

	1									
Tb927.3.800	-----	-----	-----MSESE	KIDVSGARNY	KS-----ISA	ARXKAFETGD				
PF3D7_1223700	-----	-----	-----	-----MVS	KK-----TIE	ARXKAYYNE				
PBANKA_1438600	-----	-----	-----	-----MGK	QK-----IID	ARXKAYYEGD				
ETH_00007450	-----	-----	-----MAQLMGDE	ERRDVYSATT	NA-----LQT	RFXYAMYIRA				
TgVit	-----	-----	-----MPA	SGAGYAGGSI	VPDGRTLVNC	RD-----LHK	ARXDAYQLRD			
NCLIV_039240	-----	-----	-----MPA	GGAGHAGGNI	VPDGRTLVNC	RD-----LNK	ARXDAYQLRD			
ScCCC1	MSIVALKNAV	VTLIQKAKGS	GGTSELGGSE	STPLLRGSNS	NSSRHDNLSS	SSSDIYGRX				
AtVit1	-----	-----	-----	-----	-----XMS	SEEDKITRIS				
EgVIT1	-----	-----	-----	-----	-----XMD	GANDGGNPGA				
	61								Asp43 Gly44	
Tb927.3.800	IEMSRMEHQK	HI--YKEV--	-----H----	-----NPSASD	YVKSUVFGGL	DGXIITSFTV				
PF3D7_1223700	VVLSKEAHDF	-----YHNL--	DKHGEN----	-----HNLDKD	NLKTIIFGSL	DGXIIITIFAI				
PBANKA_1438600	IEKSKEIHSH	-----YHNL--	DKHAEH----	-----HSLDKD	HLKTIIFGSL	DGXIIITIFAI				
ETH_00007450	AHQHNHHHSQ	NPGDYGELSG	VHTEAH----	-----KKTSSD	YLKAIVFGGL	DGXIVTIFAI				
TgVit	IEATRAAHS	DL--YREL	DKHESH----	-----TNTSSD	YVKAVVFGGL	DGXIVTIFAI				
NCLIV_039240	IEATRAAHS	DL--YREL	DHESH----	-----TNTSSD	YVKAVVFGGL	DGXIVTIFAI				
ScCCC1	NSAQDLENSP	MSVGKDNRRNG	DNGSDNEKAN	LGFFQSVDP	VISDLIIGLS	DGXLTPPFAL				
AtVit1	IEPEKQTL-	-----	DHHTEK----	-----HFTAGE	IVRDIIIGVS	DGXLTPPFAL				
EgVIT1	EEQQR-	-----L--	DQKHA----	-----HFTAGE	IVRDIIIGVS	DGXLTPPFAL				
	121		Gly69	Glu72	Gly76	Met80			Glu102	Glu105
Tb927.3.800	VSAAVGSNS	VASVLIFGFS	NVIADGFAMG	FGEYVSGEAE	RDNALSERXR	REEWEVENAF				
PF3D7_1223700	VSGCVGAKIT	PTQVIIIIGIG	NLFANAISM	FSEYTSSTAQ	RDFMLAEKXK	REEWEIENCP				
PBANKA_1438600	VSGCVGANIT	PAQVIIIIGVG	NLFANAISM	FSEYTSSTAQ	IDFMLAERXQ	REEWEIENCP				
ETH_00007450	VAGCVGANLH	PSKVVIIGIG	NLLADAISM	FGEFVSSAAE	DDFVKSERXD	REEWEIENCP				
TgVit	VAGCVGADLS	CSQVLMVGLG	NLLADAISM	FGEYVSAAAE	KDFVEAEKXQ	REEWEVENCP				
NCLIV_039240	VAGCVGADLS	CSQVLMVGLG	NLLADAISM	FGEYVSAAAE	KDFVEAEKXQ	REEWEVENCP				
ScCCC1	TAGLSSLG-D	AKLVITGGFA	ELISGAISM	LGGYLGAKSE	SDYYHAEVXK	KEKRKFYDNS				
AtVit1	AAGLSGANAS	SSIVLTAGIA	EVAAGAISM	LGGYLAAKSE	EDHYAREMXX	REQEIVAVP				
EgVIT1	AAGLSGANAS	SSIVLTAGIA	EVAAGAISM	LGGYLAAKSE	ADNYARELXX	REQEIIIRVP				
	181	Glu113	Glu116						Met149	Glu153
Tb927.3.800	DMEVDEMVIQI	YE--MKGLSH	EDATTIVNII	SKDKPLFVDF	MMTEELGIXI	IDTEDTHGPK				
PF3D7_1223700	SEKQEMIDI	YMN-KYKFDS	EDARNLVEIT	FRNKNFFLEH	MMSEELGLXI	VTNEDKNECL				
PBANKA_1438600	TEKQEMIDI	YIN-KYKFDS	KDAKNLVEIT	FRNKHFFLEH	MMSEELGLXI	LTNEDKSEAF				
ETH_00007450	DEEKQEMIEI	YRD-RYGFTE	EDADSLVNIT	FKYREFFVRH	MMVEELGLXM	-ATEGPS-PL				
TgVit	EEKREMEVEI	YTE-KYGF	ADAQSMVDIT	FKYKFFVQH	MMVEELGLXM	YGFDEPT-PI				
NCLIV_039240	EEKREMEVEI	YTE-KYGF	ADAQSMVDIT	FKYKFFVQH	MMVEELGLXM	YGFDEPT-PI				
ScCCC1	NLINREIEDI	LLEINPNFSD	ETIVSFIKDL	QRTPELMVDF	IIRYGRGLX-	-DEPAENREL				
AtVit1	ETEAAEVAEI	LA--QYGI	HEYSPVVAL	RKNPQAWLDF	MMRFELGLX-	-EKDPKRAL				
Eg_VIT	DTEAAEVAEI	LA--RYGI	HEYGPVVAL	RKKPQAWLDF	MMKFELGLX-	-EKDPKRAL				
	241		Asp175							
Tb927.3.800	KQGLVMFLSF	MFFGAVPLLA	Y-----LPGK	GKGIDGVFAL	XSCFLATCAL	IVLGMRLGYL				
PF3D7_1223700	KKGIIMFLSF	AVFGIIPLSA	YVAYTVFFG-	YTDYTTSTFLV	XVFISTLTTL	FILGLFKSQF				
PBANKA_1438600	KKGILMFLSF	CFFGMIPLFS	YVLYNLFFS-	AENYTSFAV	XVFISTLITL	FILGLFKSQF				
ETH_00007450	RRGAVMFASF	SIFGLLPLAG	FVAWLTLSTG	STDGHLAFAM	XACVVSIGIAL	FILGFFKGRF				
TgVit	KRGLVMFTAF	CFFGLLPLAG	FIGWVAAFGL	GAEADMAFLM	XACVVSIMTL	FILGFSKGF				
NCLIV_039240	KRGLVMFTAF	CFFGLLPLAG	FIGWVAAFGL	GAEADMAFLM	XACVVSIMTL	FVLGFSKGF				
ScCCC1	ISAVTIGGGY	LLGGLVPLVP	Y-----FF--	VSDVGTGLIY	XSIIVMVVTL	FWFQVVKTKL				
AtVit1	QSAFTIAIAY	VLGGFIPLLP	Y-----ML--	IPHAMDAVVA	XSVVITLAL	FIFGYAKGHF				
EgVIT1	QSAFTIAIAY	VLGGLVPLIP	Y-----MF--	IPVARKAVVA	XSVILTLMAL	LIFGYAKGYF				
	301									
Tb927.3.800	-----SGVS	MLRSAALMVF	NGVVSGLFSF	TXVGSLSVEHA	LRSSIEVX-					
PF3D7_1223700	-----TNQK	PITCALYMLV	NGMIAGMVPF	LXLGVVLKNN	ISEX-----					
PBANKA_1438600	-----TTQK	PIVCALSMVL	NGSIAGMLPF	LXFGVLLKTN	SGDX-----					
ETH_00007450	-----VNQS	SLKSGLLMII	NGTCAGTVAY	TXVGAALEGV	VAGTLX---					
TgVit	-----VGQN	PTKSACLAM	NGGCAGTVAY	GXVGSLLQLV	VGANLTAAX					
NCLIV_039240	-----VGQN	PTKSACLAM	NGSCAGTVAY	GXVGSLLQLA	VGANMSSGX					
ScCCC1	SMGSGSSTS	KVTEGVEMVV	VGGVAAGAAW	FXFVKLLGX-	-----					
AtVit1	-----TGSK	PLRSAFETAF	IGAIASAAAF	CXLAKVVQHX	-----					
EgVIT1	-----TDNK	PFKSALQATAL	IGAIASAAAF	GXMAKAVQSX	-----					

Figure S1. Full alignment of *T. gondii* VIT with VIT sequences from selected eukaryotes. Blue boxes represent transmembrane domains, purple indicates Asp43 and Met80 which are conserved in all VIT members and important for iron binding. Light purple indicates residues important in EgVIT iron binding which are not conserved in apicomplexan sequences. Red indicates conserved residues in the MBD important for Zn coordination. Orange residues indicate conserved 'kink-inducing' residues, important for metal binding the cytoplasmic pocket. All residue numbers from EgVIT.

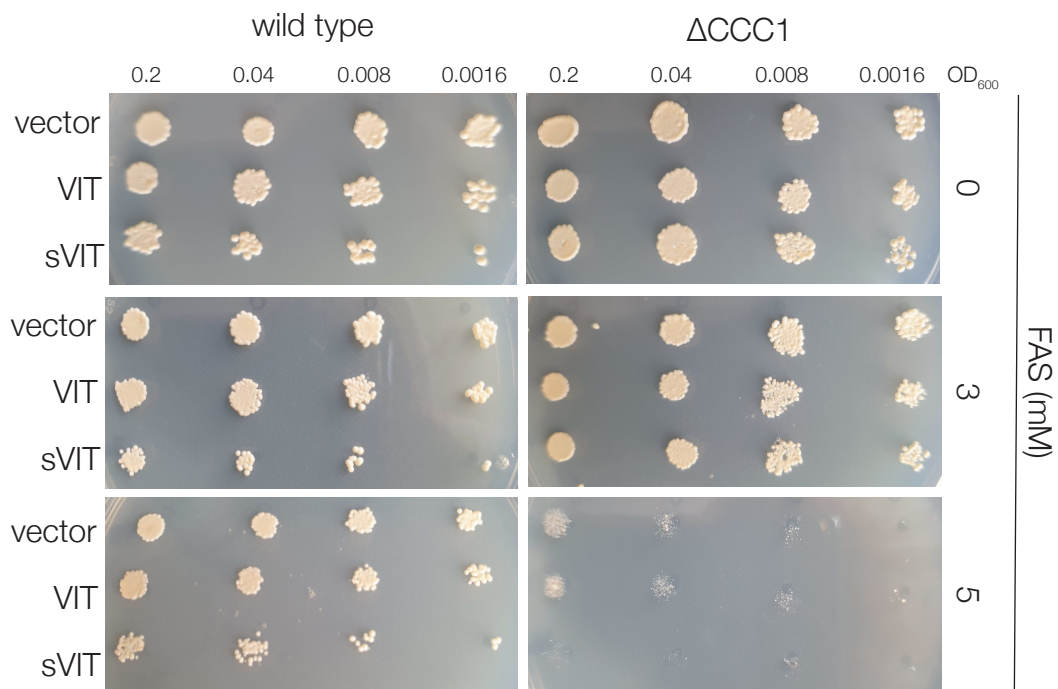


Figure S2. Complementation of yeast with TgVIT. Wild type or Δ CCC1 yeast were transfected with the empty vector or a vector expressing VIT or sVIT₆₃₋₃₁₃ (an N-terminal truncation) and plated at the indicated OD₆₀₀ dilution. While the Δ CCC1 strain behaved as expected and was unable to grow on plates containing high iron, expression of VIT or sVIT₆₃₋₃₁₃ did not complement the phenotype.

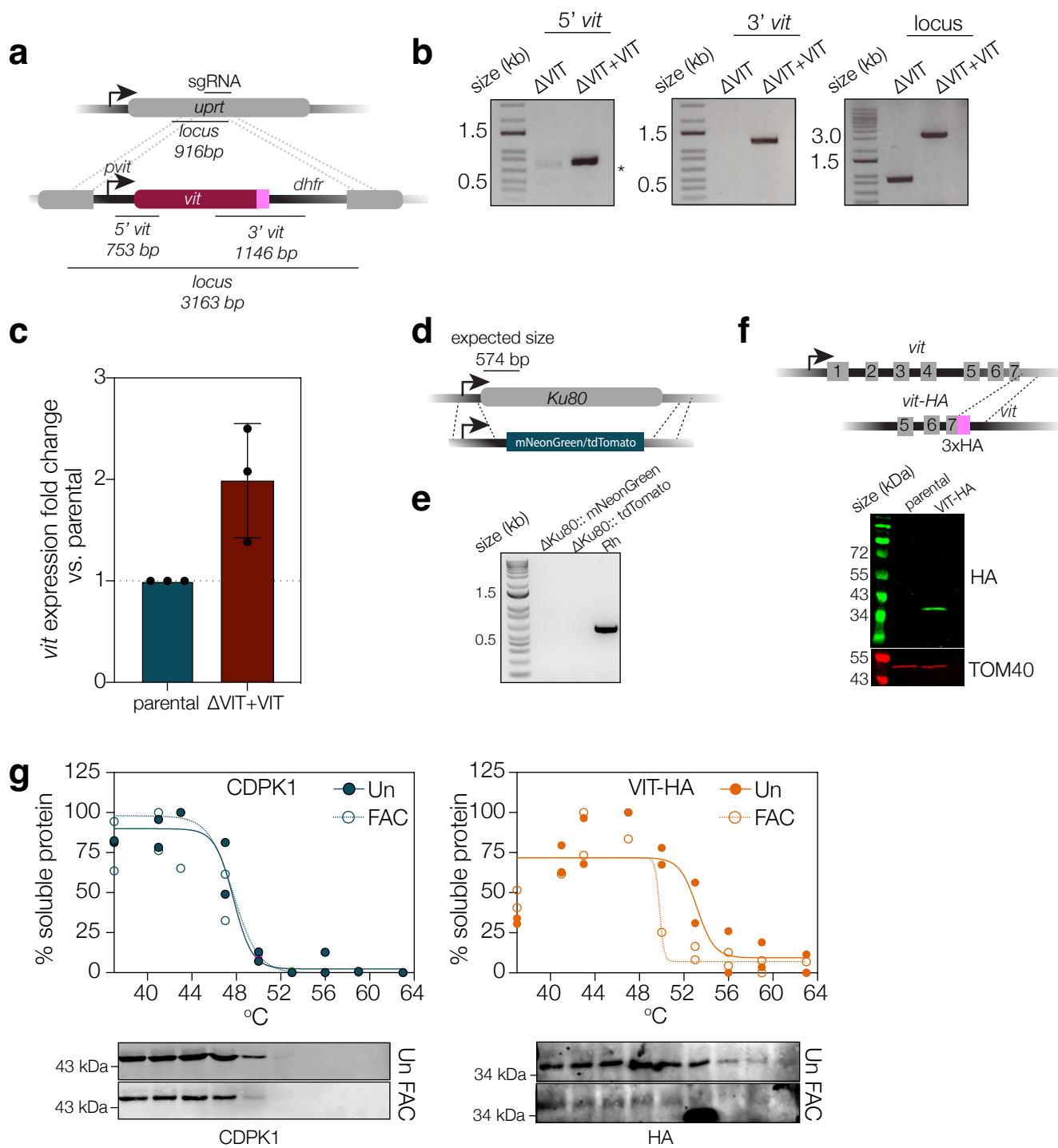


Figure S3. Complementation of Δ VIT parasite line and creation of new Δ Ku80 lines expressing mNeonGreen or tdTomato **a.** Schematic indicating the strategy used to replace the *uprt* gene with the *pvit-vit-3' dhfr* cassette. **b.** PCR confirmation of the presence of the *vit* complementation cassette and the disruption of the endogenous locus, expected sizes indicated in (a). Non-specific band marked with asterisk. **c.** qPCR of *vit* in the complemented line shows approximately double the expression compared to parental line. Results are mean \pm SD from three independent experiments. Schematic indicating the strategy for replacement of *ku80* gene by mNeonGreen or tdTomato cassettes. **e.** PCR of region indicated in (d), confirming the loss of the endogenous gene. **f.** VIT was endogenously tagged at the C-terminal with 3xHA tags. A single band at the expected size (35 kDa) was obtained. **g.** Lysates of VIT-HA parasites treated or untreated for 1 h with FAC were subjected to a range of temperatures as indicated and western blots using anti-CDPK1 and anti-HA performed. Band density plotted, normalised to 37 °C samples, points from two independent experiments.

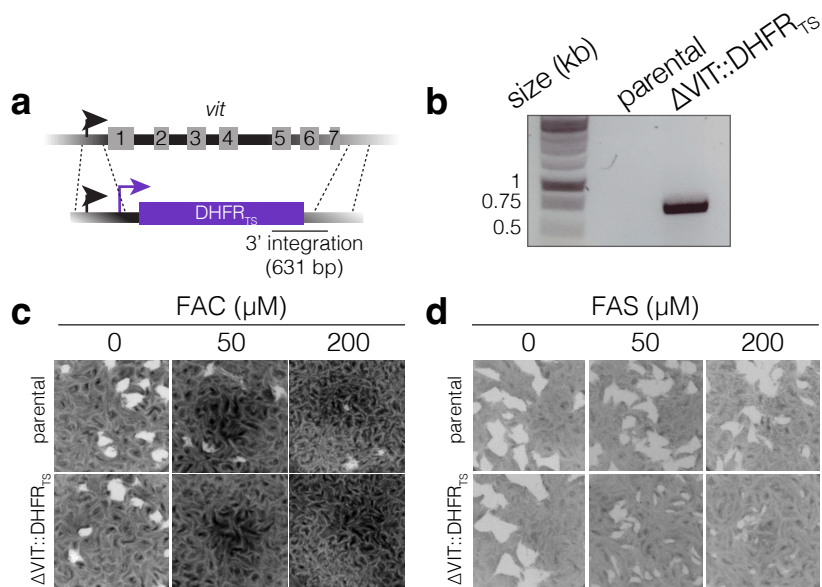


Figure S4. Construction of Δ VIT::DHFR_{TS} parasite line.
a. Schematic showing how *vit* was replaced with the DHFR cassette. **b.** PCR showing the 5' integration of the DHFR cassette. Plaque assays showing increased sensitivity of the Δ VIT::DHFR_{TS} line to excess ferric ammonium citrate (FAC) (**c**) and ferrous ammonium sulphate (FAS) (**d**) at the indicated concentration compared to the parental parasite line. Representative plaque assays from three independent replicates.

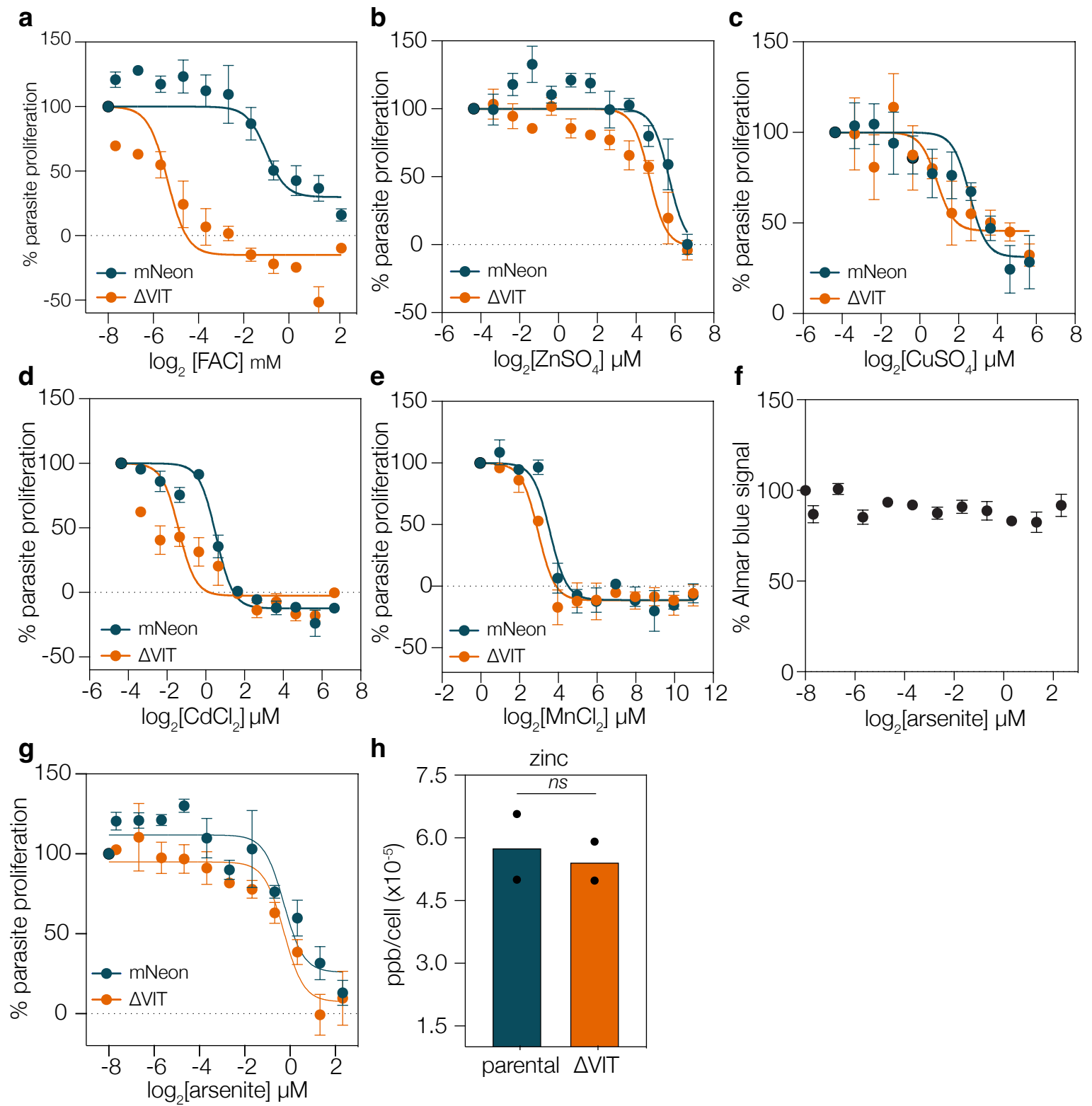


Figure S5. Dose response curves of mNeon and Δ VIT treated with excess metals. As described above, parasites were treated with the indicated concentration of FAC (a) ZnSO₄ (b), CuSO₄ (c), CdCl₂ (d), or MnCl₂ (e). All experiments are the mean of six (FAC) four (ZnSO₄) or three (CuSO₄, CdCl₂, and MnCl₂) independent experiments performed in triplicate, \pm SEM. f. Survival of uninfected HFF cells at the range of sodium arsenite concentrations used above. No change in fluorescence was seen at the concentrations used. Results are the mean $n = 3$ independent biological replicates, \pm SEM. g. Treatment of mNeon and Δ VIT parasites with sodium arsenite, a ROS generator, showed no difference in sensitivity between the two lines. Points are the mean of $n = 3$ independent biological replicates, \pm SEM. h. ICP-MS quantifying zinc (⁶⁶Zn) from parental and Δ VIT parasites. Each point represents an independent biological replicate, performed in technical duplicate. Bars at mean.

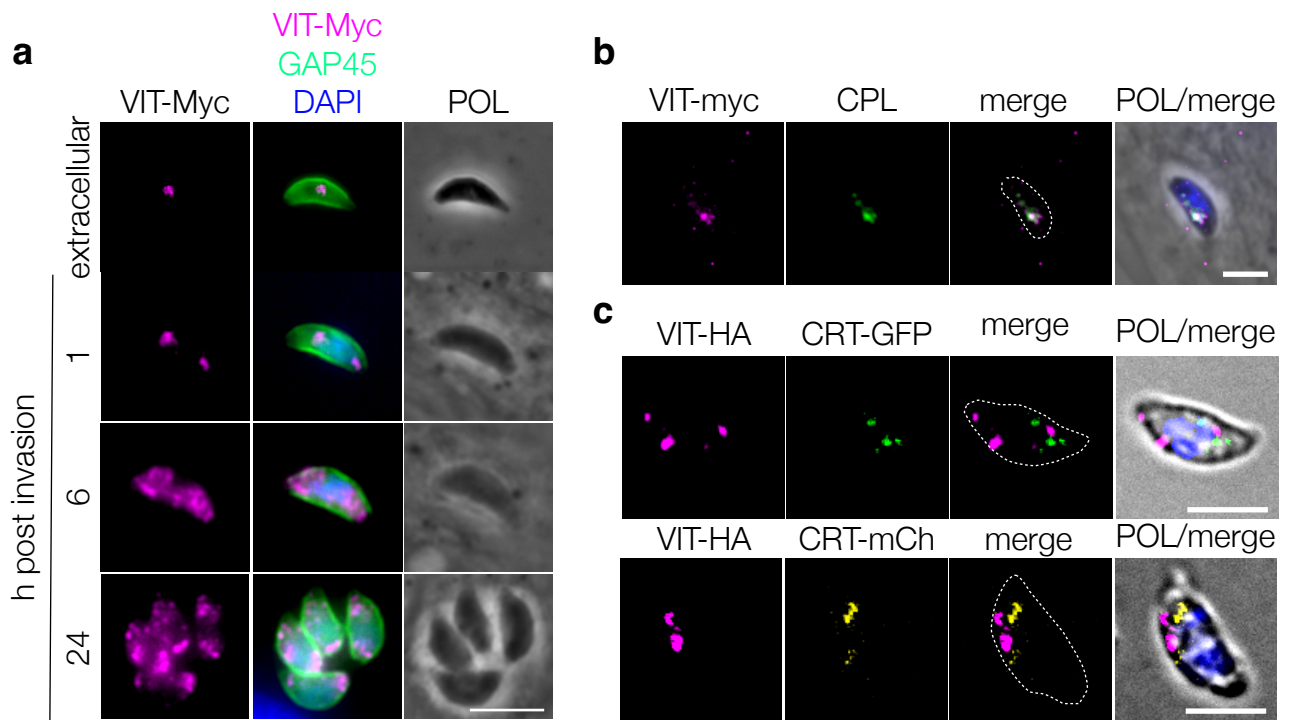


Figure S6. VIT-myc has a dynamic localisation. **a.** VIT-myc demonstrates a dynamic localisation throughout the cell cycle and fragments between 1 and 6 h post invasion. Cell periphery indicated by GAP45. Representative of three independent experiments. **b.** VIT-myc colocalizes with CPL in extracellular parasites. Representative of two independent experiments. **c.** VIT-HA does not colocalize with transiently overexpressed CRT-GFP or CRT-mCherry in extracellular parasites. Representative of one experiment. Scale bar 5 μ m.

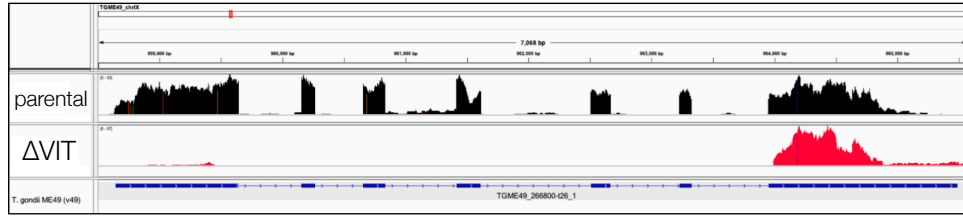
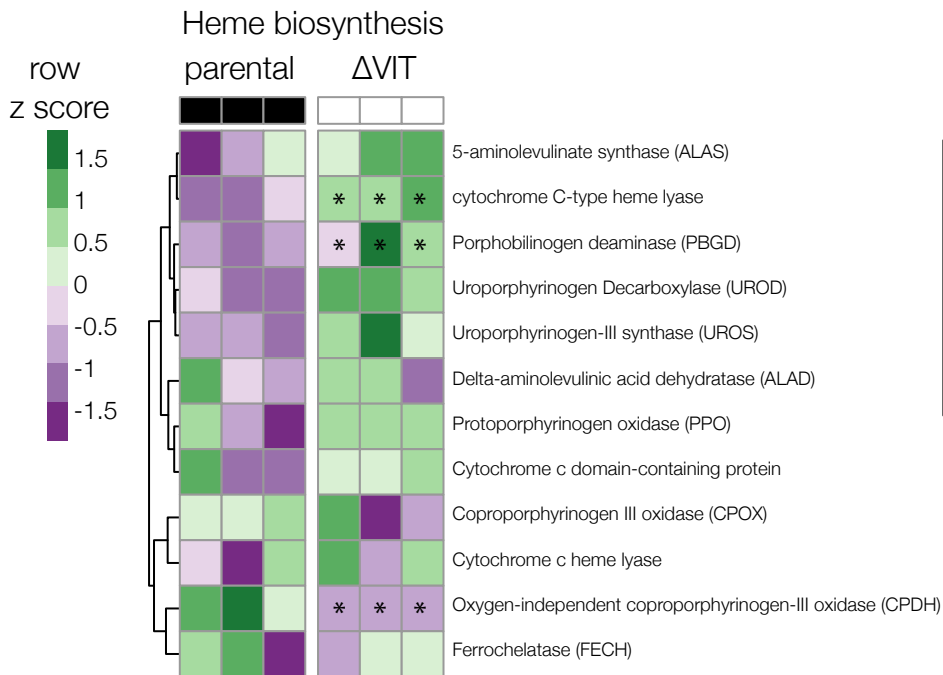
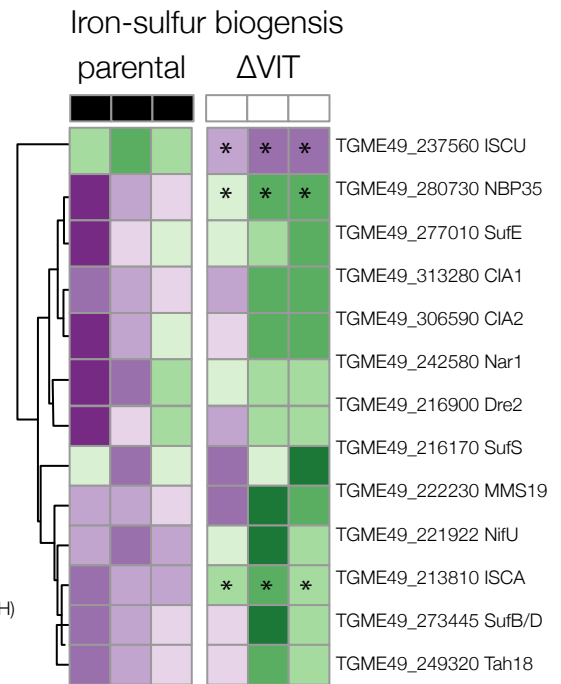
a**b****c**

Figure S7. Heatmaps of genes from major iron pathways from parental and ΔVIT parasites **a.** Mapping of all reads from the parental and ΔVIT strains to the *vit* gene. As the 5' UTR of *vit* still exists, some reads map to this region, but no reads map to the coding regions. **b.** Genes involved in the heme biosynthesis pathway, read counts normalised across rows. * indicates adj. *p* value of < 0.05 between parental and ΔVIT strain. **c.** Genes involved in Fe-S biogenesis read counts, normalised across rows. * indicates adj. *p* value from Wald test with Benjamini and Hochberg correction of < 0.05 between parental and ΔVIT strain.

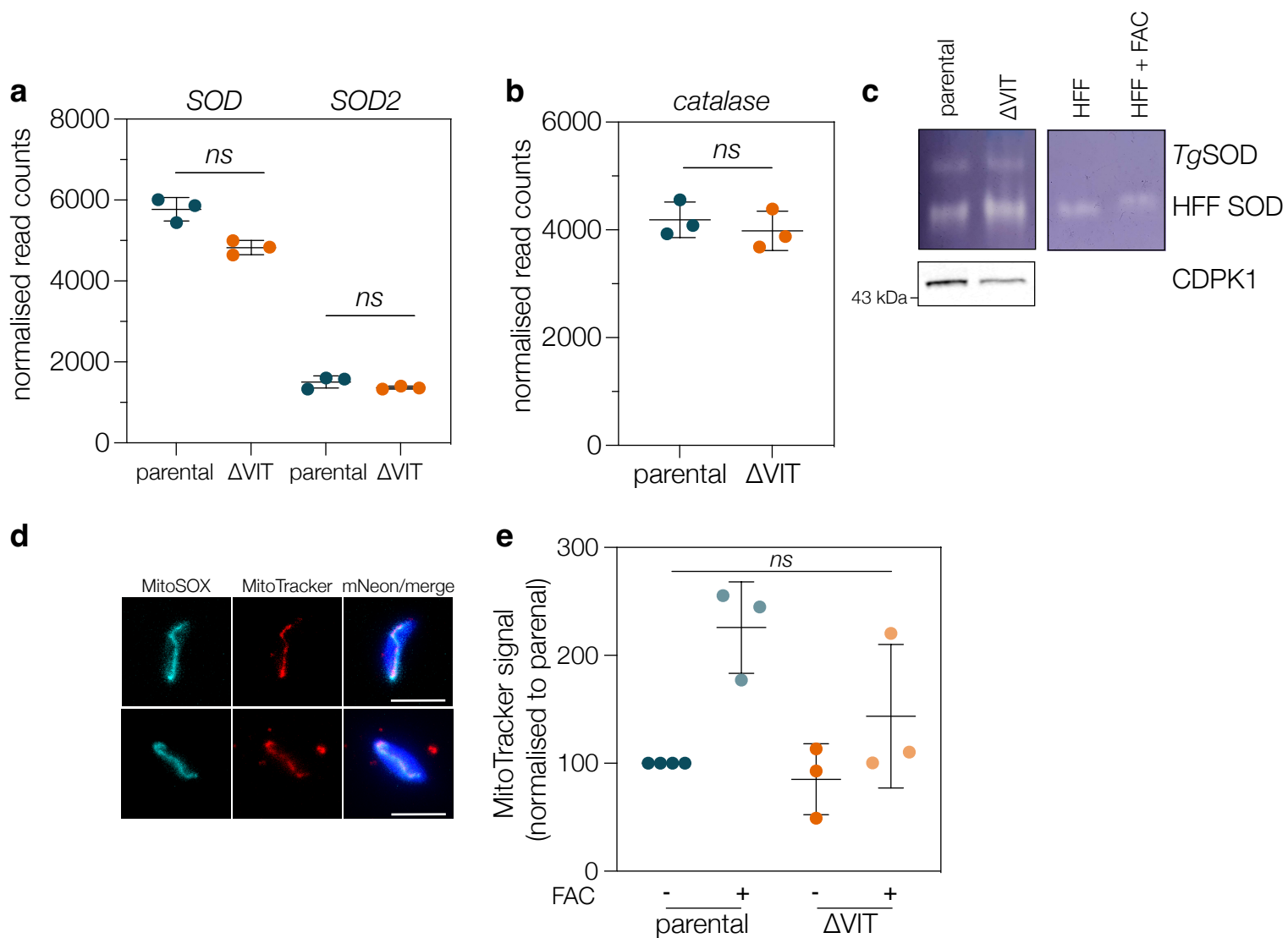


Figure S8. Δ VIT parasites do not alter transcription of oxidative stress genes **a.** Normalised read counts for *SOD* (TGME49_316310) and *SOD2* (TGME49_316330) from RNAseq. Each point represents an independent experiment, performed in duplicate. Line at mean \pm SD. *ns*-non significant from Wald test with Benjamini and Hochberg correction. **b.** Normalised read counts for *catalase* (TGME49_232250). Each point represents an independent experiment, performed in duplicate. Line at mean \pm SD. *p* value from Wald test with Benjamini and Hochberg correction. **c.** In gel activity assay for SOD, enzyme activity results in cleared areas on native gel. Host (HFF) and parasite enzymes indicated, CDPK used as a parasite loading control. No obvious differences were seen between the parental and Δ VIT parasite lines. Results representative of two independent experiments. **d.** Live mNeon parasites were co-stained with MitoSOX Red (cyan) and Mitotracker DeepRed (red) and imaged. MitoSOX and MitoTracker signals co-localised in the mitochondrion of the parasites. Scale bar 5 μ m. **e.** MitoTracker signal was measured by flow cytometry and normalised to the mNeon untreated line. Although FAC treatment led to generally increased signal, no significant differences were seen (Kruskal-Wallis test, Dunns multiple comparison correction). Each point represents an independent experiment, *n* = 3, line at mean \pm SD.

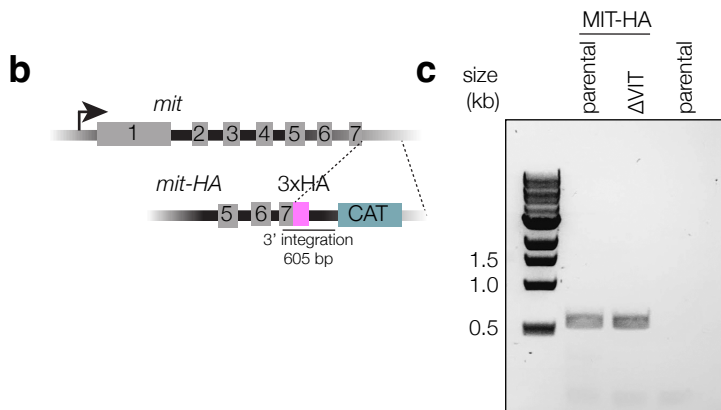
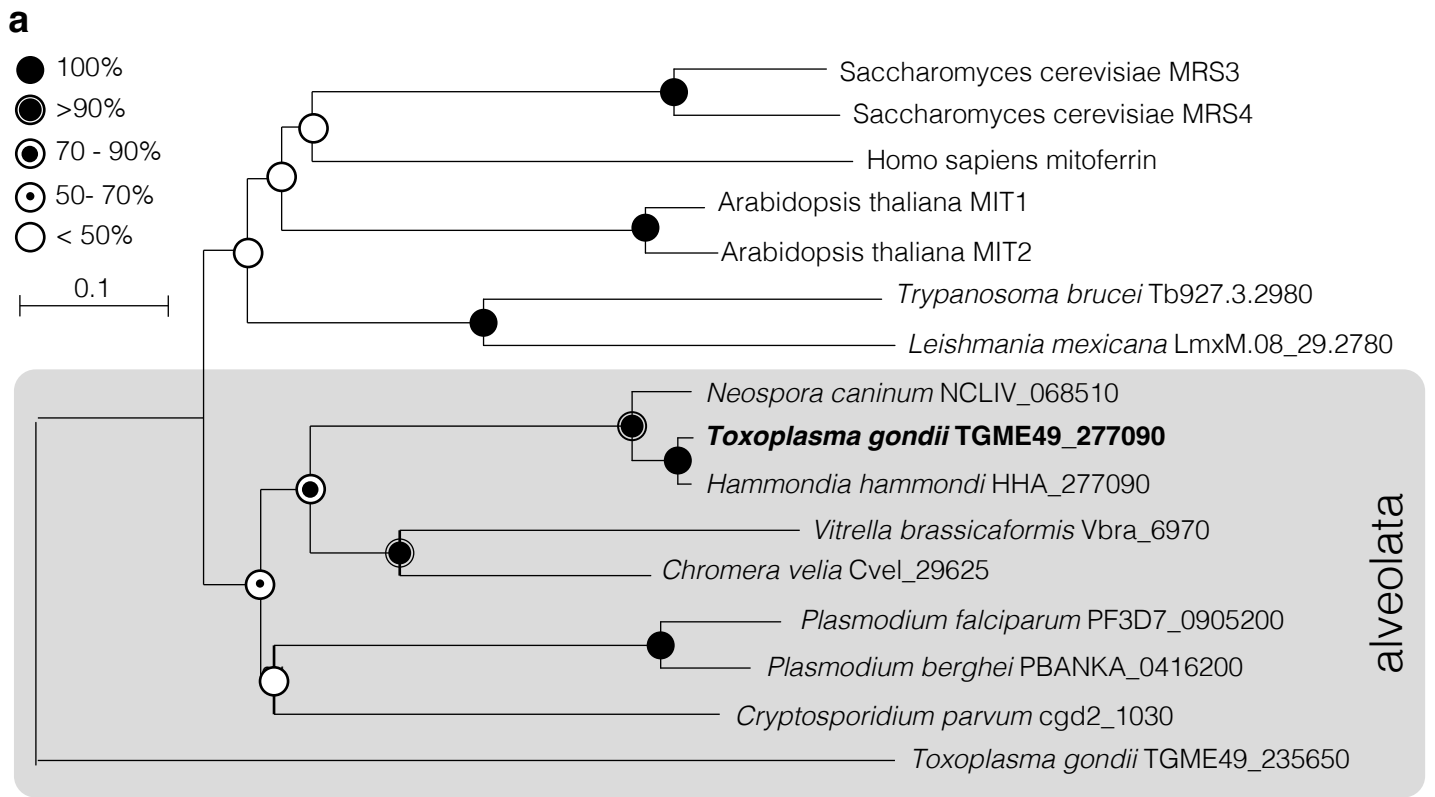


Figure S9. Identification and characterisation of *T. gondii* mitochondrial iron transporter (MIT). **a.** Phylogenetic tree of MIT sequences from a variety of species. TGME49_235650 represents an unrelated mitochondrial solute carrier. Bootstraps determined from 100 simulations. Scale indicates substitutions per site. **b.** Diagram demonstrating C-terminal HA endogenous tagging strategy. **c.** PCR confirming the integration of HA into MIT locus in the parental (Δ ku80) and Δ VIT parasite lines.

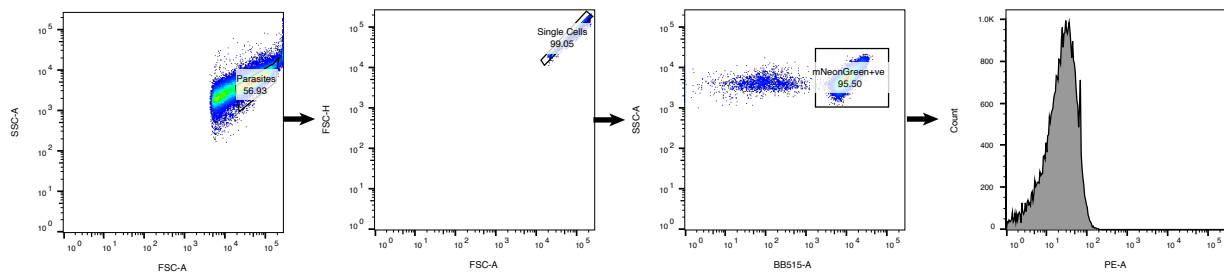


Figure S10. Example gating strategy $\Delta ku80::mNeonGreen$ parasites were stained with MitoSOX as described. Gates were drawn as indicated to isolate parasites, remove doublets and select only mNeonGreen parasites to calculate geometric MFI.