Conditional genome engineering in *Toxoplasma gondii* uncovers alternative invasion mechanisms

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We established a conditional site-specific recombination system based on dimerizable Cre recombinase–mediated recombination in the apicomplexan parasite *Toxoplasma gondii*. Using a new single-vector strategy that allows ligand-dependent, efficient removal of a gene of interest, we generated three knockouts of apicomplexan genes considered essential for host-cell invasion. Our findings uncovered the existence of an alternative invasion pathway in apicomplexan parasites.

Several strategies have been established to generate conditional knockdown mutants in *T. gondii*1,2. However, leaky expression inherent to these designs can obscure mild phenotypes. Because of this problem, the question of whether a gene of interest (GOI) is essential for function cannot be answered with certainty. The current model of gliding motility and active host-cell invasion, for example, is well supported by knockdown mutants for crucial components of the parasite’s actin-myosin motor, resulting in YFP-expressing parasites (Fig. 2a). We tested this strategy by transfecting RH DiCre parasites with a vector containing *loxP*-flanked *KillerRed*10. Parasites incubated in rapamycin exhibited a highly efficient switch from red to green fluorescence (96%), as assessed by microscopy and FACS analysis (Fig. 1f,g). The strategy allows easy visual identification of YFP-positive GOI-knockout parasites upon GOI removal, and we have used it to generate knockout mutants (data not shown). Furthermore, the *ku80::diCre* parental strain we generated improved the efficiency of homologous recombination, facilitating the engineering of conditional knockouts (Supplementary Fig. 1).

To functionally dissect critical components of the parasite’s invasion machinery, we generated conditional knockout mutants for the gliding motor myosin A (MyoA), the micronemal protein MIC2 and actin (Act1) (Fig. 2a and Supplementary Figs. 2–5). These invasion factors are considered essential for host-cell invasion, according to the interpretation of phenotypes obtained in knockdown (MyoA3 and MIC2 (ref. 8)) or overexpression (Act1 (ref. 11)) mutants. To produce the conditional *myoA* knockout parasites, we first introduced a *loxP*-flanked and *Ty*-tagged copy of *myoA* (regulated by the chimeric promoter p5RT70)12 randomly into the genome of RH DiCre parasites. The endogenous *myoA* was then replaced with the *cat* gene, producing parasites with only one copy of *myoA* (Supplementary Fig. 2).

For *mic2* and *act1*, we used the gene-swap strategy in *ku80::diCre* directly, exchanging the endogenous GOI for the gene-swap construct such that expression of the respective cDNA was under control of the endogenous promoter (Supplementary Figs. 4 and 5 and Supplementary Note). We verified all genomic modifications

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Figure 1 | Conditional Cre recombinase system in T. gondii. (a) In the DiCre system, N-terminal (N-ter) Cre (Cre recombinase amino acids 1–59) is fused to FKBP12 and C terminal (C-ter) Cre (Cre recombinase amino acids 60–343) is fused to FRB. Rapamycin dimerizes the subunits to reconstitute Cre recombinase activity. (b) Immunoblot of DiCre expression using FKBP12 and FRB antibodies in the loxP-loxZ-loxP reporter parasite line (RH BloxP). Catalase was the loading control. (c) Quantification of Cre recombinase–mediated recombination using 50 nM rapamycin or 1 µM Shield-1 for 6 h. Data from three experiments are shown, ± s.d. **P = 0.0008 in a two-tailed Student’s t-test. (d) Cre recombinase–mediated recombination in RH BloxP parasites expressing FKBP-Cre59 and FRB-Cre60 treated with 50 nM rapamycin. β-galactosidase expression was determined 72 h after induction. Data are mean values from three experiments ± s.d. ** **P < 0.0001 in a two-tailed Student’s t-test. (e) In the gene swap strategy the endogenous GOI (magenta) is replaced by the indicated cassette; the loxP-flanked GOI cDNA is followed by YFP and the selectable marker (HX). Cre recombinase–mediated excision of the GOI cDNA places YFP under control of the promoter (p5RT70), producing fluorescent knockout parasites. (f,g) Immunofluorescence (f) and FACS analysis (g) of Killer Red and YFP expression in RH DiCre transfected with loxP-KillerRed-loxP-YFP analyzed 5 d after induction with 50 nM rapamycin for 8 h (10,000 events were analyzed by FACS). Scale bars, 20 µm.

using analytical PCR on genomic DNA (Supplementary Figs. 2–5). The parasite strains containing loxP-flanked versions of the GOI (strains named loxPMyoA, loxPMic2 and loxPAct1) did not exhibit any detectable differences in the phenotype compared to wild-type parasites (Fig. 2a,b and Supplementary Figs. 2–5). In contrast, activation of Cre recombinase with 50 nM rapamycin for 4–8 h resulted in a mixed population consisting of uninduced and knockout parasites for all three lines (Fig. 2a and Supplementary Figs. 2–5). The excision efficiency was dependent on the strain: loxPMyoA, ~75% efficiency; loxPMic2, ~40%; and loxPAct1, ~20% when analyzed 24–36 h after induction. Given that knockout parasites express YFP, it is straightforward

Figure 2 | Using the DiCre system to dissect the invasion machinery. (a) Current model of the apicomplexan gliding and invasion machinery (top left) and summary of knockout parasites generated (bottom left). Immunofluorescence analysis of the indicated knockout (KO) strains using the indicated antibodies. PM, plasma membrane; MLC1, myosin light chain 1; GAP40, GAP45 and GAP50, gliding associated proteins; IMC, inner membrane complex; Rec., Recombination. Scale bars, 5 µm (top, middle) and 10 µm (bottom). (b) Plaque formation on a human foreskin fibroblast (HFF) monolayer by indicated parasite strains. Area of 15 plaques was assessed. **P < 0.0001 in a two-tailed Student’s t-test. (c) Immunofluorescence analysis of myoA KO parasites using the indicated antibodies. Scale bars, 5 µm. (d) Immunofluorescence analysis of indicated parasites using the indicated antibodies. Merge is between the respective antibody, DAPI and YFP signals. Scale bars, 5 µm. (e) Immunofluorescence analysis of act1 KO. LoxPAct1 parasites were treated with rapamycin for 4 h, inoculated on HFF cells and imaged after 24 h, 48 h and 72 h using the indicated antibodies. Parasites were artificially released after 72 h, reinculated on HFF cells and fixed after 24 h (72 h + 24 h). The apicoplast was stained with HSP60 antibody. Scale bar, 10 µm.
to perform phenotypic characterizations of the respective conditional knockout or to enrich this population using FACS (Supplementary Fig. 5 and data not shown).

Although we verified that the conditional myoA knockout had similar egress, gliding motility and host-cell invasion phenotypes to those previously described for a myoA knockout mutant (Supplementary Fig. 2), we isolated a clonal myoA knockout parasite line that can be permanently maintained in culture. Similarly, we isolated a clonal mic2 knockout parasite line, demonstrating to our knowledge for the first time that neither myoA nor mic2 are essential for parasite survival. This suggests the existence of a MIC2- and MyoA-independent invasion mechanism (Fig. 2a,b).

For both clonal knockout lines, we verified the absence of the respective genes using analytical PCR on genomic DNA and a lack of protein using immunoblots and immunofluorescence assays (Fig. 2a and Supplementary Figs. 3 and 4). In a growth assay of myoA and mic2 parasites, both knockout strains showed a substantial growth defect that was more pronounced for myoA knockouts than for control parasites (Fig. 2a,b). The reintroduction of myoA led to full complementation of myoA knockout parasites, demonstrating that the phenotype is specific (Supplementary Fig. 3). Notably, MyoA deletion did not affect secretory organelles or the localization of other glideosome components, such as the myosin light chain 1 or the gliding-associated protein 45 (Fig. 2c).

Immunofluorescence analysis of mic2 knockout parasites exhibited the absence of MIC2 (Fig. 2a), whereas other micronemal proteins AMA1 and MIC3 remained unaffected and exhibited normal localizations (Fig. 2d and Supplementary Fig. 4). As described previously for a mic2 knockdown, the processed and unprocessed forms of the MIC2 interaction partner M2AP were severely mislocalized, appearing as puncta in the parasite and in the parasitophorous vacuole (Fig. 2d and Supplementary Fig. 4).

In contrast to our success with MyoA and MIC2, we could not isolate a clonal act1 knockout line, demonstrating that actin is essential for parasite survival. We never observed plaques formed by YFP-positive act1 knockout parasites upon induction of the loxPAct1 strain (Fig. 2a and Supplementary Fig. 5). An immunofluorescence time course of YFP parasites in the induced loxPAct1 population established that Act1 protein levels were substantially decreased as early as 24 h after act1 excision, indicating host-cell invasion in a typical delayed death phenotype.

The DiCre system enables the efficient generation of conditional knockout mutants with a clear and interpretable phenotype, as background expression of the GOI can be excluded. Using three knockout examples of genes previously described as essential for host-cell invasion, we demonstrated that the parasite can use other invasion mechanisms. We found MyoA and MIC2 to be dispensable for host-cell invasion (Supplementary Discussion). Although it is possible that a few remaining undetected actin molecules could enable invasion, as we observed a substantial effect on apicoplast replication as early as 24 h after act1 excision, we consider this unlikely. Instead, we favor the interpretation that T. gondii can use an actin-MyoA-MIC2-independent mechanism to invade the host cell. Future analysis will elucidate the function of these invasion factors, previously described as essential and help to discover the minimal invasion machinery.

We have adapted the DiCre system for the malarial parasite Plasmodium falciparum (C.R. Collins, E.H. Wong, N.A., S. Mueller, M.M. and M.J. Blackman; unpublished data), demonstrating the potential of this technology to enhance all apicomplexan biological research.

METHODS

Methods and any associated references are available in the online version of the paper.

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COMPEETION FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Cloning of DNA constructs. All primers used in this study are listed in Supplementary Table 1. The FKB-Cre59 open reading frame (ORF) consists of NLS, FKBP12, linker and Cre1-59; FRB-Cre60 ORF consists of NLS, FRB T2B98L, linker and Cre60–343 (ref. 9). The respective ORFs were amplified using the primers FRB-Cre60/FKB-Cre59 forward (fw) and FRB-Cre60 reverse (rv) or FRB-Cre60/FKB-Cre59 fw and FKB-Cre59 rv and cloned into the plasmid p5RT70mycGFPpMyoAAtt-Ty-HX (ref. 15) using EcoRI and PacI restriction sites (p5RT70FKBPCre59-HX; p5RT70FBRCre60-HX). In p5RT70FKBPCre59-HX, hsgprt was exchanged for dhfr derived from pDHFR-Tsc3 (ref. 16) via SacI (p5RT70FKBPCre59-DHFR). To generate a plasmid for integration of both Cre recombinase subunits into the same genomic locus, FKB-Cre59 was amplified with the primers p5RT70-FKB-Cre59 fw and rv using p5RT70FKBPCre59-DHFR as template. The resulting p5RT70FKBPCre59 fragment was cloned into p5RT70FBRRCre60-HX using KpnI (p5RT70DiCre-HX). Finally, the hsgprt resistance gene was exchanged for dhfr via SacI. The resulting vector is referred to as p5RT70DiCre-DHFR.

To generate ploxPyoMyoAloxO-DHFR, the expression cassette p5RT70TyMyoA, which amplifies expression of N-terminal Ty-tagged MyoA, was placed between the two LoxP sites of plox-P30/11LacZ-Lox-CAT (ref. 17) using Clal and PacI. The chloramphenicol acetyltransferase (cat) selection cassette on this vector was exchanged for dhfr from pDHFR-Tsc3 using NotI and XbaI, resulting in the construct p5RT70loxPyoMyoAloxO-DHFR.

A 5′ UTR–DiCre–ku80 3′ UTR cassette was constructed to replace the hox ORF by dicre in ku80::hx strain18. The 5′ UTR and the 3′ UTR of ku80 locus were amplified using ku80 5′ UTR fw/rv or ku80 3′ UTR fw/rv primer pairs, respectively. Afterward, the amplified fragments were cloned into p5RT70ddmycGFPpMyoAAtt-Ty-HX15 using KpnI–SpeI and SpeI–SacI restriction sites. Finally, the DiCre expression construct from p5RT70DiCre-DHFR was placed between the 5′ UTR and 3′ UTR of ku80 using SpeI.

The gene swap vector p5RT70loxPKillerRedloxPYFP-HX (Supplementary Note) was generated by cloning KillerRed10 into p5RT70mycGFPpMyoAAtt-Ty-HX15. To introduce a loxP site upstream of KillerRed, the promoter region was amplified using p5RT70 fw and rv primers. The amplified promoter fragment was placed upstream of KillerRed using KpnI and Apal. To introduce a loxP site and YFP downstream of the KillerRed STOP codon, YFP was amplified from p5RT70ddYFP-CAT1 using loxPYFP fw and rv primers. The fragment was then placed downstream of KillerRed using PacI and NotI.

To generate loxPMic2loxP-YFP-HX, the parental vector p5RT70loxPKillerRedloxPYFP-HX was modified such that the mic2 3′ UTR was amplified from genomic DNA using the primer pair 3′ UTR Mic2 fw/rv, and the PCR fragment was cloned into p5RT70loxPKillerRedloxPYFP-HX via SacI. The mic2 ORF (TMGE49_201780) was amplified from cDNA using the primers Mic2 ORF fw/rv and was cloned into the parental vector p5RT70loxPKillerRedloxPYFP-HX using EcoRI and PacI. Finally, the mic2 5′ UTR containing the endogenous promoter was amplified from genomic DNA using the primer pair 5′ UTR Mic2 fw/rv and cloned into the final vector using Apal and EcoRI.

The loxPact1loxP-YFP-HX construct was generated using the strategy described for loxPMic2loxP-YFP-HX with minor alterations. First, the act1 ORF was amplified from cDNA using the primers Act1 ORF fw/rv, and then the resulting PCR product was cloned into the parental vector p5RT70loxPKillerRedloxPYFP-HX using EcoRI and PacI. To put act1 under the control of the endogenous promoter, a 2-kb fragment upstream of the start codon of act1 was amplified from genomic DNA using the oligos 3′ UTR Act1 fw/rv and cloned into the parental vector using EcoRI and PacI. Finally, the act1 3′ UTR was amplified from genomic DNA using the primer pair 3′ UTR Act1 fw/rv and cloned into the final vector using Apal and EcoRI.

Generation of parasite lines. For the LacZ reporter strain, pLox-P30/11LacZ-Lox-Tub5CAT17 was inserted randomly into RH hsgprt1 genome, and positive clones were selected on the basis of chloramphenicol resistance. Subsequently, p5RT70FKBPCre59-DHFR (30 µg DNA) and p5RT70FRBRCre60-HX (60 µg DNA) were cotransfected and selected using pyrimethamine, resulting in the strain RH hsgprt/loxPlacZloxP/FKBPCre59/FRBCre60 (referred to here as BloxP).

To generate a DiCre recipient strain expressing both subunits in the same genomic locus, p5RT70DiCre-HX was transfected into RH hsgprt– (RH hsgprt/diCre, referred to here as RH DiCre). Expression of Cre recombinase subunits was confirmed by western blot analysis with antibodies to FKBP12 (Thermo Scientific, 1:500 dilution) and FRB (Enzo Life Sciences, 1:1,000).

The ku80::dicre recipient strain was generated by replacing HX with dicre in the ku80::HX strain by homologous recombination (ku80::HX::dicre, referred to here as ku80::dicre). The 5′ UTR–DiCre–ku80 3′ UTR cassette was transfected into ku80::HX strain, and subsequently ku80::dicre parasites were selected using 6-thioxanthine to remove HX. Integration of dicre into the ku80 locus was confirmed by analytical PCR on genomic DNA using ku80::HX fw (1) and ku80 rv (1) primer pair to check for the presence of hX in the ku80 locus.

The conditional myoA knockout (KO) strain (RH DiCre/myoA::cat/loxPyoMyoAloxP, referred to here as loxPMyoA) was generated in three steps. Initially, a loxP-flanked transgenic copy of myoA was randomly integrated into the RH DiCre recipient strain. To this end, the plasmid p5RT70loxPTyMyoAloxP-DHFR, which confers pyrimethamine resistance, was transfected. Then, the endogenous copy of myoA was replaced with the cat selection cassette by double homologous recombination using pTGMyoA-KO-CAT3.

The resulting loxPMyoA strain carries only one copy of myoA, which was confirmed by PCR with the primers MyoA fw and rv that amplify DNA between two exons of myoA. Additionally, western blotting using anti-Ty and anti-MyoA antibodies was performed. The loxP-flanked myoA can be excised by adding rapamycin (50 nM for 8 h), resulting in the myoA null mutant (RH DiCre/myoA::cat/tymyoA–, referred to here as myoA KO). Confirmation of the site-specific recombination leading to the excision of myoA was confirmed by PCR using the following primer sets: MyoA fw (1) and MyoA rv (1′) and UpstreamLoxP fw (2) and DownstreamLoxP rv (2′). All assays were performed 96 h after induction of Cre-mediated recombination. Reintroduction of the expression vector p5RT70loxPTyMyoAloxP-DHFR was achieved in absence of drug selection to complement the loss of myoA, resulting in the parasite line RH DiCre/myoA::cat/tymyoA–/loxPyoMyoAloxP (referred to here as loxPMyoA comp).
The conditional mic2 knockout strain (ku80::diCre/endogenous mic2::loxpNic2loxp, referred to here as loxPMic2) was generated by transfecting 60 μg of the plasmid loxPMic2loxpPYFP-HX into the ku80::diCre parasites to replace the endogenous copy of mic2, and parasites containing stable integration of this construct were selected using xanthine (XAN) and mycophenolic acid (MPA) as previously described. The resulting loxPMic2 strain carries only one copy of mic2, which can be excised by adding rapamycin (50 nM in DMSO for 4 h before washout) to generate the mic2 null mutant (ku80::diCre/mic2′, referred to here as mic2 KO). The clonal mic2 KO line was isolated by performing serial dilutions on the clonal induced loxPMic2 strain after 4-h induction and subsequent removal of rapamycin. Analytical PCR on genomic DNA using the primers intMic2ORF fw (1) and intMic2ORF rv (1′) established the presence or absence of the mic2 ORF. PCR using primers Mic2 ORF (2) and Mic2 vector 5′ rv (2′) confirmed the 5′ UTR integration in both the loxPMic2 and mic2 KO parasites and Cre recombinase-mediated recombination in the mic2 KO parasites. The 3′ UTR integration was checked using the primers HX fw (3) and Mic2 3′ UTR rv (3′). Additionally, western blotting using anti-MIC2 and anti-aldolase antibodies was performed.

The conditional act1 KO strain (ku80::diCre/endogenous act1::loxPAct1loxP, referred to here as loxPAct1) was generated by transfecting 50 μg of the plasmid loxPAct1loxPYFP-HX into the ku80::diCre parasites to replace the endogenous copy of act1. After transfection, parasites were selected for stable integration using XAN and MPA as described previously. After the selection process, the parasite pool was serially diluted to isolate single clones. The resulting loxPAct1 strain carries only the Cre recombinase–inducible copy of act1, allowing excision of act1 upon rapamycin addition (50 nM in DMSO for 2–4 h before washout) to generate the act1 null mutant (ku80::diCre/act1′, referred to here as act1 KO). To confirm that the endogenous act1 genomic DNA was replaced by the introduced act1 cDNA, analytical PCR was performed using the primers Act1 ORF fw (1) and Act1 ORF rv (1′). Confirmation of 5′ UTR integration and site-specific recombination leading to the excision of act1 was achieved by PCR using the oligos Act1 5′ UTR fw (2) and YFP rv. The 3′ UTR integration into the correct locus was analyzed using the primers HX fw (3) and Act1 3′ UTR rv (3′).

Parasite lines, transfections and selection. T. gondii tachyzoites (RH hxgprt+) were cultured in human foreskin fibroblast (HFF) cells and maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FCS, 2 mM glutamine and 25 μg ml−1 gentamicin. To generate stable transformants, 1 × 106 of freshly lysed RH hxgprt+ parasites were transfected with 60 μg linearized DNA by electroporation. Selection on the basis of chloramphenicol (1 μM in EtOH)20 pyrtrimethamine (1 μM in EtOH)13, mycophenolic acid (12.5 mg ml−1 in MeOH), xanthine (20 mg ml−1 in 1 M KOH)19 and 6-thioxanthine (25 mg ml−1 in 0.3 M NaOH) resistance were performed as described earlier.

PCR, immunoblotting and immunofluorescence analysis. To extract genomic DNA from T. gondii to use as a PCR template, we pelleted parasites and swashed them in PBS. DNA extraction was performed using Qiagen DNEasy blood and tissue kit, according to manufacturer’s protocol. Western blot samples were obtained by spinning down extracellular parasites and incubating them with RIPA buffer (50 mM Tris-HCl, pH 8; 150 mM NaCl; 1% Triton X-100; 0.5% sodium deoxycholate; 0.1% SDS; 1 mM EDTA) for 20 min on ice to lyse the parasites. Afterward, samples were centrifuged for 30 min at 14,000 rpm at 4 °C, and Laemmli buffer (63 mM Tris HCl; 10% glycerol; 2% SDS; 0.0025% Bromophenol Blue; pH 6.8) was added to the supernatant. Unless indicated otherwise, 106 parasites were loaded onto an SDS acrylamide gel and immunoblotting was performed as described previously. For immunofluorescence analysis, infected HFF monolayers grown on coverslips were fixed in 4% paraformaldehyde for 20 min at room temperature; they were then blocked and permeabilized (2% BSA and 0.2% Triton X-100 in PBS). The staining was performed using different combinations of primary antibodies for 1 h, then secondary Alexa Fluor 488–, Alexa Fluor 350– or Alexa Fluor 594–conjugated antibodies for another 45 min (1:1,000–1:3,000, Invitrogen Molecular Probes).

Equipment and settings. For image acquisition, z stacks of 2-μm increments were collected using a UPLSAPO 100× oil (1.40 NA) objective on a DeltaVision Core microscope (Image Solutions, Applied Precision, GE) attached to a CoolSNAP HQ2 CCD camera. Deconvolution was performed using SoftWoRx Suite 2.0 (Applied Precision, GE) and further processed using ImageJ and Adobe Photoshop. Image acquisition was also conducted using a 100× oil objective on a Zeiss Axioscope 2 MOT+ microscope attached to a Hamamatsu Orca-ER digital CCD camera using OpenLab 5.5.2 software (Improvision) and further processed using ImageJ and Adobe Photoshop.

β-galactosidase analysis. To determine the efficiency of DiCre, BlocP parasites were transfected with 50 nM rapamycin or 1 μM Shield-1 for 6 h and grown under normal growth conditions for 48 h. Freshly lysed parasites were then added onto a monolayer of HFF and fixed 20 h later. The presence of β-galactosidase was assessed by adding X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) as substrate. Intracellular parasites were then fixed in 4% paraformaldehyde for 20 min and subsequently washed three times in PBS. Afterward, the samples were incubated with the staining solution for 30 min at 37 °C. The staining solution contains 6 mM potassium ferricyanide, 6 mM potassium ferrocyanide, 2 mM MgCl2, 0.2% Igepal, 0.1% Na deoxycholate, 1:40 dilution of X-gal solution (40 mg ml−1 in N,N-dimethylformamide) in PBS. Afterward, the samples were rinsed 5 times in PBS. The experiment was performed in triplicate, and 200 vacuoles were counted using a 100× objective.

Invasion assay. To investigate the invasion rates of the loxPMyoA parasites (uninduced and induced), parasites were treated with 50 nM rapamycin in DMSO for 8 h and grown in HFF cell monolayers for 96 h. Afterward, uninduced and induced parasites were scratched and filtered, and 1 × 105 parasites were inoculated onto HFF monolayers per well. Parasites were allowed to invade for 1 hr at normal growth conditions (37 °C, 5% CO2), extracellular parasites were washed away with PBS and parasites were incubated for another 24 h. Cells were fixed in 4% paraformaldehyde for 20 min, then immunostained with anti-IMC1 and anti-Ty.
antibodies. The number of invaded parasites was counted in 20 fields using a 100× objective and calculated as a percentage value of uninduced loxPMyoA parasites normalized to 100%.

**Egress assay.** We grew $4 \times 10^5$ parasites (loxPMyoA uninduced and induced 96 h earlier) in HFF monolayers on coverslips for 36 h. Medium was exchanged for pre-warmed, serum-free DMEM supplemented with 2-µM A23187 (in DMSO) to artificially induce egress. After 10 min, cells were fixed and stained with antibodies as indicated. Two hundred vacuoles were counted for each condition in three assays.

**Plaque assay.** We added 1,000 freshly lysed parasites to a confluent monolayer of HFF cells and incubated them for 5 d. The HFF monolayer was washed in PBS and fixed in ice-cold methanol for 20 min. Afterward, the HFFs were stained with Giemsa (1:10 in water) for 45 min and finally washed in PBS (3×, 5 min each). As methanol destroys the YFP signal, the actI KO plaque assays were imaged without fixation to differentiate between YFP-positive and YFP-negative parasites. To quantify the relative plaque size of the indicated strains after 5 d on HFF monolayers, the average size of 15 plaques was determined and displayed relative to ku80::dicre or RH hgxprt−± s.d. Plaque area was assessed using ImageJ 1.34r software.

**FACS analysis.** Freshly lysed parasites (mixed population expressing p5RT70loxPKillerRedloxPYFP-HX) were pelleted and washed once in PBS. Parasites were resuspended in 1 ml of 4% paraformaldehyde and incubated in the dark for 20 min. The suspension was then centrifuged and washed again in PBS. Finally, FACS buffer (1% FCS in PBS supplemented by 1 mM EDTA) was added to achieve a final concentration of $1 \times 10^6$ parasites ml$^{-1}$. We counted 10,000 events using FACSCalibur Becton Dickinson LSRII. FSC and SSC were detected as small- and large-angle scatter of the 488-nm laser. KillerRed was excited by the 561-nm laser and detected by a 585/15 band pass filter. The YFP was excited by the 488-nm laser and detected by a 530/30 band pass filter and 505-nm long pass filters. For quantification, total number of fluorescent parasites was considered 100%.

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