Supplemental Files

Materials and Methods

Mosquito strains. The *Anopheles gambiae* G3 and the *An. stephensi* Nijmegen strains were used. Mosquitoes were reared at 27 °C and 80% humidity on a 12-h light-dark cycle under standard laboratory conditions.

Quantitation of gene expression and midgut bacteria- A. gambiae midguts were isolated at different times post-feeding, placed into 50 µl RNAlater (Ambion) frozen in liquid nitrogen and stored at -80°C. Poly(A) mRNA was isolated using Oligotex-dT beads (Qiagen), following the manufacturer's instructions. First-strand cDNA was synthesized using random hexamers and Superscript II reverse transcriptase (Invitrogen). Gene expression was assessed by SYBR green quantitative real-time PCR (qPCR) (DyNAmo HS; New England Biolabs) in a Chromo4 system (Bio-Rad). PCR involved an initial denaturation at 95ºC for 5 min, 44 cycles of 10 sec at 94°C, 20 sec at 56°C, and 30 sec at 72°C. Fluorescence readings were taken at 72°C after each cycle. A final extension at 72°C for 5 min was completed before deriving a melting curve (70–95°C) to confirm the identity of the PCR product. qRT-PCR measurements were made in duplicate. Relative quantitation results were normalized with *A. gambiae* ribosomal protein S7 mRNA as internal standard and analyzed by the $2^{-\Delta\Delta Ct}$ method (*1*). For complete list of qRT-PCR primers and accession numbers see Table S2. Bacteria quantitation was done on cDNA samples or genomic DNA (Qiagen) using universal eubacteria primers to amplify 16S rRNA F- TCCTACGGGAGGCAGCAGT and R-GGACTACCAGGGTATCTAATCCTGTT (*2*).

dsRNA synthesis. A 462 bp fragment of the IMPer gene was amplified using the primers (5' to 3') F- TCGGTGCTGGAAAAGGATGG and R-GTTGGCGCGAGCTAAACACG and cloned into the pCR®II-TOPO® vector. T7 promoters were incorporated onto the ends of this fragment by amplifying the cloned insert using the following primers M13F-GTAAAACGACGGCCAGT and M13R-CTCGAGTAATACGACTCACTATAGGGCAGGAAACAGCTATGAC. The PCR product was used as template to synthesize dsRNA *in vitro* using the MEGAscript RNAi kit (Ambion, Austin, TX). dsRNA was further purified with water and concentrated to 3 μg/μl using a Microcon YM-100 filter (Millipore). A similar strategy was used to generate dsNOS, dsDUOX, and dsLacZ using the following primers: NOS-Fw-GGTGTTCTCGATCGCGTGTTCTT and NOS-Rev-CGCAGCGTCAGCATGTATTTCTC. Duox-Fw-GCCCGCAGGAAGTTCGTCAAAAA and Duox-R-TCGCATCATCTCGCTCAGTTCTCC, LacZ-Fw-GAGTCAGTGAGCGAGGAAGC and LacZ-Rev-TATCCGCTCACAATTCCACA (using pCR®II-TOPO® vector as a template).

Gene silencing in adult female mosquitoes. Female mosquitoes were injected with 69 nl of a 3 μg/μl solution of dsRNA (207 ng dsRNA) from the gene of interest into the hemocoel 3-4 days post-emergence (PE). For IMPer and NOS silencing, mosquitoes were injected with dRNA 3-4 days post-emergence (PE) and blood fed 24-36h after injection. For Duox silencing, 1-2 day-old females were injected and blood fed 3-4 days

later. Control mosquitoes were injected with dsLacZ at similar times as the experimental group. Females were either fed on a mouse infected with *P. berghei*, or were infected by feeding on a *P. falciparum* gametocyte culture provided in an artificial feeding system. The silencing efficiency in the midgut of blood-fed females, relative to controls injected with dsLacZ (100% expression) was: 84-90% for IMPer , 60-65% for NOS and 77-80% for Duox.

Peroxidase activity in the midgut. Females were fed on a sugar cube and water containing Penicillin (100 units/ml) and Streptomycin (0.1 mg/ml) since the first day of emergence until they were fed on a healthy mouse. For whole tissue mounts, blood-fed midguts were dissected 1h or 12h post-feeding, fixed for 1 min in 4% paraformaldehyde in PBS and opened longitudinally to remove the blood meal. Samples were incubated at room temperature with 2.5 mM 3,3´-Diaminobenzidine (DAB) (Sigma), 10 mg/ml 3-amino-1,2,4-triazole (AT) (catalase inhibitor) and 1 mM H_2O_2 (Sigma) in PBS (pH 6.5) and continuously observed under the microscope. The reaction was stopped by fixing the stained midguts for 20 min with 4% paraformaldehyde. To obtain tissue sections, whole midguts, were fixed for 5 min in 4% paraformaldehyde and stained using the same DAB solution as above. Tissues were embedded in paraffin, sectioned longitudinally and stained with toluidine blue. For the silencing experiments, either LacZ or IMPer dsRNA was injected 1 day before blood feeding.

Recombinant IMPer protein expression and antibody production. Primers were designed to amplify a short (695 bp) fragment and a long fragment (1,506 bp) of IMPer from midgut cDNA isolated 12 h post-feeding. Using the following primers (5' to 3'): F-CTGAGCTTTACAGCGGATGCT and R-TCATTAGACCGAGGCATCGTAGTCGT and F-GAGGCCCCAGTATGTGACGAT and R-TCATTACTGATCCAGCAGCAGGCAAG to generate recombinant proteins of 29.2 and 59.6 kDa, respectively. Both PCR products were cloned into a pCR®T7/CT-TOPO plasmid (Invitrogen, San Diego, CA), sequenced and expressed in BL21(DE3)pLysE *E. coli* (Invitrogen). The expressed proteins included a C-terminal His-tag and were purified using Ni-NTA columns and an imidazole gradient. The purified proteins were mixed and used to generate polyclonal antisera in rabbits.

Western Blot analysis. Midguts of blood fed females were collected at different times after feeding. The blood meal was removed and the tissue homogenized in PBS buffer containing protease inhibitors (Complete protein inhibitor cocktail tablests, Roche, Mannheim, Germany). The homogenate equivalent to two midgut was loaded in each lane. Samples were subjected to SDS gel electrophoresis under non-reducing conditions. Proteins were transferred to a nitrocellulose membrane that was treated with 1 mM levamisol (Sigma) in Tris-buffered saline (TBS), pH 7.5 for 20 min to inhibit endogenous phosphatase activity. All antibody incubations were

done in a 6% bovine serum albumin (BSA) and 0.5% gelatin solution in TBS. The anti-IMPer polyclonal anti-serum was used at a 1:5,000 dilution and detected with alkaline phosphatase substrates.

Infection of Mosquitoes with P. berghei. Mosquito females were infected with *P. berghei* by feeding them on anesthetized infected Balb/C mice. The infectivity of the mice was established by determining the parasitemia and by performing an exflagellation assay as described previously (*3*). In all the studies, mice having 2-3 exflagellations/field under 40X objective were used to infect mosquitoes. Blood-fed mosquitoes were kept at 21 °C and 80% humidity. *P. berghei* infections were performed using the ANKA 2.34 strain or a transgenic *GFP-P. berghei* strain (GFP-CON transgenic 259cl2 strain (*4*)), and infection phenotypes determined 7-8 days PI. *P. berghei* midgut infection 30h and 48 h PI was quantified by immunofluorescence using mouse anti-Pbs21 antibody as previously described (*5*). The distribution of parasite numbers in individual mosquitos between the control and experimental groups were compared using the Kolmogorov-Smirnov test (KS test). All phenotypes were confirmed in 2-3 independent experiments. All mosquitoes fed on uninfected or Plasmodium-infected mice were kept at 21ºC.

Experimental infection of mosquitoes with P. falciparum. *A. gambiae* (G3) female mosquitoes were infected artificially by membrane feeding with a *P. falciparum* gametocyte culture. *P. falciparum* 3D7 strain was maintained in O^+ human erythrocytes using RPMI 1640 medium supplemented with 25 mM HEPES, 50 mg/L hypoxanthine, 25 mM $NaHCO_3$ and 10%(v/v) heat-inactivated type O^+ human serum (*6*) (*7*).

Gametocytogenesis was induced as previously described (*8*). Mature gametocyte cultures (stages IV and V) that were 14-16 days-old were used to feed mosquitoes in 37°C warmed membrane feeders for 30 min and blood-fed mosquitoes were kept at 26ºC. Midguts were dissected 8 days PI and oocysts stained with 0.05% (w/v) mercurochrome in water and counted by light microscopy. The distribution of parasite numbers in individual mosquitos between control and experimental groups were compared using the Kolmogorov-Smirnov test (KS test).

Statistical analysis. For those data sets that were not normally distributed (oocysts intensities and dextran fluorescence in hemolymph), two-sample comparisons were done using two different non-parametric tests, the Kolmogorov-Smirnov (KS) and the Mann-Whitney test (GraphPad, Prism 5.01). Both detected the same significant differences. Four-sample comparisons were done using the nonparametric Fruskal-Wallis test and medians were compared using the Dunn's test (GraphPad, Prism 5.01). The values were also normalized by transforming them to a "natural logarithm" scale and analyzed by ANOVA. Multiple comparisons of the means of the normalized data using the Tukey test confirmed the results obtained with the Fruskal-Wallis test. Survival curves were analyzed with GraphPad Prism 5.01 using Log-rank(Mantel-Cox) test, and found not to be statistically different. Pair-wise comparisons of mRNA expression levels were done using Student's T-test and the ANOVA-Tukey tests were used to compare multiple means.

Midgut dextran permeability assay. *A. gambiae* G3 5 day-old females were fed a mixture of 40% (v/v) human RBC O+, 50% human serum O+, and 10% of fluorescein isothiocyanate 4kD Dextran (FD4, SIGMA) in water (36mg/600ul) using an artificial membrane feeding system. Mosquito hemolymph was collected 18 hr post-feeding by puncturing the anterior abdomen and flushing with 4µl of PBS injected in the thorax. Fluorescence in hemolymph was measured in a Nanodrop ND 3300 fluorospectrometer. Results from three independent experiments were pooled. To administer oral antibiotics, females were fed on a sugar cube and water containing Penicillin (100 units/ml) and Streptomycin (0.1 mg/ml) since the first day of emergence until they were blood-fed.

Immunofluorescence of midguts with anti-dityrosine antibodies. Control or silenced mosquitoes were fed 40% (v/v) human O+ RBC in 60% human O+ serum using an artificial membrane feeding system and kept at 26ºC. Midguts were dissected 14h post feeding and fixed for 20 sec in 4% paraformaldehyde in PBS. The bolus was removed and the cleaned midgut epithelium fixed for 30 min. Midguts were incubated in 1% BSA in PBS (PB) for 1hr and incubated with anti-3,3'-Di-Tyrosine antibody (1:200 dilution in PB) (JaICA, Nikken Seil Co, Ltd., Shizouka, Japan) for 2 hr. Midguts were washed three times with PB (15 min each) and incubated with the secondary antibody (Alexa-555 Anti-mouse, Molecular Probes, antibody at 1:500 dilution in PBS,) for one hour, rinsed 2 times (20 min each) with PB and once with PBS and then incubated with Alexa-488 labeled phalloidin (Molecular probes) (1:40 dilution in PB) for 20 min. Midugts were then mounted in Vectashield containing DAPI (Vector Laboratories, Inc.). Microsocopy was carried out using a Leica SP2 confocal microscope.

Microarray Analysis. Anopheles gambiae G3 mosquitoes (5-6 day-old) were fed a BSA solution (20% in PBS in 10 mM sodium bicarbonate) with or without heat killed *E. coli* (equivalent of 2.5 ml of culture 0.8 OD) and kept at 27ºC. Pools of 10 mosquito midguts were dissected after 3h and placed in 50 µl of ice cold RNA later (Ambion) and kept at -70 ºC until processed. Total RNA was extracted with the RNeasy Mini kit (Qiagen). RNA integrity was confirmed using the Agilent Bioanalyzer 2100 system. cDNA and biotin-labeled cRNA were synthesized using the Affymetrix one cycle target labeling assay (Affymetrix). Labeled targets were hybridized to the GeneChip Plasmodium falciparum/Anopheles gambiae genome array (Affymetrix). Hybridization was done at 45 ºC for 48 hours. Upon completion of the fluidics process, each chip was scanned using the Affymetrix 7Gplus GeneChip scanner to create the image files. GeneChip Operative Software (GCOS v1.4) was used to convert the image files to cell intensity data (CEL) files. To generate the expression (CHP) files, CEL files were normalized using the GCOS Software scaling method. Further analysis was carried out using the GeneSpring GX 7.3 software (Agilent). Significant differences in gene expression between treatments were identified using the Student's t-test ($p < 0.05$). A hierarchical cluster of differentially expressed genes was done using the Pearson's correlation. This analysis generated a dendrogram of the biological replicates. As expected, replicate samples for each treatment cluster together (Fig S3 A). Up-regulation of putative immune genes was confirmed by qPCR as described above (Fig. S4). The complete data set for this microarray experiment has been submitted to the NCBI Gene Expression Omnibus (GEO) database.

Notes and References

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9. Author Contributions: S.K. designed and carried out all experiments involving the IMPer gene, A. M-C. identified midgut immune markers by microarray analysis, generated the *P. falciparum* gametocyte cultures, designed and carried out all experiments involving the Duox gene, performed the Dextran permeability experiments and confocal detection of the dityrosine network. L. G. provided assistance phenotyping *P. berghei* infections. J. R. carried out histological stainings to localize IMPer activity in midgut sections. C. B-M. contributed to the experimental design, the interpretation of results and wrote the manuscript.