Some strains of the protozoan parasite *Toxoplasma gondii* (such as RH) are virulent in laboratory mice because they are not restricted by the Immunity-Related GTPase (IRG) resistance system in these mouse strains. In some wild-derived Eurasian mice (such as CIM) on the other hand, polymorphic IRG proteins inhibit the replication of such virulent *T. gondii* strains. Here we show that this resistance is due to direct binding of the IRG protein Irgb2-b1CIM to the *T. gondii* virulence effector ROP5 isoform B. The Irgb2-b1 interface of this interaction is highly polymorphic and under positive selection. South American *T. gondii* strains are virulent even in wild-derived Eurasian mice. We were able to demonstrate that this difference in virulence is due to polymorphic ROP5 isoforms that are not targeted by Irgb2-b1CIM, indicating co-adaptation of host cell resistance GTPases and *T. gondii* virulence effectors.
In the co-evolutionary process of host–pathogen interaction, adaptation to local conditions is critical. Infectious agents are a constant threat to multicellular organisms, and all metazoan organisms have evolved immune defense mechanisms to combat virulent microbes. Immune defense mechanisms emerge from selective pressures that microbes impose; invasive microbes, in turn, evolve to avoid or counteract immune effector mechanisms long enough to allow for efficient transmission. The host and the pathogen undergo co-adaptation at the molecular level. These equilibria are unstable and their conditions vary locally.

Toxoplasma gondii is an obligate intracellular protozoan parasite belonging to the phylum Apicomplexa. It is distantly related to the genus Plasmodium, the causative agent of malaria. Unlike Plasmodium, however, T. gondii has an extraordinarily broad host range, with all true cats (Felidae) as definitive hosts and all warm-blooded animals, including birds and humans, as intermediate hosts. About one-fourth of the human population is infected with T. gondii, although local rates vary considerably. A few lineages predominate in Europe and North America and these canonical strains differ markedly in virulence in laboratory strains of mice. Virulent strains (e.g., restriction fragment length polymorphism (RFLP) genotype 10, previously called type I) are usually lethal following inoculation with even a single parasite, whereas the LD50 (lethal dose, 50%) of avirulent strains (e.g., RFLP genotypes 1, 2, 3, previously called types II and III) ranges between 10 and 105 in laboratory mice. These virulence differences are due to a small number of polymorphic genes within the parasite genomes. However, in other parts of the world, genetically highly diverse T. gondii strains have been isolated and especially in South America the majority of isolates is associated with high mortality rates in laboratory mice.

Immunity-Related GTPases (IRG) drive an essential mechanism of early cell-autonomous resistance against intracellular pathogens in mice. IRG proteins are represented by about 20 single coding units in the C57BL/6 (BL/6) mouse genome and multiple members are highly inducible by interferon-γ (IFNγ). The typical IRG gene has one or two short 5′-untranslated exons followed by a single long exon encoding the entire open reading frame. Four IRG genes depart from this structure, with two adjacent long exons each encoding a full-length IRG polypeptide, resulting in expression of proteins carrying two IRG domains joined by a short linker, subsuming a single promoter, the so-called tandem IRG proteins.

Knockout (ko) mouse strains lacking single or multiple IRG members have consistently shown higher susceptibility to infection with normally avirulent T. gondii strains. Following infection of an IFNγ-stimulated cell of a laboratory mouse, guanosine triphosphate (GTP)-activated effector IRG proteins begin to accumulate at the T. gondii parasitophorous vacuolar membrane (PVM) within minutes after invasion. Premature activation in uninfected cells is prevented by the three regulator IRG proteins, Irgm1, 2 and 3, which keep the effector IRG proteins in a guanosine diphosphate (GDP)-bound inactive conformation at endogenous cellular membranes until infection. The loading of effector IRG proteins is cooperative and hierarchical, with two family members serving as pioneers for members loading later in the hierarchy. IRG protein accumulation is a prerequisite for subsequent disruption of the PVM, which is in turn invariably followed by death of the parasite and subsequent necrotic death of the host cell. A mechanoochemical effector function of IRG proteins is suggested, by analogy to the dynamin, to be responsible for the ruffling, vesiculation and ultimately disruption of the PVM observed at the microscopic level.

Virulence of T. gondii can be directly correlated with inactivation of the IRG resistance system. The initial loading of the PVM with IRG proteins is markedly reduced in virulent compared to avirulent strains. To preserve the integrity of the PVM, T. gondii has evolved several polymorphic virulence effectors that are injected from secretory organelles (rhoptries and dense granules) directly into the cytosol during and after host cell invasion. Genetic screens showed that the polymorphism in the rhoptry-derived ROP5 and ROP18 components of a secreted threonine kinase complex accounts for the differences in virulence between T. gondii strains in infected laboratory mice. Several T. gondii effectors have been shown to inactivate mouse effector IRG proteins, thus preserving integrity of the PVM.

Recently, we showed considerable polymorphism in IRG proteins among several wild and wild-derived inbred mouse strains. The wild-derived Mus musculus castaneus strain CIM from South India counters effectors of Eurasian virulent strains, leading to encystment, and therefore potential transmission, of virulent parasites. In breeding experiments the resistance of CIM mice could be mapped to highly polymorphic IRG genes located on chromosome 11. Within this locus, one of the most polymorphic family members is the tandem IRG protein Irgb2-b1. We could show that in transiently transfected BL/6 cells, overexpression of Irgb2-b1CIM can rescue the effector IRG protein, Irga6BL/6CIM, from ROP5/ROP18/GRA7-mediated phosphorylation. However, these observations did not show that protection of Irga6 is sufficient to enable wild-derived CIM mice themselves to fully resist infection by T. gondii virulent strains. Indeed, in BL/6 mice, resistance is only partially lost after deletion of Irga6BL/6CIM, suggesting that in the case of CIM mice, protection of Irga6CIM by Irgb2-b1CIM may not be sufficient to explain full resistance against virulent Eurasian T. gondii strains.

In the present study, we show that Irgb2-b1CIM is the CIM-inherited element largely responsible for resistance against virulent Eurasian T. gondii strains. We demonstrate efficient binding of Irgb2-b1CIM only to virulent T. gondii-derived ROP5B but not ROP5A and ROP5C. The interface necessary for ROP5 binding is located within the N-terminal portion of Irgb2-b1CIM and encompasses structural motifs that were previously shown to be under positive selection. Our findings are consistent with critical responsibility of ROP5 for the heightened virulence of T. gondii type I strains against laboratory Mus musculus, and inevitably suggest that other ROP pseudokinases may not play a significant role in virulence/avirulence behavior in mice against T. gondii. Furthermore, and supporting this last contention, we provide evidence that virulence of T. gondii strains from South America in CIM mice is due to a mismatch between Irgb2-b1CIM and ROP5 isoforms. These results provide further evidence that T. gondii virulence and mouse resistance follow some form of allelomatching evolutionary dynamics.

Results

Irgb2-b1CIM protects from virulent T. gondii strain infections. Our own recent work demonstrates that the highly polymorphic Irgb2-b1CIM tandem gene may be responsible for resistance of wild-derived CIM mice against virulent Eurasian T. gondii strains. In transiently transfected IFNγ-induced BL/6 cells, expression of Irgb2-b1CIM protected endogenous Irga6BL/6CIM from ROP5/ROP18/GRA7-mediated phosphorylation by the virulent strain RH. High levels of Irgb2-b1CIM at the PVM of T. gondii virulent strains were accompanied by absence of Irga6BL/6CIM phosphorylation. To test definitively the relevance of Irgb2-b1CIM for the resistance of CIM cells against virulent T. gondii, we generated two independent lines of Irgb2-b1CIM ko cells (T17 and i3) by CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9) nickase technology. Loss of Irgb2-b1CIM was confirmed in lysates of
IFNγ-stimulated cells by western blot with an antibody specific for the C terminus of tandem IRG proteins (Fig. 1a) and Irgb2-b1CIM could not be detected by immunofluorescence on vacuoles of RHΔhxgprt strain T. gondii in ko cells (Fig. 1b, representative images are shown in Supplementary Fig. 1). Expression levels of IRG effector proteins Irga6, Irgb6, Irgb10 and Irgd, necessary for full resistance against avirulent strains of T. gondii in laboratory mice16, were not found to be different in CIM wild-type (wt) cells compared with Irgb2-b1CIM ko cells (Fig. 1a).

Phosphorylation of Irga6BL/6 in susceptible BL/6 cells by the ROPS/ROP18/GRA7 kinase complex of virulent T. gondii strains occurs mainly at two threonine residues (T102 and T108) in the G-domain39,40. In wt CIM cells, Irga6CIM phosphorylation by RHΔhxgprt strain T. gondii is almost completely inhibited (Fig. 1c); however, in the two Irgb2-b1CIM ko CIM cell lines, the frequencies of vacuoles carrying pT108-phosphorylated Irga6CIM are comparable to frequencies in BL/6 cells (Fig. 1c, representative images are shown in Supplementary Fig. 2). The ability of IFNγ-induced CIM wt and Irgb2-b1CIM ko cells to control replication of RH-YFP was compared in infected cells by flow cytometry (Fig. 1d, gating strategy and representative images are shown in Supplementary Fig. 3) and incorporation of β-H-uracil (Supplementary Fig. 4). In both assays, Irgb2-b1CIM ko cells lost the ability to restrict T. gondii virulent strain replication shown by CIM wt cells. The Irgb2-b1CIM ko cell line T17 was complemented with Irgb2-b1CIM and Irgb2-b1CIM expression was confirmed by western blot of detergent lysates (Supplementary Fig. 5). In unstimulated cells, Irgb2-b1CIM expression is only detectable in complemented but not wt cells (left hand panel), whereas upon stimulation with IFNγ, Irgb2-b1CIM levels are similar in CIM wt and complemented cells (right hand panel). Low Irgb2-b1 expression levels in BL/6 wt cells have been described earlier21, and Irgb2-b1CIM ko cells show no signs of Irgb2-b1CIM expression. Complementation of ko cells with Irgb2-b1CIM restored resistance against virulent T. gondii RH-YFP to wt levels (Fig. 1e).

Irgb2-b1CIM is mainly associated with ROP5. Several published results have demonstrated a specific molecular interaction between the ROP5 pseudokinase component of the ROP5/ROP18/GRA7 kinase complex of virulent Eurasian T. gondii strains, and the IRG effector, Irga6BL/638,41,43,47. A structurally similar interaction has been proposed between Irgb2-b1CIM and Irga6CIM38,42,43. A yeast two-hybrid (Y2H) assay allows direct screening for interaction of two proteins. Therefore, Irgb2-b1CIM and virulent RHΔhxgprt T. gondii isoforms ROP5A/B/C were expressed as N-terminal fusion proteins with the Gal4 DNA-binding (BD) or Gal4 activation domain (AD) in a yeast reporter strain. Colony growth on selective medium indicated direct interaction of Irgb2-b1CIM with ROP5B. Significantly less colony growth was observed for ROP5C, and no interaction with ROP5A was detectable (Fig. 2c). In the protein-fragment complementation assay (PCA), binding of Irgb2-b1CIM to ROP5 isoforms B and C could be reproduced and, also in this case, binding to ROP5B was significantly stronger than to ROP5C (Fig. 2d).

The Irgb2-b1CIM interface for ROP5B binding is under positive selection. Many IRG proteins are highly polymorphic in Mus musculus, but only the N-terminal unit of the Irgb2-b1 tandem, Irgb2, has been under recent positive selection21. A significant evolutionary hotspot encompassing putative structural motifs ad and H4 spans Irgb2 nucleotides 500 to 700. Both ad and H4 constitute essential parts of the Irga6BL/6 interface for ROP5 binding38,47 and we considered that these motifs probably also participate in Irgb2-b1CIM binding to ROP5. Initially, the N-terminal moiety Irgb2CIM and C-terminal moiety Irgb1CIM were expressed separately as GST-fusion proteins and applied in a pull-down approach with T. gondii virulent tachyzoite detergent lysates. Binding of Irgb2CIM but not Irgb1CIM to T. gondii-derived ROP5 (Fig. 3a) strongly suggests that the interface for ROP5 binding is located in the polymorphic N-terminal part of Irgb2-b1CIM. Input of GST-fusion proteins (Fig. 3a, upper left hand panel) and protein amounts in the tachyzoite lysate used in the pull-down (Fig. 3a, right hand panels) is shown. To demonstrate that ad and H4 of Irgb2CIM are responsible for ROP5 recognition, a chimeric Irgb2-b1 protein was generated and tested for ROP5 binding. The Irgb2-b1Chimera encompasses a modified Irgb2CIM, where ad and H4 were replaced with the respective BL/6 sequences, followed by full-length Irgb1BL/6 (Fig. 3b).

In the PCA, binding of the Irgb2-b1Chimera to ROP5B was almost completely abrogated, whereas binding to ROP5C was even more pronounced than wt Irgb2-b1CIM binding to ROP5C (Fig. 3c). These results were confirmed in a Y2H approach. Only ROP5C but not ROP5B binding to Irgb2-b1Chimera could be observed (Fig. 3d). Binding of Irgb2-b1Chimera to ROP5C was also reflected in recruitment to the T. gondii virulent strain-derived PVM. After complementation of Irgb2-b1CIM ko cells, the vacuolar intensities of Irgb2-b1Chimera were significantly lower than Irgb2-b1CIM, but still detectable (Fig. 3e). We could not observe differences in numbers (protein intensities are not considered in these analyses) of Irgb2-b1CIM and Irgb2-b1Chimera-positive vacuoles (Fig. 3f) confirming the association of both proteins with different ROP5 isoforms.

ROP5B is the main isoform responsible for T. gondii virulence in vitro. Binding of Irgb2-b1Chimera to ROP5C stimulated us to reinvestigate binding of Irgb2-b1BL/6 to virulent strain-derived ROP5 isoforms. In the PCA, only binding of Irgb2-b1BL/6 to ROP5C, but not to ROP5B or ROP5A, could be detected (Fig. 4a); these results were further confirmed by Y2H analysis (Fig. 4b). Surprisingly, Irgb2-b1BL/6 binding to ROP5C could not be
**Fig. 1** Irgb2-b1CIM is largely responsible for resistance against virulent *T. gondii* strains. a Western blot of detergent lysates from CIM wild-type (wt) and Irgb2-b1CIM knockout (ko) diaphragm-derived cells (DDCs) (T17 and i3) stimulated for 24 h with 200 U ml⁻¹ interferon-γ (IFNγ). The signal representing Irgb2-b1CIM in wt cells is lost in both ko cell lines (upper panel). Expression levels of all other Immunity-Related GTPases (IRG) proteins are unchanged in ko cell lines compared with wt cells (middle panels). Actin serves as loading control (lower panel). b, c Frequency of vacuoles positive for Irgb2-b1CIM (b) or Irga6CIM phosphorylated at T108 p(T108)Irga6CIM (c) detected by immunofluorescence with anti-p(T108)Irga6CIM or anti-Irgb2-b1CIM antibodies in IFNγ-induced (200 U ml⁻¹) wt CIM and BL/6 or Irgb2-b1CIM ko CIM DDCs infected for 2 h with RHΔhxgprt. Error bars indicate the mean and standard error of the mean (SEM) of three (b) or four (c) independent experiments (about 100 vacuoles were counted per experiment). One-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison was used to test differences between groups; ****p < 0.0001; **p < 0.0025; n.s. not significant. b No Irgb2-b1CIM positive vacuoles were found in Irgb2-b1CIM ko cells. c Frequencies of p(T108)Irga6CIM-positive vacuoles in ko cells are significantly increased in comparison with wt cells. d, e CIM DDCs were induced with 100 U ml⁻¹ IFNγ for 24 h and infected with *T. gondii* RH-YFP at a multiplicity of infection (MOI) of 1 or 3. Intracellular parasite growth was determined by flow cytometry 24 h post infection as described in Methods. Error bars indicate the mean and SEM of three independent experiments. One-way ANOVA followed by Tukey’s multiple comparison was used to test differences between groups; ****p < 0.0001; n.s. not significant. d Control of *T. gondii* replication is lost in wt BL/6 and Irgb2-b1CIM ko CIM DDCs compared with wt CIM DDCs. e Control of *T. gondii* replication is restored to wt levels (wt CIM DDCs) in Irgb2-b1CIM ko CIM DDCs complemented with Irgb2-b1CIM (T17 + Irgb2-b1CIM).
detected in the pull-down with a GST-tagged fusion protein (Fig. 2b) but this proved to be due to discrimination between ROP5 isoforms by the antibody used in subsequent western blot analysis since, after overexpression of single FLAG-tagged ROP5 isoforms in transiently transfected cells, the anti-ROP5-specific antibody 3E2 detects ROP5A and ROP5B but not ROP5C in the western blot of detergent cell lysates. Protein expression was verified with an anti-FLAG antibody, with calnexin levels serving as loading control (Supplementary Fig. 6). Phylogenetic analysis of Irgb2 sequences21 reveals the allelic diversity between GRA7 Irgb2-b1
laboratory and wild-derived mice (Fig. 4e, upper panel). An alignment of amino acids 167 to 233 encompassing putative structural motifs ad and H4 highlight the polymorphic residues within this region (Fig. 4e, lower panel). To confirm the different binding specificities for ROP5B in infected cells, we used RHΔrop5 parasites expressing virulent allelic isoforms ROP5A and ROP5B with HA- or FLAG-epitope tag (RHΔROP5A and FLAG-ROP5B)49. We can demonstrate ROP5B binding to Irgb2-b1 CIM but not Irgb2-b1BL/6 or Irgb2-b1Chimera by co-immunoprecipitation from detergent lysates of cells infected with T. gondii RHΔrop5+ A/B expressing HA-ROP5A and FLAG-ROP5B.49 We can demonstrate ROP5B binding to Irgb2-b1 CIM but not Irgb2-b1BL/6 or Irgb2-b1Chimera by co-immunoprecipitation from detergent lysates of cells infected with T. gondii RHΔrop5+ A/B (Supplementary Fig. 7) again suggesting that residues within this polymorphic hotspot determine the interaction. Protein amounts in the lysates used for co-immunoprecipitation are shown (Supplementary Fig. 7, middle and right hand panels).

Irgb2-b1BL/6 binding to ROPSC can account for the residual protein amounts at the PVM in T. gondii virulent strain-infected Irgb2-b1 CIM ko cells complemented with Irgb2-b1BL/6 (Fig. 4c).

The possibility that Irgb2-b1BL/6 and Irgb2-b1Chimera interaction with ROPSC might contribute to parasite control was investigated by flow cytometry of infected cells. Expression of these particular proteins in Irgb2-b1 CIM ko cells did not result in increased growth inhibition of virulent T. gondii compared to CIM wt cells or Irgb2-b1 CIM ko cells complemented with Irgb2-b1 CIM (Fig. 4d), suggesting that ROPSC does not contribute significantly to virulence of Eurasian T. gondii strains in CIM cells.

T. gondii strains from South America are not restricted by Irgb2-b1 CIM. T. gondii isolates from South America are genetically highly diverse, and the majority is associated with high mortality rates in laboratory mice. We compared virulence of T. gondii strains VAND and AS28, both originating from South America, in CIM mice. Unlike virulent Eurasian T. gondii strains, VAND (Fig. 5a) and AS28 (Fig. 5b) are lethal in CIM mice.

T. gondii VAND-derived ROP5 isoforms are not targeted by Irgb2-b1 CIM. The first step to identify the molecular constituents that account for virulence of T. gondii strains from South America in CIM mice was to determine activity of the ROP5/ROP18/GRA7 kinase complex on Irga6 CIM. We assessed Irga6 CIM phosphorylation in T. gondii-infected CIM cells by immunofluorescence analysis. Mean p(T108)Irga6 CIM intensities were increased in CIM cells infected with T. gondii VAND, but not with AS28, compared with RHΔhxgprt (Fig. 6a). Since high p(T108)Irga6 levels have already been shown to correlate with low Irgb2-b1 intensities21, we considered the possibility that Irgb2-b1 CIM levels on VAND-derived vacuoles in CIM cells might be lower than on RHΔhxgprt. This, in turn, would suggest that Irgb2-b1 CIM interacts inefficiently with ROP5 variants expressed by VAND. We therefore determined vacuolar Irgb2-b1 CIM intensities in T. gondii RHΔhxgprt-, VAND- and AS28-infected CIM cells. Irgb2-b1 CIM intensities at vacuoles of both AS28 and VAND were clearly decreased relative to RHΔhxgprt (Fig. 6b), representative images are shown in Supplementary Fig. 8). Indeed, in the case of VAND, Irgb2-b1 CIM levels were similar to those observed at vacuoles of RHΔrop5 parasites21, suggesting that VAND ROP5 does not interact at all with Irgb2-b1 CIM. These results were confirmed in pull-down experiments from tachyzoite lysates with GST-tagged Irgb2-b1 CIM; binding of Irgb2-b1 CIM to ROP5 was reduced in case of T. gondii AS28 and not detectable in case of T. gondii VAND (Fig. 6c). Input of GST-Irgb2-b1 CIM (Fig. 6c, upper left hand panels) and protein amounts in the tachyzoite lysates used in the pull-down (Fig. 6c, right hand panels) is shown. These results correlate well with results obtained by immunofluorescence analysis (Fig. 6b). Equal ROP5 expression levels and recognition of AS28 and VAND ROP5 variants by the anti-ROP5 antibody used for western blot analysis could be confirmed after immunoprecipitation from extracellular tachyzoite lysates (Supplementary Fig. 9). Recently we demonstrated that T. gondii GRA7 is associated with ROP543 but surprisingly, VAND GRA7 was detected in the GST-Irgb2-b1 CIM pull-down in the absence of ROP5 binding (Fig. 6c). This proved to be due to a direct interaction between VAND GRA7 and empty GST beads (Supplementary Fig. 10a). Further evidence that GRA7 is not a functional inhibitor of VAND ROP5 association with Irgb2-b1 CIM was provided by Y2H analysis. Here, in the absence of VAND GRA7, none of the VAND ROP5 isoforms, ROP5A, B1, B2 and B3, interacted with Irgb2-b1 CIM (Fig. 6d), suggesting that an intrinsic polymorphism of ROP5VAND is responsible for its failure to interact with Irgb2-b1 CIM. Moreover, the YTH results confirm that absolute absence of ROP5VAND binding to Irgb2-b1 after pull-down (Fig. 6c) is not simply due to failure of the antibody used in the western blot to recognize ROP5VAND isoforms. ROP5 uses a surface for binding to Irga6BL/6 that is highly polymorphic and under positive selection49. When T. gondii RH and VAND rop5 alleles are compared, several amino acid substitutions within this region can be found41,50 (Fig. 6e). We created a ROP5B VAND mutant carrying the ROP5RH interface for Irga6BL/6 binding (ROP5BChimera) and investigated binding to Irgb2-b1 CIM. In the PCA assay, binding of ROP5B VAND to Irgb2-b1 CIM is significantly reduced compared to ROP5BChimera and Irgb2-b1 CIM (Supplementary Fig. 11).
Discussion

Restriction of *T. gondii* growth in mice is dependent on IFNγ-inducible IRG and GBP (guanylate-binding protein) proteins that accumulate at the PVM leading to its rupture, death of the parasite and necrotic death of the host cell\(^1\).\(^6\),\(^1\).\(^1\) *T. gondii* virulent strains are able to overcome cell-autonomous resistance by secretion of effector proteins that inactivate IRG and GBP protein function\(^5\),\(^2\),\(^5\),\(^3\). Certain *T. gondii* strains of the RFLP genotype 10 (formerly type I) are highly virulent for laboratory mice but are well resisted in CIM wild-derived mice. In the latter, polymorphic
IRG proteins on Chr11 were shown to protect CIM mice from T. gondii virulent strain infections and the highly polymorphic tandem IRG protein Irgb2-b1_CIM was implicated\(^2\). In this study, we have shown by gene deletion and complementation that polymorphic variation in Irgb2-b1 is indeed responsible for the restriction of virulent T. gondii strains in CIM cells and the failure of restriction in C57BL/6 (BL/6) cells (Fig. 7). Two independent Irgb2-b1_CIM ko CIM cell lines lost the ability to control T. gondii virulent strain replication compared to wt cells. Complementation of ko cells with Irgb2-b1_CIM restored resistance to wt levels (Fig. 1e). Phosphorylation of Irga6 is a good indicator for parasite virulence\(^{39,40}\) and loss of parasite control in the absence of Irgb2-b1_CIM is reflected in elevated numbers of p(T108)Irga6_CIM-positive vacuoles upon virulent strain infection (Fig. 1c).

In our earlier study, overexpression of Irgb2-b1_CIM in transiently transfected BL/6 cells rescued endogenous Irga6\(_{BL/6}\) from ROP5/ROP18/GR A7-mediated phosphorylation. In the same study Irgb2-b1_CIM was itself shown to be phosphorylated by virulent ROP5/ROP18 but this phosphorylation did not inhibit its PVM accumulation. Surprisingly and in stark contrast to other IRG proteins, vacuolar accumulation of Irgb2-b1_CIM is strictly dependent on T. gondii-derived ROP5; lower ROP5 levels correlate with decreased amounts of Irgb2-b1_CIM\(^{21}\). Based upon these results, we investigated binding of Irgb2-b1_CIM to ROP5 as the underlying molecular mechanism of CIM-inherent resistance. Here we demonstrate direct binding of Irgb2-b1_CIM to T. gondii-derived ROP5. The result provides corroborative evidence that Irgb2-b1_CIM traps ROP5, leading to diversion of the ROP18 kinase function from Irga6 to Irga6-b1, and in that way confers cell-autonomous resistance against virulent T. gondii strains. Interestingly, binding of Irgb2-b1_CIM to ROP5 is isoform specific. The impact of ROP5 isoforms on T. gondii virulence has been investigated in former studies. Single or pair-wise combinations of ROP5 isoforms were tested to rescue the virulence phenotype in RH\_Apop5 organisms. Complementation with one or two copies of rop5\(_{Arii}\) only partially rescued virulence and only rop5\(_{Arii}\) in combination with rop5\(_{Brii}\) displayed a phenotype indistinguishable from parental strain infections\(^{49}\). Otherwise, when ROP5\(_{Arii}\) or ROP5\(_{Cbi}\) was expressed alone in a T. gondii avirulent genetic background, Irgb6\(_{BL/6}\) loading was decreased compared to wt strain infections. However, none of these ROP5 isoforms increased parasite virulence in mice\(^{41}\). The effect of ROP5\(_{Brii}\) alone on T. gondii virulence has never been investigated. In all our assays, efficient binding of Irgb2-b1_CIM could be observed only to ROP5\(_{Brii}\) but not to ROP5\(_{Rbi}\) or ROP5\(_{Arii}\). Irgb2-b1_CIM\(_{BL/6}\) was strongly associated with ROP5\(_{Cbi}\) at the PVM. In VAND-infected IFNγ-induced
CIM cells, the absence of Irgb2-b1\textsubscript{CIM} on the vacuole was associated with elevated levels of p(T108)Irga6\textsubscript{CIM}. In case of AS28, the Irgb2-b1\textsubscript{CIM} loading phenotype was intermediate between RH\textsubscript{Δhxgprt} and VAND, but perhaps sufficient to account for the complete absence of Irga6\textsubscript{CIM} phosphorylation (Fig. 6a, b). Consistently, limited binding of AS28-derived ROP5 to Irgb2-b1\textsubscript{CIM} could be detected, whereas no interaction with Irgb2-b1\textsubscript{CIM} for any of the VAND ROP5 isoforms was visible in our assays (Fig. 6). In summary, in VAND infections, escape from Irgb2-b1\textsubscript{CIM} binding conclusively results in increased Irga6\textsubscript{CIM} phosphorylation and is therefore a very likely explanation for virulence of \textit{T. gondii} VAND in CIM cells and mice (Fig. 7).
**Fig. 4** Irgb2-b1<sub>BL/6</sub> directly binds ROP5C. **a** Protein-fragment complementation assay. Proteins were fused to N-terminal (BlaN) or C-terminal (BlaC) fragments of TEM-1 β-lactamase. One-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison was used to test differences between groups; ****p < 0.0001; n.s. not significant. The kinetic of the reaction is shown for one representative experiment (left hand panel). Irgb2-b1<sub>BL/6</sub> interacts with *T. gondii* ROPSC but not ROPSB and ROPSA. **b** Yeast two-hybrid. Proteins were expressed as fusion with transcriptional activation domain (AD) from pGAD-C3 or DNA-binding domain (BD) from pGBD-C3. Colony growth is indicative of Irgb2-b1<sub>BL/6</sub> and Irgb2-b1<sub>Chimera</sub> interaction with ROP5B and Irgb2-b1<sub>CIM</sub> interaction with ROPSC and Irgb2-b1<sub>CIM</sub> interaction with ROP5B. Bold black lines separate samples from negative controls. **c** Intensities of individual vacuoles detected by immunofluorescence with anti-Irgb2-b1-specific anti-serum in interferon-γ (IFN-γ)-induced (200 U ml<sup>−1</sup>) cells infected for 2 h with RHΔhxgprt. Kruskal-Wallis test followed by Dunn’s multiple comparisons was used to test differences between groups; ****p < 0.0001; n.s. not significant. Intensities of Irgb2-b1<sub>BL/6</sub> and Irgb2-b1<sub>Chimera</sub> are reduced at RHΔhxgprt-derived vacuoles. **d** Polymorphic Irgb2-b1<sub>CIM</sub> interface mediating ROP5B binding is crucial for *T. gondii* control in vitro. CIM diaphragm-derived cells (DDCs) were induced with 100 U ml<sup>−1</sup> IFN-γ for 24 h and infected with *T. gondii* RH-YFP at a multiplicity of infection (MOI) of 1 or 3. Parasite growth was determined by flow cytometry 24 h post infection. One-way ANOVA followed by Dunnett’s multiple comparisons was used to test differences between CIM wild-type (wt) and other cells; ***p < 0.0001; **p < 0.001; ***p < 0.0008; **p < 0.006; n.s. not significant. Control of *T. gondii* virulent strain replication is lost in Irgb2-b1<sub>CIM</sub> knock-out (KO) CIM DDCs complemented with Irgb2-b1<sub>Chimera</sub>. **e** Upper panel, phylogenetic analysis and maximum likelihood tree of Irgb2 sequences (CIM, *M. m. castaneus*; CAST, *M. m. castaneus*; LAB, *M. m. domesticus*; PWK, *M. m. musculus*; SPRET, *Mus spretus*). LAB indicates the allele shared by all laboratory mice tested so far, including C57BL/6<sup>−/−</sup>. Only bootstrap values above 50 are shown. Lower panel, alignment of Irgb2 amino acids M167 to P233 encompassing putative structural motifs ad and H4. Polymorphic sites are highlighted in blue. Error bars indicate the mean and SEM or standard deviation of three independent experiments.

**Fig. 5** South American *T. gondii* VAND and AS28 evade growth restriction in CIM mice. CIM wild-type (wt) (**a**, n = 25; **b**, n = 19) and C57BL/6 (**a**, n = 15; **b**, n = 10) mice were infected by intraperitoneal injection of 1000 *T. gondii* VAND (**a**) or 100 AS28 (**b**) strain parasites and survival monitored for 15–20 days. Data shown are combined from three (**a**) or two (**b**) independent experiments.

Decreased vacuolar Irgb2-b1<sub>CIM</sub> levels in *T. gondii* AS28 infections, on the other hand, do not result in increased Irga6<sub>CIM</sub> phosphorylation, implicating either different molecular virulence mechanisms not associated with the IRG system or possibly different effector IRG protein targets, not including Irga6.

It would appear that the allelic diversity of the tandem IRG protein Irgb2-b1 present in laboratory or some wild-derived strains such as CIM does not include a sequence capable of fully inhibiting the ROP5/ROP18/GRA7 virulence kinase complex of VAND and probably other South American strains. It is interesting to consider whether indeed a suitable Irgb2-b1<sup>−/−</sup> allele exists anywhere in the mouse species, and if not, what other, presumably South American, intermediate host species are capable of attenuating the extreme virulence of these *T. gondii* strains. For the time being, it seems legitimate to consider that the ROP5/ROP18/GRA7 virulence kinase complex is specifically directed against IRG effector proteins. Everything points to accurate molecular complementarity as the basis for the function of the kinase complex, both at the level of the ROP5/IRG interface and the accurate targeting of the rhoptry kinase to the sensitive threonines in the IRG switch region. Although GBP have been demonstrated to be associated with resistance to *T. gondii* in mice, there is presently no indication that alleles of the ROP5/ROP18/GRA7 system can also be deployed to directly target and phosphorylate this entirely different protein family. Indeed, there is already some evidence that the striking differential virulence of *T. gondii* strains in mice is not reflected in comparable differences of virulence in humans, where the IRG system has been lost. Lethality of *T. gondii* for healthy humans is exceedingly rare, and the main differential pathologies seem to be associated primarily with damage to the optic system. A reasonable hypothesis is that the ROP5/ROP18/GRA7 kinase system has specifically evolved to attenuate an attack by effector proteins of the IRG system on early stages of tachyzoite expansion. The opposed polymorphisms connecting virulence and resistance suggest complex evolutionary dynamics beyond the species level and on a global scale. While it is evident that a big part of the resistance component is due to IRG proteins, it is unlikely that IRG proteins of *Mus musculus*, an Old World species, are alone responsible for the binding specificities of ROP5 evolved in *T. gondii*, a parasite almost certainly of New World origin. It is more likely that Irgb2-b1<sup>−/−</sup> alleles of evolutionarily significant New World mammals will turn out to be the key resistance factors against New World *T. gondii* strains.

**Methods**

**Propagation of *T. gondii***. Tachyzoites of *T. gondii* strains RHΔhxgprt<sup>Δ</sup>, RH-YFP<sup>Δ</sup>, ME49<sup>Δ</sup>, RHΔrop577 VAND<sup>S</sup> and AS28<sup>S</sup> 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 or pull-down experiments.

**Cell culture.** HEK293T cells (ATCC, CRL-3216) and wt or ko diaphragm-derived cells (DDCs) derived from C57BL/6 and CIM mice<sup>21</sup> were maintained by serial passage in Dulbecco’s modified Eagle’s medium, high glucose (Invitrogen Life Technologies) supplemented with 2 mM l-glutamine, 1 mM sodium pyruvate, 1× Minimum Essential Medium non-essential amino acids, 100 U ml<sup>−1</sup> penicillin, 100 mg ml<sup>−1</sup> streptomycin (PAA) and 10% fetal calf serum (FCS, Biochrom).
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$^{-1}$ penicillin, 100 mg ml$^{-1}$ streptomycin and 5% FCS. All cells were mycoplasma-free and regularly tested by PCR$^{61}$.  

**Immunological reagents.** Immunoreagents used in this study were: 3E2 mouse monoclonal antibody against ROP5 isoforms$^{62}$, affinity-purified rabbit sera 87558 against (pT108)Irga6 (1:8000)$^{39}$, 10E7 mouse monoclonal antibody (1:2000)$^{63}$ against Irga6, B34 mouse monoclonal antibody (1:2000)$^{64}$ against Irgb6, 940/6 rabbit antiserum (1:2000)$^{43}$ against Irgb10, 2078 rabbit antiserum (1:1000)$^{65}$ against Irgd, 954/1-C15A rabbit antiserum (1:8000 in western blot; 1:4000 in immunofluorescence)$^{21}$ against a conserved Irgb-tandem C-terminal peptide, 3.1.2 (1:500) and 2.4.21 (1:1000) rat monoclonal antibodies against $T$. gondii GRA7$^{43}$, anti-GST goat antiserum (1:1000 GE Healthcare 27457701), anti-FLAG mouse monoclonal antibody (1:1000, Sigma Aldrich F3165), mouse monoclonal anti-actin.
**Fig. 6** T. gondii VAND-derived ROP5 escapes targeting by Irgb2-b1CIM. a p(T108)Irga6CIM protein intensities are increased at VAND-derived vacuoles. Interferon-γ (IFNγ)-induced CIM diaphragm-derived cells (DDCs; 200 U ml⁻¹) were infected for 2 h with indicated T. gondii strains and individual p(T108)Irga6CIM-positive vacuoles identified with 558 p(T108)Irga6CIM-specific antisera. Error bars indicate the mean and SEM of three independent experiments. Kruskal–Wallis test followed by Dunn’s multiple comparisons was used to test differences between groups; ****p < 0.0001; n.s. not significant. b Irgb2-b1CIM intensities are reduced at VAND- and AS28-derived vacuoles. IFNγ-induced CIM DDCs (200 U ml⁻¹) were infected for 2 h with indicated T. gondii strains and individual Irgb2-b1CIM-positive vacuoles identified with anti-Irgb2-b1CIM-specific antisera. Error bars indicate the mean and SEM of three independent experiments. Kruskal–Wallis test followed by Dunn’s multiple comparisons was used to test differences between groups; ****p < 0.0001; **p < 0.006. c In vitro pull-down with recombinant GST-Irgb2-b1CIM fusion protein and T. gondii tachyzoite detergent lysates. Pull-down of ROP5 by Irgb2-b1CIM is reduced with AS28 and completely lost in case of T. gondii tachyzoite lysates compared to RHΔhxgprt-derived ROP5 (middle left hand panel). VAND GRA7 pull-down is not dependent on ROP5 (lower panel). The upper panel indicates input of GST-Irgb2-b1CIM in the pull-down. The right hand blot shows ROP5 (upper panel) and GRA7 (lower panel) levels in tachyzoite lysates. All tracks were run on a single gel; vertical white lines indicate excision of irrelevant tracks. d VAND ROP5 isoforms ROP5A, ROP5B1, ROP5B2 and ROP5B3 do not directly interact with Irgb2-b1CIM or VAND GRA7 in a yeast two-hybrid approach. Proteins were expressed either as fusion to a transcriptional activation domain (AD) from pGAD-C3 or to a DNA-binding domain (BD) from pGBD-C3. Colony growth under 3DO conditions is indicative of protein/protein interaction. Bold black line separates samples from negative controls. e Upper panel, alignment of T. gondii RH and VAND ROP5 amino acid sequences that represent a polymorphic hotspot and have been shown to be involved in binding to Irga6BL/C. Polymorphic sites are highlighted in blue. Lower panel, phylogenetic analysis and maximum likelihood tree of T. gondii RH and VAND rop5 sequences. Only maximum likelihood bootstrap values above 50 are shown

![Image 1](https://example.com/image1.png)

**Fig. 6** Model for Irgb2-b1-mediated control of T. gondii infection in wild-derived mice. a In laboratory mice, effector proteins from canonical virulent T. gondii strains (like RH) specifically phosphorylate certain Immunity-Related GTPases (IRG) proteins, thereby inhibiting oligomerisation and destruction of the parasitophorous vacuolar membrane (PVM). Two rhoptry kinases, ROP18 and ROP17, have been demonstrated to preferentially phosphorylate Irga6BL/CIM. Consequently, effector IRG proteins are free to accumulate around the PVM, resulting in growth control, encystment and transmission of the parasite. Genetically more diverse T. gondii strains, like T. gondii VAND from South America, express polymorphic ROP5 variants that are not targeted by Irgb2-b1CIM. Consequently, effector IRG proteins such as Irga6BL/CIM are phosphorylated and inactivated by a T. gondii VAND kinase complex. Infected animals die shortly after parasite challenge. Molecular interaction between ROP18 and ROP5 or Irga6 and ROP5 within the VAND kinase complex awaits experimental confirmation.

![Image 2](https://example.com/image2.png)

**Lysate preparation from free tachyzoites and infected cells.** The 10⁻²⁵ × 10⁶ free T. gondii tachyzoites or 2.5 × 10⁶ DDCs seeded in 10 cm plates were 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 10 and washed trifold with phosphate-buffered saline (PBS), and 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.

**Immunoprecipitation and pull-down analysis.** For immunoprecipitation experiments, postnuclear lysates were incubated with the indicated antibodies o/n at 4 °C followed by an additional 1 h of incubation with 100 µl 1:1 (lysis buffer) bead suspension of protein A-Sepharose (Amersham) resin. For pull-down experiments, 100 or 200 pmol (Fig. 3a) of purified GST or GST-fusion proteins were mixed with 100 µl 1:1 bead suspension of glutathione sepharose 4B (GE

![Image 3](https://example.com/image3.png)
Healthcare) resin in 500 µl PBS/2 mM dithiothreitol (DTT) for 1 h at 4°C. The resin was washed trifold in ice-cold lysis buffer containing 2 mM DTT without detergent pre-incubated with 800 µl postnuclear T. gondii lysate or at 4°C.

Beads were washed trifold with lysis buffer and either stored at –80°C or immediately boiled in sample buffer (80 mM Tris/HCl (pH 6.8), 5 mM EDTA, 4% SDS, 34% sucrose, 40 mM DTT, 0.002% bromphenol blue) for 5 min at 95°C and subjected to SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) and western blot. Uncropped images of all western blots are provided in the Source Data file.

**T. gondii replication assay.** T. gondii proliferation in infected DCCs was determined by incorporation of 1H-uracil flow cytometry.

For the uracil incorporation assay, cells were grown for 24 h in the presence of IFNy or left unstimulated, and then infected with T. gondii RH-YFP at different MOIs for an additional 24 h. Cultures were subsequently labeled with 1H-uracil (0.3 µCi per well) for 24 h, harvested on glass fiber filters and radioactivity incorporated into proliferating T. gondii DNA determined in a beta scintillation spectrometer.

For flow cytometry, 1 × 10^5 DCCs seeded in 12-well plates and stimulated with 100 U ml^−1 IFNy for 24 h were infected with RH-YFP. At 24 h post infection, cells were trypsinized, washed 2x with PBS containing 3% FCS (PBS/FCS) and resuspended in 400 µl PBS/FCS containing 1% paraformaldehyde (PFA). After 15 min of incubation at room temperature, fixed cells were washed 2x with PBS/FCS, resuspended in 400 µl PBS/FCS and analyzed by FACSCanto II flow cytometer (BD Biosciences). For each sample, 20,000 events were recorded. Further analysis was performed using FlowJo vX 10.0.7 Software. Percental inhibition of 1H-uracil replication was defined as follows: 100 – (mean IFNy-stimulated /mean unstimulated) × 100.

**Generation of Irgb2-b1 ko cells.** 2 × 10^5 C57DIC DDCs were seeded in 6-well plates and cotransfected with CRISPR/Cas9 plasmids (see Plasmid constructs) according to the Lipofectamine 3000 protocol (Invitrogen). At 48 h post transfection, 1 cell per 100 µl was seeded into a well of a 96-well plate and each well observed for several days to contain only one single colony. Single colonies were transferred to 24-well plates and detergent lysis of IFN-γ-induced (200 U ml^−1) cells 24 h later analyzed by western blot for Irgb2-b1CIM expression.

**Mice virulence assay.** Female and male mice (25 Mus musculus domesticus, C57BL/6, 44 Mus musculus castaneus, CIM) with ages ranging from 2 to 4 months were infected intraperitoneally with 300 µl or 100 µl of PBS containing freshly harvested tachyzoites of indicated T. gondii strains. Mice were killed at the indicated days post infection and tested for seroconversion using the Toxocell Latex Kit (Biokit). Data shown are combined from independent experiments.

**Expression and purification of GST-fusion proteins.** Recombinant GST-Irgb2-b1_CIM, GST-Irgb2-b1BL/6 and GST-Irgb1 protein were generated from pGEX-4T-2-Irgb2-b1_CIM and pGEX-4T-2-Irgb1 protein were generated from pGW1H-Irgb2-b1CIM and pGW1H-Irgb2-b1BL/6. Irgb2-b1_CIM and Irgb2-b1BL/6 and Irgb1 protein were expressed in E. coli BL21 (DE3) and purified using a nickel affinity column (GE Healthcare) in PBS/2 mM DTT. Proteins were eluted with 150 µl wash buffer (Promega) containing protease inhibitor cocktail (Roche). After 45 min of incubation on ice and centrifugation for 30 min at 15,000 × g and 4°C, 50 µl of supernatants were mixed with 15 µl nitrocefin (Abcam), 15 µl H2O and 120 µl PBS in a 96-well plate. The β-lactamase-mediated hydrolysis of nitrocefin was measured by the change of absorbance at 495 nm at intervals of 8–9 s for 30 cycles. In the presence of a standard substrate concentration, the actual nitrocefin hydrolysis rate is dependent on the amounts of reconstituted Bla, consequently on the interaction between the fusion proteins. Therefore, to determine the strength of the interaction, the nitrocefin hydrolysis rates, expressed in mAU min^−1, were calculated for the linear phase of the reaction and compared to each other and the background rates, which were observed upon transfection of the respective fusion proteins alone.

**Plasmid constructs.** The pGEX-4T-2-Irgb2-b1, pGEX-4T-2-Irgb1 and pGEX-4T-2-Irgb2 constructs allowing expression of recombinant GST-Irgb2-b1, GST-Irgb2 or GST-Irgb1 protein were generated from pGW1H-Irgb2-b1CIM and pGW1H-Irgb2-b1BL/6 and ligated into pGAD-C3, pGBD-C3 and BlaW/BlacW. The complete coding sequences of VAND isoforms were subcloned from IDT (Integrated DNA Technologies) vectors into pGAD-C3 and pGBD-C3. VAND grw7 was amplified from T. gondii strain VAND genomic DNA and ligated into pGAD-C3 or pGBD-C3.

**Lentiviral transduction.** Lentiviral transduction was applied to generate Irgb2-b1CIM cells overexpressing Irgb2-b1 variants. For this purpose, gag-pol-expressing and env-expressing plasmids were co-transfected with the plasmid carrying the gene of interest into HEK293T cells that have been grown to a density of 70% in a 10 cm plate. At 24 h post transfection, the medium was exchanged and cells incubated for additional 24 h. The supernatant was filtered and transferred to Irgb2-b1CIM cells (T17) that have been seeded 1 day before in a 6 cm plate and grown to a density of 70%. After 24 h, cells were harvested and transferred into appropriate cells culture flasks with medium containing 1-5 µg ml^−1 puromycin for selection of transduced cells.

**Statistics.** All statistical analyses were performed using GraphPad Prism 7.0 software (GraphPad). P values were determined by an appropriate statistical test. Statistical differences in IRG protein intensities between groups at single T. gondii-derived intracellular vacuoles were determined using a two-tailed Student’s t-test. One-way analysis of variance (ANOVA) followed by Tukey’s multiple comparisons or Kruskal–Wallis test followed by Dunn’s multiple comparisons were used to test differences in IRG protein frequencies or intensities between more than two groups at T. gondii-derived intracellular vacuoles respectively. Statistical differences for T. gondii replication analysis by FACS were determined using one-way ANOVA followed by Tukey’s or Dunnett’s multiple comparisons. In case of PCA assays, one-way ANOVA followed by Tukey’s multiple comparison was used to test differences between groups. All error bars indicate the mean and standard error of the mean (SEM) or standard deviation of at least three independent experiments. The p values < 0.05 were considered to be significant.

**Ethics statement.** All experiments with mice at the University of Cologne were conducted under the regulations for animal experiments in accordance with guidelines of 800 µl postnuclear T. gondii lysate or at 4°C.

**Immunocytochemistry.** CIM wt and ko DDCs grown on coverslips were infected with T. gondii strains at MOI of 5 for 2 h, washed with PBS, fixed in PBS/4% PFA for 20 min at room temperature and permeabilized in PBS/0.1% saponin for 10 min at room temperature before immunostaining. Microscopy and image analysis were performed blind on coded slides. Intracellular parasites were identified from the pattern of T. gondii GRA7 staining.

**Yeast two-hybrid assay.** Saccharomyces cerevisiae strain PJ69-4a was incubated with 1 µg of plasmid DNA (pGAD-C3 or pGBD-C3 containing the indicated genes) in transformation buffer (50% PEG 3350, 0.2 M LiAc, 0.5 mg ml^−1 single-stranded DNA, 0.1 M DTT) 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 replicated again on double dropout media before OD_600 measurement of single colonies resuspended in liquid triple dropout media (SD/-Leu/-Trp/-His). Same amount of material was plated on triple dropout media containing 1 mM 3-AT and incubated for 5 to 10 days at 30°C.

**Protein-fragment complementation assay.** The PCA is based on split TEM-1 β-lactamase (Bla) of E. coli. Two fragments of the reporter protein (Bla) were fused to two homologous interaction partners. The individual Bla fragments are non-functional unless proximity upon interaction of the fused proteins is restored. 7.5 × 10^5 HEK293T cells seeded in 6-well plates were co-transfected with 1 µg respective plasmid DNA using Lipofectamine 3000 reagent following the manufacturer’s instructions (Invitrogen). At 24 h post transfection, cells were transferred to 150 µl lysis buffer (Promega) containing protease inhibitor cocktail (Roche). After 45 min of incubation on ice and centrifugation for 30 min at 15,000 × g and 4°C, 50 µl of supernatants were mixed with 15 µl nitrocefin (Abcam), 15 µl H2O and 120 µl PBS in a 96-well plate. The β-lactamase-mediated hydrolysis of nitrocefin was measured by the change of absorbance at 495 nm at intervals of 8–9 s for 30 cycles. In the presence of a standard substrate concentration, the actual nitrocefin hydrolysis rate is dependent on the amounts of reconstituted Bla, consequently on the interaction between the fusion proteins. Therefore, to determine the strength of the interaction, the nitrocefin hydrolysis rates, expressed in mAU min^−1, were calculated for the linear phase of the reaction and compared to each other and the background rates, which were observed upon transfection of the respective fusion proteins alone.

**All PCA assays were carried out three times and the differences between average hydrolysis rates were compared to evaluate the strength of the interactions.**
upon request. Associated raw data for Figs. 2, 3, 4, 6 and Supplementary Figures 4, 5, 6, 7, 9, 10, 11 can be found in the source data file.

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Author contributions

T.S., M.M.L. and U.B.M. conceived the study; T.S., M.M.L., U.B.M., I.Z., S.S., P.W., C.C., C.A., S.K.-W., N.L. and Z.R. designed experiments; T.S., M.M.L., U.B.M., I.Z., S.S., P.W., C.C., C.A., S.K.-W. and N.L. performed the experiments; T.S., M.M.L., U.B.M., Z.R., J.C.H. and M.S. evaluated the data; T.S., M.M.L. and U.B.M. wrote the manuscript.

Additional information

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