The *Toxoplasma gondii* rhoptry protein ROP18 is an Irga6-specific kinase and regulated by the dense granule protein GRA7

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Introduction

Hosts and pathogens impose intense selection pressures on each other, resulting in dynamic changes in the genetic structure of populations and rapid co-evolutionary change in molecules contributing to virulence and resistance. As JBS Haldane (1949) predicted, much polymorphic variation in protein sequence will arise from such interaction. A detailed analysis of polymorphic molecules involved in resistance and virulence provides insight into functional mechanisms, with potential implications for disease control. In this study, we analyse the biochemistry of an important polymorphic virulence complex secreted into mammalian cells by the ubiquitous protozoan pathogen *Toxoplasma gondii* (*T. gondii*).

*T. gondii* is an obligate intracellular parasite able to establish a productive infection in a remarkably broad range of mammalian and avian intermediate hosts, including man. Sexual reproduction occurs only in its primary host, i.e. all members of the family of true cats (*Felidae*). Because of their abundant worldwide distribution and sympathy with domestic cats, house mice are probably important intermediate hosts for *T. gondii* (Lilue et al., 2013). Nevertheless, some strains of *T. gondii* are highly virulent for laboratory mice. Virulence in laboratory mice is associated with inactivation of the immunity-related GTPase (IRG) resistance system (Taylor, 2007; Taylor et al., 2007). IRG proteins represent a complex family of polymorphic interferon gamma (IFN-γ)-inducible GTPases (Boehm et al., 1998; Martens and Howard, 2006). About 20 IRG genes are encoded within the genome of C57BL/6 mice, located in two adjacent clusters on chromosome 11 and one cluster on chromosome 18 (Bekpen et al., 2005). Four of these genes are transcribed as adjacent pairs, resulting in expression of proteins carrying two IRG domains, the so-called tandem IRG proteins (Lilue et al., 2013). Based upon a unique substitution within the first nucleotide binding motif (GX₄GK/MS), IRG proteins can be classified into two subfamilies. Activated GTP-bound effector IRG proteins (the GKS group) accumulate in high densities at the parasitophorous vacuolar membrane (PVM) of avirulent *T. gondii* type II strains early after invasion, leading to PVM rupture and parasite clearance (Martens et al., 2005; Ling et al., 2006; Zhao et al., 2009b). The loading is cooperative...
and hierarchical, with two family members serving as pioneers for members loading later (Khaminets et al., 2010). Premature activation of IRG effectors on cellular endomembranes is prevented by three regulatory IRG proteins, Irgm1–Irgm3 (the GMS group), which keep the effector proteins in a GDP-bound inactive conformation at endogenous cellular membranes (Hunn et al., 2008; Halder et al., 2013; Marie-Biresev et al., in preparation). Exactly how IRG proteins contribute to disruption of the PVM is not known, but their essential, non-redundant function in early resistance of mice to avirulent *T. gondii* type II strains has been unambiguously established (Taylor et al., 2000; Collazo et al., 2001; Liesenfeld et al., 2011).

With *T. gondii* virulent type I strains, the initial loading of the PVM with IRG proteins is markedly reduced (Zhao et al., 2009c; Khaminets et al., 2010). In order to preserve the integrity of the PVM, *T. gondii* has evolved several polymorphic effector proteins (Hunter and Sibley, 2012; Alaganan et al., 2014; Etheridge et al., 2014). Genetic screens revealed that polymorphism in effectors accounts for the differences in virulence between *T. gondii* strains in infected mice (Saeij et al., 2006; Taylor et al., 2006; Saeij et al., 2007; Behnke et al., 2011). Different polymorphic rhoptry proteins were shown to be important for inhibition of the IRG resistance system. The catalytically active kinase ROP18 of *T. gondii* virulent type I strains phosphorylates IRG proteins at highly conserved threonines in the switch I region of the nucleotide binding site (Fentress et al., 2010; Steinfeldt et al., 2010). Phosphorylation of multiple family members could be demonstrated, but the target sites identified in Irga6 most closely match the ROP18 substrate phosphorylation motif (Lim et al., 2013). Certain *T. gondii* avirulent type II strains also express a virulent allelic form of ROP18 (Saeij et al., 2006; Niedelman et al., 2012) but fail to restrict IRG protein accumulation at the PVM to an extent that allows survival in infected mouse cells (Zhao et al., 2009b). We could demonstrate that for efficient phosphorylation, IRG proteins have to be kept in the inactive GDP-bound conformation (Fleckenstein et al., 2012). This conformation is facilitated by direct binding of the pseudokinase ROP5 to IRG proteins (Fleckenstein et al., 2012; Niedelman et al., 2012). Although not apparent for an IRG-specific function of ROP5 in our own experiments (Fleckenstein et al., 2012), an interaction of ROP5 with ROP18 was reported to control *T. gondii* virulence by up-regulation of ROP18 activity (Behnke et al., 2012). The rop5 locus is represented by a cluster of tandemly repeated genes encoding three different ROP5 isoforms, ROP5A, ROP5B and ROP5C, each containing multiple copies, and all isoforms are predicted to be catalytically inactive (El Hajj et al., 2006; Reese and Boothroyd, 2011). Individual sequences of these rop5 paralogues are almost identical within strains but differ considerably between *T. gondii* types I, II and III strains (Behnke et al., 2011; Reese et al., 2011; Niedelman et al., 2012). In wild-derived mice, this two-component ROP5/ROP18 parasite virulence system is counteracted by polymorphic tandem IRG proteins (Lilue et al., 2013), most likely due to active decoying of ROP5 and ROP18 leaving Irga6 unphosphorylated. Another active rhoptry kinase, ROP17, was demonstrated only recently to phosphorylate Irg6 and Irgb6 in vitro. ROP17 shows a preference for Irgb6, and unlike ROP18, kinase activity is independent from ROP5 (Etheridge et al., 2014).

In this study, we show that the dense granule protein GRA7 (Bonhomme et al., 1998; Fischer et al., 1998) is directly associated with ROP5 and is essential for efficient phosphorylation of Irga6. Consequently, the loss of GRA7 can be correlated directly with parasite virulence in *vitro* and *in vivo*. We conclusively demonstrate that GRA7 is an Irga6-specific *T. gondii* effector and propose a model explaining the molecular function of GRA7 by regulation of ROP18 kinase activity. This model is supported by our finding that Irga6 is also the only specific target of ROP18 among IRG proteins. Clarification of this Irga6-specific function of ROP18 *in vivo* suggests that other yet unknown *T. gondii* virulence effectors may be strictly specific for other IRG proteins.

**Results**

**GRA7 is a component of the IRG-targeting ROP5/ROP18 kinase complex**

In pull-down experiments from detergent lysates of L929 fibroblasts infected with virulent RH-YFP type I strain tachyzoites, several *T. gondii* proteins could be identified by liquid chromatography–tandem mass spectrometry (LC-MS/MS) to interact with glutathione S-transferase (GST)-Irga6 (Fleckenstein et al., 2012; Howard and Steinfeldt, unpublished results). To find which of these proteins might represent IRG-related *T. gondii* effector proteins, we first determined the composition of Irga6-specific complexes under non-denaturing conditions. After separation by blue native (BN) polyacrylamide gel electrophoresis (PAGE), LC-MS/MS analysis from similar pull-down experiments revealed that some of these proteins are indeed components of the same complex (Table S1). In addition to all ROP5 isoforms and ROP18, tryptic peptides were identified unambiguously corresponding to the dense granule protein GRA7. However, GRA7 was not found in pull-down approaches using detergent lysates of *T. gondii* RH:rop5 strain. This result suggests ROP5 is required in an Irga6/GRA7 complex. The LC-MS/MS results obtained after BN-PAGE were confirmed by Western blot analysis (Fig. 1). ROP5 binding to Irga6 was unchanged in the absence of GRA7.
(RHΔgra7), but no GRA7 could be detected when RHΔrop5 parasites were applied in the same pull-down approach. In parallel, GST alone was used and provided control for specificity (Fig. 1A). Equal protein amounts in tachyzoite lysates were confirmed in the supernatants after pull-down with GST (Fig. 1B).

The results shown in Fig. 1 indicate either that the interaction between Irga6 and GRA7 is indirect and mediated by ROP5 or that direct binding of GRA7 to Irga6 requires a conformational change of the GTPase induced by direct binding of ROP5 (Fleckenstein et al., 2012).

**GRA7 is directly associated with ROP5**

We directly looked for GRA7 binding to ROP5, which we immunoprecipitated using a GRA7-specific antibody from *T. gondii* tachyzoite lysates or lysates of infected L929 cells that were stimulated with IFNγ or left untreated. Subsequent Western blot analysis using a ROP5-specific antibody revealed interaction of the parasite proteins in both tachyzoite lysates (Fig. 2A) and lysates from infected cells (Fig. 2B). In the latter case, prestimulation with IFNγ (middle panel) had no clearly visible effect on the intensity of GRA7 binding to ROP5. The impact of ROP5 on vacuolar GRA7 accumulation was investigated by signal intensity measurements at the PVM. In mouse embryonic fibroblasts (MEFs) stimulated with IFNγ and subsequently infected with *T. gondii*, the mean signal intensities of GRA7 were clearly decreased at RHΔrop5-derived vacuoles compared with RHΔhxgprt (Fig. 2C), indicating that association of a significant proportion of GRA7 with the *T. gondii* PVM is dependent on ROP5 in infected cells.

In summary, these results demonstrate that recruitment of GRA7 to the *T. gondii* kinase complex is dependent on binding to ROP5 but does not necessarily indicate a direct binary interaction.

In a yeast two-hybrid assay, *T. gondii* proteins GRA7 and ROP5A/B/C were expressed as N-terminal fusions with the Gal4 DNA-binding or Gal4 activation domain in a yeast reporter strain. Colony growth on selective medium allowed us to screen for direct protein–protein interactions. In this way, direct binding of GRA7 to all three ROP5 isoforms could be shown. No colony growth was observed for the negative controls (Fig. 2D).

**GRA7 does not contribute to Irga6/ROP5/ROP18 complex assembly**

In the following analysis, the presence of GRA7 as a basic requirement for the association of different complex members was investigated. Our pull-down results already demonstrated no differences in ROP5 binding to Irga6 in the presence or absence of *T. gondii* GRA7 expression (Fig. 1). Stimulation of ROP18 kinase activity was suggested earlier to be an important molecular function of ROP5 (Behnke et al., 2012). We analysed ROP5 immunoprecipitations from detergent tachyzoite lysates of different *T. gondii* strains for binding of ROP18. Western blot analysis with a ROP18-specific antiserum demonstrated binding of ROP5 to ROP18 in *T. gondii* RHΔhxgprt and RHΔgra7 detergent lysates (Fig. 3A, left-hand panel). No difference in binding of ROP5 to ROP18 in the absence of GRA7 was observed. Equal protein amounts in tachyzoite detergent lysates were confirmed in the supernatants after immunoprecipitation (Fig. 3A, right-hand panel). These results indicate that GRA7 is probably
not required for the association of the ROP5/ROP18 complex.

Interestingly, after immunoprecipitation of GRA7 from *T. gondii* detergent lysates, Western blot analysis with a ROP18-specific antiserum revealed interaction of ROP18 with GRA7 only in the absence of ROP5 (Fig. 3B, upper panel). Efficiency of immunoprecipitation (GRA7) in the case of RHΔhxgprt and RHΔrop5 is indicated (Fig. 3B, lower panel). To investigate a direct interaction of GRA7 and ROP18, proteins were expressed as N-terminal fusions with the Gal4 DNA-binding (ROP18) or Gal4 activation domain (GRA7) in a yeast reporter strain. Direct binding of GRA7 to ROP18 is indicated by colony growth on selective medium compared with the negative controls (Fig. 3C).

GRA7 is required for efficient phosphorylation of Irga6

The association of GRA7 with ROP5 (Fig. 2) and ROP18 (Fig. 3) indicated that GRA7 is a component of
the *T. gondii* kinase complex. In the following, we were able to demonstrate that GRA7 indeed contributes to phosphorylation and inactivation of the IRG resistance system.

MEFs stimulated with IFNγ for 24 h were infected with *T. gondii* RHΔhxgprt, RHΔrop5 or RHΔgra7 strain for a further 2 h. Western blot analysis of detergent lysates indicated diminished phosphorylation of Irga6 at T108 [(pT108)Irga6], one of the ROP18 target threonines, in the absence of GRA7 expression compared with parental strain-infected cells. As expected, no phosphorylation was detectable in the absence of ROP5 (Fig. 4A, right-hand panel). ROP5 and GRA7 expression levels in the lysates of infected cells served as infection control (Fig. 4A, left-hand panels), and calnexin (Fig. 4A, upper panels) provided loading controls. Expression levels of ROP18 and ROP5 were comparable in RHΔhxgprt and RHΔgra7 tachyzoite detergent lysates (Fig. S1).

The reduced phosphorylation of Irga6 in RHΔgra7 strain-infected cells seen in Western blot was confirmed by fluorescence signal intensity measurements at individual vacuoles in MEFs 2 h post-infection. Compared with parental strain-infected cells, mean signal intensities of (pT108)Irga6 were significantly decreased in RHΔgra7-infected MEFs (Fig. 4B). These results clearly indicate that GRA7 is an important component within the *T. gondii* kinase complex, contributing to phosphorylation of Irga6 by ROP5 and ROP18 (Fentress et al., 2010; Steinfeldt et al., 2010; Fleckenstein et al., 2012).

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**Fig. 3.** GRA7 and ROP5 interact with ROP18.

A. ROP18 co-immunoprecipitates with ROP5 from *T. gondii* tachyzoite detergent lysates. Co-immunoprecipitation of ROP18 with ROP5 (upper panel) from RHΔhxgprt and RHΔgra7 using 3E2 ROP5-specific monoclonal antibody. The lower panel indicates equivalent co-immunoprecipitation of GRA7 from RHΔhxgprt and RHΔrop18. The right-hand blot indicates ROP5 (upper panel) and GRA7 (lower panel) levels in the supernatants after immunoprecipitation.

B. ROP18 co-immunoprecipitates with GRA7 from *T. gondii* tachyzoite detergent lysates. Co-immunoprecipitation of ROP18 with GRA7 (upper panel) is only visible from RHΔrop5 but not RHΔhxgprt, RHΔgra7 or RHΔrop18 using 3.1.2 GRA7-specific monoclonal antibody. The lower panel indicates equivalent levels of GRA7 in the immunoprecipitations. Asterisks indicate heavy chain of the respective antibody used for immunoprecipitation. White spaces indicate excision of irrelevant tracks.

C. GRA7 and ROP18 directly interact in a yeast two-hybrid approach. Cotransformants grown on double-dropout (2DO) plates were replica-plated under 2DO (SD/-Leu/-Trp) and triple-dropout (3DO, SD/-Leu/-Trp/-His) conditions. Colony growth under 3DO conditions is indicative of a GRA7:ROP18 interaction.
GRA7 contributes to inhibition of vacuolar IRG protein accumulation

With *Toxoplasma gondii* virulent type I strains, IRG protein accumulation at the PVM is markedly reduced compared with type II or III avirulent strains (Zhao et al., 2009a; 2009c; Khaminets et al., 2010). The lack of efficient Irga6 phosphorylation by *T. gondii* RHΔgra7 (Fig. 4) predicted increased IRG protein amounts at the PVM relative to RHΔhxgprt. We therefore determined recruitment of certain IRG proteins to the PVM in MEFs 2 h post-infection. Compared with RHΔhxgprt, signal intensities (Figs 5A, B and S2) and frequencies of loaded vacuoles (Fig. S2) of Irga6, Irgb6, Irgb10 and Irgd were significantly increased in RHΔgra7-infected cells.

Growth restriction of *T. gondii* by IFNγ in infected cells (Zhao et al., 2009b) is directly associated with inhibition of efficient IRG protein loading onto the PVM. We measured intracellular proliferation of *T. gondii* RHΔgra7 in bone marrow-derived macrophages (BMMs) and MEFs in vitro by incorporation of 3H-uracil. The inability of RHΔgra7 parasites to prevent IRG protein accumulation at the PVM (Figs 5A, B and S2) was reflected in significantly greater inhibition of intracellular replication relative to *T. gondii* RHΔhxgprt upon stimulation with IFNγ (Fig. 5C). Therefore, the impact of GRA7 on IRG protein accumulation is directly correlated with control of parasite replication in cells in vitro.

We next investigated the contribution of GRA7 to *T. gondii* virulence in mice. The virulence difference between RHΔhxgprt and RHΔgra7 cannot be resolved in C57BL/6 mice (Alaganan et al., 2014) (Fig. 6D). However, the wild-derived Indian mouse strain CIM is considerably more resistant to type I *T. gondii* strains (Lilue et al., 2013). The median lethal dose of RHΔhxgprt injected intraperitoneally in these mice was determined to be between 1 × 10⁵ and 1 × 10⁶ parasites (Fig. S3). Almost all CIM mice infected with 5 × 10⁵ RHΔhxgprt tachyzoites died within 25 days post-infection, whereas in RHΔgra7 and RHΔrop18 infections, time to death was slightly delayed and more mice survived the infection (Fig. 5D). Slightly reduced virulence of RHΔgra7 was also reported by Alaganan et al. (2014) in CD-1 mice.

The impact of GRA7 on IRG protein accumulation is Irga6-specific

Among all IRG-related *T. gondii* effectors identified so far, ROP5 had the largest effect on vacuolar IRG protein accumulation. We therefore wanted to compare the impact of GRA7 with ROP5. Whereas signal intensities of Irgb6 and Irgb10 at RHΔgra7-derived vacuoles were found to be intermediate between vacuoles derived from RHΔrop5 and RHΔhxgprt, the vacuolar loading phenotype of Irga6

![A. Western blot of detergent lysates from C57BL/6 MEFs stimulated for 24 h with 200 U ml⁻¹ of IFNγ and infected for 2 h with *T. gondii* RHΔrop5, RHΔhxgprt or RHΔgra7. The signal representing phosphorylation of Irga6 at T108 [p(T108)Irga6] upon RHΔhxgprt infection is clearly reduced upon infection with RHΔgra7 (middle right panel). Signals for ROP5 (middle left panel) and GRA7 (lower panel) indicate equivalent levels of infection. Band intensities relative to calnexin were determined using IMAGEJ software. Asterisk indicates unspecific proteins. Calnexin (upper panels) served as loading control. B. In MEFs, induced for 24 h with 200 U ml⁻¹ of IFNγ and infected for 2 h with *T. gondii* RHΔhxgprt or RHΔgra7, signal intensities of p(T108)Irga6 at the PVM were determined using anti-p(T108)Irga6-specific antibody. One representative out of two independent experiments is shown (40 vacuoles counted).](image-url)
was very similar in RHΔgra7-infected and RHΔrop5-infected cells (Fig. 6A). Based on the fact that GRA7 is directly associated with ROP5 (Fig. 2), RHΔrop5 is a functional RHΔrop5/gra7 strain in terms of Irga6 phosphorylation. Because the impact of single (RHΔgra7) and functional double knockout (ko) mutant (RHΔrop5/gra7) on Irga6 but not Irgb6 or Irgb10 loading is exactly the same, these results suggested an Irga6-specific function of GRA7.

To confirm an Irga6-specific function of GRA7, fluorescence signal intensities of Irgb6 and Irgb10 at the PVM were determined in Irga6 ko MEFs. No difference in the mean total protein amounts of either Irgb6 or Irgb10 could be observed in these cells infected with T. gondii RHΔhxgprt or RHΔgra7 and individual IRG proteins identified with mouse monoclonal antibody 10D7 (Irga6) and B34 (Irgb6) or 940/6 (Irgb10) and 2078 (Irgd) rabbit antisera. The fluorescent signal intensities of each IRG protein were determined on coded slides in at least two independent experiments. One representative experiment is shown, and horizontal lines indicate means (30–35 vacuoles counted).

C. C57BL/6 BMMs were induced with 1, 10 or 100 U ml⁻¹ of IFNγ for 24 h and infected with T. gondii RHΔhxgprt or RHΔgra7 at an MOI of 0.3. Intracellular parasite growth was calculated by incorporation of 3H-uracil 24 h post-infection as described in Experimental procedures. The percentages are averages of triplicates, and the error bar represents the standard deviation for these averages.

D. CIM mice were infected by intraperitoneal injection of 5 × 10⁵ T. gondii RHΔhxgprt (n = 7), RHΔgra7 or RHΔrop18 (n = 8) strain tachyzoites, and survival was monitored for 45 days.
Fig. 6. GRA7 is an Irga6-specific *T. gondii* virulence effector.

A. IFNγ-induced (200 U ml⁻¹) wt MEFs were infected for 2 h with *T. gondii* RHΔhxgprt, RHΔrop5 or RHΔgra7 and individual IRG protein positive vacuoles evaluated, 30 vacuoles counted. One representative out of two independent experiments is shown.

B. IFNγ-induced (200 U ml⁻¹) wt or Irga6 ko MEFs were infected for 2 h with *T. gondii* RHΔhxgprt, RHΔrop5 or RHΔgra7 and individual IRG protein positive vacuoles evaluated, 50 vacuoles counted. One representative out of two independent experiments is shown.

C. wt and Irga6 ko MEFs were induced with 10 U ml⁻¹ of IFNγ for 24 h and infected with *T. gondii* RHΔhxgprt, RHΔgra7 or RHΔrop5 at an MOI of 1.

D. Virulence of *T. gondii* RHΔhxgprt, RHΔgra7 and RHΔrop5 in wt and Irga6 ko mice. C57BL/6 wt (n = 10) and Irga6 ko (n = 10) mice were infected by intraperitoneal injection of 50 *T. gondii* RHΔhxgprt, RHΔgra7 or 200 RHΔrop5 strain parasites and survival monitored for 36 days.
amounts of Irgb6 and Irgb10 at RHΔgra7-derived vacuoles in wt MEFs (Fig. 6A) are dependent only on elevated levels of Irga6, a cooperative interaction documented earlier (Khaminets et al., 2010).

The Irga6 specificity of T. gondii GRA7 was confirmed by intracellular proliferation in Irga6ΔΔko MEFs. In contrast to its attenuated virulence phenotype in wt cells (Figs. 6C, and 5C), proliferation in Irga6ΔΔko MEFs (Fig. 6C) was not efficiently restricted by IFNγ, and T. gondii RHΔgra7 almost behaved like virulent RHΔhxgprt in these cells. According to its vacuolar IRG protein loading phenotype (Fig. 6B), proliferation of RHΔrop5 was still strikingly inhibited in the presence of IFNγ in Irga6ΔΔko MEFs (Fig. 6C). We next investigated T. gondii virulence in vivo. After challenge with RHΔgra7, Irga6ΔΔko and wt mice succumbed to infection within ten days whereas almost all wt and ko mice challenged with RHΔrop5 parasites survived infection (Fig. 6D).

**Irga6 is the main target of ROP18 among IRG proteins in BL/6 mice**

The importance of GRA7 for full ROP18 kinase activity (Fig. 4) and its apparent specificity for Irga6 (Fig. 6) stimulated us to investigate whether the overall impact of ROP18 on the IRG resistance system (Fentress et al., 2010; Steinfeldt et al., 2010; Behnke et al., 2012; Fleckenstein et al., 2012; Niedelman et al., 2012) is also due only to specific inhibition of Irga6.

Fluorescence signal intensities and frequencies of loaded vacuoles of Irgb6 and Irgb10 were determined at the PVM in wt and Irga6ΔΔko MEFs 2 h post-infection with wt RHΔhxgprt or RHΔrop18. As shown earlier, Irgb6 and Irgb10 loading was substantially higher on RHΔrop18 than on vacuoles. However, the increase in loading intensity and number of positive vacuoles associated with loss of ROP18 was significantly diminished in Irga6ΔΔko cells (Figs 7A and S5). These results differ slightly from the complete extinction of Irgb6 and Irgb10 loading to RHΔgra7-derived vacuoles in Irga6ΔΔko MEFs (Figs 7A, 6B and S5). In summary, these results indicate that the enhanced loading of Irgb6 and Irgb10 at the PVM in RHΔrop18-infected wt MEFs is largely an indirect effect of increased Irga6 amounts. In contrast, deletion of ROP5 resulted only in a marginal decrease of intensities (Fig. 7A) and frequencies (Fig. S5) of Irgb6 and Irgb10 at the T. gondii PVM in Irga6ΔΔko cells relative to wt cells.

The pattern of vacuolar IRG protein accumulation is directly correlated with control of proliferation in infected cells upon stimulation with IFNγ (Zhao et al., 2009c). The avirulent phenotype of RHΔrop18 in infected wt MEFs was reversed to a virulent phenotype in Irga6ΔΔko cells and in this way very similar to RHΔgra7, whereas proliferation of RHΔrop5 was still efficiently inhibited in the absence of Irga6 expression relative to wt cells (Fig. 7B).

The Irga6 deficiency also affects virulence of T. gondii RHΔrop18 in vivo. After challenge with RHΔrop18, Irga6ΔΔko mice succumbed to infection within 10 days, whereas in wt mice, time to death was significantly delayed (Fig. 7C).

With respect to IRG protein inactivation, these results are consistent with ROP18 being an Irga6-specific kinase.

**Discussion**

Virulence of T. gondii in mice is adjusted by dozens of parasite proteins injected into the host cell cytosol at the onset and after host cell invasion (Hunter and Sibley, 2012; Bougdour et al., 2013; Braun et al., 2013; Alaganan et al., 2014; Etheridge et al., 2014). The rhoptry proteins ROP5 and ROP18 have been shown to inhibit the IRG resistance system by phosphorylation of highly conserved threonine residues in the switch I region of the nucleotide binding site (Fentress et al., 2010; Steinfeldt et al., 2010; Behnke et al., 2012; Fleckenstein et al., 2012; Niedelman et al., 2012). In this study, we could confirm that the dense granule protein GRA7 is an additional component of the T. gondii ROP5/ROP18 kinase complex (Alaganan et al., 2014). GRA7 is directly associated with ROP5 and required for efficient phosphorylation of Irga6. In a recent study, direct GRA7 : Irga6 association was observed with purified proteins in vitro in the presence of GTP (Alaganan et al., 2014). However, in our pull-down analyses, GRA7 binding to Irga6 was always dependent on ROP5 regardless of whether additional GTP was added (data not shown) or not. Moreover, at the PVM of T. gondii RHΔrop5 in IFNγ-stimulated MEFs, GRA7 intensities were significantly, although not completely, reduced (Fig. 2C). A fraction of GRA7 is associated with the vacuolar network of tubular membranes rather than the PVM (Bonhomme et al., 1998), and in addition, binding of GRA7 to ROP2 and ROP4 was shown (Dunn et al., 2008), perhaps explaining the only partial decrease of vacuolar GRA7 amounts in the absence of ROP5.

In addition to Irga6, also Irgb6 and Irgb10 have been shown to be phosphorylated upon infection with T. gondii virulent type I strains in cells of laboratory mice (Fentress et al., 2010; Steinfeldt et al., 2010; Behnke et al., 2012; Lilue et al., 2013), and inactivation by the ROP5/ROP18/ GRA7 kinase complex is accompanied by reduced accumulation at the PVM (e.g. Fig. 7A). However, our data show that the effect of GRA7 and ROP18 on other IRG proteins is mediated through Irga6. Thus, attenuation of the kinase complex by loss of GRA7 (in RHΔgra7) or ROP18 (in RHΔrop18) caused significant increases in signals at the PVM of Irgb6, Irgb10 and Irgd in wt MEFs but had little or no effect on PVM accumulation in Irga6ΔΔko cells.
Likewise, RH\textsubscript{Δgra7} and RH\textsubscript{Δrop18} were converted from parasite strains with an attenuated virulence phenotype in wt cells to fully virulent strains in Irga6\textsubscript{ko} cells (Figs 6 and 7). Strikingly and in contrast to RH\textsubscript{Δrop5}, RH\textsubscript{Δrop18} is fully virulent in Irga6\textsubscript{ko} mice (Figs 6D and 7C). These results illustrate the interdependent accumulation of IRG proteins at the \textit{T. gondii} PVM documented earlier (Khaminet\textsubscript{et al.}, 2010) and clearly demonstrate a molecular function of GRA7 and ROP18 that is specific for Irga6. The fact that loading of Lrgb6 and Lrgb10 protein positive vacuoles were analysed. One representative out of two independent experiments is shown (30 vacuoles counted).

B. wt and \textit{Irga6} ko MEFs were induced with 10 U ml\textsuperscript{-1} IFN\textgamma for 24 h and infected with \textit{T. gondii} RH\textsubscript{Δhxgprt}, RH\textsubscript{Δgra7}, RH\textsubscript{Δrop18} or RH\textsubscript{Δrop5} at an MOI of 1.

C. Virulence of \textit{T. gondii} RH\textsubscript{Δrop18} in wt and \textit{Irga6} ko mice. C57BL/6 wt (\(n = 8\)) and \textit{Irga6} ko (\(n = 9\)) mice were infected by intraperitoneal injection of 200 \textit{T. gondii} RH\textsubscript{Δrop18} strain parasites, and survival was monitored for 60 days.

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B. wt and \textit{Irga6} ko MEFs were induced with 10 U ml\textsuperscript{-1} IFN\textgamma for 24 h and infected with \textit{T. gondii} RH\textsubscript{Δhxgprt}, RH\textsubscript{Δgra7}, RH\textsubscript{Δrop18} or RH\textsubscript{Δrop5} at an MOI of 1.

C. Virulence of \textit{T. gondii} RH\textsubscript{Δrop18} in wt and \textit{Irga6} ko mice. C57BL/6 wt (\(n = 8\)) and \textit{Irga6} ko (\(n = 9\)) mice were infected by intraperitoneal injection of 200 \textit{T. gondii} RH\textsubscript{Δrop18} strain parasites, and survival was monitored for 60 days.
mice after infection with the same number of parasites. However, the double deletion mutant RHΔgra7Δrop18 had a completely avirulent phenotype in C57BL/6 wt mice (Alaganan et al., 2014). This result is unexpected, if both components contribute to virulence exclusively through Irga6 because resistance of C57BL/6 mice even to avirulent type II strains is only partially lost by deletion of Irga6 (Liesenfeld et al., 2011). These seemingly paradoxical results are however consistent with the idea that Irga6 is a major player in resistance of laboratory mice against type I strain RHΔhxgprt attenuated by loss of ROP18/GRA7 (Alaganan et al., 2014), while the residual resistance of Irga6 ko laboratory mice to type II ME49 is largely mediated by other IRG proteins. However, virulence of single deletion mutants RHΔgra7 and RHΔrop18 is similarly attenuated relative to RHΔhxgprt in the highly resistant C57 mouse strain (Fig. 5D), as expected if GRA7 function is required for full ROP18 kinase activity. The different virulence phenotypes of T. gondii RHΔgra7 in wild-derived mice (this study) and inbred and outbred laboratory strains of mice (this study and Alaganan et al., 2014) could be explained by genetic differences but need further investigation. Although no IFN-γ-mediated control of T. gondii RHΔgra7 replication was observed previously (Alaganan et al., 2014), attenuated virulence of RHΔgra7 compared with wt infections could be well established in this study in bone marrow-derived macrophages (Fig. 5C) and fibroblasts (Figs 6C and 7B). These different observations might be explained by different cells or methods used to determine parasite replication.

No differences were observed in binding of ROP5 either to Irga6 or ROP18 in the absence of GRA7 (Figs 1 and 3A), indicating that the presence of GRA7 does not contribute significantly to the strength of the interaction between different members of the complex. Surprisingly, we were able to demonstrate GRA7 binding to ROP18 only in the absence of ROP5. It is conceivable that, in the presence of ROP5, the interface necessary for GRA7:ROP18 interaction is either occupied or not fully accessible because of steric hindrances upon binding of ROP5. We also have to consider that our assay might not be sensitive enough to detect such an interaction in the presence of ROP5. Therefore, we cannot exclude at this point that direct binding of GRA7 is crucial for ROP18 activity even in the presence of ROP5. However, the whole scenario of IRG protein inactivation is played out at the PVM (Fentress et al., 2012). It is therefore possible that the interplay of certain components is indeed GRA7-dependent but could not be revealed in our in vitro systems. Furthermore, the existence of additional yet unidentified parasite proteins important for inactivation of Irga6 cannot be excluded.

The interaction between Irga6 and ROP5 has been crystallized from purified components (Reese et al., 2014) and does not require post-translational modification of either protein. Yet, both phosphorylation and glycosylation have been reported for members of the ROP5/ROP18/GRA7 complex (Dunn et al., 2008; Luo et al., 2011; Treeck et al., 2011). It is possible that some of these modifications may be involved in the interactions between components of the kinase complex. In a recent study, Lim et al. (2013) identified a potential ligand-binding pocket within the crystal structure of the ROP18 kinase domain adjacent to the active site and critical for ROP18-mediated virulence. The existence of this ligand-binding pocket, which contained a sucrose molecule derived from the crystallization buffer, might be indicative of co-translational or post-translational modification of GRA7 that could play a role in T. gondii virulence in general and ROP18 activation especially.

This study shows that GRA7 is a component of the T. gondii ROP5/ROP18 complex necessary to achieve efficient ROP18 kinase activity for the phosphorylation and consequent inactivation specifically of Irga6 (Fig. 8). Our results confirm Irga6 as an important target of T. gondii to promote parasite virulence. This is consistent with a former study that demonstrated a significant role for Irga6 in the control of T. gondii avirulent type II strain infection in vivo (Liesenfeld et al., 2011). By a specific attack on this highly conserved member (Lilue et al., 2013) of the family of IRG proteins, the complex pattern of IRG protein loading onto the T. gondii PVM is significantly disturbed and guarantees a growth advantage in vitro and in vivo. Just as Irga6 could be identified in this study as the only ROP18-specific target among IRG proteins, ROP17 has recently been found to be a significant virulence factor preferably phosphorylating Irgb6, but this action is reported to be independent of ROP5 (Etheridge et al., 2014). These findings strongly suggest that other T. gondii rhoptry effectors might have co-evolved to specifically inactivate other IRG proteins (Fig. 8). Future experiments will identify these genetic matches to better understand the inhibition of vacuolar IRG protein accumulation by virulent T. gondii strains to guarantee parasite survival in mice. These experiments document the intimacy and complexity of the co-evolving resistance–virulence contest played out between T. gondii and its mouse host. However, here we have considered only the interaction of one strain of T. gondii and its virulence mutants with the IRG proteins of the C57BL/6 strain of mouse. Yet the IRG proteins are themselves at least as polymorphic as the virulence factors that target them (Lilue et al., 2013), and how these structural variants contribute to adaptive outcomes for host and pathogen in the wild remains an important area for future research.
Experimental procedures

Propagation of T. gondii

Tachyzoites of T. gondii strains RHΔhxgprt, RHΔgra7, RHΔrop5 and RHΔrop18 were cultivated in confluent monolayers of human foreskin fibroblasts (HS27, ATCC CRL-1634), harvested and immediately used for infection of cells or lysed for subsequent immunoprecipitation, pull-down experiments or Western blot analysis.

Cell culture

L929 mouse fibroblasts and wt or Irga6 ko MEFs derived from C57BL/6 mice were maintained by serial passage in Dulbecco’s modified Eagle medium (DMEM), high glucose (Invitrogen Life Technologies) supplemented with 2 mM of L-glutamine, 1 mM of sodium pyruvate, 1× minimal essential medium non-essential amino acids, 100 U ml⁻¹ of penicillin, 100 mg ml⁻¹ of streptomycin (PAA) and 10% fetal calf serum (FCS, Biochrom). C57BL/6 BMFs were cultured in DMEM, high glucose containing 10% L929 P2 cell-conditioned medium and supplements indicated earlier.

IRG protein expression was induced with 200 U ml⁻¹ of IFNγ (Cell Concepts) for 24 h before infection. Human foreskin fibroblasts (HS27, ATCC CRL-1634) were maintained in Iscove’s modified Dulbecco’s medium, high glucose (Invitrogen Life Technologies) supplemented with 100 U ml⁻¹ of penicillin, 100 mg ml⁻¹ of streptomycin and 5% FCS.

Immunological reagents

Immunoreagents used in this study were 3E2 mouse monoclonal antibody against ROP5 isoforms (Leriche and Dubremetz, 1991), anti-ROP18 rat antiserum (El Hajj and Dubremetz, unpublished), affinity-purified rabbit sera 87558 against (pT108)Irga6 (Steinfeldt et al., 2010), 10D7 and 10E7 mouse monoclonal antibodies (Papic et al., 2008) or 165 rabbit antiserum (Martens et al., 2004) against Irga6, B34 mouse monoclonal antibody (Carlow et al., 1998) against Irgb6, 940/6 rabbit antiserum (unpublished) against Irgb10, 2078 rabbit antiserum (Martens et al., 2004) against Irgd, 3.1.2 and 2.4.21 rat monoclonal antibodies against T. gondii GRA7 (unpublished), and rabbit anti-calnexin antiserum (Calbiochem).

Alexa 488-labelled and Alexa 555-labelled goat anti-rabbit, donkey anti-mouse and goat anti-rat fluorescent antisera (Molecular Probes), donkey anti-rabbit horseradish peroxidase (HRP, GE Healthcare), goat anti-rat HRP (Jackson Immuno Research Laboratories) and goat anti-mouse HRP (Pierce) polyclonal antibodies were used as secondary reagents.

Postnuclear lysate preparation from free tachyzoites and infected cells

25–50 x 10⁶ free T. gondii tachyzoites or 2.5 x 10⁶ MEFs seeded in 10 cm plates, stimulated with 200 U ml⁻¹ IFNγ for 24 h, subsequently infected for 2 h with T. gondii at a multiplicity of infection (MOI) of 7 and washed threefold with PBS were lysed in 800 μl NP-40-lysis buffer [0.1 % NP-40, 150 mM NaCl, 20 mM Tris/HCl (pH 7.6), 5 mM MgCl2 supplemented with protease inhibitors (Roche)] for 2 h under constant rotation at 4°C. Postnuclear lysates were subjected to immunoprecipitation or pull-down analysis.

5 x 10⁵ extracellular T. gondii tachyzoites or 4 x 10⁵ MEFs seeded in 6 cm plates, stimulated with 200 U ml⁻¹ IFNγ for 24 h, subsequently infected for 2 h with T. gondii at a MOI of 7 and washed once with PBS were lysed in 50 to 100 μl NP-40-lysis buffer (0.5 % NP-40) for 1 h on ice. Postnuclear lysates were boiled in sample buffer and subjected to SDS-PAGE and Western blot.

Immunoprecipitation and pull-down analysis

Postnuclear lysates were incubated with the indicated antibodies for 2 h at 4°C followed by an additional 1 h incubation with 100 μl of 1:1 (lysis buffer) bead suspension of protein A-sepharose (Amersham) resin or used in pull-down experiments as described earlier (Fleckenstein et al., 2012). Beads were washed threefold with lysis buffer and either stored at −80°C or immediately boiled in sample buffer [80 mM of Tris/HCl (pH 6.8), 5 mM of EDTA,
4% SDS, 34% sucrose, 40 mM of dithiothreitol and 0.002% bromophenol blue] for 5 min at 95°C and subjected to SDS-PAGE and Western blot or BN-PAGE.

**Toxoplasma gondii** replication assay

*T. gondii* proliferation in infected MEFs or BMMs was determined by incorporation of [3H]-uracil as described earlier (Köhnen-Waisman and Howard, 2007; Liesenfeld *et al.*, 2011).

**Mice virulence assay**

Mice were infected intraperitoneally with 300 μl of PBS containing freshly harvested tachyzoites of indicated *T. gondii* strains. Survivors were sacrificed at the indicated days post-infection and tested for seroconversion using the Toxocell Latex Kit (Biokit, Barcelona, Spain).

**Expression and purification of recombinant Irga6**

Recombinant protein GST-Irga6 was expressed and purified as described previously (Fleckenstein *et al.*, 2012).

**Immunocytochemistry**

Fixation and staining of C57BL/6 MEFs grown on coverslips was performed as described earlier (Steinfeldt *et al.*, 2010), and microscopy and image analysis were performed blind on coded slides essentially according to Khaminets *et al.* (2010). Intracellular parasites were identified from phase contrast images or from the pattern of *T. gondii* GRA7 staining.

**Yeast two-hybrid assay**

*Saccharomyces cerevisiae* strain PJ69-4α was incubated with 1 μg of plasmid DNA (pGAD-C3 or pGBD-C3 containing the indicated genes) in transformation buffer (40% polyethylene glycol 3350, 0.2 M of LiAc, 0.5 mg ml⁻¹ of single-stranded DNA and 0.1 M of dithiothreitol) for 30 min at 42°C. Cotransformants were selected by plating on double-dropout media (SD/-Leu/-Trp). Colonies grown on double-dropout media were replica-plated on triple-dropout media (SD/-Leu/-Trp/-His) containing 0.5 mM of 3-amino-1,2,4-triazole.

**Expression constructs**

The pGEX-4T-2-Irga6 construct allowing expression of recombinant GST-Irga6 protein was generated earlier (Uthaiah *et al.*, 2003). The complete coding sequences of mature ROP5 isoforms or GRA7 were amplified by polymerase chain reaction from *T. gondii* strain RHΔhxgprt genomic DNA and ligated into pGAD-C3 or pGBD-C3 (both James *et al.*, 1996). The pGBD-C3-ROP18 construct was generated earlier (Steinfeldt *et al.*, 2010).

**Statistics**

Significant differences in IRG protein intensities and frequencies at single *T. gondii*-derived intracellular vacuoles were determined using Student’s *t*-test under the assumption of unequal variance and with a two-tailed test. The *p*-values are displayed by *p* < 0.05 (single asterisk), *p* < 0.01 (double asterisk) or *p* < 0.001 (triple asterisk) in the respective figures.

**Quantification of band intensities in Western blot**

Intensities of bands in Fig. 4A were quantified from scanned images using IMAGEJ software (National Institutes of Health).

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**Ethics statement**

Animal experimentation: All experiments with mice were conducted under the regulations and protocols for animal experimentation in accordance with the guidelines of the European Commission (Directive 2010/63/EU) and approved by the local government authorities (Bezirksregierung Köln, Germany), LANUV Nordrhein-Westfalen permit no. 44.07.189.

**Author contributions**

The author(s) have made the following declarations about their contributions: T. H., U. B. M., J. C. H. and T. S. conceived and designed the experiments; T. H., U. B. M., S. K. W., J. C. H. and T. S. performed the experiments; T. H., U. B. M., S. K. W., J. C. H. and T. S. analysed the data; J. C. H. contributed reagents/materials/analysis tools; and T. H., U. B. M., J. C. H. and T. S. wrote the paper.

**Conflict of interest**

The authors declare that no conflicts of interest exist.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Table S1.** *T. gondii* proteins in complex with GST-Irga6

**Fig. S1.** Determination of protein levels for *T. gondii* strains. Western blot of *T. gondii* RHΔhxgprt, RHΔgra7 and RHΔrop18 tachyzoite detergent lysates. Relative levels of ROP18 (middle left panel), ROP5 (middle right panel) and GRA7 (lower panels) were determined using specific antibodies and calnexin (upper panels) served as loading control.

**Fig. S2.** Vacular IRG protein loading is increased in the absence of GRA7. IFNγ-induced MEFs (200 U ml−1) were infected for 2h with *T. gondii* RHΔhxgprt or RHΔgra7, and individual IRG protein positive vacuoles identified with mouse monoclonal antibody B34 (Irgb6) or 940/6 (Irgb10) and 2078 (Irgd) rabbit antisera. (A–C) Upper panels: phase contrast images. Lower panels: fluorescent images of vacuoles (white arrows) from RHΔhxgprt or RHΔgra7 loaded with Irgb6 (A, green), Irgb10 (B, green) and Irgd (C, green). Nuclei stained with 4',6-diamidino-2-phenylindole (blue). (D) The percentage of IRG protein positive vacuoles was evaluated by visual inspection of coded slides. The results of two independent experiments are shown (100 vacuoles counted).

**Fig. S3.** Determination of *T. gondii* RHΔhxgprt median lethal dose in CIM mice. CIM mice were infected by intraperitoneal injection of indicated numbers of *T. gondii* RHΔhxgprt strain tachyzoites, and survival was monitored for 80 days. NMRI mice infected with 1000 *T. gondii* tachyzoites served as control to confirm RHΔhxgprt strain virulence.

**Fig. S4.** IRG protein levels in C57BL/6 wt and *Irag6* ko MEFs. Western blot of detergent lysates from C57BL/6 wt and *Irag6* ko MEFs stimulated for 2h with 200 U ml−1 of IFNγ or left untreated. Monoclonal antibodies 10E7 (Irga6, lower left panel) and B34 (Irgb6, lower middle panel) or rabbit antiserum 940/6 (Irgb10, lower right panel) were
used to determine relative IRG protein levels. The upper band of a doublet in the case of Irgb10 could represent phosphorylated protein detected in non-infected cells (Steinfeldt et al., 2010). Calnexin (upper panels) served as loading control.

**Fig. S5.** Frequencies of IRG positive vacuoles in infected wt and *Irga6* ko MEFs. After infection of IFN-γ-induced (200 U ml⁻¹) wt or *Irga6* ko MEFs for 2 h with *T. gondii, RHΔhxgprt, RHΔgra7, RHΔrop18* or RHΔrop5 individual IRG protein positive vacuoles were analysed. The increase in numbers of Irgb6 and Irgb10 positive RHΔgra7-derived and RHΔrop18-derived vacuoles in wt MEFs is strongly inhibited in *Irga6* ko MEFs. The number of Irgb6 and Irgb10 positive RHΔhxgprt-derived vacuoles is also slightly reduced in *Irga6* ko compared with wt MEFs, but no significant difference was observed for RHΔrop5 when comparing both cell types. The results of three independent experiments are shown (150 vacuoles counted).