Supplemental methods

Generation of CRISPR/CAS9 plasmids.

Using RH genomic DNA as template and 5'pU6-SacI and 3'-gRNA-SacI as primers, the *T. gondii* U6 promoter (TgU6) was PCR amplified, SacI digested and ligated into SacI digested pSAG1-Ble-SAG1 vector. The 3'-gRNA-SacI primer was designed to contain the *UPRT* targeting guide RNA and scaffold sequences such that the resulting plasmid (pSAG1-U6::sgUPRT) expressed a *UPRT* targeting single guide RNA (sgUPRT) driven by the U6 promoter. Subsequently, a PCR product containing CAS9-NLS-GFP amplified from pMJ920 (1) using the primers 5'-CAS9-NsiI and 3'-CAS9-PacI was digested with NsiI and PacI and cloned into pSAG1-U6::sgUPRT to generate the *UPRT* targeting CRISPR plasmid pSAG1::CAS9-U6::sgUPRT (Table S1).

All other CRISPR plasmids were generated by replacing the *UPRT* targeting gRNA in pSAG1::CAS9-U6::sgUPRT with specified gRNA sequences using Q5 DNA polymerase mutagenesis (New England Biolabs, Ipswich, MA). These Q5 mutagenesis reactions used a generic revise primer 3'-ROP18-gRNA and unique forward primers (Table S2) to specify the guide RNA sequences: 5'-ROP18-gRNA was used to make the *ROP18* targeting CRISPR plasmid pSAG1::CAS9-U6::sgROP18; 5'-UPRT-OT1 and 5'-UPRT-OT2 were used to generate the *UPRT* off-target CRISPR plasmids pSAG1::CAS9-U6::sgUPRT-OT1 and pSAG1::CAS9-U6::sgUPRT-OT2, respectively.

Generation of disruption and deletion plasmids for *UPRT*.

The plasmid pUPRT::DHFR-I used for inserting *DHFR* into the *UPRT* gene was constructed by Gateway cloning (Life Technology, Grand Island, NY). A 750 bp fragment directly upstream the gRNA target in the *UPRT* gene was PCR amplified using the primers 5'-B4-UpgRNA and 3'-B1r-UpgRNA and cloned into pDONR-P4P1r using a BP reaction to generate the entry vector pDONR-P4P1r-UpgRNA. Similarly, a 900 bp fragment downstream the gRNA target in the *UPRT* gene was PCR amplified using the primers 5'-B2r-DngRNA and 3'-B3-DngRNA and cloned into pDONR-P2rP3 to generate pDONR-P2rP3-DngRNA. The pyrimethamine resistance

cassette *DHFR* (DHFR*) was PCR amplified from pDHFR-TS (2) using the primers 5'-B1-DHFR and 3'-B2-DHFR and ligated into pDONR221 to give pDONR-DHFR. Subsequently, these three entry vectors were used in a LR ligation reaction with the destination vector pDEST-R4R3 to generate pUPRT::DHFR-I. The plasmid pUPRT::DHFR-D was also made by three-fragments Gateway cloning, using pDEST-R4R3 and the entry vectors (Table S1) that were generated as follows: a ~1.0 Kb fragment corresponding to the 5'-UTR of *UPRT* was PCR amplified using the primers attB4-UPRT 5' KO - forward and attB1r-UPRT 5' KO - reverse and cloned into pDONR-P4P1r to get pDONR-P4P1r-U5UPRT as previously described (3); the 3'-UTR (~1.0 Kb) of the *UPRT* gene was PCR amplified using the primers attB2r-UPRT 3' KO – forward and attB3 UPRT 3' KO - reverse and cloned into PDONR-P2rP3 to produce pDONR-P2rP3-U3UPRT (3); the third entry vector pDONR-DHFR was described above.

Generation of plasmids for deletion and complementation of *ROP18*.

To disrupt *ROP18* in GT-1, we designed a CRISPR plasmid (pSAG1::CAS9-U6::sgROP18) expressing a *ROP18* targeting sgRNA, as described above. Meanwhile we also generated a homology template (pROP18::DHFR) to promote insertion of *DHFR** into the *ROP18* locus. The plasmid pROP18::DHFR was made by Gateway cloning using the destination vector pDEST-R4R3 and the following entry vectors: pDONR-P4P1r-UpROP18 (Table S1) was generated by PCR amplification (using the primers 5'-B4-UpROP18 and 3'-B1r-UpROP18) of a 820 bp fragment upstream of the gRNA target in *ROP18* and subsequent BP cloning of this fragment into pDONR-P4P1r. pDONR-P2rP3-DnROP18 was made by PCR amplification (using the primers 5'-B2r-DnROP18 and 3'-B3-DnROP18) and BP cloning of a 850 bp fragment downstream the gRNA target in *ROP18* into pDONR-P2rP3. pDONR-DHFR that was described above. The orientation and nucleotide sequences of all inserted fragments were confirmed by Sanger sequencing (Genewiz Inc., South Plainfield, NJ). The plasmid used for *ROP18* complementation (pUPRT::IMC1-ROP18) was described previously (3).

References

1. **Jinek M, East A, Cheng A, Lin S, Ma E, Doudna J.** 2013. RNA-programmed genome editing in human cells. Elife **2:**e00471.
2. **Donald RG, Roos DS.** 1993. Stable molecular transformation of Toxoplasma gondii: a selectable dihydrofolate reductase-thymidylate synthase marker based on drug-resistance mutations in malaria. Proc Natl Acad Sci U S A **90:**11703-11707.
3. **Behnke MS, Fentress SJ, Mashayekhi M, Li LX, Taylor GA, Sibley LD.** 2012. The polymorphic pseudokinase ROP5 controls virulence in Toxoplasma gondii by regulating the active kinase ROP18. PLoS Pathog **8:**e1002992.