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Supporting Information for

Trichomonas vaginalis extracellular vesicles upregulate and directly transfer adherence factors promoting host cell colonization

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This PDF file includes:

Supplemental Methods

Figures S1 to S8

Tables S1-2

Legends for Movies S1

Legends for Datasets S1 to S11

Other supporting materials for this manuscript include the following:

Movies S1

Datasets S1 to S11

Supplemental Methods

Generation of transgenic parasites. Nucleofection of *T. vaginalis* strain G3 with 15 μgs of the resulting vectors or an empty vector control was done as described previously (52). Transfectants were selected and maintained using 100 μgs/ml G418. CLP and CLP-MUT transgenic parasites were generated using vectors previously described (52). The constructs were transfected into *T. vaginalis* strain G3, and parasites containing the constructs were selected using 100 μgs/ml G418. All primers used for cloning are listed in (*SI Appendix*, Table S1).

PCR verification of CLP and CLP-MUT transgenic parasites. To confirm the presence of the CLP and CLP-MUT gene in our transgenic parasites, total genomic DNA was isolated and then used in a PCR reaction with primers pMN_Out_REV-gactccactcaagacgtcac and pMN_aSCS_FWD-aacaggagaaacgattgcccc.

Immunofluorescent verification of CLP, HPB2, and HPB3 overexpressing parasites. For immunofluorescence assays, CFSE-labeled *Trichomonas* were fixed with 4% paraformaldehyde for 15 minutes. The parasites were then permeabilized and blocked for 60 minutes with 0.1–0.2% (vol/vol) Triton-X 100 in PBS (pH 7.4), with 3% (wt/vol) goat serum. Cells were then incubated with rabbit anti-HA (1:500) (Cell Signaling Technologies; Cat. #3724S) overnight at 4°C, followed by incubation with anti-rabbit CF583R at 1:500 (Biotium; Cat. #20793-50μL) and Hoechst (Biotium; Cat. #40044) for 1 hour at room temperature.

Western blot verification of CLP and CLP-MUT transgenic parasites. Mouse anti-GFP polyclonal antibody (1:1,000; Clontech) and anti-TVGAPDH (1:10,000; Cocalico Biologicals) were used as the primary antibodies, and horseradish peroxidase (HRP)-conjugated anti-mouse (1:25,000) and anti-rabbit (1:25,000) antibodies (Jackson Laboratory) were used as the secondary antibodies. A total of 1×10^6 parasites were taken from *T. vaginalis* cultures and washed with 1x PBS plus 5% sucrose plus 1x Halt protease inhibitor cocktail (Thermo Fisher Scientific). The cells

were then lysed in RIPA buffer (Thermo Fischer Scientific) plus 1x Halt protease inhibitor cocktail (Thermo Fisher Scientific). The protein concentration was quantified using Pierce BCA Kit (Thermo Scientific #23225) and equal amounts of protein were loaded from each sample.

Extracellular vesicle uptake assay. Parasites were spun and resuspended in Dulbecco's Gibco Phosphate Buffered Saline (DPBS #14190-144) at $1x10^6$ cells/mL. CFSE-labeled TvEVs were added to parasites, incubated at 37°C for the indicated timepoints and fixed in 100 µLs 4% PFA for 15 minutes at room temperature, then resuspended in 1 mL 1x DBPS for FACS analysis or imaging on Operetta® CLS™ platform (48). FACS analysis was performed using the LSRII Analyzer. Cells were first gated to include all cells and discard cellular debris. The second gate included only single cells and discarded all doublets. The third gate quantified the percent CFSE positive cells based on the unlabeled negative control. Percent uptake was then calculated by dividing the number of CFSE positive parasites by the number of total parasites multiplied by 100. MFI for CFSE is calculated from the total population of parasites (P2 gate). To measure TvEV uptake using the Operetta® CLS™ platform, we additionally stained the fixed cells with ZombieRed (Biolegend) (1:1000) for 30 minutes at room temperature, washed and resuspended in 1x PBS. Cells were then plated onto a 96-well black, clear bottom plate and imaged. Images were analyzed using Harmony™ software. Total parasites were identified and enumerated via ZombieRed staining using the standard nuclei identification module. CFSE positive parasites were identified using the Harmony™ identify population tool. Fluorescent intensity cutoffs were set at 1,000 and length and width cutoffs were set at 5 µms and 2 µms respectively to filter out small auto-fluorescent debris. Percent uptake was then calculated by dividing the number of CFSE positive parasites by the number of total parasites multiplied by 100. MFI for CFSE is calculated from the total population of parasites identified using zombieRED.

Adherence assay. 1×10^6 CFSE-labeled *T. vaginalis* parasites were incubated with the indicated amount of TvEVs for 30 minutes and then 5×10^4 parasites were added to confluent monolayers of BPH-1s for 1 hour. Unattached parasites were washed off and cells were fixed in 4%

111 formaldehyde in 1x PBS for 20 minutes at room temperature. Plates were imaged using the
112 Operetta® CLS™ platform, high throughput microplate imaging system and analyzed using
113 Harmony™ software. CFSE positive parasites were identified using the Harmony™ identify cells
114 tool. Fluorescent intensity cutoffs were set at 1,000 and length and width cutoffs were set at 5 µms
115 and 2 µms respectively to filter out small auto-fluorescent debris. The % attachment was
116 determined as the number of parasites in the well divided by input (5×10^4) multiplied by 100.
117
118 **Aggregation assay.** G3 parasites (1 x 10^6 cells/mL) were spun down at 3200 rpm for 10 minutes.
119 Pellets were resuspended in 1 mL 1x DPBS. Parasites were labeled with CFSE (1:1000) and
120 TvEVs were added at 20 µgs/mL or BSA as a control. Parasites were vortexed briefly and
121 transferred to a black, clear-bottom 96-well plate (100 µLs/well) in triplicate. Plates were incubated
122 at 37°C for 30 minutes. Afterwards, 100 µLs of 8% PFA was added (final 4% PFA concentration)
123 to fix the cells and incubated at room temperature for 15 minutes. Plates were spun at 300 rpm for
124 5 minutes before removing PFA and adding 100 µLs of 1x DPBS for imaging. Plates were imaged
125 using the Operetta® CLS™ platform, high throughput microplate imaging system and analyzed
126 using Harmony™ software. CFSE-positive parasites were identified using the Harmony™ identify
127 cell tool. Fluorescent intensity cutoffs were set at 1,000 and length and width cutoffs were set at 5
128 µms and 2 µms respectively to filter out small auto-fluorescent debris. Parasites 'clumps' were
129 counted as an aggregate if they exceeded 250 µms in diameter. I
130
131 **Immunofluorescence for dSTORM.** dSTORM imaging of parasite incubated with TvEVs was
132 performed as follows. *T. vaginalis* strain G3 was incubated with 50 µgs/mL of CLP-EVs or WT-EVs,
133 as a negative control, for 30 minutes. Parasites were then fixed and stained using dSTORM
134 Discovery Kit with Strong Fixative (Onibio) using fixative b protocol. Briefly, parasites were fixed
135 with 4% PFA-0.2% glutaraldehyde for 10 minutes followed by quenching with Discovery Kit
136 quenching solution for 7 minutes. Parasites were then blocked with 5% BSA in 1x PBS for 1 hour
137 at room temperature. Parasites were not permeabilized to ensure any CLP signal originated from
138 the surface of the parasite rather than reflecting CLP that had been internalized. Parasites were

then incubated in Mouse anti-GFP polyclonal antibody (1:1,000; Clontech) overnight at 4°C, followed by incubation with goat anti-mouse Alexa Fluor 647 (1:500; Invitrogen Cat. # A-21235) for 1 hour at room temperature. Parasites were then imaged on a Nanoimager (Onibio) at 100x magnification.

Supplemental Figures

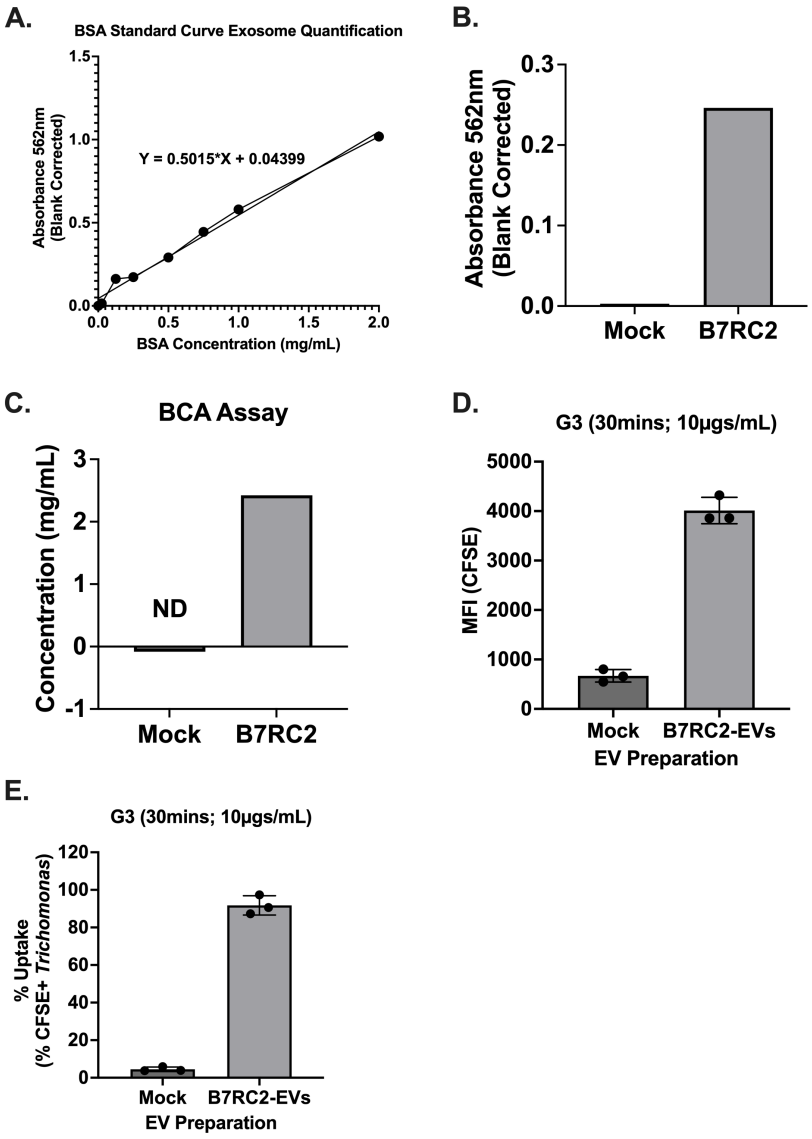

Figure S1. Validation of TvEV isolation method. (A) Bicinchoninic acid (BCA) assay standard curve using bovine serum albumin as a standard for calculating protein concentration of isolated TvEVs. (B) Absorbance values for either a mock (Diamond's media alone) or TvEV preparation. (C) Calculated concentration of either mock (Diamond's media alone) or TvEV preparation in milligrams per milliliters using equation generated in (A) multiplied by 6 to account for dilution factor. (D and E) Mean Fluorescent Intensity (MFI) and Percent uptake of *T. vaginalis* strain G3 fed either a mock preparation or TvEVs for 30mins. For mock treated parasites equivalent volumes of mock

preparation were used as the amount of protein was below the detectable limit for BCA analysis. Dots = mean ± SD. N = 3 wells/experiment, 3 experiments total.

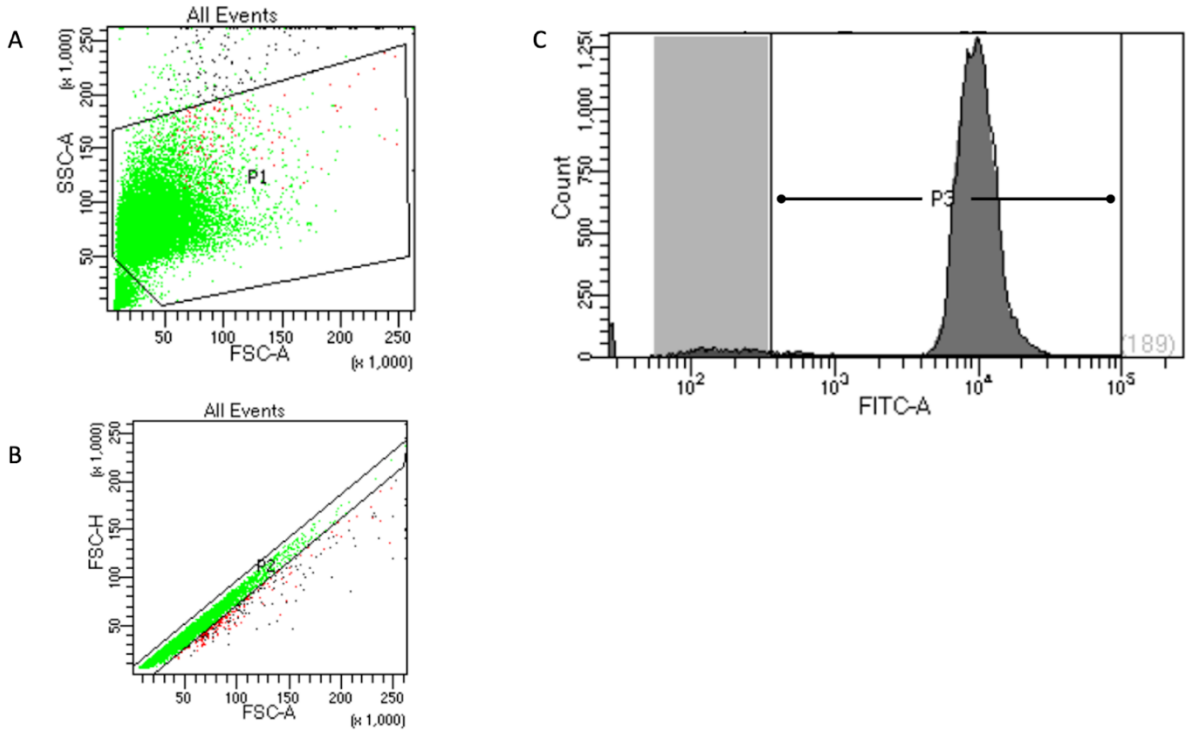

Figure S2. Example of gating scheme for Figure 1. (A) P1 – Parasites were first gated on forward and side scatter. (B) P2 – Doublets were excluded so that single parasites were counted. (C) P3 – TvEV fed parasites were then identified by CFSE positivity (labeled FITC-A on Y-axis). P3 events (CFSE positive parasites) were used to calculate % positive of population divided by the total number of parasite events recorded (Gate P2). MFI values were calculated from the entire P2 population.

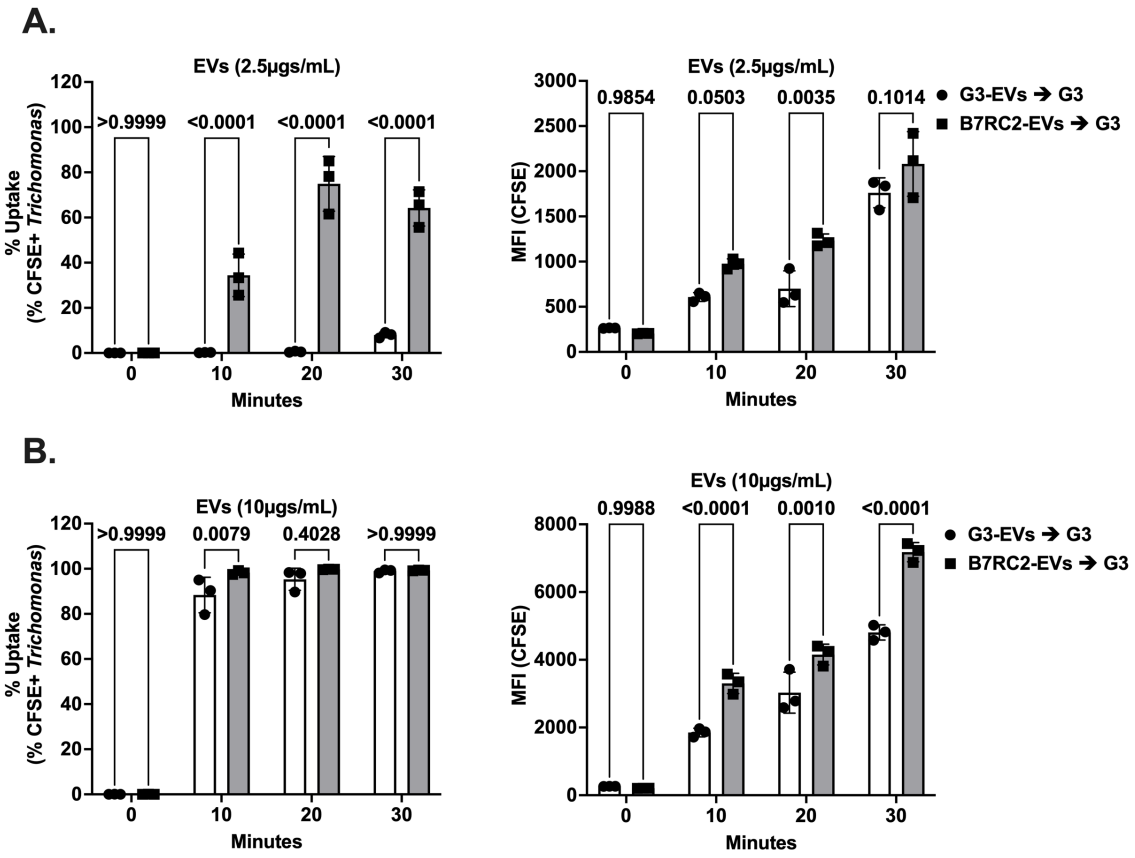

Figure S3. B7RC2-EVs are taken up more efficiently than G3-EVs. (A, B) Quantification of % uptake and total MFI between G3-EVs and B7RC2-EVs at (A) 2.5 µgs/mL and (B) 10 µgs/mL. Bars, mean ± SD. N = 3 wells/experiment, 3 experiments total. Numbers above bars indicate p-values for two-way ANOVA, Dunnett's multiple comparisons test compared to G3-EVs.

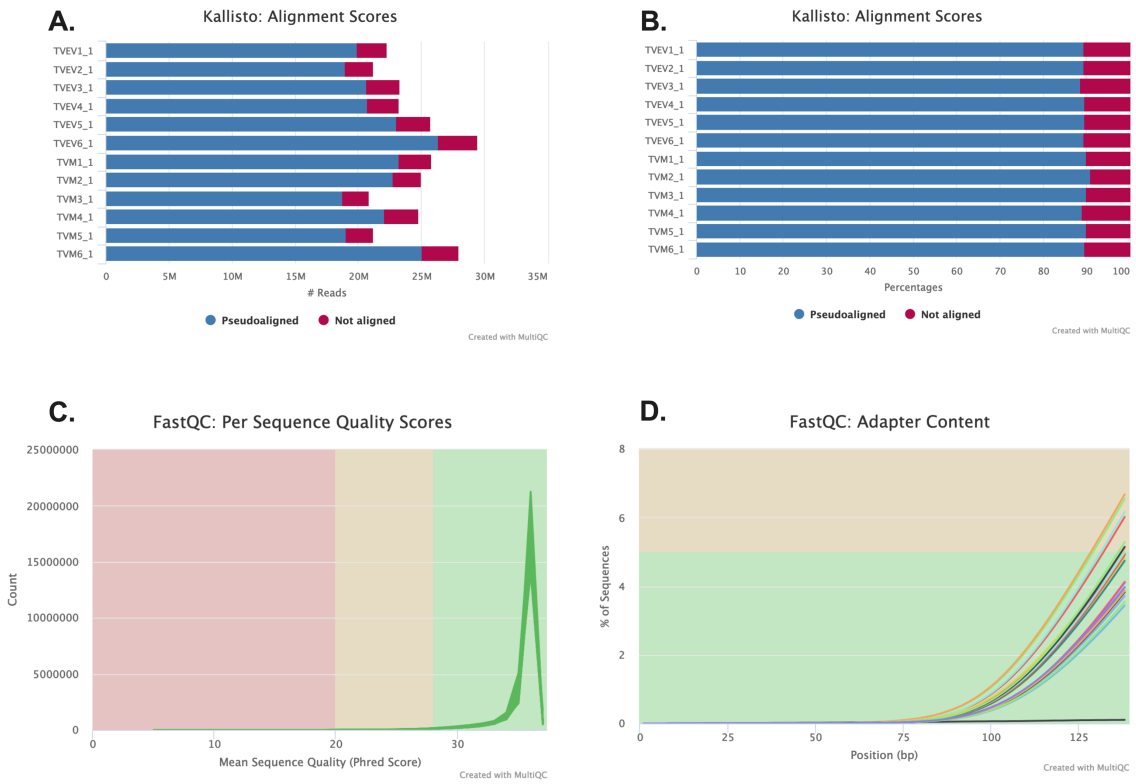

Figure S4. MultiQC summary of RNA-seq data. (A and B) Summary of number of reads and percentage of reads for each sample that pseudoaligned to the *T. vaginalis* G3 transcriptome available on TrichDB (https://trichdb.org/common/downloads/release-64/TvaginalisG32022/fasta/data/). (C and B) Per sequence Phred score and adapter content for each sample summarized using MultiQC.

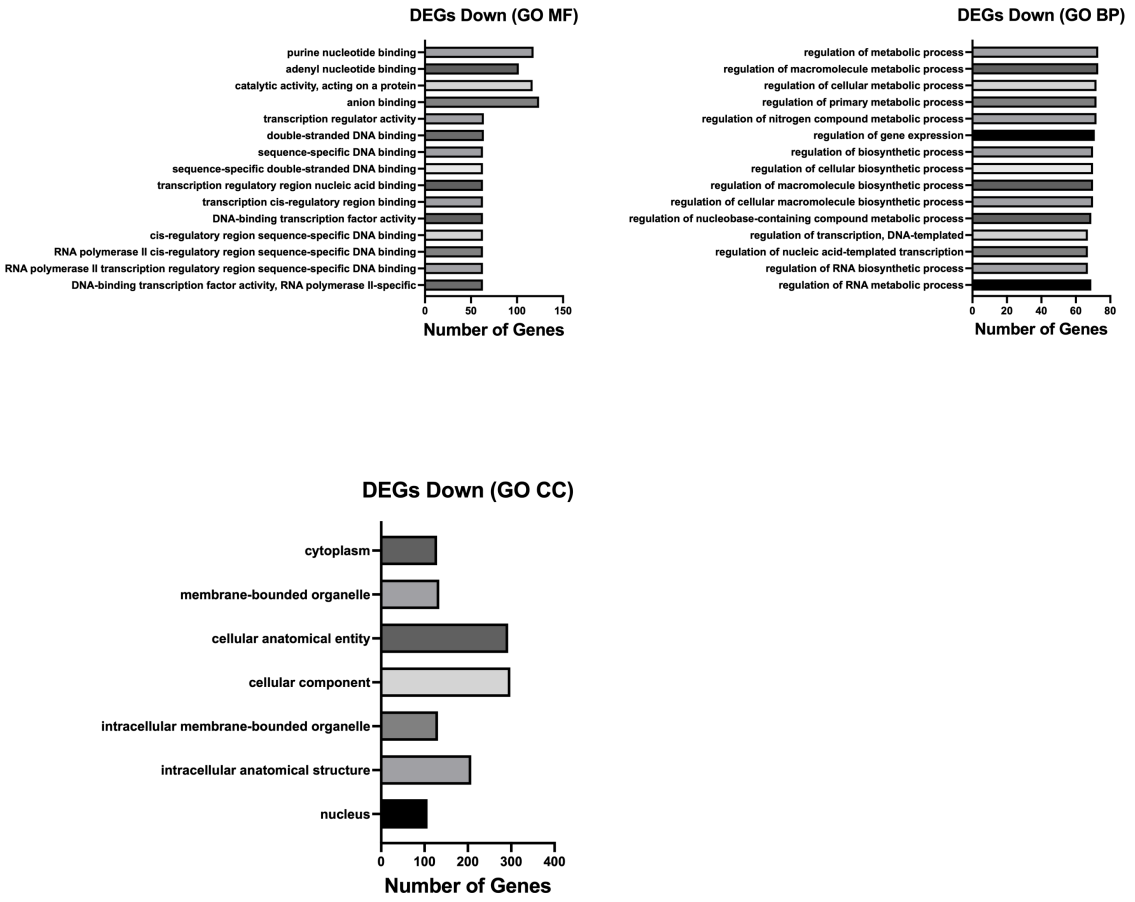

Figure S5. GO enrichment summary of down-regulated DEGs from RNA-seq data. Number of *T. vaginalis* genes in each significantly enriched GO annotation for molecular function (MF), biological process (BP), and cellular compartment (CC). Full set of GO annotations can be found in Dataset S3-5.

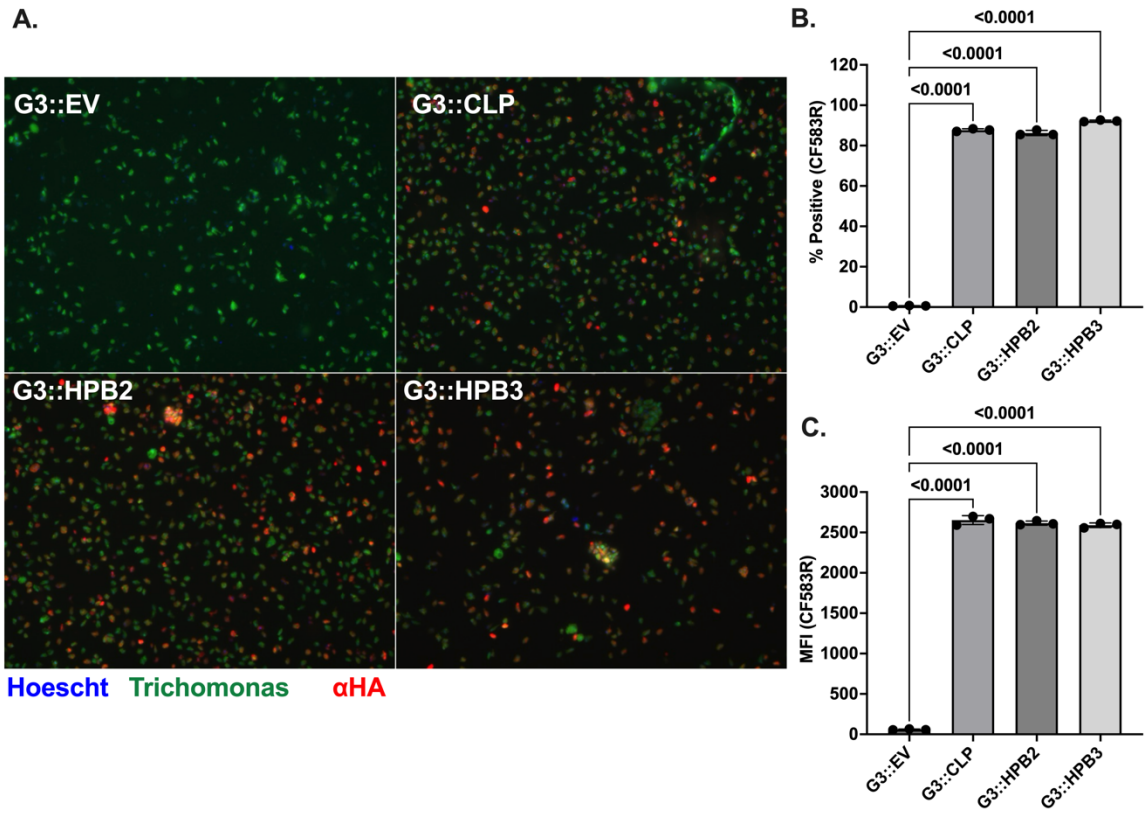

Figure S6. Overexpression of CLP, HPB2, and HPB3 in *T. vaginalis* strain G3. (A) Representative IFA of HA-tag on CLP, HPB2, and HPB3. Images depict Hoechst (Blue), CFSE-labelled *T. vaginalis* strain G3 (Green) and CF583R conjugated secondaries for the HA-tag on each of the above indicated proteins (Red). Quantification of % of parasites positive for CF583R signal. (C) MFI of parasites stained for HA-tag on each of the above indicated proteins. (B and C) Bars, mean ± SD. N = 3 wells/experiment, 3 experiments total. Numbers above bars indicate p-values for one-way ANOVA, Dunnett's multiple comparisons test compared to G3::EV.

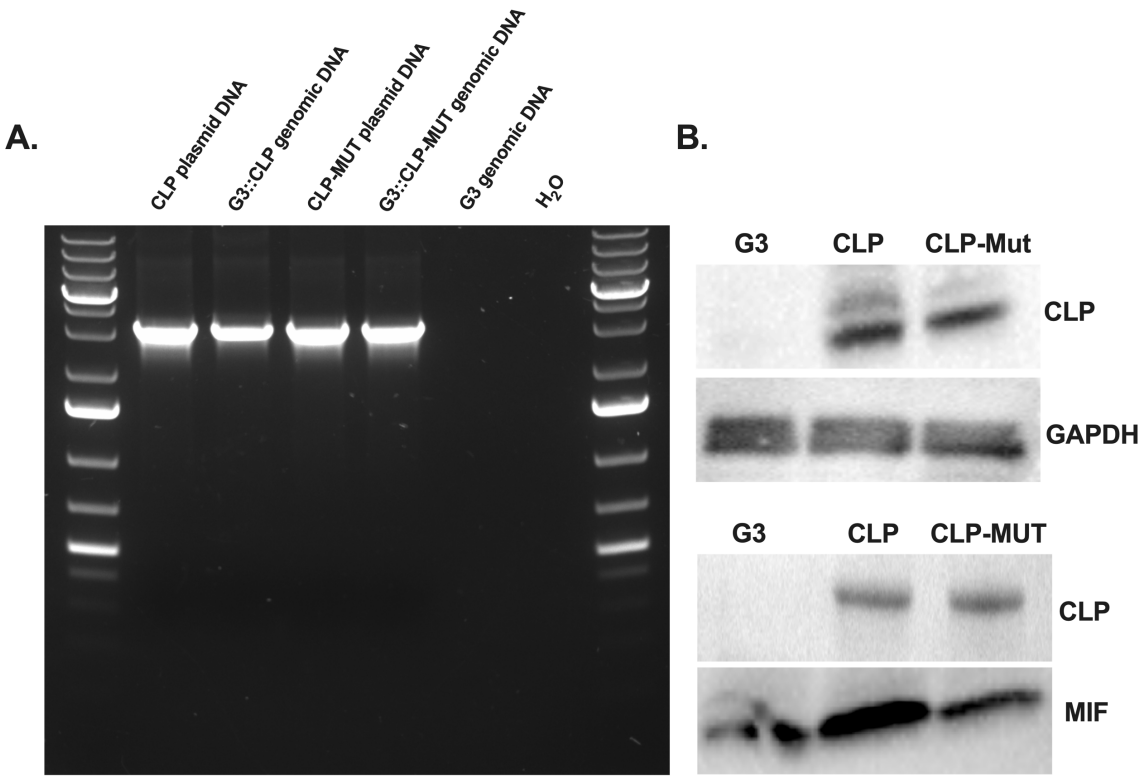

Figure S7. PCR confirmation of CLP and CLP-MUT G3 strains. (A) Expected size = 2970bp. (B) *Top*, Western blot analysis of CLP overexpression in *T. vaginalis* strain G3. *Bottom*, Western blot analysis of CLP overexpression in TvEVs derived from indicated strains. GPDH is used as host cell protein loading control and MIF is used as a TvEV resident protein control.

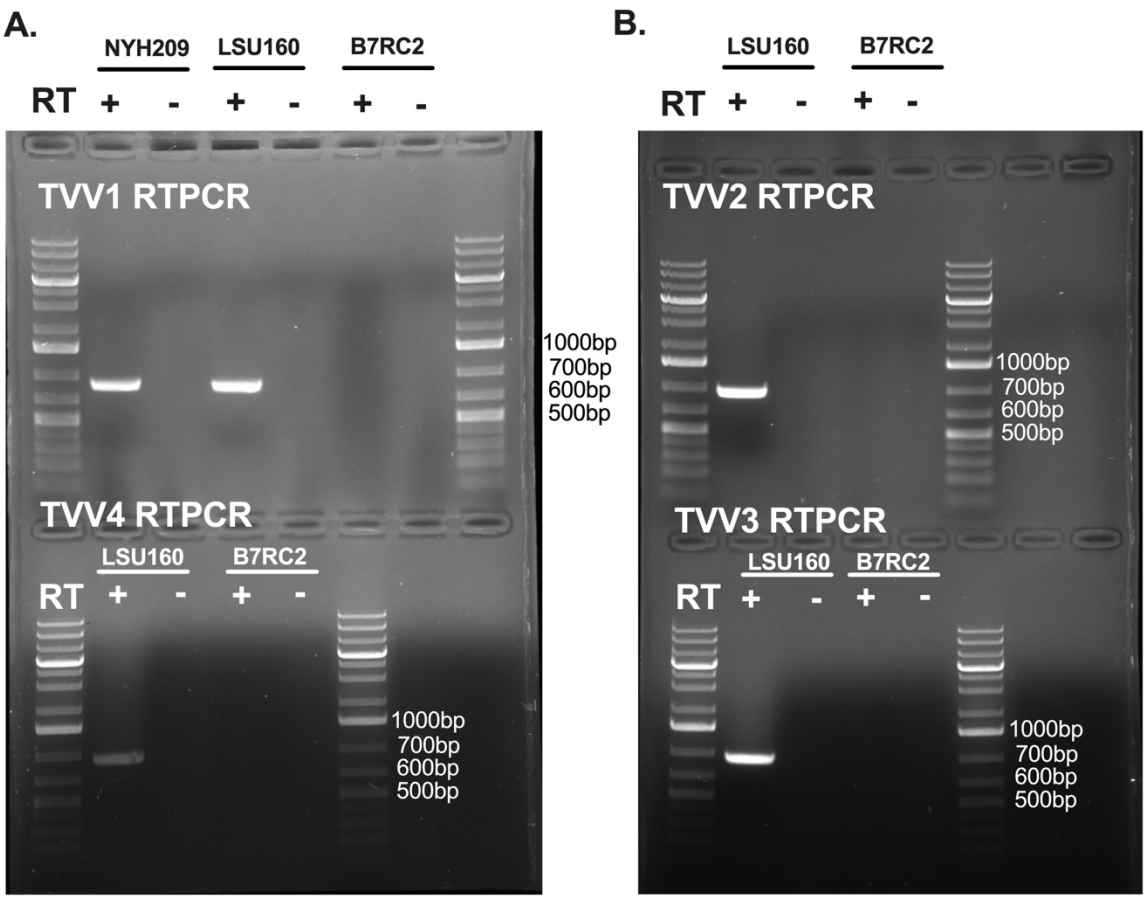

Figure S8. RT-PCR screen for *Trichomonas vaginalis* virus (TVV1-4) in *T. vaginalis* strain B7RC2. (A) RT-PCR screen for TVV1, or 4 in *T. vaginalis* strains NYH209 (positive control containing TVV1), LSU160 (positive control containing both TVV1 & 4) and B7RC2. Expected size for TVV1 = 652bp. Expected size for TVV4 = 700bp. (B) RT-PCR screen for TVV2 and TVV3 in *T. vaginalis* strains LSU160 (positive control containing both TVV2 & 3) and B7RC2. Expected size for TVV2 and TVV3 = 680bp. Positive signals for TVV1-4 are observed in the reverse transcriptase (RT)+ samples for positive control strains. No signal is observed for TVV1-4 in B7RC2.

Table S1. List of Top 10 most significantly up-regulated DEGs.

Gene ID (2022 Reference)	Gene ID (2013 Reference)	Product Description	log2(FC)	padj
TVAGG3_0964910	TVAG_097730	Secreted RxLR effector peptide	1.53946691	4.06E-09
TVAGG3_0232100	TVAG_157940	heteropolysaccharide binding	1.15253797	4.19E-09
TVAGG3_0392810	TVAG_185520	bacterial hemolysins family	1.36428372	4.19E-09
TVAGG3_0969590	TVAG_222040	heteropolysaccharide binding	1.71079909	4.19E-09
TVAGG3_0163930	TVAG_154680	heteropolysaccharide binding	1.28699709	5.25E-09
TVAGG3_0430810	TVAG_239840	Major Facilitator Superfamily protein family	1.10461903	1.05E-08
TVAGG3_0606520	TVAG_237690	actin filament capping	1.03031125	1.26E-08
TVAGG3_0583720	TVAG_393390	unspecified product (cadherin-like protein)	0.89773958	1.39E-08
TVAGG3_0410780	TVAG_414870	unspecified product	1.1369581	1.58E-08

Table S2. Primers used throughout this study. Sequences listed 5'-3'

Primer Name	Sequence	Use
pMN_Out_REV	GACTCCACTCAAGACGTCAC	PCR verification in Figure S6
pMN_aSCS_FWD	AACAGGAGAAACGATTGCCCC	PCR verification in Figure S6
pMNeo_IF_FWD	TACCCATACGATGTTCCAGATTACG	In-fusion cloning for vector (pMNeo) backbone
pMNeo_IF_REV	ATGATGTGAAGTGAACAAAGCC	In-fusion cloning for vector (pMNeo) backbone
CLP_pMNeo_IF_FWD	TTCACTTCACATCATATGATTTGGACTTTTTTATTGCAGG	In-fusion cloning for CLP
CLP_pMNeo_IF_REV	AACATCGTATGGGTACTTTCTAAGCCAAAGAATTATTACT	In-fusion cloning for CLP
HPB2_pMNeo_IF_FWD	TTCACTTCACATCATATGATCTCAGCAACATCAGTAAATG	In-fusion cloning for HPB2
HPB2_pMNeo_IF_REV	AACATCGTATGGGTATATTCTGTGTGAATCGCGAAC	In-fusion cloning for HPB2
HPB3_pMNeo_IF_FWD	TTCACTTCACATCATATGATCTCCACATCAGCCAATGGA	In-fusion cloning for HPB3
HPB3_pMNeo_IF_REV	AACATCGTATGGGTATATTCTGTGGGAATCACGAACGAGT	In-fusion cloning for HPB3
TVV1_FWD	CACTTGACCGGATCTTGCCTCGCAT	RT-PCR screen for TVV1 Figure S8
TVV1_REV	TATTACGGGGCCACAACGTACAGCT	RT-PCR screen for TVV1 Figure S8
TVV2_FWD	TACTAATAGTGACTGCGGTCCCTGA	RT-PCR screen for TVV2 Figure S8
TVV2_REV	GATCCGTAGATTTGGCTCATCGCCA	RT-PCR screen for TVV2 Figure S8
TVV3_FWD	CTGACTCAGTCAGACCAGTCCAAAC	RT-PCR screen for TVV3 Figure S8

TVV3_REV	CCTTGCGTTGGATGATGTCCATGTA	RT-PCR screen for TVV3 Figure S8
TVV4_FWD	ACCTGCCCAGTTTTCGATGGTTCACA	RT-PCR screen for TVV4 Figure S8
TVV4_REV	GTGAGGCTTTGCGGATGAGGCCT	RT-PCR screen for TVV4 Figure S8

Movie S1 (separate file). Exoglow™ Green-labeled TvEVs are internalized by *T. vaginalis*. Time-lapse video showing the uptake of fluorescently labeled G3-EVs by *T. vaginalis* strain G3.

Dataset S1 (separate file). Gene expression data for *T. vaginalis* treated with TvEVs compared to mock.

Dataset S2 (separate file). Down-regulated DEGs for *T. vaginalis* treated with TvEVs compared to mock.

Dataset S3 (separate file). Gene Ontology for Molecular Function of down-regulated DEGs.

Dataset S4 (separate file). Gene Ontology for Biological Process of down-regulated DEGs.

Dataset S5 (separate file). Gene Ontology for Cell Compartment of down-regulated DEGs.

Dataset S6 (separate file). Up-regulated DEGs for *T. vaginalis* treated with TvEVs compared to mock.

Dataset S7 (separate file). Gene Ontology for Molecular Function of up-regulated DEGs.

Dataset S8 (separate file). Gene Ontology for Biological Process of up-regulated DEGs.

Dataset S9 (separate file). Gene Ontology for Cell Compartment of up-regulated DEGs.

Dataset S10 (separate file). Protein abundance data for B7RC2-EVs vs NYH209-EVs.

Dataset S11 (separate file). Differentially abundant proteins B7RC2-EVs vs NYH209-EVs.