LETTER

Genetic modification of the diarrhoeal pathogen Cryptosporidium parvum

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Recent studies into the global causes of severe diarrhoea in young children have identified the protozoan parasite Cryptosporidium as the second most important diarrhoeal pathogen after rotavirus1–3. Diarrhoeal disease is estimated to be responsible for 10.5% of overall child mortality4. Cryptosporidium is also an opportunistic pathogen in the contexts of human immunodeficiency virus (HIV)-caused AIDS and organ transplantation5,6. There is no vaccine and only a single approved drug that provides no benefit for those in gravest danger: malnourished children and immunocompromised patients7,8. Although there is no tissue culture system for continuous passage, Cryptosporidium development can be observed for 2–3 days by infecting human ileocecal adenocarcinoma cells (HCT-8)9. To achieve transfection, sporozoites were excysted from oocysts purified from the faeces of experimentally infected calves using a protocol that mimics stomach and intestinal passage11, and then electroporated before infection of HCT-8 cells (Fig. 1a). The transfection plasmids used here flanked a variety of reporter genes with candidate C. parvum 5’ and 3’ regulatory sequences derived from highly expressed housekeeping genes. We observed significant reporter activity 48 h after transfection using plasmids carrying nanoluciferase (Nluc; Fig. 1b), a small ATP-independent enzyme from deep sea shrimp12, but not firefly luciferase or fluorescent proteins. Nluc luminescence correlated with the number of parasites and the amount of DNA used for transfection. Luminescence was also shown to require the presence of parasite-specific promoter elements and the introduction of DNA into parasites and not host cells (Fig. 1).

Figure 1 | Transfection of C. parvum. a, Schematic overview. C. parvum sporozoites were prepared from oocysts purified from infected calves and electroporated in the presence of plasmid DNA before infection of HCT-8 cells (Eno, flanking sequence from the C. parvum enolase gene). b–j, Luminescence measurements (the means of three technical replicates, standard deviation (s.d.) shown as error bars) of C. parvum (b–e, h–j, blue), T. gondii (f), or human HCT-8 cells (g) transfected with Nluc expression plasmids. b–d, C. parvum transfection requires electroporation (b) of DNA (c) into parasites (d). e, f, h, Transfection also requires plasmids to carry parasite-specific promoter sequences (e, f; testing C. parvum (Cp) and T. gondii (Tg) promoters in both parasites), and is susceptible to the Cryptosporidium drug nitazoxanide (h). g, Lipofection of HCT-8 cells with the original Nluc plasmid pNL1.1 (Promega), but not derived parasite vectors, results in luciferase activity in the host alone. Choice of promoter (f; enolase (Eno), aldolase (Aldo), 5-tubulin 5’ (Tub) (the 3’ untranslated region (UTR) was uniformly from the enolase gene)) or codon composition (j; Nluc optimized to 35% GC (oNluc)) influences expression level in C. parvum. Note automatic gain adjustment of luminescence measurements; units are not comparable between panels. Independent biological experiments were repeated three times, and representative data are shown.

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and shows strong codon bias. We also noted a preference for A over T in the enolase promoter to be strongest. The flanking sequences from different parasites were found to have different effects. Translational fusions were constructed placing Neo at the amino or carboxy terminus of Nluc. Nluc–Neo shows luciferase activity, albeit at a reduced level when compared to Nluc alone. C. parvum transfected with Nluc (blue) or Nluc–Neo (red) were grown in different concentrations of paromomycin. Luciferase activity for each plasmid was normalized to its drug-free level. d. CRISPR/Cas9 plasmid for C. parvum. Flag, epitope tag; nls, nuclear localization signal; ribo, ribosomal protein L13A 3."}

Furthermore, reporter signal was ablated by the anti-parasitic drug nitazoxanide. Transient transfection of C. parvum sporozoites and grown for 2 days in the presence of paromomycin. b. Translational fusions were constructed placing Neo at the amino or carboxy terminus of Nluc. Nluc–Neo shows luciferase activity, albeit at a reduced level when compared to Nluc alone. c. C. parvum transfected with Nluc (blue) or Nluc–Neo (red) were grown in different concentrations of paromomycin. Luciferase activity for each plasmid was normalized to its drug-free level. d. CRISPR/Cas9 plasmid for C. parvum. Flag, epitope tag; nls, nuclear localization signal; ribo, ribosomal protein L13A 3.}

In an effort to enhance efficiency we evaluated different electroporation devices, electrical wave programs and buffer compositions. Upon reinfection, parasites show strong drug resistance (e) and luciferase activity (f). In repeat experiments we noted that luciferase is detectable as early as 6 days after transfection in the faeces of the first infected mouse (Extended Data Fig. 2). e. Protein extracts from oocysts were analysed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and western blot using an antibody against Neo (rabbit anti-neomycin phosphotransferase II; EMD Millipore). Predicted molecular mass of the Nluc–Neo fusion protein is 54.1 kDa. f. Immunofluorescence staining using anti-Neo (mouse anti-Neo; Jackson ImmunoResearch) and treated as indicated. Emergence of paromomycin resistance required the small intestine by surgery (Extended Data Fig. 2) and mice were treated with paromomycin. Oocysts were purified from the faeces and used to infect cultures or mice by oral gavage. b. Quantitative PCR of C. parvum DNA isolated from faeces of mice infected with transfected sporozoites (four mice per group) and treated as indicated. Emergence of paromomycin resistance required the small intestine by surgery (Extended Data Fig. 2) and mice were treated with paromomycin. Oocysts were purified from the faeces and used to infect cultures or mice by oral gavage. b. Quantitative PCR of C. parvum DNA isolated from faeces of mice infected with transfected sporozoites (four mice per group) and treated as indicated. a. Outline of the selection strategy. Transfected sporozoites were injected into the small intestine by surgery (Extended Data Fig. 2) and mice were treated with paromomycin. Oocysts were purified from the faeces and used to infect cultures or mice by oral gavage. b. Quantitative PCR of C. parvum DNA isolated from faeces of mice infected with transfected sporozoites (four mice per group) and treated as indicated. Emergence of paromomycin resistance required the small intestine by surgery (Extended Data Fig. 2) and mice were treated with paromomycin. Oocysts were purified from the faeces and used to infect cultures or mice by oral gavage. b. Quantitative PCR of C. parvum DNA isolated from faeces of mice infected with transfected sporozoites (four mice per group) and treated as indicated. Emergence of paromomycin resistance required the small intestine by surgery (Extended Data Fig. 2) and mice were treated with paromomycin. Oocysts were purified from the faeces and used to infect cultures or mice by oral gavage. b. Quantitative PCR of C. parvum DNA isolated from faeces of mice infected with transfected sporozoites (four mice per group) and treated as indicated. Emergence of paromomycin resistance required the small intestine by surgery (Extended Data Fig. 2) and mice were treated with paromomycin. Oocysts were purified from the faeces and used to infect cultures or mice by oral gavage. b. Quantitative PCR of C. parvum DNA isolated from faeces of mice infected with transfected sporozoites (four mice per group) and treated as indicated.
We thus constructed translational fusions between the 
Nluc reporter resistance in culture is complicated by the lack of continuous growth. 

When *C. parvum* treatment and renders the repaired gene resistant to further Cas9 cutting. 

To build a *C. parvum* CRISPR/Cas9 system, we constructed a plasmid in which the *C. parvum* U6 RNA promoter drives a guide RNA cassette and the *Streptococcus pyogenes* Cas9 is flanked by parasite regulatory sequences (Fig. 2d). To test this system, we conducted a Cas9-dependent DNA repair experiment (Fig. 2e–g). We introduced a stop codon into the Nluc reporter that ablated luciferase activity (Dead Nluc). We then targeted the dead gene with a guide RNA, and provided a short double-stranded template for repair that restores read-through translation and renders the repaired gene resistant to further Cas9 cutting. When *C. parvum* sporozoites are co-transfected with a specific guide, luciferase activity is restored (*P* = 0.0006, unpaired *t*-test). No change is observed with no or off-target guides.

Interferon-γ knockout mice are susceptible to *C. parvum* infection through oral inoculation of oocysts. However, infection with free sporozoites is less effective, probably due to stomach passage. We developed a surgical protocol to inject transfected sporozoites directly into the small intestine to maximize infection (Extended Data Fig. 2). When mice were killed 24 h after infection, luciferase activity was observed in scrapings of the intestinal epithelium. We also established an effective treatment protocol using paromomycin supplementation of the drinking water (Extended Data Fig. 3).

Next, we infected mice by surgery with transfected sporozoites and treated them with paromomycin as indicated (Fig. 3 and Extended Data Fig. 4; four mice per group). Faeces were collected every 3 days and oocyst shedding was measured by quantitative polymerase chain reaction (PCR) targeting the *C. parvum* 18S ribosomal RNA locus. Mice infected with parasites transfected with the Nluc–Neo plasmid that did not receive drug shed high numbers of oocysts and remained infected for the 30 days observed (Fig. 3b, blue). Those infected with parasites that received the Nluc plasmid (lacking the Neo gene; Fig. 3b, green) were rapidly cured by drug treatment. Those transfected with Nluc–Neo alone and drug treated were also cured (infection may persist slightly longer). In contrast, infection with parasites carrying the Nluc–Neo plasmid and the Cas9 plasmid (Fig. 3b, red; Cas9 target detailed later) rapidly rebounded to levels similar to untreated mice. Oocysts emerging from selection were purified from faeces and used to infect mice that were again treated with paromomycin; wild-type oocysts were used in parallel (100,000 oocysts per mouse by gavage). While paromomycin treatment cured infection with wild-type parasites, transgenic parasites showed immediate robust drug resistance (Fig. 3c). When these oocysts were probed by western blot with anti-Neo antibody, we detected a band consistent with an Nluc–Neo fusion protein.

Purified oocysts were also used to infect cell cultures, and processed for immunofluorescence after 2 days. Transgenic but not wild-type intracellular parasite stages showed fluorescence when probed with antibodies specific for either Neo or Nluc (Fig. 3f and data not shown). These cultures also displayed strong luciferase activity not observed in wild type. This activity exceeded that previously observed in transient transfection experiments by five orders of magnitude on a per-cell basis. We assessed whether these organisms could be suitable for drug-screening assays by infecting 96-well plates with 1,000 oocysts per well and measured luciferase after 48 h. Infected wells were clearly distinguishable from uninfected wells (Δ*F* > 0.6; *n* = 20). Similarly, wells treated with nitazoxanide showed significant growth inhibition (*P* = 0.0036, unpaired *t*-test). Luciferase also provided a convenient way to assess the infection state of animals. We sampled 10 mg of faeces from mice diagnosed in parallel by PCR and found this assay to be sensitive, specific and faster than PCR (Fig. 3d). We note that Nluc expression remains stable when parasites are propagated in mice in the absence of paromomycin (Extended Data Fig. 5).

*Cryptosporidium* is remarkably resistant to antifolates, a mainstay of treatment against other apicomplexans, and this resistance has been attributed to differences in the target enzyme dihydrofolate reductase-thymidylate synthase (DHFR-TS)⁹. However, *Cryptosporidium* is

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**Figure 4** | Targeted deletion of *C. parvum* TK. **a**, Owing to a horizontal gene transfer, *C. parvum* has two pathways to synthesize dTMP: TK and DHFR-TS. DHF, dihydrofolic acid; THF, tetrahydrofolic acid; dUMP, uridine monophosphate. **b**, Map of the *C. parvum* TK locus, the targeting plasmid and the predicted modified locus. Primers and ampiclon sizes of diagnostic PCR products are indicated (Ins, insertion). **c**, Primer analysis using genomic DNA from wild-type (WT) and transgenic parasites (Nluc–Neo, oocysts purified from faeces of infected mice shown in Fig. 3c; CDS, coding sequence). Primer sequences are provided in Supplementary Table 1. **d**, Quantification of EdU-labelling experiments (meronts with four or more nuclei were scored, two biological repeats, *n* = 105 each sample, error bars are s.d.). **e**, Representative fluorescence micrographs are shown. Antibody to *C. parvum* tryptophan synthase B was used to identify parasites (green). **f**, Trimethoprim treatment of wild-type (blue) and Nluc–Neo transgenic (red) parasites. Wild-type parasites were measured in transient transfection assays with Nluc plasmid (*n* = 3, technical replicates, error bars are s.d.). The assay shown was conducted in the presence of 10 μM thymidine to avoid indirect host cell toxicity⁹ (experiments without thymidine produced indistinguishable results). Experiments were repeated three times and representative data are shown.

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unique among apicomplexans in that it acquired a thymidine kinase (TK) by horizontal gene transfer from bacteria. We hypothesized that TK may also contribute to Cryptosporidium antifolate resistance by providing an alternative route to thymidine monophosphate (dTMP) to the disease, infection is rarely detected in older children. This is consistent with infection studies in people and animals suggesting the presence limits the efficacy of antifolate therapy in Cryptosporidium. We show that major hurdles towards genetic analysis and manipulation for cryptosporidiosis can be overcome by maximizing the efficiency of each step of the process and by focusing on in vivo propagation and selection. There is an urgent need for new anti-parasitic drugs. Cryptosporidium is not susceptible to drugs widely used against related pathogens, which reflects substantial differences in its metabolism and metabolite uptake. Luciferase reporter parasites enable phenotypic screening in culture and animals with sufficient sensitivity and specificity to warrant a comprehensive effort to discover novel compounds. Gene deletion now permits biological target validation. Genetic modification may also allow the construction of attenuated parasites as a potential oral vaccine. While infants and toddlers are highly susceptible to the disease, infection is rarely detected in older children. This is consistent with infection studies in people and animals suggesting the development of anti-parasitic and anti-disease immunity. A better understanding of the mechanisms underlying disease and protection will be required to design and produce such a vaccine.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Supplementary Information is available in the online version of the paper.

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Author Contributions S.V. developed the transfection and luciferase assay; M.C.P., A.S., C.F.B. and S.V. developed the transfection and luciferase assay; M.C.P., A.S. and C.F.B. provided bioinformatics support. S.V., M.C.P., A.S. and C.F.B. conducted animal experiments and genotypic and phenotypic characterization. S.V., M.C.P., A.S. and C.F.B. conceived the study and B.S. wrote the manuscript with contributions from S.V., M.P. and A.S.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare competing financial interests: details are available in the online version of the paper. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to B.S. (striepen@uga.edu).
METHODS

C. parvum reporter and drug resistance vectors. C. parvum transfection vectors were derived from plasmid pHeB920 modified and modified to contain C. parvum promoter and 5’ and 3’ untranslated messenger RNA regions. We mined the genome and a variety of expression data sets collectively available through CryptoDB (http://www.cryptodb.org)21 to identify genes that are highly expressed across the life cycle. Promoters and 5’ UTRs of the enolase (cgd5_1960), 3'-ubiquitin (cgd4_2860), and aldolase (cgd1_3020) genes and 3’ UTRs of enolase (51 bp), 3'-ubiquitin (97 bp) or ribosomal protein L13A (cgd5_970, UTR 211 bp) were amplified from genomic DNA by PCR (see Supplementary Table 1 for a list of primer sequences and restriction sites used). Nluc was amplified from pNLL1 (Promega Corporation), firefly luciferase and different fluorescent protein genes were amplified from vectors used for T. gondii22-24. The neomycin resistance gene was amplified from plasmid pNeo4 (ref. 15) (a gift from J. Gaertig, University of Georgia) and introduced 5’ or 3’ of Nluc in a plasmid with enolase regulatory sequences. To target the TK gene, regions flanking the gene were amplified and introduced into the Nluc-Neo vector (the promoter but not the 3’ UTR was retained).

C. parvum CRISPR/Cas9 genome editing. Human codon-optimized Streptococcus pyogenes Cas9 (hsSpCas9) carrying a Flag tag and N- and C-terminal nuclear localization signals was amplified from pX330 (ref. 35) and introduced into the Aldolase-Nluc-ribo vector replacing the Nluc. A guide RNA cassette was synthesized containing the C. parvum U6 promoter identified by genome searches using known structural RNA sequences from Plasmidium falciparum26, two inverted BbsI restriction sites to facilitate guide cloning, a trans-activating CRISPR RNA (tracrRNA) consensus sequence and a terminator (poly T) sequence, and was introduced into the Cas9 plasmid.

For transient transfection experiments, electroporated sporozoites suspended in 200 μl PBS containing sterile food colouring dye as tracer. After injection, suicide was performed to close the peritoneum. Mice were administered 0.01–0.02 ml per gram body weight of warm lactated Ringer’s solution subcutaneously after surgery. Meloxicam analgesic was also administered to the mice after surgery. At completion of the procedure, the eye ointment was wiped off and the vaporizer was turned off and the mice were allowed to breathe the oxygen supply gas until they began to wake. Mice were placed in a recovery area until ambulatory and exhibiting normal respiration and were watched for 2 h after surgery. Incision sites were monitored daily until fully healed (10-14 days). Twenty-four hours after surgical infection, water in mouse cages was replaced with distilled H2O containing 16 mg ml-1 paromomycin, a concentration we determined to deliver a daily dose of 40 mg kg-1 paromomycin to each mouse (Extended Data Fig. 3). Mice were randomly assigned to groups before surgery. A sample size of four animals per treatment group was judged to be sufficiently large enough to draw appropriate conclusions. All mice survived surgery and were included in the results reported here. Investigators were not blinded to group allocation during the experiments.

Mouse faeces collection and storage. Faecal samples were collected from mice (typically four mice per cage) starting 3 days after infection every third day for up to a month. Mice were transferred to a fresh, sterile cage for 2–3 h, and faeces from the cage were collected, pooled, and stored at 4 °C.

Luciferase assay. For transient transfection experiments, electroporated sporozoites were added to 70% confluent HCT-8 culture and infection was allowed to proceed at 37 °C for 48 h. Media was removed from wells and 200 μl of NanoGlo lysis buffer supplemented with NanoGlo substrate (1:50, Promega Corporation) was added to each well. Cells were scraped and the lysate was transferred to white 96-well plates and luminescence was measured using a Synergy H4 Hybrid Microplate Reader (BioTek Instrument, Inc.). For drug assays with purified sterile transgenic oocysts, the culture supernatant was collected after 48 h from 96-well plates. An equal amount of supernatant and NanoGlo lysis buffer with substrate was combined and luminescence was measured.

For luciferase measurement from mouse faecal samples, 20 mg of faeces was weighed into a 1.5-ml microcentrifuge tube and homogenized in 1 ml of lysis buffer (50 mM Tris-HCl, 10% glycerol, 1% Triton-X, 2 mM dithiothreitol (DTT), 2 mM EDTA) using 10–15 glass beads (3 mm) and a vortex mixer for 1 min, followed by clarification of lysate by brief centrifugation. One-hundred microlitres of lysate was mixed with an equal volume of NanoGlo Luciferase Buffer (prepared with 1:50 dilution of substrate) and luminescence was measured as described.

High-throughput imaging assay for parasite growth. For drug assays we used either luciferase activity or a 96-well infection and imaging protocol29 using a BD Pathway instrument. Parasites and host cells were quantified using an ImageJ macro adapted from ref. 39. The ratio of parasites to host nuclei was determined for each sample image and normalized to untreated controls.

For oocyst quantification by high-throughput microscopy, we weighed collected mouse faeces and diluted in PBS (5 μl mg-1). Samples were incubated at 95 °C for 10 min, vortexing every 2 min at high speed. Large debris was allowed to settle for 10 min, then 10 μl of the suspension were mixed with 990 μl PBS and 1 μl of fluoroscein isothiocyanate (FITC)-conjugated goat polyclonal anti-Cryptosporidium antibody (GeneTex). After 1 h at room temperature, the sample was centrifuged at 2,000g for 15 min. The pellet was suspended in 200 μl PBS and transferred to a 96-well plate for microscopy. Plates were imaged using BD Pathway and oocysts were counted.
Centrifuged for 3 min at 16,000 g of this preparation was overlaid onto 0.8 ml of 1.15 specific gravity CsCl, and collected from the supernatant and suspended in 0.85% saline solution. 0.5 ml Substrate (Thermo Pierce) and exposure to film. Equal loading of blots was confirmed or corrected as described. No animals were excluded from experimental measurements.

### Western blotting

For western blot analysis, oocysts from wild-type and transgenic Nluc–Neo parasites were excysted as described earlier and sporozoites were lysed in SDS sample buffer. Protein extract from 10^7 sporozoites was loaded per lane and subjected to electrophoresis on a precast Any kD Mini-PROTEAN TGX gel (Bio-Rad). Blots were blocked and probed with an anti-neomycin phosphotransferase II antibody (EMD Millipore) at 1:1,000 dilution followed by detection with ECL Western Blotting Reagents (Bio-Rad). Antibodies used were anti-mouse or anti-rabbit conjugated to Alexa488 or Alexa546 (Molecular Probes, Life Technologies) at a dilution of 1:1,000. DNA was visualized with DAPI (2 mg ml^-1). Images were collected on an Applied Precision Delta Vision inverted epifluorescence microscope at the UGA Biomedical Microscopy Core, deconvolved and adjusted for contrast using SoftWoRx software. Statistical methods. All bar graphs depict the mean with standard deviations shown as error bars. Unless indicated otherwise, graphed data represent three technical replicates; each experiment was repeated at least twice and representative data are shown. No statistical tests were used to predetermine sample size. Unpaired t-tests were used appropriately to determine statistical significance and a P value <0.05 was considered significant. Assumptions for statistical tests were confirmed or corrected as described. No animals were excluded from experimental measurements.

### Oocyst purification from mouse faeces

Oocysts were purified from faeces using sucrose suspension followed by a caesium chloride centrifugation step. Each sample was diluted in 10 ml water, 1 ml of eluate was used for qRT–PCR along with 10 ml primers targeting Cryptosporidium 18S rRNA and SYBR Master Mix (Life Technologies) for detection. Each qRT–PCR reaction was normalized using an eight-point standard curve (faecal DNA purified from uninfected mouse faeces spiked with known amounts of oocysts) for each set of samples.

### Statistical methods

All bar graphs depict the mean with standard deviations shown as error bars. Unless indicated otherwise, graphed data represent three technical replicates; each experiment was repeated at least twice and representative data are shown. No statistical tests were used to predetermine sample size. Unpaired t-tests were used appropriately to determine statistical significance and a P value <0.05 was considered significant. Assumptions for statistical tests were confirmed or corrected as described. No animals were excluded from experimental measurements.
Extended Data Figure 1 | Optimization of sporozoite transfection. a, Ten-million sporozoites prepared in either cytomix (BTX) or Lonza Buffers SE, SF or SG (4D Nucleofection) were combined with 10 μg DNA (Eno_Nluc-GS-Nluc_Eno). Samples were electroporated using previously determined settings for BTX (1,500 V, 25 Ω, 25 μF) or various program settings for 4D Nucleofection as indicated. Parasites were added to cultures of HCT-8 cells and luciferase activity was read after 48 h. Bars represent average of two technical replicates. b, Transfection was further optimized by comparing the best preliminary settings (buffers SF and SG; programs EH 100 and EO 100) with additional pulse programs as indicated. Transfection was carried out as in a. Bars represent average of two technical replicates. c, Electroporation systems (BTX and 4D Nucleofection) were compared using the same number of C. parvum sporozoites and quantities of DNA using buffers and conditions optimized in a and b. Bars represent average of three technical replicates. Note about tenfold enhancement of transient transfection using 4D Nucleofection. The impact of electroporation on stable transformation cannot be assessed in this setup and may be higher. Experiments in a and b were done once for the purpose of optimization, while c was repeated three times; a single representative experiment is shown.
Extended Data Figure 2 | Direct surgical injection of transfected *C. parvum* sporozoites into the small intestine. Mice are shaved and anaesthetized with isofluorane (3% initially, then maintained at 1.5% for the surgery). The abdominal skin is disinfected with Betadine and a small incision is made into the peritoneum. Forceps are used to grasp the small intestine and 100 μl of PBS containing $10^7$ transfected *C. parvum* sporozoites is injected into the lumen. The peritoneum and the abdominal skin are each sutured with 4-0 polydioxanone and mice are injected with meloxicam (1 mg kg$^{-1}$) subcutaneously. Each procedure takes around 15 min, and mice recover rapidly.
Extended Data Figure 3 | Optimization of paromomycin treatment of infected mice. a, Dosing of mice accounting for drug concentration, animal weight, and measured daily water consumption. At 16 mg ml\(^{-1}\) each mouse received 40 mg paromomycin daily (dotted line). b, This dose was found to be sufficient to decrease oocyst shedding in treated mice to background. By day 7 mice without paromomycin treatment shed large amounts of oocysts when compared to untreated mice. Treated mice showed no shedding above background. Oocysts were enumerated by high-throughput imaging assay. Five mice were analysed individually with two technical replicates.
Extended Data Figure 4 | Mouse model for selection of stable *C. parvum* transgenics. Repeat of the experiment described in Fig. 3b. **a**, Measurement of *C. parvum* infection using faecal PCR. **b**, Luminescence measurements. Note increasing luminescence from day 6 in parasites that received resistance and Cas9 plasmids. Mice were infected in groups of four per cage and pooled faeces was analysed for each cage (each measurement represents three technical replicates).
Extended Data Figure 5 | *C. parvum* maintains the stable transgene when passed serially in mice without paromomycin treatment. a, Mice were infected orally with 100,000 transgenic oocysts. b, c, Infected mice were then treated with paromomycin (b) or left untreated (c). Oocysts were purified from faecal collections by sucrose flotation and CsCl centrifugation, and used to infect a second cohort of mice. Again, each mouse received 100,000 transgenic oocysts and mice were treated or not. Faeces were tested for luminescence every 3 days. Each reading represents the pooled faecal sample from five mice with three technical replicates.