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Identification and analysis of novel *Toxoplasma* virulence factors

(トキソプラズマ新規病原性因子の探索と機能解析)

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ABSTRACT 要旨

Toxoplasma gondii, which can infect virtually all warm-blooded cells, secretes various virulence factors which subvert the interferon-gamma (IFN- γ)-dependent immune response. While these secreted proteins have been extensively characterized, the parasite-intrinsic mechanisms underlying virulence remain unclear. I investigated the roles of non-secretory proteins of *T. gondii* in immunosuppression and identified a transcription factor, TgIWS1, and a deubiquitinase, TgJosephin, as novel suppressors of IFN- γ -driven immunity.

TgIWS1 deficiency in *T. gondii* led to increased accumulation of the host IFN- γ -inducible protein IRGB6 around the parasite and loss of virulence in wild-type mice. In contrast, TgIWS1-deficient parasites retained normal virulence in mice lacking the IFN- γ receptor (IFN γ R). Furthermore, lack of TgIWS1 reduced the mRNA expression of ROP18, a secretory kinase that inhibits IRGB6, and ectopic expression of ROP18 recovered virulence in TgIWS1-deficient parasites.

Deletion of deubiquitinase TgJosephin resulted in attenuated virulence in wild-type mice but not in mice lacking IFN γ R. Moreover, TgJosephin expression was sustained by TgRad23, a DNA repair and protein shuttling protein. Notably, TgJosephin depletion led to increased ubiquitination of subpellicular microtubule protein 1 (SPM1), a stabilizing component of the parasite microtubules, and mutating its ubiquitination sites restored virulence in the absence of TgJosephin.

Taken together, these results suggest the TgIWS1–ROP18 pathway and TgRad23–TgJosephin–SPM1 pathway as parasite-intrinsic mechanisms important for resistance to IFN- γ -mediated immunity.

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MAIN 本文

General Introduction

Toxoplasma gondii is a protozoan parasite that can virtually infect all warm-blooded animals and is estimated to have infected one third of the global population (1). Although most human infections are asymptomatic, infection during pregnancy or in immunocompromised individuals may result in severe toxoplasmosis (2). Understanding the molecular mechanisms of *T. gondii* virulence is important for treatment and prevention of toxoplasmosis (3).

Upon invading a host cell, *T. gondii* forms a parasitophorous vacuole (PV) and proliferates within (1). Host cells express various interferon-gamma (IFN- γ)-induced genes, which facilitate cell-autonomous parasite growth inhibition by nitric oxide production and tryptophan degradation (4, 5) and parasite killing by p47 immunity-related GTPase (IRG) activity on the PV membrane (6–8). IRGB6 has been shown to initially accumulate on the *T. gondii* PVM, followed by other IRGs such as IRGA6 and IRGB10, p65 guanylate-binding proteins (GBPs), p62/Sqstm1, and ubiquitin (12, 13), eventually leading to PV rupture and parasite death.

The highly virulent Type I strains of *T. gondii* secrete various virulence factors from rhoptries and dense granules across the PV membrane to evade host immunity (9). Rhoptry kinases, such as ROP17, ROP18, and ROP5, are secreted into the host cell cytoplasm and localized at the PVM to directly phosphorylate and inactivate IFN-inducible GTPases to inhibit their accumulation on the *T. gondii* PVM (10, 11). Loss of ROP18 from a virulent Type I *T. gondii* strain results in decreased virulence in mice (11, 12). While functions of these secreted proteins are widely studied, their regulators and non-secretory contributors to virulence are largely uninvestigated.

An *in vitro* genome-wide loss-of-function screen in a Type I *T. gondii* strain identified >350 genes that determine fitness in IFN- γ -activated murine macrophages (19). Of the highly ranked genes, those encoding putative dense granule proteins were characterized to determine their roles in immunosuppression (19), while most of the others were left uncharacterized. In the first part of my study, I investigated roles of several Type I *T. gondii* proteins encoded by uncharacterized genes in suppression of IRG-dependent host defense. I found that a putative transcription factor called TgIWS1 was important for ROP18-mediated virulence of Type I *T. gondii*. Loss of TgIWS1 in Type I parasites led to markedly increased accumulation of IFN-inducible GTPases at the PVM, and severely reduced parasite virulence in mice. Global gene profile analysis revealed that TgIWS1 deficiency resulted in downregulation of various genes including ROP18. Moreover, ROP18 overexpression in TgIWS1-deficient *T. gondii* lowered recruitment of IFN-inducible GTPases to the PVM and restored *in vivo* virulence. Taken together, the *T. gondii* transcription factor TgIWS1 is important for ROP18 mRNA expression to subvert host cell-autonomous immunity mediated by IFN-inducible GTPases.

On the other hand, an *in vivo* CRISPR screen was performed on the Type I *T. gondii* genome using wild-type and IFN- γ -deficient mice, listing candidates for immunosuppressive genes organized by putative subcellular localization (13, 14). Apart from known secretory proteins, highly ranked candidates included genes in the endomembrane-nucleus sublibrary and the metabolism-related sublibrary (**Fig. 6A and B**). In the second part of my study, I investigated four non-secretory candidates and found that TgJosephin, a highly conserved deubiquitinase, was important for virulence in wild-type mice but not mice lacking the IFN- γ receptor (IFN γ R). In addition, TgJosephin expression was dependent on TgRad23, and loss of TgJosephin led to increased ubiquitination of a microtubule protein SPM1. These results suggest that *T. gondii* suppresses host IFN- γ -dependent immunity through regulation of SPM1 ubiquitination by TgRad23 and TgJosephin.

Results Part 1: *Toxoplasma* TgIWS1 Determines Fitness in Interferon- γ -Activated Host Cells and Mice by Regulating ROP18 mRNA Expression.

IRGB6 loading was increased on the PVM of TgIWS1-deficient parasites

Except for GRA family protein genes GRA45, GRA22, TGGT1_263560, and TGGT1_269950, candidate genes that determine *T. gondii* fitness in IFN- γ -stimulated murine macrophages have not been tested for IRG recruitment (15). Along with ROP proteins secreted from rhoptries, GRA proteins of dense granules have been extensively studied for their roles in parasite virulence (16). Aiming to find new mechanisms of *T. gondii* virulence, I investigated non-GRA genes TGGT1_269620 (encoding an DNA repair-like protein, TgDRL1), TGGT1_314500 (protease, TgSUB2), TGGT1_276940 (putative ribosome-associated membrane protein, TgRAMP4), and TGGT1_227560 (putative transcription factor, TgIWS1) from among the candidates (15).

To explore possible roles of these non-GRA proteins in subversion of host IFN- γ -induced cell-autonomous immunity, I generated Type I single knockout parasites lacking TgDRL1, TgSUB2, TgRAMP4, and TgIWS1 by CRISPR/Cas9 genome editing (**Fig. S1A-B**). Consistent with previous CRISPR screening reports (15, 17), Type I *T. gondii* lacking TgDRL1, TgSUB2, TgRAMP4, or TgIWS1 formed plaques in a manner similar to wild-type parasites (**Fig. 1B**).

IRGB6 is known to be the first IRG recruited onto the *T. gondii* PVM (7, 8). I tested the mutant parasites for IRGB6 recruitment to the PVM in IFN- γ -stimulated mouse embryonic fibroblasts (MEFs) (**Fig. 1C-D**). The percentages of IRGB6-coated PVM were comparable to those for wild-type parasites for the TgDRL1-, TgSUB2-, and TgRAMP4-deficient parasites. Notably, however, TgIWS1-deficient parasites showed higher levels of IRGB6 recruitment to the PVM than the wild-type or the other gene-disrupted parasites (**Fig. 1D**). To assess whether the increased IRGB6 accumulation on the PVM of TgIWS1-deficient parasites was due to the lack of TgIWS1, I complemented TgIWS1 into the endogenous locus in TgIWS1-deficient parasites (**Fig. S1C**) and tested the IRGB6 loading onto the PVM. The percentage of IRGB6 recruitment to the PVM of $\Delta iws1$ parasites reconstituted with TgIWS1 was significantly less than that for the TgIWS1-deficient parasites (**Fig. 1E**), indicating that TgIWS1, but not TgDRL1, TgSUB2, or TgRAMP4, is involved in subversion of the IRGB6-dependent anti-*T. gondii* response of host cells.

TgIWS1 deficiency increased effector recruitment to the PVM

IRGB6 recognizes the *T. gondii* PVM and subsequently elicits downstream recruitment of other effectors such as IRGA6, IRGB10, GBPs, and p62/Sqstm1 (7, 8). Therefore, I next assessed the recruitment of effectors other than IRGB6 to the PVM of wild-type and TgIWS1-deficient parasites. Percentages of PVs coated with IRGA6 and IRGB10 were higher for TgIWS1-deficient parasites than for wild-type parasites in IFN- γ -stimulated MEFs (**Fig. 2A-B**). In addition to IRGs, GBP2, ubiquitin, and p62/Sqstm1 proteins were more frequently detected on the PVM of TgIWS1-deficient parasites than on the PVM of wild-type parasites (**Fig. 2A-B**). Moreover, complementation of TgIWS1 in TgIWS1-deficient parasites markedly decreased the recruitment of IRGA6, IRGB10, and p62/Sqstm1 to the PVM (**Fig. 2C**). Collectively, these results demonstrate that *T. gondii* TgIWS1 negatively regulates host IFN- γ -inducible GTPase-dependent antiparasitic responses.

TgIWS1 deficiency reduced virulence *in vivo*.

TgIWS1 deficiency in Type I *T. gondii* led to defective suppression of IFN-inducible GTPase-mediated cell-autonomous immunity in MEFs, prompting me to examine the effect of TgIWS1 deficiency on *in vivo* virulence (**Fig. 3**). Wild-type mice were intraperitoneally infected with luciferase-expressing wild-type or TgIWS1-deficient *T. gondii* and examined for parasite spreading using an *in vivo* imaging system (**Fig. 3A**). Strong abdominal luciferase signals were detected in the mice infected with wild-type parasites; however, the luciferase signals in mice infected with TgIWS1-deficient parasites were

remarkably decreased (**Fig. 3A**). I next assessed parasite burdens in tissues from mice infected with wild-type or TgIWS1-deficient parasites. At 6 days post-infection, higher parasite burdens were detected in peritoneal cavities and liver, mesenteric lymph nodes (MLN), spleen, and lungs of wild-type parasite-injected mice than TgIWS1-deficient parasite-injected mice (**Fig. 3B**). I next compared the survival of wild-type parasite-infected mice and TgIWS1-deficient parasite-infected mice. Wild-type mice infected with TgIWS1-deficient parasites tended to survive for longer than those infected with wild-type parasites (**Fig. 3C**). Furthermore, TgIWS1 complementation in TgIWS1-deficient parasites restored parasite spread and *in vivo* virulence (**Fig. 3D and 3E**). Moreover, both IFN- γ receptor (IFN γ R)-deficient mice infected with TgIWS1-deficient parasites and wild-type mice infected with wild-type parasites died within ten days after infection (**Fig. 3C**). Taken together, these results indicate that *T. gondii* TgIWS1 is important for suppression of IFN- γ -dependent host defense *in vivo*.

ROP18 mRNA was decreased in TgIWS1-deficient parasites.

I next explored the molecular mechanism(s) by which TgIWS1 suppresses host IFN-inducible GTPase-mediated cell-autonomous immunity. IWS1 was shown to be a transcription elongation factor in yeast (18). Therefore, I compared global gene expression profiles in freshly isolated tachyzoites of wild-type and TgIWS1-deficient Type I *T. gondii*. RNA-Seq analysis demonstrated that hundreds of genes were differentially expressed between wild-type and TgIWS1-deficient parasites (**Fig. 4A**), with upregulation of 101 genes and downregulation of 103 genes in TgIWS1-deficient parasites relative to the wild-type ($p < 0.05$ and $|\log_2 \text{fold-change}| > 1$) (**Fig. 4B**). TgIWS1 mRNA was the most differentially expressed gene between wild-type *T. gondii* and TgIWS1-deficient parasites (**Fig. 4C and Table S2**). Notably, mRNA encoding the virulence factor ROP18, which was shown to inactivate IFN-inducible GTPases (10, 11), was among the genes highly differently expressed between wild-type *T. gondii* and TgIWS1-deficient parasites (**Fig. 4C and Table S2**). Along with TgIWS1 mRNA, TgIWS1-deficient parasites had dramatically decreased levels of ROP18 mRNA as shown by quantitative RT-PCR (**Fig. 4D**). Furthermore, complementation of TgIWS1 restored mRNA expression of ROP18 and TgIWS1 (**Fig. 4D**), suggesting that TgIWS1 is required for ROP18 mRNA expression.

Ectopic ROP18 expression overcame TgIWS1 deficiency.

It was unclear whether the reduction of ROP18 mRNA expression accounted for the defective suppression of host IFN-inducible GTPase-mediated cell-autonomous immunity in $\Delta iws1$ parasites. To clarify the causal relationship, I examined whether TgIWS1-independent ROP18 overexpression could overcome the effect of TgIWS1 deficiency. A plasmid vector that allowed ectopic expression of ROP18 was transfected into TgIWS1-deficient parasites. Wild-type-level ROP18 mRNA expression was confirmed in TgIWS1-deficient parasites transfected with the ROP18-expressing vector ($\Delta iws1::ROP18$) while still lacking TgIWS1 expression (**Fig. 5A**). Then I compared recruitment of IFN-inducible GTPases, ubiquitin, and p62/Sqstm1 to the PVM of wild-type, $\Delta iws1$, and $\Delta iws1::ROP18$ parasites. PVs coated with IRGB6, IRGA6, IRGB10, Gbp2, ubiquitin, and p62/Sqstm1 were abundant in TgIWS1-deficient parasites and sparse in $\Delta iws1::ROP18$ parasites and wild-type parasites (**Fig. 5B**). Parasite viability in IFN- γ -stimulated MEFs was measured using a luciferase reporter assay, relative to that in unstimulated MEFs. TgIWS1 deficiency reduced parasite resistance to the IFN- γ + environment, which was then overcome by ROP18 overexpression (**Fig. 5C**). Taken together, these results demonstrate that ectopic ROP18 expression in $\Delta iws1$ parasites compensated for the susceptibility to IFN-inducible GTPase-mediated cell-autonomous immunity *in vitro*.

Finally, I assessed whether the TgIWS1-independent ROP18 expression could also restore *in vivo* virulence. To assess the parasite spread, wild-type mice were intraperitoneally infected with luciferase-expressing wild-type parasites, TgIWS1-deficient parasites, or $\Delta iws1::ROP18$ parasites. $\Delta iws1::ROP18$

parasites showed stronger luciferase signals TgIWS1-deficient parasites, but weaker than wild-type parasites (**Fig. 5D**). Consistent with the *in vivo* parasitic spread, deaths of mice infected with $\Delta iws1::ROP18$ parasites were delayed compared to those infected with wild-type parasites, but all died within 20 days while most mice infected with $\Delta iws1$ parasites survived for over 30 days (**Fig. 5E**). Collectively, these data show that TgIWS1-independent ROP18 overexpression considerably, though not completely, restored the *in vivo* virulence of TgIWS1-deficient *T. gondii*.

TgIWS1 indirectly promotes ROP18 mRNA expression.

To assess whether TgIWS1 controls ROP18 mRNA expression directly or indirectly, I conducted genome-wide chromatin immunoprecipitation coupled with high-throughput sequencing (ChIP-seq) based upon the binding site information for TgIWS1 in the *T. gondii* genome. First, I generated *T. gondii* expressing the N-terminally Venus-tagged TgIWS1 (**Fig. S2A**). The Venus-TgIWS1 was detected solely in the parasitic nucleus but not in the cytosol, whereas Venus expressed alone was observed in both regions (**Fig. S2B**). Next, I performed ChIP-seq using an anti-GFP (green fluorescent protein) antibody. The obtained DNA was sequenced with the NovaSeq 6000 sequencer, and reads were mapped onto the *T. gondii* GT1 genome sequence. Three independent ChIP-seq analyses identified peaks in the same regions on the genome (**Fig. S2C**), suggesting high reproducibility in the replicates. No peaks were observed on the promoter, coding, and 5'- or 3'-untranslated regions of the ROP18 gene (**Fig. S2D**), indicating that the Venus-TgIWS1 does not directly bind with the ROP18 gene region. I found 453 common peaks on the *T. gondii* GT1 genome in all three replicates (ChIP-seq, data not shown). Furthermore, putative TgIWS1 direct target genes with the common peaks included nine genes encoding known AP2 family transcription factors (**Table S3**). Among them, TgAP2VIII-7 mRNA expression was 23% lower in TgIWS1-deficient parasites compared with wild-type parasites (**Table S2, Table S3**). Taken together, the ChIP-seq analyses indicated that TgIWS1 indirectly influences ROP18 mRNA expression.

Figure 1. TgIWS1 deletion increased loading of IRGB6 onto *T. gondii* PVM.

(A) Schematic representation of the *in vitro* CRISPR screen by Wang et al.(15). *T. gondii* mutants made with a gRNA library were passaged in HFFs with pyrimethamine and then in BMDMs. Mutations were identified as: insignificant gene mutations (green); mutants of genes important for resistance to IFN- γ (red), and mutants of genes important for fitness BMDMs (blue).

(B) Optical microscope images of plaques in MEFs. Plaques: arrowheads.

(C) Confocal microscope images represent the localization of IRGB6 (red) to *T. gondii* vacuoles (green), and DAPI (blue) at 2 h post-infection of WT or mutant parasites in MEFs treated with 10 ng/ml IFN- γ .

(D) Percentages of IRGB6-coated PVs in WT or mutant parasites corresponding to (C).

(E) Confocal microscope images (left) and the graphs (right) represent the localization of IRGB6 (red) to *T. gondii* vacuoles (green), and DAPI (blue) at 2 h post-infection of $\Delta iws1$ or TgIWS1-rescued parasites in MEFs treated with 10 ng/ml IFN- γ .

(C-E) All images are representative of three independent experiments. White arrowheads indicate PVs. Scale bars: 10 μ m. A total of 100 PVs were counted in each experiment. Indicated values are means $\pm$ SD of three biological replicates. * $p < 0.05$, ** $p < 0.01$, N.S. not significant.

Fig 1

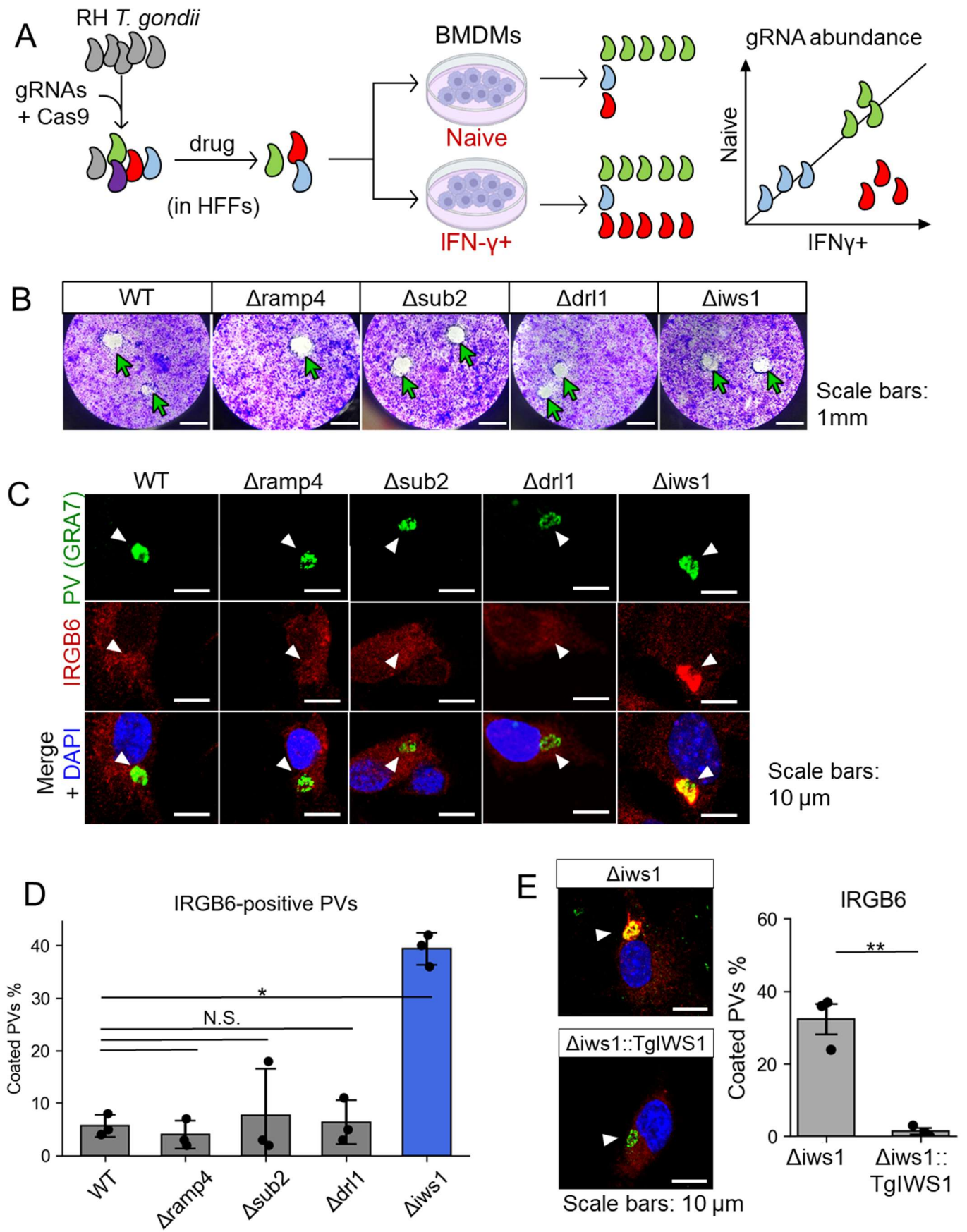
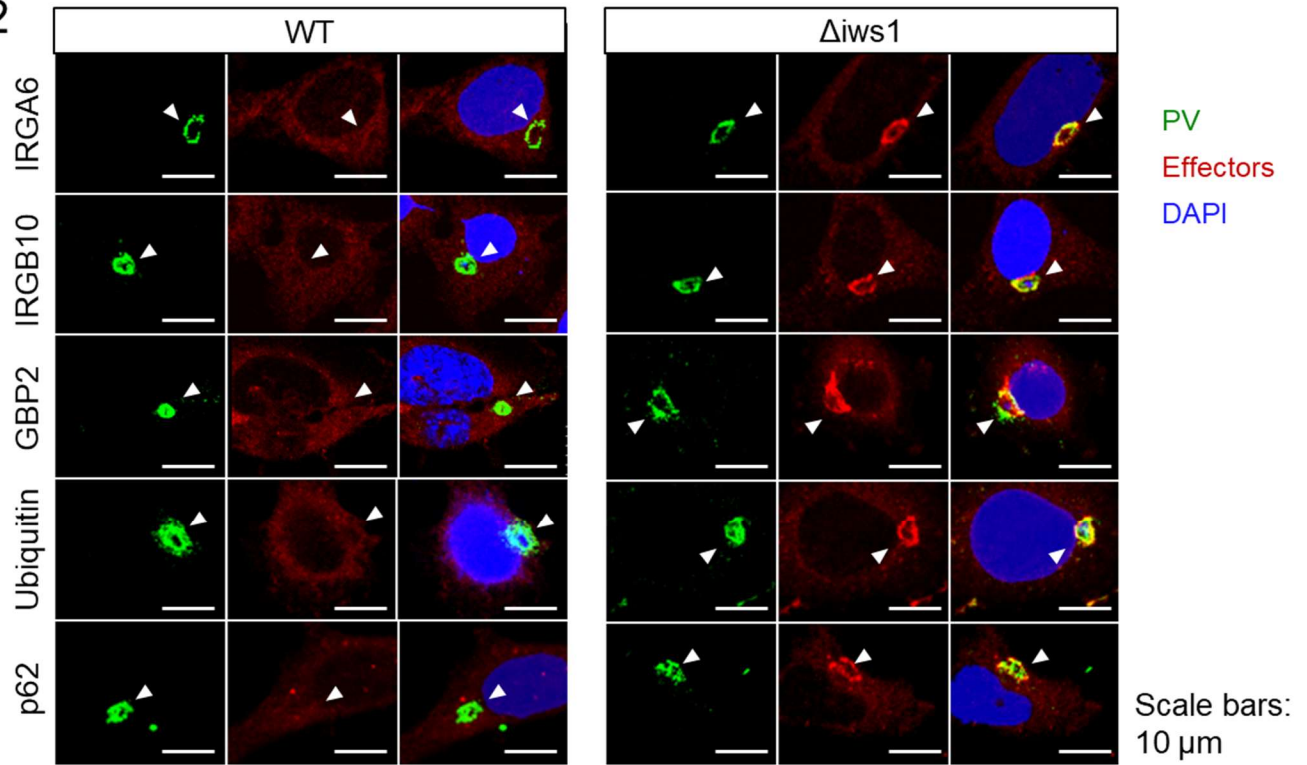
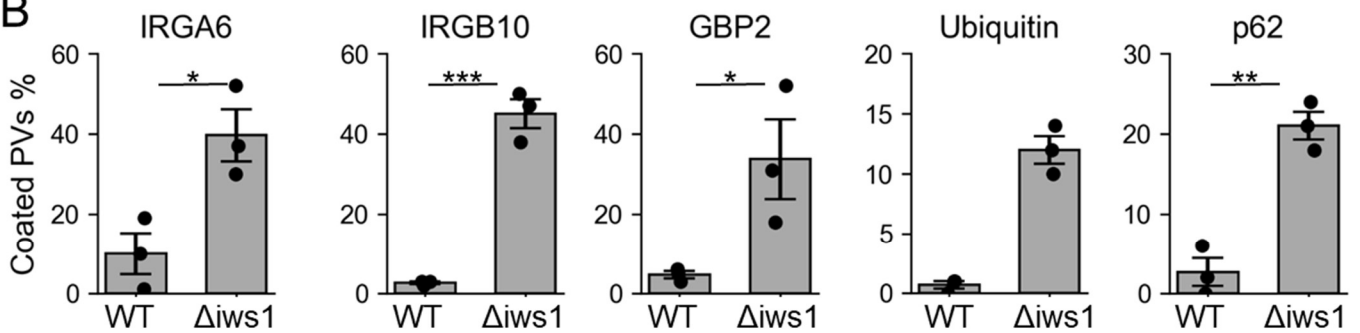


Fig 2

A



B



C

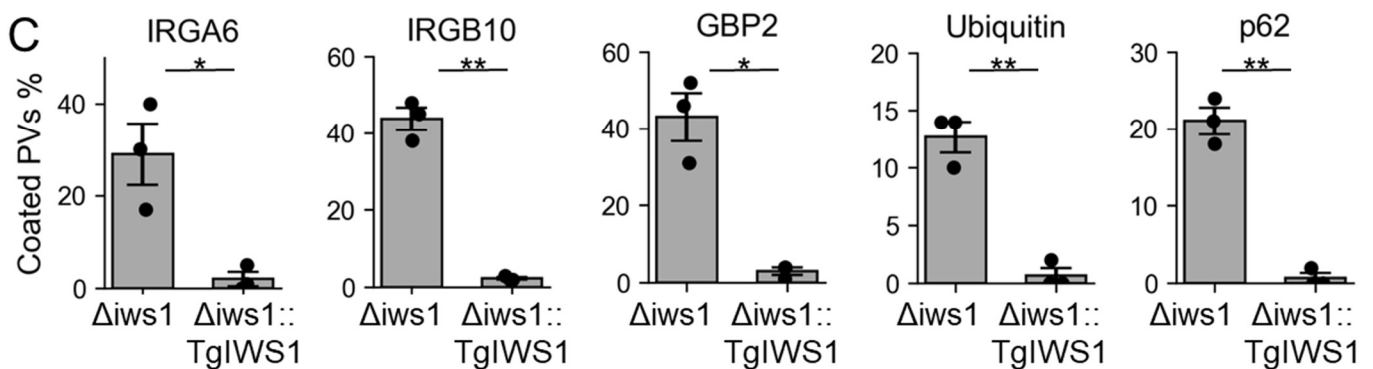


Figure 2. TgIWS1 deletion increased loading of IFN- γ -inducible factors onto *T. gondii* PVM.

(A, B) Confocal microscope images (A) and the graphs (B) represent the localization of IRGA6, IRGB10, Gbp2, ubiquitin, and p62 (red) to *T. gondii* vacuoles (green), and DAPI (blue) at 2 h post-infection of WT or $\Delta iws1$ parasites in MEFs treated with 10 ng/ml IFN- γ . White arrowheads: PVs

(C) Recruitment rate of the indicated host effectors on PVs of $\Delta iws1$ or TgIWS1-rescued parasites at 2 h post-infection of $\Delta iws1$ or TgIWS1-rescued parasites in MEFs treated with 10 ng/ml IFN- γ .

Total of 100 PVs were counted in each experiment. Indicated values are means $\pm$ SD of three biological replicates. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig 3

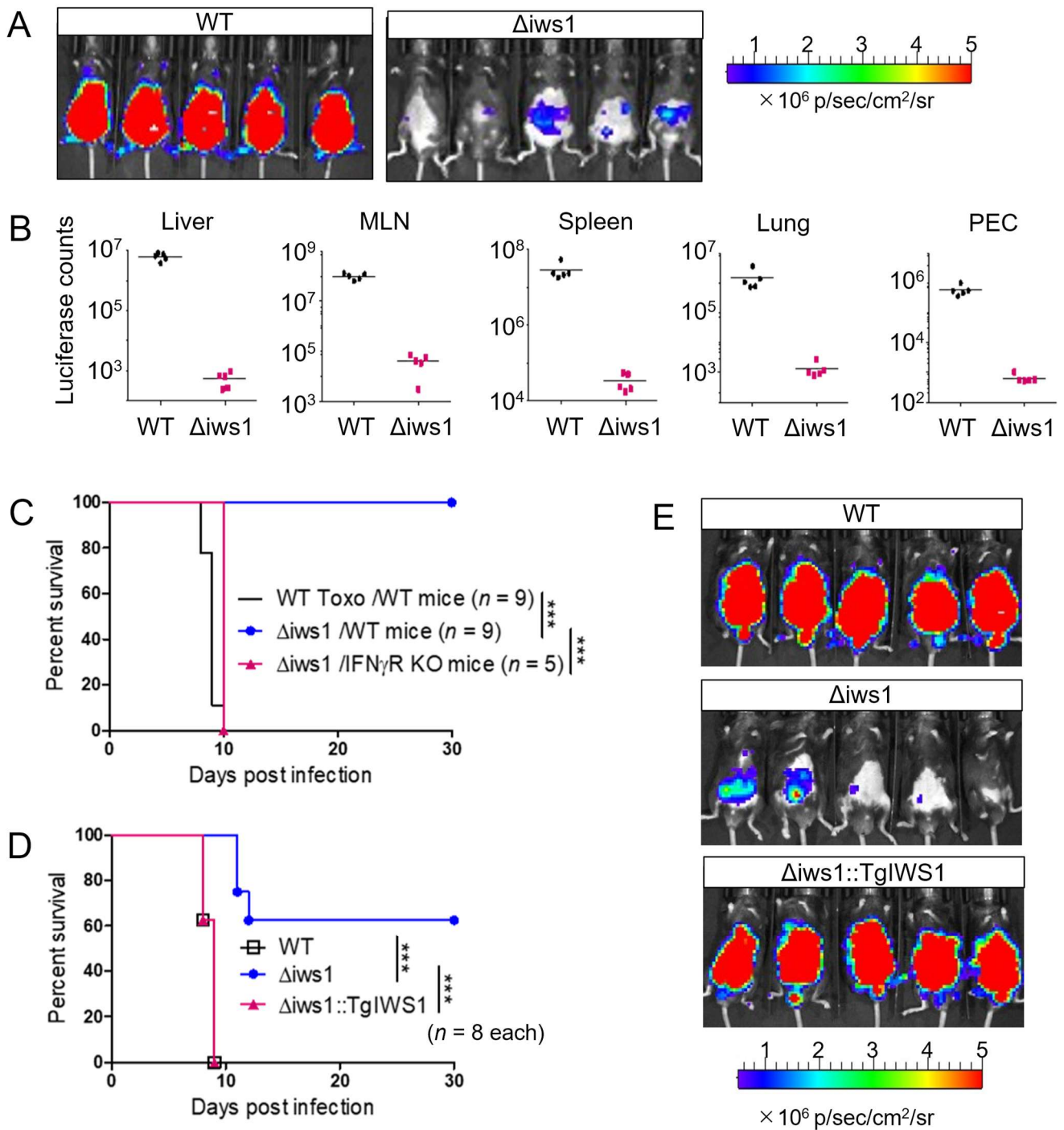


Figure 3. Loss of TgIWS1 reduced virulence in vivo.

(A) Bioluminescence imaging of mice infected with WT or $\Delta iws1$ parasites at day 6 post infection.(B) Luciferase counts in indicated organs of mice infected with WT or $\Delta iws1$ parasites ($n = 5$).(C) Survival curves of WT mice or IFN γ R-deficient mice infected with WT parasites or $\Delta iws1$ parasites.(D) Survival curves and (E) bioluminescence images (day 6 post infection) of WT mice infected with WT parasites, $\Delta iws1$ or TgIWS1-rescued $\Delta iws1$ parasites.*** $p < 0.001$, N.S. not significant.

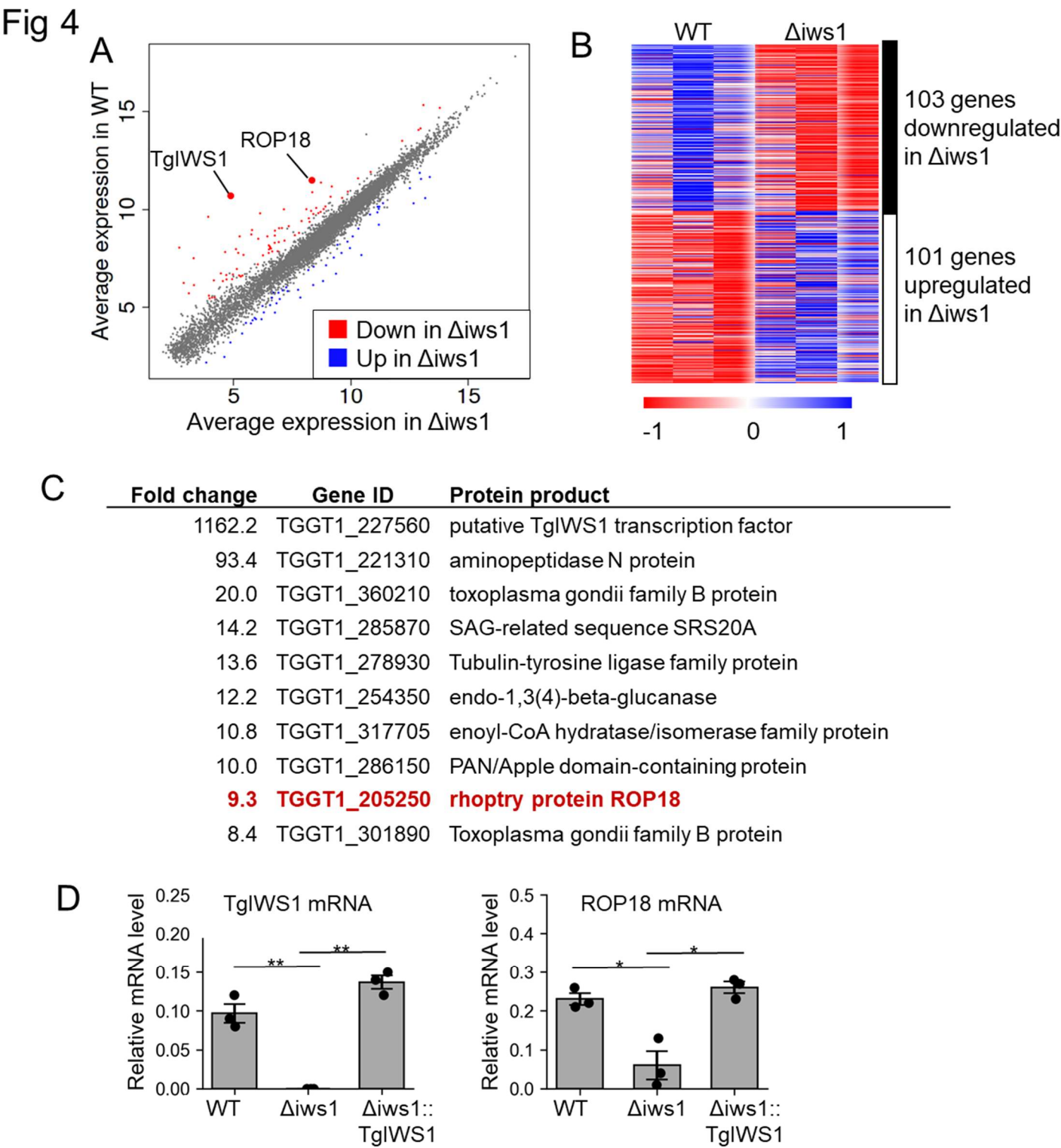


Fig 5

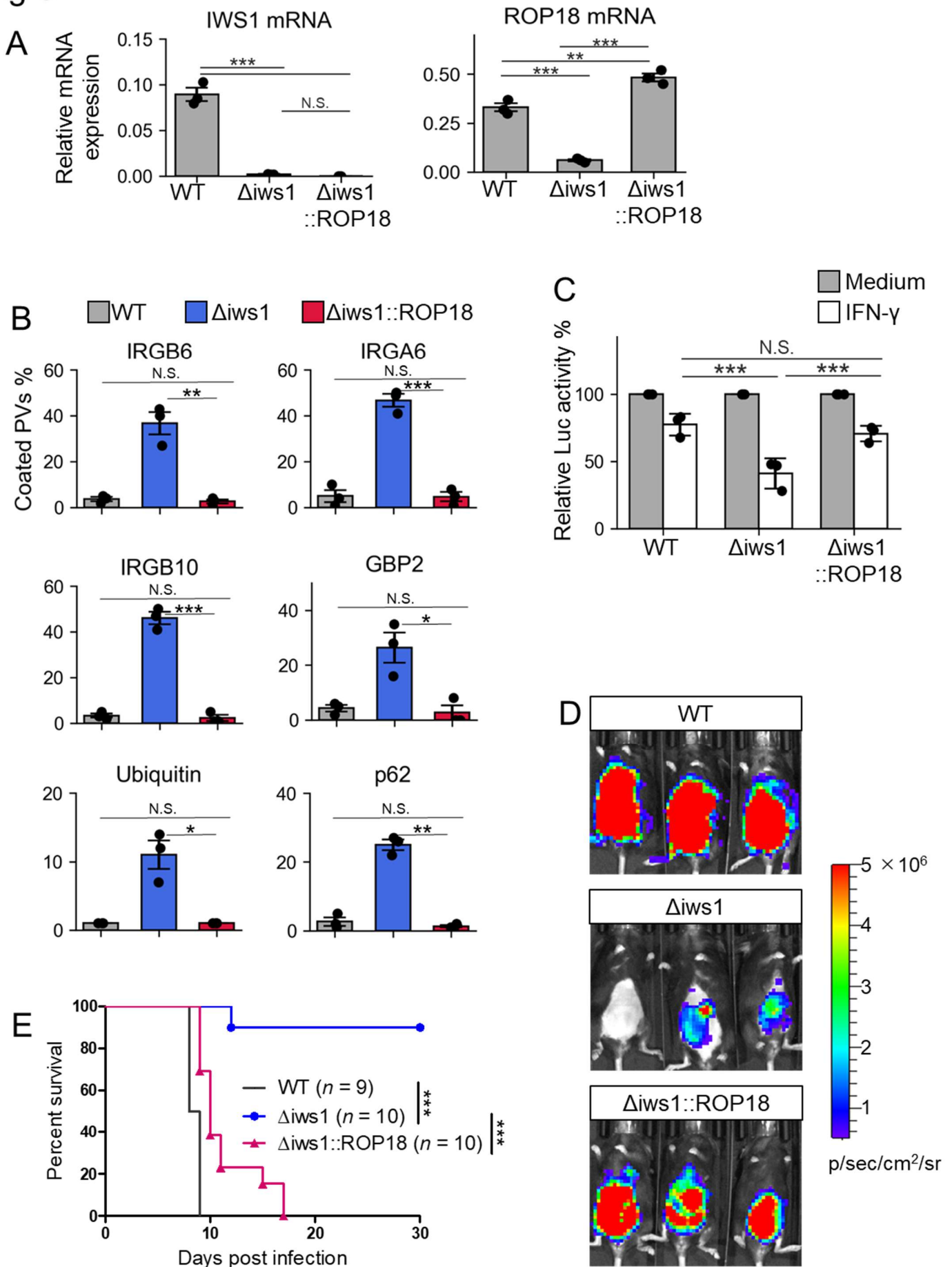


Figure 5. Overexpression of ROP18 in Δ iws1 parasites restored virulence phenotypes in TgIWS1-deficient parasites.

- (A) Quantitative RT-PCR analysis of WT, Δ iws1 or ROP18-overexpressing Δ iws1 parasites (Δ iws1::ROP18). Gene expression was normalized relatively to TgACT1.
- (B) Percentages of PVs coated with IRGA6, IRGB10, Gbp2, ubiquitin and p62 at 2 h post-infection of parasites in MEFs pre-treated with 10 ng/ml IFN- γ . Total of 100 PVs were counted in each experiment. White arrowheads indicate PVs. Scale bars, 10 μ m.
- (C) Relative luciferase counts of IFN- γ -treated or untreated cells infected with WT, Δ iws1, or Δ iws1::ROP18 parasites. Data shown are relative luciferase counts in IFN- γ -treated vs untreated samples of indicated parasite lines.
- (D) Bioluminescence images at day 6 post infection and (E) survival curves of mice infected with WT, Δ iws1 or Δ iws1::ROP18 parasites.

Indicated values are means $\pm$ SD of three biological replicates (A, B, C). * p < 0.05, ** p < 0.01, *** p < 0.001, N.S. not significant.

Results Part 2: TgJosephin And TgRad23 Are Important For Anti-IFN- γ Virulence via Deubiquitination of SPM1 In Toxoplasma.

TgRad23 and TgJosephin deletion reduced virulence *in vivo*.

Our CRISPR/Cas9 *in vivo* screen using wild-type and IFN γ R-knockout mice (**Fig. 6A**) revealed a signal peptidase (TGGT1_214090) named TgRqc2 (19) assigned to the non-chromatin region of the nucleus, along with three putative metabolism-related proteins TgRad23 (TGGT1_295340), TgJosephin (TGGT1_229650), and F-actin capping protein subunit beta TgCapZB (TGGT1_219290) assigned to the parasite cytosol as candidates for virulence factors against IFN- γ -mediated immunity (**Fig. 6B**).

To investigate possible roles of TgRqc2, TgRad23, TgJosephin, and TgCapZB, I generated individual knockout Type I parasites with CRISPR/Cas9 genome editing (**Fig. S3A, B**). First, parasite replication and plaque formation in unstimulated MEF monolayers was examined to assess whether the mutations affected parasite growth *in vitro*. All mutants showed normal replication in MEFs for 48 hours (**Fig. 6C**). However, parasites lacking the TgCapZB (Δ 219290) produced smaller plaques in eight days compared to the others, suggesting its vital role in *in vitro* growth (**Fig. 6D, Fig. S6A**). Since the aim of this study was to analyze their roles as virulence factors, the TgCapZB was omitted from further experiments.

Next, to assess whether these mutations affected parasite virulence *in vivo*, wild-type mice were infected with 10^3 wild-type or mutant tachyzoites via footpad injection. While mice infected with wild-type or TgRqc2-deficient strains all died within 17 days post infection, mice infected with TgRad23 or TgJosephin deficient strains showed significantly prolonged survival (**Fig. 6E**). Taken together, these results suggest that TgRad23 and TgJosephin are novel virulence factors.

TgJosephin and TgRad23 are important for suppression of IFN γ -dependent immunity.

To understand how TgJosephin and TgRad23 contribute to *T. gondii* virulence *in vivo*, I compared the survival of wild-type mice and mice lacking the IFN- γ receptor (IFN γ R) infected with parasites lacking TgJosephin or TgRad23. For both TgRad23 and TgJosephin mutants, all IFN γ R knockout mice died within 17 days post infection while wild-type mice survived after 20 days (**Fig. 7A**). This suggests that TgJosephin and TgRad23 are important for specifically suppressing IFN- γ -mediated immunity.

TgRad23 and TgJosephin are homologous to mammalian Rad23A/B and Ataxin-3 (20–22), (**Fig. S5**). Rad23A/B participates in nucleotide excision repair and protein shuttling to the proteasome (23, 24), while ataxin-3 is a deubiquitinase known for its mutation that causes a neurodegenerative disorder called Machado–Joseph disease, or spinocerebellar ataxia type 3 (25). The roles of their apicomplexan counterparts in virulence are relatively understudied. Deletion of UBL-UBA shuttle proteins including TgRad23 results in accumulation of ubiquitinated proteins (26). Ataxin-3, a homologue of TgJosephin, is known to interact with Rad23, and knockdown of Rad23 results in lower levels of ataxin-3 protein in mammalian cells and *Drosophila* (27), indicating that Rad23 maintains Ataxin-3 protein expression. To investigate possible connections between the *T. gondii* counterparts TgRad23 and TgJosephin, a TgRad23-deficient strain was generated using Type I parasites expressing an HA tag at the C-terminal region of the endogenous TgJosephin gene (**Fig. 7B**). HA-tagged TgJosephin detection was diminished in the TgRad23-deficient strain, suggesting that TgRad23 maintains TgJosephin protein abundance (**Fig. 7C**).

TgJosephin suppresses an IRG-independent immune pathway.

To further investigate the mechanism by which TgJosephin inhibits IFN- γ -dependent immunity, I first tested *in vitro* markers of the cell autonomous immunity in wild-type and Δ josephin parasites. TgJosephin-deficient parasites did not show increased loading of IFN- γ -inducible host factors IRGB6, IRGA6, p62, GBP1, GBP2, and GBP1-5 on PV membranes compared to wild-type parasites, suggesting that TgJosephin controls an immune suppression mechanism independent of IFN-inducible GTPase

inhibition (**Fig. 8A**).

I then examined the virulence of TgJosephin-deficient parasites in wild-type mice or mice lacking IFN γ R, inducible nitric oxide synthase (iNOS), or indoleamine 2,3-dioxygenase (IDO). In 30 days post infection, 80% of wild-type mice survived while all mice lacking IFN γ R died, and mice lacking iNOS and IDO were reduced to 33% and 50%, respectively (**Fig. 8B**). Compared to wild-type mice, IFN γ R- and iNOS-deficient mice were shown here to be significantly more susceptible to Δ Josephin parasites.

These results suggest that TgJosephin suppresses the growth inhibitory function mediated by iNOS, not PV coating and destruction by IFN-inducible GTPases.

The deubiquitinase catalytic domain is important for TgJosephin-mediated virulence.

Previous studies in mammalian cells indicate that functions of Josephin family proteins include deubiquitination, aggresome formation, and docking of substrates to the proteasome (24, 28, 29). TgJosephin has the conserved catalytic domain (the Josephin domain), three ubiquitin-interacting motifs, and a UBX domain, generally found in Cdc48/p97 cofactor proteins involved in various cellular functions (30) (**Fig. 9A, S5B**).

I aimed to confirm that the catalytic activity was critical to TgJosephin-mediated virulence. I searched for function-compromising mutation sites of the TgJosephin protein by alignment with mammalian homologues using MEGA11(31). Cysteine 24 of TgJosephin aligned with the active-site cysteine 14 of human Ataxin-3 and cysteine 24 of human Josephin2, which are crucial for their catalytic enzyme activity (32–34).

To investigate whether mutation of this cysteine 24 to serine (C24S) affected *T. gondii* virulence *in vivo*, Δ Josephin parasites were complemented with a FLAG-tagged TgJosephin or TgJosephin^{C24S} cDNA in their putative uracil phosphoribosyltransferase FUR1 (UPRT) gene region and injected into footpads of wild-type mice (**Fig. 9A, D**). Mice infected with parasites expressing wild-type TgJosephin all died within 16 days. On the other hand, mice infected with parasites expressing TgJosephin^{C24S} survived for 20–30 days (**Fig. 9D**). These results demonstrate that the deubiquitinase activity of TgJosephin is important for its role in immunosuppression.

SPM1 ubiquitination was increased in TgJosephin-deficient parasites.

I searched for virulence factors among substrates of TgJosephin by comparison of ubiquitinated peptide abundances in wild-type and Δ Josephin parasites isolated from Vero or IFN- γ -stimulated MEF cells using ubiquitin-targeted mass spectrometry. No significant changes in ubiquitination of known virulence factors such as GRA proteins and ROP proteins were found between wild-type and Δ Josephin strains. Meanwhile, the highest increase rate of ubiquitination by TgJosephin deletion was found in microtubule-associated protein SPM1 (**Fig. 10A and Table S4**).

To investigate the significance of SPM1 ubiquitination in parasite virulence, I generated SPM1-knockout (Δ spm1) and SPM1-TgJosephin-double knockout (DKO) parasites and complemented these with a tagged SPM1 or the mutant SPM1^{KR}, of which all the detected ubiquitin target lysine residues were mutated to arginine (**Fig. 10B–C and Fig. S3D**). Expression of SPM1 and SPM1^{KR} was confirmed by western blotting and RT-qPCR (**Fig. 10D, Fig. S3C**).

SPM1 is a component of the subpellicular microtubules of apicomplexans that is important for *T. gondii* viability and resistance of microtubules against detergent treatment *in vitro* (35, 36). There are currently no reports on the role of *T. gondii* SPM1 in IFN- γ -stimulated cells or immunosuppression *in vivo*. Interestingly, deletion of SPM1 resulted in a mild virulence defect in wild-type mice, for which SPM1 and the mutant SPM1^{KR} could equally complement (**Fig. 10E**).

Next, to address whether increased ubiquitination of SPM1 affected immunosuppression in TgJosephin-deficient parasites, wild-type mice were infected with Δ Josephin or Δ spm1 Δ Josephin (DKO)

strains complemented with SPM1 or SPM1^{KR}. Notably, all mice infected with parasites lacking TgJosephin and expressing the normal SPM1 survived for over 30 days after infection, while mice infected with the SPM1^{KR}-expressing strain died within 20 days (**Fig. 10F**). These data suggest that SPM1 ubiquitination is generally harmless to the parasite but can impede its virulence in TgJosephin-deficient conditions.

Figure 6. TgRad23 and TgJosephin deletion reduced virulence *in vivo*.

- (A) Schematic representation of the *in vivo* CRISPR screen made using <https://BioRender.com>. *T. gondii* mutants made with a gRNA library were selected in Vero cells with pyrimethamine and subcutaneously injected in footpads of mice. Mutations of parasites retrieved from the spleen were identified as of insignificant genes (green); genes important for parasite fitness *in vitro* (purple), *in vivo* (blue), and resistance to IFN- γ -dependent immunity (red).
- (B) Scatterplot showing *in vivo* fitness scores for each gene in WT and IFN γ R-deficient mice in the indicated hyperLOPIT-determined sub-libraries. Newly reported genes are marked in blue. ROP18 is marked in black as a control gene.
- (C) *In vitro* replication assay. 100 PVs per strain were counted for each experiment to compare parasite numbers per PV. $n = 3$.
- (D) Quantification of plaque sizes in five parasite strains in three experiments. “Strain name (total plaque number in three experiments)” and plaque size (mm²) are shown. The three experiments are distinguished by color. Data was compared with the Mann–Whitney test. *** $p < 0.001$.
- (E) Survival analysis of WT mice infected with the indicated parasites. *** $p < 0.001$.

Fig 6

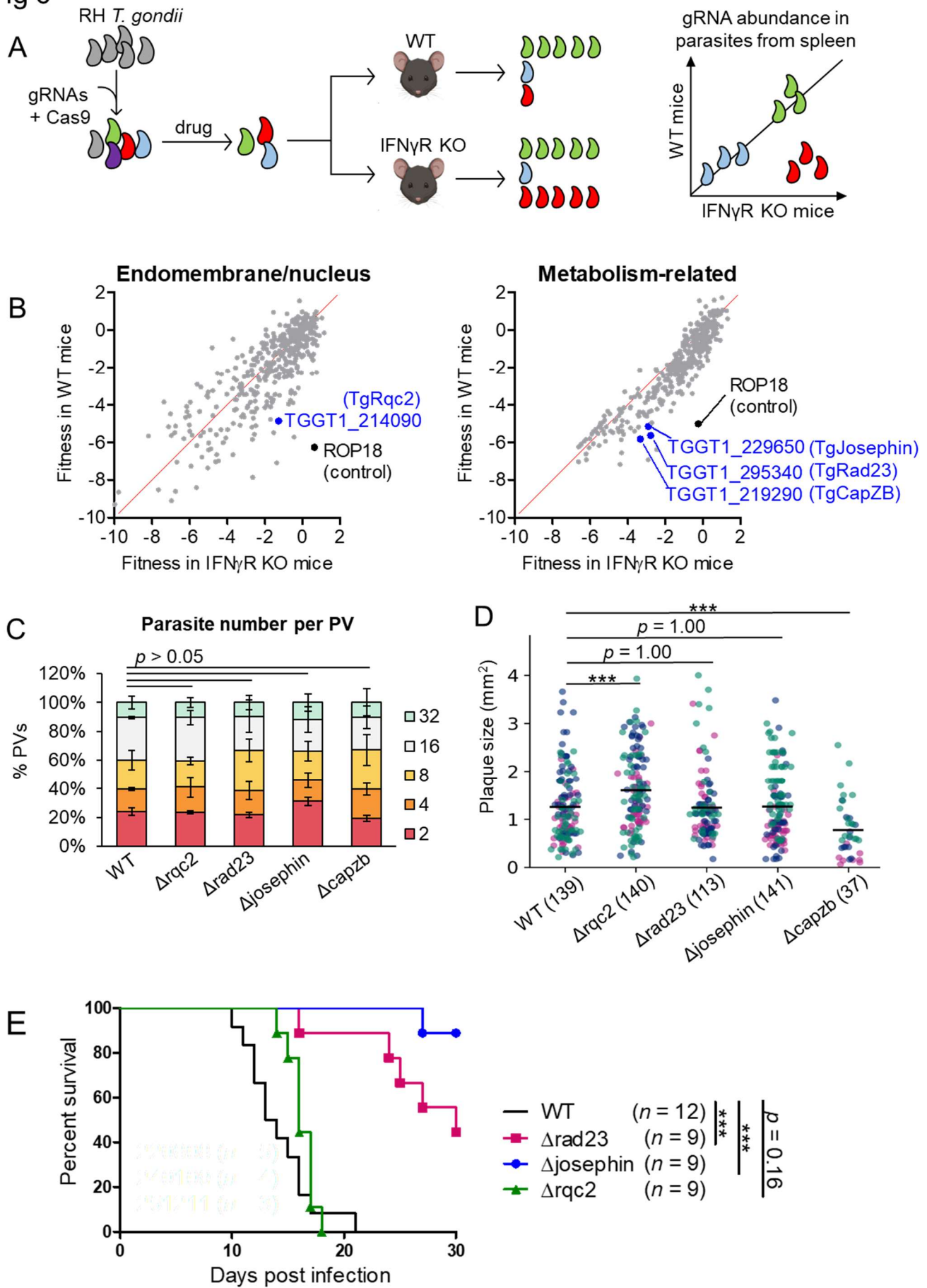


Fig 7

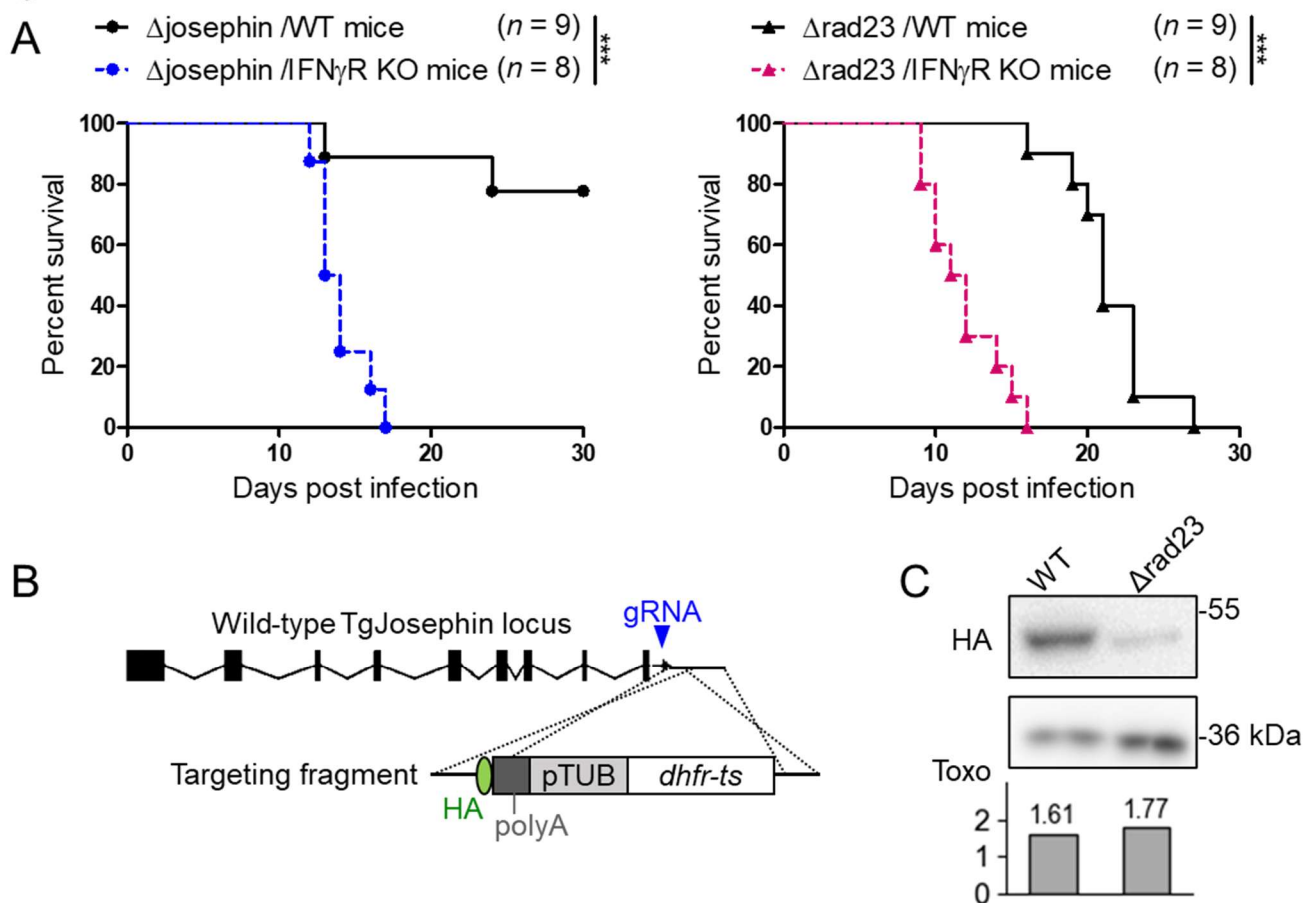


Figure 7. TgJosephin and TgRad23 are important for suppression of IFN- γ -dependent immunity. (A) Survival analyses of WT or IFN γ R-deficient mice infected with TgRad23- or TgJosephin-deficient parasites. *** $p < 0.001$.

(B) C-terminal tagging of TgJosephin in the endogenous TgJosephin gene region with an HA tag-containing gene cassette.

(A) Western blot of WT and Δ rad23 parasites expressing an endogenous TgJosephin tagged with HA, stained with anti-HA and anti-Toxo antibodies. Bar graph: HA/Toxo signal intensity ratio of each lane.

Fig 8

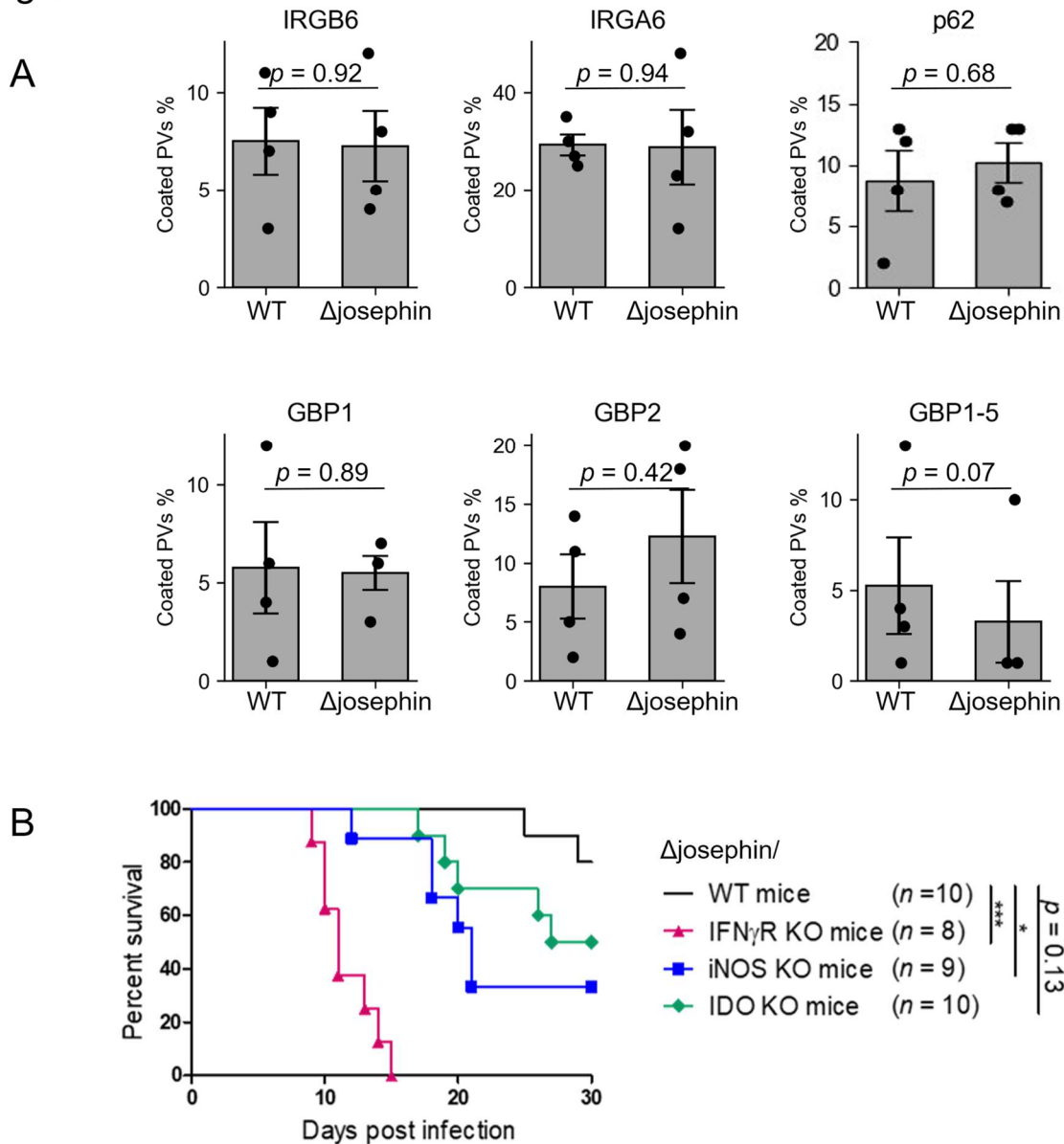


Figure 8. TgJosephin deletion did not increase loading of IFN- γ -induced proteins onto *T. gondii* PVs.

(A) Graphs represent the percentage of IRGB6, IRGA6, p62, GBP1, GBP2, and GBP1-5-coated PVs (marked with GRA5 or GRA17) at 2 hours post infection with WT or Δ josephin parasites in MEFs treated with 10 ng/mL IFN- γ . 100 PVs were counted in each experiment. Mean $\pm$ SEM of four biological replicates are shown.

(B) Survival analysis of WT mice or mice lacking IFN γ R, iNOS, or IDO infected with TgJosephin-deficient parasites.

* $p < 0.05$; *** $p < 0.001$; N.S., not significant.

Fig 9

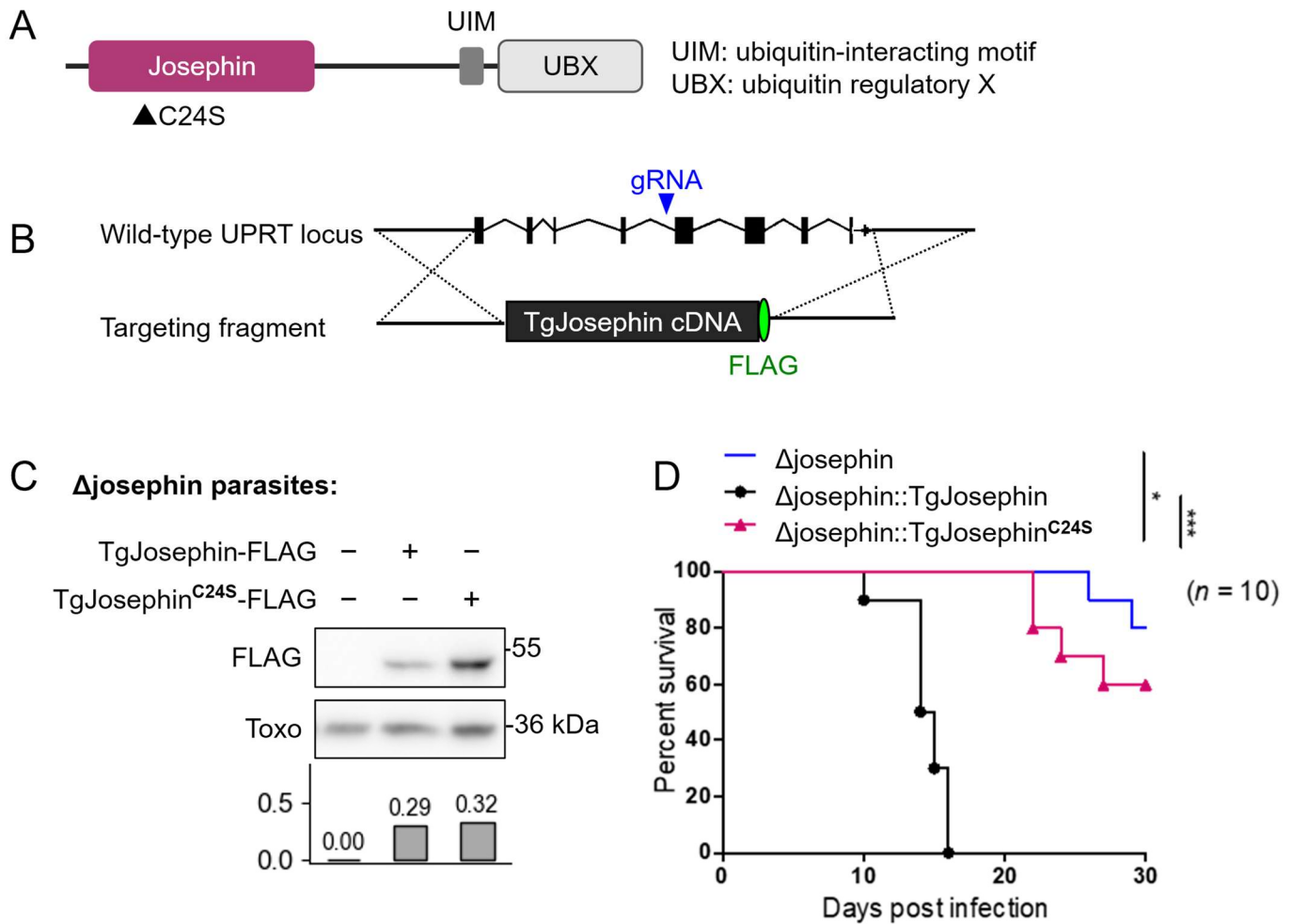


Figure 9. The deubiquitinase catalytic domain is important for TgJosephin-mediated virulence.

(A) The Josephin domain, UIM (ubiquitin-interacting motif), and UBX (ubiquitin regulatory X) domain of TgJosephin with the C24S mutation site indicated below.

(B) Complementation of TgJosephin in the UPRT (putative uracil phosphoribosyltransferase FUR1) locus of the ΔJosephin strain.

(C) Western blot analysis of FLAG-tagged TgJosephin in ΔJosephin parasites with an anti-Toxo antibody detection as a positive control. Bar graph: FLAG/Toxo signal intensity ratio is shown for each lane.

(D) Survival analysis of mice infected with TgJosephin-deficient parasites with or without complementation with TgJosephin or TgJosephin^{C24S}. ** $p < 0.01$; N.S., not significant.

Fig 10

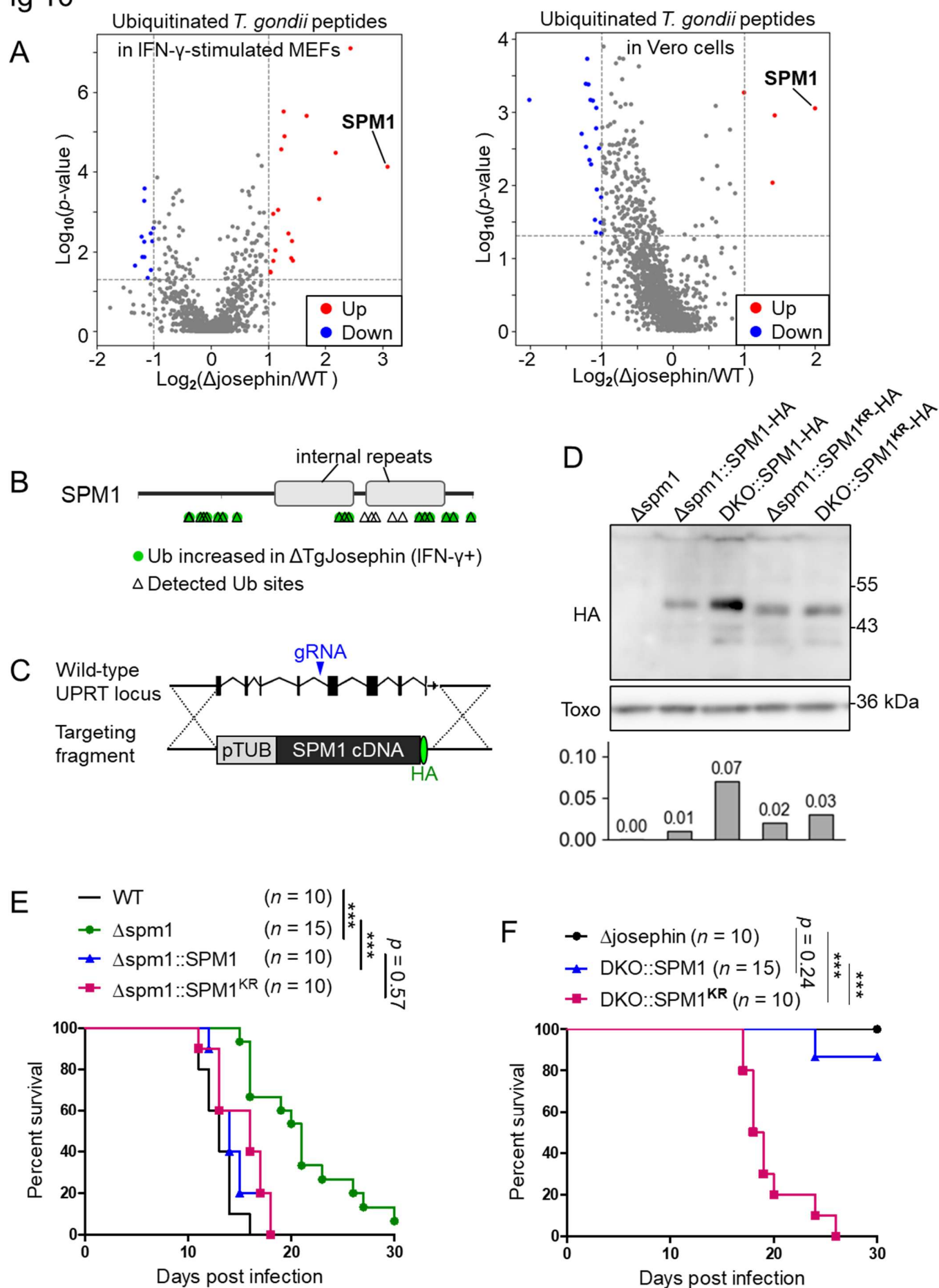


Figure 10. SPM1 ubiquitination was increased in TgJosephin-deficient parasites.

(A) Volcano plots of differentially ubiquitinated peptides between WT and Δ josephin parasites extracted from IFN- γ -stimulated MEF cells (left) and Vero cells (right). Abundance of each peptide was determined with ubiquitin-targeted mass spectrometry across five biological replicates each. Each dot represents a *T. gondii* peptide.

(B) Schematic representation of the *T. gondii* SPM1 protein domain structure predicted by SMART. Arrowheads represent lysine residues detected as ubiquitination sites by ubiquitin-targeted mass spectrometry. Sites with higher ubiquitination rates in Δ josephin parasites compared to WT parasites ($p < 0.05$, student's t test) are marked in green.

(C) Complementation of SPM1 in the UPRT locus of the Δ spm1 strain. pTUB: TGME49_316400 alpha tubulin TUBA1 promoter region.

(D) Western blot analysis of HA-tagged SPM1 with anti-Toxo antibody detection as a positive control. Parasite samples blotted with a mouse anti-HA antibody (HA.11) following immunoprecipitation with a rabbit anti-HA antibody. Bar graph: HA/Toxo signal intensity ratio is shown for each lane.

(E) Survival analysis of mice infected with WT parasites, Δ spm1 parasites, or Δ spm1 parasites complemented with SPM1 or SPM1^{KR}.

(F) Survival analysis of mice infected with Δ josephin or DKO parasites complemented with either SPM1 or SPM1^{KR}.

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; N.S., not significant. DKO: Δ spm1 Δ josephin double knockout.

Discussion

My research suggests two novel anti-IFN γ virulence mechanisms of *T. gondii*: regulation of ROP18 expression by TgIWS1 and deubiquitination of SPM1 by TgJosephin maintained by TgRad23.

In the first part, I have searched *T. gondii* factors that can inhibit IRGB6-dependent cell-autonomous response in genes that confer fitness in IFN- γ -stimulated murine cells (15). Among the candidate proteins besides putative dense granule (GRA) proteins, I have identified a putative TgIWS1 transcription elongation factor as a new inhibitor of IRGB6-dependent anti-*T. gondii* cell-autonomous immunity. Moreover, the *T. gondii* TgIWS1 was important for ROP18 mRNA expression. IWS1 was originally discovered as an RNA polymerase II elongation factor in yeast (37). IWS1 is shown to interact with the conserved elongation factor Spt6 (also known as SUPT6) to regulate H3K36 trimethylation and play crucial roles in mRNA processing and export (18). Currently, it remains unclear whether the *T. gondii* IWS1 (TgIWS1) also functions as a transcription elongation factor. TgIWS1 might indirectly induce ROP18 mRNA by expressing other transcription factors that directly transcribe ROP18 mRNA.

Of the three major strain types of *T. gondii*, Type I is virulent while Type II and III are avirulent. TgIWS1 of Type I (TGGT1_227560) and Type II (TGME49_227560) differ by one amino acid and mRNA expression is slightly greater in Type I than in Type II (Davis and Roos, unpublished (2008), ToxoDB and RT-qPCR; data not shown), yet the transcript levels of ROP18 are comparable between those types (38). Meanwhile, although ROP18 mRNA expression is greater in Type I than in Type III (38), the TgIWS1 amino acid sequences (TGGT1_227560, TGVEG_227560) are identical, and the TgIWS1 transcript abundance in Type I is comparable with (Davis and Roos, unpublished (2008), ToxoDB) or slightly less than (RT-qPCR; data not shown) in Type III. From these data, TgIWS1 transcript levels and polymorphism are unlikely to determine of differences in virulence or ROP18 expression across strains.

While Δ iws1::ROP18 parasites appeared to fully reconstitute suppression of IRG/GBP/ubiquitin/p62 recruitment to the parasite *in vitro* and considerably rescued virulence *in vivo*, infection of the Δ iws1::ROP18 parasites still exhibited a minor prolonged survival period in mice. In addition, IFN γ R-deficient mice were susceptible to TgIWS1-deficient parasites in a similar time course to wild-type mice infected with wild-type *T. gondii*. These results indicate that a TgIWS1-dependent gene product(s) independent of ROP18 is necessary for full wild-type virulence. Among mRNAs reduced by over 1.5-fold in TgIWS1-deficient parasites, genes encoding SAG-related sequence SRS29B (TGGT1_233460) and two hypothetical proteins (TGGT1_280375 and TGGT1_289740) were listed as genes that significantly confer fitness in IFN- γ -stimulated murine macrophages albeit without high confidence (15). Although their roles in *T. gondii* virulence are uncertain, these proteins possibly suppress an IRG/GBP-independent pathway in the IFN- γ -mediated immune response.

ROP18 has TgIWS1 for the transcriptional production machinery. Similarly, other ROP and GRA virulence effectors may have their own or shared production and trafficking machineries within this pathogen like TgASP5, which is a parasite Golgi-resident protease that cleaves several GRAs to localize the PVM and host nucleus (39).

From the *in vivo* CRISPR/Cas9-screened candidates, I have found TgJosephin as a novel virulence factor of *T. gondii*. I aimed to discover unknown pathways and molecules in immunosuppression by screening gene knockout strains with parasite proliferation in mice and survival of infected mice. I selected candidates predicted as proteins resident in the parasite nucleus or cytosol, and unlikely to bind host factors as conventional virulence factors do. My study suggests that TgJosephin is important for adequate virulence in wild-type mice but not in IFN γ R-deficient mice, and TgRad23 is important for abundant TgJosephin expression. The relatively mild virulence impairment of Δ rad23 parasites may be attributed to inadequate expression of TgJosephin, whose total absence leads to a sharper loss of

virulence. As TgJosephin deletion did not increase host IRG and GBP recruitment on PVs, TgJosephin likely suppresses a pathway of the IFN- γ -mediated response independent of IFN-inducible GTPases. Furthermore, deubiquitination activity of TgJosephin was suggested as the key to its role in virulence, and TgJosephin deficiency causes increased ubiquitination of SPM1, an impediment to *T. gondii* virulence in mice.

I propose that TgJosephin, maintained by TgRad23, inhibits a pathway in the IFN- γ response apart from PV coating by IFN-inducible GTPases, by deubiquitination and clearance of abnormal SPM1. The mechanism by which SPM1 deubiquitination by TgJosephin may suppress host IFN- γ -dependent immunity remains unclear. Ubiquitination of SPM1 could possibly alter the morphology and functions of subpellicular microtubules, which could influence motility and invasion (40).

As both inhibitory and promotive traits of Josephin family deubiquitinases in protein degradation are discussed (41–43), lack of deubiquitination of SPM1 by TgJosephin could lead to scarce SPM1 expression due to increased degradation, or an abnormal pool of ubiquitinated SPM1. Given that the SPM1-HA signal in DKO::SPM1 parasites was no weaker than in Δ spml::SPM1, Δ spml::SPM1^{KR}, or DKO::SPM1^{KR} (**Fig. 10D**), wild-type SPM1 expression while lacking TgJosephin expression may not result in over-degradation of SPM1 but in accumulation of ubiquitinated SPM1.

Known virulence proteins were not prominently affected by TgJosephin deletion according to bulk RNA sequencing (**Table S5**), ubiquitination (**Table S4**) and whole-cell mass spectrometry results (**Table S6**). Still, I cannot exclude the possibility that multiple substrates of TgJosephin are involved. In Δ josephin parasites compared to wild-type parasites, while SPM1 had an outstanding number of increased ubiquitination sites in both Vero cells and stimulated MEFs, SPM2 (TGGT1_286590), hypothetical protein (TGGT1_220510), and HMG (high mobility group) box domain-containing protein (TGGT1_263720) also had increased ubiquitination at more than one lysine residue (**Table S4**). Since SPM2 is also localized in subpellicular microtubules (19), its overubiquitination in TgJosephin deficiency may have similar consequences on parasite virulence as SPM1. The TGGT1_263720 is homologous to HMGB1, a DNA binder that functions as a transcriptional regulator in the nucleus (44, 45). A bulk RNA sequencing experiment showed that some genes were upregulated or downregulated in the Δ josephin strain compared to the wild-type (data not shown), all that seemed irrelevant to immunosuppression, while the possibility of unknown virulence mechanisms by these genes cannot be excluded.

Given that TgJosephin deletion did not affect patterns of PV coating by IRGB6, IRGA6, p62, or GBP1/2/1-5 or parasite viability for up to 48 hours in IFN- γ -stimulated murine bone-marrow macrophages (BMDMs) (luciferase reporter assay; data not shown), Δ josephin parasites in wild-type mice have likely succumbed to IFN- γ -driven growth inhibition instead of cell-autonomous cytotoxicity. The difference between mice lacking IFN γ R and iNOS or IDO in **Fig. 8B** suggests that TgJosephin represses multiple pathways in the IFN- γ -induced response including nitric oxide production by iNOS and possibly tryptophan starvation by IDO. Although IDO (IDO1 and IDO2) deficiency did not significantly increase susceptibility to Δ josephin parasites, simultaneous deletion of iNOS and IDO may affect host survival to a degree comparable to deletion of IFN γ R.

IFN- γ -dependent nitric oxide increase has been reported *in vitro* (46, 47), and a recent study showed evidence for iNOS-dependent nitration around *T. gondii* in infected cells (48). Sodium nitroferricyanide(III) dihydrate (SNP) has been used to mimick cellular nitric oxide production (49–51). When tachyzoites were exposed to 0~2 mM SNP prior to MEF infection to test their sensitivity to nitric oxide, I found no significant difference between wild-type and Δ josephin parasites (**Fig. S6B**). This could suggest that interference of TgJosephin in the iNOS-mediated pathway precedes nitric oxide metabolism in the parasite, though I am yet to establish a method to study the importance of TgJosephin in virulence-related phenotypes *in vitro*.

My findings suggest the importance of TgIWS1 epigenetic regulation and the TgRad23-TgJosephin-SPM1 axis in suppression of IFN- γ -driven immunity. It is worth noting that, unlike typical virulence factors of rhoptries and dense granules, TgIWS1, TgRad23, and TgJosephin have homologues in mammals. These atypical virulence factors highlight the need to extend our study of *T. gondii* immunosuppression to genes conserved beyond apicomplexans and genes with basic functions.

Materials and Methods

Cell culture and mice

C57BL/6NCrSlc (C57BL/6N) mice were purchased from SLC. Mice lacking IFN γ R were described previously (52). For CRISPR/Cas9 targeting of iNOS and IDO, T7 promoter was added to the gRNA templates (iNOS_gRNA1 and iNOS_gRNA2 for iNOS, IDO1_gRNA and IDO2_gRNA for IDO) using KOD FX NEO. These gRNA and Cas9 mRNA were purified and used to generate iNOS- or IDO-deficient mice as done previously for GNAQ-deficient mice (53). All animal experiments were conducted with the approval of the Animal Research Committee of Research Institute for Microbial Diseases in Osaka University.

T. gondii and mammalian cells were cultured at 37°C in 5% CO₂. *T. gondii* strains were maintained in Vero cells by bi-weekly passage in RPMI (Nacalai Tesque) supplemented with 2 % heat-inactivated fetal calf serum (FCS; JRH Bioscience), 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Nacalai Tesque). All parasites used were tachyzoites of RH Δ hxgprt Δ ku80 and its derivatives.

Mouse embryonic fibroblasts (MEFs) were grown in DMEM (Nacalai Tesque) supplemented with 10 % FCS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Nacalai Tesque).

Murine macrophages were induced from the marrow of the pelvis, femur, and tibia. Bone marrow was collected and washed with phosphate buffered saline (PBS) before and after Ammonium-Chloride-Potassium (ACK) lysing Buffer suspension and cultured for six days in 70% RPMI 1640-based medium (containing 10 % FCS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin) + 30% L-cell supernatant. For iNOS detection, cells were stimulated with 1 μ g/ml lipopolysaccharide (LPS) overnight on the 6th day (Fig. S4).

Reagents

Goat polyclonal antibody against IRGB6 (TGTP; sc-11079) was purchased from Santa Cruz Biotechnology, Inc. Rabbit polyclonal anti-GBP2 and mouse monoclonal anti-p62 (H00008878-M01J) antibodies were obtained from Proteintech and Abnova, respectively. Recombinant mouse IFN- γ was purchased from PeproTech. Other reagents are listed in Table S1.

Plasmid construction for generation of KO *T. gondii* strains

For construction of the CRISPR/Cas9 plasmids targeting TgIWS1 (TGGT1_227560), TgSUB2 (TGGT1_314500), TgRAMP4 (TGGT1_276940), TgDRL1 (TGGT1_269620), TgRqc2 (TGGT1_219290), TgRad23 (TGGT1_295340), TgJosephin (TGGT1_229650), TgCapZB (TGGT1_219290), and SPM1 (TGGT1_263520), two oligonucleotide primers (TgIWS1_gRNA1_F and TgIWS1_gRNA1_R, TgIWS1_gRNA2_F and TgIWS1_gRNA2_R for TgIWS1, TgSUB2_gRNA1_F and TgSUB2_gRNA1_R, TgSUB2_gRNA2_F and TgSUB2_gRNA2_R for TgSUB2, TgRAMP4_gRNA1_F and TgRAMP4_gRNA1_R, TgRAMP4_gRNA2_F and TgRAMP4_gRNA2_R for TgRAMP4, TgDRL1_gRNA1_F and TgDRL1_gRNA1_R, TgDRL1_gRNA2_F and TgDRL1_gRNA2_R for TgDRL1, TgRqc2_gRNA1_F and TgRqc2_gRNA1_R, TgRqc2_gRNA2_F and TgRqc2_gRNA2_R for TgRqc2, TgRad23_gRNA1_F and TgRad23_gRNA1_R, TgRad23_gRNA2_F and TgRad23_gRNA2_R for TgRad23, TgJosephin_gRNA1_F and TgJosephin_gRNA1_R, TgJosephin_gRNA2_F and

TgJosephin_gRNA2_R for TgJosephin, TgCapZB_gRNA1_F and TgCapZB_gRNA1_R, TgCapZB_gRNA2_F and TgCapZB_gRNA2_R for TgCapZB, SPM1_gRNA1_F and SPM1_gRNA1_R, SPM1_gRNA2_F and SPM1_gRNA2_R for SPM1) containing gRNA sequences were annealed and cloned into the BsaI cleavage site of the Cas9-pU6-Universal vector.

Targeting fragments containing a floxed HXGPRT cassette between two 60-bp-genomic regions outside the PAM sequences were amplified using primers TgIWS1_targeting_F and TgIWS1_targeting_R, TgSUB2_targeting_F and TgSUB2_targeting_R, TgRAMP4_targeting_F and TgRAMP4_targeting_R, TgDRL1_targeting_F and TgDRL1_targeting_R, TgRqc2_targeting_F and TgRqc2_targeting_R, TgRad23_targeting_F and TgRad23_targeting_R, TgJosephin_targeting_F and TgJosephin_targeting_R, TgCapZB_targeting_F and TgCapZB_targeting_R, SPM1_targeting_F and SPM1_targeting_R.

Primer sequences are listed in **Table S1**. Primers and gRNA were constructed using *T. gondii* GTI genome sequences on ToxoDB.org (54).

Gene-targeted CRISPR/Cas9 genome editing of *T. gondii*

In all transfection procedures of *T. gondii*, tachyzoites were filtered and resuspended in Cytomix (10 mM KPO₄, 120 mM KCl, 0.15 mM CaCl₂, 5 mM MgCl₂, 25 mM HEPES, 2 mM EDTA) supplemented with 2 mM ATP (Wako) and 5 mM Glutathione (Nacalai Tesque). Cells were electroporated with GENE PULSER II (Bio-Rad Laboratories). Mutant parasites were selected with drugs in Vero culture or by sorting, then separated into clones by limiting dilution in MEF monolayers in 96-well plates.

Deletion of *T. gondii* genes by CRISPR/Cas9 genome editing

The RHΔhxgprtΔku80 strain was transfected with 50 μg gRNA1 and 50 μg gRNA2 CRISPR plasmids along with the PCR-amplified targeting fragment for each gene. Mutants were passaged in 25 μg/ml mycophenolic acid (Sigma) and 50 μg/ml xanthine (Wako). Mutant clones were obtained by limiting dilution and screened by quantitative RT-PCR, using primers listed in **Table S1**.

For the Δspm1Δjosephin double knockout, the HXGPRT cDNA was deleted in Δspm1 parasites using a Cre-YFP vector. Δspm1Δhxgprt cells were selected by 80 μg/ml 6-Thioxanthine (Santa Cruz) and used for TgJosephin gene deletion.

Cells expressing the endogenously tagged TgJosephin-HA were used for TgRad23 deletion (**Fig. 7B**).

Overexpression of ROP18 in TgIWS1-deficient *T. gondii*

The TgIWS1-deficient parasites lacking HXGPRT were filtered, washed and resuspended in Cytomix. Parasites were mixed with 50 μg of the ROP18 expression vector containing ROP18 cDNA (18), in which 1 kb promoter of the SAG1 (TG GTT1_233460 SAG-related sequence SRS29B) was fused to the coding sequence of ROP18 cDNA and followed by the polyA sequence and HXGPRT expression cassette in pBlueScript plasmid vector (Figure S2B), and supplemented with 2 mM ATP, 5mM GSH. Parasites were electroporated by GENE PULSER II. Selection by growth for 14 days in 25 μg/ml mycophenolic acid (Sigma) and 50 μg/ml xanthine (Wako) were used to obtain stably resistant clone. And then parasites were plated in limiting dilution in 96-well plates to isolate single clones. To confirm the expression of the gene encoding ROP18, I analyzed mRNA of ROP18 from the rescued parasites by quantitative RT-PCR using primers listed in **Table S1**.

Protein tagging in the endogenous TgJosephin gene region

An HA tag sequence conjoined with the polyA tail and promoter regions of TGME49_316400 alpha tubulin TUBA1 and DHFR-TS cDNA sequences was inserted in the C-terminal region of the endogenous TgJosephin gene locus of Δjosephin parasites.

Complementation of TgJosephin and SPM1 in TgJosephin- and SPM1-deficient *T. gondii*

T. gondii cDNA sequences were obtained from ToxoDB.org (54). To complement TgJosephin-deficient parasites, full-length TgJosephin cDNA with or without the C24S mutation was amplified with PCR, using the RHΔhxgprtΔku80 cDNA as template, and inserted into the pBlunt vector.

The cDNA fragments were cleaved and inserted into the pBlueScript plasmid vector containing the 3-terminal and 5-terminal UTRs of the putative uracil phosphoribosyltransferase FUR1 (UPRT) gene.

To complement SPM1- deficient parasites, full-length SPM1 cDNA was amplified with PCR, using the RH *T. gondii* cDNA as template, and inserted into the pBlunt vector. A synthetic mutant SPM1^{KR} cDNA was ordered from FASMAC. 20 lysine residues detected as ubiquitination sites by mass spectrometry (Table S4) were substituted with arginine (Fig. S3).

The SPM1 and SPM1^{KR} cDNA were cleaved and inserted into the pBlueScript plasmid vector containing the promoter and polyA tail regions of TGME49_316400 alpha tubulin TUBA1 and the 3-terminal and 5-terminal UTRs of the UPRT gene.

TgJosephin- and SPM1- deficient *T. gondii* parasites were transfected with plasmids containing UPRT_gRNA and their respective cDNA fragments (50 µg each).

Transfected parasites were selected with 10µg/ml fluorodeoxyuridine (FUDR) (Wako).

PV coating assay

MEFs were seeded at 1.0×10^5 cells per well in a 6-well plate containing coated coverslips and treated with 10 ng/ml mouse IFN-γ for 18-20 hours at 37 °C. Cells were infected with *T. gondii* at MOI = 4 and incubated at 37 °C for 2 hours.

Cells were fixed with 4% formaldehyde in PBS for 10 min, permeabilized with 0.002% digitonin in PBS for 10 min, and blocked with 8 % FCS in PBS for 10 min. Cells were co-stained with antibodies against a *T. gondii* marker (GRA2/5/7/17) and a host effector (IRGB6, IRGA6, IRGB10, p62, GBP1, GBP2, GBP1-5, or ubiquitin) for 1 hour, followed by incubation with Alexa 488-, Alexa 594-conjugated secondary antibodies and 49,6-diamidino-2-phenylindole (DAPI) for 30 min in the dark. Coverslips were mounted onto glass slides with Perma Fluor and analyzed with confocal laser microscopy (Olympus FV3000 IX83).

All procedures following the 2-hour incubation were done at room temperature. A total of 100 PVs were counted in each experiment. Student's t test was used to compare percentages of coated PVs of two groups on GraphPad Prism 9. For three groups, ordinary one-way ANOVA was used.

Assessment of *T. gondii* virulence in mice

Part 1 (Fig. 3, 5): Mice were intraperitoneally infected with 1.0×10^3 *T. gondii* tachyzoites in 200 µl PBS per mouse. Survival rates were measured daily for 25 days.

Part 2 (Fig. 6-10): Mice were infected with 1.0×10^3 *T. gondii* tachyzoites in 50 µl PBS per mouse by footpad injection. Survival was monitored for 30 days post-infection.

Kaplan–Meier survival curves are shown; statistical significance was determined using the log-rank (Mantel–Cox) test.

In vivo measurement of parasites by bioluminescent imaging

Mice were intraperitoneally infected with 1.0×10^3 *T. gondii* tachyzoites in 200 µl PBS per mouse. In vivo imaging was done on day 6 post infection. Mice were intraperitoneally injected with 3 mg D-luciferin (Promega) in 200 µl PBS and anaesthetized for 10 mins with isoflurane (Dainippon Sumitomo Pharma), and used for photonic emission detection with IVIS-Spectrum and Living Image Software (Xenogen).

In vitro measurement of *T. gondii* numbers by luciferase assay

MEFs were seeded 1.0×10^5 cells per well in a 12-well plate in DMEM containing 10% FCS ±10 ng/ml

IFN- γ and incubated in 37 °C for 18-20 hours. Luc-expressing tachyzoites were added at MOI 1 and incubated in 37°C for 24 hours.

For experiments using BMDMs, BMDMs were seeded at 1.0×10^6 cells/well in a 6-well plate in RPMI containing 5% L-cell supernatant and 10% FCS ± 10 ng/ml IFN- γ . Luc-expressing tachyzoites were added at MOI 1 and incubated in 37°C for 48 hours.

Cells were collected and centrifuged (7,000 rpm for 5 min at 4°C) and suspended in 100 μ l 1 $\times$ Promega Passive Lysis Buffer, followed by sonication for 30s. Cells were then centrifuged (14,000 rpm for 5 min at 4°C), and 5 μ l of supernatants were mixed with 50 μ l LARII and used for Luciferase activity detection with Promega GLOMAX20/20 luminometer. The data represented show luciferase activity relative to that in untreated MEFs. Differences in the *T. gondii* inhibition activity between IFN- γ activated vs non-activated or SNP treated vs untreated conditions were subjected to two-way ANOVA with Tukey's multiple comparisons test to analyze the difference between genotypes.

Treatment of *T. gondii* with SNP and extracellular nitrite measurement

2×10^6 parasites/ml were incubated at 37 °C for 30 min in serum-free DMEM containing 0-2 mM SNP and then centrifuged (2,000 rpm for 5 min at 25°C). Supernatants were used for nitrite measurement with Griess Reagent Kit (Dojindo Laboratories). Cell fractions were used for MEF infection (MOI 1) for luciferase assay.

Organ luciferase assay

Mice were dissected on day 6 post infection. Peritoneal exudate cells (PECs) were collected with PBS. Organs were homogenized in 1x Promega Passive Lysis Buffer. 200 μ l of PEC and organ homogenates were collected and sonicated for 30s and centrifuged for 10 min at 13000 rpm at 4 °C. 10 μ l of each supernatant was mixed with 50 μ l LARII (Promega Dual-Luciferase Reporter Assay System) and used for Luciferase activity detection with Promega GLOMAX20/20 luminometer.

Gene expression measurement by quantitative PCR

Tachyzoites were pelleted and used for total RNA extraction with QIAGEN RNeasy Mini Kit. RNA samples were incubated at 70°C for 2-3 mins and used to synthesize cDNA with thermoscientific Verso cDNA Synthesis Kit. RT-PCR was performed with the synthesized cDNA and Promega GoTaq qPCR Master Mix in BIO-RAD CFX Connect Real-Time System. Expression data was taken by the $\Delta\Delta C_t$ method relative to TgACT1 (TGGT1_209030) expression levels.

Ordinary one-way ANOVA was used to compare ROP18 or TgIWS1 expression between three different genotypes.

Plaque assay

Two hundred tachyzoites were added onto a primary MEF monolayer in a 6-well plate and cultured for 8 days. Cells were washed with PBS, fixed for 30 min with 4% formaldehyde, then stained with 1% crystal violet. Photos were taken at 4 $\times$ magnification with an optical microscope (Olympus CKX53).

Bulk RNA-seq analysis

For RNA-seq analysis, total RNA was extracted from cells with an miRNeasy Mini kit (Qiagen) following the manufacturer's instruction. Library preparation was performed using a TruSeq stranded mRNA sample prep kit (Illumina, San Diego, CA) according to the manufacturer's instructions. Sequencing was performed on an Illumina NovaSeq 6000 sequencer (Illumina) in the 101-base single-end mode. Sequenced reads were mapped to the *Toxoplasma gondii* GT1 reference genome sequences (GCA_000149715.2_TGGT1) using HISAT2 version 2.1.0 (28), and the reads aligned to the *Toxoplasma* reference genome were counted using featureCounts (29). Raw count data were analyzed using iDEP.92

(30) and GSEA software ver. 4.1.0. (31). Genes with $FDR < 0.05$ and $|\log_2FC| > 1$ in were considered as differentially expressed genes (DEGs). For GSEA, gene sets were obtained from published data. Heatmaps visualizing the normalized gene expression levels on a scale of rawmin to rawmax for each gene were generated using Morpheus (<https://software.broadinstitute.org/morpheus>).

Mass spectrometry

Quantitative ubiquitylome analysis was performed based on a previously reported method (PMID: 31953384) with minor modifications. WT and Δ josephin parasites were cultured at 37°C for two days in Vero cells, unstimulated MEFs, or MEFs stimulated overnight with 10 ng/mL IFN- γ . Parasites were filtered through a 5 μ m filter and washed with HEPES-buffered saline. Then, parasites were lysed in guanidine-TCEP buffer (6 M guanidine-HCl, 100 mM HEPES-NaOH, pH7.5, 10 mM TCEP, 40 mM chloroacetamide). Following heat denaturation and sonication, proteins were purified by methanol–chloroform precipitation and resuspended in 100 μ L of 0.1% RapiGest SF (Waters) in 50 mM triethylammonium bicarbonate. After sonication and heating at 95°C for 10 min, proteins were digested with 2 μ g of MS-grade trypsin (Thermo Fisher Scientific) at 37°C overnight.

The resulting peptide solutions were heated again at 95°C for 10 min, diluted 6-fold with HBS (50 mM HEPES-NaOH, pH7.5, 150 mM NaCl), and centrifuged. The supernatants were incubated with PTMScan HS K- ϵ -GG remnant magnetic immunoaffinity beads (Cell Signaling Technology) at 4°C for 2 h with rotation. The beads were washed four times with HBS and resuspended in 100 μ L of 100 mM HEPES-NaOH, pH8.0. On-antibody tandem mass tag (TMT) labeling was performed by adding 0.25 mg of TMTpro 18-plex reagents (Thermo Fisher Scientific) and incubating at 25°C for 10 min with shaking. The reaction was quenched by adding 4 μ L of 5% hydroxylamine. After two washes with Milli-Q water, the beads from all conditions were pooled, and peptides were eluted by incubating three times with 200 μ L of 60% acetonitrile (ACN) and 0.1% trifluoroacetic acid (TFA). The combined eluates were evaporated, diluted to 120 μ L with 10 mM ammonium formate (pH 9.0) in 2% ACN, and fractionated using offline high-pH reversed-phase chromatography on a Vanquish DUO UHPLC system (Thermo Fisher Scientific). Peptides were separated into 48 fractions, which were consolidated into 12 fractions. Each fraction was evaporated and dissolved in 3% ACN and 0.1% TFA.

LC-MS/MS analysis of the resulting peptides was performed on an EASY-nLC 1200 UHPLC connected to a Q Exactive Plus mass spectrometer through a nanoelectrospray ion source (Thermo Fisher Scientific). The peptides were separated on a C18 reversed-phase column (75 μ m $\times$ 150 mm; Nikkyo Technos) with a linear gradient of 4–32% ACN for 0–100 min, followed by an increase to 80% ACN for 10 min and a final hold at 80% ACN for 10 min. The mass spectrometer was operated in data-dependent acquisition mode with the top 10 MS/MS method. MS1 spectra were measured with a resolution of 70,000, an automatic gain control (AGC) target of 3e6, and a mass range of 375–1400 m/z. HCD MS/MS spectra were acquired at a resolution of 35,000, AGC target of 1e5, isolation window of 0.7 m/z, maximum injection time of 200 ms, and normalized collision energy of 32. The dynamic exclusion was set to 20 s. Raw data were directly analyzed against *T. gondii* GT1 protein data (ToxoDB release 61) using Proteome Discoverer 2.5 (Thermo Fisher Scientific) with the Sequest HT search engine for identification and TMT quantification.

The search parameters were as follows: (a) trypsin as an enzyme with up to two missed cleavages, (b) precursor mass tolerance of 10 ppm, (c) fragment mass tolerance of 0.02 Da; (d) TMTpro of peptide N-terminus and carbamidomethylation of cysteine as fixed modifications, and (e) oxidation of methionine, TMTpro of lysine, and di-glycine of lysine as variable modifications. Peptides were filtered at a false-discovery rate of 1% using the Percolator node. Four or five biological replicates per group were analyzed with Student's t test. Four or five biological replicates per group were analyzed with Student's t test.

Immunoprecipitation

Cells were lysed in lysis buffer (1% NP-40, 150 mM NaCl, 20 mM Tris-HCl, pH 7.5) containing 1% protease inhibitor cocktail (Nacalai Tesque). Cell lysates were mixed with Protein G Sepharose™ 4 Fast Flow (Cytiva) by rotation for 30 min. Lysates were centrifuged and supernatants were mixed with an antibody (1 µg) by rotation overnight. Protein G Sepharose™ 4 Fast Flow was added to the samples and rotated for 1 hour, then washed with lysis buffer.

Western blotting

Cells were lysed in lysis buffer containing 1% protease inhibitor cocktail (Nacalai Tesque). Cell lysates or immunoprecipitated samples were separated by SDS-PAGE and transferred to Immobilon®-P PVDF Membranes (Millipore) and blotted with the indicated antibodies in 5% skim milk (Difco Laboratories). Membranes were immersed in Luminata Forte Western HRP substrate (Millipore) and used for signal detection by ImageQuant™ LAS 4000 (GE Healthcare). The *Toxoplasma gondii* (1.B.516): sc-73210 antibody by Santa Cruz Biotechnology, Inc. (referred to as “Toxo” in figures) was used as a loading control for *T. gondii* samples.

Abbreviations

BMDM: bone marrow-derived macrophage
 ChIP-seq: chromatin immunoprecipitation coupled with high-throughput sequencing
 CRISPR: clustered regularly interspaced short palindromic repeats
 DAPI: 4',6-diamidino-2-phenylindole
 DKO: double knockout
 DMEM: Dulbecco's modified eagle medium
 DRL1: DNA repair-like 1
 FCS: Fetal calf serum
 GBP: Guanylate Binding Protein
 hyperLOPIT: hyper localization of organelle proteins by isotope tagging
 IDO: indoleamine 2,3-dioxygenase
 IFNγR: interferon-gamma receptor
 IFN-γ : interferon-gamma
 iNOS: inducible nitric oxide synthase
 IRG: immunity-related GTPase
 IWS1: Interacts With Spt6 1
 KO: knockout
 LPS: lipopolysaccharide
 MEF: mouse embryonic fibroblast
 MOI: multiplicity of infection
 PBS: phosphate buffered saline
 PV: parasitophorous vacuole
 PVM: parasitophorous vacuole membrane
 RAMP4: ribosome-associated membrane protein 4
 RT-PCR: reverse transcription polymerase chain reaction
 SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
 SPM1: subpellicular microtubule protein 1
 SUB2: Subtilisin 2
T. gondii: *Toxoplasma gondii*
 UPRT: uracil phosphoribosyltransferase

WT: wild-type

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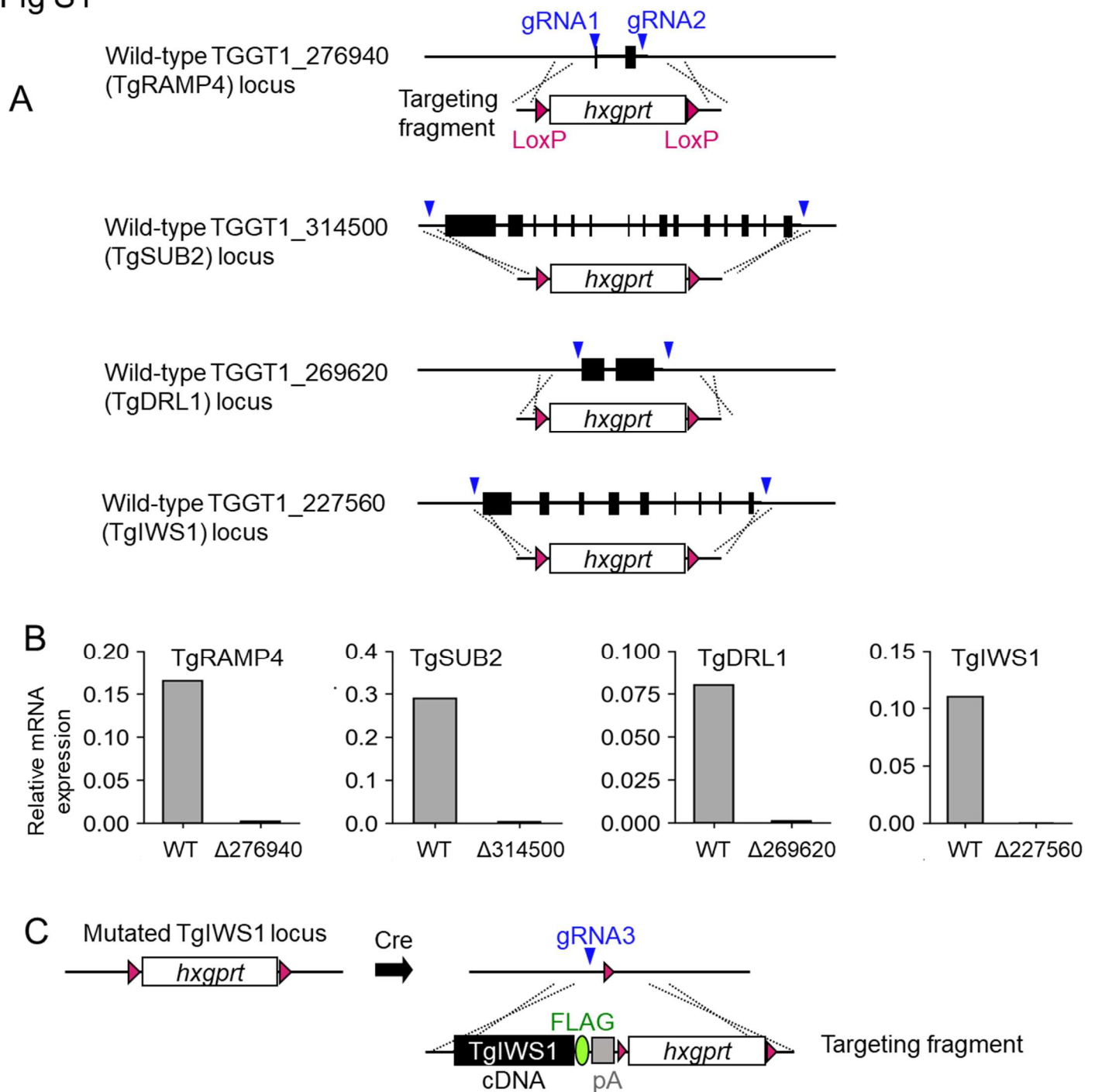
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Supplemental information

Fig S1

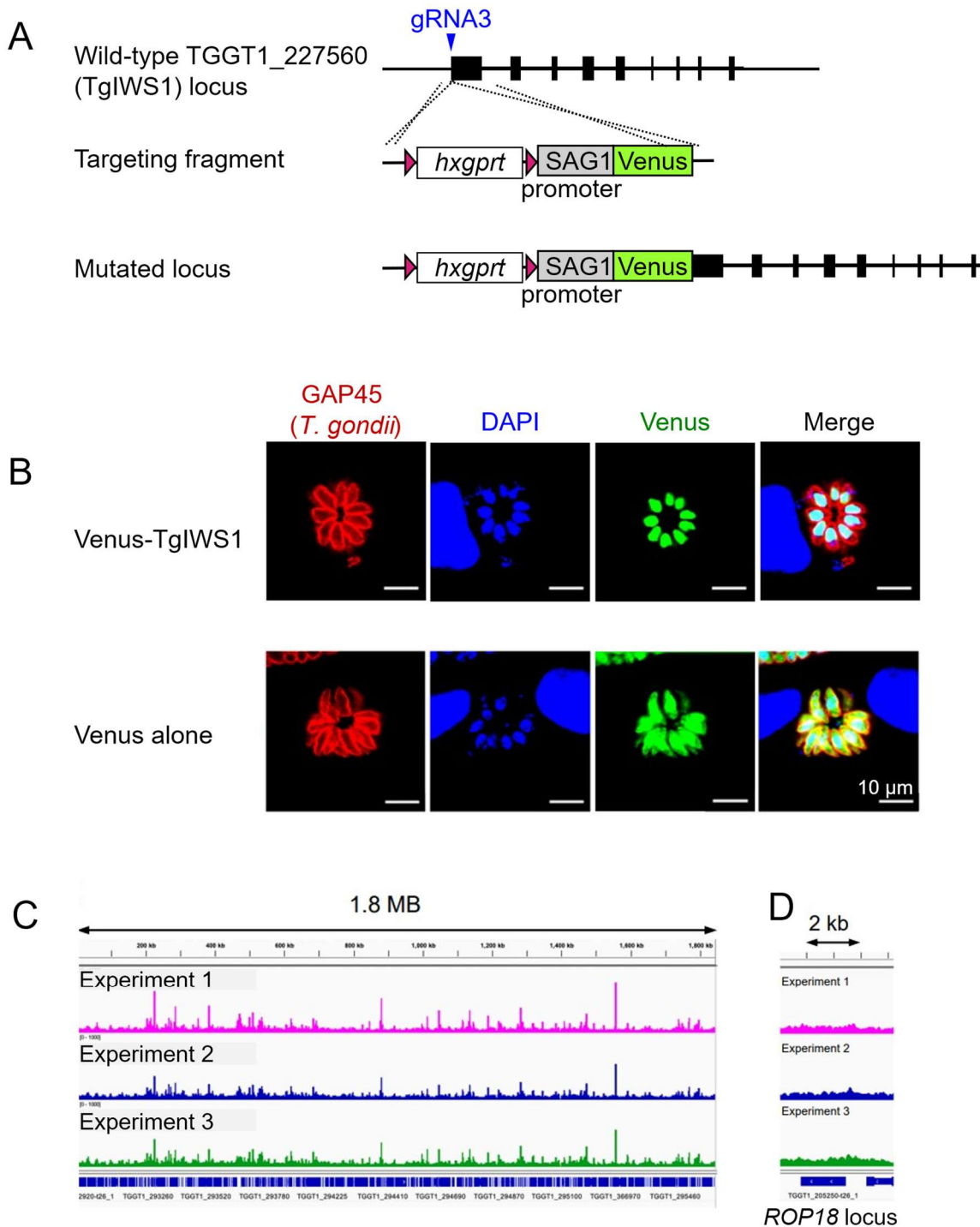


Supplementary Figure S1. Mutation of *T. gondii* genes (TgRAMP4, TgSUB2, TgDRL1, TgIWS1) (A) Deletion of genes. The wild-type locus of each gene was cleaved by CRISPR/Cas9 at the indicated gRNA target sites, and a PCR fragment containing the *hxgprt* cDNA with two flanking loxP sequences was inserted.

(B) Quantitative RT-PCR analysis of parasites. Gene expression levels of each gene in WT and KO parasites were normalized relative to the expression of TgACT1.

(C) Complementation of Δ iws1 parasites. The *hxgprt* cassette in the endogenous TgIWS1 locus was deleted with Cre transfection in the presence of 6-thioxanthine. A PCR fragment containing a TgIWS1 cDNA copy with a polyA signal and the *hxgprt* cassette was inserted following Cas9 cleavage of the indicated region (arrowhead).

Fig S2



Supplementary Figure S2. Nuclear localization of Venus-TgIWS1 and ChIP-seq analysis.

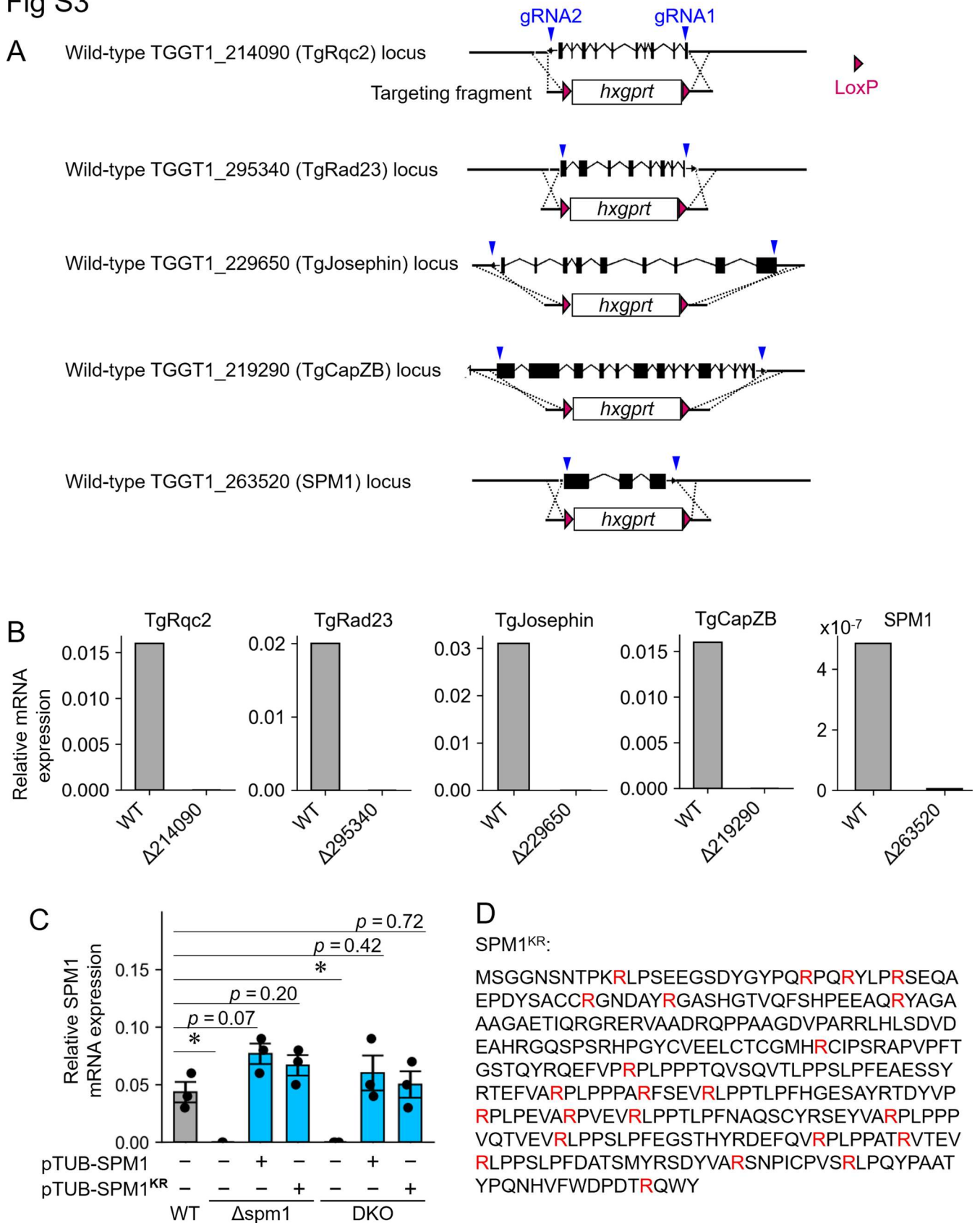
(A) Insertion of the HXGPRT/SAG1p-Venus cassette into the N terminus of TgIWS1 by genome editing.

(B) Confocal microscope images represent the localization of Venus (green) to *T. gondii* (red) and DAPI (blue) at 2 h post-infection with Venus-TgIWS1 parasites or control parasites expressing Venus alone.

Scale bars = 10 μ m.

(C-D) Comparison of the mapped views of Venus-TgIWS1 peaks obtained in three independent experiments. Reads were mapped on the reference sequence of a region of *T. gondii* GT1 chromosome Ia (C) and the ROP18 gene locus (D). Images were made in the integrative genomics viewer.

Fig S3



Supplementary Figure S3. Mutation of genes in *T. gondii* (TgRqc2, TgRad23, TgJosephin, TgCapZB, SPM1)

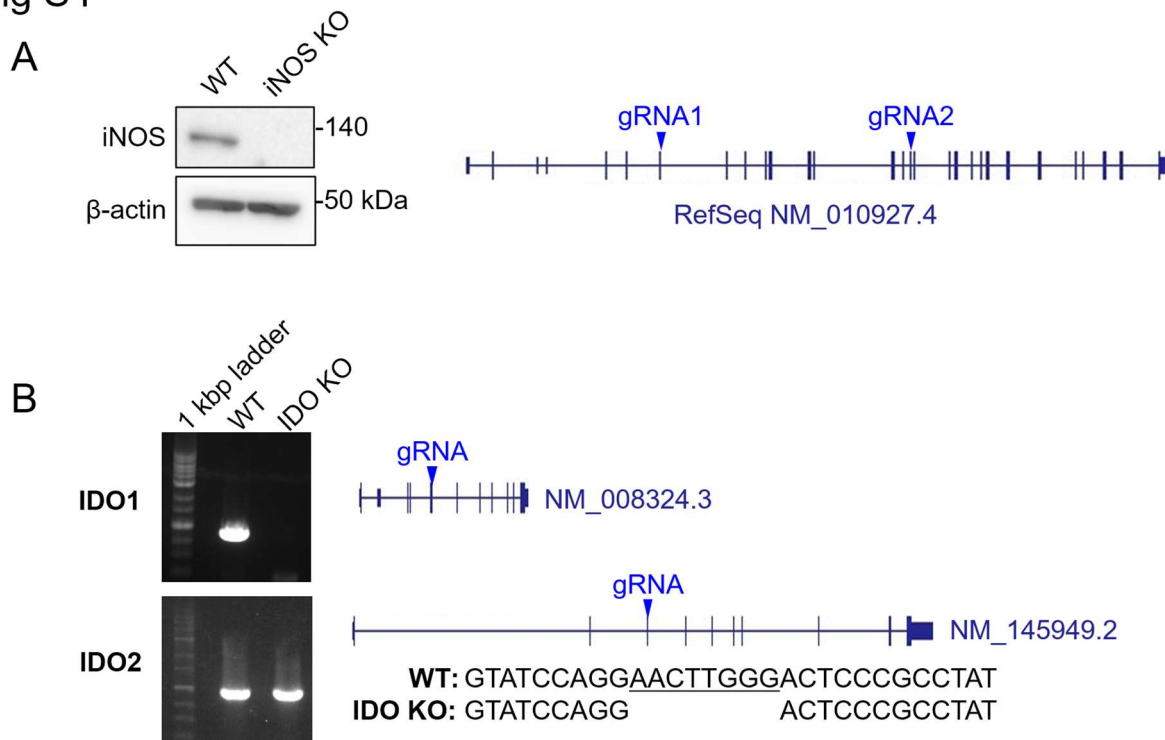
(A) Deletion of genes. The WT locus of each gene was cleaved by CRISPR/Cas9 at the indicated gRNA target sites (54), and a PCR fragment containing the HXGPRT cDNA with two flanking LoxP sequences was inserted.

(B) RT-qPCR analysis of parasites. Expression levels of the indicated genes in parental (WT) and mutant parasites were normalized to TgACT1 mRNA.

(C) RT-qPCR analysis of WT, Δ spm1, and Δ spm1 Δ josephin DKO parasites. mRNA levels of SPM1/SPM1^{KR} expressed by the endogenous promoter (grey) or the TUBA1 promoter (blue) were normalized to TgACT1 mRNA. $n = 3$; * $p < 0.05$.

(D) Amino acid sequence of SPM1^{KR}. Lysine-to-arginine substitutions are highlighted in red.

Fig S4

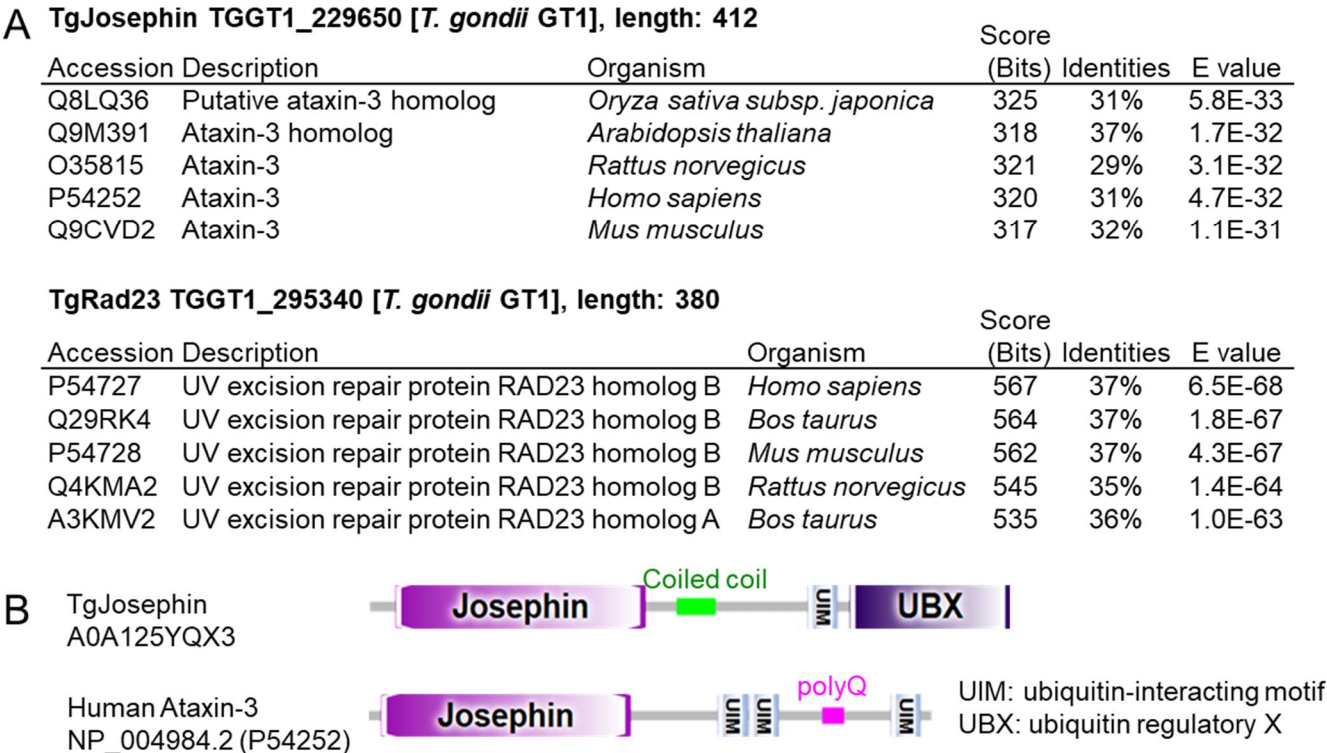


Supplementary Figure S4. IDO/iNOS deletion in mice.

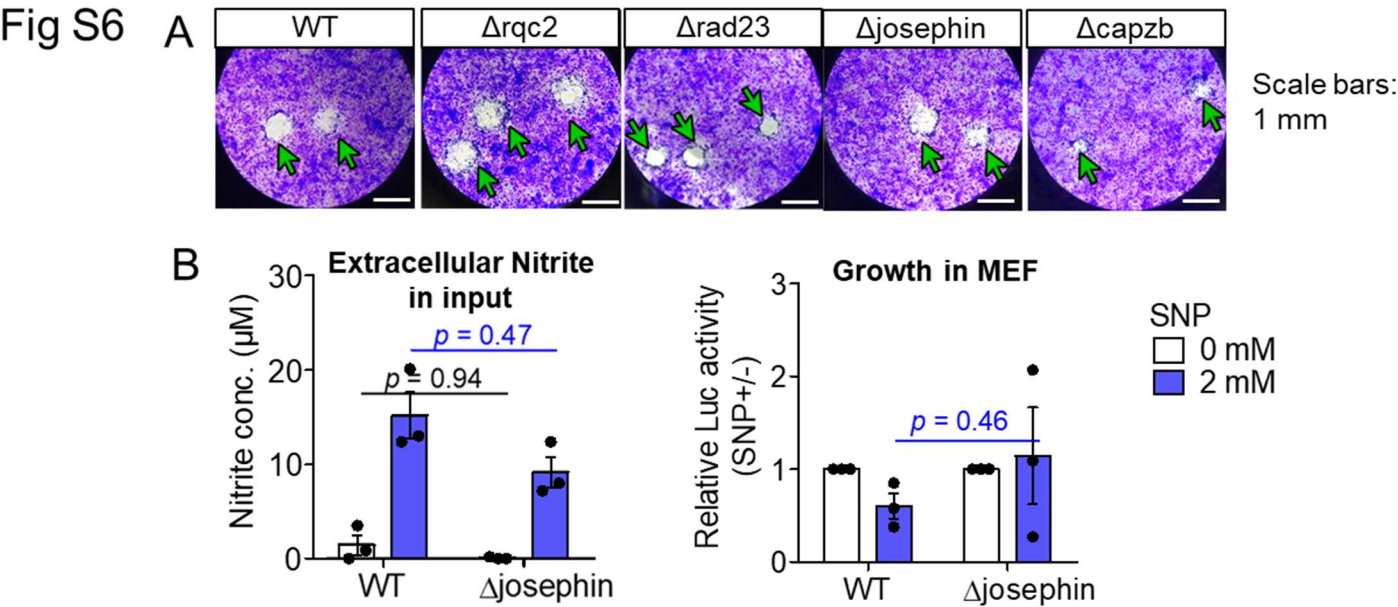
(A) iNOS expression in mice: western blotting analysis of 1 μ g/ml LPS-stimulated BMDMs induced from WT and iNOS KO mice (left). The gRNA target site on the iNOS gene is shown (right) (55, 56).

(B) Gel electrophoresis images of PCR products made from WT and IDO-deficient mouse tail fibroblast genome templates (left). 0.9-1.0 kbp exon regions including the CRISPR/Cas9 target sites in IDO1 and IDO2 were amplified (right). The underlined sequence at the IDO2 cleavage site was absent in IDO KO samples, leading to a codon frame shift (55, 56).

Fig S5



Supplementary Figure S5. Homologues of TgRad23 and TgJosephin in model organisms. (A) The top five hits for *T. gondii* TgJosephin and TgRad23 protein sequences by PSIBLAST 2.14.0+. Database: uniprotkb_swissprot (57) (B) Protein domains of TgJosephin and human Ataxin-3 predicted with SMART (58).



Supplementary Figure S6. (A) Optical microscope images of plaques made by *T. gondii* in MEFs. Arrowheads: plaques. (B) Effect of extracellular nitric oxide on parasites *in vitro*. Left: nitrite measurement of parasite supernatants (2×10^6 parasites/mL) after incubation in serum-free DMEM containing 2 mM SNP for 30 min. Right: Relative luciferase counts of parasites grown in MEFs (MOI = 1) for 24 hours after exposure to 2 mM SNP. Data shown are luciferase count ratios of 2 mM SNP-treated/untreated (0 mM) samples for each parasite line. Bar graphs represent mean $\pm$ sem.

Table S1A. Primers used in this study

Primer names	F Sequences (5'-3')	R Sequences (5'-3')	Resulting plasmids and descriptions
iNOS_gRNA1	TTAATACGACTCACTATAGGGAGTTCATCAACCAGTATTAGTTTTAGAGCTAGAAATAGCAAGTTAAAAT		iNOS gRNA1
iNOS_gRNA2	TTAATACGACTCACTATAGGTGAAGCACTAGCCAGGGACCGCTTTTAGAGCTAGAAATAGCAAGTTAAAAT		iNOS gRNA2
mIDO1_gRNA	TTAATACGACTCACTATAGGCTTCTCTGAGAGCTCGCAGTGTTTTAGAGCTAGAAATAGCAAGTTAAAAT		mIDO1 gRNA
mIDO1_screen_F/R	CTCACTGCCACTAGCTAACATGGTG	CGCACATGTAGAGGTCAGAGGGAAA	Genotyping
mIDO2_gRNA	TTAATACGACTCACTATAGGTGTTGAGGTATCCAGGAACCTGTTT TAGAGCTAGAAATAGCAAGTTAAAAT		mIDO2 gRNA
mIDO2_screen_F/R	GCATCATGTGTCATCTAGCCTTTGTATC	TTATTACAGCTGGAGGCTGACAGTCTG	Genotyping
SPM1_cDNA_F/SPM1-HA_R	agatctACCATTGTCGGGAGGAAAC	GCGGCCGCTAagcgtaatctggaacatcgatgggtaCCACTGTT	SPM1 cDNA
SPM1_gRNA1_F/R	aagttgCACACCCAAGAAGTTGCCGTg	aaaacAACTTCTTGGGTGTGTTTGAc	SPM1 gRNA1
SPM1_gRNA2_F/R	aagttgAAACACAGCTTTTCTGGGACGg	aaaacGGTCCCAGAAACGTGGTTCCa	SPM1 gRNA2
SPM1_targeting_F/R	CATTTACGCCACGCCAGAAAATGTCGGGAGGAAAC TCAAAACACACCCAAGAAGTTGCCGTATAACTTCGTA TAGCATACATTATACGAAGTTATagcttgatcagcacgaaac cttgca	CAGTGCATGCTATTGCTCACATAACAAGCCCCGAGGT TAGTACCACTGTTTCGTGTCCGATAAAGTTCTGTATAATGT ATGCTATACGAAGTTATgatccccctccaccgcggtgtcact	Generation of Δ263520 (Δspm1) parasites
TgACT1_qPCR_F/R	AAGCTTTTGCTACATCGCCCT	CCAACGGTGATGATGTTGCC	Quantitative RT-PCR
TgCapZB_gRNA1_F/R	aagttgTTCCGCGGTCCCGGCAGCGAg	aaaaCTCGCTGCCGGGACCGGCCAAc	TgCapZB gRNA1
TgCapZB_gRNA2_F/R	aagttgGTCCTGGCGGGCGCAGCATGg	aaaacCATGCTGCGCCCGCCAGGACc	TgCapZB gRNA2
TgCapZB_qPCR_F/R	GAGTCTGTGCATGTGGTGGA	TCGCTTGTTCGAGTGAGCAT	Quantitative RT-PCR
TgCapZB_targeting_F/R	ACGAACCGGAGCACCTGGAACAAGGGAGGTGAGAA GCTGCTGCGGAGCGCCAGGTTTGTGCATAACTTCG TATAGCATACATTATACGAAGTTATagcttgatcagcacg aaccttgca	TCCGTGCGCTTCTCAATTGTCGCTTCCCGACCTCGGTG CGAGAAAGGACCTCGAGCCCGGATAAAGTTCTGTATAAT GTATGCTATACGAAGTTATgatccccctccaccgcggtgtcact	Generation of Δ219290 (Δcapzb) parasites
TgDRL1_gRNA1_F/R	AAGTTgggtgagtcgtcggtcccgcgG	AAAACcgggggaccgagactcacaccA	TgDRL1 gRNA1
TgDRL1_gRNA2_F/R	AAGTTgctgctgagctgcccgaagG	AAAACTtcgggaggttcagcgacgcA	TgDRL1 gRNA2
TgDRL1_qPCR_F/R	AAAATGTGTGGTGGTCCCGA	TCGGTTTCTGACAAGGACGG	Quantitative RT-PCR
TgDRL1_targeting_F/R	acatgagcgcgtcagcgcgttcgtctgcgaaaattctcgtgtgagtcctgg tcccgatATAACTTCGTATAGCATACATTATACGAAGT TATagcttgatcagcacgaaaccttgca	gggaggggaagagtcgtcgaacgggtaacgtagaattcagtcctcgccggagt gcatcgcATAACTTCGTATAATGATGCTATACGAAGTTAT gatccccctccaccgcggtgtcact	Generation of Δ269620 (Δdrl1) parasites
TgIWS1_cDNA_F/R	AGATCTACCATGGCGGACGCGGGAGACGAGGGAG ATGCCCTCAGGGCAA	ttaataaCTActgtgctcatgctttgtagtcGAGGCCACGTCCTTCA ATTGAAACCTTCTGCGCTCTGGTCGGCTT	TgIWS1 cDNA
TgIWS1_complement_F/R	CTCGGACCTCGTTTTTTTTTCCGGAATCTCTTGCCCT AAGCGTTCTGTGACGCGTCTCGAGTaccATGGCGGA CGCGGGAGACGAGGGAGATGCCCTCAGGGCAAAT GCAGGAGGATCACCC	caagtctctaattgaacaccttcgctgcttgcgtcggtccgaactgatcgtgcac taCTAGAAGTGTGGATCAGATCCCCCTCGGGGGGG CAAGAATTGTGTTAACCGGTTTCGAC-	TgIWS1 complementation in Δ227560 (Δiws1) parasites
TgIWS1_complement_gRNA_F/R	AAGTTggtagcgcgtctcgatgatggG	AAAACcctactcgcagacgcgttcaccA	gRNA3 for TgIWS1_complementation
TgIWS1_gRNA1_F/R	AAGTTgtagtcggcgagcgggagacgG	AAAACcgtctcccgctcgcgcatacA	TgIWS1 gRNA1
TgIWS1_gRNA2_F/R	AAGTTggcgagtgcttcaccggttcaG	AAAACTgaacgcgtgaagactctgcacA	TgIWS1 gRNA2
TgIWS1_NVenus_KI_F/R	GGACCTCGTTTTTTTTCCGGAATCTCTTGCCCTAAG CGTTCGTGACGCGTCTCGAGTATGtgatcagcacgaaac cttgcaatcaaacccgcccggaagatcgcgtctgtgctgtt	tggtctgggtagctctcgtcattggcccgagggcgtcctcctcgtctcccgctcc gcCAGCTCGTCCATGCCGAGAGTGATCCCGCGCGCGG TCACGAAGCTCCAGCAGGACCATGTG	Generation of the N-terminal-Venus-TgIWS1 KI parasites
TgIWS1_qPCR_F/R	AATGCGGCAAAGGAAACGAC	GAGTGGAGGCATCGGACATT	Quantitative RT-PCR
TgIWS1_targeting_F/R	ccggaactcttgccctaagcgttgcgtgacgcgtcgtcgtgatggcggaacg gggagacgATAACTTCGTATAGCATACATTATACGAAG TTATagcttgatcagcacgaaaccttgca-	caagtctctaattgaacaccttcgctcgtcgtcggtccgaactgatcgtgcac caATAACTTCGTATAATGATGCTATACGAAGTTATgatcc ccctccaccgcggtgtcact	Generation of Δ227560 (Δiws1) parasites
TgJosephin_cDNA_F/R	agatctaccATGGCTATCCGAGCTGCGCGGCCGT		TgJosephin cDNA
TgJosephin_CHA_KI_F/R	GCGTGTGGTTTTCAGGGTATTCGATCAAGGATCG AGAGCTCGAGGAGCTGGGCTTICAAAGACGCGCAAC AGCTGATGATGATGCATCTGtaccatacagatgttccagattac gcttagGCTGATCAGCCTCGACTGTGCCTTCTAGTT	GGATGTAAGTTACAGCTCCACCATGCATGCAGAGTGA AGCAGAGAAAAGCGTTTGATTTItaGACAGCCATCTCCA TCTGGATTCTCGTGTGCGGGACGTAGCCACGACCTC AAA	Generation of the C-terminal-HA-TgJosephin KI parasites
TgJosephin_CHA_KI_gRNA_F/R	aagttgTCATCAGCTGTTTCGCGTCTTg	aaaacAAGACGCGAACAGCTGATGAc	TgJosephin KI gRNA
TgJosephin_gRNA1_F/R	aagttgGTGGTGCTACTGGGAGAAGCAc	aaaacCGTCTTCCCCAGTACACCACc	TgJosephin gRNA1
TgJosephin_gRNA2_F/R	aagttgGATCGAGAGCTCGAGGAGCTg	aaaacAGCTCCTCGAGCTCTCGATCca	TgJosephin gRNA2
TgJosephin_qPCR_F/R	CACAGCCGAGAGGAGGGATTTC	TCCAAGTTGTACCAGGTGCC	Quantitative RT-PCR
TgJosephin_targeting_F/R	CGCGGCCATGGCTATCCGAGCTGCGCGGCCGTG TCCGGCGTGGTGTAAGTGGGAGAAGCAATAAAGTTCCG TATAGCATACATTATACGAAGTTATagcttgatcagcacg aaaccttgca	AGAAAAGCGTTTGATTTTACAGATGCATCATCATCAG CTGTTTCGCGTCTTTGGAGGCCCATAACTTCGTATAATG TATGCTATACGAAGTTATgatccccctccaccgcggtgtcact	Generation of Δ229650 (Δjosephin) parasites
TgJosephin-FLAG_C24S_F	GAGAAGCAGGGCGCGAACCCGATGtCGCCTTGC ACTGCATCAACTCGCTG		TgJosephin cDNA
TgJosephin-FLAG_C24S_R		CAGCGAGTTGATGCAGTGCAGGCGgACATGCGGTTT GCGCCCTGCTTCTC	TgJosephin cDNA
TgJosephin-FLAG-PacI_R		ttaataaCTActgtgctcatgcttctttgtagtcCAGATGCATCATCATC AGCTGTTTCG	TgJosephin cDNA
TgJosephin-HA-PacI_R		TTAATTAActaagcgttaacttggaacatcgatgggtaCAGATGCATC ATCATCAGCTGTTTCG	TgJosephin cDNA
TgRad23_gRNA1_F/R	aagttgCAATGAGGAGGCGGAGCTCGg	aaaaCGAGCTCCGCTCTCTATTGc	TgRad23 gRNA1
TgRad23_gRNA2_F/R	aagttgACGGCTTCATCTTTCTGTTc	aaaacGAACAGAAAGATGAAGCCGTc	TgRad23 gRNA2
TgRad23_qPCR_F/R	GCGACCTCAGAGGCTCTCAG	AAATCCCATGGCGACCAAGT	Quantitative RT-PCR
TgRad23_targeting_F/R	GGCAGCGGAGGACAAATGAAGCTGCGCATCCGGAC GCTGAGCAATGAGGAGGCGGAGCTCGATAAAGTTCCG GTATAGCATACATTATACGAAGTTATagcttgatcagcacg aaaccttgca	CATGCTTACGCGGAGAAAACAAGACACCCGGGAGA CTGACGCGAGGAGGCGGATCAATATAAAGTTCTGTATAAT GTATGCTATACGAAGTTATgatccccctccaccgcggtgtcact	Generation of Δ2295340 (Δrad23) parasites
TgRAMP4_gRNA1_F/R	AAGTTggctgctgtgctccagcaaG	AAAACTgctggaagccaagcagaccA	TgRAMP4 gRNA1
TgRAMP4_gRNA2_F/R	AAGTTgggagcggttcaactattatGt	AAAACacataatagttgaacgtcccaA	TgRAMP4 gRNA2
TgRAMP4_qPCR_F/R	GTGGCAATGTTCCGGAAGAGC	AATCGCAGATGCCACAAACGA	Quantitative RT-PCR
TgRAMP4_targeting_F/R	agtgactctcttttttccctcagctttagcaaggaattgtgtcgttgcctccag caaATAACTTCGTATAGCATACATTATACGAAGTTAT agcttgatcagcacgaaaccttgca	tcgtgaagtcgaggaactctgctgctactgatccttatttcgacggtgattccc aATAACTTCGTATAATGATGCTATACGAAGTTATgatccc cctccaccgcggtgtcact	Generation of Δ276940 (Δramp4) parasites
TgROP18_qPCR_F/R	ACTGCATCTGGTGCAAGGAA	AGAGGCTTCGGATGAGGTCT	Quantitative RT-PCR
TgRqc2_gRNA1_F/R	aagttgACTGGACGTCGGGCTCTCGg	aaaaCGAGAGCCCGGACGCTCCAGTc	TgRqc2 gRNA1
TgRqc2_gRNA2_F/R	aagttgCATCCAGAGCAGCTGACGGg	aaaacCCGTGACGCTGCTTCTGGATGc	TgRqc2 gRNA2
TgRqc2_qPCR_F/R	GAAAGGCGGGAGTTGAAGGA	TTTGCTGCATTGTTCCGTG	Quantitative RT-PCR
TgRqc2_targeting_F/R	GGTGGAGCGCTGCATTGCTGCGCCCGTGCCTCC GCGGTTCCCTGCGTTTCTTCTTGTGCTATAAAGTTCTG ATAGCATACATTATACGAAGTTATagcttgatcagcacgaa accttgca	GGGGCTGAGACACTTTGCTGCATGTTTACTCCTCAGCT GTGACGGTCTTTTTCTCTGCTATAAAGTTCTGTATAATGT ATGCTATACGAAGTTATgatccccctccaccgcggtgtcact	Generation of Δ214090 (Δrqc2) parasites
TgSPM1_qPCR_F/R	CGTTCAACGCTCAGTCTTGC	AGCTTCAACCTCAACGGTCTG	Quantitative RT-PCR
TgSUB2_gRNA1_F/R	AAGTTgctcatttaggtaccgaagaG	AAAACTcttcggtgacctaaatgagcA	TgSUB2 gRNA1
TgSUB2_gRNA2_F/R	AAGTTgacgagaatgagctgtagcctG	AAAACaggtagcagactcattctgtcA	TgSUB2 gRNA2
TgSUB2_qPCR_F/R	TGGAGCACGCATCAAAAGAT	CCAAAGCTCTGGGTTTTGCC	Quantitative RT-PCR
TgSUB2_targeting_F/R	tgtagacgtactgggacgtatgatccttccccaccttgctcatttaggtcac cgaagaATAACTTCGTATAGCATACATTATACGAAGTT ATagcttgatcagcacgaaaccttgca	aatatcaacacgccttcaaggcggttcgcatagactgcgcacacgccttc taccataATAACTTCGTATAATGATGCTATACGAAGTTATgat ccccctccaccgcggtgtcact	Generation of Δ214500 (Δsub2) parasites
UPRT_gRNA_F/R	aagttGGGCGTCTCGATTGTGAGAGCg	aaaacGCTCTCACAAATCGAGACGCCca	UPRT gRNA (Tachibana et al. 2024)

Table S1B. Reagents and tools used in this study (IFA: immunofluorescence, WB: western blot, IP: immunoprecipitation)

Product	catalog no.	Provider	Purpose
4',6-diamidino-2-phenylindole (DAPI)	11034-56	Nacalai Tesque	IFA
Anti-10D7 antibody (IRGA6)	-	Dr. J. C. Howard	IFA
Anti-GRA17 rabbit pAb antibody	-	Our laboratory	IFA
Anti-GRA5 Antibody (TG 17.113)	A1299-50	BioVision	IFA
Anti-SQSTM1 mAb antibody (p62)	H00008878-M01J	Abnova	IFA
AlexaFluor 594 donkey anti-goat IgG (H+L) antibody	A11058	invitrogen	IFA
CF488A Donkey anti-mouse IgG antibody	20014	BIOTIUM	IFA
CF488A Donkey anti-rabbit IgG antibody	20015	BIOTIUM	IFA
CF594 Donkey anti-mouse IgG antibody	20115	BIOTIUM	IFA
CF594 Donkey anti-rabbit IgG antibody	20152	BIOTIUM	IFA
GBP1 rabbit pAb antibody	-	Dr. E. Frickel	IFA
GBP2 Rabbit PolyAb antibody	11854-1-AP	Proteintech	IFA
Anti-HA.11 Epitope Tag Antibody	901514	BioLegend	IFA/WB
Anti-TGTP(A-20) antibody (IRGB6)	sc-11079	Santa Cruz Biotechnology	IFA
GBP5 antibody (G-12): sc-166960 (GBP1-5)	sc-166960	Santa Cruz Biotechnology	IFA
Anti-β-Actin antibody, Mouse monoclonal	A1978	Sigma-Aldrich	WB
Goat anti-mouse IgG (H+L) HRP antibody	G21040	invitrogen	WB
Goat anti-rabbit IgG HRP (H+L) antibody	G21234	invitrogen	WB
HA tag Polyclonal antibody	51064-2-AP	Proteintech	WB
Purified Mouse Anti-iNOS/NOS Type II antibody	610328	BD Biosciences	WB
Toxoplasma gondii (1.B.516) antibody	sc-73210	Santa Cruz Biotechnology	WB
Monoclonal ANTI-FLAG® M2 antibody produced in mouse	F1804	Sigma-Aldrich	WB
Ammonium-Chloride-Potassium Lysing Buffer (ACK buffer)			Bone marrow
Verso cDNA Synthesis kit	AB1453B	Thermo Fisher Scientific	cDNA
Dulbecco's Modified Eagle Medium (DMEM)	08458-16	Nacalai Tesque	Cell culture
L cell supernatant, filtered	-		Cell culture
Penicillin-Streptomycin Mixed Solution (P/S)	26253-84	Nacalai Tesque	Cell culture
Phosphate Buffered Saline(10x)(pH 7.4) (PBS)	27575-31	Nacalai Tesque	Cell culture
RPMI 1640 with L-Gln, liquid	30264-56	Nacalai Tesque	Cell culture
heat-inactivated fetal calf serum (FCS)		JRH Bioscience	Cell culture, IFA
lipopolysaccharide from E. coli 055:B5 (LPS)	L2880	Sigma Aldrich	Cell stimulation
Mouse IFN-gamma Recombinant Protein (IFN-γ)	315-05	PeproTech®	Cell stimulation
2'-Deoxy-5-fluorouridine (FUDR)	D2235	Tokyo Chemical Industry	Drug selection
6-Thioxanthine (6TX)	sc-472656A	Santa Cruz	Drug selection
Mycophenolic Acid (MPA)	M3536	Sigma Aldrich	Drug selection
Pyrimethamine	P7771	Sigma Aldrich	Drug selection
Xantine	245-00011	FUJIFILM Wako	Drug selection
Digitonin	12333-51	Nacalai Tesque	IFA
Formaldehyde Solution Concentration: 36-38% (PFA)	16223-55	Nacalai Tesque	IFA
Mountant, PermaFluor 30ml	TA-030-FM	Epredia	IFA
Protein G Sepharose™ 4 Fast Flow	17-0618-05	Cytiva	IP
Promega Dual-Luciferase Reporter Assay System (LAR II)	E1910	Promega	Luc assay
Sodium nitroferricyanide(III) dihydrate (SNP)	228710	Sigma Aldrich	Nitric Oxide donor
NO2/NO3 Assay Kit-C II (Colorimetric) (Griess Kit)	344-07991	Dojindo Laboratories	Nitrite measurement
Crystal violet	031-04852	FUJIFILM Wako	Plaque staining
10 mM KPO4, 120 mM KCl, 0.15 mM CaCl2, 5 mM MgCl2, 25 mM HEPES, 2 mM EDTA (Cytomix)			Plasmid Transfection
Adenosine 5'-Triphosphate Disodium Salt n-Hydrate (ATP)	017-09673	Wako	Plasmid Transfection
RNeasy Mini Kit	74004	QIAGEN	RNA extraction
TruSeq stranded mRNA sample prep kit		Illumina	RNA-seq
GoTaq qPCR Master Mix (SYBR)		Promega	RT-PCR
Glutathione(Reduced Form), free acid (GSH)	17050-14	Nacalai Tesque	Transfection
20 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl, 1% NP-40, pH 7.5 (TNE lysis buffer)			WB
19% Tric-HCl (pH7.6), 30% Glycerol, 0.06 g/ml SDS (Sample Buffer)			WB
30(w/v)%-Acrylamide/Bis Mixed Solution(29:1) (R1)	06141-35	Nacalai Tesque	WB
BD Difco Skim milk (Skim milk)	232100	Difco Laboratories	WB
Luminata Forte Western HRP substrate (HRP substrate)		Millipore	WB
N,N,N',N'-Tetramethylethylenediamine (TEMED)	33401-72	Nacalai Tesque	WB
Polyoxyethylene Sorbitan Monolaurate (Tween®20)	28353-85	Nacalai Tesque	WB
protease inhibitor cocktail	25955-11	Nacalai Tesque	WB
Protein Ladder One Plus, Triple-color for SDS-PAGE	19593-25	Nacalai Tesque	WB
1kbp DNA Ladder One	08232-85	Nacalai Tesque	DNA
2-Mercaptoethanol (2ME)	21438-82	Nacalai Tesque	WB, cell culture
MEGAclear kit		Life Technologies	Mouse gene mutation
MEGAsortscript T7		Life Technologies	Mouse gene mutation
Gene Prep Star PI-480α		Kurabo	DNA extraction
Applied Biosystems 3500/3500xL Genetic Analyzer		Thermo Fisher Scientific	DNA sequencing
FLUOVIEW FV3000 IX83		Olympus	IFA
GLOMAX20/20 luminometer		Promega	Luc assay
Olympus CKX53		Olympus	optical microscopy
Gene Pulser II		Bio-Rad	Plasmid transfection
Gene Pulser II cuvette		Bio-Rad	Plasmid transfection
NovaSeq 6000 sequencer		Illumina	RNA-seq
TruSeq stranded mRNA sample prep kit		Illumina	RNA-seq
CFX Connect Real-Time System		Bio-Rad	RT-PCR
GraphPad Prism9		MDF	Statistical analysis
ImageQuant™ LAS 4000		GE Healthcare	WB
FACS Aria III		BD Biosciences	Cell sorting
mMESSAGE mMACHINE T7 ULTRA kit		Life Technologies	Mouse gene mutation

Table S2. Bulk RNA-sequence results of WT vs Δiws1 parasites.

Genes with a |fold change| > 2 and a Student's t-test p-value below 0.05 between WT and Δiws1 (hypothetical proteins omitted) are shown.

WT/Δiws1				Δiws1 (n = 3)		WT (n = 3)	
Fold Change	p-value	gene name	product	Normalized FPKM	Geometric Mean	Normalized FPKM	Geometric Mean
1162.2	0.00	TGGT1_227560	putative IWS1 transcription factor	0.0	0.0	116.5	116.2
93.4	0.00	TGGT1_221310	aminopeptidase N protein	0.3	0.3	23.8	23.8
20.0	0.05	TGGT1_360210	toxoplasma gondii family B protein	1.5	0.0	12.4	12.2
14.2	0.00	TGGT1_285870	SAG-related sequence SRS20A	7.9	6.7	97.2	95.0
13.6	0.00	TGGT1_278930	Tubulin-tyrosine ligase family protein	1.4	1.3	17.4	17.4
12.2	0.00	TGGT1_254350	endo-1,3(4)-beta-glucanase	0.3	0.3	4.2	4.0
10.8	0.00	TGGT1_317705	enoyl-CoA hydratase/isomerase family protein	4.0	3.8	41.8	41.0
10.0	0.00	TGGT1_286150	PAN/Apple domain-containing protein	2.0	1.9	20.0	19.6
9.3	0.00	TGGT1_205250	rhoptry protein ROP18	20.6	20.2	197.1	188.6
8.4	0.02	TGGT1_301890	Toxoplasma gondii family B protein	0.3	0.0	2.4	2.1
8.1	0.00	TGGT1_307030	Purine nucleoside phosphorylase	3.4	3.0	24.7	24.7
8.0	0.00	TGGT1_203720	vitamin k epoxide reductase family protein	9.5	9.1	75.3	72.7
6.2	0.00	TGGT1_285470	patched family protein	6.9	6.7	41.4	41.4
6.1	0.00	TGGT1_229140	MaoC family domain-containing protein	1.4	1.4	8.3	8.3
5.6	0.00	TGGT1_306338B	putative dynein gamma chain, flagellar outer arm	3.8	3.7	20.9	20.8
4.8	0.01	TGGT1_239090	SAG-related sequence SRS23	11.5	10.5	51.2	50.5
4.7	0.02	TGGT1_312930	putative cystathione gamma lyase	2.7	2.5	13.3	12.0
4.6	0.00	TGGT1_204050	subtilisin SUB1	366.1	361.6	1678.9	1678.4
4.6	0.01	TGGT1_278120	SCP family extracellular subfamily protein	1.1	1.0	5.0	4.8
4.4	0.02	TGGT1_220080	Toxoplasma gondii family C protein	14.1	12.5	57.0	55.3
4.3	0.00	TGGT1_323020	KRUF family protein	8.6	8.3	36.4	36.1
4.3	0.01	TGGT1_203580	NAD-binding domain 4 domain-containing protein	0.2	0.1	0.6	0.6
3.9	0.01	TGGT1_280570	SAG-related sequence SRS35A	6.8	6.8	28.4	26.3
3.8	0.00	TGGT1_283450	Toxoplasma gondii family B protein	2.8	2.7	10.8	10.4
3.8	0.00	TGGT1_226310	zinc finger (CCCH type) motif-containing protein	3.3	3.3	12.5	12.3
3.7	0.02	TGGT1_272240	Toxoplasma gondii family D protein	0.3	0.3	1.1	1.1
3.6	0.05	TGGT1_365980	putative transmembrane protein	1.7	1.4	5.3	5.1
3.4	0.00	TGGT1_275360	SAG-related sequence SRS47B	0.3	0.3	0.9	0.9
3.3	0.04	TGGT1_205402	SNF2 family N-terminal domain-containing protein	0.1	0.0	0.5	0.5
3.3	0.05	TGGT1_266860	BTB/POZ domain-containing protein	2.4	2.2	7.8	7.3
3.1	0.01	TGGT1_236990	beta-ketoacyl synthase, N-terminal domain-containing protein	1.7	1.7	5.7	5.4
2.9	0.00	TGGT1_214580	tetratricopeptide repeat-containing protein	7.8	7.8	22.6	22.5
2.8	0.01	TGGT1_230830	ATPase family associated with various cellular activities (AAA) domain-containing protein	3.7	3.5	9.8	9.8
2.7	0.00	TGGT1_205265	transporter, cation channel family protein	1.3	1.3	3.5	3.5
2.6	0.00	TGGT1_233480	SAG-related sequence SRS29C	1419.7	1411.1	3676.4	3667.6
2.5	0.00	TGGT1_235515	MORN repeat-containing protein	9.7	9.7	24.5	24.4
2.4	0.01	TGGT1_278110	1,3-beta-glucan synthase component protein	14.6	14.4	35.2	34.3
2.4	0.01	TGGT1_205398	putative DNA repair and recombination protein RAD54	0.4	0.3	0.9	0.8
2.4	0.03	TGGT1_257945	3'5'-cyclic nucleotide phosphodiesterase domain-containing protein	2.1	2.0	4.8	4.7
2.2	0.00	TGGT1_228500	3'5'-cyclic nucleotide phosphodiesterase domain-containing protein	0.2	0.2	0.5	0.5
2.0	0.00	TGGT1_246660	AP2 domain transcription factor AP2XII-3	0.1	0.1	0.2	0.2
2.0	0.03	TGGT1_254850	putative myosin heavy chain	2.0	1.9	3.9	3.9
-2.0	0.00	TGGT1_215500	TPR repeat-containing protein	30.7	30.6	15.1	15.1
-2.1	0.00	TGGT1_259530	GalNac	38.2	38.0	18.5	18.4
-2.1	0.02	TGGT1_312100	plasma membrane-type Ca(2+)-ATPase A1 PMCAA1	22.5	22.3	11.0	10.8
-2.1	0.00	TGGT1_278390	Toxoplasma gondii family A protein	47.1	46.7	22.6	22.4
-2.1	0.00	TGGT1_248810	nuclear factor NF7	166.0	163.6	78.1	78.0
-2.1	0.00	TGGT1_305460	putative methionine aminopeptidase 2	568.8	562.8	264.9	262.8
-2.2	0.00	TGGT1_216140	tetratricopeptide repeat-containing protein	146.2	143.8	66.4	66.4
-2.3	0.03	TGGT1_238210	EGF family domain-containing protein	3.0	2.9	1.3	1.2
-2.4	0.04	TGGT1_233770B	calcium-translocating P-type ATPase, PMCA-type protein	0.8	0.7	0.3	0.3
-2.4	0.03	TGGT1_275370	SAG-related sequence SRS47C	9.8	9.2	3.9	3.9
-2.5	0.03	TGGT1_266335	Toxoplasma gondii family A protein	0.3	0.2	0.0	0.0
-2.5	0.05	TGGT1_253380	AP2 domain transcription factor AP2III-2	27.8	26.6	11.2	10.4
-2.7	0.00	TGGT1_286120	prolyl endopeptidase	321.1	319.2	118.6	118.5
-2.7	0.04	TGGT1_243170	Toxoplasma gondii family A protein	0.4	0.3	0.1	0.0
-2.8	0.00	TGGT1_265542	protein phosphatase 2C domain-containing protein	0.3	0.3	0.1	0.1
-3.0	0.03	TGGT1_280420	HEAT repeat-containing protein	0.6	0.6	0.2	0.2
-3.0	0.01	TGGT1_230905	hydrolase, alpha/beta fold family protein	4.4	4.4	1.5	1.5
-3.1	0.01	TGGT1_232400	PAN domain-containing protein	10.6	10.5	3.5	3.4
-3.1	0.01	TGGT1_270580	HECT-domain (ubiquitin-transferase) domain-containing protein	0.8	0.8	0.3	0.2
-3.3	0.02	TGGT1_223920	rhoptry neck protein RON3	190.1	186.0	59.9	55.6
-3.3	0.00	TGGT1_294570	rhodanese family domain-containing protein	0.4	0.4	0.1	0.1
-3.4	0.02	TGGT1_306450	putative short chain dehydrogenase family protein	13.8	12.5	3.7	3.7
-3.5	0.01	TGGT1_202285	EGF family domain-containing protein	0.4	0.4	0.1	0.0
-3.5	0.02	TGGT1_278380	Toxoplasma gondii family A protein	4.6	4.1	1.2	1.2
-3.6	0.01	TGGT1_279460	Toxoplasma gondii family B protein	2.3	2.3	0.7	0.6
-3.6	0.00	TGGT1_219240	Peptidyl-tRNA hydrolase PTH2 domain-containing protein	64.6	64.3	17.7	17.7
-3.9	0.00	TGGT1_293480	MoeA N-terminal region (domain I and II) domain-containing protein	28.0	27.5	7.0	7.0
-4.0	0.00	TGGT1_320480	Rab11b	348.1	341.5	86.0	85.6
-4.2	0.01	TGGT1_212740	aromatic amino acid hydrolase AAH2	0.5	0.4	0.0	0.0
-4.3	0.02	TGGT1_410590	cAMP-dependent protein kinase	0.6	0.5	0.1	0.0
-4.4	0.00	TGGT1_294600	Toxoplasma gondii family D protein	0.4	0.4	0.0	0.0
-5.0	0.00	TGGT1_312570	CMGC kinase, MAPK family (ERK) MAPK-1	165.2	164.9	33.6	33.3
-5.5	0.01	TGGT1_314250	bradyzoite rhoptry protein BRP1	66.5	59.8	11.6	10.9
-6.2	0.00	TGGT1_315220	rhoptry protein ROP14	309.5	303.5	50.4	49.3
-6.3	0.01	TGGT1_292375	KRUF family protein	18.4	15.7	2.5	2.5
-7.2	0.01	TGGT1_253790	zinc finger (CCCH type) motif-containing protein	37.6	33.9	5.1	4.7
-7.8	0.05	TGGT1_304450	cation-transporting ATPase	2.1	1.4	0.2	0.2
-13.3	0.00	TGGT1_247560	putative dynein light chain protein	2.2	2.2	0.2	0.2

Table S3. List of putative target genes of TglWS1 (hypothetical proteins omitted)

ID	annotation	ID	annotation
TGGT1_263090	14-3-3 protein	TGGT1_318230	phosphoglycerate kinase PGKI
TGGT1_319650	3' exoribonuclease family, domain 1 domain-containing protein	TGGT1_254690	phospholipase/carboxylesterase
TGGT1_318675	3'5'-cyclic nucleotide phosphodiesterase domain-containing protein	TGGT1_258410	photosensitized INA-labeled protein PHIL1
TGGT1_214440	4'-phosphopantetheinyl transferase superfamily protein	TGGT1_280380	poly(ADP-ribose) glycohydrolase
TGGT1_219180	acetyltransferase, GNAT family protein	TGGT1_236010	prenylcysteine oxidase
TGGT1_260550	acetyltransferase, GNAT family protein	TGGT1_293690	profilin PRF
TGGT1_209030	actin ACT1	TGGT1_247460	proliferating cell nuclear antigen PCNA1
TGGT1_220400	actin depolymerizing factor ADF	TGGT1_201630A	protein phosphatase 2C domain-containing protein
TGGT1_215480	Adenosine/AMP deaminase domain-containing protein	TGGT1_220610	protein phosphatase 2C domain-containing protein
TGGT1_240960	AIG2 family protein	TGGT1_244450	protein phosphatase 2C domain-containing protein
TGGT1_250360	alpha/beta hydrolase fold domain-containing protein	TGGT1_276920	protein phosphatase 2C domain-containing protein
TGGT1_231640	alveolin domain containing intermediate filament IMC1	TGGT1_230380	protein translocation complex, SEC61 gamma subunit
TGGT1_230210	alveolin domain containing intermediate filament IMC10	TGGT1_252640	P-type ATPase PMA1
TGGT1_239770	alveolin domain containing intermediate filament IMC11	TGGT1_260600	Pumilio-family RNA binding repeat-containing protein
TGGT1_260540	alveolin domain containing intermediate filament IMC14	TGGT1_271888	putative 3-ketoacyl-CoA reductase
TGGT1_231630	alveolin domain containing intermediate filament IMC4	TGGT1_249900	putative adenine nucleotide translocator
TGGT1_220270	alveolin domain containing intermediate filament IMC6	TGGT1_279450	putative adenylosuccinate synthetase
TGGT1_299020	AP2 domain transcription factor AP2III-4	TGGT1_224110	putative adhesion regulating molecule region protein
TGGT1_318610	AP2 domain transcription factor AP2IV-3	TGGT1_217890	putative alkyl hydroperoxide reductase/ Thiol specific antioxidant/ Mal allergen
TGGT1_318470	AP2 domain transcription factor AP2IV-4	TGGT1_224460	putative aminopeptidase n
TGGT1_267460	AP2 domain transcription factor AP2IX-1	TGGT1_204400	putative ATPase synthase subunit alpha
TGGT1_240900	AP2 domain transcription factor AP2VI-2	TGGT1_221620	putative beta-tubulin
TGGT1_282210	AP2 domain transcription factor AP2VIIa-8	TGGT1_201380	putative chorismate synthase
TGGT1_269010	AP2 domain transcription factor AP2VIII-7	TGGT1_219750	putative cytochrome c
TGGT1_215150	AP2 domain transcription factor AP2X-9	TGGT1_293390	putative cytochrome C-type heme lyase
TGGT1_309410	AP2 domain transcription factor AP2XI-1	TGGT1_214970	putative DNA replication licensing factor
TGGT1_250800	AP2 domain transcription factor AP2XII-8	TGGT1_262480	putative dynein light chain roadblock-type 2
TGGT1_300130	apical membrane antigen 1 domain-containing protein	TGGT1_294800A	putative elongation factor 1-alpha (EF-1-ALPHA)
TGGT1_270510	asparaginyl-tRNA synthetase (NOB+tRNA synthase)	TGGT1_235540	putative eukaryotic initiation factor-2 beta
TGGT1_205420	aspartate-semialdehyde dehydrogenase	TGGT1_250770	putative eukaryotic initiation factor-4A
TGGT1_304630	ATG C terminal domain-containing protein	TGGT1_210760	putative glutamine amidotransferase
TGGT1_202580	ATPase, AAA family protein	TGGT1_281490	putative glutamine amidotransferase subunit pdxt
TGGT1_314250	bradyzoite rhoptry protein BRP1	TGGT1_251680	putative histamine-releasing factor
TGGT1_206590	calcium-dependent protein kinase CDPK2A	TGGT1_288740	putative L-asparaginase
TGGT1_233770B	calcium-translocating P-type ATPase, PMCA-type protein	TGGT1_208730	putative microneme protein
TGGT1_356400	cAMP-dependent protein kinase	TGGT1_221190	putative mrna cleavage factor family protein
TGGT1_410590	cAMP-dependent protein kinase	TGGT1_247470	putative nucleolar protein
TGGT1_293810	carboxyvinyl-carboxyphosphonate phosphorylmutase	TGGT1_224850	putative polyadenylate binding protein
TGGT1_247230	centrin	TGGT1_318660	putative PP2C
TGGT1_311720	chaperonin protein BiP	TGGT1_243950	putative prohibitin
TGGT1_293770	chitinase-like protein CLP1	TGGT1_279390	putative proliferation-associated protein 2G4
TGGT1_290730	CS domain-containing protein	TGGT1_276940	putative ribosome associated membrane protein RAMP4
TGGT1_279430	cwf18 pre-mRNA splicing factor protein	TGGT1_251690	putative seryl-tRNA synthetase, cytoplasmic
TGGT1_313600	DDHD domain-containing protein	TGGT1_306450	putative short chain dehydrogenase family protein
TGGT1_226250	DEAD (Asp-Glu-Ala-Asp) box polypeptide DDX3X	TGGT1_409570	putative toxoplasma gondii family A protein
TGGT1_241140	DEAD/DEAH box helicase domain-containing protein	TGGT1_363940	putative transmembrane protein
TGGT1_263820	DEAD/DEAH box helicase domain-containing protein	TGGT1_410370	putative transmembrane protein
TGGT1_297880	dense granule protein DG32	TGGT1_411410	putative transmembrane protein
TGGT1_212410	dense granule protein GRA11	TGGT1_262960	putative U1 snRNP-associated protein Usp106
TGGT1_237800	dense granule protein GRA11	TGGT1_208570	putative ubiquitin conjugating enzyme E2
TGGT1_275850	dense granule protein GRA12	TGGT1_280770	regulator of chromosome condensation (RCC1) repeat-containing protein
TGGT1_288650	dense granule protein GRA12	TGGT1_200290	rhomboid protease ROM1
TGGT1_239740	dense granule protein GRA14	TGGT1_212910	rhomboid protease ROM3
TGGT1_275470	dense granule protein GRA15	TGGT1_269885A	rhoptry metalloprotease toxolysin TLN1
TGGT1_227620	dense granule protein GRA2	TGGT1_295125	rhoptry protein ROP4
TGGT1_275440	dense granule protein GRA6	TGGT1_308090	rhoptry protein ROP5
TGGT1_203310	dense granule protein GRA7	TGGT1_215775	rhoptry protein ROP8
TGGT1_222170	dense-granule antigen DG32	TGGT1_243730	rhoptry protein ROP9
TGGT1_270650	deoxyribose-phosphate aldolase	TGGT1_289530	ribosomal protein RPL19
TGGT1_243680	dihydrodipicolinate reductase	TGGT1_250810	ribosomal protein RPL35
TGGT1_261540	DNA-directed RNA polymerase I RPAC2	TGGT1_300190	ribosomal protein RPL37A
TGGT1_232050	DnaJ domain-containing protein	TGGT1_245460	ribosomal protein RPS8
TGGT1_294330	EGF family domain-containing protein	TGGT1_205410	RNA pseudouridine synthase superfamily protein
TGGT1_321800	EGF family domain-containing protein	TGGT1_223855	RNA recognition motif-containing protein
TGGT1_249030	endonuclease/exonuclease/phosphatase family protein	TGGT1_262620	RNA recognition motif-containing protein
TGGT1_214320	facilitative glucose transporter GT1	TGGT1_409850	SAG-related sequence protein SRS47A
TGGT1_242380	fatty acid elongase	TGGT1_360840	SAG-related sequence protein SRS48E
TGGT1_292110	formate/nitrite transporter protein	TGGT1_362620	SAG-related sequence protein SRS48E
TGGT1_206430	formin FRM1	TGGT1_222370	SAG-related sequence SRS13
TGGT1_216670	FUSE-binding protein 2 / KH-type splicing regulatory protein	TGGT1_254060	SAG-related sequence SRS14A
TGGT1_249850	GAP40 protein	TGGT1_320250	SAG-related sequence SRS15A
TGGT1_314240	general transcription factor IIIC polypeptide 3 GTF3C3	TGGT1_319360	SAG-related sequence SRS17A
TGGT1_271970	glideosome-associated protein with multiple-membrane spans GAPM	TGGT1_238440	SAG-related sequence SRS22A
TGGT1_294200	glucose-6-phosphate 1-dehydrogenase	TGGT1_238460	SAG-related sequence SRS22B

Table S4. Mass spectrometry of ubiquitinated *T. gondii* peptides.
The top 30 (Δ josephin>WT) in MEF (IFN γ +)- and Vero-derived results are shown.

Parasites grown in MEF

			Δ josephin/WT Abundance Ratio (Red: >2)		Δ josephin/WT P-Value (Yellow: < 0.05):	
Position in Protein	gene	transcript product	IFN γ -	IFN γ +	IFN γ -	IFN γ +
33-68	TGGT1_263520	microtubule associated protein SPM1	15.5	8.5	0.00	0.00
183-193	TGGT1_255890	pyridine nucleotide-disulfide oxidoreductase domain-containing p	4.0	5.4	0.00	0.00
121-133	TGGT1_263720	HMG (high mobility group) box domain-containing protein	7.5	4.5	0.00	0.00
528-548	TGGT1_221950	putative spliceosome-associated protein	1.6	4.2	0.99	0.31
134-145	TGGT1_263720	HMG (high mobility group) box domain-containing protein	2.5	3.7	0.02	0.00
391-399	TGGT1_220510	hypothetical protein	3.5	3.2	0.00	0.00
41-55	TGGT1_315570	hypothetical protein	2.9	2.7	0.18	0.02
57-78	TGGT1_286600	hypothetical protein	1.4	2.7	0.30	0.01
385-397	TGGT1_220510	hypothetical protein	3.2	2.7	0.01	0.01
33-51	TGGT1_263520	microtubule associated protein SPM1	4.6	2.6	0.00	0.00
345-354	TGGT1_286590	microtubule associated protein SPM2	3.2	2.4	0.00	0.00
11-32	TGGT1_263520	microtubule associated protein SPM1	2.3	2.4	0.00	0.00
134-145	TGGT1_263720	HMG (high mobility group) box domain-containing protein	4.1	2.3	0.00	0.00
313-327	TGGT1_263520	microtubule associated protein SPM1	3.3	2.3	0.00	0.00
1800-1819	TGGT1_250780	Zn-finger in ubiquitin-hydrolases domain-containing protein	2.4	2.2	0.05	0.01
530-537	TGGT1_212980	hypothetical protein	1.3	2.1	0.88	0.20
200-207	TGGT1_218520	microneme protein MIC6	1.3	2.1	0.36	0.02
88-106	TGGT1_263720	HMG (high mobility group) box domain-containing protein	3.0	2.1	0.00	0.00
49-58	TGGT1_239330	ribosomal protein RPL37	2.8	2.1	0.00	0.03
942-952	TGGT1_215950	hypothetical protein	1.2	2.1	0.94	0.03
141-152	TGGT1_267430	DnaJ domain-containing protein	1.3	2.0	0.75	0.12
320-333	TGGT1_235340	hypothetical protein	1.8	2.0	0.07	0.01
52-63	TGGT1_311000	hypothetical protein	1.5	2.0	0.52	0.34
265-279	TGGT1_315690	DnaJ domain-containing protein	1.6	2.0	0.16	0.01
15-37	TGGT1_240810	hypothetical protein	1.7	2.0	0.47	0.26
46-68	TGGT1_263520	microtubule associated protein SPM1	3.9	2.0	0.01	0.02
183-193	TGGT1_255890	pyridine nucleotide-disulfide oxidoreductase domain-containing p	3.8	1.9	0.00	0.00
272-294	TGGT1_304490	hypothetical protein	1.3	1.9	0.85	0.18
477-487	TGGT1_290270	SPRY domain-containing protein	1.4	1.9	0.35	0.35
12-28	TGGT1_263520	microtubule associated protein SPM1	3.6	1.9	0.00	0.00

Parasites grown in Vero

Position	Master Protein Descriptions		Abundance Ratio	P-Value
33-51	TGGT1_263520	microtubule associated protein SPM1	4.0	0.00
29-45	TGGT1_263520	microtubule associated protein SPM1	2.7	0.00
28-42	TGGT1_305520	ribosomal protein RPS2	2.6	0.01
345-354	TGGT1_286590	microtubule associated protein SPM2	2.0	0.00
313-327	TGGT1_263520	microtubule associated protein SPM1	1.8	0.01
391-397	TGGT1_220510	hypothetical protein	1.8	0.07
7-29	TGGT1_289750	ribosomal-ubiquitin protein RPL40	1.8	0.16
300-308	TGGT1_313390	ribosomal protein RPL6	1.8	0.68
392-399	TGGT1_220510	hypothetical protein	1.8	0.14
11月28日	TGGT1_263520	microtubule associated protein SPM1	1.8	0.00
400-414	TGGT1_220510	hypothetical protein	1.7	0.01
126-161	TGGT1_247230	centrin	1.7	0.43
170-177	TGGT1_207950	hypothetical protein	1.7	0.31
87-92	TGGT1_249850	GAP40 protein	1.6	0.07
134-144	TGGT1_263720	HMG (high mobility group) box domain-containing protein	1.6	0.09
115-127	TGGT1_306190	hypothetical protein	1.6	0.43
173-183	TGGT1_225790	PDI family protein	1.6	0.01
281-298	TGGT1_263520	microtubule associated protein SPM1	1.6	0.05
249-266	TGGT1_263520	microtubule associated protein SPM1	1.5	0.01
12-29	TGGT1_289750	ribosomal-ubiquitin protein RPL40	1.5	0.03
315-339	TGGT1_261780	microneme protein MIC7	1.5	0.00
350-362	TGGT1_205300	hypothetical protein	1.5	0.84
652-669	TGGT1_290290	ubiquitin-activating enzyme E1 family protein	1.5	0.82
1-27	TGGT1_289750	ribosomal-ubiquitin protein RPL40	1.5	0.19
910-918	TGGT1_232510	hypothetical protein	1.5	0.67
44-52	TGGT1_249250	ribosomal protein RPL35A	1.5	0.03
43-55	TGGT1_254600	ubiquitin family protein	1.5	0.68
154-164	TGGT1_250870	DHHC zinc finger domain-containing protein	1.4	0.76
318-325	TGGT1_288360	tryptophanyl-tRNA synthetase (TrpRS2)	1.4	0.96
44-56	TGGT1_295340	UV excision repair protein Rad23 protein	1.4	0.69

Table S5. Bulk RNA sequencing in results in WT and Δ josephin parasites. Genes with a |fold change| > 2 and a Student's t-test p-value below 0.05 between WT and Δ josephin (hypothetical proteins omitted)

Δjosephin				WT		Δjosephin/WT				Δjosephin		WT			
Fold Change	p-value	gene name	product	Normalized d FPKM	Geometric Mean	Normalized d FPKM	Geometric Mean	Fold Change	p-value	gene name	product	Normalized FPKM	Geometric Mean	Normalized FPKM	Geometric Mean
24.5	0.02	TGGT1_270580	HECT-domain (ubiquitin-transferase) domain-containing protein	22.8	22.7	1.6	0.9	2.7	0.02	TGGT1_253930	GCC2 and GCC3 domain-containing protein	40.4	39.2	15.0	14.3
15.6	0.05	TGGT1_270595	UBA/TS-N domain-containing protein	8.8	8.6	1.1	0.5	2.7	0.02	TGGT1_295720	sulfite oxidase, putative	19.3	19.1	7.4	7.0
9.0	0.03	TGGT1_202285	EGF family domain-containing protein	7.9	7.6	1.1	0.8	2.7	0.01	TGGT1_269600	biotin-requiring enzyme domain-containing protein	42.0	41.8	15.9	15.4
9.0	0.00	TGGT1_294570	rhodanese family domain-containing protein	1.7	1.6	0.2	0.2	2.7	0.01	TGGT1_263380	Dullard family phosphatase domain-containing protein	5.9	5.9	2.3	2.2
8.6	0.02	TGGT1_266270	short-chain-acyl-CoA dehydrogenase	10.7	10.7	1.6	1.3	2.6	0.00	TGGT1_219832	cyclin-dependent kinase regulatory subunit protein	16.2	16.2	6.2	6.2
8.1	0.01	TGGT1_282230	sulfate adenyllyltransferase/adenyllysulfate kinase	2.7	2.7	0.4	0.3	2.6	0.05	TGGT1_207875	GCC2 and GCC3 domain-containing protein	4.8	4.7	2.0	1.8
8.0	0.01	TGGT1_202280	WD domain, G-beta repeat-containing protein	43.1	42.8	6.3	5.4	2.5	0.00	TGGT1_257720	proton ATPase, putative	5.6	5.5	2.2	2.2
7.6	0.04	TGGT1_230905	hydrolase, alpha/beta fold family protein	30.1	30.0	5.4	4.0	2.5	0.05	TGGT1_289240	DnaJ domain-containing protein	0.9	0.9	0.4	0.3
7.5	0.03	TGGT1_294820	type I fatty acid synthase, putative	4.8	4.6	0.8	0.6	2.5	0.02	TGGT1_230555	adenylate and guanylate cyclase catalytic domain-containing protein	2.4	2.3	1.0	0.9
7.3	0.02	TGGT1_211270	sushi domain (scr repeat) domain-containing protein	5.8	5.7	0.9	0.8	2.5	0.01	TGGT1_297780	ATPase/histidine kinase/DNA gyrase B/HSP90 domain-containing protein	18.1	18.0	7.5	7.3
7.3	0.04	TGGT1_255710	ULK kinase	3.1	3.0	0.6	0.4	2.5	0.01	TGGT1_267460	AP2 domain transcription factor AP2IX-1	2.3	2.2	0.9	0.9
7.1	0.01	TGGT1_229140	MaoC family domain-containing protein	61.3	61.1	9.8	8.6	2.4	0.02	TGGT1_295750	IgA-specific serine endopeptidase	0.4	0.3	0.1	0.1
6.8	0.02	TGGT1_284602	E1-E2 ATPase subfamily protein	2.6	2.6	0.5	0.4	2.4	0.03	TGGT1_244850	GMC oxidoreductase	1.9	1.9	0.8	0.8
6.7	0.01	TGGT1_207005	SAG-related sequence SRS48Q	0.8	0.7	0.0	0.0	2.4	0.02	TGGT1_232450	SWI2/SNF2-containing protein RAD54	6.6	6.6	2.9	2.8
6.6	0.00	TGGT1_293480	MoeA N-terminal region (domain I and II) domain-containing protein	28.0	26.4	4.0	4.0	2.4	0.01	TGGT1_306450	short chain dehydrogenase family protein, putative	7.9	7.7	3.3	3.3
6.6	0.01	TGGT1_237800	dense granule protein GRA11	25.9	22.9	3.7	3.5	2.4	0.01	TGGT1_235860	subtilisin SUB11	3.8	3.8	1.7	1.6
6.4	0.01	TGGT1_284598	haloacetal dehalogenase family hydrolase domain-containing protein	82.8	82.8	15.2	12.9	2.3	0.00	TGGT1_313950	GMC oxidoreductase	5.6	5.5	2.4	2.3
6.2	0.01	TGGT1_315560	ATP-binding cassette G family transporter ABCG77	1.9	1.8	0.3	0.3	2.3	0.00	TGGT1_204360	subtilisin SUB4	10.8	10.7	4.6	4.6
5.7	0.02	TGGT1_276960	tetratricopeptide repeat-containing protein	9.5	9.4	1.9	1.7	2.3	0.01	TGGT1_260480	leucine rich repeat-containing protein	4.4	4.3	1.9	1.9
5.6	0.01	TGGT1_239020	ABC transporter transmembrane region domain-containing protein	49.3	49.3	9.9	8.7	2.3	0.00	TGGT1_314340	Sodium:neurotransmitter symporter family protein	1.0	1.0	0.4	0.4
5.5	0.01	TGGT1_202140	enoyl-CoA hydratase/isomerase family protein	1.1	1.1	0.2	0.2	2.3	0.04	TGGT1_201850	WD domain, G-beta repeat-containing protein	3.4	3.2	1.5	1.4
5.4	0.03	TGGT1_207665	kinesin motor domain-containing protein	6.9	6.2	1.3	1.2	2.2	0.00	TGGT1_240370	Toxoplasma gondii family E protein	0.2	0.2	0.0	0.0
5.2	0.00	TGGT1_253110	kinesin motor domain-containing protein	1.5	1.5	0.3	0.3	2.2	0.01	TGGT1_235650	carrier superfamily protein	8.3	8.3	3.8	3.7
5.2	0.00	TGGT1_213780	radical SAM domain-containing protein	56.8	56.8	11.4	11.0	2.2	0.05	TGGT1_219070	cyclic nucleotide-binding domain-containing protein	21.8	21.8	10.5	9.8
5.0	0.01	TGGT1_205430	isovaleryl-CoA dehydrogenase	4.6	4.3	0.9	0.9	2.2	0.00	TGGT1_234570	sterol carrier protein-2 HAD-2SCP-2	3.9	3.9	1.8	1.8
5.0	0.02	TGGT1_296640	SAG-related sequence SRS48E	0.6	0.5	0.0	0.0	2.2	0.04	TGGT1_243720	peroxisomal biogenesis factor PEX11	8.8	8.7	4.1	3.9
4.6	0.02	TGGT1_221830	subtilisin SUB12	1.7	1.7	0.4	0.4	2.2	0.03	TGGT1_301890	Toxoplasma gondii family B protein	203.3	202.0	96.0	91.8
4.5	0.05	TGGT1_269120	oxidoreductase, putative	0.8	0.7	0.2	0.1	2.1	0.04	TGGT1_284670	flagellar associated protein	0.6	0.6	0.3	0.3
4.3	0.04	TGGT1_288050	intraflagellar transport protein 172, putative	1.9	1.8	0.5	0.4	2.1	0.05	TGGT1_215785	hoptry protein ROP2A	0.5	0.5	0.2	0.2
4.3	0.00	TGGT1_270210	kinesin motor domain-containing protein	18.6	18.5	4.6	4.3	2.1	0.00	TGGT1_310160	argonaute AGO	12.0	12.0	5.7	5.7
4.2	0.00	TGGT1_212970	protein kinase (incomplete catalytic triad)	7.1	7.0	1.7	1.7	2.1	0.00	TGGT1_234440	aminotransferase, putative	13.9	13.8	6.7	6.6
4.1	0.00	TGGT1_230460	Dpy-30 motif protein	18.5	18.5	4.5	4.5	2.1	0.04	TGGT1_255620	B9-C2 domain-containing protein	6.6	6.6	3.3	3.2
4.0	0.05	TGGT1_221550	CMGC kinase, MAPK family, MEK kinase-related (incomplete catalytic triad)	2.6	2.4	0.7	0.6	2.1	0.01	TGGT1_318275	peptidyl-prolyl cis-trans isomerase, FKBP-type domain-containing protein	7.4	7.2	3.5	3.5
4.0	0.03	TGGT1_286932	ATPase family associated with various cellular activities (AAA) domain-containing protein	4.7	4.7	1.4	1.2	2.1	0.05	TGGT1_206650	zinc finger, c2h2 type domain-containing protein	2.1	2.0	1.0	1.0
3.9	0.02	TGGT1_307460	Toxoplasma gondii family B protein	6.8	6.4	1.8	1.6	2.0	0.02	TGGT1_233140	deoxyuridine 5'-triphosphate nucleotidohydrolase, putative	38.9	38.6	19.4	19.0
3.9	0.01	TGGT1_207970	HEAT repeat-containing protein	10.9	10.8	2.9	2.7	2.0	0.04	TGGT1_257470	myosin J	5.1	5.0	2.6	2.5
3.9	0.00	TGGT1_328400	aminotransferase, class V family protein	0.4	0.4	0.0	0.0	2.0	0.01	TGGT1_253030	glycosyl hydrolase, family 31 protein	20.4	20.3	10.2	10.1
3.9	0.00	TGGT1_293800	transmembrane protein 65, putative	3.6	3.4	0.9	0.9	-2.0	0.05	TGGT1_233920	zinc finger, C3HC4 type (RING finger) domain-containing protein	21.1	20.9	44.4	42.2
3.9	0.02	TGGT1_269930	calcium binding egf domain-containing protein	29.7	29.5	8.4	7.6	-2.1	0.02	TGGT1_216830	DTW domain-containing protein	10.9	10.8	22.8	22.3
3.8	0.01	TGGT1_272260	Dpy-30 motif protein	0.7	0.7	0.2	0.2	-2.1	0.04	TGGT1_293280	cyclin protein	23.5	23.2	49.9	47.9
3.8	0.01	TGGT1_315845	kinesin motor domain-containing protein	1.1	1.1	0.3	0.3	-2.2	0.02	TGGT1_306620	AP2 domain transcription factor AP2IX-9	64.9	64.2	142.7	139.1
3.8	0.00	TGGT1_211910	kinesin-related protein 3A, putative	0.4	0.4	0.1	0.1	-2.2	0.03	TGGT1_318610	AP2 domain transcription factor AP2IV-3	3.5	3.4	8.0	7.7
3.7	0.00	TGGT1_206570	kinesin, putative	0.4	0.4	0.1	0.0	-2.4	0.01	TGGT1_244180	microneme-like protein	0.2	0.2	0.4	0.4
3.7	0.02	TGGT1_258508	3'-cyclic nucleotide phosphodiesterase domain-containing protein	1.5	1.4	0.4	0.4	-2.5	0.01	TGGT1_208730	microneme protein, putative	88.7	86.5	216.5	214.6
3.6	0.03	TGGT1_219590	RuvB family 1 protein	88.3	77.6	21.5	21.4	-2.6	0.04	TGGT1_256040	PA14 domain-containing protein	1.4	1.3	3.8	3.5
3.6	0.00	TGGT1_238210	EGF family domain-containing protein	3.2	3.1	0.9	0.9	-2.7	0.03	TGGT1_247310	cpw-wpc domain-containing protein	0.5	0.5	1.3	1.2
3.5	0.00	TGGT1_277970	dolichol-phosphate-mannose synthase family protein	24.5	24.5	7.0	7.0	-2.7	0.02	TGGT1_249000	regulator of chromosome condensation (RCC1) repeat-containing protein	6.2	6.1	17.6	16.5
3.5	0.00	TGGT1_224840	3'-cyclic nucleotide phosphodiesterase domain-containing protein	23.0	22.8	6.6	6.6	-2.7	0.01	TGGT1_321480	SAG-related sequence SRS12B	3.4	3.3	9.4	9.1
3.4	0.00	TGGT1_223060	MORN repeat-containing protein	36.5	36.3	11.2	10.8	-2.7	0.04	TGGT1_207610	hoptry kinase family protein ROP36 (incomplete catalytic triad)	0.8	0.8	2.3	2.1
3.3	0.01	TGGT1_309410	AP2 domain transcription factor AP2XI-1	29.2	28.5	8.9	8.5	-2.7	0.03	TGGT1_205662	LCCL domain-containing protein	0.7	0.7	2.1	2.0
3.3	0.03	TGGT1_257130	zinc finger (CCCH type) motif-containing protein	3.0	2.9	1.0	0.9	-2.8	0.03	TGGT1_319360	SAG-related sequence SRS17A	14.2	13.1	37.1	36.6
3.3	0.00	TGGT1_277950	lipase	3.8	3.8	1.2	1.1	-2.8	0.04	TGGT1_272510	aspartyl protease	0.5	0.5	1.5	1.4
3.2	0.01	TGGT1_221890	WD domain, G-beta repeat-containing protein	3.0	2.9	1.0	0.9	-2.9	0.03	TGGT1_283530	small rab-related GTPase	1.1	1.1	3.4	3.2
3.2	0.01	TGGT1_278910	O-acetylserine (thiol) lyase 2, putative	5.1	5.1	1.6	1.6	-3.0	0.01	TGGT1_295640	peptidase family M13 protein	0.1	0.1	0.4	0.4
3.2	0.03	TGGT1_237860	protein kinase domain-containing protein	9.9	9.7	3.4	3.0	-3.0	0.00	TGGT1_224770	SAG-related sequence SRS40D	0.1	0.1	0.3	0.3
3.2	0.01	TGGT1_272200	AGC kinase	1.4	1.3	0.5	0.4	-3.0	0.03	TGGT1_269680	acyl-CoA carboxyltransferase beta chain, putative	1.6	1.5	4.7	4.5
3.1	0.00	TGGT1_238200	alpha/beta hydrolase fold domain-containing protein	8.0	8.0	2.6	2.5	-3.2	0.00	TGGT1_264485	AP2 domain transcription factor AP2IX-3	2.3	2.2	7.3	7.1
3.1	0.01	TGGT1_269035	nucleoside diphosphate kinase	3.6	3.6	1.2	1.2	-3.4	0.01	TGGT1_243190	Toxoplasma gondii family A protein	0.1	0.1	0.4	0.3
3.1	0.04	TGGT1_239530	alanine-glyoxylate aminotransferase	8.1	7.6	2.7	2.5	-3.7	0.00	TGGT1_295472	C2 domain-containing protein	31.0	30.5	112.3	111.5
3.0	0.00	TGGT1_211370	tetratricopeptide repeat-containing protein	5.1	5.1	1.8	1.7	-4.2	0.02	TGGT1_295468	C2 domain-containing protein	15.8	14.3	61.4	60.2
2.9	0.02	TGGT1_200130	Toxoplasma gondii family C protein	76.5	75.3	27.0	25.5	-4.7	0.02	TGGT1_278430	Toxoplasma gondii family A protein	0.1	0.1	0.7	0.6
2.8	0.00	TGGT1_222340	NOL 1/NOP2/sun family protein	7.8	7.8	2.8	2.7	-7.3	0.01	TGGT1_298090	Toxoplasma gondii family B protein	0.1	0.0	1.0	0.9
2.8	0.04	TGGT1_294320	Oxysterol-binding protein	1.2	1.1	0.4	0.4	-58.1	0.00	TGGT1_229650	TgJosephin	0.3	0.3	16.2	15.9

Table S6. Quantification of proteins in WT and Δ josephin parasites by data-independent acquisition (DIA).

Proteins with an |Abundance ratio| > 2 and P-value < 0.05 between WT and Δ josephin samples are shown.

Protein FDR Confidence: Combined	ToxoDB ID	Description	Δ josephin/WT Abundance Ratio	P- Value
High	TGGT1_411200	SAG-related sequence protein SRS48Q	5.42	0.00
Low	TGGT1_245530	hypothetical protein	5.06	0.00
High	TGGT1_238200	alpha/beta hydrolase fold domain-containing protein	4.08	0.00
High	TGGT1_215150	AP2 domain transcription factor AP2X-9	3.16	0.00
High	TGGT1_200320	hypoxanthine-xanthine-guanine phosphoribosyl transferase HXGPRT	3.01	0.00
High	TGGT1_237090	AP2 domain transcription factor AP2X-5	2.94	0.00
High	TGGT1_294820	putative type I fatty acid synthase	2.80	0.00
High	TGGT1_229140	MaoC family domain-containing protein	2.79	0.00
High	TGGT1_276155	hypothetical protein	2.76	0.00
High	TGGT1_411240	toxoplasma gondii family B protein	2.74	0.00
Medium	TGGT1_221270	oxidoreductase, 2OG-Fe(II) oxygenase family protein	2.71	0.05
High	TGGT1_252210	pentatricopeptide repeat domain-containing protein	2.69	0.00
High	TGGT1_207130	SAG-related sequence SRS49A	2.64	0.00
Medium	TGGT1_265070	hypothetical protein	2.53	0.02
High	TGGT1_301210	putative NAD(P) transhydrogenase subunit beta	2.46	0.00
High	TGGT1_207150	SAG-related sequence SRS49C	2.44	0.00
Low	TGGT1_309880	hypothetical protein	2.26	0.01
High	TGGT1_269340	hypothetical protein	2.26	0.00
High	TGGT1_248610	hypothetical protein	2.26	0.00
High	TGGT1_202430	hypothetical protein	2.20	0.00
High	TGGT1_280580	SAG-related sequence SRS35B	2.20	0.00
High	TGGT1_309930	melibiase subfamily protein	2.20	0.00
High	TGGT1_278030	hypothetical protein	2.17	0.00
High	TGGT1_278815	hypothetical protein	2.16	0.00
High	TGGT1_223900	hypothetical protein	2.03	0.00
High	TGGT1_239600	roptry kinase family protein ROP23 (incomplete catalytic triad)	2.00	0.00
High	TGGT1_292280	SAG-related sequence SRS36D	0.49	0.00
High	TGGT1_294830	methyltransferase domain-containing protein	0.47	0.00
High	TGGT1_218310	microneme protein MIC14	0.47	0.00
High	TGGT1_286020	hypothetical protein	0.47	0.00
Low	TGGT1_301260	hypothetical protein	0.46	0.00
High	TGGT1_315410	SAG-related sequence SRS53F	0.45	0.00
High	TGGT1_244130	hypothetical protein	0.42	0.00
High	TGGT1_321800	EGF family domain-containing protein	0.40	0.00
High	TGGT1_268970	hypothetical protein	0.39	0.00
High	TGGT1_244120	hypothetical protein	0.38	0.00
High	TGGT1_229650	josephin protein (TgJosephin)	0.23	0.00
High	TGGT1_218340	hypothetical protein	0.18	0.00

ACCOMPLISHMENTS 業績

令和 3-4 年度

- Hashizaki E, Sasai M, Okuzaki D, Nishi T, Kobayashi T, Iwanaga S, Yamamoto M, 2023. *Toxoplasma* TgIWS1 Determines Fitness in Interferon- γ -Activated Host Cells and Mice by Indirectly Regulating ROP18 mRNA Expression. *mBio* 14:e03256-22.
- 「トキソプラズマの新規核内タンパク質による宿主免疫抑制機構の解明」 第 91 回日本寄生虫学会 (2022)
- Subversion of host defense system by a *Toxoplasma* nuclear protein, 第 20 回あわじ感染と免疫国際フォーラム (2022)
- 「転写因子 IWS1 によるトキソプラズマ病原性因子の遺伝子発現制御」 第 77 回日本寄生虫学会・西日本支部大会 (2022)
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- 令和4年度大阪大学女子大学院生優秀研究賞 受賞

令和 5-8 年度

- 「トキソプラズマ新規抗宿主免疫病原性因子の同定と解析」 第 78 回日本寄生虫学会西日本支部大会 (2023)
- 「トキソプラズマ新規抗宿主免疫因子の同定と機能解析」 第 93 回寄生虫学会合同大会 (2024)
- Identification and function analysis of novel anti-host immunity factors of *Toxoplasma*, 17th International Congress on Toxoplasmosis (2024)
- Identification and analysis of novel *Toxoplasma* factors against host immunity, AMED SCARDA「ワクチン開発のための世界トップレベル研究開発拠点の形成事業」若手(合同)シンポジウム (2024)
- 「トキソプラズマ新規病原性因子 TgJosephin の機能解析」 第 80 回日本寄生虫学会西日本支部大会 (2025)
- Hashizaki E, Tachibana Y, Sasai M, Kosako H, Okuzaki D, Yamamoto M, TgJosephin and TgRad23 are Important for Anti-IFN- γ Virulence via Deubiquitination of SPM1 in *Toxoplasma*. *mSphere* (2025-in revision)
- TgJosephin and TgRad23 are Important for Anti-IFN- γ Virulence via Deubiquitination of SPM1 in *Toxoplasma*, 令和 7 年度微生物病研究所研究業績発表会ポスター賞 受賞 (2026)