Supplementary Information for

"A broadly active fucosyltransferase ***LmjFUT1*** whose mitochondrial localization and activity is essential in parasitic ***Leishmania*****"**

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Supplemental results.

Efforts to detect potential *FUT1*-dependent products in *Leishmania major*. We surveyed unsuccessfully for *FUT1*-dependent products within the parasite mitochondrion, comparing WT and Δ*fut1*^{S} parasites. No mitochondrial specific-labelling was observed with fluorescent lectins from *Aleuria aurantia* (AAL), *Ulex europaeus* (UEA) or Isolectin B4 (IB4) (1), or with antisera to anti-blood group antigen H type 1 (2) or anti Glcβ1-3 Fucα1- (provided by L. Mahal and L. Zhang, New York University). For both lectins and antibodies, often labelling outside of the mitochondrion was observed, possibly arising from one of the other secretory pathway glycosyltransferases (Table 1). Bioorthogonal labelling with 6-alkynyl fucose showed no differences, although labelling was seen in a region in the vicinity of the Golgi/flagellar pocket in both. Such labelling, was absent in the Δ*lpg2*$^{-}$ mutant lacking the Golgi GDP-Man/ L-Fuc/D-Arap transporter (3-5), suggesting that 6-alkymyl fucose had been activated to GDP-6-alkynyl-Fuc and trafficked to the secretory pathway for use by other FUTs therein (Table 1).

Detailed Materials and Methods.

***Leishmania* culture and transfection**. *L. major* strain FV1 (LmjF or WT; WHO code MHOM/IL/80/Friedlin) was grown at 26°C in M199 medium (U.S. Biologicals) containing 10% heat-inactivated fetal bovine serum and other supplements and transfected by electroporation using a high voltage protocol as described (6, 7). Following transfection, cells were allowed to grow for 16-24 h in M199 medium and then plated on semisolid media containing 1% Nobel

agar (Fisher) and appropriate selective drugs (30 μg/ml puromycin, 100 μg/ml nourseothricin, 10 μg/ml phleomycin, or 10 μg/ml G418). Individual colonies were picked and grown in liquid medium in same drug concentration as used in plates. Clones were maintained in selective medium for less than 10 passages before use, prior to which selective drug was removed for one passage. In some studies parasites were grown in Schneider's medium containing 10 % heat-inactivated fetal bovine serum.

Molecular constructions and primers. Molecular constructs are described in detail in Table S1 and oligonucleotide primers are described in Table S2. Molecular constructs were confirmed by restriction mapping, sequencing, and functional testing. Molecular methods were performed as described (30).

Homologous replacement of chromosomal FUTs, often in the presence of ectopically expressed *FUTs*. For *FUT1,* pXNGPHLEO-*FUT1* (B7073) was first transfected to LmFV1 WT, yielding WT / +pXNGPHLEO-*FUT1*. The presence of plasmid was confirmed by PCR and FACS analysis for the presence of *PHLEO* and GFP markers, respectively. The targeting fragments *FUT1*::*SAT* and *FUT1*::*PAC* were liberated from pGEM-*FUT1-SAT* (B7081) or pGEM-*FUT1-PAC* (B7079) by digestion with BamHI and HindIII, and then sequentially transfected into WT/ +pXNGPHLEO-*FUT1* with nourseothricin or puromycin selection, to generate Δ*fut1*::*PAC*/Δ*fut1*::*SAT/ +pXNGPHLEO-FUT1* (termed Δ*fut1¯/ +pXNGPHLEO-FUT1*). For plasmid shuffling experiments, Δ*fut1¯/ +pXNG-FUT1* was further transfected with episomal pIR1NEO (B6483) into which test *FUT1* genes had been inserted (Table S1).

A similar protocol was followed for *FUT2*, substituting pXNGPHLEO-FUT2 and targeting fragments *FUT2*::*HYG* and *FUT2*::*PAC* (Table S1, Fig. S2), to generate Δ*fut2*::*PAC*/ Δ*fut2*::*HYG* / +*pXNGPHLEO-FUT2*, hereafter termed Δ*fut2*¯ / +*pXNGPHLEO-FUT2*.

Deletion of both chromosomal copies of *SCAL* was accomplished by successive introduction of targeting fragments *SCAL*::*HYG* and *SCAL*::*BSD* (Table S1; Fig. S1), yielding Δ*scal*::*HYG*/ Δ*scal*::*BSD* , hereafter termed Δ*scal*¯.

Plasmid segregation or shuffling by single cell sorting. Δ*fut1*¯ parasites bearing pXNGPHLEO-FUT1 alone (segregation) or additionally expressing test FUT1 sequences from pIR1NEO (shuffling) were subjected to flow cytometry and single cell cloning to recover GFP+ cells (bright) or GFP-negative (dim), the latter representing potential segregants or 'shuffles'. Prior to flow cytometry, cells were incubated in M199 medium without phleomycin for 24 h. washed with phosphate-buffered saline (PBS), and filtered through CellTrics 50 μm filters to remove particulates. Single cell sorting of GFP "bright" or "dim" cells was performed with appropriate gates (Fig. 2C illustrates typical gating settings) using a Dako MoFlo high-speed cell sorter, with single cells selected by stringent gating on forward and side scatter parameters. Single cells were placed into individual wells of 96-well plates, each containing 150 μl M199 or Schneider medium. Plates were incubated at 26° C for at least 2 weeks and parasite growth assessed.

Subcellular localization of FUT1. Logarithmic phase parasites expressing FUT-HA were collected by centrifugation and resuspended in DMEM media containing Mitotracker Red CMXRos (Invitrogen; 50 nM) for 15 min. Parasites were then washed in DMEM and fixed in

4% paraformaldehyde (in PBS) for 5 min, washed in PBS, and deposited on coverslips by centrifugation. The coverslips were then incubated in blocking buffer (PBS containing 0.1% (v/v) Triton-X-100 and 5% (v/v) normal goat sera) for 30 min prior to staining with a rabbit anti-HA polyclonal antibody (Invitrogen, diluted 1:100 in blocking buffer) for 1 h. Following extensive washing in PBS, the coverslips were then stained with an Alexa 488 goat anti-rabbit antibody (Invitrogen, diluted 1:1000 in blocking buffer).

Immunoelectron Microscopy. Parasites were fixed in 4% paraformaldehyde/0.05% glutaraldehyde (Polysciences Inc., Warrington, PA) in 100 mM PIPES/0.5 mM $MgCl_2$, pH 7.2 for 1 hr at 4°C. Samples were then embedded in 10% gelatin and infiltrated overnight with 2.3 M sucrose/20% polyvinyl pyrrolidone in PIPES/$MgCl_2$ at 4°C. Samples were trimmed, frozen in liquid nitrogen, and sectioned with a Leica Ultracut UCT cryo-ultramicrotome (Leica Microsystems Inc., Bannockburn, IL). 50 nm sections were blocked with 5% FBS/5% NGS and subsequently incubated with rabbit anti-HA antibody (H 6906, Sigma, St. Louis, MO) for 1 hr, followed by secondary anti-rabbit IgG antibody conjugated to 18nm colloidal gold (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 1 hr. Labeling experiments were conducted in parallel with controls omitting the primary antibody, which were consistently negative. Sections were washed in PIPES buffer followed by a water rinse, and stained with 0.3% uranyl acetate/2% methyl cellulose. Samples were viewed with a JEOL 1200EX transmission electron microscope (JEOL USA Inc., Peabody, MA). For quantitation, images from sections intersecting the mitochondrion, cytosol and nucleus were chosen; for each compartment, the area was measured using Image J and beads were counted. Bead counts were normalized to the relative area of each compartment measured across all sections (approximately 1:3:2 for mitochondrion: cytosol: nucleus).

Transmission electron microscopy. Parasites were fixed with 4% paraformaldehyde (PFA) and 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 4 h at room temperature (RT). The samples were then washed in 0.1 M phosphate buffer, postfixed in 2% OsO4, and encapsulated in agarose. This was followed by dehydration in ascending grades of ethanol, infiltration, embedding in an Epon 812-araldite plastic mixture, and polymerization at 60°C for 24 h. Ultrathin sections (50 to 70 nm) were obtained using an ultramicrotome (Leica Ultracut UCT; Leica Microsystems GmbH, Wetzlar, Germany) and picked up onto 200 mesh copper grids. The sections were double stained with uranyl acetate and lead citrate and observed under a FEI Tecnai-12 twin transmission electron microscope equipped with a SIS MegaView II CCD camera at 80 kV (FEI Company, Hillsboro, OR, USA).

Measurement of mitochondrial membrane potential (ΔΨm) in live *Leishmania* promastigotes. Tetramethylrhodamine ethylester perchlorate (TMRE, Molecular Probes, Inc., Eugene OR, USA) was prepared at a concentration of 1 mM in DMSO and stored at -20°C. *L. major* promastigotes were washed once in PBS, resuspended at 2×10^6 cells/ml in PBS containing 100 nM TMRE, incubated for 15 min at room temperature, and analyzed by flow cytometry on the FL2-H channel. Baseline TMRE was recorded and expressed as mean fluorescence in arbitrary units (a.u.). Subsequently, carbonyl cyanide m-chlorophenylhydrazone (CCCP, Sigma) was added to the cells (300 mM final concentration) and TMRE fluorescence was measured at various times up to 60 min.

Protein expression and purification. GST- and His-tagged recombinant *FUT1* constructs were transformed to BL21 (DE3) *E. coli* strain. Cells were grown at 37 ºC until OD_{600} reached about 0.5, when 50 µM isopropyl-β-D-thiogalactopyranoside (IPTG) was added, and cultures were

further incubated at 15 ºC for 16 h. Cells were harvested by centrifugation and incubated 1 hr on ice in 10 mM TrisHCl, pH 7.5, 0.15 M NaCl, 1 mM DTT, 1 mg/ml lysozyme (Sigma) with EDTA-free Complete Protease Inhibitor Tablet (Roche), before disrupting by sonication. The soluble fraction was obtained by centrifugation at 17,000 × *g*, 4 ºC for 30 min. For GST-tagged protein purification, the supernatant was incubated with Glutathione Sepharose 4B resin (GE Healthcare) for 1 h at 4 ºC. The bound protein was washed with 10 bed columns of 10 mM TrisHCl, 0.15 M NaCl followed by elution using 50 mM Tris, 50 mM reduced glutathione, 0.15 M NaCl, 5 mM DTT, 0.1% Triton X-100, pH 8.5. For His-tagged PTR1-FUT1, supernatant was incubated with Ni-NTA resin (Qiagen) for 1 h at 4 ºC. The bound protein was washed with 10 bed columns of 10 mM TrisHCl, pH 8.0, 0.15 M NaCl, 50 mM imidazole followed by elution using 10 mM TrisHCl, 0.15 M NaCl, 250 mM imidazole, 5 mM DTT, 1% Triton X-100. The yield of the recombinant protein was estimated by Qubit 2.0 (Invitrogen) and the degree of purification by SDS-PAGE analysis.

Fucosyltransferase activity assays. Enzymatic activity was determined by GDP-Glo bioluminescent GDP detection assay with GDP-Fucose (Promega Corporation, Madison, WI USA). Aliquots of 3 µg of the affinity purified recombinant proteins were incubated with 600 µM GDP-Fuc, 10 mM acceptor **(**β-D-Gal-(1→3)-D-GlcNAc**;** lac-N-biose or LNB), N-acetyllactosamine (LacNAc), β-Galactose, or Galβ1,3GalNAc) or 1.7 mM peptide from mHSP70 or mHSP60 (EWKYVSDAEKE or SKELESLANDS, respectively; Peptide2go, Manassas, VA) in 20 mM sodium phosphate, 25 mM KCl, 5 mM $MgCl_2$, pH 6.0 or 40 µM acceptor (TSR or EGF repeats, provided by R. Haltiwanger, U. Ga.) in 50 mM imidazole-HCl (pH 7.0), 50 mM $MnCl_2$, in a final volume of 25 µl for 5 min at 37 ºC. Reactions were

terminated by boiling for 5 min before 25 µl of GDP detection regent was added and incubated at RT for 1 h. Luminescence was recorded using a Microplate Luminometer or IVIS imaging system (Perkin Elmer, IL)

For peptide mass spectrometry, a reaction mix of LmFUT1 with the mHSP70 peptide above was acidified with trifluoroacetic acid (TFA) to a final concentration of 1%. Peptides were bound to porous graphite carbon micro-tips (Glygen) (PMID 22338125) and were eluted with 60% acetonitrile in 0.1% TFA, and dried in a Speed-Vac after adding TFA to 5%. and dissolved in 2.7 µL of 1% formic acid. Samples were analyzed by LC-MS, using an 75 µm i.d. × 50 cm Acclaim® PepMap 100 C18 RSLC column (Thermo-Fisher Scientific) equilibrated with 1% formic acid and a gradient from 1% formic acid to acetonitrile/1% formic acid and a Q Exactiv™ Quadrupole-Orbitrap™ mass spectrometer. The acquisition of the Higher-energy collisional dissociation (HCD) spectra were triggered from the values corresponding to the expected charge states of the doubly and triply charged protonated molecular ions for the unmodified and fucosylated EWKYVSDAEKE peptide (*m/z* = 692.325, 461.888, 765.354 and 510.572). The MS1 scan was acquired in the Orbitrap mass analyzer with an AGC target value of 3e6 over a scan range of *m/z* = 150 – 2000 at a mass resolving power set to 70,000 (at *m/*z = 200). The unprocessed LC-MS data were analyzed using SKYLINE (version 3.6.9). It should be noted that HCD can lead to significant loss of some post-translational modifications, leading to an underestimation or in some cases complete loss (8).

Enzymatic assay of HA-tagged FUT1 expressed in *Leishmania* and bound to anti-HA beads. Assays and resolution of products by HPTLC were performed as described (9). Briefly, *Leishmania* lysates were incubated with anti-HA-beads (Pierce) in the presence of 1 µCi

GDP[^{3}H]Fuc (American Radiochemicals) and 1 mM LNB in 50 mM TrisHCl, 25 mM KCl, pH 7.2 in a final volume of 25 µl for 2 h at 37°C. Reactions were then cooled on ice, water added to 200 µl final volume, and desalted on a mixed-bed column made of 100 µl each Chelex100 (Na^+) over Dowex AG50 (H^+) over Dowex AG3 (OH^-) over QAE-Sepharose A25 (OH^-). The products were freeze dried, resuspended in water and dried in a Speedvac concentrator, and dissolved in 20% 1-propanol (HiPerSolv Chromanorm, VWR). They were then separated by HPTLC using 10 cm Si-60 plates (Merck) and separated using 1-propanol : acetone : water 9:6:4 (v:v:v) as mobile phase. Plates were sprayed with En^{3}hance® (PerkinElmer) and the materials visualized by fluorography at -80°C using Biomax XAR film (Kodak) with an intensifying screen.

Lectins, antibodies, and 6-alkynyl labelling. For lectin-mediated imaging, WT, ***Δfut1^{s}***, or ***Δlpg2^{-}*** parasites were grown in the presence of 100 µM to 1mM L-fucose. After 3 days, 2 x 10^6 parasites were washed, fixed onto glass coverslips, and incubated with 17 µg/mL fluorescein labeled *Aleuria aurantia* lectin (AAL; Vector Laboratories FL-1319), 20 µg/mL *Ulex Europaeus* Agglutinin I (UEAI; Vector Laboratories FL-1061), or 5µg/mL *Griffonia simplicifolia* IB4-Alexa 488 lectin (Thermo Fisher; kindly provided by Igor Almeida) at room temperature in the dark for one hour. For antibody-mediated imaging, fixed parasites were incubated with 5µg/mL Blood Group Antigen H (O) Type 1 monoclonal antibody (Invitrogen 13-9810-82), washed three times with 1x PBS and incubated with 1:100 α-mouse secondary antibody conjugated to Alexa 488 (Invitrogen, A11029) each for 1 hour at room temperature. All imaging was performed using a Zeiss LSM880 Confocal Laser Scanning Microscope with Airyscan.
For biorthogonal labeling, parasites were inoculated into M199 medium at 1 x 10^5/mL (WT or ***Δlpg2^{-}***) or 3.8 x 10^6 / ml (***Δfut1^{s}***) and grown for three days in the presence/absense of 100µM alkyne fucose (Invitrogen C10264), after which parasites were washed in PBS. Prior to confocal

microscopy, click labeling was done using azide-488 (Invitrogen A10266) and the Click-iT™ Cell Reaction Buffer Kit (Invitrogen C10269). For western blotting, 1 x 10^6 labelled parasites were lysed 10 mM Tris pH7.5, 150 mM NaCl, 1% NP-40, and Roche cOmplete EDTA free proteinase inhibitor cocktail (11836170001). Click labeling with biotin azide (Invitrogen B10184) was performed using the Click-iT™ Protein Reaction Buffer Kit (Invitrogen C10276). Proteins were separated using a 10% SDS-PAGE gel and transferred onto a PVDF membrane which was subsequently probed with 1:5,000 fluorescent streptavidin probe (Li-Cor 925-32230). Antisera against *L. major* histone H2A was prepared as described and used as a sample loading control (10). Western blotting with anti-GlcFuc antibody (kindly provided by Lara Mahal) was performed with lysed samples from WT, *Δfut1*^{s}**,** or *Δlpg2*$^{-}$ parasites grown with or without 1mM fucose as described above; a positive control of 336 ng BSA-GlcFuc was included. The membrane was incubated in using 1µg/mL anti-GlcFuc for 1 hour at room temperature, washed, and probed with 1:5,000 donkey anti-chicken secondary antibody for 1 hour at room temperature (Li-Cor 925-68075). All western blot imaging was performed using the Li-Cor system.

SI References

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Figure S1

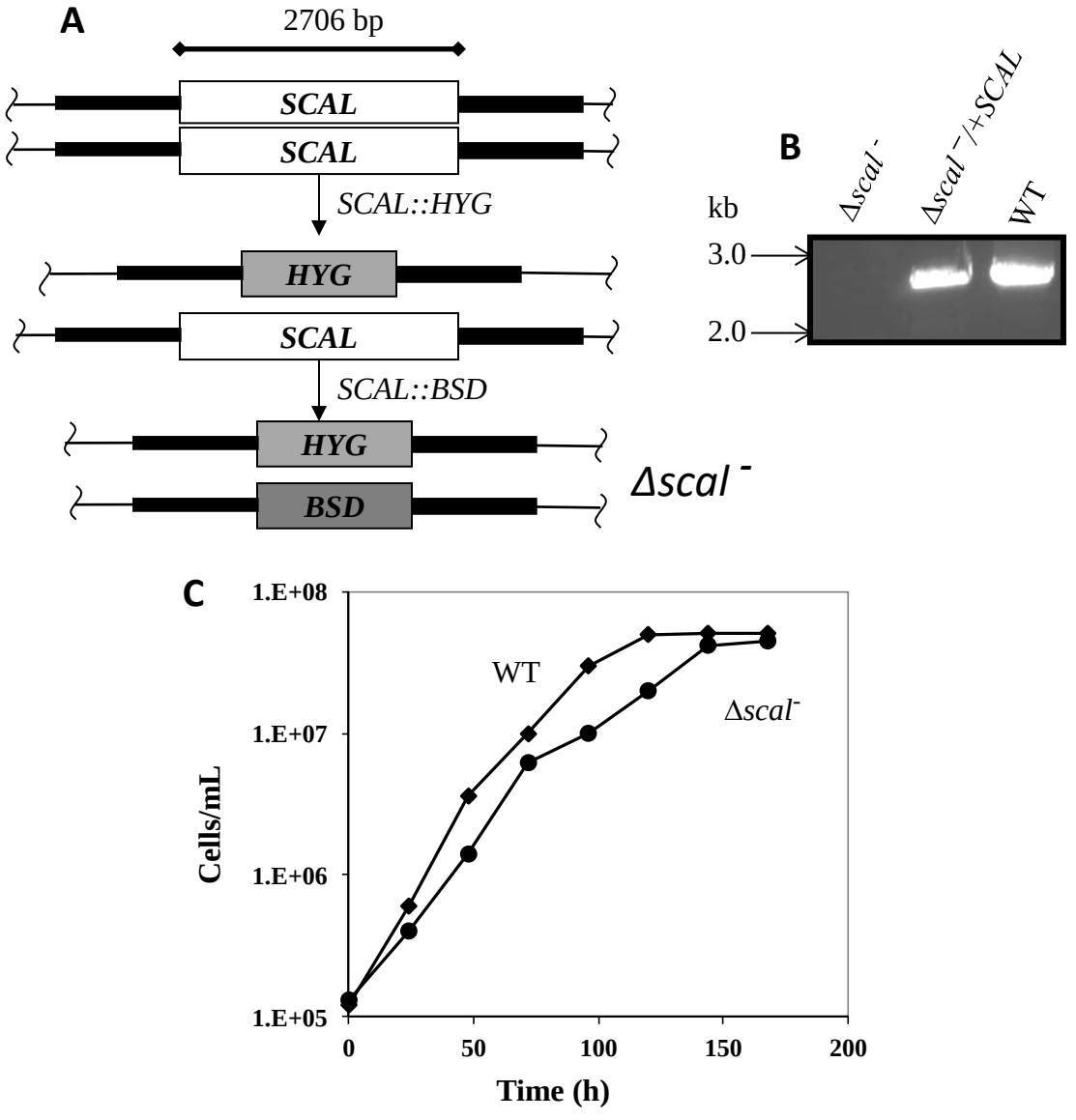


Figure S1. The *SCAL* gene is not essential in *L. major* promastigotes

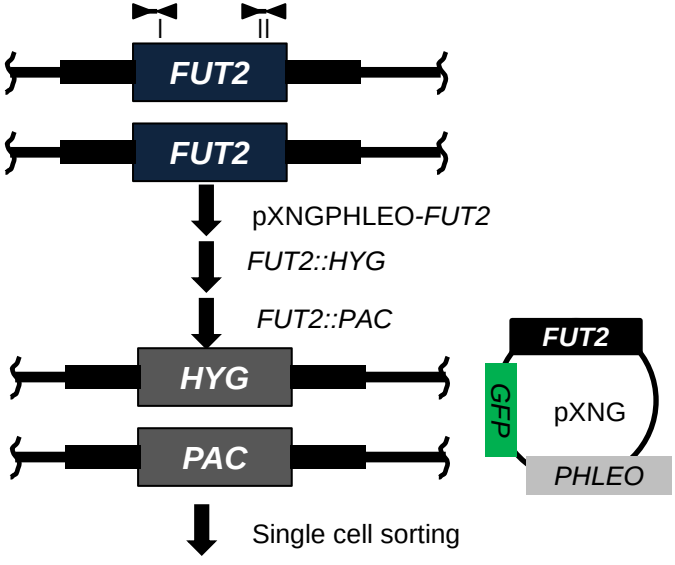
A. Schematic of the *SCAL* locus showing flanking regions used for homologous gene replacement (dark flanking bars), the SCAL ORF used for PCR tests with primers 3840/3841, and the *SCAL::HYG* or *SCAL::BSD* drug resistance cassettes. WT *L. major* were successively transfected and selected for the *SCAL*::*HYG* and *SCAL*:*BSD* targeting fragments, leading to complete loss of *SCAL (*Δ*scal*⁻*).*

B. PCR with *SCAL* ORF primers showing loss of *SCAL* in Δ*scal* ⁻ and retention in WT. and restoration in Δ*scal* ⁻/ +pXNGSAT-*SCAL*

C. Growth of WT and Δ*scal*⁻ in M199 medium.

Figure S2

A



B

	Wells exhibiting growth	Retention of pXNGPHLEO –*FUT2*
"dim"	**245/288** (85%)	**1/24** (4%)
"Bright" (control)	**177/192** (92%)	**12/12** (100%)

C

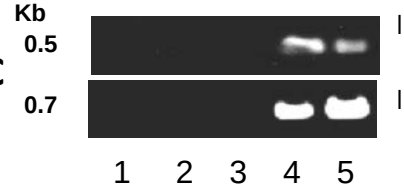


Figure S2. The *FUT2* gene is not essential in *L. major* promastigotes.

A. Diagram of the *FUT2* locus and PCR primers used, and the scheme for introduction of episomal FUT2 followed deletion of both chromosomal alleles. Heavy black bars depict the flanking sequences used in targeting constructs.

B. Results of plasmid segregation tests; survival of "dim" cells and loss of pXNGPHLEO-*FUT2* established the viability of the Δ*fut2*$^{-}$ cells.

C. PCR to confirm loss of all *FUT2* sequences in Δ*fut2*$^{-}$. Lanes 1-3, 3 different Δ*fut2*$^{-}$ clones; lane 4, Δ*fut2*$^{-}$ /+pIR1SAT-*FUT2*; lane 5, WT. Primer pairs I (3990/3839) and II (3957/3958) probe the left and right ends of the *FUT2* ORF.

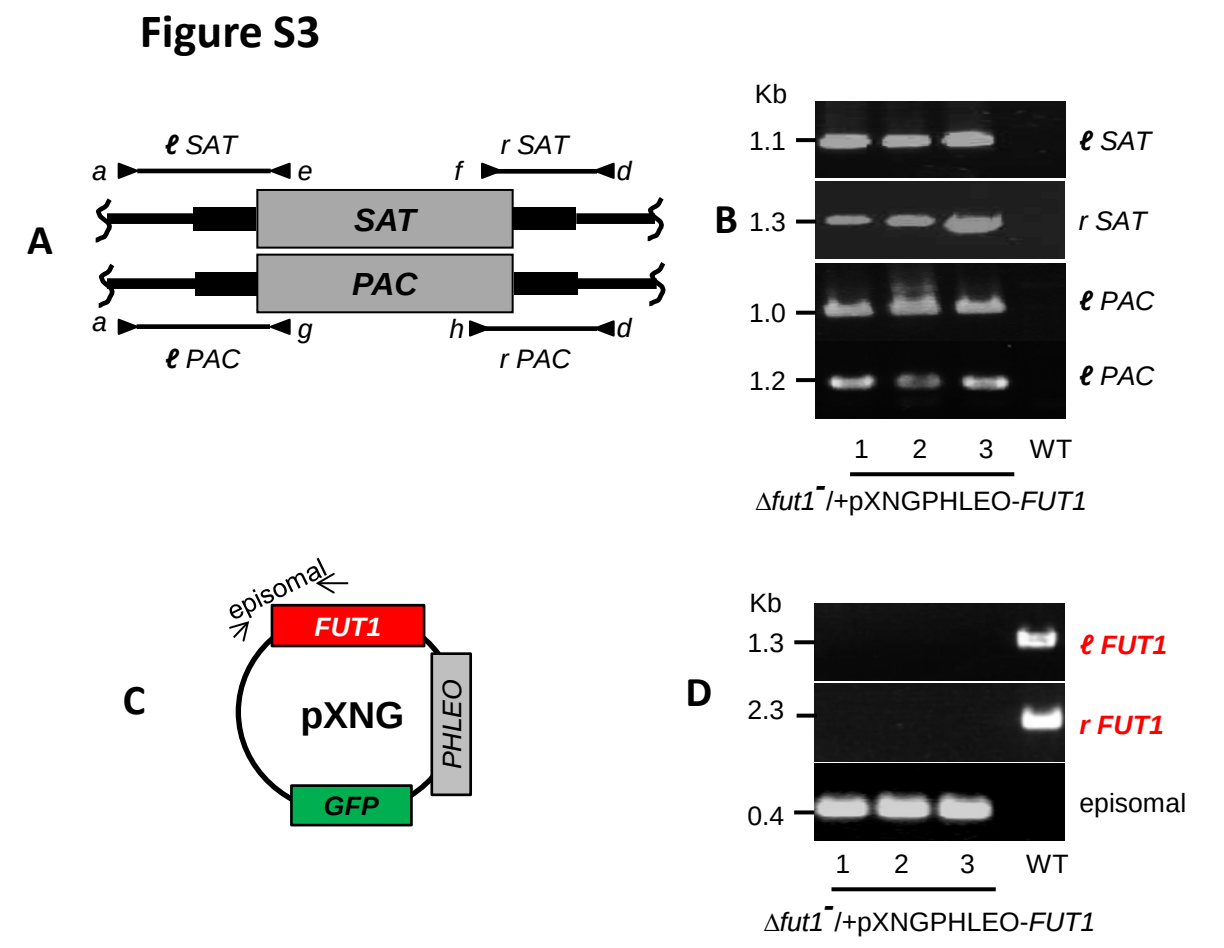


Fig. S3 PCR verification tests of *fut1*⁻/+pXNGPHLEO-*FUT1*

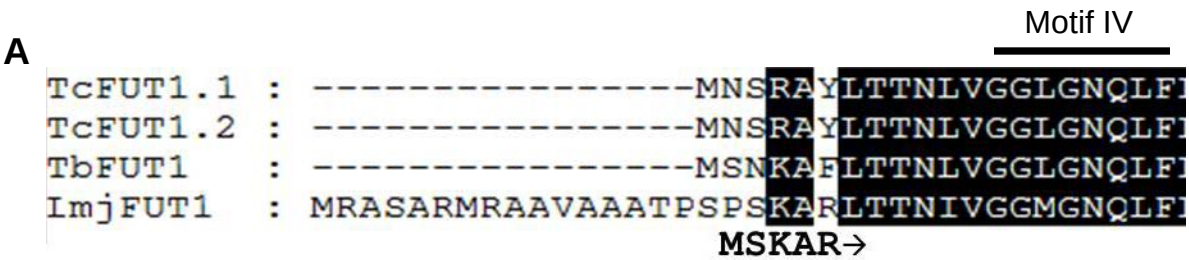
A. Schematic of chromosomal loci with *FUT1:SAT* and F*UT1:PAC* replacements. The location of PCR primer pairs used to establish both planned chromosomal replacements is shown. Black bars represent flanking regions used for homologous recombination.

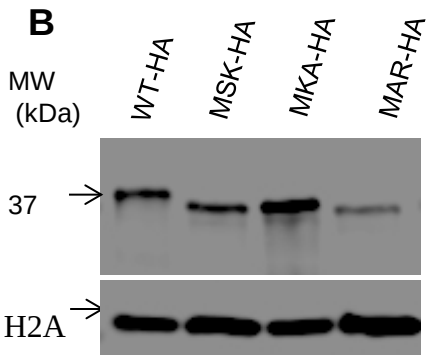
B. PCR tests of planned SAT and PAC replacements depicted in panel A. Lanes 1-3 contain three different Δ*fut1*⁻/+pXNGPHLEO-*FUT1* clones; lane 4, WT.

C. Schematic of the pXNGPHLEO-*FUT1* episome. The location of the episomal-specific *FUT1* PCR primers is indicated.

D. PCR tests confirming expected presence or absence of pXNGPHLEO-*FUT1*; lanes and samples are the same as in panel B.

Figure S4





C Δ*fut1*$^{-}$/+pXNG-*FUT1/pIR1-(mutant)*

↓ Plasmid shuffling

mutant	"dim" wells exhibiting growth	Retention of pXNGPHLEO –*FUT1*
MSK	**242/288*0.96** (84%)	**0/6** (0%)
MKA	**12/288*0.90** (4%)	**3/12** (25%)
MAR	**6/288*0.87** (2%)	**6/6** (100%)

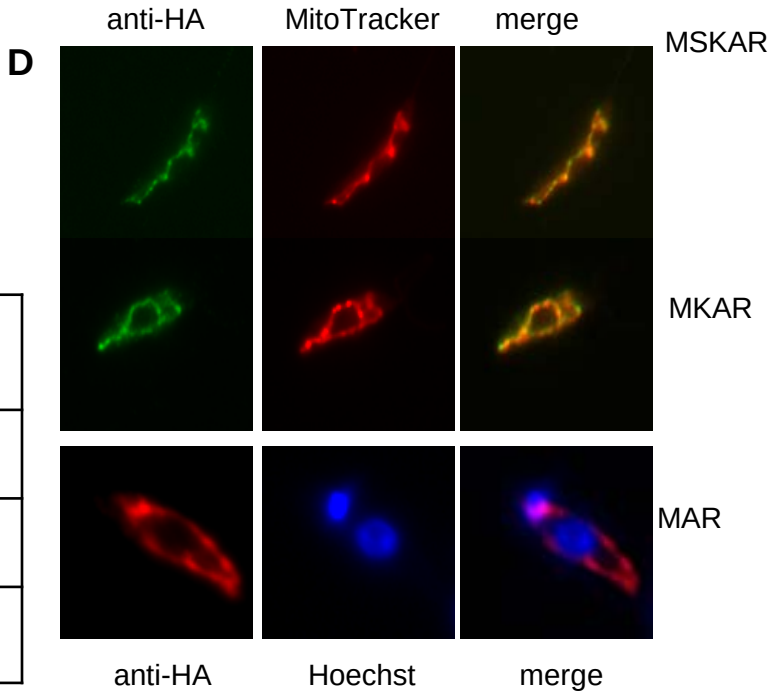


Figure S4. Functionality and mitochondrial localization of N-terminal deletions of LmFUT1

Panel A. Alignment of N-termini of FUT1s from Trypanosomatids; beneath the LmjFUT1 the starting sequences of the three truncation mutants tested are shown.

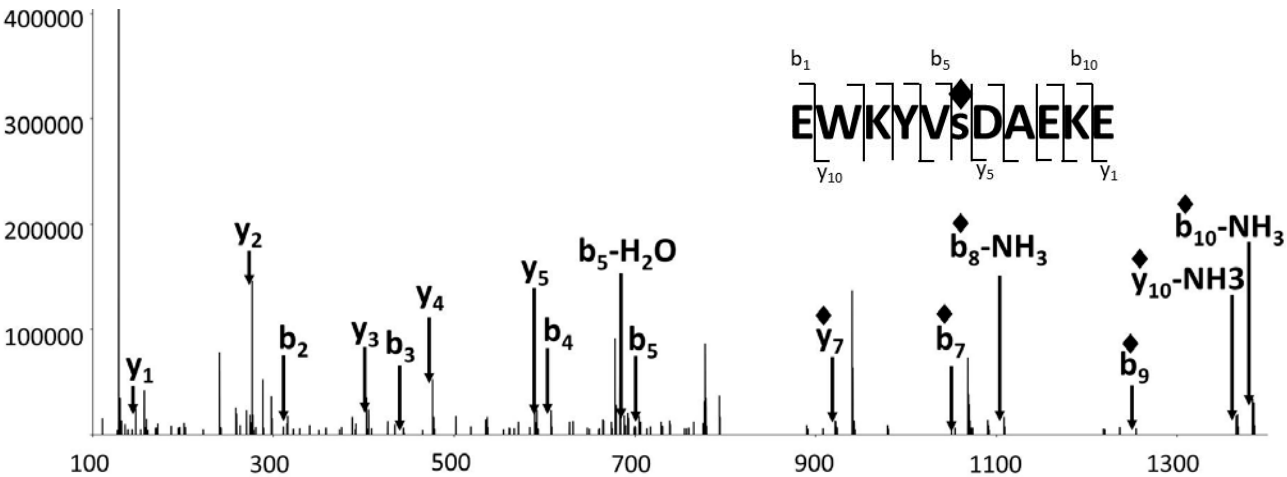
Panel B. Western blot analysis with anti-HA antisera of whole cell lysates from tagged truncated FUT1 mutants (Δ*fut1*$^{-}$/+pXNG-FUT1/pIR1-TRU-HA). Lane 1: WT-HA , Lane 2: MSK-HA, Lane 3: MKA-HA, and lane 4: MAR-HA .

Panel C. Plasmid shuffling to test the function of truncated LmjFUT1s (TRUs).

Panel D. Indirect immunofluorescence of truncated LmjF mutants described in panel C incubated with anti-HA antibody (green or red, column a , Mitotracker Red CMXRos (red, column b for MSKAR and MKAR mutants), or Hoechst dye (blue, column b for MAR mutant); merged fluorescence images are shown in panel c. For MSKAR and MKAR mutants, Pearson's correlation coefficient with Mitotracker was 0.95 and 0.93.

Figure S5. Tandem MS analysis

A. HCD spectrum of the EWKYVS(Fuc)DAEKE peptide product formed after fucosylation by recombinant LmjFUT1 *in vitro,* acquired from the doubly charged precursor ion at *m/z* = 765.355. Most of the unmarked ions could be assigned to loss of water and ammonia from the parent fragment ions as listed in panel B. ♦ marks fucose-containing ions.

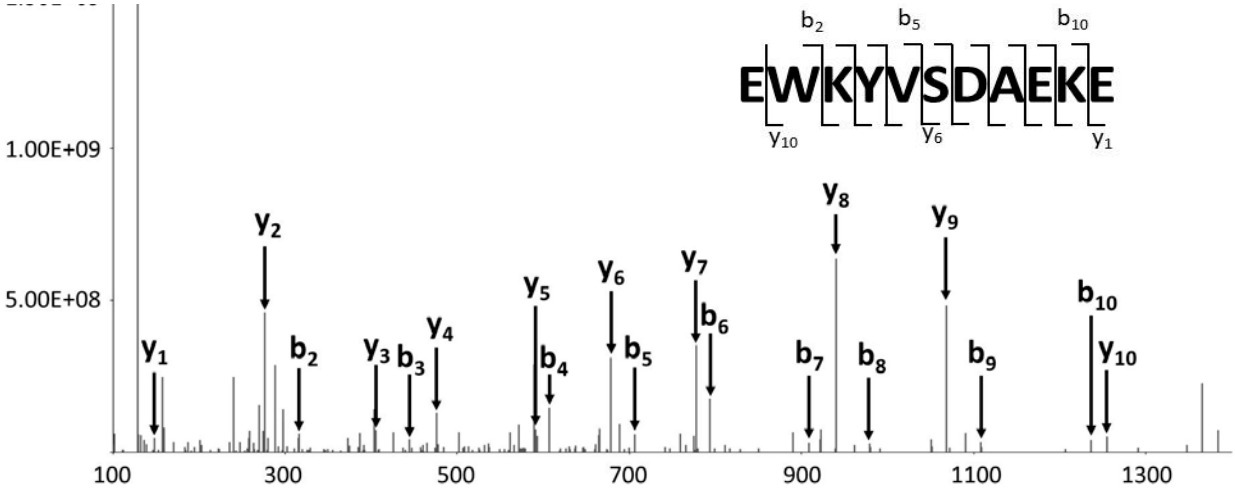


B. Assigned fragmentation ions and mass accuracy from tandem MS of the EWKYVS(Fuc)DAEKE peptide shown in panel A.
♦ marks fucose-containing ions.

ion	*m/z* (Theoretical)	m/z (Observed)	ppm
b_4	607.288	607.289	2.6
♦-b_7	1054.473	1054.48	3.0
♦-b_9	1254.552	1254.559	5.2
b_2-H_2O	298.119	298.119	0.7
b_3-H_2O	426.214	426.212	3.3
b_4-H_2O	589.277	589.278	2.0
b_5-H_2O	688.345	688.342	4.8
♦-b_{10}-NH_3	1365.621	1365.627	4.5
y_1	148.060	148.061	1.4
y_2	276.155	276.156	0.4
y_3	405.200	405.199	2.5
y_4	476.235	476.235	1.0
y_5	591.262	591.264	3.9
♦-y_7	923.420	923.418	2.5
y_1-H_2O	130.050	130.05	0.8
y_2-H_2O	258.145	258.145	1.9
y_3-H_2O	387.187	387.188	1.8
y_2-NH_3	259.129	259.128	1.2
♦-y_{10}-NH_3	1383.632	1383.637	4.0

Figure S5. Tandem MS analysis (continued)

C. HCD spectrum of the EWKYVSDAEKE peptide, acquired from the doubly charged precursor ion at *m/z* = 692.325. Most of the unmarked ions could be assigned to loss of water and ammonia from the parent fragment ions as listed in panel D.



D. Assigned fragmentation ions and mass accuracy from tandem MS of the EWKYVSDAEKE peptide shown in panel C.

ion	*m/z* (Theoretical)	m/z (Observed)	ppm
b_3	444.224	444.226	-5
b_4	607.288	607.288	-0.8
b_8	979.452	979.454	-1.5
b_9	1108.495	1108.493	1.3
b_{10}	1236.59	1236.59	-0.6
b_2-H_2O	298.119	298.119	-2.3
b_3-H_2O	426.214	426.215	-2.1
b_4-H_2O	589.277	589.278	-2.2
b_5-H_2O	688.345	688.346	-1.2
b_7-H_2O	890.404	890.408	-4.4
b_8-H_2O	961.441	961.444	-2.4
b_9-H_2O	1090.484	1090.479	4.4
b_6-NH_3	776.361	776.363	-1.7
y_1	148.060	148.060	0.7
y_2	276.155	276.156	-1.1
y_3	405.198	405.198	-1.2
y_4	476.235	476.236	-2.7
y_5	591.262	591.261	1.5
y_6	678.294	678.294	-0.1
y_7	777.362	777.364	-1.5
y_8	940.426	940.427	-1.4
y_9	1068.521	1068.526	-5
y_{10}	1254.6	1254.598	1.7
y_2-H_2O	258.145	258.146	-3.5
y_3-H_2O	387.187	387.188	-1.8
y_4-H_2O	458.225	458.227	-4.6
y_9-H_2O	1050.51	1050.511	-0.9
y_{10}-H_2O	1236.59	1236.59	-0.6
y_2-NH_3	259.129	259.130	-2.7

Figure S6

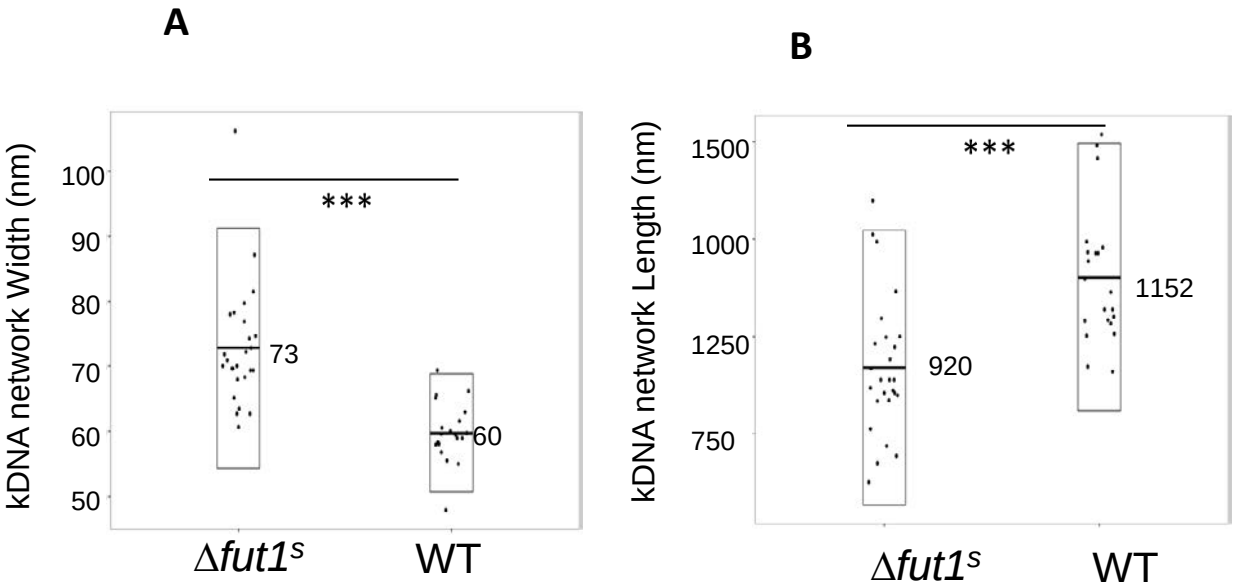


Figure S6. Measurement of the kDNA network dimensions in WT and Δ*fut1*^{s}.

The kDNA network width and length were measured from transmission EM images using Image J; each dot represents one network. A, width; B, length. ***, p value < 0.001

Table S1. Expression and gene targeting constructs used in this study

Construct Name	Lab strain	Description	Reference or method of construction (for primer sequences seeTable S2)
Leishmania or *E. coli* vectors			
pXNGPHLEO	B6432	*Leishmania* expression vector, PHLEO^R	Guo, H. *et a* l (2017), *J. Biol. Chem.* 292: 10696–10708.
pXNGSAT	B5840	*Leishmania* expression vector, SAT^R	pXNGPHLEO with SAT ORF replacing PHLEO ORF
pIR1NEO	B6483	*Leishmania* expression vector, NEO^R (episomal, or integrating follow *Swa* I digestion)	Robinson K.A., Beverley, S.M. (2003); *Mol Biochem Parasitol* 128: 217–228
pIR1SAT	B3541	*Leishmania* expression vector, SAT^R (episomal, or integrating follow *Swa* I digestion)	Capul AA, *et al* (2007), *J. Biol. Chem.* 282: 14006–14017
pGEX-3X	B6011	*E. coli* expression vector; Amp^R	Smith D.B., Johnson K.S (1988), *Gene* 67:31-40.
pET-16B		*E. coli* expression vector; Amp^R	Studier, F.W. (2018), *Current Protocols in Molecular Biology* (2018) 124:e63
pGEM-T		*E. coli* TA cloning vector; Amp^R	http://www.promega.com
constructs for expression in *Leishmania*			
pGEM-FUT1	B6552	LmjFUT1 ORF donor	PCR with primers 3955/3956 from LmFV1 genomic DNA, insert by TA cloning into pGEM-T
pXNGPHLEO-FUT1	B7073	*Leishmania* expression vector for LmjFUT1	insertion of BglII -digested ORF from pGEM-FUT1 into BglII site of pXNGPHLEO
pIR1NEO-FUT1-HA	B7147	*Leishmania* expression vector for LmjFUT1-HA	PCR LmjF ORF using primers 3955/5645 adding C-terminal HA tag with LmjFV1 DNA template; insert into BglII site of pIR1NEO
pIR1NEO-TbFUT1	B7148	*Leishmania* expression vector for TbFUT1	PCR of TbFUT1 ORF from T.brucei strain 427 genomic DNA with primers 5642/5643, digested with XmaI and inserted into XmaI site of pIR1NEO
pIR1NEO-TbFUT1-HA	B7198	*Leishmania* expression vector for TbFUT1-HA	PCR of TbFUT1 from *T.brucei* strain 427 genomic DNA with primers 5642/6193 adding C-terminal HA tag with T.brucei strain 427 DNA, inserted into pIR1NEO
pIR1NEO-MSK-FUT1-HA	B7197	*Leishmania* expression vector for truncated LmjFUT1 mutant MSK-FUT1-HA	PCR with primers 6192/5645from LmFV1 genomic DNA, digested with BglII, and inserted into BglII site of pIR1NEO
pIR1NEO-MKA-HA	B7321	*Leishmania* expression vector for truncated LmjFUT1 mutant MKA-FUT1-HA	PCR with primers 6761/5645from LmFV1 genomic DNA, digested with BglII and inserted into BglII site of pIR1NEO
pIR1NEO-MAR-HA	B7322	*Leishmania* expression vector for truncated LmjFUT1 mutant MAR-FUT1-HA	PCR with primers 6762/5645 from LmFV1 genomic DNA, digested with BglII and inserted into BglII site of pIR1NEO
pIR1NEO-MTP-HA	B7299	*Leishmania* expression vector for LmjFUT1 mutant MTP-HA with mutated MTP	PCR with primers 6741/5645from LmFV1 genomic DNA, digested with BglII and inserted into BglII site of pIR1NEO
pIR1NEO-BLOCK-HA	B7326	*Leishmania* expression vector for LmjFUT1 mutant BLOCK-HA with blocked MTP	*PTR1* and *FUT1* were PCR amplified using primer pairs 2577/6795 and 6794/5645; products were mixed and amplified using primers SMB 2577/5645; large product , digested with BglII, and inserted into BglII site of pIR1NEO
pIR1NEO-CAT-HA	B7329	*Leishmania* expression vector for LmjFUT1 catalytic mutant CAT-HA	R298A site directed mutant using primer 5895 with pGEM-FUT1; released by BglII and isnerted into BglII site of pIR1NEO
pGEM-FUT2	B6553	LmjFUT2 ORF donor	PCR with primers 3990/3958 from LmjFV1 genomic DNA, insert by TA cloning into pGEM-T
pIR1SAT-FUT2	B6553	*Leishmania* expression vector for LmjFUT2	insertion of BglII-digested ORF from pGEM-FUT2 into BglII site of pIR1SAT
pGEM-SCAL	B6476	LmjSCAL ORF donor	PCR with primers 3840/3841 from LmjFV1 genomic DNA, insert by TA cloning into pGEM-T
pXNGSAT-SCAL	B6554	*Leishmania* expression vector for LmjSCAL	insertion of BglII digested ORF from pGEM-SCAL into BglII site of pXNGSAT
knockout constructs			note: fusion PCR was performed as described by Murta *et al* 2009 *Molec. Micro* . 71:1386
pGEM-FUT1-SAT	B7081	pGEM-T containing Δfut1::SAT targeting fragment	Fusion PCR product bearing FUT1 5' flanking region (primers 3763/5321), SAT ORF (primers 2630/2631), and 3' flanking region (primers 3765/3766), inserted in pGEM-T
pGEM-FUT1-HYG	B7080	pGEM-T containing Δfut1::HYG targeting fragment	Fusion PCR product bearing FUT1 5' flanking 900 nt (primers 3763/5321), HYG ORF (primers 2561/2662), and 3' flanking 700 nt (primers 3765/3766
pGEM-FUT1-PAC	B7079	pGEM-T containing Δfut1::PAC targeting fragment	Fusion PCR product bearing FUT1 5' flanking 900 nt (primers 3763/5321), PAC ORF (primers 2557/2558), and 3' flanking 700 nt (primers 3765/3766)
pGEM-FUT2-HYG	B7226	pGEM-T containing Δfut2::HYG targeting fragment	Fusion PCR product bearing FUT2 5' flanking region with primers 3767/3987, HYG ORF (primers 2561/2562), and 3' flanking region (primers 3769/3770) inserted in pGEM-T; released by BamHI_HindII_DraI
pGEM-FUT2-PAC	B6548	pGEM-T containing Δfut2::PAC targeting fragment	Fusion PCR product bearing FUT2 5' flanking region with primers 3767/3987, PAC ORF (primers 2557/2558), and 3' flanking region (primers 3769/3770), inserted in pGEM-T; released by BamHI+HindII
pGEM-SCAL-HYG	B6438	pGEM-T containing Δscal::HYG targeting fragment	Fusion PCR product bearing SCAL 5' flanking region with primers 3759/3760, HYG ORF (primers 2561/2562), and 3' flanking region (primers 37613769/3762) inserted in pGEM-T; released by BamHI+HindIII digestion
pGEM-SCAL-BSD	B6475	pGEM-T containing Δscal::BSD targeting fragment	Fusion PCR product bearing SCAL 5' flanking region with primers 3767/3987, BSD ORF (primers 3763/5321), and 3' flanking region (primers 3769/3770) inserted in pGEM-T; released by BamHI+HindIII
constructs for expression of WT and mutant LmjFUT1 in *E.coli*			
pGEX-FUT1	B7230	pGEX derivative containing FUT1; AmpR	FUT1 ORF from pIR1NEO-FUT1-HA was released by BglII digestion and inserted into BamHI site of pGEX-3X
pGEX-CAT-MUT	B7600	pGEX derivative containing mutant of FUT1 (R297A); AmpR	FUT1(R297A) ORF from pIR1NEO-CAT-HA was released by BglII digestion and inserted into BamHI site of pGEX-3X
pGEX-MTP-MUT	B7604	pGEX derivative containing mutant FUT1 (2 Glu replaced first two Arg); AmpR	FUT1 ORF from pIR1NEO-MTP-MUT-HA was released by BglII digestion and inserted into BamHI site of pGEX-3X
pET-BLOCK-MUT	B7632	pET16B derivative containing PTR1-FUT1; AmpR	PTR-FUT1 was PCRed from pIR1NEO-BLOCK-HA with primers 7693/3956, digested with NdeI_BalII and inserted into Nde-BamHI digested pET16b

Table S2. Oligonucleotide primers Guo *et al* 2021

Primer number	Sequence (5'-3')	added restriction sites	Purpose
SMB2555	*GGTAACGGTGCGGGCTGACG* CCACCATGGGCCAAGCCTTTGTCTCA		forward primer to amplify *BSD* with linker at 5'
SMB2556	*CGAGATCCCACGTAAGGTGC* TTAGCCCTCCCACACATAACCAGAG		reverse primer to amplify *BSD* with linker at 5'
SMB2557	*GGTAACGGTGCGGGCTGACG* CCACCATGACCGAGTACAAGCCC		forward primer to amplify *PAC* with linker at 5'
SMB2558	*CGAGATCCCACGTAAGGTGC* TCAGGCACCGGGCTTGCG		reverse primer to amplify *PAC* with linker at 5'
SMB2561	GGTAACGGTGCGGGCTGACGCCACCATGAAAAGCCTGAACTC		forward primer to amplify HYG with linker at 5'
SMB2562	CGAGATCCCACGTAAGGTGCCTATTCCTTTGCCCTCG		reverse primer to amplify HYG with linker at 5'
SMB2577	TTAGATCTCCACCATGACTGCTCCGACCGTGC	BglII	forward primer to amplify ORF of *PTR1*
SMB2601	ATCCAATGTTTCCGCCACCTGCTC		l-SAT FUT1 primer e (Fig. S3)
SMB2609	TACTCTCGGCGGCATTGACCTGTT		r-SAT primer f (Fig. S3)
SMB2630	GGTAACGGTGCGGGCTGACGCCACCATGAAGATTTCGGTGATCC		forward primer to amplify SAT with linker at 5'
SMB2631	CGAGATCCCACGTAAGGTGCTTAGGCGTCATCCTGTGCTCC		reverse primer to amplify SAT with linker at 5'
SMB2888	ACGTCGAGTGCCCGAAGGAC		r-PAC primer h (Fig. S3)
SMB2889	ACCGTGGGCTTGTACTCGG		l-PAC primer g (Fig. S3)
SMB3176	CAGCAGCCCTCTATATACCCGC		episomal pXNG-FUT forward primer (Fig. S3)
SMB3759	AAGCTTAGTTCGACAGCAGCCAAAGC	HindIII	forward primer to amplify 5' flanking region of *SCAL* with linker at 5'
SMB3760	*CGTCAGCCCGCACCGTTACC* GATGATGCGTGACCAATAATGGC		reverse primer to amplify 5' flanking region of *SCAL* with linker at 5'
SMB3761	*GCACCTTACGTGGGATCTCG* GCTTCGCGACCCAGCCTCGAGCC		forward primer to amplify 3' flanking region of *SCAL* with linker at 5'
SMB3762	GGATCCGAGGAAGGTGGAGAGGGAGC	BamHI	reverse primer to amplify 3' flanking region of *SCAL* with linker at 5'
SMB3763	AAGCTTCGGACGTGTGGGCTTCTGTTCG	HindIII	forward primer to amplify 5' flanking region of *LmjFUT1*
SMB3765	*GCACCTTACGTGGGATCTCG* TCGAGGTGAACCGAGGGAGG		forward primer to amplify 3' flanking region of *LmjFUT1* with linker at 5'
SMB3766	GGATCCATATCAAGGTGACGGGGAGT	BamHI	reverse primer to amplify 3' flanking region of *LmjFUT1*
SMB3767	AAGCTTCTCGCTACATTGCCCGTGTA	HindIII	forward primer to amplify 5' flanking region of *LmjFUT2*
SMB3769	*GCACCTTACGTGGGATCTCG* CATCACTGGCCCGAGTACGC		forward primer to amplify 3' flanking region of *LmjFUT2* with linker at 5'
SMB3770	GGATCCGGGCGCCGCGGACGAAGAAG	BamHI	reverse primer to amplify 3' flanking region of *LmjFUT2*
SMB3791	CACGGGATCCGTCGCTCTGCCGCTCTGTTGGCA		RT-PCR to identify correct start codon of *LmjFUT2*
SMB3796	GCACGCCGTAATGACCGAGG		episomal pXNG-FUT reverse primer (Fig. S3)
SMB3839	CCGCGCCGCGCACAGTGACGATAACAAGG		reverse primer for FUT2 ORF left side (Fig. S2)
SMB3840	CCCTGATCACCACCATGCAGGACATCGCTTCAACCC	BclI	forward primer to amplify ORF of *LmjSCAL*
SMB3841	CCCTGATCATCACATGTAACCCTTGTACAGCA	BclI	reverse primer to amplify ORF of *LmjSCAL*
SMB3955	CCCAGATCTCCACCATGAGAGCTTCTGCTCGGATGCG	BglII	forward primer to amplify ORF of *LmjFUT1*
SMB3956	CCCAGATCTTCAGATGAGTATCCATCCAGGG	BglII	reverse primer to amplify ORF of *LmjFUT1*
SMB3957	CCCAGATCTCCACCATGATGAAGCGTCGTACAGGCG	BglII	forward primer for FUT2 ORF right side (Fig. S2)
SMB3958	CCCAGATCTTTAGGCGCCCCCTGAAGCCCGCG	BglII	reverse primer for pXNGPHLEO-FUT2 ORF right side (Fig. S2)
SMB3987	*CGTCAGCCCGCACCGTTACC* GCAGCATCGCTCGTGTCGATGTAGC		reverse primer to amplify 5' flanking region of *LmjFUT2* with linker sequece at 5'
SMB3988	GGGTGCTTTTGTCAGCTGACTCGC		l-SAT FUT1 primer a = l-PAC = l-WT (Fig. S3)
SMB3990	CCCAGATCTCCACCATGCCTGATAATAGATACGGC	BglII	forward primer for pXNGPHLEO-FUT2 ORF left side (Fig. S2)
SMB5321	*CGTCAGCCCGCACCGTTACC* GGACCGCGACGGCGCGTGAGCAATTGAGC		reverse primer to amplify 5' flanking region of *LmjFUT1* with linker sequece at 5'
SMB5511	ACTGTCATAGAGGCAGCCGTTC		r-SAT primer d - r-PAC = r-WT (Fig. S3)
SMB5642	CCCGGGCCACCATGAGCAACAAGGCATTCCTAAC	XmaI	forward primer to amplify ORF of *TbFUT1*
SMB5643	CCCGGGCTACAGGAGGATCCAGTCGTTAC	XmaI	reverse primer to amplify ORF of *TbFUT1*
SMB5645	AGATCTTCA**AGCGTAATCTGGAACATCGTATGGGTA**GATGAGTATCCATCCAGGG	BglII	reverse primer to amplify ORF of *LmjFUT1* with HA tag
SMB5895	GCCCTGCACGTGGCGCGCGGCGACTA		site-directed -mutagenesis primer for *LmjFUT1*
SMB5922	AGATCTCCACCATGGGCAACCAGCTTTTCCTCG	BglII	forward primer to amplify ORF of *LmjFUT1* using the third start codon
SMB5966	AGATCTCCACCATGCGTGCTGCCGTCGCAGCAGCGAC	BglII	forward primer to amplify ORF of *LmjFUT1* using the second start codon
SMB6192	AGATCTCCACCATGTCGAAAGCACGCCTCACAAC	BglII	forward primer to amplify ORF of *LmjFUT1* from starting from MSK
SMB6193	CCCGGGTCA**AGCGTAATCTGGAACATCGTATGGGTA**CAGGAGGATCCAGTCGTTAC	XmaI	reverse primer to amplify ORF of *TbFUT1* with HA tag
SMB6741	AGATCTCCACCATGgagGCTTCTGCTgagATGCGTGCTGCCGTCGCAGCAGCG	BglII	forward primer to amplify ORF of *LmjFUT1* using two GLU codon (gag) to replace ARG codon(AGA)
SMB6761	AGATCTCCACCATGAAAGCACGCCTCACAACTAAC	BglII	forward primer to amplify ORF of *LmjFUT1* from starting from MKA
SMB6762	AGATCTCCACCATGGCACGCCTCACAACTAACATTG	BglII	forward primer to amplify ORF of *LmjFUT1* from starting from MAR
SMB6794	ATGAGAGCTTCTGCTCGGATGCGTG		forward primer to amplify ORF of *LmjFUT1*
SMB6795	*CACGCATCCGAGCAGAAGCTCTC* ATGGCCCGGGTAAGGCTG		reverse primer to amplify ORF of *PTR1* with complementary sequence of 6794
SMB7693	GGAATTCCATATGACTGCTCCGACCGTGCCG	NdeI	forward primer to amplify ORF of *PTR1*