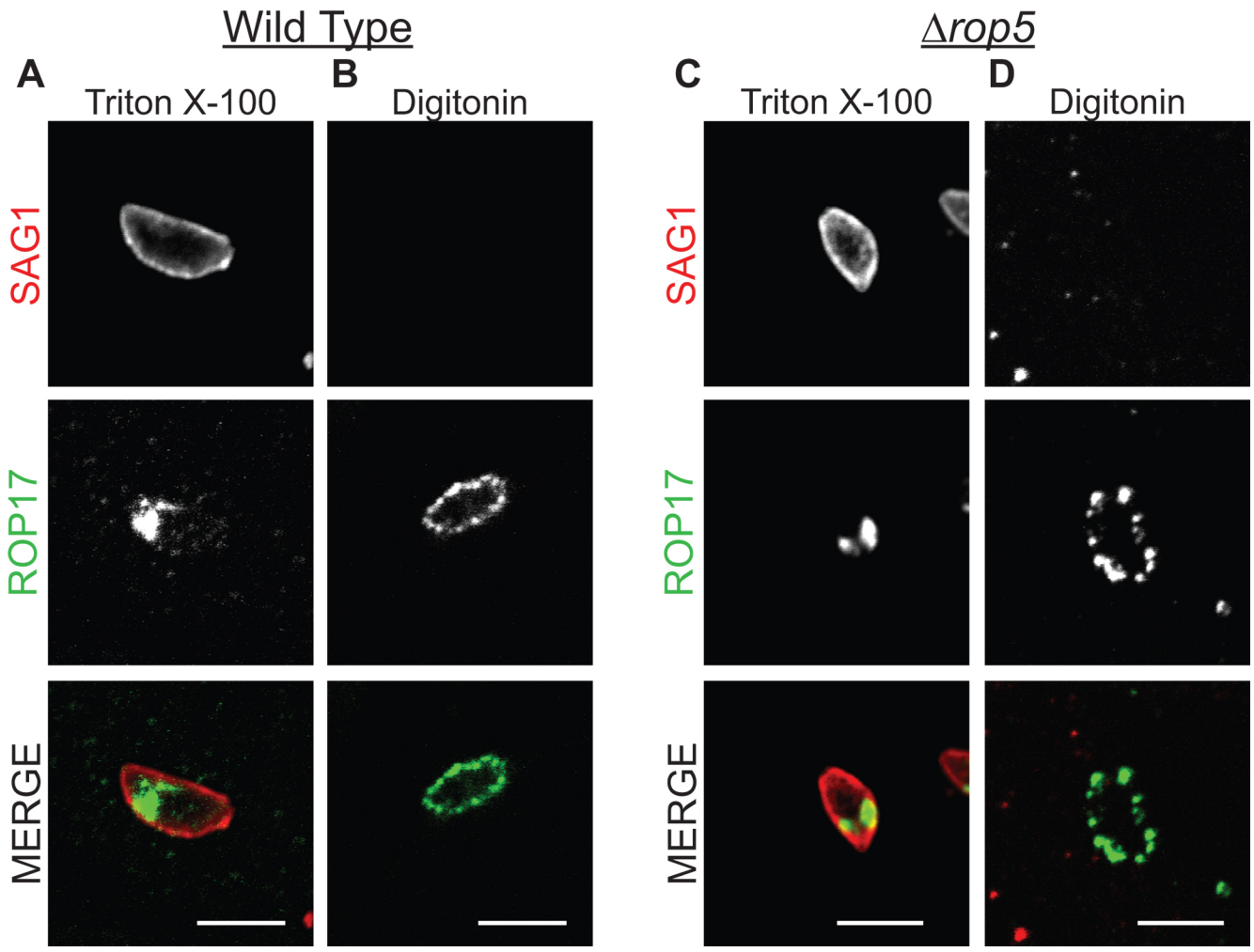


Figure S1. Localization of ROP17 to the cytosolic face of the PVM. Related to Figure 2. RH*Δku80* (A-B) and RH*Δku80Δrop5* (C-D) *T. gondii* parasites were used to challenge HFF monolayers for 30 min. Cells were fixed, and either fully permeabilized with 0.1% Triton X-100 (A and C), or partially permeabilized with 0.002% digitonin (B and D) to reveal ROP17's intracellular location in the rhoptries and the cytosolic face of the PVM, respectively. Samples were incubated with mouse anti-ROP17, visualized by goat anti-mouse IgG conjugated to Alexa 488 (green), and rabbit antisera to SAG1, visualized by goat anti-rabbit IgG conjugated to Alexa 594 (red). Absence of surface antigen 1 (SAG1) staining was used to confirm PVM integrity as seen in B and D. (Scale bars = 5 microns.).

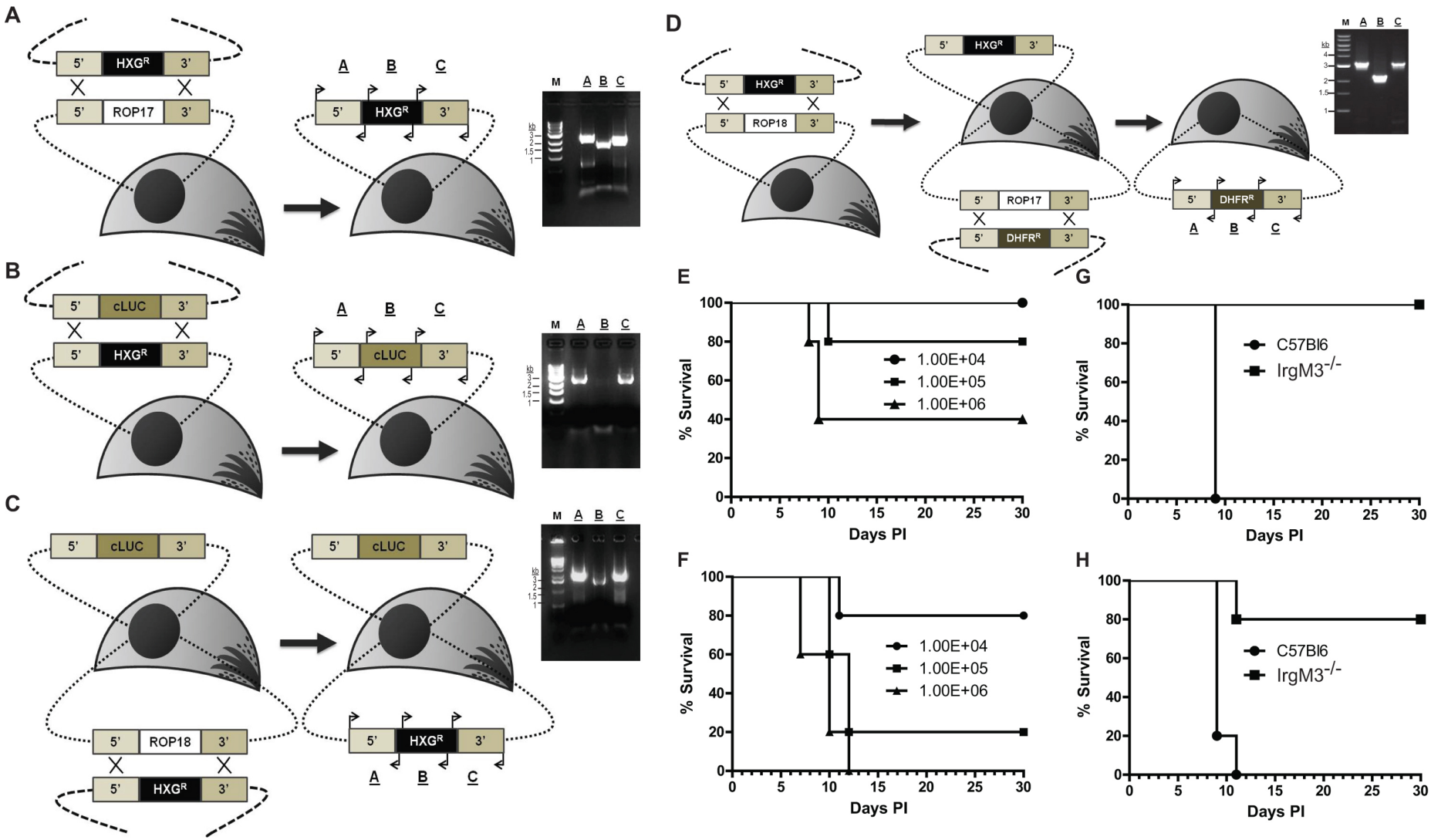


Figure S2. Generation of the RH*Δku80Δrop17Δrop18* and reverse double-deletion (RH*Δku80Δrop18Δrop17*) mutants.

Related to Figure 4. Using the RH*Δku80* cell line, a double crossover deletion of ROP17 was generated (A) followed by removal

of the drug selection marker HXGPRT via negative selection deletion to replace with the drug marker with the Click-Luciferase (cLUC) gene (B). The HXGPRT drug marker was recycled to delete the ROP18 gene and generate the double deletion cell line (C). To generate the reverse deletion mutant (D), RH*Δku80Δrop18* parasites were transfected with a ROP17 deletion construct containing pyrimethamine-resistant DHFR for selection and individual clones were isolated. Primers used in diagnostic PCR are shown in the diagram and a representative gel is shown for each step. (E-F) Two individually derived reverse double deletion mutants (RH*Δku80Δrop18Δrop17* (a) and (b) respectively) were inoculated ip into CD-1 mice at different parasite doses (10^4,10^5, and 10^6) and virulence monitored for 30 days. Mean values shown per group (n = 5). The individual clones (a) and (b) used in (E-F) were used to infect parental C57Bl/6 and IrgM3$^{-/-}$ mice to demonstrate equivalent virulence attenuation relative to the original double deletion mutant RH*Δku80Δrop17Δrop18*. (E-F) Representative experiment.

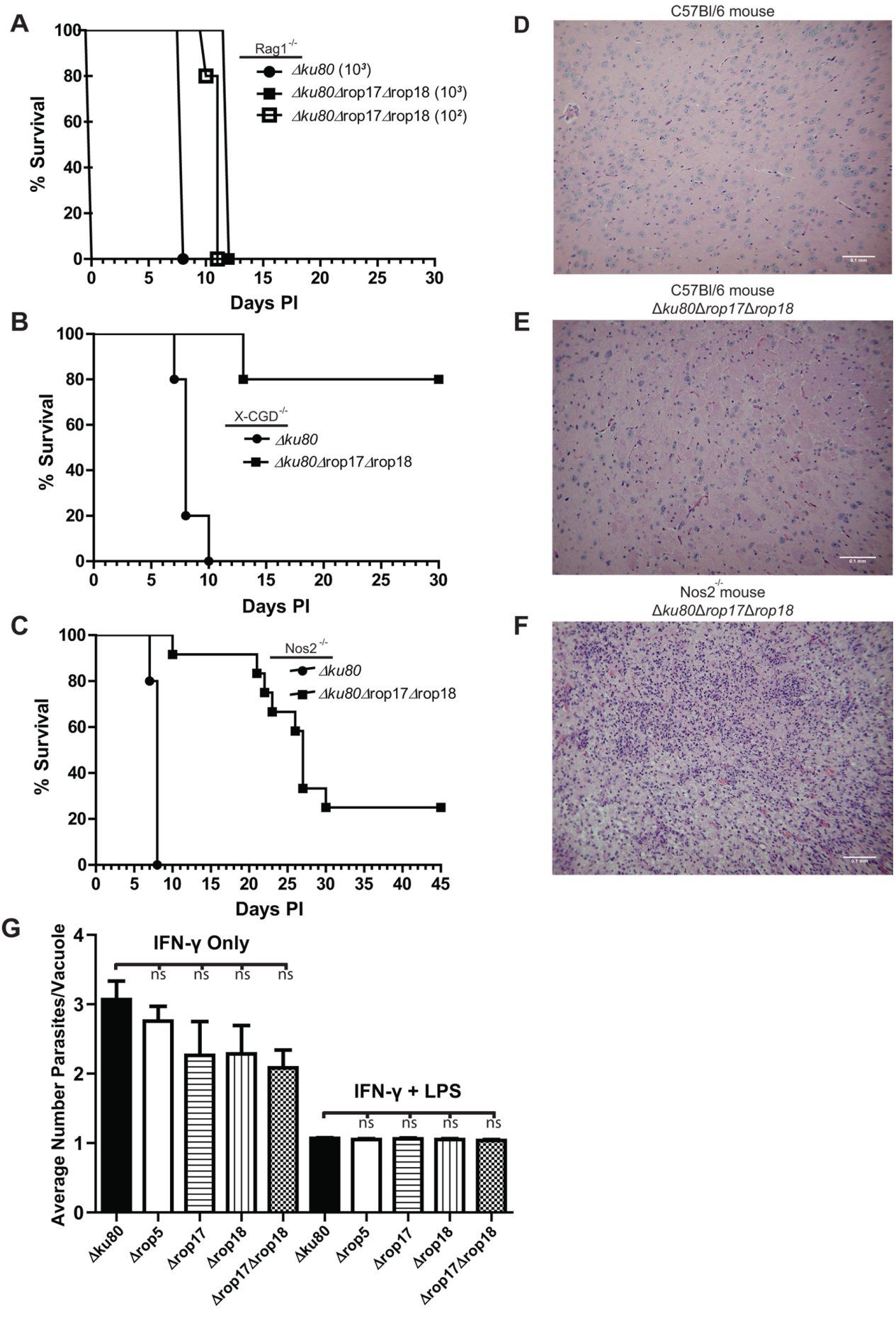

A
% Survival
Days PI
Rag1-/-
Δku80 (10³)
Δku80Δrop17Δrop18 (10³)
Δku80Δrop17Δrop18 (10²)
B
% Survival
Days PI
X-CGD-/-
Δku80
Δku80Δrop17Δrop18
C
% Survival
Days PI
Nos2-/-
Δku80
Δku80Δrop17Δrop18
D
C57Bl/6 mouse
E
C57Bl/6 mouse
Δku80Δrop17Δrop18
F
Nos2-/- mouse
Δku80Δrop17Δrop18
G
Average Number Parasites/Vacuole
IFN-γ Only
IFN-γ + LPS
ns
Δku80
Δrop5
Δrop17
Δrop18
Δrop17Δrop18

Figure S3. IFN-γ signaling is the major pathway for control of RH*Δku80Δrop17Δrop18* parasites. Related to Figure 5. (A) $Rag^{-/-}$ mice were inoculated ip with 10^3 parental RH*Δku80* or 10^3 and 10^2 *Δku80Δrop17Δrop18* double deletion parasites (n = 5 per group) and survival was followed for 30 days. (B) X-CGD mutant mice (n = 5) were inoculated ip with 10^3 parental *Δku80* or *Δku80Δrop17Δrop18* deletion parasites. Mouse survival was monitored for 30 days. (C) $Nos2^{-/-}$ mice were inoculated ip with 10^3 parental *Δku80* (n = 5) and 10^3 *Δku80Δrop17Δrop18* (n = 12) and monitored for 45 days post infection. Data are representative of two or more experiments with a similar outcome. Parental line C57Bl/6 (D-E) and $Nos2^{-/-}$ (F) mice were inoculated ip with 10^3 *Δku80Δrop17Δrop18* parasites and the infection was allowed to proceed for 30 days to resolve the acute infection. At day 30, two surviving mice from each group were sacrificed and brains were removed, fixed in formaldehyde, and processed for histology. Hematoxylin and eosin stained slides of the brain hemispheres at the level of the thalamus were used to capture representative images. Scale bars = 0.1 mm. Representative experiment. (G) RAW 264.7 mouse macrophages activated with IFN-γ (100 units) alone or IFN-γ (100 units) and LPS (0.1 ng/ml)) were challenged with *T. gondii* parasite strains and infection was allowed to proceed for 20 hr. Monolayers were fixed and permeabilized with 0.05% saponin and stained with rabbit antisera to SAG1, visualized by goat anti-rabbit IgG conjugated to Alexa 594 (red). The number of parasites per vacuole was counted and averaged for each parasite strain under both conditions. Means ± SEM, n = 2 samples each, from three combined experiments. Student's *t*-test, ns, non-significant.

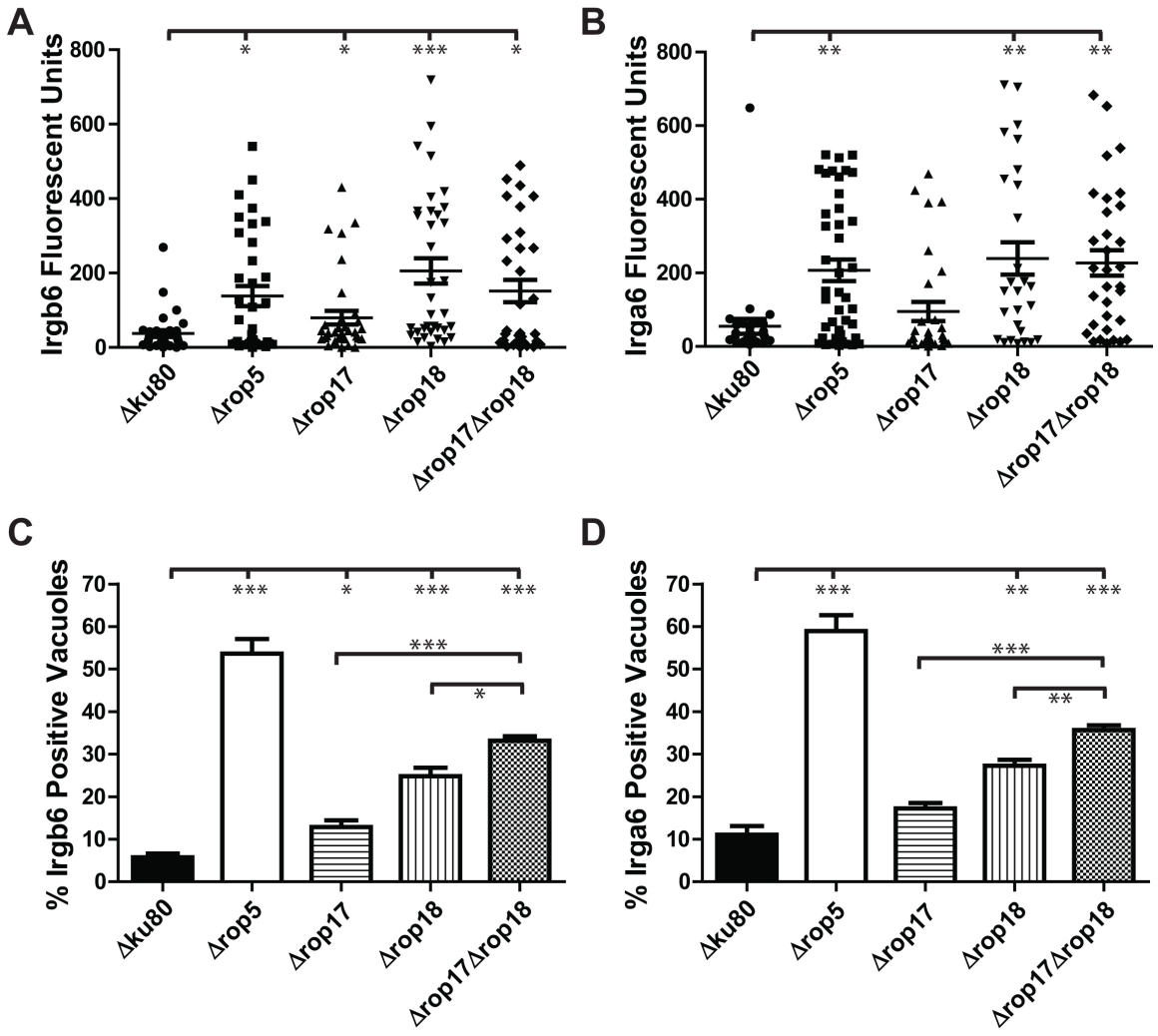
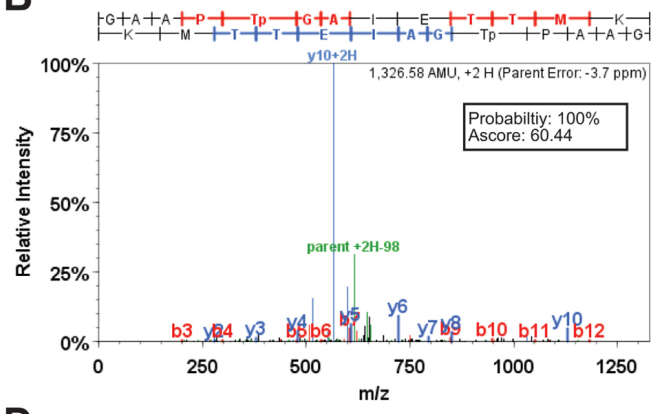


Figure S4. Quantification of Irgb6 and Irga6 loading onto parasite-containing vacuoles. Related to Figure 5. (A-B) ROP kinase deletion mutants were used to infect activated RAW 264.7 mouse macrophages for 30 min followed by fixation and permeabilization with 0.05% saponin. Cells were stained with rabbit polyclonal anti-Irgb6 or mAb 10D7 Irga6, mAb Tg17-113 to GRA5 or rabbit polyclonal anti-GRA7 and DAPI. IRG loading intensity on the PVM was quantified as described in the methods from approximately 30 vacuoles per mutant. (C-D) Activated RAW 264.7 mouse macrophages were infected *in vitro* for 2 hr and processed for IF microscopy, as described above. The percentage of Irgb6 (Rb anti-Irgb6) (C) or Irga6 (mAb 10D7) (D) positive vacuoles was determined by comparison to staining with the PVM markers GRA5 or GRA7, respectively. Samples were imaged using Alexa Fluor conjugated secondary antibodies. Means ± SEM, n = 3 samples each, from three combined experiments. (A,B) Kruskal-Wallis test with Dunn's post test, (C,D) Student's *t*-test, **P* ≤ 0.05, ***P* ≤ 0.01 and ****P* ≤ 0.005.

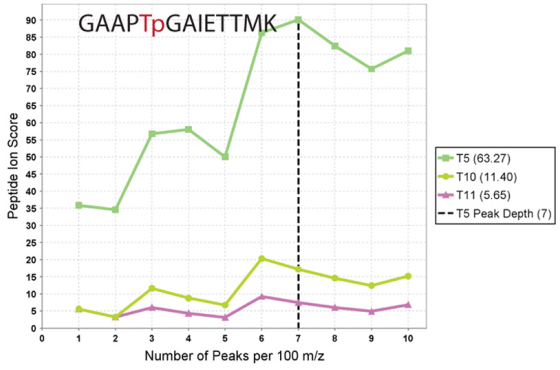
A

	T102		T108	
	ROP17	ROP18	ROP17	ROP18
Irga6	91	86.9	21.7	96
Irga1	100	73.3	26.6	93
Irga2	100	100	13	95.6
Irga3	N/A	N/A	47.8	100
Irga4	N/A	N/A	15.3	100
Irga7	N/A	N/A	18.5	100
Irga8	N/A	N/A	57.1	100
Irgb1	82	48.4	27.2	51.5
Irgb2	93	62.9	74	93
Irgb3	93	64.2	10.7	82.1
Irgb4	88	62.5	9.3	81.2
Irgb5	93	60.6	39.3	75.7
Irgb6	78	16.6	12.5	96
Irgb8	92	61.5	11.5	80.7
Irgb9	93	70	33.3	86.6
Irgb10	N/A	N/A	15.3	85
Irgc	100	60.8	17.3	69.5
Irgd	N/A	N/A	76.4	88.2
Irgm1	83	65.2	21.7	78.2
Irgm2	82	63.6	27.2	22.7
Irgm3	80	70	20	50

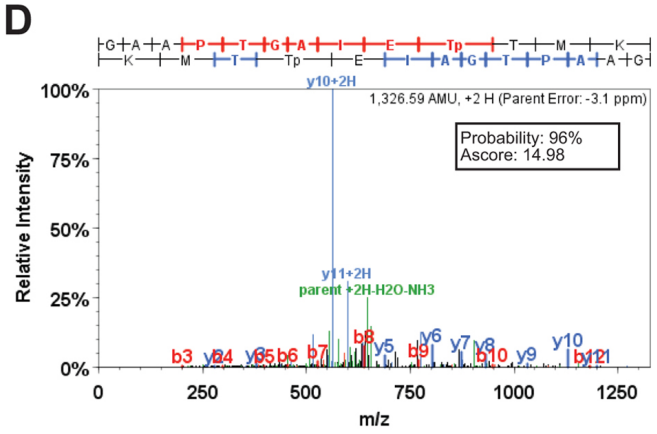
B



C



D



E

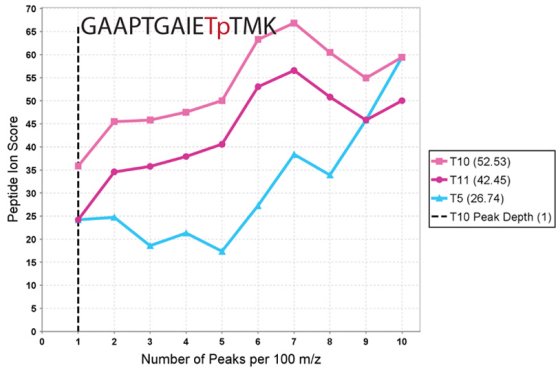
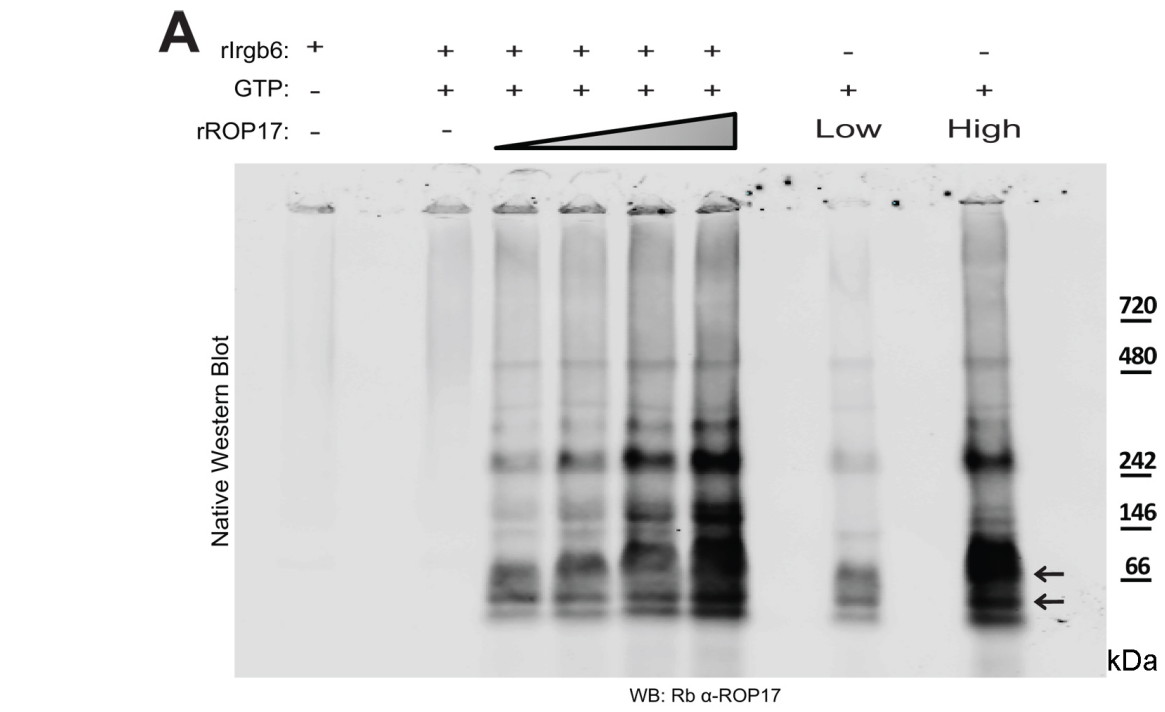


Figure S5. Theoretical and empirical preference comparison of the Thr102 or Thr108 motif in the mouse IRG protein family by ROP kinases. Related to Figure 6. (A) Each member of the IRG protein family listed were parsed into a series of 10 letter words (10 amino acid peptides) with Threonine (Thr) at the fifth position for every Thr present in each protein. Peptides were ranked in descending order of predicted preference by ROP17 and ROP18 by "prophet" analysis with 100% being the most preferred peptide and 0% being the least preferred peptide in an individual protein based on the probability score matrix. Thr102 and Thr108 peptide substrates preferred by ROP17 are highlighted in blue and those preferred by ROP18 in red. The percentage preference for these residues within each individual protein is listed. Proteins lacking a Thr at a particular conserved position are denoted by (N/A). (B-E) The LC-MS/MS analysis was further tested for the confidence of the phosphorylation localization using Scaffold PTM. Peptide ion scores calculated on the most relevant peaks to differentiate the phosphosites were used to determine the probability that a phosphorylation event occurred specifically at either Thr102 (denoted as T5) (B-C) or Thr107 (denoted as T10) (D-E). The Thr108 (denoted as T11) is also listed but no phosphorylation of this residue was observed in the analysis. The confidence level and Ascore for each phosphopeptide is listed for each site in (B and D). In Scaffold PTM, the Ascore utilizes the optimal peak depth for each spectra that best separates the best score from the second best score (denoted by the dashed vertical line in C and E). This method allows the Ascore calculation to account for varying noise levels in different spectra.


A
rIrgb6: + + + + + + - -
GTP: - + + + + + + +
rROP17: - - Low High
Native Western Blot
720
480
242
146
66
kDa
WB: Rb α-ROP17

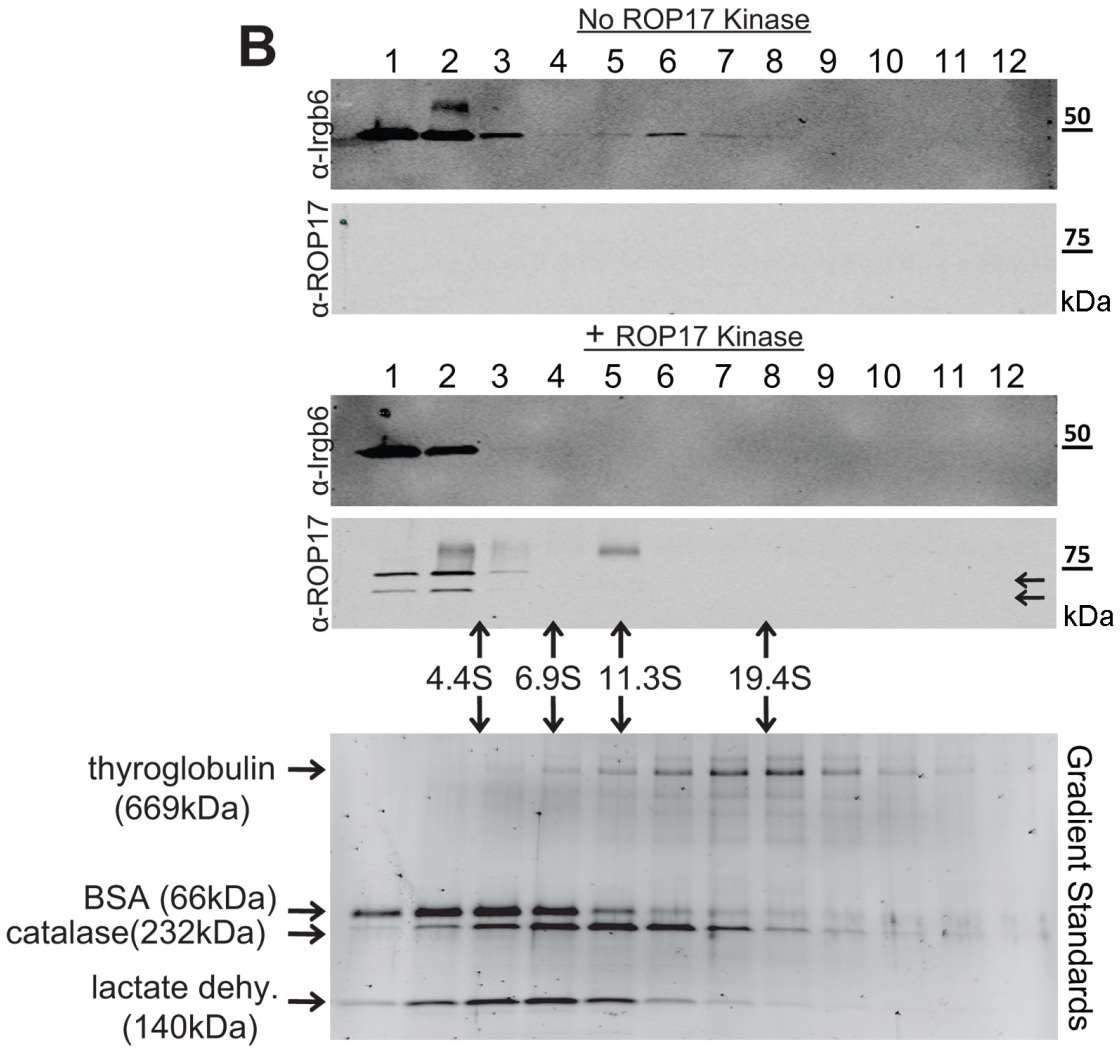

B
No ROP17 Kinase
1 2 3 4 5 6 7 8 9 10 11 12
α-Irgb6
50
α-ROP17
75
kDa
+ ROP17 Kinase
1 2 3 4 5 6 7 8 9 10 11 12
α-Irgb6
50
α-ROP17
75
kDa
4.4S 6.9S 11.3S 19.4S
thyroglobulin (669kDa)
BSA (66kDa)
catalase(232kDa)
lactate dehy. (140kDa)
Gradient Standards

Figure S6. Contribution of rROP17 to phosphorylation and Irgb6 polymer disassembly. Related to Figure 6. (A) *In vitro* kinase reaction combining rROP17 and rIrgb6 (as shown in Figure 6G) was carried out for 30 min at 37°C followed by Native-PAGE gel (8-16%) separation and Western blotting for ROP17. Arrows indicate major and minor species of recombinant ROP17 protein. Additional lanes containing rROP17 without rIrgb6 both at the low (0.5 µg) and high (4 µg) concentrations used in Figure 6G are shown. (B) Irgb6 subjected to a kinase reaction with or without rROP17 for 30 min at 37°C was fractionated on a 10-30% glycerol gradient. Gradient fractions were resolved on 8-16% SDS-PAGE gels, and probed with antibodies against Irgb6 and ROP17. Arrows indicate major/minor species of purified recombinant ROP17. Western blots were imaged on the ODYSSEY imager using LI-COR specific secondary antibodies. Sedimentation coefficients were determined using standards run in parallel. Lower gel stained with SYPRO Ruby and imaged on a Bio-Rad Gel Doc using UV transillumination.

Table S1 Common Contaminants of Rhoptry Complexes. Related to Table 1.

Protein ID *T. gondii* [a]	Name/Motif	Signal Peptide [c]	ROP5-TAP	ROP18-TAP	ROP17-TAP
TGGT1_072750	selenophosphate synthase	no	12(14) [b]	10(10)	ND
TGGT1_101680	microtubule-binding protein	no	9(9)	9(9)	ND
TGGT1_018920	hypothetical	no	8(10)	13(15)	ND
TGGT1_073240	3', 5'-cyclic nucleotide phosphodiesterase	no	8(9)	6(7)	ND
TGGT1_056160	60S ribosomal protein L4	no	8(9)	9(13)	ND
TGGT1_051860	40S ribosomal protein S7	no	8(8)	9(14)	ND
TGGT1_020420	40S ribosomal protein S4	no	6(11)	6(13)	ND
TGGT1_114810	40S ribosomal protein S3A	no	6(7)	11(14)	ND
TGGT1_050780	hypothetical	no	6(7)	6(9)	ND
TGGT1_006930	60S ribosomal protein L13	no	6(6)	7(8)	ND
TGGT1_088710	hypothetical	no	6(6)	9(11)	ND
TGGT1_088710	hypothetical	no	6(6)	9(11)	ND
TGGT1_025360	heat shock protein 60	no	5(9)	ND	7(10)
TGGT1_024480	40S ribosomal protein S9	no	5(6)	7(10)	ND
TGGT1_072950	KH domain	no	5(5)	ND	6(21)
TGGT1_069190	DEAD/DEAH box helicase	no	ND	15(17)	12(16)
TGGT1_071470	hypothetical	no	ND	8(9)	9(14)

[a] The cut-off for inclusion of individual proteins into this list was set at five or more unique peptide matches with a probability of 0.95 or above. [b] Numbers of unique peptides is indicated and those in parenthesis indicate the number of spectra detected for each protein. [c] This list includes all proteins lacking a signal peptide. ND, none detected.

Table S2 Strains used in this study. Related to Experimental Procedures.

Parental Strain	Strain Genotype	Drug Selection	Strain Description
GT1 Type I	GT1 Type 1::Tet-Repressor-YFP::CAM	chloramphenicol (Cm)	Parental line expressing the tetracycline respressor fused to YFP and used in generation of all successive inducible TAP-tag expression strains.
GT1 Type I	GT1 Type 1::Tet-Repressor-YFP::Cam::ROP5-TAP::Ble	Cm and phleomycin (Ble)	Parasite strain expressing the TAP-tagged ROP5 gene.
GT1 Type I	GT1 Type 1::Tet-Repressor-YFP::Cam::ROP17-TAP::Ble	Cm and Ble	Parasite strain expressing the TAP-tagged ROP17 gene.
GT1 Type I	GT1 Type 1::Tet-Repressor-YFP::Cam::ROP18-TAP::Ble	Cm and Ble	Parasite strain expressing the TAP-tagged ROP18 gene.
GT1 Type I	GT1 Type 1::Tet-Repressor-YFP::Cam::ROP17D/A-TAP::Ble	Cm and Ble	Parasite strain expressing the TAP-tagged ROP17 gene with a catalytic aspartate mutated to alanine to inactivate the enzyme and create a catalytically dead mutant.
GT1 Type I	GT1 Type 1::Tet-Repressor-YFP::Cam::mCherry-TAP::Ble	Cm and Ble	Parasite strain expressing the TAP-tagged mCherry gene to serve as a negative control for specificity of the TAP-tag.
RH Type I lacking Ku80 gene (VBC)	Δ*ku80*/Δ*rop17*::HXGPRT^{R}	Mycophenolic Acid/Xanthine (MPA/Xa)	Knockout line of ROP17. Used as the parental line in subsequent parasite lines.
RH Type I lacking Ku80 gene (VBC)	Δ*ku80*/Δ*rop17*::cLUC	Negative Selection of HXGPRT: 6-Thioxanthine	Generated as an intermediate strain to recycle the HXGPRT drug selection marker for the subsequent ROP18 knockout line.
RH Type I lacking Ku80 gene (VBC)	Δ*ku80*/Δ*rop17*::cLUC/Δrop18::HXGPRT^{R}	(MPA/Xa)	Knockout line of ROP17 and ROP18 (double deletion strain).
RH Type I lacking Ku80 gene (VBC)	Δ*ku80*/Δ*rop17*::ROP17/DHFR^{R}/Δ*rop18*::HXGPRT	Pyrimethamine (Pyr)	Complemented cell line containing ROP17 under its endogenous promoter.

Table S3 Primers used in this study. Related to Experimental Procedures.

Application	Plasmid	Name	Sequence, 5'-3'	Polarity
Tet-On-Tap				
	RPS13+Tet-Operator	A7	GACGGTACCAGCTTGTCAGTGCATGACACA	sense
	RPS13+Tet-Operator	A24	CTACAATTGCGCCATTTTGACGGTGACGAAGCCACCTGAGGAAGACGCAGAG	anti-sense
	MCS+TAP-tag	A9	GATCAATTGCCTAGGAAGCTTCTCGAGGGATC	sense
	MCS+TAP-tag	A10	CGATTAATTAATCAGGTTGACTTCCCCGCGG	anti-sense
	SAG/Ble/SAG drug marker	A11	GATACTAGTGCTCTTCAAGGTTTTACATCCGTTGCC	sense
	SAG/Ble/SAG drug marker	A12	GTAACTAGTGAAGAGCTCGGGGGGGCAAGAA	anti-sense
Mutagenesis				
	C1418G	A15	CACTATCGAAAAGGATTCGTAGGTTTCTATCCTCTGC	mutate
	G1565C	A16	GAATCCGAGAGAGGCATCCAACCCACCGA	mutate
	C1990G	A17	ATCAGTGATAGAGATCTGTAGATTCCTCGACGGGT	mutate
	G3040C	A18	CCCCCCGAGGGGCATCCACTAGTGC	mutate
	G3460C	A19	GGAGCTTTACCATGCGTCTACAAGGTGGATGC	mutate
	C4574G	A20	CCGAGCTCTTCACTAGTTGTAGAGCGGCC	mutate
	C1307A ROP17 D/A- TAP-tag mutant	E1	CAACAAGAAAATTTTGCAGTTTCACAGCGCCATGAACAAGTCCGAA	mutate
TAP-Expression				
	ROP5-TAP	B5	CATAAGCTTATGGCGACGAAGCTCGCTAGACTAGCC	sense
	ROP5-TAP	B6	GATGGATCCAGCGACTGAGGGCGCAGCA	anti-sense
	ROP17-TAP	A35	GTAAAGCTTATGGAGTTGGTGTTGTGCTTTGTGATAATAAC	sense
	ROP17-TAP	A36	GATGGATCCCTCCTTCTGTAATAAAGCCGCCTC	anti-sense
	ROP18-TAP	A33	GTAAAGCTTATGTTTTCGGTACAGCGGCCACC	sense
	ROP18-TAP	A34	GATGGATCCTTCTGTGTGGAGATGTTCCTGCTG	anti-sense
KO Constructs				
	ROP17-KO B4-B1r upstream-flank	C26	GGGGACAACTTTGTATAGAAAAGTTGGGAACTGCGCGAGACCTAAAGCTG	sense
	ROP17-KO B4-B1r upstream-flank	C27	GGGGACTGCTTTTTTGTACAAACTTGGGCGAGTCACGGACGAAGCTTCAAC	anti-sense
	ROP17-KO B2r-B3 downstream-flank	C28	GGGGACAGCTTTCTTGTACAAAGTGGGATGCGGTCAATGCAACAAGAAAG	sense
	ROP17-KO B2r-B3 downstream-flank	C29	GGGCAGGACTGTGTACCACGGAATGCAACTTTATTATACAAAGTTGTCCCC	anti-sense
	ROP18-KO B4-B1r upstream-flank	C22	GGGGACAACTTTGTATAGAAAAGTTGGTTCCGTCTCCAGGTCTCGAAGAG	sense
	ROP18-KO B4-B1r upstream-flank	C23	GGGGACTGCTTTTTTGTACAAACTTGCACAACTTTCACACAAACTGGAC	anti-sense
	ROP18-KO B2r-B3 downstream-flank	C24	GGGGACAGCTTTCTTGTACAAAGTGGGAACGTGGCGGTGCCGCCCTACCG	sense
	ROP18-KO B2r-B3 downstream-flank	C25	GGGGACAACTTTGTATAATAAAGTTGGTACAAGAGATATACAAGGACAGCTGGAAC	anti-sense
***E. coli* Expression**				
	pET22b expression construct primer	rROP17 For	CATATGCTTCTTTTACGGTCACCAACGA	sense
	pET22b expression construct primer	rROP17Rev	AAGCTTCTCCTTCTGTAATAAAGCCGCCT	anti-sense
Screening Primers				
	Diagnostic ROP17 Knockout upstream integration	A64	CAATACCTGCACAGCATTGCCGAGCTGACAG	sense
	Diagnostic ROP17 Knockout upstream integration	A65	GTCGAACAAAGCACGGAGGAGAGACGGAAAG	anti-sense
	Diagnostic ROP17 Knockout downstream integration	A66	ACACCATTTGCAGATCGATAATCTGCGACCGC	sense
	Diagnostic ROP17 Knockout downstream integration	A67	GCGTGATAGTGTTCTCCCGATTCTGAGAACAGTCG	anti-sense
	Diagnostic middle HXGPRT drug cassette integration	A26	GATGAATTCCAGCACGAAACCTTGCATTCAAAC	sense
	Diagnostic middle HXGPRT drug cassette integration	A14	CATACTAGTTTCAATGATCCCCCTCCACCGCGG	anti-sense
	Diagnostic ROP18 Knockout upstream integration	C80	GGTATATGTGGAGGCACGCATGTTATGCGTGTGTATG	sense
	Diagnostic ROP18 Knockout upstream integration	A65	GTCGAACAAAGCACGGAGGAGAGACGGAAAG	anti-sense
	Diagnostic ROP18 Knockout downstream integration	A66	ACACCATTTGCAGATCGATAATCTGCGACCGC	sense
	Diagnostic ROP18 Knockout downstream integration	C81	CACGAATGCGAAATTGGCATCCAGACACACAACGAAAC	anti-sense
	Diagnostic ROP17 cleanup upstream integration	A64	CAATACCTGCACAGCATTGCCGAGCTGACAG	sense
	Diagnostic ROP17 cleanup upstream integration	C42	CCTCCAAAGGATGGAGAGGCTCAGGGCC	anti-sense
	Diagnostic ROP17 cleanup downstream integration	C43	GATTACCTGGCTGAACGTGTGAGCCATACTAAG	sense
	Diagnostic ROP17 cleanup downstream integration	A67	GCGTGATAGTGTTCTCCCGATTCTGAGAACAGTCG	anti-sense

Table S4 Plasmids used in this study. Related to Experimental Procedures.

Common Name	Description	Source	Backbone
pTet07-Sag1-myc-GFP-Sag1	Vector containing SAG1 5' and 3' UTR's expressing a myc-tagged GFP. This vector is used in the Tet-Off (TATi) *T. gondii* lines where tetracycline turns off expression.	In-house	N/A
Tet-On-TAP	In house generated TAP-tagging vector used to express Rhoptry kinases under a tet-inducible promoter.	In-house	pTet07-Sag1/mycGFP/Sag1
TubYFP/Tr-sagCAT	Vector containing the Tet-repressor-YFP fusion construct under the Tubulin promoter.	Dick Schaap	N/A
pRS13subIV-23LacZ	Vector containing *T. gondii* RPS13 promoter with Tet-operator sites inserted provided Tetracycline inducible expression. This promoter was integrated into the Tet-On-TAP vector.	Dick Schaap	N/A
Gateway 3 Fragment Cloning	Commercially available kit containing 3 pDONR plasmids (P4P1r, P1P2, P2P3) that were used in the generation of the knockout constructs used in this study. Three fragments containing an 5' flanking region, a drug marker and 3' flanking region were combined into the destination vector (R4R3) making the completed knockout vector.	Invitrogen	N/A
Gateway 4 Fragment Cloning	Commercially available kit containing 4 pDONR plasmids (P1P5r, P5P4, P4rP3r, P3P2) that could be combined in the R1R2 destination vector. This kit was used to generate the ROP17 complementation vector.	Invitrogen	N/A
pET22b-rROP17	*E. coli* protein expression vector used to generate recombinant ROP17. The ROP17 full-length gene was amplified from *T. gondii* RH cDNA. Coding sequence starts from amino acid 22 in order to remove the signal sequence. Gene was cloned between NdeI/HindIII on vector pET22b.	Novagen	pET22b

Supplementary Experimental Procedures

In Vivo Infections Studies

Animals were maintained in an Association for Assessment and Accreditation of Laboratory Animal Care approved facility and the Institutional Care Committee at Washington University in St. Louis, School of Medicine, approved all protocols. Outbred CD-1 and C57BL/6 mice were purchased from Charles River Laboratories. Ifnγr1$^{-/-}$, Rag1$^{-/-}$, and Nos2$^{-/-}$ mice, all on a C57BL/6 background, were obtained from Dr. Herbert Virgin (Washington University). Mice deficient in the superoxide-generating NADPH-oxidase gp91subunit, referred to as X-CGD mice, on a C57BL/6 background were obtained from Dr. Mary C. Dinauer (Washington University). IrgM3$^{-/-}$ mice on a C57BL/6 background were obtained from Dr. Greg Taylor (Duke University). Knockout mice lines were bred locally at Washington University. Age and sex- matched mice were challenged by intraperitoneal (ip) injection with parasites and cumulative mortality determined for 30 days post injection as a measure of acute virulence (Su et al., 2002). To examine pathology in chronically infected mice, mouse brains were fixed in 10% neutral buffered formalin, dehydrated in ethanol, embedded in paraffin and 5 micron sections stained with hematoxylin and eosin (H&E).

Parasite and Cell Culture

T. gondii tachyzoites were serially passaged in human foreskin fibroblast (HFF) monolayers cultured in complete medium (Dulbecco's Modified Eagles Medium (DMEM)(Invitrogen) and supplemented with 10% FBS (HyClone), 2 mM L-glutamine, 10 mM HEPES (pH 7.5) and 20 µg/ml gentamicin) (referred to as complete medium) at 37°C in 5% CO_2. Stable transgenic parasites were selected in chloramphenicol (20 mg/ml)(Sigma), phleomycin (Ble) (5 mg/ml)(Invitrogen), anhyrdrous tetracycline (Tet) (0.15 mg/ml)(Clontech), pyrimethamine (Pyr) (3 mM)(Sigma), mycophenolic acid (25 µg/ml), and xanthine (50 µg/ml) (MPA/Xa). Negative selection to remove the HXGPRT maker was performed by growth in complete medium supplemented with 1% dialyzed FBS (Invitrogen), and 6-thioxanthine (340 mg/ml). Cultures were negative for mycoplasma contamination using the e-Myco plus mycoplasma PCR detection kit (Boca Scientific).

Recombinant Protein Purification

Genomic sequences of *ROP17* from the types I, II, and III lineages (www.Toxodb.org) were analyzed for cumulative values of polymorphisms using the Synonymous Nonsynonymous

Analysis Program (SNAP). The *ROP17* coding sequence (including amino acids 22-608) (TgGT1_011620) was amplified by genomic DNA from type I RH strain of *T. gondii* using iProof high-fidelity polymerase (Bio-Rad), cloned into pET22b, and expressed in *E. coli* BL21 (DE3) V2RpAcYc-LIC+LamP-phosphatase cells (obtained from Ray Hui, SGC). The ROP17 protein was C-terminally tagged with His6 (ROP17His6) and *E. coli* cultures were grown to an OD of 0.5 followed by induction with 1 mM IPTG at 15^{o}C in for 16 hr. Clarified bacterial lysates were incubated with HIS-Select Nickel Affinity Gel (Sigma), eluted in 50 mM of imidazole, and dialyzed in 20 mM Tris-HCl pH 8.0, 250 mM NaCl and 10 mM $MgCl_2$. Purified ROP17 was stored in 20% glycerol at -80°C. Proteins were resolved on SDS-PAGE stained with SYPRO-Ruby and concentration determined by comparison to BSA standards (Invitrogen). Polyclonal antibodies to ROP17 were generated in CD1 mice by subcutaneous (SC) injections of purified recombinant ROP17 (rROP17) mixed with Freund's Complete Adjuvant in the first injection and Freund's Incomplete Adjuvant in the subsequent boosts.

Immunofluorescence Microscopy

To examine the distribution of proteins in fully permeabilized cells, parasite-infected monolayers were fixed with 4% formaldehyde in PBS for 10 min, permeabilized with 0.1% Triton X-100 for 10 min at room temperature, and blocked in 10% FBS for 15 min. For selective staining of the PVM, parasite-infected monolayers were fixed with 4% formaldehyde in PBS for 10 min, permeabilized with 0.002% digitonin, and blocked in 10% FBS for 15 min. For evacuole formation, parasites were preteated with cytohalasin D (1 μM for 10 min) and used to challenge monolayer of HFF cells for 30 min at 37^{o}C, as described previously (Håkansson et al., 2001). Monolayers were then fixed with 4% formaldehyde in PBS for 10 min, permeabilized using 0.05% saponin, and blocked in 10% FBS for 15 min, all at 4°C. Samples were incubated with primary antibodies, washed in PBS, and incubated with secondary antibodies (goat anti-mouse IgG or goat anti-rabbit IgG) conjugated to Alexa fluor dyes (Invitrogen). Following staining, the slides were mounted with Prolong Gold and examined with a Zeiss laser scanning confocal microscope LSM510 (Carl Zeiss, Inc.) using a 63X NA 1.6 oil immersion lens and Z-stack images were acquired at 0.36 μm intervals. Images were processed with Photoshop CS4.

Clearance Assay

RAW 264.7 mouse macrophages were seeded onto 96-well clear, flat-bottom plates and were infected with freshly egressed parasites propagated in HFF cells, as described above.

Macrophages were activated with IFN-γ (150 units/ml) (R&D Systems) and LPS (10 ng/ml) derived from *E. coli* 055:B5 (Sigma L2880) for 24 hr prior to infection. RAW cells were challenged with parasites in complete medium at 37^{o}C 5% CO_2 for 30 min, washed to remove extracellular parasites, and fixed with 4% formaldehyde either at 1 hr or returned to culture in complete medium and fixed at 20 hr. Cells were permeabilized with 0.1% Triton-X100 and blocked with 1% FBS and 1% normal goat serum. Parasite burden was assessed by in-cell Western blot analysis by probing with rabbit anti-GRA7 antibodies followed by secondary antibody staining with (donkey anti-rabbit IgG) (LI-COR Biosciences) and imaged on the ODYSSEY infrared imager (LI-COR Biosciences). Host cells were normalized via staining with wheat germ agglutinin conjugated to Alexa fluor 680 dye (Invitrogen) that is compatible with the ODYSSEY imager. The percent of initial infection was determined through comparison of infection levels between 1 hr and 20 hr post infection. Experiments were repeated three or more times, with each sample run in duplicate.

Recruitment of IRGs to the PVM

RAW 264.7 mouse macrophages were seeded onto 24-well plates containing coverslips and activated with IFN-γ (100 units/ml) and LPS (0.1 ng/ml) for 24 hr. Cells were infected with parasites by incubation in complete medium at 37^{o}C, 5% CO_2 for 30 min. Monolayers were washed and then either fixed immediately or returned to culture for 90 min (2 hr time point). Monolayers were fixed in 4% formaldehyde in PBS, permeabilized with 0.05% saponin for 10 min at room temperature, and blocked in 5 % FBS and 5% normal goat serum. *T. gondii* containing vacuoles were stained with mouse mAb Tg17–113 against dense granule protein 5 (GRA5) (Charif et al., 1990) and Irgb6 was localized via staining with rabbit anti-Irgb6 (Henry et al., 2009) followed by Alexa-conjugated secondary antibodies. In parallel, Irga6 was localized by staining with mouse mAb10D7 anti-Irga6 antibody (Papic et al., 2008) and parasite-containing vacuoles were labeled with rabbit anti-GRA7 antibodies (Dunn et al., 2008), followed by Alexa-conjugated secondary antibodies. The numbers of positive vacuoles were determined by counting of 10 fields using a 40x objective from 3 coverslips in 3 separate experiments.

To determine the intensity of IRG loading onto vacuoles, IF images were acquired at fixed exposure levels and TIFF files were analyzed for fluorescence intensity using Zeiss LSM510 software. Four independent measurements were recorded for IRG loading intensity (2 polar points and 2 medial points). Proximal background fluorescence measurements were taken for

each point. Following background correction, the four point recordings were averaged for each vacuole measurement. Approximately 30 vacuoles per mutant were recorded.

Parasite Replication Assay

RAW 264.7 mouse macrophages were seeded onto 24-well plates containing coverslips. Cells were activated either with IFN-γ alone (100 units/ml) or with IFN-γ (100 units/ml) and LPS (0.1 ng/ml) for 24 hr. Cells were infected with parasites by incubation in complete medium at 37^{o}C, 5% CO_2 for 30 min followed by washing and return to culture for 20 hr. Monolayers were fixed in 4% formaldehyde in PBS, permeabilized with 0.05% saponin for 10 min at room temperature, and blocked in 5 % FBS and 5% normal goat serum. Parasite-containing vacuoles were stained with mouse anti-SAG1 monoclonal antibody DG52 followed by Alexa-conjugated secondary antibodies. The number of parasites per vacuole was determined by counting of 10 fields using a 40x objective from 2 coverslips in 3 separate experiments. The average number of parasites per vacuole was determined for each mutant

Extract Preparation and Gradient Analysis

Toxoplasma gondii whole cell extract was generated by sonicating freshly egressed tachyzoites in extraction buffer (20 mM Tris-HCl pH 7.6, 150 mM KCl, 10 mM $MgCl_2$, 1 mM EDTA, 0.4% NP-40, cOmplete™ EDTA free protease inhibitor cocktail (Roche) and 1 mM DTT) and adjusted to a concentration of 0.3 mg /ml. Extracts were centrifuged in the TLA100.3 rotor at 60,000 rpm (156,424*g*) for 10 min and the soluble portion retained for further analysis. A 10-30% linear glycerol gradient was generated using the Gradient Master gradient maker (BioComp Instruments). Clarified parasite lysate (200 uL) was loaded onto the top of the gradient and centrifuged in a Beckman SW41 rotor at 111,000*g* for 20 hr. Gradients were fractionated from the top and sedimentation values were determined using BSA (4.4S, 66 kDa), lactate dehydrogenase (6.9S, 140 kDa), catalase (11.3S, 232 kDa), and thyroglobulin (19.4S, 669 kDa) as standards (Sigma).

Immunoprecipitation (IP) and Western Blotting

Freshly harvested parasites were lysed in extraction buffer and clarified by centrifugation as described previously above. For IP's, antibodies were added to clarified extracts and incubated at 4°C for 2 hr then incubated with Protein G Dynabeads (Invitrogen) for 2 hr at 4°C with rotation. Beads were washed with extraction buffer 3 times with 10 min incubations at 4°C. Samples were eluted using denaturing SDS-PAGE sample buffer, resolved on 8-16% acrylamide gels, and transferred to nitrocellulose. For comparison, *T. gondii* parasite lysates

(5 x 10^6 parasites/lane) were resolved on SDS-PAGE denaturing gels and transferred to nitrocellulose. Blots were blocked in 5% milk in Tris-buffered saline (TBS) pH 7.5. Western blots were probed with rabbit (Rb) anti-Irgb6 (Henry et al., 2009), Rb anti-ROP5 (Behnke et al., 2011), Rb anti-ROP18 (Taylor et al., 2006) mouse (Mo) anti-ROP17, mouse mAb anti-MIC2 (6D10) (Wan et al., 1997) or Rb anti-*T. gondii* aldolase (TgALD1) (Jewett and Sibley, 2003). Primary antibodies were incubated in either 3% milk in TBS or 3% BSA in TBS for 1 hr at room temperature followed by washing in TBS-Tween (0.5%). Blots were incubated with secondary antibodies conjugated to IRDye 680CW (donkey anti-rabbit IgG) or IRDye 800CW (donkey anti-mouse IgG) (LI-COR Biosciences) and signals detected using the ODYSSEY infrared imager (LI-COR Biosciences). Images were processed and analyzed using the ODYSSEY infrared imaging system software.

In Vitro Kinase Assays

Kinase activity was tested on heterologous substrates including Casein-a (1 µg/reaction) (EMD Chemicals), dephosphorylated myelin basic protein (dMBP) (0.5-10 µg/reaction) (Millipore), or on recombinantly expressed and purified Irgb6 and Irga6 (2 µg/reaction), as described previously (Fentress et al., 2010). Briefly, Irgb6 and Irga6 full coding sequences were *E. coli* expressed with an N-terminal fusion to GST followed by purification with glutathione bound agarose beads (GE Healthcare). Proteins were specifically eluted with PreScission Protease enzyme (GE Healthcare) to release the GST tag that was removed by binding to glutathione beads. Kinase reactions were conducted in 25 mM Tris-HCl pH 7.5, 15 mM $MgCl_2$ and 2 mM $MnCl_2$. containing 10 µCi of ^{32}P γ-ATP (specific activity: 3,000 Ci/mmol) (Perkin Elmer, Inc) in addition to 33 µM cold ATP (Sigma-Aldrich). Reactions were allowed to proceed at 30°C for 40 min, samples were solubilized in denaturing SDS PAGE sample buffer, resolved by PAGE, and either dried or transferred to nitrocellulose before imaging with an FLA5000 phosphorimager (FujiFilm).

Native Gel Kinase Assay

Purified recombinant Irgb6 protein (2 µg / reaction) was used in a kinase reaction conducted in 25 mM Tris–HCl pH 7.5, 15 mM $MgCl_2$ 2 mM $MnCl_2$, 1 mM DTT in the presence of 65 µM ATP (Sigma-Aldrich), in the presence or absence of 10 mM GTP (Sigma). Recombinant ROP17 kinase was added to the reaction at doses of 0, 0.5, 1, 2 or 4 µg / reaction. Kinase reactions were allowed to proceed at 37°C for 30 min, diluted with 2x Native sample buffer (Invitrogen), and loaded onto an 8-16% PAGE gel. Native Mark (Invitrogen) was loaded as a size marker.

The samples were run at 4°C for 20 hr at 40 v, followed by blotting onto nitrocellulose for 8 hr at 50 v. A portion of the reaction products were denatured by boiling with 2X SDS sample buffer followed by separation on an SDS-PAGE gel to provide a control for equal loading. Irgb6 was visualized using Rb anti-Irgb6 (Henry et al., 2009) and secondary LI-COR antibodies. Threonine phosphorylation by ROP17 was observed by probing with Rb anti-phosphothreonine antibody (Cell Signaling) and visualized with LI-COR secondary antibodies. ROP17 was visualized by probing with Rb anti-ROP17 (Covance) followed by staining with LI-COR secondary antibodies. Blots were imaged on the LI-COR ODYSSEY Imager.

Glycerol Gradient Analysis of Recombinant Proteins

Kinase reactions using rROP17 and rIrgb6 used in the native gel analysis were prepared as described above and loaded onto 10-30% glycerol gradients in the presence of aluminum fluoride to stabilize the IRG oligomer as described (Papic et al., 2008). Gradients were centrifuged in a Beckman SW41 rotor at 111,000*g* for 10 hr. Gradients were fractionated from the top and sedimentation values were determined using BSA (4.4S, 66 kDa), lactate dehydrogenase (6.9S, 140 kDa), catalase (11.3S, 232 kDa), and thyroglobulin (19.4S, 669 kDa) as standards (Sigma). Samples were resolved on 8-16% SDS-PAGE gels followed by blotting to nitrocellulose. Blots were probed with Rb anti-Irgb6 and Rb anti-ROP17 followed by staining with LI-COR secondary antibodies. Blots were imaged on the LI-COR ODYSSEY Imager.

Motif Analysis

The phosphorylation site consensus for ROP18 was determined using a positional scanning peptide array incubated with kinase (8 ng/µl), ^{32}P γ-ATP (50 μM at 30 μCi/ml) for 2 hr at 30°C, as described (Turk et al., 2006). Aliquots were spotted onto streptavidin-coated membranes (Promega), washed, air-dried, and exposed to a phosphor imaging screen. Radiolabel incorporation into each peptide was quantified with QuantityOne software (BioRad), and the data were normalized as described (Turk et al., 2006). Phospho-blot data were normalized to adjust the values for Thr (multiplied by 0.5) and converted to a WebLogo (Crooks et al., 2004). The normalized values from the ROP17 phosphopeptide array were used to create a Gribskov protein profile suitable for use with “prophet” available from EMBOSS (Rice et al., 2000). Mouse IRG family members were searched using the ROP17 motif to determine preferred ten-letter words centered on all possible Thr residues. The top five scoring T words from each IRG protein were then submitted to MEME (Bailey et al., 2006) to produce up to three distinct MEME logos.

Generation of Knockout and Tagged Parasite Lines

Rhoptry kinase genes of interest were PCR-amplified from the GT1 strain gDNA, cloned into the Tet-repressible expression construct, and expression was induced with 0.15 µg/ml anhydrous tetracycline (Tet). Knockout (KO) and cleanup constructs were generated using the 3-fragment Gateway^{TM} system (Invitrogen) where homologous regions upstream and downstream of the target gene were cloned into the pDONR vectors as well as the drug selection marker HXGPRT. The full KO vectors containing a drug selection marker flanked by regions homologous to the target gene were transfected into the RHΔ*ku80* parasite background (Huynh and Carruthers, 2009). Generation of cell lines replacing the drug marker with Click-Luciferase (cLUC) were carried out in the same manner. Following drug selection, site-specific integration and gene deletion was monitored by PCR screening and Western blotting. Mutations in ROP17 were introduced with the QuickChange II XL mutagenesis kit (Stratagene). A full list of primers, plasmids, and strains is provided in Tables S2-4.

Generation of Tet-On-TAP Cloning Vector. The pTet07-SAG1/myc-GFP/SAG1 vector was digested with KpnI/PacI restriction enzymes followed by phosphatase treatment. The RPS13 promoter containing integrated Tet-operator sites was amplified with 5' KpnI and 3' MfeI restriction sites that include a *T. gondii* specific Kozak sequence and Alanine as the 2nd amino acid to maximize expression ((gtcaaa: Kozak) and (atggcg: start Methonine and Alanine)) sequence in the 3' end before MfeI and Start codon. The multiple cloning site (MCS) and TAP-tag were amplified with 5' MfeI and 3' PacI cut sites. A 3 fragment ligation was carried out with the digested vector, RPS13 promoter and MCS-TAP-tag fusion. The assembled vector was digested with SpeI followed by phopshatase treatment and gel purification. A phleomycin (ble) resistance gene driven by SAG1 promoters was amplified with 5' and 3' SpeI cut sites and the drug marker was ligated into the Tet-On-TAP vector. The drug cassette was checked for orientation via sequencing/digestion. A total of 6 additional sites were mutated to remove duplicate restriction sites in the MCS. Primers A7/A24 were used to amplify the RPS13 promoter with Tet-Operators (Table S3). Primers A9/10 were used to amplify the MCS-TAP-tag fusion (Table S3). Primers A11/A12 were used to amplify the SAG/Ble/SAG drug marker cassette (Table S3). Redundant restriction sites were removed using the mutagenesis primers A15, A16, A17, A18, A19 and A20 (Table S3). Amplification of Rhoptry kinases for cloning into Tet-On-TAP vector: ROP5 (B5/B6), ROP17 (A35/A36), ROP18 (A33/A34).

Tandem Affinity Purification (TAP)

For scale up, L929 cells from four T175 flasks grown to confluency were trypsinized and transferred to a 1 L spinner flask (Bellco Biotechnology) grown in suspension in 500 ml of complete medium. The cell spinner was operated between 50-60 revolutions per min max. When cells reached a concentration of 5 x 10^5 cells/ml, they were infected at a multiplicity of infection of 10:1 as follows. The host cells and *T. gondii* were centrifuged together at 900*g* at 4°C for 10 min and then transferred back into the spinner flask. To monitor the efficiency of infection, samples of infected host cells were taken at 24 and 36 hr. Assessment of the percentage of infected cells was done via flow cytometry to detect the constitutively expressed Tet-Repressor fused to YFP. An infection rate of 80-90% provided optimal yields. Parasites were induced to express the TAP-tagged construct 2 hr after the infection step by addition of 0.15 µg/ml Tet. At 48 hr, the cells were centrifuged at 1,000*g* at 4°C for 10 min and the pellet passed through a 22 gauge needle 3 times to lyse any remaining host cells. Parasites were washed 2 times with cold PBS to remove contaminating debris. Approximately 4 x 500 ml spinner flask infections were used per TAP purification procedure that yielded 3 to 5 grams w/w parasite material. For the TAP-purification, parasites were lysed by sonication in extraction buffer (50 mM Tris-HCl pH 7.6, 150 mM KCl, 1 mM EDTA, 1 x cOmplete EDTA free protease inhibitor (Roche) and 0.5% NP-40. The extract was centrifuged in a TLA100.3 rotor at 90,000 rpm (437,003*g*) for 10 min in a Beckman Optimal TL Ultracentrifuge. The soluble portion was recovered and the pellet was re-extracted with sonication as above with an equal volume of extraction buffer without NP-40. The pellet extract was centrifuged again and the soluble portion was removed and combined with the initial soluble portion to lower the final concentration of NP-40 to approximately 0.2%. The extract was applied to Fast Flow Agarose-IgG beads (GE Healthcare) at 4°C and rocked for 2 hr. The agarose beads were captured in a disposable Pierce 1 ml column and washed with IgG binding buffer (20 mM Tris-HCl pH 7.4, 150 mM KCl, 1 mM EDTA, and 0.1% NP-40). The TAP-tagged protein was eluted with 100 units of TEV protease (Invitrogen) onto calmodulin bound agarose beads (Agilent) in the presence of calmodulin binding buffer (20 mM Tris-HCl pH 7.6, 150 mM KCl, 0.1% NP-40, 10 mM B-mercaptoethanol, 1 mM MgOAc, 1 mM imidazole and 2 mM $CaCl_2$). After binding, the column was washed with calmodulin binding buffer and the sample eluted with calmodulin elution buffer (20 mM Tris-HCl pH 7.6, 150 mM KCl, 0.1% NP-40, 10 mM B-mercaptoethanol, 1 mM imidazole, 1 mM EDTA and 3 mM EGTA). Sample fractions were then either run on SDS-PAGE gels, precipitated for MS analysis preparation, or stored in 10% glycerol at -80°C.

MS Analyses

Fractions from calmodulin column were mixed with 1/5th vol of 100% trichloric acid and 0.5% deoxycholate, incubated on ice for 30 min, and centrifuged for 30 min at 18,000*g* at 4 °C. The pellet was washed with ice-cold acetone and air-dried. A two-step protocol with LysC and trypsin proteases was carried out. The pellet was re-suspended in 25 µl of LysC buffer (8 M urea, 100 mM Tris-HCl (pH 8.5)), LysC added to a 1:100 mass ratio and incubated at 37 °C for 4 h. For trypsin digestion, the mixture was supplemented with 75 µl of 50 mM NH_4HCO_3 (pH 7.8), trypsin added to a 1:100 mass ratio, and digestion continued overnight. Peptides were purified using Vivapure C18 microspin columns (Vivascience Corp.). Eluted samples were reduced by vacuum evaporation to 1 µl and 10 µl of 2% ACN + 0.1% formic acid was added. Peptides were analyzed using an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Rockford, USA) coupled with a nanoLC Ultra (Eksigent, Dublin, USA). Each sample (5 µL) was loaded onto a trap column (C_{18} PepMap100, 300 µm ×1 mm, 5 µm, 100 Å, Dionex, Sunnyvale, USA) at a flow rate of 4 µL/min for 5 min. Peptide separation were carried out on a C_{18} column (Acclaim PepMap C18, 15 cm × 75 µm × 3 µm, 100 Å, Dionex) at a flow rate of 0.26 µL/min. Peptides were separated using a 38 min linear gradient ranging from 2% to 65% B (mobile phase A, 0.1% formic acid; mobile phase B, 0.1% formic acid in ACN). The mass spectrometer was operated in positive ionization mode. The MS survey scan was performed in the FT cell from a mass range of 300 to 2000 m/z. The resolution was set to 60,000 @ 400 m/z and the automatic gain control (AGC) was set to 500,000 ions. CID fragmentation was used for MS/MS and the 20 most intense signals in the survey scan were fragmented. Detection was done in the ion trap with an isolation window of 1.5 m/z and a target value of 10,000 ions. Fragmentation was performed with normalized collision energies of 35% and activation time of 30 ms. Dynamic exclusion was performed with a repeat count of 1 and exclusion duration of 50 sec, and a minimum MS signal for triggering MS/MS is set to 5,000 counts.

Data was processed using Mascot Distiller v2.2 and searched using Mascot Daemon 2.2 (Matrix Science, London, UK). Searches were performed against the *T. gondii* database (v6.1) and NCBI nr database (Jan 2011; 12679685 entries) using the following settings: trypsin as cleavage enzyme; one missed cleavage; oxidation (M) and deamidation (N/Q) as variable modifications. The mass error tolerance for precursor ions was set to 15 ppm and 0.8 Da for

fragment ions. The data was submitted to Scaffold 3.3 (Proteome Software, Portland, OR) to validate MS/MS based peptide and protein identifications. Cutoffs for peptide identifications were set at > 80.0%, and protein identifications > 99.0% probability with at least 2 identified peptides. Direct identification of phosphorylated sites was based on > 95% localization probability.

Statistical Analyses

Statistical analyses were conducted with GraphPad (Prism5) using nonparametric tests (i.e. Kruskal-Wallis with Dunn's post test comparison), or Student's *t*-tests (2-tailed test of unpaired samples with equal variance). $*P \leq 0.05$ was considered the minimum cutoff for significance and individual values are reported in the figure legends.

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