

ATF6 β is a host cellular target of the *Toxoplasma gondii* virulence factor ROP18

Masahiro Yamamoto,^{1,3} Ji Su Ma,^{1,3} Christina Mueller,⁵
 Naganori Kamiyama,^{1,3} Hiroyuki Saiga,^{1,3} Emi Kubo,¹ Taishi Kimura,^{1,3}
 Toru Okamoto,⁴ Megumi Okuyama,^{1,3} Hisako Kayama,^{1,3}
 Kisaburo Nagamune,⁶ Seiji Takashima,² Yoshiharu Matsuura,⁴
 Dominique Soldati-Favre,⁵ and Kiyoshi Takeda^{1,3}

¹Department of Microbiology and Immunology and ²Department of Cardiovascular Medicine, Graduate School of Medicine;

³Laboratory of Mucosal Immunology, World Premier International Research Center Immunology Frontier Research Center; and ⁴Department of Molecular Virology, Research Institute for Microbial Diseases; Osaka University, Suita, Osaka 565-0871, Japan

⁵Department of Microbiology and Molecular Medicine, University Medical Center, University of Geneva, 1211 Geneva 4, Switzerland

⁶Department of Parasitology, National Institute of Infectious Diseases, Shinjuku-ku, Tokyo 162-8640, Japan

The ROP18 kinase has been identified as a key virulence determinant conferring a high mortality phenotype characteristic of type I *Toxoplasma gondii* strains. This major effector molecule is secreted by the rhoptries into the host cells during invasion; however, the molecular mechanisms by which this kinase exerts its pathogenic action remain poorly understood. In this study, we show that ROP18 targets the host endoplasmic reticulum-bound transcription factor ATF6 β . Disruption of the ROP18 gene severely impairs acute toxoplasmosis by the type I RH strain. Because another virulence factor ROP16 kinase modulates immune responses through its N-terminal portion, we focus on the role of the N terminus of ROP18 in the subversion of host cellular functions. The N-terminal extension of ROP18 contributes to ATF6 β -dependent pathogenicity by interacting with ATF6 β and destabilizing it. The kinase activity of ROP18 is essential for proteasome-dependent degradation of ATF6 β and for parasite virulence. Consistent with a key role for ATF6 β in resistance against this intracellular pathogen, ATF6 β -deficient mice exhibit a high susceptibility to infection by ROP18-deficient parasites. The results reveal that interference with ATF6 β -dependent immune responses is a novel pathogenic mechanism induced by ROP18.

CORRESPONDENCE

Kiyoshi Takeda:

ktakeda@ongene.med.osaka-u.ac.jp

Abbreviations used: BMDC, BM-derived DC; ERAD, ER-associated degradation; GST, glutathione S-transferase; HA, hemagglutinin; HFF, human foreskin fibroblast; IRG, immunity-related GTPase; KD, kinase dead; MOI, multiplicity of infection; MPA, mycophenolic acid; PFA, paraformaldehyde; PV, parasitophorous vacuole; PVM, PV membrane; UPR, unfolded protein response; UPRE, UPR element.

Toxoplasma gondii causes life-threatening toxoplasmosis in immunocompromised individuals such as those suffering from AIDS or being treated by chemotherapy (Montoya and Remington, 2008). As a member of the phylum of Apicomplexa, *T. gondii* is an obligate intracellular parasite, defined by the presence of an apical complex including secretory organelles such as rhoptries and micronemes (Joynson and Wreghitt, 2001). During invasion, *T. gondii* delivers numerous effector molecules into the forming parasitophorous vacuole (PV) and the host cytoplasm to co-opt the host cell for growth and survival (Boothroyd and Dubremetz, 2008).

T. gondii is divided into three major lineages (types I, II, and III) in addition to exotic

strains (Ajzenberg et al., 2004; Dardé, 2008). Although type II parasites are the most prevailing opportunistic strains, infection with type I strains appears to be responsible for encephalitis in AIDS patients, ocular toxoplasmosis, and congenital hydrocephalus, whereas infection with type III strains seldom results in disease manifestations (Howe and Sibley, 1995; Boothroyd and Grigg, 2002). In terms of the strain-dependent phenotypes, virulence in mice has been well characterized, although the median lethal dose of type II or III parasites ranges from 10^2 to 10^5 , and the lethal dose of the most virulent type I strain is one (10^0) parasite (Sibley and Boothroyd, 1992; Sibley and Howe, 1996;

T. Okamoto's present address is The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria 3052, Australia.

© 2011 Yamamoto et al. This article is distributed under the terms of an Attribution-Noncommercial-Share Alike-No Mirror Sites license for the first six months after the publication date (see <http://www.rupress.org/terms>). After six months it is available under a Creative Commons License (Attribution-Noncommercial-Share Alike 3.0 Unported license, as described at <http://creativecommons.org/licenses/by-nc-sa/3.0/>).

Dubremetz, 2007). Previous forward genetic studies, in which types I/II and III were intercrossed to explore genes responsible for virulence, culminated in the identification of *ROP18* as the dominant candidate gene (Saeij et al., 2006; Taylor et al., 2006).

ROP18 is a Ser/Thr kinase related to the *ROP2* subfamily, secreted by the rhoptries into the PV and host cytosol. Its action as effector molecule is anticipated to modulate host factors by an as yet unknown mechanism (El Hajj et al., 2006;

Taylor et al., 2006; Dubremetz, 2007). Most recently, *ROP18* has been shown to target a member of IFN-inducible small GTPases (immunity-related GTPases [IRGs]), *Irgb6* (Fentress et al., 2010; Steinfeldt et al., 2010), indicating that interference with the innate function of *Irgb6* is a key mechanism by which *ROP18* mediates virulence at an early stage after infection. *ROP16*, another *ROP2* subfamily member, was previously identified as virulence determinant, distinguishing how type I/III and II strains activate Stat3/6 during parasite

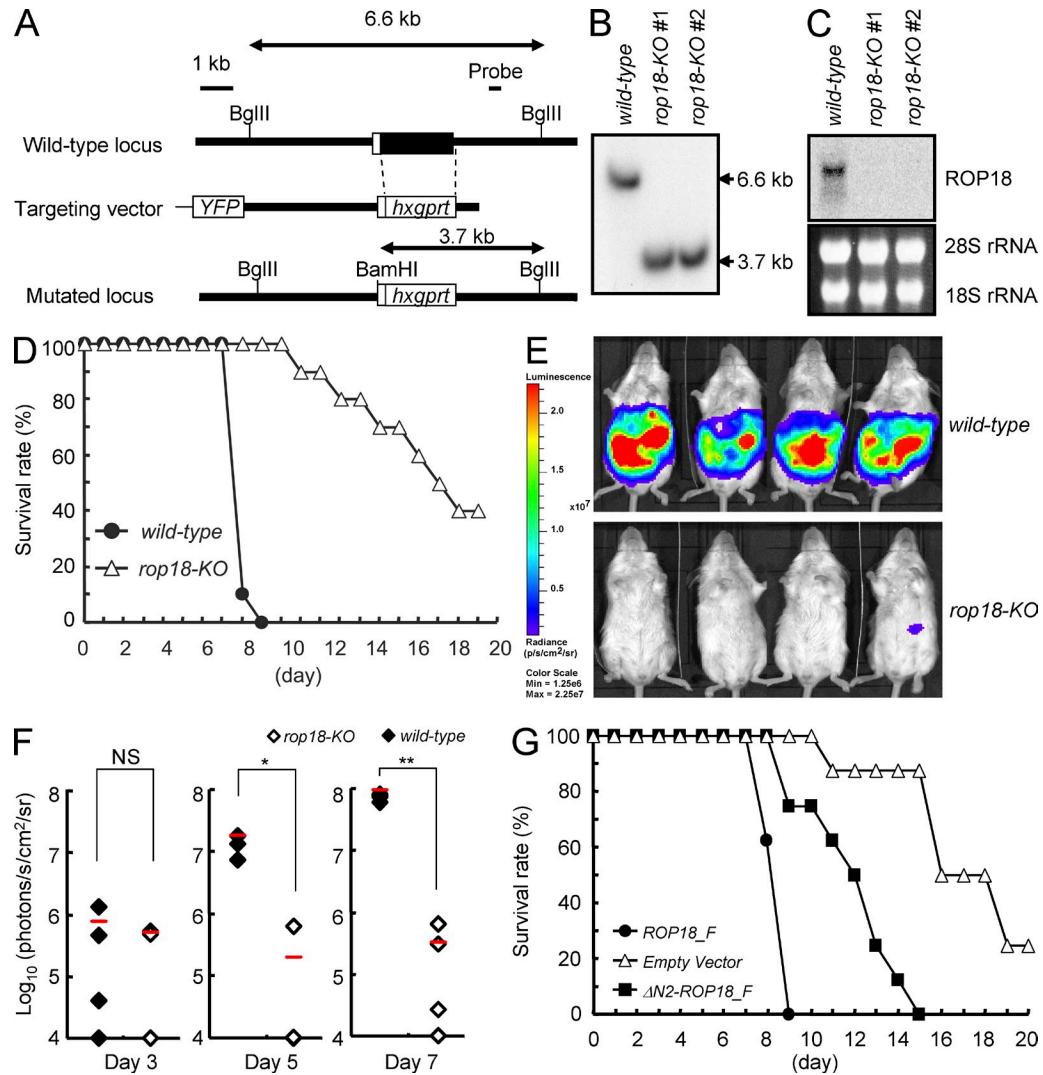


Figure 1. The N terminus of ROP18 is required for acute virulence in type I *T. gondii*. (A) The structure of the *ROP18* gene, the targeting vector, and the predicted disrupted gene. Closed boxes denote the exons. (B) Southern blot analysis of WT or two lines of *rop18*-KO parasites. 30 μ g total genomic DNA was extracted from parasites, digested with BglII-BamHI, electrophoresed, and hybridized with the radiolabeled probe indicated in A. Southern blotting yielded a single 6.6-kb band for the WT and a 3.7-kb band for the disrupted locus. (C) Northern blot analysis on 10 μ g total parasite RNA separated on a gel, transferred to a nylon membrane, and hybridized with ROP18 probe. The 28S and 18S ribosomal RNA is shown as the loading control (bottom). (D) BALB/c mice ($n = 10$) were infected with 10^3 WT or *rop18*-KO parasites, and the survival rates were monitored for 20 d. (E) BALB/c mice ($n = 4$) were infected with 10^3 WT or *rop18*-KO luciferase-expressing parasites, and the progress of infection was assessed by bioluminescence imaging at day 6 after infection. The color scale indicates photon emission during a 60-s exposure. (F) Total photon emission analysis from BALB/c mice ($n = 4$) infected with 10^3 WT or *rop18*-KO luciferase-expressing parasites at days 3, 5, or 7 after infection. Abdominal photon emission was assessed during a 60-s exposure. The red bars show means of the four samples. *, $P < 0.05$; **, $P < 0.001$. (G) BALB/c mice ($n = 8$) were infected with 10^3 *rop18*-KO parasites complemented with the indicated vectors, and the survival rates were monitored for 20 d. (B–G) Data are representative of two independent (B, C, E, and F) or cumulative percentages of two independent (D and G) experiments.

infection (Saeij et al., 2006, 2007). Subsequently, this effector molecule was shown to directly phosphorylate Stat3 and Stat6, and the N-terminal extension of ROP16 was demonstrated to play a key role in the interaction with the substrates (Yamamoto et al., 2009; Ogawa et al., 2010; Ong et al., 2010). Like ROP16, ROP18 exhibits an uncharacterized N-terminal extension (El Hajj et al., 2006).

To explore the function of the N terminus of ROP18, we first generated ROP18-deficient type I parasites and confirmed the *in vivo* significance of ROP18 in the type I parasite-mediated virulence. Complementation experiments showed that the N-terminal portion of ROP18 is required for the full recovery of virulence in *rop18*-KO parasites. A yeast two-hybrid screening and biochemical experiments found that the host factor ATF6 β is a binding partner with the N-terminal extension of ROP18. This association led to a proteasome-dependent degradation of ATF6 β in a kinase activity-dependent manner. Consistent with a defensive function of ATF6 β against the parasite, ATF6 β -deficient mice were generated and shown to be highly susceptible to *rop18*-KO but not WT parasites. Collectively, these results identified ATF6 β as one of the host cellular factors targeted by ROP18 in the context of acute pathogenesis by the type I *T. gondii* strain.

RESULTS

The N terminus of ROP18 is involved in acute virulence in type I parasites

To determine whether ROP18 contributes to the high mortality rate of type I strains in mice, we disrupted the *ROP18* gene in the type I RH strain by reverse genetics and isolated several independent clones (Fig. 1, A and B). Northern blot analysis confirmed the absence of *ROP18* messenger RNAs in the clones (Fig. 1 C). The parasite lytic cycle as measured by plaque assay and intracellular growth on human foreskin fibroblasts (HFFs) was unaffected in these mutants as compared with WT parasites (Fig. S1, A and B). The recruitment of host organelles such as the mitochondria and ER around the PV was unaltered (unpublished data). We first challenged BALB/c mice with WT or *rop18*-KO parasites and monitored animal survival for 20 d. All mice infected with WT parasites died within 9 d. In contrast, mice infected with *rop18*-KO parasites showed a considerably lower mortality rate (Fig. 1 D). Luciferase-expressing WT or *rop18*-KO parasites were generated to visualize the *in vivo* parasite burden in BALB/c mice. A significantly stronger signal was detected in the ventral side of mice infected with WT parasites than those with *rop18*-KO parasites (Fig. 1 E). Moreover, the photon flux measurement during the course of infection increased notably faster and demonstrated a two log difference between WT and *rop18*-KO parasites at 7 d after infection (Fig. 1 F). Given the importance of the N-terminal extension for ROP16, we complemented *rop18*-KO parasites with full-length Flag-tagged (F) ROP18 (*ROP18_F* strain), the ROP18 mutant deleted in residues 147–164 of the N-terminal portion ($\Delta N2$ -*ROP18_F* strain), or the empty vector (*Empty vector* strain) and tested the recovery of the acute virulence phenotype

(Fig. S2, A and B). Expression of *ROP18_F* fully restored acute virulence, whereas the *Empty vector* parasites exhibited an avirulent phenotype comparable with the parental *rop18*-KO parasites. The recovery of the virulence in $\Delta N2$ -*ROP18* parasites was only partial compared with the *ROP18_F* strain (Fig. 1 G). These data formally establish that ROP18 is a virulence factor of acute toxoplasmosis in type I strain and highlight the important contribution of the N-terminal extension of ROP18 for the full manifestation of the virulence phenotype.

Enhanced type I immune responses in mice infected with *rop18*-KO parasites

We next compared type I immune responses in the infected BALB/c mice because IFN- γ , mainly produced by Th1-polarized CD4 and CD8 T cells, plays a critical role in the control of acute toxoplasmosis (Subauste and Remington, 1991; Shirahata et al., 1994; Yap and Sher, 1999). 6 d after infection, CD4 and CD8 T cells from the spleens of mice demonstrating no abnormalities in appearance and no alteration in splenic cellularities and were tested for IFN- γ production by anti-CD3 treatment (Fig. S3, A–C). Compared with T cells from mice infected with WT parasites, CD4 and CD8 T cells from those infected with *rop18*-KO parasites displayed dramatically higher IFN- γ production in response to anti-CD3 (Fig. 2, A and B). To analyze antigen-specific IFN- γ production, we next compared the production from T cells stimulated with heat-killed *T. gondii* in the presence of DCs. Even in this condition, CD4 and CD8 T cells from mice infected with *rop18*-KO parasites produced higher concentrations of IFN- γ than those infected with WT parasites (Fig. 2, A and B). Collectively, these results show that ROP18 critically contributes to the suppression of the host type I immunity during infection with the virulent type I strain.

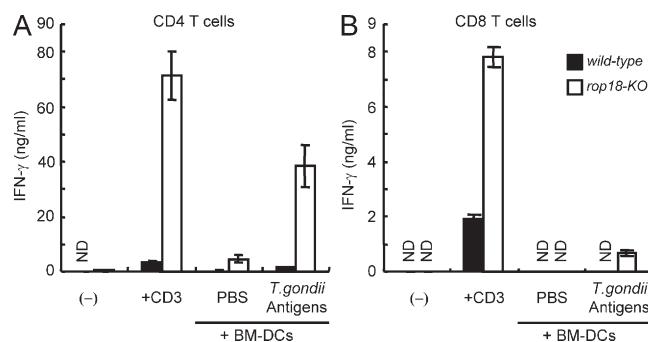
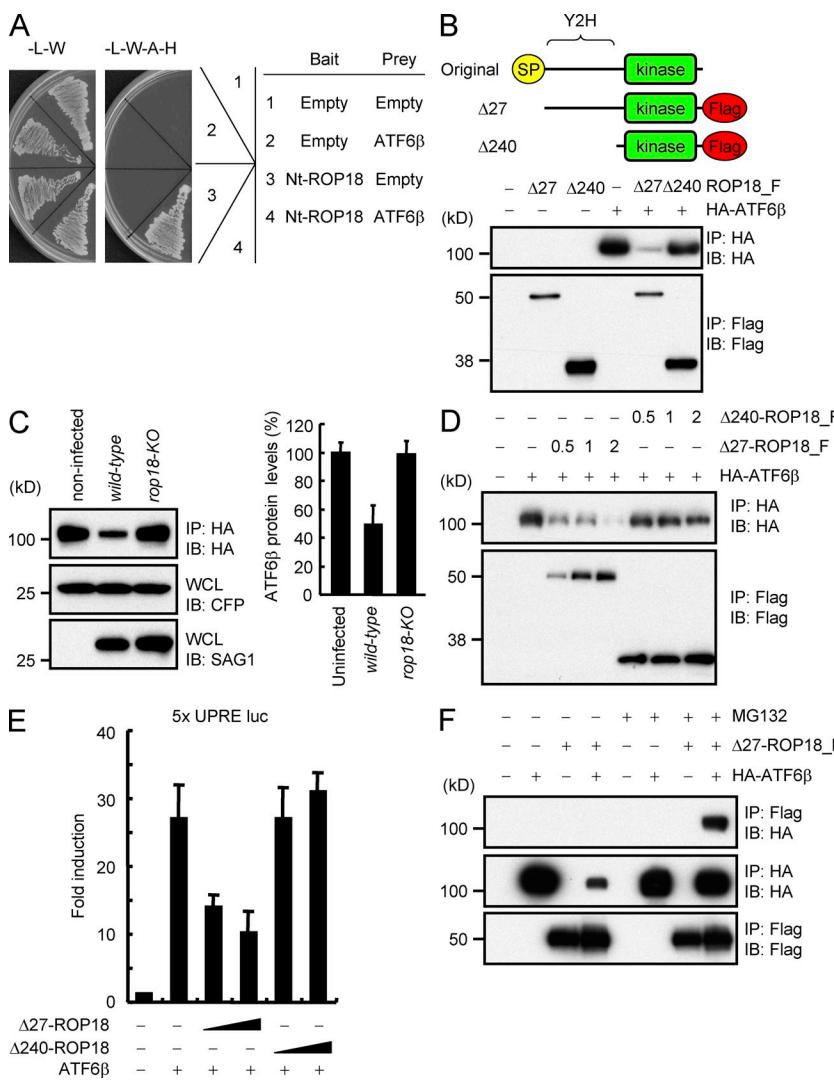


Figure 2. Enhanced IFN- γ production from T cells in mice infected with *rop18*-KO parasites. (A and B) CD4 (A) or CD8 (B) T cells from BALB/c ($n = 3$) mice were cultured in the presence of 5 μ g/ml plate-bound anti-CD3 for 24 h or in the presence or absence of *T. gondii* antigen-pulsed BMDCs for 24 h. Concentration of IFN- γ in the culture supernatants was measured by ELISA. Indicated values are means \pm SD of triplicates. Data are representative of three independent experiments. ND, not detected.

ROP18 interacts with ATF6 β and mediates its degradation

The N-terminal extension of ROP18 interacts with the host factors Stat3/Stat6 (Yamamoto et al., 2009; Ogawa et al., 2010). Therefore, a yeast two-hybrid screening was undertaken to identify potential host partners interacting with the N-terminal portion of ROP18 (Nt-ROP18). ATF6 β , a component of the ER membrane-bound transcription factor implicated in stress response, was found as the predominant hit, and its association with Nt-ROP18 was first confirmed in yeast cells (Fig. 3 A). Moreover, Nt-ROP18 and ATF6 β failed to interact with SV40 large T antigen and p53 used as negative controls, respectively, demonstrating that Nt-ROP18 specifically associates with ATF6 β in yeast (Fig. S4 A). To further assess the specificity of this interaction, we expressed Flag-tagged ROP18 lacking its signal peptide (Δ 27-ROP18_F) or a larger N-terminal deletion of ROP18 (Δ 240-ROP18_F) together with hemagglutinin

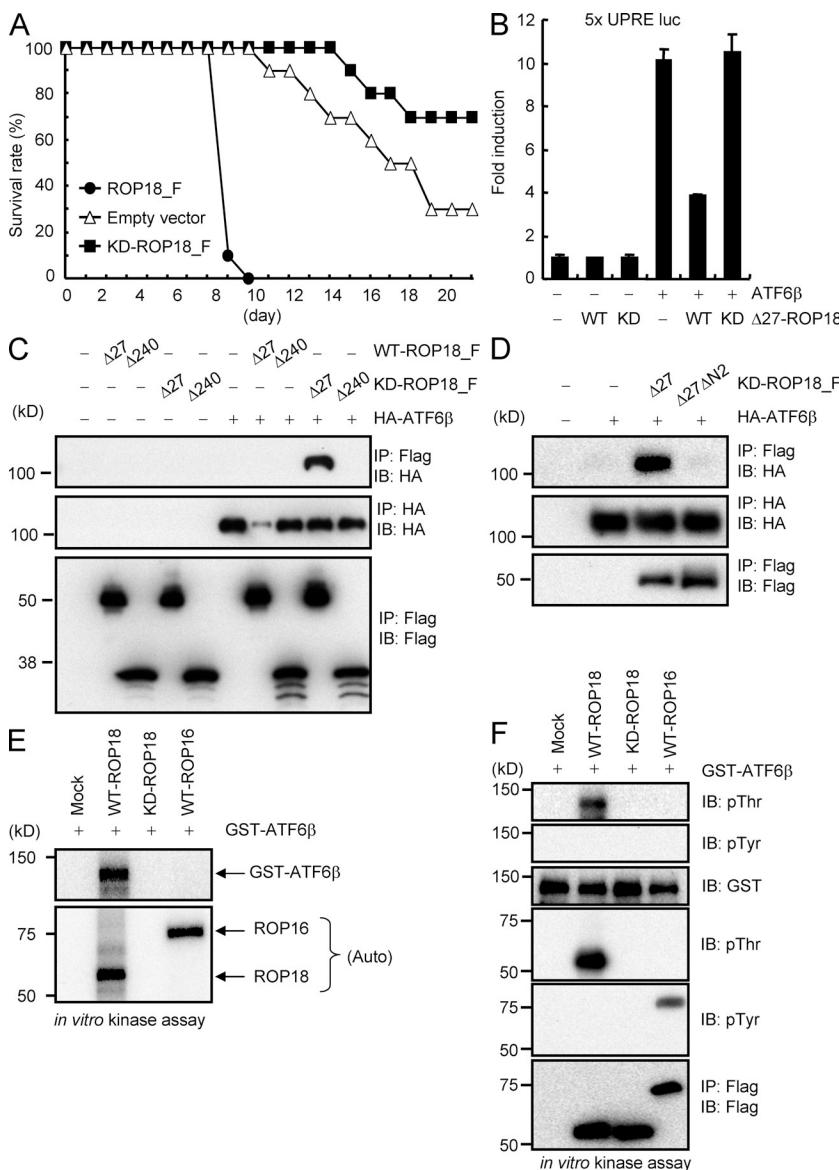
(HA)-tagged ATF6 β in mammalian 293T cells. Interestingly, we failed to detect an interaction between Δ 27-ROP18_F and HA-ATF6 β , but we noticed that overexpression of Δ 27-ROP18_F but not Δ 240-ROP18_F resulted in a dramatic reduction in the level of HA-ATF6 β (Fig. 3 B). To determine whether parasite infection also caused a drop in HA-ATF6 β levels, 293T cells expressing HA-ATF6 β fused to CFP (with a self-cleavage signal [T2A] in between to produce the same level of both proteins) were similarly infected with WT or *rop18*-KO parasites (Fig. S4 B). The level of ATF6 β protein was consistently reduced in cells infected with WT but not with *rop18*-KO parasites (Fig. 3 C). Moreover, Δ 27-ROP18_F but not Δ 240-ROP18_F decreased the ATF6 β level in a dose-dependent fashion in dually transfected 293T cells (Fig. 3 D). ATF6 β is a component of the ER stress response transcription factor that activates expression of genes harboring an unfolded protein response



293T cells transiently cotransfected with 2 μ g Flag-tagged ROP18 and/or 2 μ g HA-tagged ATF6 β in the absence or presence of 10 μ M MG132 for the last 12 h were immunoprecipitated with the indicated antibodies and detected by Western blot. (A–F) Data are representative of three (B and D–F) and two (A and C) independent experiments.

Figure 3. Identification of ATF6 β as a ROP18-interacting protein. (A) Plasmids expressing Nt-ROP18 fused to the GAL4 DNA-binding domain or an empty vector were cotransfected with a plasmid expressing ATF6 β fused to the GAL4 transactivation domain or an empty vector. Interactions were detected by the ability of cells to grow on medium lacking Ade, Trp, Leu, and His (-L-W-A-H). Growth of cells on plates lacking Trp and Leu (-L-W) was indicative of the efficiency of the transfection. (B) Lysates of 293T cells transiently cotransfected with 2 μ g of the indicated Flag-tagged ROP18 and/or 2 μ g HA-tagged ATF6 β expression vectors were immunoprecipitated with the indicated antibodies and detected by Western blot with the indicated antibodies. The top images denote the structure of ROP18 variants used in this study. WT, original ROP18; SP, signal peptide; Y2H, the region used as the bait in the yeast two-hybrid screen. (C) The 293T cells transfected with 0.1 μ g plasmids for tandem expression of HA-tagged ATF6 β and T2A-CFP were infected with the indicated parasites at an MOI of 10. 24 h after infection, the cells were lysed and subjected to Western blot or immunoprecipitation (IP) with anti-HA (left). The expression levels of HA proteins were normalized against CFP (right). Error bars represent means \pm the variation range of duplicates. IB, immunoblot; WCL, whole cell lysates. (D) Lysates of 293T cells transiently cotransfected with the indicated volumes of the Flag-tagged Δ 27- or Δ 240-ROP18 and/or 2 μ g HA-tagged ATF6 β expression vectors were immunoprecipitated with the indicated antibodies and detected by Western blot with the indicated antibodies. (E) 293T cells were transfected with the ATF6 β -dependent luciferase reporter together with the indicated expression vectors. Luciferase activities were expressed as fold increases over the background levels shown by lysates prepared from mock-transfected cells. Error bars represent means \pm SD of triplicates. (F) Lysates of

(UPR) element (UPRE) in their promoters (Wang et al., 2000; Yoshida et al., 2001). Overexpression of HA-ATF6 β resulted in the activation of a UPRE-containing luciferase reporter in 293T cells; however, the coexpression of $\Delta 27$ -ROP18_F but not $\Delta 240$ -ROP18_F down-regulated the ATF6 β -dependent activation in a dose-dependent manner (Fig. 3 E). To determine whether the ROP18-dependent decrease in ATF6 β protein level is mediated by the proteasome, HA-ATF6 β was coexpressed with $\Delta 27$ -ROP18_F in the presence of the proteasome inhibitor MG132. Under this condition, the level of ATF6 β was stabilized, and the interaction between $\Delta 27$ -ROP18 and ATF6 β could be monitored by coimmunoprecipitation (Fig. 3 F). Together, these data support the view that ROP18 associates with ATF6 β and targets it to a proteasome-dependent degradation pathway.



The kinase activity of ROP18 is required for ATF6 β degradation

ROP18 was previously shown to be a secreted active protein kinase in vitro and in vivo (Taylor et al., 2006; El Hajj et al., 2007). To assess whether the kinase activity of ROP18 plays a role in type I parasite-mediated pathogenicity, we complemented the *rop18*-KO parasites with WT (ROP18_F) or kinase-dead (KD; KD-ROP18_F) constructs (Fig. S2, B and C) and tested the virulence of the corresponding parasites in BALB/c mice (Fig. 4 A). Mice infected with *ROP18*_F resulted in 100% lethality within 10 d. In contrast, most of mice infected with *KD-ROP18*_F survived throughout the tested period. These results are consistent with a previous study, which reported that an avirulent type III strain expressing the *ROP18* but not the KD form of the type I strain can acquire virulence, pointing out the essential role of kinase activity of

ROP18 for acute virulence (Taylor et al., 2006). To determine whether the kinase activity is involved in the ROP18-mediated degradation of ATF6 β , mammalian expression vectors for KD-ROP18_F, ATF6 β , and UPRE-containing luciferase reporter were cotransfected into 293T cells and followed by luciferase assays (Fig. 4 B). Overexpression of KD-ROP18_F failed to suppress ATF6 β -mediated gene activation. Moreover, the

Figure 4. Essential role of ROP18 kinase activity in ATF6 β degradation. (A) BALB/c mice ($n = 8$) were infected with 10^3 *rop18*-KO parasites complemented with the indicated vectors, and the survival rates were monitored for 20 d. (B) 293T cells were transfected with the ATF6 β -dependent luciferase reporter together with the indicated expression vectors. Luciferase activities were expressed as fold increases over the background levels as shown by lysates prepared from mock-transfected cells. Error bars represent means \pm SD of triplicates. (C and D) Lysates of 293T cells transiently cotransfected with 2 μ g of the indicated Flag-tagged ROP18 and/or 2 μ g of HA-tagged ATF6 β expression vectors were immunoprecipitated with the indicated antibodies and detected by Western blot. (E and F) 293T cells were transiently transfected with Flag-tagged ROP18WT, ROP18KD, or ROP16WT. Cell lysates were immunoprecipitated with anti-Flag and subjected to an in vitro kinase reaction in the presence of GST-ATF6 β . Proteins were separated on SDS-PAGE, followed by autoradiography (E) or Western blot (F) to analyze ATF6 β phosphorylation or the autop phosphorylation of ROP18 and ROP16. For the detection of phosphorylated or unphosphorylated GST-ATF6 β and Flag-tagged proteins by Western blot, anti-pThr/anti-pTyr, anti-GST, and anti-Flag were used, respectively. IB, immunoblot; IP, immunoprecipitation. (A–F) Data are representative of three (B) or two (A and C–F) independent experiments.

ectopic expression of ROP18_F but not KD-ROP18_F led to HA-ATF6 β degradation. KD-ROP18_F coprecipitated with ATF6 β , as previously observed in the presence of MG132 (Figs. 3 F and 4 C). These results establish a connection between the kinase activity of ROP18 and ATF6 β degradation. To examine which portion of the N terminus of ROP18 interacts with ATF6 β , vectors expressing the KD versions Δ 27- or Δ 240-ROP18_F were introduced into 293T cells together with ATF6 β . HA-ATF6 β coprecipitated with Δ 27-KD-ROP18_F but not with Δ 240-KD-ROP18_F (Fig. 4 C). To refine the analysis, Δ 27 Δ N2-ROP18_F was cotransfected and shown to fail to coprecipitate with ATF6 β (Fig. 4 D), indicating that the N-terminal 147–164 portion of ROP18 determines the binding to ATF6 β . To challenge the possibility that ATF6 β is a substrate for ROP18, we performed an *in vitro* kinase assay using recombinant glutathione S-transferase (GST)-tagged ATF6 β as a substrate. Immunoprecipitates from 293T cells expressing Flag-tagged ROP18WT, ROP18KD, or ROP16WT were incubated with GST-ATF6 β in the presence of radiolabeled ATP. Phosphorylation of GST-tagged ATF6 β was detectable in the presence of ROP18WT but not with ROP18KD or ROP16WT (Fig. 4 E). To determine which amino acids on ATF6 β are phosphorylated by ROP18, we relied on anti-phospho-Thr (anti-pThr), anti-pSer, and anti-pTyr. GST-tagged ATF6 β was detected only in the presence of ROP18WT with anti-pThr, whereas anti-pSer and anti-pTyr gave no signal (Fig. 4 F and not depicted). In contrast, anti-pTyr detected autophosphorylation of ROP16WT (Fig. 4 F), correlating with the Tyr kinase activity of ROP16 (Yamamoto et al., 2009; Ong et al., 2010). Collectively, these results show that ROP18 phosphorylates at least one Thr residue on ATF6 β , which leads to its degradation.

The C-terminal portion of ATF6 β associates with ROP18

To characterize the region on ATF6 β implicated in binding to ROP18, deletion mutants of ATF6 β lacking both the transmembrane and the C-terminal portion (Δ CA Δ TM) or the C terminus alone (Δ C) were generated (Fig. 5 A). Activation of the UPRE-containing reporter by Δ CA Δ TM-ATF6 β was unaffected by the presence of ROP18_F, even at high doses (Fig. 5 B). Moreover, KD-ROP18_F coprecipitated with the HA-tagged full-length ATF6 β but not with the Δ C form (Fig. 5 C), suggesting that the C-terminal portion of ATF6 β

is implicated in the association with ROP18. Intriguingly, the family members of ATF6 proteins are anchored in the membrane of the ER with their C-terminal region predicted to localize in the lumen (Stirling and O'Hare, 2006). If this topology holds true for ATF6 β , it would imply that ROP18 needs to gain access to ATF6 β in the host ER. To examine whether ROP18 localizes to the ER, YFP-tagged ATF6 β and T2A_CFP-tagged KD-ROP18 were bicistronically cloned and coexpressed with an ER-localized RFP in 293T cells (Fig. S5, A and B). Live microscopy revealed colocalization of ER-localized RFP and CFP-ROP18, suggesting that ROP18 is in close proximity with ER-resident proteins. The host ER has recently been reported to fuse with the PV membrane (PVM; Sinai et al., 1997; Goldszmid et al., 2009), and thus it is plausible that ER-localized ATF6 β in the vicinity of PVMs might be targeted by ROP18. Indeed, when HFFs infected with parasites were analyzed by electron microscopy, PVMs not only associated with, but also appeared to directly fuse with the host ER (Fig. S5 C). These results indicate that the functional ROP18 kinase is necessary to trigger degradation of ATF6 β and suggest that ROP18 might colocalize with ATF6 β in the host ER or at the PVM.

Defensive function of ATF6 β against infection of *rop18*-KO parasites

Given the correlation between the ability of ROP18 to induce ATF6 β degradation and manifestation of virulence in type I parasites, we hypothesized that mice infected with *rop18*-KO parasites are resistant, at least in part, because of a protective effect dependent on ATF6 β . To test this possibility under physiological conditions, we generated mice lacking the *Atf6* β gene (Fig. S6, A–C). Homozygous mice with the *Atf6* β -deleted allele were born at the expected Mendelian ratio and grew healthy in specific pathogen-free conditions, as previously reported for another line of ATF6 β -deficient mice (Yamamoto et al., 2007). In terms of the cellularity of immune cells under unstimulated conditions, ATF6 β -deficient mice behaved similarly to WT mice (unpublished data). When

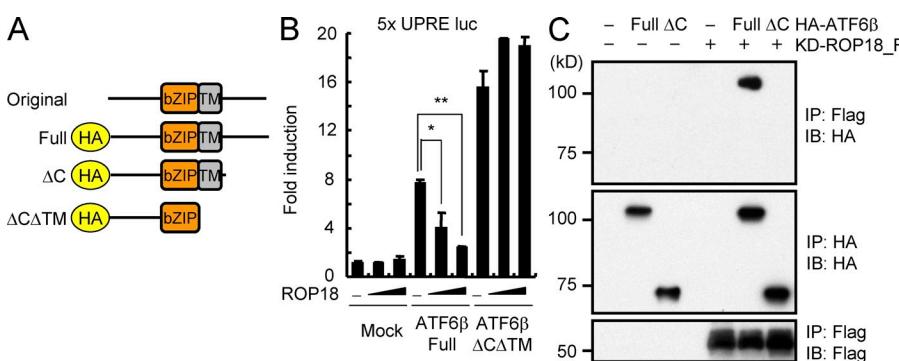
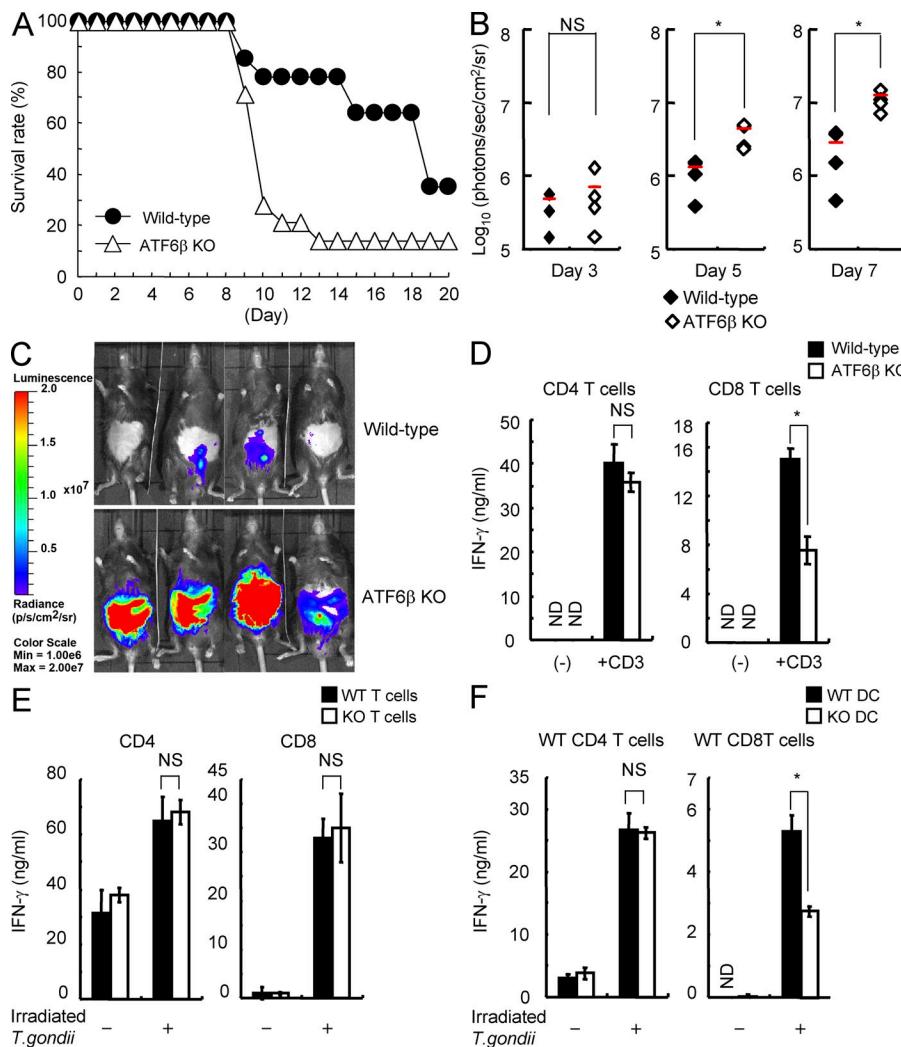


Figure 5. The C-terminal portion of ATF6 β interacts with ROP18. (A) HA-tagged ATF6 β variants. bZIP, basic Leu zipper; TM, transmembrane. (B) 293T cells were transfected with the ATF6 β -dependent luciferase reporter together with the indicated expression vectors. Luciferase activities were expressed as fold increases over the background levels as shown by lysates prepared from mock-transfected cells. Error bars represent means \pm the variation range of duplicates. *, P < 0.03; **, P < 0.001. (C) Lysates of 293T cells transiently cotransfected with 2 μ g of the indicated Flag-tagged ROP18 and/or 2 μ g of the indicated HA-tagged ATF6 β expression vectors were immunoprecipitated with the indicated antibodies and detected by Western blot. IB, immunoblot; IP, immunoprecipitation. (B and C) Data are representative of three (C) or two (B) independent experiments.

challenged with parasites, ATF6 β -deficient mice were more susceptible to *rop18*-KO parasites than WT mice (Fig. 6 A). In sharp contrast, the susceptibility of both genotypes of mice infected with WT parasites was comparable (Fig. S6 D). Moreover, ATF6 β -deficient mice similarly succumbed to infection with complemented *rop18*-KO parasite lines (Fig. S6 E). The CTG strain was used as a natural *rop18*-KO parasite and shown to cause a higher mortality rate in ATF6 β -deficient mice than in WT mice (Fig. S6 F). To follow more accurately the course of infection, WT and ATF6 β -deficient mice were infected with 10^3 luciferase-expressing *rop18*-KO parasites, and the kinetics of infection were monitored by in vivo imaging. Marked increments of *rop18*-KO parasites were observed at days 5, 7, and 8 in ATF6 β -deficient mice compared with WT mice (Fig. 6, B and C). To assess whether type I immune responses in ATF6 β -deficient mice were affected, the T cell responses were examined at day 6 after infection with *rop18*-KO parasites. The cellularities after the parasite challenges were unchanged in WT and ATF6 β -deficient mice (unpublished data). Under these conditions, a significantly reduced IFN- γ response to anti-CD3 was observed in CD8 T cells but not in

CD4 T cells (Fig. 6 D). Next, we examined whether the defective IFN- γ production from ATF6 β -deficient cells is intrinsic or extrinsic to CD8 T cells. WT DCs were infected with irradiated *rop18*-KO parasites in vitro, and then intraperitoneally injected into WT or ATF6 β -deficient mice. 6 d after the injection, splenic CD4 and CD8 T cells were isolated and co-cultured with WT DCs noninfected or infected with *rop18*-KO parasites, and the supernatants were analyzed for IFN- γ production. Comparable IFN- γ production was observed in WT and ATF6 β -deficient CD8 T cells, suggesting that the defective IFN- γ production in ATF6 β -deficient CD8 T cells may be extrinsic to the T cells (Fig. 6 E). Next, to test whether APCs determine the phenotype, we collected splenic T cells from WT mice infected with *rop18*-KO parasites 6 d after infection and co-cultured with WT or ATF6 β -deficient DCs noninfected or infected with *rop18*-KO parasites. We found that CD8 T cells with the infected ATF6 β -deficient DCs produced significantly lower amounts of IFN- γ than those with WT DCs (Fig. 6 F). Thus, ATF6 β in DCs is responsible for the CD8 T cell-mediated host defense against *rop18*-KO parasites.



ATF6 β -independent response to the parasite infection

$\Delta N2\text{-}ROP18_F$, which are insensitive to the ATF6 β -mediated host response, moderately restored in vivo virulence (Fig. 1 G), suggesting that ATF6 β -independent host responses may be functional to eliminate the parasites. Because ROP18 is shown to inactivate IRGs by the direct Thr phosphorylation (Fentress et al., 2010; Steinfeldt et al., 2010), we postulated the involvement of IRGs in the moderate host resistance in WT mice infected with $\Delta N2\text{-}ROP18_F$. To assess whether $\Delta N2\text{-}ROP18$ inactivates IRG, macrophages treated with IFN- γ were infected with *ROP18_F*, $\Delta N2\text{-}ROP18_F$, *KD-ROP18_F*, or *EMPTY* parasites, and the interaction of an IRG Irgb6 with the ROP18 variants was examined by an immunoprecipitation assay (Fig. 7 A). $\Delta N2\text{-}ROP18_F$ as well as *ROP18_F* and *KD-ROP18_F* associated with Irgb6 in inflammatory macrophages. Next, we tested whether $\Delta N2\text{-}ROP18$ mediates Thr phosphorylation on Irgb6 by an in vitro kinase assay. Immunoprecipitates of parasites expressing $\Delta N2\text{-}ROP18_F$ and *ROP18_F* but not *KD-ROP18_F* induced Thr phosphorylation of Myc-Irgb6-YFP but not Myc-YFP (Fig. 7 B and not depicted), indicating that activity of the IRG can be blocked by $\Delta N2\text{-}ROP18$. Finally, to more carefully dissect contribution of the ATF6 β -independent host response, ATF6 β -deficient mice were infected with *rop18*-KO parasites complemented with ROP18 variants at a low dose (10^2 tachyzoites), and the parasite loads in the peritoneal

cavities and IFN- γ production from splenic T cells were examined. Although the parasite number in mice infected with *Empty vector* was less than those in mice with *ROP18_F* or $\Delta N2\text{-}ROP18_F$ parasites at day 3 after infection, it was almost comparable at day 6. In contrast, the number of parasites in mice infected with *KD-ROP18_F* was consistently lower throughout the course of the experiment (Fig. 7 C). In terms of IFN- γ production, although the CD8 T cell-mediated production was unaltered, the production from CD4 T cells in mice infected with *ROP18_F* or $\Delta N2\text{-}ROP18_F$ was markedly reduced compared with that in mice with *KD-ROP18_F* or *Empty vector* (Fig. 7 D). Thus, these results suggest that the ATF6 β -independent response regulates CD4 T cell-mediated IFN- γ production against *rop18*-KO parasites.

DISCUSSION

In this study, we provide the first genetic and biochemical evidence that the host cellular protein, ATF6 β , is targeted for degradation by ROP18, a key virulence factor in *T. gondii*. ATF6 β is a member of the ATF6-related family of transcription factors that have been shown to operate in the UPR (Yoshida et al., 2001; Yamamoto et al., 2007). Recently, UPR-related molecules such as XBP-1 in *Caenorhabditis elegans* or mammals and a plant ATF6-related molecule were shown to be involved in host

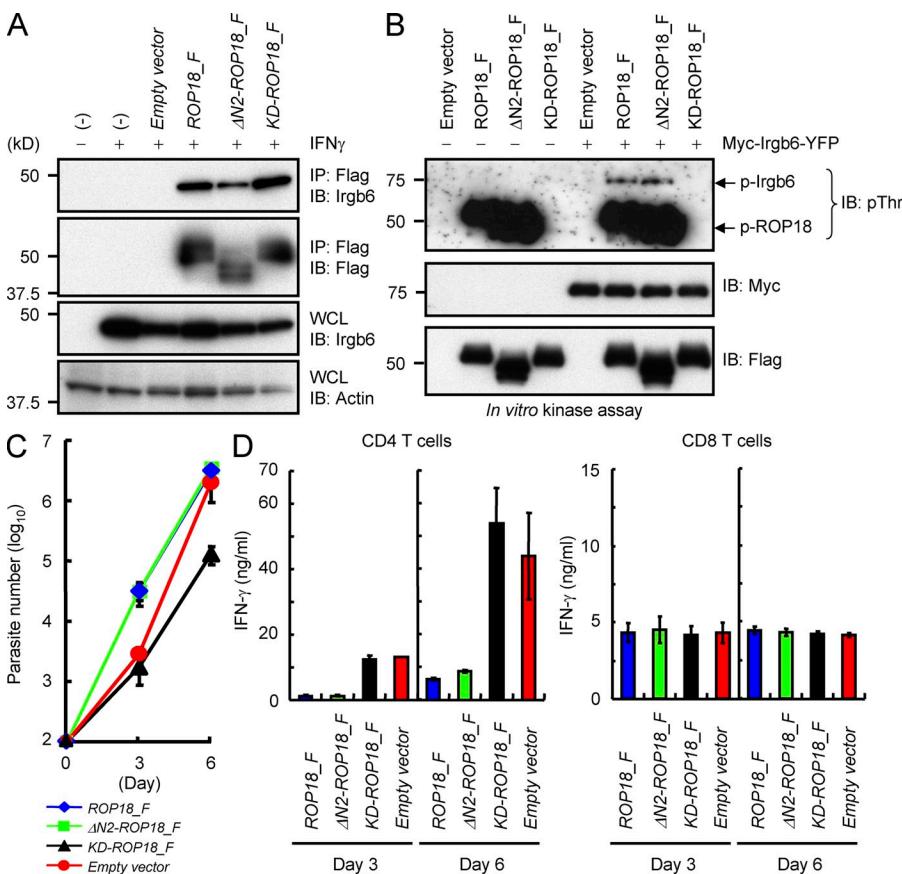


Figure 7. Parasite-induced ATF6 β -independent host response. (A) Peritoneal macrophages treated with 30 ng/ml IFN- γ for 24 h were infected with the indicated parasites. Lysates of the infected cells were immunoprecipitated with anti-Flag and detected by Western blot with the indicated antibodies. IB, immunoblot; IP, immunoprecipitation; WCL, whole cell lysate. (B) 293T cells were transiently transfected with empty or Myc-tagged Irgb6-YFP vectors. Cell lysates were immunoprecipitated with anti-Myc and subjected to an in vitro kinase reaction in the presence of anti-Flag immunoprecipitates of the indicated parasites. Proteins were separated on SDS-PAGE, followed by Western blot to analyze Irgb6 phosphorylation or the autophosphorylation of ROP18. For detection of phosphorylated or unphosphorylated Myc-Irgb6-YFP and Flag-tagged proteins by Western blot, anti-pThr, anti-Myc, and anti-Flag were used, respectively. (C and D) ATF6 β -deficient mice ($n = 3$) intraperitoneally infected with a dose of the indicated 10^2 parasites. Peritoneal fluids were collected at the indicated days after infection, and the parasite numbers were counted by plaque forming assays (C). CD4 or CD8 T cells from spleens of mice infected with the indicated parasites were cultured in the presence of 5 μ g/ml plate-bound anti-CD3 for 24 h. Concentration of IFN- γ in the culture supernatants was measured by ELISA. Indicated values are means \pm SD of triplicates. (A–D) Data are representative of two independent experiments.

defense against pathogens (Tateda et al., 2008; Martinon et al., 2010; Richardson et al., 2010). In this study, we report the protective effect of ATF6 β against *rop18*-KO but not WT parasite infection. It remains to be determined whether ATF6 β also participates in host defense against other pathogens and whether other ATF6 family members including ATF6 α and Creb3 (also known as Luman) are targeted by ROP18 (Liang et al., 2006; Zhang and Kaufman, 2008).

Our data suggest that ATF6 β deficiency in DCs rather than in T cells is responsible for the defective production of IFN- γ in CD8 T cells, yet a formal proof would require the conditional ablation of ATF6 β in these cell types. Given that ATF6 β acts as transcription factor, it will be important to determine which of the ATF6 β -regulated genes mediate DC activation leading to CD8 T cell responses.

DCs treated with an inhibitor of the ER-associated degradation (ERAD) system were shown to fail to induce CD8 T cell response by cross-presentation (Goldszman et al., 2009). The close association and possible fusion of host ER with the PV of *T. gondii* could cause an ER overload leading to ERAD activation (Sinai et al., 1997; Goldszman et al., 2009). Interestingly, the ATF6 family members regulate the transcription of a subset of ERAD components (Wu et al., 2007), so it is conceivable that type I virulent *T. gondii* strains might target ATF6 β in DCs (or other APCs such as macrophages) to down-regulate UPR-mediated host defense (Blanchard and Shastri, 2010). The data presented in this study are consistent with the previous findings that (part of) host ERs fuse with PVs during *T. gondii* infection (Goldszman et al., 2009; Melo et al., 2010). However, studies showing that some ER-resident proteins colocalized with the PVMs do not constitute direct evidence for the host ER–PV fusion, and thus more in depth analysis is required to support this model.

ATF6 β -deficient mice are highly susceptible to *rop18*-KO parasites with high parasite burdens possibly caused by defective IFN- γ responses of CD8 T cells. However, T cells from dying ATF6 β -deficient mice (for instance, at days 9–10 after infection), in which the spleen was about to be autodigested and partly adhesive to other organs, secreted extremely high concentrations of IFN- γ even under nonstimulated conditions (unpublished data), which is indicative of an overreacting immune responses just before death. Thus, high susceptibility to the parasites might be primarily attributable to the excessive parasite proliferation and terminally dysregulated immune pathology.

Previous studies reported that ROP18-overexpressing parasites exhibited accelerated growth (Taylor et al., 2006; El Hajj et al., 2007). In contrast, the *rop18*-KO parasites showed normal intracellular growth in this study. It is plausible that overexpression of ROP18 impacts proliferation by acting on additional host cell substrates that are not essential to promote normal growth of *rop18*-KO parasites.

Expression of Δ N2-ROP18_F, which lacks the ATF6 β -binding region, moderately restores the virulence of *rop18*-KO parasites. Because this ROP18 mutant does not associate with ATF6 β , ATF6 β -mediated host defense might be functional in Δ N2-ROP18_F parasite infection. Nevertheless, Δ N2-ROP18_F

parasites are not avirulent, indicating the existence of an additional ROP18-mediated but ATF6 β -independent virulence mechanism. Δ N2-ROP18_F lacks 17 aa in the N terminus of ROP18, which corresponds to helix 2 of the ROP5 N-terminal extension that is essential for its localization to the PV (Reese and Boothroyd, 2009). Similarly, ROP18 is injected into the host cytosol during invasion and is ultimately found at the PV (Boothroyd and Dubremetz, 2008). The Δ N2-ROP18_F strain might incapacitate ATF6 β -independent innate immune responses, including IRG-mediated resistance which is operative soon after the parasite invasion (Hunn et al., 2008; Zhao et al., 2009; Khaminets et al., 2010). Indeed, ROP18 has recently been shown to target the member of IRGs, Irgb6 (Fentress et al., 2010; Steinfeldt et al., 2010). In this study, we characterized ATF6 β -independent immune responses in ATF6 β -deficient mice infected with Δ N2-ROP18_F, which inactivated Irgb6. Compared with infection of EMPTY or KD-ROP18_F, Δ N2-ROP18_F infection resulted in much reduced CD4 T cell-mediated IFN- γ production, suggesting that IRGs may be involved in activation of the ATF6 β -independent CD4 T cell response. However, this assumption is contradictory to previous findings that the Stat1–IRG axis is not required for the development of Th1 immunity (Taylor et al., 2000; Collazo et al., 2001; Lieberman et al., 2004). This may be caused by strain-dependent differential host innate immune responses (Robben et al., 2004). Previous studies used type II parasites, whereas this study used type I parasites. IRG-deficient mice with high numbers of type II parasites are shown to culminate in higher concentrations of IL-12 in vitro and in vivo than that in WT mice, possibly leading to the development of apparently normal Th1 immunity that can compensate for the defect in the potential IRG-mediated CD4 T cell phenotype (Taylor et al., 2000; Collazo et al., 2001). Alternatively, the other ATF6 β /IRG-independent ROP18-mediated effector mechanisms may suppress the CD4 T cell response. It is of interest in the future to revisit the potential involvement of IRGs in the development of CD4 T cell response using relatively immune-silent but IRGs-sensitive type I *rop18*-KO or type III parasites.

Despite the CD4 T cell-mediated IFN- γ production and the successful suppression of *rop18*-KO parasite (*Empty vector*) increment at the early stage after infection, ATF6 β -deficient mice allowed the parasites to proliferate at the late stage and eventually succumbed. The CD8 T cell-mediated response is defective in ATF6 β -deficient mice. Moreover, mice deficient in CD8 but not CD4 T cell functions are highly susceptible to acute toxoplasmosis (Casciotti et al., 2002; Combe et al., 2005; Lu et al., 2009). Therefore, even though the ATF6 β -independent mechanism activates the CD4 T cell-mediated immunity that contributes to host resistance at early stages, it might be ultimately incompetent to counteract the acute parasite infection under condition of the defective CD8 T cell responses. In contrast, considering that the *rop18*-KO parasites (*Empty vector*) are not as virulent as the Δ N2-ROP18_F in WT mice, the ATF6 β -independent CD4 T cell response

may considerably enhance host resistance under the normal CD8 T cell conditions. Previous studies established that interference with the innate function of Irgb6 is an important mechanism of ROP18-mediated virulence especially at an early stage after infection (Fentress et al., 2010; Steinfeldt et al., 2010). Furthermore, our study reveals that ROP18 also targets ATF6 β -dependent CD8 T cell-mediated acquired immune responses that act at a later stage after infection. Thus, ROP18 may continue to disarm host immunity during the acute pathogenesis by targeting different host factors at each stage.

In conclusion, this study identifies ATF6 β as a novel and unexpected substrate for ROP18. This finding nicely complements the recently published study to establish that the kinase activity of ROP18 is essential for the mortality phenotype and may be required for the suppression of both the ATF6 β -dependent and -independent host defense mechanisms (Taylor et al., 2006). Interestingly, KD-ROP18_F expression in *rop18*-KO parasites appeared to reduce virulence. Because ROP18 belongs to the ROP2 subfamily consisting of >30 members including 17 active kinases (Peixoto et al., 2010), it is possible that expression of KD-ROP18 might have a dominant-negative effect, suppressing the action of other family members implicated in virulence. Whether other ROP2 subfamily members are important for the type I parasite-mediated virulence in concert with ROP18 should be examined in the future. Intriguingly, type II parasites are shown to be avirulent in spite of normal expression levels of ROP18. Given that ROP16 does not act positively in the virulence (Saeij et al., 2006), type II parasites might be defective in other ROP2 subfamily members.

In summary, ATF6 β has been associated in this study for the first time with a resistance mechanism against an intracellular pathogen. ROP18, an effector molecule that constitutes the major virulence factor *T. gondii* type I strain, controls ATF6 β degradation. In the future, the in vitro assay system based on the UPR-containing reporter may facilitate the development of a new pharmaceutical drug targeting ROP18 that can block acute toxoplasmosis.

MATERIALS AND METHODS

Cells, mice, and parasites. BALB/c and outbred CD1 ICR mice (6–8 wk old) were obtained from SLC. All animal experiments were conducted with the approval of the Animal Research Committee of the Graduate School of Medicine at Osaka University. RH Δ hxgpert and its derivatives of *T. gondii* were maintained in Vero or HFFs by biweekly passage in RPMI (Nacalai Tesque) supplemented with 2% heat-inactivated FCS (JRH Biosciences), 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Invitrogen). The CTG strain was provided by D. Sibley (Washington University School of Medicine, St. Louis, MO) and maintained in Vero cells in the RPMI media.

Reagents. Antibodies against *T. gondii* major surface antigen 1 (SAG1), anti-TGTP (Irgb6), anti-Myc, and HA probe were obtained from Santa Cruz Biotechnology, Inc. Anti-pTyr and anti-pThr were obtained from Cell Signaling Technology. Anti-Flag and anti-pSer were obtained from Sigma-Aldrich. Anti-ROP1 was provided by J.F. Dubremetz (Université de Montpellier 2, Montpellier, France). For indirect immunofluorescence, anti-HA rat antibody was obtained from Roche. MG132 was obtained from EMD.

Generation of RH Δ hxgpert *T. gondii*-expressing OVA and a fusion protein of RFP and luciferase. To express luciferase, RFP, and OVA, we constructed a plasmid harboring p30-OVA and luciferase-RFP fusion proteins (pOVRFPluc). The luciferase and RFP fragments were obtained by PCR using primers OVA_F and OVA_R, with plasmid pGL3 as the template or primers RFP_F and RFP_R using pDsRed-express (Takara Bio Inc.) as the template, respectively. The information for all primers used in this study is listed in Table S1. The EcoRI-XbaI fragment of luciferase and the XbaI-PacI fragment of RFP were cloned into the EcoRI-PacI-digested sag1-ROP16HA-3UTR plasmids (Yamamoto et al., 2009). The NotI fragment of sag1-Luciferase/RFP-3UTR was cloned into the site of p30-OVA plasmids (a gift from D.S. Roos, University of Pennsylvania, Philadelphia, PA), leading to the generation of the OVRFPluc plasmid. 100 μ g of the OVRFPluc plasmids was transfected with RH Δ hxgpert parasites, and the stable transformants were selected in RPMI media containing 20 μ M chloramphenicol (Nacalai Tesque), as p30-OVA plasmids contained a cassette for the chloramphenicol resistance gene (Pepper et al., 2004). RFP-positive parasites were selected by fluorescent microscopy and tested for in vitro and in vivo luciferase activity. Three clones were isolated, and we observed comparable in vitro growth and in vivo virulence to each other and the parental line. Whether the parasite line, which was used for generation of *rop18*-KO parasites, expresses OVA has not been tested in this study.

Generation of ROP18-deficient type I *T. gondii*. Genomic DNA containing the *ROP18* gene was isolated by PCR using primers R18KOLA_F and R18KOLA_R to generate a 5.0-kb-long fragment. Primers R18KOSA_F and R18KOSA_R generated a 1.0-kb fragment. The gene encoding *T. gondii* *ROP18* consists of a single exon. The targeting vector (pKO-ROP18) was constructed by replacing the entire coding sequence of *ROP18* with the *HXGPRT* gene expression cassette (p2855). Outside the targeting vector, a YFP expression vector was ligated using a NotI site for the negative selection of random integration. 100 μ g of the targeting vector linearized by ScaI was transfected into tachyzoites of the normal RH Δ hxgpert parental strain or OVRFPluc RH Δ hxgpert parasites as previously described (Yamamoto et al., 2009). After 25 μ g/ml mycophenolic acid (MPA; Sigma-Aldrich) and 25 μ g/ml xanthine (Wako Chemicals USA) selection for 14 d, MPA/xanthine-resistant colonies were sorted using a FACSaria (BD) to isolate YFP-negative parasites. The MPA/xanthine-resistant and YFP-negative parasites were subjected to limiting dilution to isolate the clones. A total of 142 clones were selected and screened by PCR for detecting homologous recombinants using primers DHFRrc01 (from the DHFR promoter of the *HXGPRT* expression vector) and R18ex01 (genomic sequence outside the short fragment of the *ROP18* locus), to detect homologous recombinants. This resulted in the isolation of four clones using normal RH Δ hxgpert parasites and two homologous recombinants using OVRFPluc RH Δ hxgpert parasites. Subsequently, genomic DNA of WT and ROP18-deficient parasites was extracted and subjected to Southern blot analysis using a DNA probe, which was generated by PCR using primers SB_F and SB_R. Additionally, to confirm the disruption of the gene encoding ROP18, we analyzed total RNA from WT and *rop18*-KO parasites by Northern blot using a DNA probe, which was generated by PCR using primers NB_F and NB_R.

Mammalian expression plasmids. The C-terminal Flag-tagged fragments of ROP18 lacking the N-terminal signal peptide were amplified using primers R18frg2common_F and R18frg2common_R. The series of ROP18 variants were amplified using primers R18D27frg1_F for Δ 27, R18D240frg1_F for Δ 240, primers for Δ 27 Δ N2 described in Table S1, and the common primer using genomic DNA from the RH strain and were ligated into the BamHI and NotI sites of a pcDNA vector. The kinase-inactive ROP18 mutant containing point mutations was generated using the primers D394A_F and D394A_R, and expression plasmids were generated using a site-directed mutagenesis kit (Agilent Technologies). The sequences of all constructs were confirmed with a genetic analyzer (ABI PRISM; Applied Biosystems). Human ATF6 β cDNA was amplified using primers ATF6 β _full_R for Full, ATF6 β _DC_R for Δ C, ATF6 β _DCDTM_R for Δ C Δ TM, and the common primer

ATF6 β _common_F using human BM cDNA as the template and then ligated into the EcoRI and NotI sites of a pcDNA vector for the N-terminal HA-tagged proteins (Invitrogen). Murine Irgb6 cDNA was amplified using primers Irgb6_F and Irgb6_R using IFN- γ -treated mouse macrophage cDNA as the template and then ligated into the EcoRI and XhoI sites of a pcDNA vector for the N-terminal HA-tagged and C-terminal YFP-tagged proteins. To express HA-hATF6 β and CFP bicistronically using a self-cleavage T2A peptide (Holst et al., 2006), an expression vector for HA-hATF6 β _T2ACFP was generated by ligation with a pcDNA vector for the N-terminal HA and EcoRI-XhoI fragment of hATF6 β , which was generated by PCR using primers ATF6 β _common_F and ATF6 β _full_XhoI_R. T2ACFP was generated by PCR using primers T2ACFP_R and T2ACFP_F using a CFP vector as the template. The expression vector for HA_hATF6 β _YFP-T2ACFP_KDROPI8_Flag was constructed by cloning the EcoRI-XhoI fragment of hATF6 β . The Sall-SacI fragment of YFP was amplified using primers YFP_F and YFP_R, using a YFP vector as the template. The SacI-BamHI fragment of T2ACFP and the BamHI-NotI fragment of KD-ROPI8-Flag were inserted into the EcoRI-NotI site of the pcDNA vector for the N-terminal HA tag. By placing the T2A peptide between HA-hATF6 β (or HA-hATF6 β -YFP) and CFP (or CFP-KD-ROPI8-Flag), we obtained independent and reliable expression of both proteins. The ER-localizing RFP vector pDsRed2-ER was purchased from Takara Bio Inc.

Generation of transgenic parasites. To complement the *rop18*-KO parasites, we generated an N-terminal signal peptide-containing ROP18, capable of being processed in the parasite, by PCR using the primer ParasiteR18frg1_F for ROP18-Flag (ROP18_F) or KD-ROPI8_F and expressed the ROP18-F proteins driven by the sag1 promoter containing a pyrimethamine resistant gene cassette in the *rop18*-KO strain (Yamamoto et al., 2009). AN2-ROPI8 lacking residues 147–164 of the N-terminal portion was generated by ligation of two fragments: one from the EcoRI-NotI fragment of ROP18_F and the other generated using primers ParasiteR18frg1F, DN2(Δ HX2)_F, and DN2(Δ HX2)_R. We generated this vector by digesting the pyrimethamine resistance cassette of the p2854 plasmid with NotI and XhoI and ligated this into a Klenow-treated pBluescript sag1-ROPI8_F vector. A series of ROP18_F vectors or the empty p2854 vector for the generation of the *Empty vector* strain were transfected into tachyzoites of the *rop18*-KO parasites. We selected for parasites stably expressing the complemented ROP18_F constructs using 3 μ M pyrimethamine (Sigma-Aldrich) selection and subjected these to limiting dilution as described previously (Yamamoto et al., 2009).

Generation of ATF6 β -deficient mice. The *Atf6 β* gene was isolated from genomic DNA extracted from embryonic stem cells (V6.5) by PCR using LA Taq (Takara Bio Inc.). The targeting vector (pKOATF6 β) was constructed by replacing a 3.0-kb fragment encoding the exons of ATF6 β with a neomycin-resistance gene cassette (*neo*) and a herpes simplex virus thymidine kinase gene driven by the PGK promoter, for negative selection. Genomic DNA containing the murine *Atf6 β* gene was isolated by PCR amplification using primers ATF6 β KO_LA_F and ATF6 β KO_LA_R to generate a 5.0-kb fragment or primers ATF6 β KO_SA_F and ATF6 β KO_SA_R to generate a shorter 1.0-kb fragment. After the targeting vector was transfected into embryonic stem cells, colonies resistant to both G418 and ganciclovir were selected and screened by PCR and Southern blotting. Homologous recombinants were microinjected into C57BL/6 female mice, and heterozygous F1 progenies were intercrossed to obtain ATF6 β -deficient mice. ATF6 β -deficient mice and their WT littermates from these intercrosses were used for experiments.

Yeast two-hybrid analysis. Yeast two-hybrid screening was performed as described with the Matchmaker two-hybrid system (Takara Bio Inc.). For construction of the bait plasmid, the cDNA fragment coding the N-terminal portion (aa 35–235) of ROP18 was amplified using primers ROP18Y2H_F and ROP18Y2H_R and cloned in frame into the GAL4 DNA-binding domain of pGBTKT7. Yeast strain AH109 was transformed with the bait plasmid

plus the human BM Mate & Plate cDNA library (Takara Bio Inc.). After the screening of 10^7 clones, we finally obtained 10 positive clones and recovered the pGAD library from individual clones and expanded in *Escherichia coli*. The inserted cDNA was sequenced and characterized with the BLAST program, resulting in the identification of ATF6 β (nine clones) and HLA-DPA1 (one clone). Because the number of positive clones of ATF6 β was much greater than that of HLA-DPA1 and because HLA-DPA1 was difficult to be linked to the mouse homologue correctly compared with ATF6 β , we selected ATF6 β in the following study.

Luciferase reporter assay. The 5 \times UPRE luciferase reporter was generated by the synthesis of a 312-bp fragment containing five tandem ATF6 binding sites (Invitrogen), with the MluI-Sall fragment inserted into the MluI-XhoI site of the pGL3 luciferase reporter as described previously (Wu et al., 2007; Yamamoto et al., 2007). The reporter plasmids were transiently cotransfected into 293T cells with the control *Renilla* luciferase expression vectors using Lipofectamine 2000 reagent (Invitrogen). Luciferase activities of total cell lysates were measured using the Dual-Luciferase Reporter Assay System (Promega) as described previously (Yamamoto et al., 2006).

Statistical analysis. The unpaired Student's *t* test was used to determine statistical significance among experimental data.

Measurement of IFN- γ by ELISA. CD4 and CD8 T cells were obtained by positive selection using anti-CD4 (L3T4) and anti-CD8a (Ly-2) magnetic beads (Miltenyi Biotech) from splenocytes of mice at day 6 after infection with parasites and cultured for 24 h in 96-well plates (10^5 cells per well) precoated with 5 μ g/ml anti-CD3 or preincubated with 10^5 BM-derived DCs (BMDCs) pulsed with heat-killed *T. gondii* antigens, which were prepared by incubation of 10^7 parasites at 56°C for 1 h. For the generation of BMDCs, BM cells were isolated from femurs and cultured with RPMI 1640 supplemented with 10% fetal bovine serum and 10 ng/ml GM-CSF (PeproTech) with medium replaced every 2 d. Concentration of IFN- γ in the culture supernatant was measured by ELISA according to the manufacturer's instructions (R&D Systems), as described previously (Yamamoto et al., 2003).

Western blot analysis and immunoprecipitation. The 293T cells and parasites were lysed in a lysis buffer (1% Nonidet P-40, 150 mM NaCl, and 20 mM Tris-HCl, pH 7.5) containing a protease inhibitor cocktail (Roche). The cell lysates were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes. For immunoprecipitation, cell lysates were pre-cleared with protein G-Sepharose (GE Healthcare) for 2 h and then incubated with protein G-Sepharose containing 1.0 μ g of the indicated antibodies for 12 h with rotation at 4°C. The immunoprecipitants were washed four times with lysis buffer and eluted by boiling with Laemmli sample buffer. The eluates were separated by SDS-PAGE, transferred to polyvinylidene fluoride membranes, and subjected to Western blot analysis as described previously (Yamamoto et al., 2004).

Flow cytometric analysis. 2×10^6 splenocytes were stained with PE-conjugated anti-B220, -CD8, or -CD11c, FITC-conjugated anti-CD3 ϵ or -CD4, and APC-conjugated anti-Gr1. Stained cells were analyzed on a FACSCantoII (BD) and using FlowJo Software (Tree Star).

Microscopic analysis. The 293T cells were transfected with 2 μ g of expression vectors for HA_ATF6 β _YFP-T2ACFP-tagged KDR0PI8_Flag or ER-DsRed. At 24 h after transfection, cells were analyzed using a fluorescence microscope (IX71; Olympus).

Immunofluorescence analysis. HFF cells infected with parasites were fixed for 8 min in PBS containing 4% paraformaldehyde (PFA)/0.05% glutaraldehyde. Cells were permeabilized with PBS containing 0.2% Triton X-100 (PBS/TX) and then blocked with 2% BSA in PBS/TX. Subsequently, cells were incubated with anti-Rop1 rabbit antibody (1:200) and anti-Flag mouse

antibody (1:6,000; Sigma-Aldrich) for 1 h at room temperature, followed by incubation with Alexa Fluor 488-conjugated goat anti-rabbit IgG antibody (Invitrogen) and Alexa Fluor 594-conjugated goat anti-mouse IgG antibody (Invitrogen) for 45 min at room temperature in the dark. Finally, the immunostained cells were mounted with Fluoromount-G (SouthernBiotech) on glass slides and analyzed using a fluorescence microscope (Axioskop 2; Carl Zeiss) equipped with a color charge-coupled device camera (AxioCam HR; Carl Zeiss).

Plaque assay. The WT and *rop18*-KO parasites were used to infect monolayers of HFFs seeded in 24-well plates. After incubation for 8–9 d at 37°C, cells were fixed with 4% PFA and 0.05% glutaraldehyde in PBS, followed by staining with Giemsa for 10 min.

Assessment of intracellular growth. Host cells seeded on 24-well immunofluorescence assay plates were inoculated with freshly released WT or *rop18*-KO parasites. At 24 h after infection, parasites were fixed with 4% PFA. Immunofluorescence assays were performed using α-TgGAP45 antibody, and parasites were counted on at least 100 vacuoles for each strain.

Electron microscopy analysis. Monolayers of HFFs were infected with *rop18*-KO parasites. Samples were collected at 4 h after infection and processed for electron microscopy using routine techniques. In brief, parasite pellets were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, post-fixed in osmium tetroxide, dehydrated in ethanol, and treated with propylene oxide before embedding in Spurr's epoxy resin. Thin sections were stained with uranyl acetate and lead citrate before examination with an electron microscope (1200EX; JEOL).

In vivo imaging analysis. Mice were intraperitoneally infected with 10³ freshly egressed tachyzoites resuspended in 100 μl PBS, with assessment of bioluminescence performed on the indicated days after infection. For the detection of bioluminescence emission, mice were intraperitoneally injected with 3 mg D-luciferin in 200 μl PBS (Promega), maintained for 5 min to allow for adequate dissemination of luciferin, and subsequently anaesthetized with isoflurane (Dainippon Sumitomo Pharma). At 10 min after injection of D-luciferin, photonic emissions were detected using an in vivo imaging system (IVIS 100; Xenogen) and Living image software (Xenogen).

Recombinant ATF6β purification. GST-tagged ATF6β cDNA was subcloned into pFAST-Bac (Invitrogen) for expression in Sf9 insect cells. Proteins were purified in accordance with the manufacturer's instruction as described previously (Yamamoto et al., 2006).

In vitro kinase assay. 5 × 10⁶ 293T cells (6-cm dish) were transiently transfected with a total of 4 μg of either empty vector or the indicated plasmids (4 μg of Flag-tagged ROP18WT, ROP18KD, or ROP16WT), using Lipofectamine 2000 as specified by the manufacturer. Cells were harvested 24 h after transfection, lysed, and then immunoprecipitated with protein G-Sepharose together with 1.0 μg anti-Flag M2 mAb (Sigma-Aldrich) for 12 h by rotation. The beads were washed four times with lysis buffer and another three times with kinase assay buffer (30 mM MOPS, pH 7.5, 50 mM NaCl, 10% glycerol, 10 mM MgCl₂, and 10 mM MnCl₂). The immunoprecipitates were incubated with 1 μg GST-ATF6β and 10 mCi γ-[³²P]ATP (GE Healthcare) or 5 μM cold ATP (Wako Chemicals USA) to phosphorylate GST-ATF6β, ROP18WT, ROP18KD, or ROP16WT at 30°C for 30 min. In the experiments for Fig. 7 B, immunoprecipitates of the parasites complemented with the ROP18 variants, which were prepared by immunoprecipitation of the parasite lysates by anti-Flag, were incubated with anti-myc immunoprecipitates of 293T cells transfected with control- or Myc-tagged Irgb6-YFP fusion vectors in the presence with 5 μM cold ATP in the kinase reaction buffer at 30°C for 30 min with gentle agitation. Kinase reactions were stopped by the addition of Laemmli sample buffer and were separated on a 5–20% polyacrylamide gradient gel. For radioactive samples, gel was detained, dried, and exposed to x-ray film. For cold samples, gel was detained

and subjected to Western blot to detect unphosphorylated or phospho-GST-tagged ATF6β and Flag-tagged ROP18WT, ROP18KD, or ROP16WT by anti-GST, anti-pThr, anti-pSer, anti-pTyr, and anti-Flag, respectively.

Immunological experiments. To assess the phenotype in T cells (Fig. 6 E), DCs were generated from BMs of two WT mice cultured in GM-CSF. 2 × 10⁷ DCs were collected at day 6 after cultivation and infected with irradiated *rop18*-KO parasites at multiplicity of infection (MOI) = 1 for 12 h. The cells were collected at 1,100 rpm and extensively washed with PBS three times at 4°C. 1,000,000 DCs were intraperitoneally injected into the littermates of WT or ATF6β-deficient mice (*n* = 2 for each group; *n* = 4 total for two independent experiments). 6 d after injection, CD4 or CD8 T cells were isolated by the positive selection of splenic T cells. The T cells (10⁵ cells) were co-cultured with WT DCs (5 × 10⁴ cells), which were freshly isolated from the littermate WT mouse and cultivated for 6 d in the presence of GM-CSF and uninfected or infected with irradiated *rop18*-KO parasites for 12 h at MOI = 1 for 48 h. The supernatants were collected and tested for ELISA to measure the IFN-γ concentration.

To analyze the phenotype of DCs for the ability of IFN-γ production from T cells (Fig. 6 F), CD4 and CD8 T cells were isolated from WT mice infected with 10³ *rop18*-KO parasites 6 d after the infection. The T cells (10⁵ cells) were co-cultured with DCs (5 × 10⁴ cells), which were freshly isolated from the littermate WT or ATF6β-deficient mice (*n* = 2 for each group; *n* = 4 total for two independent experiments), and cultivated for 6 d in the presence of GM-CSF and uninfected or infected with irradiated *rop18*-KO parasites for 12 h at MOI = 1 for 48 h. The supernatants were collected and tested for ELISA to measure the IFN-γ concentration.

Quantification of parasite loads. The parasites in the peritoneal cavities were enumerated as described previously (Robben et al., 2005). In brief, total peritoneal contents were collected by injection of 5 ml of FCS-free RPMI into the peritoneal cavities of infected mice. Aliquots of the recovered fluids were added onto confluent monolayer of mouse embryonic fibroblasts in 96-well culture plates with 10-times serial dilutions from 10⁰ to 10⁵ and 4 wells of each dilution. 4 d after infection, the numbers of plaques were counted to calculate the total parasite numbers in the original peritoneal cavities.

Online supplemental material. Fig. S1 shows parasitological analysis of *rop18*-KO type I *T. gondii*. Fig. S2 demonstrates complementation of *rop18*-KO parasites by various ROP18 mutants. Fig. S3 exhibits splenic cellularity in mice infected with WT or *rop18*-KO parasites. Fig. S4 shows specific interaction of Nt-ROP18 with ATF6β in yeasts and similar infection levels between WT and *rop18*-KO parasites in 293T cells. Fig. S5 demonstrates ER localization of ROP18 and PVM-ER fusion. Fig. S6 describes the strategy for generation of ATF6β-deficient mice and shows comparable susceptibility in WT or ATF6β-deficient mice infected with WT *T. gondii*. Table S1 provides a list of the primers used in this study. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20101660/DC1>.

We thank C. Hidaka and M. Yasuda for excellent secretarial assistance, Y. Magota for technical assistance, and members of K. Takeda's laboratory for discussions. We are grateful to W. Daher for technical assistance and stimulating discussions. We also thank Professor D. Sibley for providing the CTG strain.

This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology, the Strategic International Cooperative Program (Research Exchange Type), the Japan Science and Technology Agency, the Takeda Science Foundation, the Kanae Foundation for the Promotion of Medical Science, the Cell Science Research Foundation, the Ichiro Kanehara Foundation, the Kato Memorial Bioscience Foundation, and the Uehara Memorial Foundation. C. Mueller is supported by the Japanese-Swiss Science and Technology Program.

The authors have no conflicting financial interests to declare.

Submitted: 11 August 2010

Accepted: 23 May 2011

REFERENCES

Ajzenberg, D., A.L. Bañuls, C. Su, A. Dumètre, M. Demar, B. Carme, and M.L. Dardé. 2004. Genetic diversity, clonality and sexuality in *Toxoplasma gondii*. *Int. J. Parasitol.* 34:1185–1196. doi:10.1016/j.ijpara.2004.06.007

Blanchard, N., and N. Shastri. 2010. Topological journey of parasite-derived antigens for presentation by MHC class I molecules. *Trends Immunol.* 31:414–421. doi:10.1016/j.it.2010.08.004

Boothroyd, J.C., and J.F. Dubremetz. 2008. Kiss and spit: the dual roles of *Toxoplasma* rhoptries. *Nat. Rev. Microbiol.* 6:79–88. doi:10.1038/nrmicro1800

Boothroyd, J.C., and M.E. Grigg. 2002. Population biology of *Toxoplasma gondii* and its relevance to human infection: do different strains cause different disease? *Curr. Opin. Microbiol.* 5:438–442. doi:10.1016/S1369-5274(02)00349-1

Casciotti, L., K.H. Ely, M.E. Williams, and I.A. Khan. 2002. CD8(+)T-cell immunity against *Toxoplasma gondii* can be induced but not maintained in mice lacking conventional CD4(+) T cells. *Infect. Immun.* 70:434–443. doi:10.1128/IAI.70.2.434–443.2002

Collazo, C.M., G.S. Yap, G.D. Sempowski, K.C. Lusby, L. Tessarollo, G.F. Woude, A. Sher, and G.A. Taylor. 2001. Inactivation of LRG-47 and IRG-47 reveals a family of interferon γ -inducible genes with essential, pathogen-specific roles in resistance to infection. *J. Exp. Med.* 194:181–188. doi:10.1084/jem.194.2.181

Combe, C.L., T.J. Curiel, M.M. Moretto, and I.A. Khan. 2005. NK cells help to induce CD8(+)T-cell immunity against *Toxoplasma gondii* in the absence of CD4(+)T cells. *Infect. Immun.* 73:4913–4921. doi:10.1128/IAI.73.8.4913–4921.2005

Dardé, M.L. 2008. *Toxoplasma gondii*, “new” genotypes and virulence. *Parasite*. 15:366–371.

Dubremetz, J.F. 2007. Rhoptries are major players in *Toxoplasma gondii* invasion and host cell interaction. *Cell. Microbiol.* 9:841–848. doi:10.1111/j.1462-5822.2007.00909.x

El Hajj, H., E. Demey, J. Poncet, M. Lebrun, B. Wu, N. Galéotti, M.N. Fournaux, O. Mercereau-Puijalon, H. Vial, G. Labesse, and J.F. Dubremetz. 2006. The ROP2 family of *Toxoplasma gondii* rhoptry proteins: proteomic and genomic characterization and molecular modeling. *Proteomics*. 6:5773–5784. doi:10.1002/pmic.200600187

El Hajj, H., M. Lebrun, S.T. Arold, H. Vial, G. Labesse, and J.F. Dubremetz. 2007. ROP18 is a rhoptry kinase controlling the intracellular proliferation of *Toxoplasma gondii*. *PLoS Pathog.* 3:e14. doi:10.1371/journal.ppat.0030014

Fentress, S.J., M.S. Behnke, I.R. Dunay, M. Mashayekhi, L.M. Rommereim, B.A. Fox, D.J. Bzik, G.A. Taylor, B.E. Turk, C.F. Lichti, et al. 2010. Phosphorylation of immunity-related GTPases by a *Toxoplasma gondii*-secreted kinase promotes macrophage survival and virulence. *Cell Host Microbe*. 8:484–495. doi:10.1016/j.chom.2010.11.005

Goldszman, R.S., I. Coppens, A. Lev, P. Caspar, I. Mellman, and A. Sher. 2009. Host ER-parasitophorous vacuole interaction provides a route of entry for antigen cross-presentation in *Toxoplasma gondii*-infected dendritic cells. *J. Exp. Med.* 206:399–410. doi:10.1084/jem.20082108

Holst, J., K.M. Vignali, A.R. Burton, and D.A. Vignali. 2006. Rapid analysis of T-cell selection in vivo using T cell-receptor retrogenic mice. *Nat. Methods*. 3:191–197. doi:10.1038/nmeth858

Howe, D.K., and L.D. Sibley. 1995. *Toxoplasma gondii* comprises three clonal lineages: correlation of parasite genotype with human disease. *J. Infect. Dis.* 172:1561–1566. doi:10.1093/infdis/172.6.1561

Hunn, J.P., S. Koenen-Waisman, N. Papic, N. Schroeder, N. Pawlowski, R. Lange, F. Kaiser, J. Zerrahn, S. Martens, and J.C. Howard. 2008. Regulatory interactions between IRG resistance GTPases in the cellular response to *Toxoplasma gondii*. *EMBO J.* 27:2495–2509. doi:10.1038/embj.2008.176

Joynson, D.H.M., and T.J. Wreggitt, editors. 2001. *Toxoplasmosis: A Comprehensive Clinical Guide*. Cambridge University Press, Cambridge. 410 pp.

Khaminets, A., J.P. Hunn, S. Koenen-Waisman, Y.O. Zhao, D. Preukschat, J. Coers, J.P. Boyle, Y.C. Ong, J.C. Boothroyd, G. Reichmann, and J.C. Howard. 2010. Coordinated loading of IRG resistance GTPases on to the *Toxoplasma gondii* parasitophorous vacuole. *Cell. Microbiol.* 12:939–961. doi:10.1111/j.1462-5822.2010.01443.x

Liang, G., T.E. Audas, Y. Li, G.P. Cockram, J.D. Dean, A.C. Martyn, K. Kokame, and R. Lu. 2006. Luman/CREB3 induces transcription of the endoplasmic reticulum (ER) stress response protein Herp through an ER stress response element. *Mol. Cell. Biol.* 26:7999–8010. doi:10.1128/MCB.01046-06

Lieberman, L.A., M. Banica, S.L. Reiner, and C.A. Hunter. 2004. STAT1 plays a critical role in the regulation of antimicrobial effector mechanisms, but not in the development of Th1-type responses during toxoplasmosis. *J. Immunol.* 172:457–463.

Lu, F., S. Huang, and L.H. Kasper. 2009. The temperature-sensitive mutants of *Toxoplasma gondii* and ocular toxoplasmosis. *Vaccine*. 27:573–580. doi:10.1016/j.vaccine.2008.10.090

Martinon, F., X. Chen, A.H. Lee, and L.H. Glimcher. 2010. TLR activation of the transcription factor XBP1 regulates innate immune responses in macrophages. *Nat. Immunol.* 11:411–418. doi:10.1038/ni.1857

Melo, M.B., P. Kasperkowitz, A. Cerny, S. Koenen-Waisman, E.A. Kurt-Jones, E. Lien, B. Beutler, J.C. Howard, D.T. Golenbock, and R.T. Gazzinelli. 2010. UNC93B1 mediates host resistance to infection with *Toxoplasma gondii*. *PLoS Pathog.* 6:e1001071. doi:10.1371/journal.ppat.1001071

Montoya, J.G., and J.S. Remington. 2008. Management of *Toxoplasma gondii* infection during pregnancy. *Clin. Infect. Dis.* 47:554–566. doi:10.1086/590149

Ogawa, M., M. Yamamoto, and K. Takeda. 2010. Role of *Toxoplasma gondii* rhoptry kinase ROP16 in Stat6 activation. *Med. J. Osaka Univ.* 53:1–4.

Ong, Y.C., M.L. Reese, and J.C. Boothroyd. 2010. *Toxoplasma* rhoptry protein 16 (ROP16) subverts host function by direct tyrosine phosphorylation of STAT6. *J. Biol. Chem.* 285:28731–28740. doi:10.1074/jbc.M110.112359

Peixoto, L., F. Chen, O.S. Harb, P.H. Davis, D.P. Beiting, C.S. Brownback, D. Ouloguem, and D.S. Roos. 2010. Integrative genomic approaches highlight a family of parasite-specific kinases that regulate host responses. *Cell Host Microbe*. 8:208–218. doi:10.1016/j.chom.2010.07.004

Pepper, M., F. Dzierszinski, A. Crawford, C.A. Hunter, and D. Roos. 2004. Development of a system to study CD4(+)T-cell responses to transgenic ovalbumin-expressing *Toxoplasma gondii* during toxoplasmosis. *Infect. Immun.* 72:7240–7246. doi:10.1128/IAI.72.12.7240–7246.2004

Reese, M.L., and J.C. Boothroyd. 2009. A helical membrane-binding domain targets the *Toxoplasma* ROP2 family to the parasitophorous vacuole. *Traffic*. 10:1458–1470. doi:10.1111/j.1600-0854.2009.00958.x

Richardson, C.E., T. Kooistra, and D.H. Kim. 2010. An essential role for XBP-1 in host protection against immune activation in *C. elegans*. *Nature*. 463:1092–1095. doi:10.1038/nature08762

Robben, P.M., D.G. Mordue, S.M. Truscott, K. Takeda, S. Akira, and L.D. Sibley. 2004. Production of IL-12 by macrophages infected with *Toxoplasma gondii* depends on the parasite genotype. *J. Immunol.* 172:3686–3694.

Robben, P.M., M. LaRegina, W.A. Kuziel, and L.D. Sibley. 2005. Recruitment of Gr-1⁺ monocytes is essential for control of acute toxoplasmosis. *J. Exp. Med.* 201:1761–1769. doi:10.1084/jem.20050054

Saeij, J.P., J.P. Boyle, S. Coller, S. Taylor, L.D. Sibley, E.T. Brooke-Powell, J.W. Ajioka, and J.C. Boothroyd. 2006. Polymorphic secreted kinases are key virulence factors in toxoplasmosis. *Science*. 314:1780–1783. doi:10.1126/science.1133690

Saeij, J.P., S. Coller, J.P. Boyle, M.E. Jerome, M.W. White, and J.C. Boothroyd. 2007. *Toxoplasma* co-opts host gene expression by injection of a polymorphic kinase homologue. *Nature*. 445:324–327. doi:10.1038/nature05395

Shirahata, T., T. Yamashita, C. Ohta, H. Goto, and A. Nakane. 1994. CD8⁺ T lymphocytes are the major cell population involved in the early gamma interferon response and resistance to acute primary *Toxoplasma gondii* infection in mice. *Microbiol. Immunol.* 38:789–796.

Sibley, L.D., and J.C. Boothroyd. 1992. Virulent strains of *Toxoplasma gondii* comprise a single clonal lineage. *Nature*. 359:82–85. doi:10.1038/359082a0

Sibley, L.D., and D.K. Howe. 1996. Genetic basis of pathogenicity in toxoplasmosis. *Curr. Top. Microbiol. Immunol.* 219:3–15.

Sinai, A.P., P. Webster, and K.A. Joiner. 1997. Association of host cell endoplasmic reticulum and mitochondria with the *Toxoplasma gondii*

parasitophorous vacuole membrane: a high affinity interaction. *J. Cell Sci.* 110:2117–2128.

Steinfeldt, T., S. Könen-Waisman, L. Tong, N. Pawlowski, T. Lamkemeyer, L.D. Sibley, J.P. Hunn, and J.C. Howard. 2010. Phosphorylation of mouse immunity-related GTPase (IRG) resistance proteins is an evasion strategy for virulent *Toxoplasma gondii*. *PLoS Biol.* 8:e1000576. doi:10.1371/journal.pbio.1000576

Stirling, J., and P.O'Hare. 2006. CREB4, a transmembrane bZip transcription factor and potential new substrate for regulation and cleavage by S1P. *Mol. Biol. Cell.* 17:413–426. doi:10.1091/mbc.E05-06-0500

Subauste, C.S., and J.S. Remington. 1991. Role of gamma interferon in *Toxoplasma gondii* infection. *Eur. J. Clin. Microbiol. Infect. Dis.* 10:58–67. doi:10.1007/BF01964408

Tateda, C., R. Ozaki, Y. Onodera, Y. Takahashi, K. Yamaguchi, T. Berberich, N. Koizumi, and T. Kusano. 2008. NtbZIP60, an endoplasmic reticulum-localized transcription factor, plays a role in the defense response against bacterial pathogens in *Nicotiana tabacum*. *J. Plant Res.* 121:603–611. doi:10.1007/s10265-008-0185-5

Taylor, G.A., C.M. Collazo, G.S. Yap, K. Nguyen, T.A. Gregorio, L.S. Taylor, B. Eagleson, L. Secret, E.A. Souton, S.W. Reid, et al. 2000. Pathogen-specific loss of host resistance in mice lacking the IFN- γ -inducible gene IGTp. *Proc. Natl. Acad. Sci. USA.* 97:751–755. doi:10.1073/pnas.97.2.751

Taylor, S., A. Barragan, C. Su, B. Fux, S.J. Fentress, K. Tang, W.L. Beatty, H.E. Hajj, M. Jerome, M.S. Behnke, et al. 2006. A secreted serine-threonine kinase determines virulence in the eukaryotic pathogen *Toxoplasma gondii*. *Science.* 314:1776–1780. doi:10.1126/science.1133643

Wang, Y., J. Shen, N. Arenzana, W. Tirasophon, R.J. Kaufman, and R. Prywes. 2000. Activation of ATF6 and an ATF6 DNA binding site by the endoplasmic reticulum stress response. *J. Biol. Chem.* 275:27013–27020.

Wu, J., D.T. Rutkowski, M. Dubois, J. Swathirajan, T. Saunders, J. Wang, B. Song, G.D. Yau, and R.J. Kaufman. 2007. ATF6 α optimizes long-term endoplasmic reticulum function to protect cells from chronic stress. *Dev. Cell.* 13:351–364. doi:10.1016/j.devcel.2007.07.005

Yamamoto, K., T. Sato, T. Matsui, M. Sato, T. Okada, H. Yoshida, A. Harada, and K. Mori. 2007. Transcriptional induction of mammalian ER quality control proteins is mediated by single or combined action of ATF6 α and XBP1. *Dev. Cell.* 13:365–376. doi:10.1016/j.devcel.2007.07.018

Yamamoto, M., S. Sato, H. Hemmi, K. Hoshino, T. Kaisho, H. Sanjo, O. Takeuchi, M. Sugiyama, M. Okabe, K. Takeda, and S. Akira. 2003. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science.* 301:640–643. doi:10.1126/science.1087262

Yamamoto, M., S. Yamazaki, S. Uematsu, S. Sato, H. Hemmi, K. Hoshino, T. Kaisho, H. Kuwata, O. Takeuchi, K. Takeshige, et al. 2004. Regulation of Toll/IL-1-receptor-mediated gene expression by the inducible nuclear protein IkappaBzeta. *Nature.* 430:218–222. doi:10.1038/nature02738

Yamamoto, M., T. Okamoto, K. Takeda, S. Sato, H. Sanjo, S. Uematsu, T. Saitoh, N. Yamamoto, H. Sakurai, K.J. Ishii, et al. 2006. Key function for the Ubc13 E2 ubiquitin-conjugating enzyme in immune receptor signaling. *Nat. Immunol.* 7:962–970. doi:10.1038/ni1367

Yamamoto, M., D.M. Standley, S. Takashima, H. Saiga, M. Okuyama, H. Kayama, E. Kubo, H. Ito, M. Takaura, T. Matsuda, et al. 2009. A single polymorphic amino acid on *Toxoplasma gondii* kinase ROP16 determines the direct and strain-specific activation of Stat3. *J. Exp. Med.* 206:2747–2760. doi:10.1084/jem.20091703

Yap, G.S., and A. Sher. 1999. Cell-mediated immunity to *Toxoplasma gondii*: initiation, regulation and effector function. *Immunobiology.* 201: 240–247.

Yoshida, H., T. Okada, K. Haze, H. Yanagi, T. Yura, M. Negishi, and K. Mori. 2001. Endoplasmic reticulum stress-induced formation of transcription factor complex ERSF including NF-Y (CBF) and activating transcription factors 6 α and 6 β that activates the mammalian unfolded protein response. *Mol. Cell. Biol.* 21:1239–1248. doi:10.1128/MCB.21.4.1239-1248.2001

Zhang, K., and R.J. Kaufman. 2008. From endoplasmic-reticulum stress to the inflammatory response. *Nature.* 454:455–462. doi:10.1038/nature07203

Zhao, Y., D.J. Ferguson, D.C. Wilson, J.C. Howard, L.D. Sibley, and G.S. Yap. 2009. Virulent *Toxoplasma gondii* evade immunity-related GTPase-mediated parasite vacuole disruption within primed macrophages. *J. Immunol.* 182:3775–3781. doi:10.4049/jimmunol.0804190