Supplemental Figure 1. Ferric reductase sequence. Alignment of *L. amazonensis LFR1* (LaLFR1), *L. major LFR1* (LmLFR1, accession no. XP_001684755), and *A. thaliana FRO1* (AtFRO1, accession no. NP_171665). Positions of amino acid identity across all three genes are shaded in black; similar residues are shaded in gray. The locations of the eleven predicted transmembrane helices of *L. amazonensis LFR1* are indicated by black lines labeled H1-H11. The conserved histidines predicted to be involved in heme binding are indicated by asterisks.

Supplemental Figure 2. Conserved motifs within LFR1 and related oxidoreductases. The figure shows alignments of motifs conserved at the amino acid level between *L. amazonensis LFR1* (LaLFR1), *L. major LFR1* (LmLFR1, accession no. XP_001684755), *A. thaliana FRO1* (AtFRO1, accession no. NP_171665), *A. thaliana FRO2* (AtFRO2, accession no. NP_171664), *P. sativum FRO1* (PsFRO1, accession no. AAK95654), *H. sapiens GP91-PHOX* (GP91-phox, accession no. P04839), and *S. cerevisiae FRE1* (ScFRE1, accession no. NP_013315). (A) Transmembrane regions containing the conserved histidines, marked by asterisks, predicted to be involved in heme binding. (B) Regions containing the FAD-binding motif (HPFT). (C) Regions containing the NAD-binding motif (CPyG) and the oxidoreductase signature sequence.

Supplemental Figure 3. Passage of *Δlfr1* parasites through mice does not restore growth in macrophages. Parasites were isolated from footpad lesions of mice as described in experimental procedures. Lesion amastigotes were transferred to promastigote growth medium and allowed to differentiate into promastigotes. BMM were infected with axenic amastigotes cultured for 48 h in amastigote growth media (MOI = 1.0) for 1 h, and fixed after the indicated periods for determining the number of intracellular parasites. Black columns, wild type *L. amazonensis*; white columns, Δ*lfr1 L. amazonensis* isolated from footpad lesions. The data represent the mean ± SD of the results of three independent experiments.

Table S1: Oligonucleotides used for PCR

Primer Name	Sequence	Description
AFPNA10	ATGCACATCGCCCCTCTGACACTC	5' *LFR1* cloning primer
AFPNA11	CTAGAAGAGGAACTCTTCCTTGTGCACC	3' *LFR1* cloning primer
AFPNA13	G**GATCC**ATGCACATCGCCCCTCTGACACTCAC	5' *LFR1* cloning primer encoding BamHI site
AFPNA14	GGCAACGTCTTCTTCTATGCGTACCTG	5' primer for *LFR1* DIG probe
AFPNA17	TTTGACGTGCACATAGAACACGGTC	3' primer for *LFR1* DIG probe
AFPNA20	G**GATCC**CTAGAAAAGAAATTCTTCCTTGTG	3' *LFR1* cloning primer encoding BamHI site
AFPNA25	GCCGTCAGCAAGCGCACGCGC	5' primer for cloning 5' UTR
AFPNA35	CTCTTTGAGCTGCTGTGCTGTTCTTG	5' nested primer for cloning 5' UTR
AFPNA36	GCCACCAATGAGGTAGCACAGCTGC	3' nested primer for cloning 5' UTR
AFPNA37	CCCTGCGTCCGCCACACCAGATAG	3' primer for cloning 5' UTR
AFPNA38	CCCGAAATCCTCGAGGCTGGACC	5' primer for cloning 3' UTR
AFPNA28	CCGAACTCGCTCGTTCGCCAGG	5' nested primer for cloning 3' UTR
AFPNA43	CCTCCCATCCCTCCATAGTGTATCTGATG	3' primer for cloning 3' UTR
AFPNA45	C**TCGCGA**CTCTTTGAGCTGCTGTGCTGTTC	5' primer for cloning 5' UTR encoding NruI site
AFPNA46	G**GATATC**CTTGGAGGTTGGCGCGGCCAAATGG	3' primer for cloning 5' UTR encoding EcoRV site
AFPNA47	G**GATATC**GCTTCGCTCATGCGCGTGCCTGTG	5' primer for cloning 3' UTR encoding EcoRV site
AFPNA48	C**AAGCTT**CCTCCCATCCCTCCATAGTGTATC	3' primer for cloning 3' UTR encoding HindIII site
AFPNA57	ATGGGATCGGCCATTGAAC	5' primer for NEO DIG probe
AFPNA58	TCAGAAGAACTCGTCAAGAAGGC	3' pimer for NEO DIG probe
AFPNA62	ATGAAAAAGCCTGAACTCACCGCG	5' primer for HYG DIG probe
AFPNA63	CTATTCCTTTGCCCTCGGACGAGTGC	3' primer for HYG DIG probe
KO1	**GATATC**TCGGCATCCATAGAGACAC	5' primer for cloning out drug resistance cassette
KO2	**GATATC**GTTACGATGTCCGTCCATTC	3' primer for cloning out drug resistance cassette
*LFR1*Fd	GCAACGTGCTTTGTCAACCTGAGA	5' primer for *LFR1* qPCR
*LFR1*Rv	ATCTCGGCATTCAGCCTCACATCT	3' primer for *LFR1* qPCR
UbiqHFd	AACGTGAACAACTGGATGTGCGTC	5' primer for ubiquitin hydrolase qPCR
UbiqHRv	ATGGTACCAAGCTTGACACATGCC	3' primer for ubiquitin hydrolase qPCR

FIGURE S1

```
                 H1                                                                                  H2
LaLFR1  MMHIAPLTLTAQLCYLIGGTVLCVFFTVFGYLVWRTQGYYQLTPEGQRRSAGFTPNGIAGITSVFTFAGLAVVLLIETTL  80
LmLFR1  -MHIAPLTLTAQLCYRIGGTVVCVFFTVFGYLVWRTQGYYELTPEGQQRSAGFTPNGIAGVTGFFTFAGLAVVLLIETTL  79
AtFRO1  ------------------------------------------------------------------------------------  0

                                                H3
LaLFR1  FYVRYAFHPSHKGRYWTMHRYASVILQAALGILYVATGVALLVKWGGNAARCAFMFCLGVDKYPETPLTRGANFNSLLSV  160
LmLFR1  FYVRYAFHPSHKGRYWTMHRYASVILQAALGILYVGTGVTLLVKWSGNSAQCAFIFCLGVDKYPKTPRTRGADFESFLSV  159
AtFRO1  ------------------------------------------------------------MGVGEMNKEVIDK--------VI  15

        H4
LaLFR1  IIFFVLVPTGLVLLYRVFTTVMLQRAAKAVRERDAASVAAGARVSSSTSDQSTGEGKTSPERVGDAIAAVATEANPMAKP  240
LmLFR1  IIFFVLVPAGLVLLYRVFTIAMLQWAEKVARECDAASVAADAQASSSASSQSTGEGTSSPGRGGGVVAAVATEANPMAKP  239
AtFRO1  KFLMMVILMGTIVIWIMMPTSTYKEIWLTSMR---------AKLGKSIYYGRPGVN------------------------  62

                                H5
LaLFR1  VSMRPFQAKSRVKQVCRLVGVLIGILVCAILSFWLPNDSRRFNSNSIASFARNSISRERRTTDTTVPEMPDAWFRTGQIR  320
LmLFR1  VSIRPFQPKSRVKQVCRLVGVLIGILVCAILSFWLPNDSRRWNPNCIASFARNGVSRERRTTNMTVPEMPDAWFRTGQIR  319
AtFRO1  ------------LLVYMFPMILLAFLGCIYLHLKKSTTVNQFNSGVEKKRAKFGALRR---------------------  108

                        H6                                  H7
LaLFR1  VSDDLTLKLFPGNVFFYAYLLMTAIVVLVLRLTQRGRYWMQRRILLCACFTYGEAAFVTATALLSVMFFIYWLHDHNYKQ  400
LmLFR1  FSDDLTLKLFPGNVFFYVYLLMTAIVVLVLRLTQRGRYWVQRRIPLCACFTCGEAAFVAATALLSVMFFIYWLHDHNYKQ  399
AtFRO1  ---PMLVNGPLGIVTVTEVMFLTMFMALLLWSLANYMYRTFVNVTSESAATDGNNLWQARLDLIAVR-------------  172

                                H8                                  H9
LaLFR1  TWTLTANESAQIEAPERWGRGLGQLAILFLSLLLLPVGRQSVVVSVLGVSRDGMLWFHRAVGYAMLAATVGHIVAFYVSY  480
LmLFR1  TWTVTANESAQIEAPERWARGLGQLAILFLSLLLLPVSRQSVVVSVLGVSRDGMLWFHRAVGYAMLAATVGHIVAFYVSY  479
AtFRO1  ----------------------IGIVGNICLAFLFYPVARGSSLLAAVGLTSESSIKYHIWLGHLVMIIFTSHGLCYFIYW  231
                                                                    *       *
                                H10                                 H11
LaLFR1  ASFGYLMQNLNTVTNRVCKKGVFDDYSVLVVTWTTWFLLIAMGIFAFNLVRRRHYELFYYTHLAATYMTLPAMVFHASAG  560
LmLFR1  ASFGYLMQNLNTVTNFVCKKGVYDDYTVLVVTWTTWFLLISMGIFALNLMRRRHYELFYYTHLAATYMTLPAMLFHASAG  559
AtFRO1  ISKNQLVSKMLEWDRTAVSN---------LAGEIALVAGLMMWVTTYPKIRRRLFEVFFYSHYLY-IVFMLFFVFHVGIS  301
                                                                        *       *

LaLFR1  WMY-MLPGMTVFLSDQLVRLWQRSAVVRVVRARVISDDTIELAFSMPGRWDMRRVHPGQYVLVCVPELTALQWHPFTLTN  639
LmLFR1  WMY-LLPGMTVLLSDQLVRLWQRSAVVRVVRARVIGDDTTELAFTVPGRWDMRRVHPGQYVLVCVPELTALQWHPFTLIN  638
AtFRO1  HALIPLPGFYIFLVDRFLRFLQSRNNVKLVSARVLPCDTVELNFSKN---PMLMYSPTSTMFVNIPSISKLQWHPFTIIS  378

LaLFR1  VVDEESVAVGSSKVSATGTVFYLHVKSMGPKTWTGRLYDLVSRG-----EEITMAVEGPCGTP-VDYRHYDHIVLVAGGI  713
LmLFR1  VVDEESAVVGSSKVSAKETVFYVHVKSMGPKTWTGRLYDLVSRG-----EEITMAVEGPCGTP-VVYRHYGNIVLVAGGI  712
AtFRO1  SSKLEPETLS------------VMIKSQG--KWSTKLYDMLSSSSSDQINRLAVSVEGPYGPSSTDFLRHESLVMVSGAS  444

LaLFR1  GATPCVSILGSLLRQCHALGHSGASPRVDLIWSTRSARHVKAVADMLQMP-MRYGNVLCQPETPTALLKKTDTYAAPEDI  792
LmLFR1  GATPCVSILGSLLRQCHALGHSGASPRVTFIWSTRSARHVKAVADMLQLP-MRCGDVVRQPATRAALVKKIDTCVAPEDV  791
AtFRO1  GITPFISIVRDLFYMSS--THKCKIPKMTLICAFKNSSDLSMLDLILPTSGLTTDMASFVDIQIKAFVTREEKTSVKEST  522

LaLFR1  TKDVRLNAEISQPANRLEQDEPLDGELGVVSEGVRNADDAVDNFFLDVYVTRVTELQQFFNGDACLGDQCSPKSFSLSPR  872
LmLFR1  TNDVRLNTEIIQPANSLEQDEPLNEEFSAVNEGVRIADDAVQNFFLDVYVTRTTELQHFSNGDACLGEQCSSTPFTPSPK  871
AtFRO1  HNRNIIKTRHFKPN---VSDQPISPILGPN-SWLCLAAILSSSFMIFIVIIAIITRYHIHP----IDQNSEKYTWAYKSL  594

LaLFR1  KGVVELDGEPGSCAEVIGSTENAFYSALSWEQKTAAAATPGPDSKANDEGEAEEEMVFSLASMGSEREDVDPTSAEDCED  952
LmLFR1  KGFVELDSKTLSCAGAMGTVQNAVDSASPWKQKTATATTTGPHGKADD---AEEAKAFSNASMGRESKGLDLTGADGCED  948
AtFRO1  IYLVSIS----------ITVVTTSTAAMLWNKKKYYAKN-------------------------------------DQ  625

LaLFR1  RIEGASESTARKKERKVVGDTASTTDEEGALVPNGLSKSTPSSATKSSEGLHD-SDGSGSGCVALKLHLGRPDVDRLIRE  1031
LmLFR1  RINGSSESTAHRMGWKVFGDAASAMDEEGAFMLNGLSRWSSSSVTKSSERLHDNSDSSGSGCVALKVHSWRPDVDRLIRE  1028
AtFRO1  YVDNLSPVIIESSPQQLISQSTDIHYGE-------------------------------------------RPNLNKLLVG  663

LaLFR1  ALKRPGSGVPKSSRLDRARTLLFVCGPN---SLVRQVTSSGAQLGVAVHQEEFCF                            1083
LmLFR1  ALKQPGSGVQESSKLDKARTLLFVCGPN---SLVRQVTASGAQLGVAVHKEEFLF                            1080
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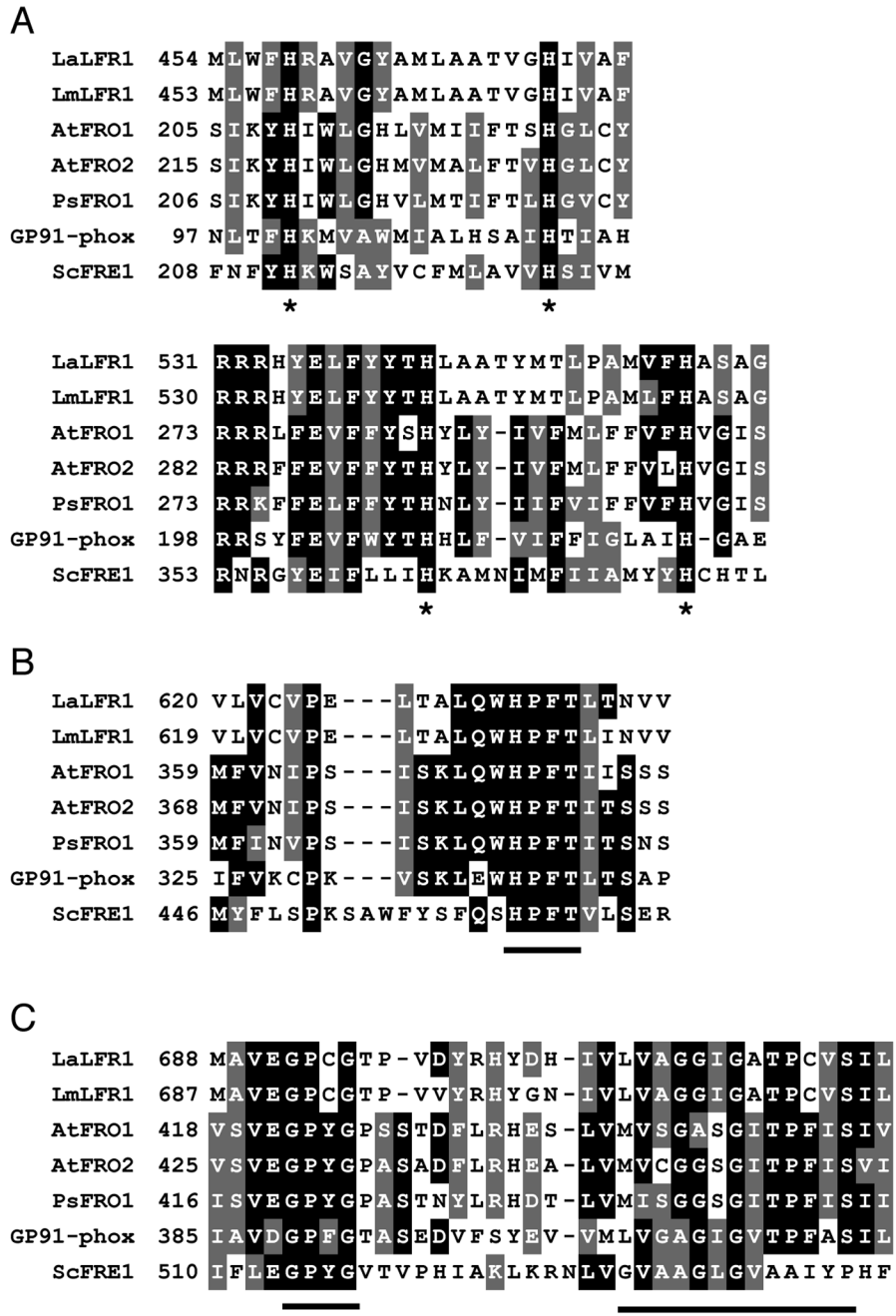
FIGURE S2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B
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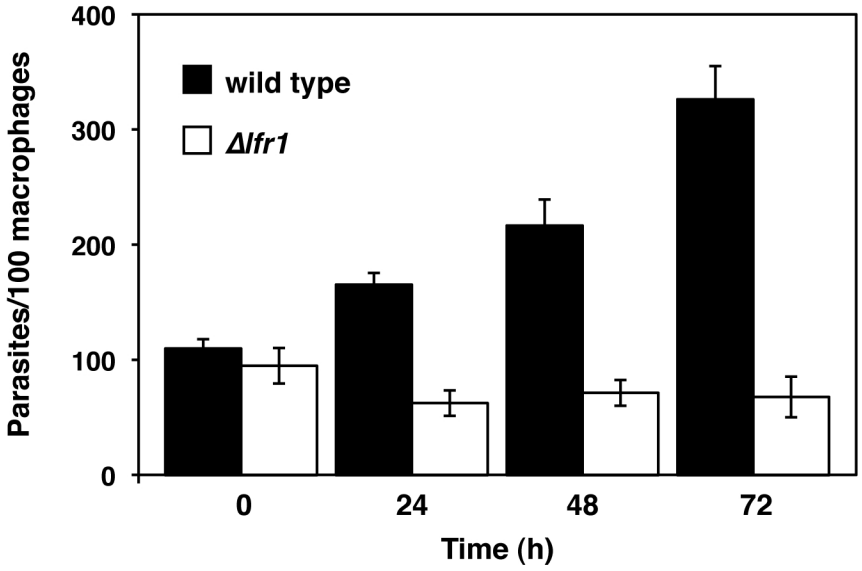
AtFRO2 425 VSVEGPYGPASADFLRHEA-LVMVCGGSGITPFISVI
PsFRO1 416 ISVEGPYGPASTNYLRHDT-LVMISGGSGITPFISII
GP91-phox 385 IAVDGPFGTASEDVFSYEV-VMLVGAGIGVTPFASIL
ScFRE1 510 IFLEGPYGVTVPHIAKLKRNLVGVAAGLGVAAIYPHF

Figure S3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