** *Tep2 in Dm*
- **GMOY013336** *Aminopeptidase in Md*
- **GMOY009375** Chitin deacetylase-like
- **GMOY009587** Pro2
- **GMOY009756** Pro3, trypsin
- **GMOY009757** *Serine protease in Md*

### B. Transporter Associated Genes

- **GMOY010253** *Sodium–coupled monocarboxylate transporter 1 in Md*
- **GMOY000121** *Trehalose transporter (Tret1-2) in Md*
- **GMOY008370** Excitatory amino acid transporter
- **GMOY010388** Minidiscs
- **GMOY000772** Major facilitator superfamily transporter
- **GMOY003579** Sodium/potassium–transporting ATPase subunit alpha
- **GMOY003126** Aquaporin
- **GMOY010965** Concentrative Na+-nucleoside cotransporter cNT1/cNT2
- **GMOY006828** *Trehalose transporter (Tret1) in Md*
- **GMOY000269** Sugar transporter
- **GMOY005074** *Ammonium transporter Rh in Dm and Md*
- **GMOY005276** *Trehalose transporter (Tret1) in Md*
- **GMOY009059** *Sodium–coupled monocarboxylate transporter 1 in Md*
- **GMOY006250** Sodium/hydrogen exchanger
- **GMOY101250** Membrane transporter
- **GMOY009338** Transporter
- **GMOY002034** Synaptic vesicle transporter
- **GMOY012079** *Drip in Dm*
- **GMOY001703** Putative zinc transporter
- **GMOY005720** Zinc transporter 35C

### C. Heme Binding & Oxidative Response

- **GMOY001150** *Cytochrome P450-305a1 in Md*
- **GMOY002937** Disembodied
- **GMOY003093** Nitric-oxide synthase
- **GMOY006213** *Cytochrome P450-4d14 in Md*
- **GMOY002158** Ubiquitin Ligase
- **GMOY003204** Cytochrome P450
- **GMOY006761** Cytochrome P450-4g1

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**Legend:**
- **Low**
- **High**
