```
L. major      1 MVFDTDFFVVNCSQMAANVLQSYIVAALHSAATQLPSSVQPYAMMLTRED    50
                ||||·|·|:||||||:|||:|||||||||||:|||||
L. donovani   1 --------------MAANALHSHIVAALHNAATKLPSSVQPYAMLLTRED    36

L. major     51 MVSTTLATAIATAVILYTVITVVLPVLRMDFYLSKLPTIKNSIPFLGHAL   100
                ||||||||||||||||||||||||||||||||||||||·||||||||||
L. donovani  37 MVSTTLATAIATAVILYTVITVVLPVLRMDFYLSKLPTIKNGIPFLGHAL    86

L. major    101 LLAGPSPWSKMSNWSLYPEKNLPQKKKSVDGPQTSRLVTYNVAGMRVIYI   150
                |||||||||||||||||||||||||||·||||||||||||||||||||||
L. donovani  87 LLAGPSPWSKMSNWSLYPEKNLPQKKKSADGPQTSRLVTYNVAGMRVIYI   136

L. major    151 NEPRLLRRVLLTHQRNYRKALAAAYKHFMCLLGTGLVTSEDEQWKKGRLL   200
                ||||||||||||||||||||||||||||||||||||||||||||||||||
L. donovani 137 NEPRLLRRVLLTHQRNYRKALAAAYKHFMCLLGTGLVTSEDEQWKKGRLL   186

L. major    201 LSHAMRIDILDSVPEMAMKAVDRILLKLDAVDAKNPSVDLNEEYRHMTLQ   250
                ||||||||||||||||||||||||||||||||||||||||||||||||||
L. donovani 187 LSHAMRIDILDSVPEMAMKAVDRILLKLDAVDAKNPSVDLNEEYRHMTLQ   236

L. major    251 VISESALSLSAEESDRIFPALYLPIVHECNKRVWAPWRAYMPFLHGSRMR   300
                ||||||||||||||||||||||||||||||||||||||||||·|||:|
L. donovani 237 VISESALSLSAEESDRIFPALYLPIVHECNKRVWAPWRAYMPFLQGSRVR   286

L. major    301 NRCLSELNKVLRDIICRRWEQRNDSNYTAKPDILALCISQIDRIDEKMIV   350
                |·||||||||||:||||||||||||·|·||||||||||||||:||||||
L. donovani 287 NHCLSELNKVLRNIICRRWEQRNDSNCTGKPDILALCISQIDRMDEKMIV   336

L. major    351 GLIDDVKTILLAGHETSAALLTFATYEVLRHPEIRQKILEEATRLFDPAR   400
                ||||||||||||||||||||||||||||||||||||||||||||||||||
L. donovani 337 GLIDDVKTILLAGHETSAALLTFATYEVLRHPEIRQKILEEATRLFDPAR   386

L. major    401 CTCTVQTRYGPRGVPALNDVRSLVWTPAVLRETLRRHSVVPLVMRYAAKD   450
                ||·||||||||||||||:||||·||||||||||||||||||||||||||
L. donovani 387 CTRTVQTRYGPRGVPAVNDVRDLVWTPAVLRETLRRHSVVPLVMRYAAKD   436

L. major    451 DVWPAADTGLDADVRIPAGCTIAVGIEGVHNNPDVWNKPEVFDPTRFIDA   500
                ||||||||||||||||||||||||||||||||||||||||||||||||||
L. donovani 437 DVWPAADTGLDADVRIPAGCTIAVGIEGVHNNPDVWNKPEVFDPTRFIDA   486

L. major    501 EIANDTNYLNQSTKDVKFAKKIDPYAFIPFINGPRNCLGQHLSMIETQVA   550
                ||||||||||||||||||||||||||||||||||||||||||||||||||
L. donovani 487 EIANDTNYLNQSTKDVKFAKKIDPYAFIPFINGPRNCLGQHLSMIETQVA   536

L. major    551 LAYMVLSYDLTIYRDPSYKGDVAAYEDAVGRHHDFIIPQVPHDGLKVWGT   600
                ||||||:|||||||||||||·||||||||||||||||||||||||||||
L. donovani 537 LAYMVLNYDLTIYRDPSYKGDAAAYEDAVGRHHDFIIPQVPHDGLKVWGT   586

L. major    601 PNKLFM       606
                ||||||
L. donovani 587 PNKLFM       592
```

Figure S1. Pair-wise alignment of *L. major* CYP5122A (LmjF.27.0090) and *L. donovani* CYP5122A1 (LdBPK_270090.1) using EMBOSS Needle. Identity: 94.4%. Similarity: 95.9%.

```
Lm   ------------------------MV---LAKKQMKIPPIHKLRPGWSAVALHTVIYLLRHDFF      37
At   ------------------------------------------------------------       0
Sc   MSAVFNNATLSGLVQASTYSQTLQNVAHYQPQLNFMEKYWAAWYSYM-------------        47
Mm   MA---TNK-SVGVFS--SASLAVEYVDSLLPENPLQEPFKNAWVYML-------------        41


Lm   TAIVATSIVQPLYHRLILQNTYLQRLSDP--ALFTLVFAVLCHCVPWAFFNGIFLFFDSI       95
At   ---------------------------------------MLLLPFMVN---TYFS---         13
Sc   -----------------NNDV--LATGLMFFLLHEFMYFFRCLPWFIIDQIPYFR-R-         84
Mm   -----------------DNYTKFQIATWGSLIVHEAIYFLFSLPGFLFQFIPYMR-K-         80
                                                      :*   .        :


Lm   HPQYGIEGLRNNALLAPLGRRMAAYKLPRKPQQLPSAALIFSTMLHTAINHYLIIPVVLY      155
At   ----------------------FVPM--QTKNNTPAAQGKCITRLLLYHFSVNLPLML        47
Sc   ----------------------WKLQ--PTKIPSAKEQLYCLKSVLLSHFLVEAIPIW       118
Mm   ----------------------YKIQ--KDKPETFEGQWKCLKKILFNHFFIQLPLIC       114
                           :          :  :            :  : *: :     :


Lm   AYLVHTNSCALRAPPPESAIVGFAPEDVVNYMSGSNLRQIPISLVTITSHFLIANVINEM      215
At   ASYPVFRA------------MG----------MRSSFP-LPSW-KEVSAQILFYFIIEDF        83
Sc   TFHPMCEK------------LG----------ITVEVP-FPSL-KTMALEIGLFFVLEDT       154
Mm   GTYYFTEF------------FN----------IPYDWERMPRWYLTLAR-CLGCAVIEDT       151
            .                   ..               .     :*       ::          ::::


Lm   GFYIVHSMLHSSPTLYRVFHKKHHMYTGTISIAAEYATPLEGILANAIPTTAYFTFMFFH      275
At   VFYWGHRILHS-KWLYKNVHSVHHEYATPFGLTSEYAHPAEILFLGFATIVGPAL-----       137
Sc   WHYWAHRLFHY-GVFYKYIHKQHHRYAAPFGLSAEYAHPAETLSLGFGTVGMPILY--VM       211
Mm   WHYFLHRLLHH-KRIYKYIHKVHHEFQAPFGIEAEYAHPLETLILGTGFFIGIVL-----       205
        .*   *  ::*      :*:  .*.  ** :     :.:  :*** * *  :   .


Lm   YTREEASKSSFVTSARAWPLFITWMWARLWETYEVHSGYCFSDTWLGKLGLLHGHRARFH      335
At   ------------TGPHLITLWLWMVLRVLETVEAHCGYHFPWSLSNFLPLYG--GADFH       182
Sc   Y----------TGKLHLFTLCVWITLRLFQAVDSHSGYDFPWSLNKIMPFWA--GAEHH       258
Mm   ------------LCDHVILLWAWVTIRLLETIDVHSGYDIPLNPLNLVPFYT--GARHH       250
                      :  *:   *: ::  :  *.** :     .       : :       *  .*


Lm   DFHHTHN---VCNYGSGL-FMDALLNTMDPYLIYRYPDKHPHTTALKEEDLKEPRDLEVG      391
At   DYHHRLLYTKSGNYSSTFVYMDWIFGTDKGYRRL---------KTLKENG---------      223
Sc   DLHHHYF---IGNYASSFRWWDYCLDTESGPEAK---------ASREERMKKRAEN---      302
Mm   DFHHMNF---IGNYASTFTWWDKLFGTDAQYHAY---------IEKSKKLGKKSD----      293
     *  **         **.* : :  *    :.*                          .:


Lm   SEAMDQVRACS      402
At   --DMKQT----      228
Sc   -NAQKKTN---      309
Mm   ----------      293
```

Figure S2. The alignment of ERG25 proteins (Lm: *L. major*; At: *Arabidopsis thaliana*; Sc: *Saccharomyces cerevisiae*; Mm: *Mus musculus*) using Clustal Omega. Asterisks (*): fully conserved residues; colons (:): highly similar residues; periods (.): weakly similar residues. *L. major* ERG25 shows 23-25% identity to other ERG25s.
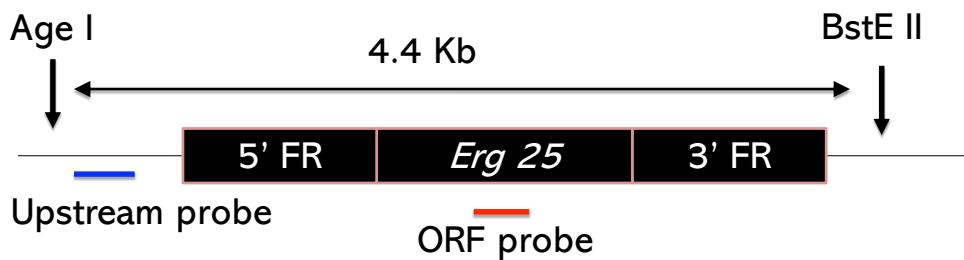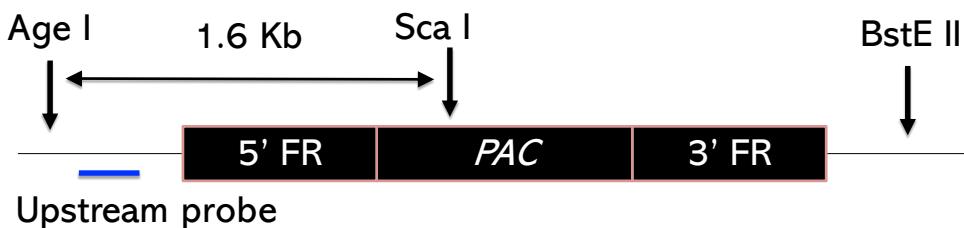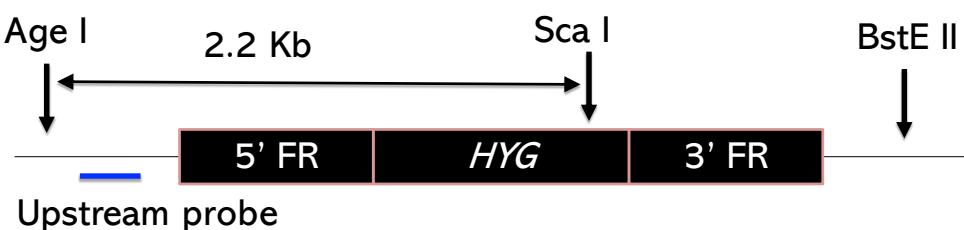
Figure S3. Schematic depiction of the Southern blot for *L. major ERG 25*. (A) The genomic locus of *ERG25* in WT *L. major* along with its open reading frame (ORF), 5' and 3' flanking regions (FR), recognition sites of Age I, BseE II and Sca I, and the positions of ORF probe and upstream probe. **(B-C)** Replacement of *ERG25* ORF with hygromycin (HYG) and puromycin (PAC) resistance genes. The expected sizes of DNA fragments recognized by the ORF probe or upstream probe were indicated.
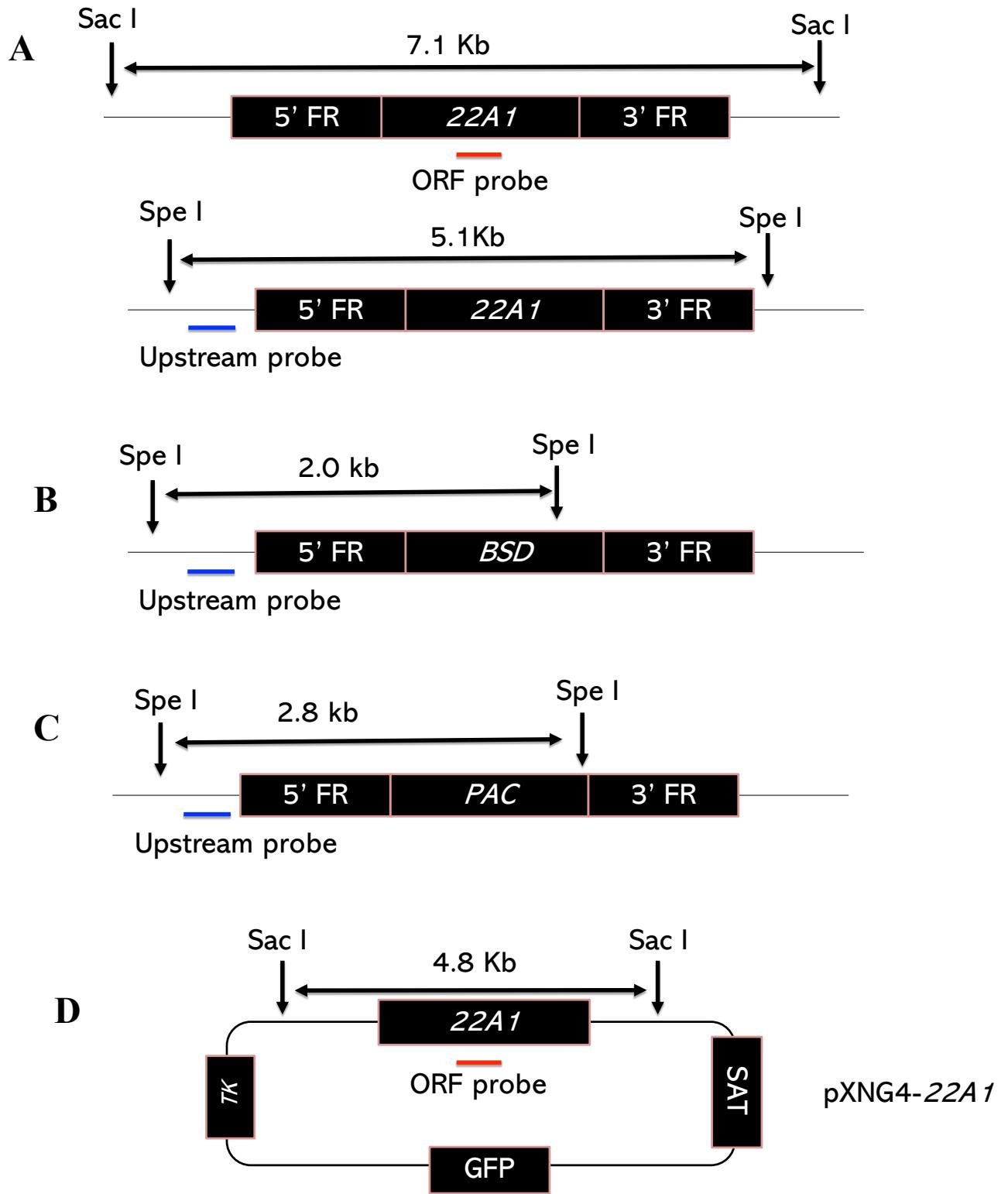
Figure S4. Schematic depiction of the Southern blot for *L. major CYP5122A1* (22A1). (A) The genomic locus of CYP5122A1 in WT *L. major* along with its open reading frame (ORF), 5' and 3' flanking regions (FR), recognition sites of Sac I and Spe I, and the positions of ORF probe and upstream probe. (**B-C**) Replacement of 22A1 ORF with blasticidin (BSD) and puromycin (PAC) resistance genes. (**D**) Episomal expression of 22A1 from pXNG4-5122A1. TK: thymidine kinase, GFP: green fluorescent protein, SAT: nourseothricin resistance gene. The expected sizes of DNA fragments recognized by the ORF probe or upstream probe were indicated.
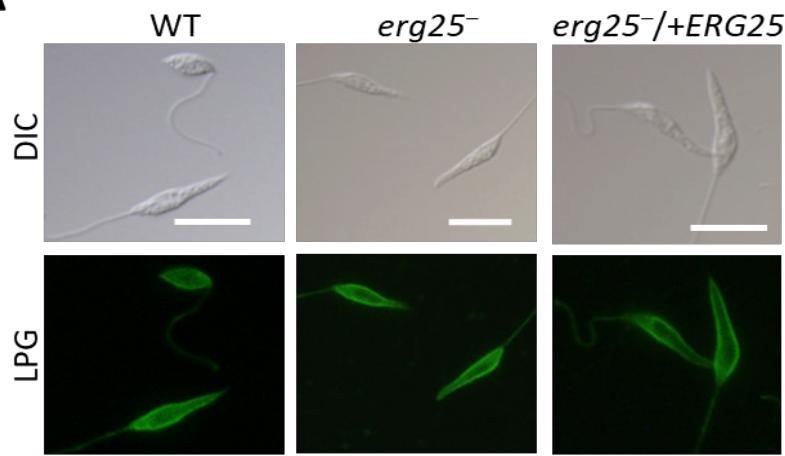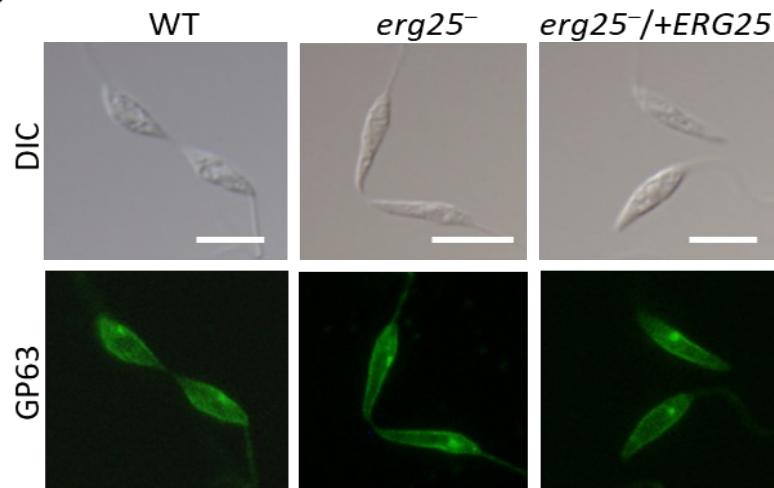
Figure S5. The expression of LPG and GP63 is not affected in *erg25⁻* promastigotes. Log phase promastigotes were labeled with mouse anti-LPG (**A**) or anti-GP63 (**B**) monoclonal antibody followed by goat anti-mouse IgG-FITC. Scale bars: 10 μm.
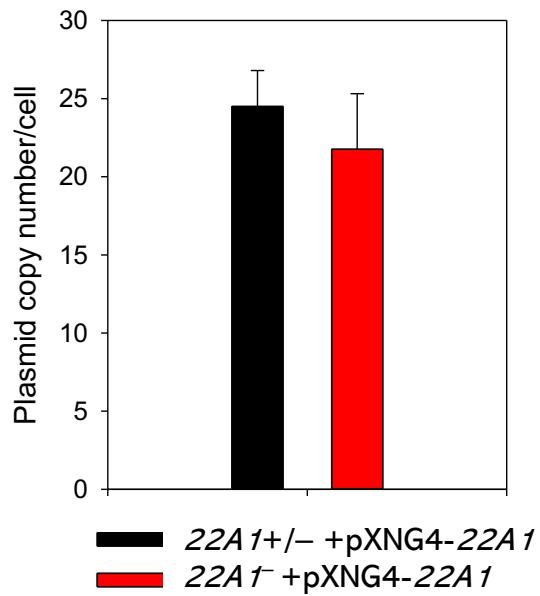
Figure S6. Plasmid levels in promastigotes. Stationary phase promastigotes of *22A1+/– +*pXNG4-*22A1* and *22A1⁻ +*pXNG4-*22A1* were cultivated in the presence of SAT and their DNA was extracted to determine the average pXNG4-Lm22A1 plasmid copy numbers per cell by qPCR.
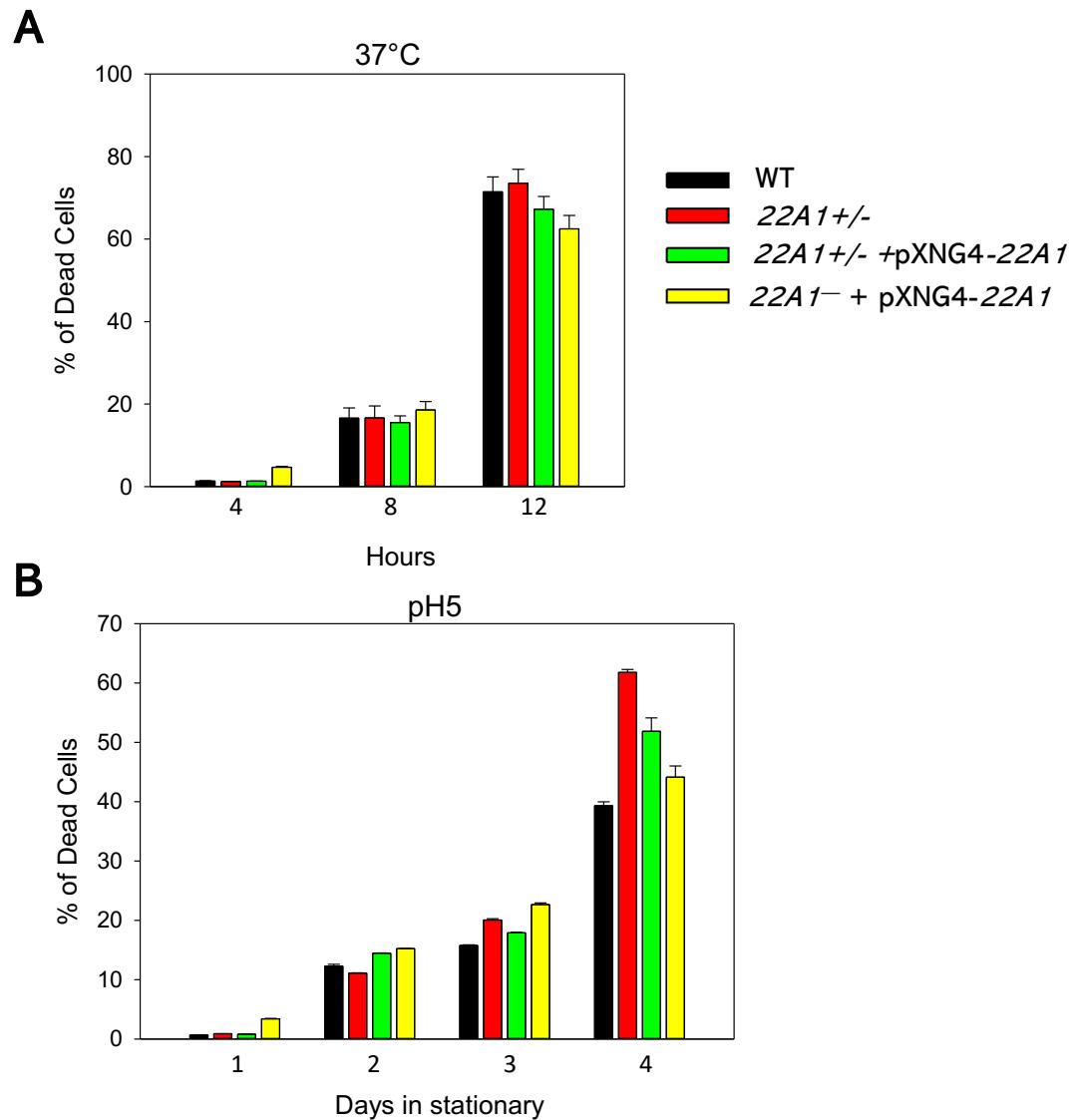
Figure S7. Stress response of *22A1* mutants. Day 1 stationary phase promastigotes were incubated under various conditions and percentages of dead cells were determined by flow cytometry at the indicated times. **A**: in complete M199, pH7.4, 37 °C. **B**: in complete M199, 27 °C, pH5.0. Error bars represent standard deviations from three experiments.
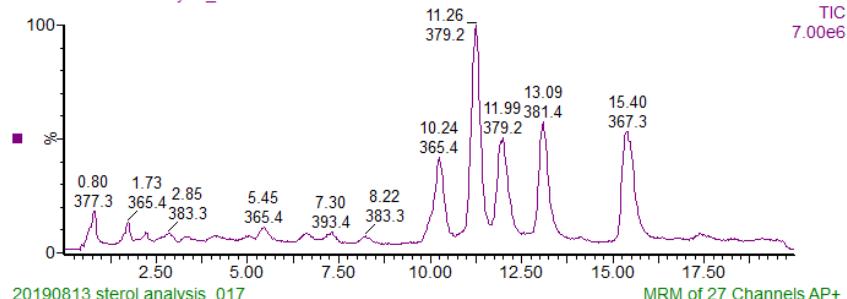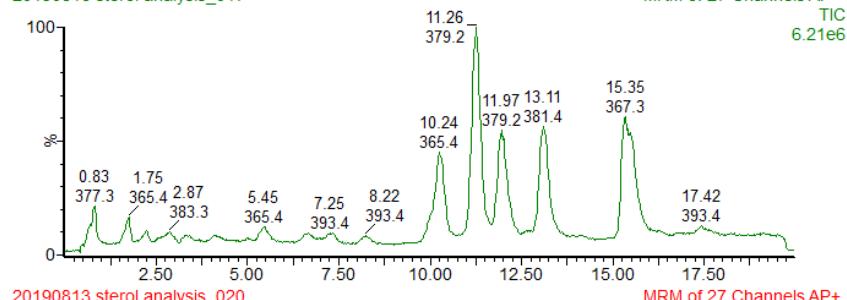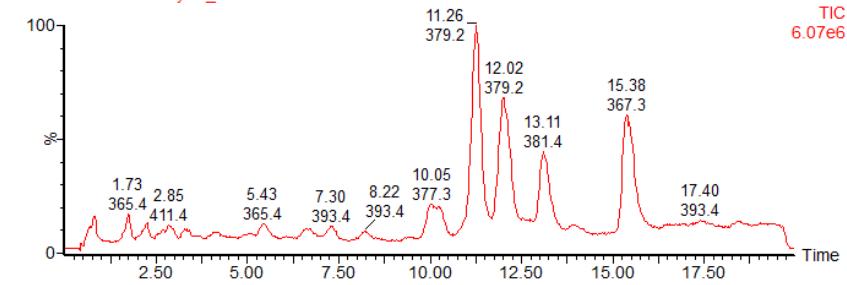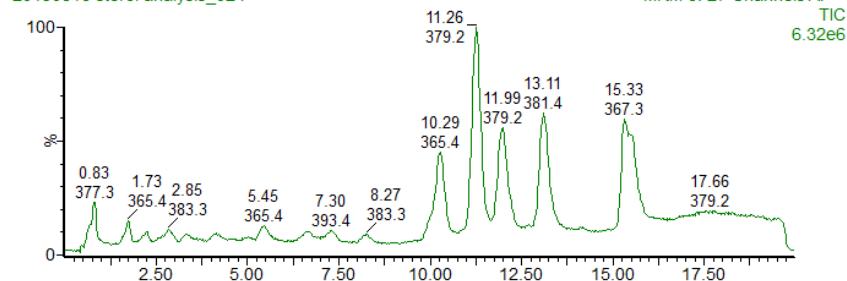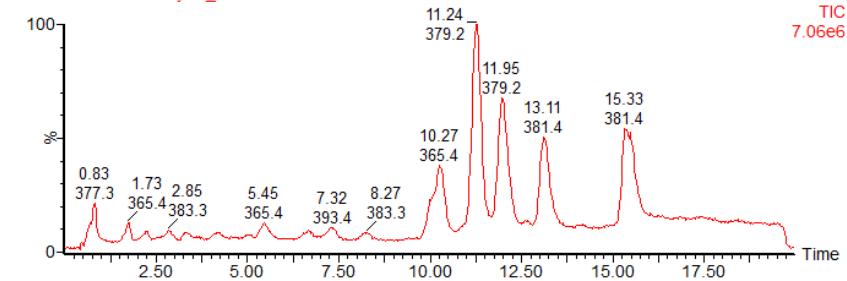
Figure S8. 22A1 half knockout and overexpression do not affect bulk sterol composition in _L. major._ Partial LC-MS chromatograms of free sterols from WT, _22A1+/- #4, 22A1+/- #4_ +pXNG4-22A1, _22A1+/- #5,_ and _22A1+/- #5_ +pXNG4-22A1 promastigotes.

A
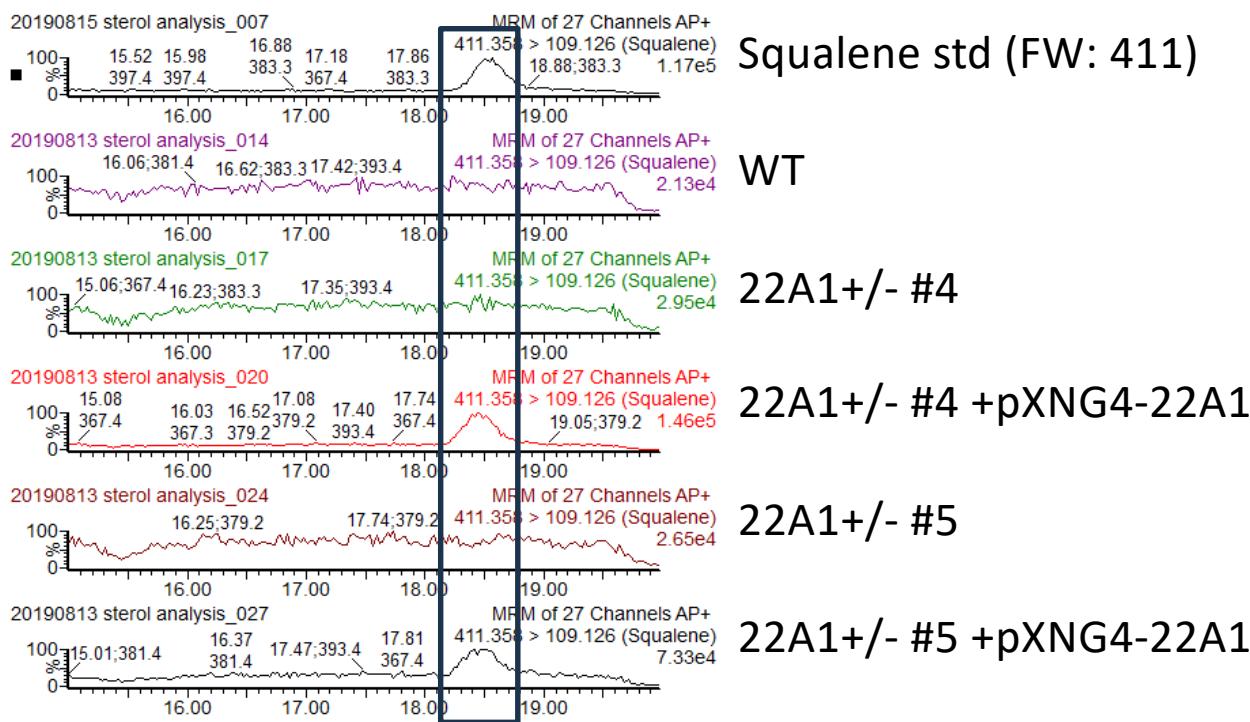
B
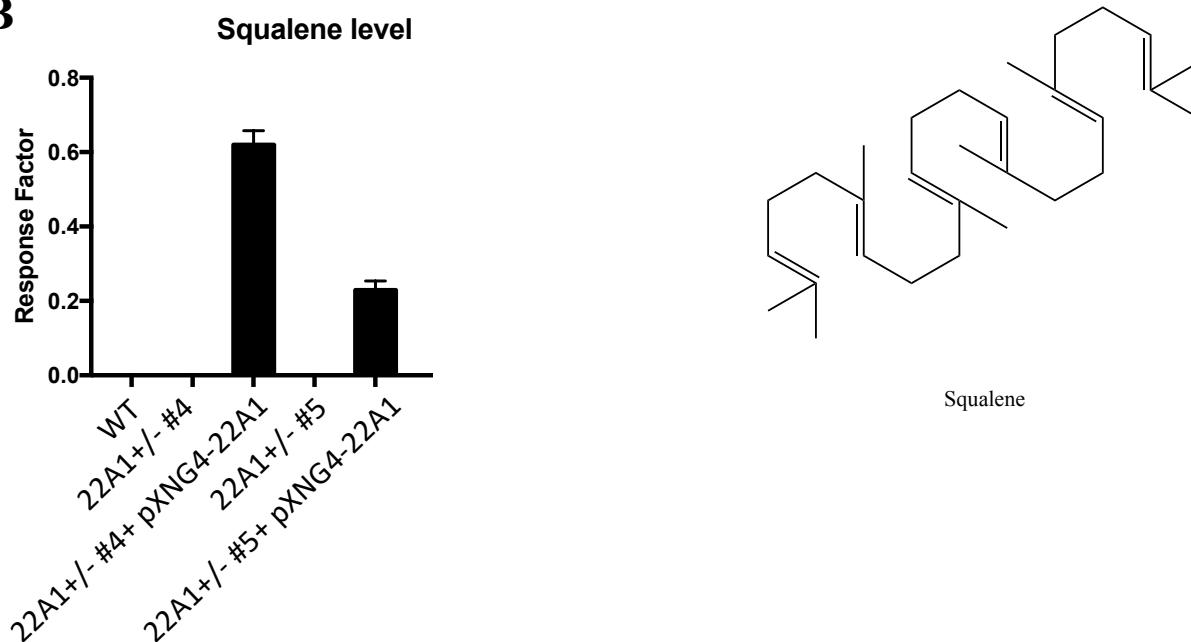
Figure S9. Squalene were detected in 22A1 overexpressing cells. (A) Partial GC chromatograms of total lipids from WT, *22A1+/- #4, 22A1+/- #4* +pXNG4-22A1, *22A1+/- #5,* and *22A1+/- #5* +pXNG4-22A1 promastigotes showed the presence of squalene in the boxed region. (**B**) The relative abundance of squalene was determined.

Table S1. List of oligonucleotides used in this study

Oligo #	Name	Purpose	Sequence
709	28s rRNA_forward	For qRT-PCR analysis of 28S rRNA	AAGATGGACCGGCCTCTAGT
710	28s rRNA_reverse	For qRT-PCR analysis of 28S rRNA	ATCCTTCCCCGCTCCAGTAT
359	5' ORF C4 methyl oxidase	To amplify the ERG25 ORF	GATCATagatct TCGATGGTGCTCGCGAAAAAGC
360	3' ORF C4 methyl oxidase	To amplify the ERG25 ORF	GATCATagatct CCTTCACGAACACGCGCGC
361	for 5' UTR C4 methyl oxidase	To amplify the 5'- flanking sequence of ERG25	GATCATgaattc TCAATGTGACTTGCGTTTAACTCCCTCC
362	rev 5' UTR C4 methyl oxidase	To amplify the 5'- flanking sequence of ERG25	GTCAGCggatccGATCTAactagt CGAGTGCCGAGGGATAAGGTGGAG
363	for 3' UTR C4 methyl oxidase	To amplify the 3'- flanking sequence of ERG25	GATCATggatcc AGGTGCTCAGGCACAAAACGACTTC
364	rev 3' UTR C4 methyl oxidase	To amplify the 3'- flanking sequence of ERG25	GATCATaagctt TCAACAGTTATGCGCATACATATTCACAG
602	Erg25_probe_F.P	To generate the ERG25 probe for Southern blot	GCGCACACCTTCAATGTG
603	Erg25_probe_R.P	To generate the ERG25 probe for Southern blot	CGAACACGACGTGTA
631	Erg25 probe out of 5'UTR F	To generate the ERG25 probe for Southern blot	CAGAGCTAAGTCGTGCTG
632	Erg25 probe out of 5'UTR R	To generate the ERG25 probe for Southern blot	GCTGTTCTGCGGATCATC
740	LmCYP-5'UTR-R	To amplify the 5'- flanking sequence of Lm22A1	GTC GCT actagt GTG AAA TGG GCG ACA AGA G
757	LmCYP-5'UTR-F	To amplify the 5'- flanking sequence of Lm22A1	CAT GCT gaattc CCT TTC TCT GTG CAC CCT TC
741	LmCYP-3'UTR-F	To amplify the 3'- flanking sequence of Lm22A1	CGG ACG actagt GGC TAG agatct AGC GAG CTG AGA ATT GCC
742	LmCYP-3'UTR-R	To amplify the 3'- flanking sequence of Lm22A1	GTC AAG aagctt GCA TTC ACG CAC GCT CTC
754	LmCYPORF-F	To amplify the Lm22A1 ORF	TCA GTA ggatcc ACC ATG GTG TTT GAC ACC GAC
755	LmCYPORF-R	To amplify the Lm22A1 ORF	GCG CT ggatcc TTACATGAACAACTTGTTCG
756	LmCYPORF-nostop-R	To amplify the Lm22A1-GFP fusion	GCG GTC gatatc CAT GAA CAA CTT GTT CGG
768	Lm22A1 ORF probe FP	To generate the Lm22A1 and Ld22A1 probe for Southern blot	GTC GCA TGC GAT GCG CAT C
769	Lm22A1 ORF probe RP	To generate the Lm22A1 and Ld22A1 probe for Southern blot	GTA GCG AGT CTG CAC GGT GC
770	Lm22A1 5'UTR probe FP	To generate the Lm22A1 probe for Southern blot	CAG TGA GCG AGA GAA CGA G
771	Lm2A1 5'UTR probe RP	To generate the Lm22A1 probe for Southern blot	CCT ACA TGA GCG TAG GAC AC