mGBP2 engages Galectin-9 and Cytoskeleton-associated Protein 4

for immunity against *Toxoplasma gondii*

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EK designed and performed most experiments; GP and KS performed and analyzed the mass spectrometry data; SH and SWP performed STED microscopy and analysis. DD and KP designed and supervised the study. EK, DD and KP wrote the paper. All authors contributed in writing, discussing and editing of the manuscript.

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34 Main Text

35 Figures 1 to 6

36 Supplementary Figures S1 to S10

37

Abstract

Guanylate binding proteins (GBPs) are large interferon-inducible GTPases, executing essential host defense activities against *Toxoplasma gondii*, an invasive intracellular apicomplexan protozoan parasite of global importance. *T. gondii* establishes a parasitophorous vacuole (PV) which shields the parasite from the host's intracellular defense mechanisms. Murine GBPs (mGBPs) recognize *T. gondii* PVs and assemble into supramolecular mGBP homo- and heterocomplexes that are required for the disruption of the membrane of PVs eventually resulting in the cell-autonomous immune control of vacuole-resident pathogens. We have previously shown that mGBP2 plays an important role in *T. gondii* immune control. Here, in order to unravel mGBP2 functions, we report Galectin-9 (Gal9) and cytoskeleton-associated protein 4 (Ckap4) as critical mGBP2 interaction partners engaged for immunity to *T. gondii*. Interestingly, Gal9 and Ckap4 also accumulate and colocalize with mGBP2 at the *T. gondii* PV. Furthermore, we could prove the requirement of Gal9 and Ckap4 for growth control of *T. gondii* by CRISPR/Cas9 mediated gene editing. These discoveries clearly indicate that mGBP2 engages Gal9 and Ckap4 and that Gal9 and Ckap4 are critical factors for the mGBP2 coordinated cell autonomous host defense mechanism against *T. gondii*.

Significance Statement

We have previously shown that mGBP2 is an essential IFN γ induced protein for defense against *Toxoplasma gondii* infection. mGBP2 is rapidly recruited to the parasitophorous vacuole (PV) of intracellular *T. gondii* and is required for the rupture and/or permeabilization of the PV membrane. Subsequently mGBP2 attacks the parasite membrane. Up to now, no data about other mGBP2 interaction partners and their molecular requirement for *T. gondii* control has been made available.

Here, we have identified Gal9 and Ckap4 as interacting proteins of mGBP2. Gal9 and Ckap4 are essential in the mGBP2 mediated attack of the *T. gondii* PV. Both proteins localize at the *T. gondii* PV in close association with mGBP2, as shown by state-of-the-art super resolution microscopy. In addition, *T. gondii* growth cannot be controlled anymore in Gal9 and Ckap4 deficient cell lines, comparable to mGBP2 deficient cells.

62 Thus, we have identified novel effector proteins in the intricate cell autonomous immune machinery against intracellular
63 pathogens.

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Introduction

Subsequent to innate immune sensing of pathogens via pattern recognition receptors, production of interferons (IFN) is a key prerequisite for the activation of cell-autonomous immunity, which is essential for the immediate restriction of microbial replication (1, 2). IFN- γ signaling induces the expression of hundreds of interferon-stimulated genes (ISGs) (3-5), which essentially contribute to innate and adaptive immune responses in immune and nonimmune cells (6, 7). Very prominent among IFN- γ ISGs are the members of several families of interferon-inducible GTPases such as the 65- to 73-kDa dynamin-related guanylate-binding proteins (GBPs) (8, 9) and the 45-kDa immunity-related GTPases (IRGs) (10, 11). Human (hGBPs) and murine GBPs (mGBPs) families consist of seven (hGBP1 to hGBP7) and eleven (mGBP1 to mGBP11) members, respectively, which are strongly upregulated in response to IFN- γ (12-14). GBPs characteristically exhibit various cellular functions encompassing antiparasitic, antiviral, and antibacterial activity, as well as inhibitory effects on cell proliferation and tumor suppression (15-26).

GBPs display a characteristic multidomain architecture including a large catalytic GTPase (G) domain and a C-terminal, helical domain (8, 27-33). Various mGBPs such as mGBP1, mGBP2, and mGBP7 are especially important effector molecules for the resistance against *Toxoplasma gondii*, an intracellular apicomplexan protozoan parasite of global importance (16, 25, 34-39). After the active invasion of host cells, *T. gondii* forms a nonfusogenic cytoplasmic membranous structure called the parasitophorous vacuole (PV) in which the parasite efficiently replicates (37, 40, 41). mGBPs are intricately involved in IFN- γ induced growth restriction and killing of the *T. gondii* in murine macrophages, fibroblasts and astrocytes (16, 20, 25, 36, 38, 42). In order to execute their antimicrobial function, mGBPs can specifically associate with pathogen containing compartments of intracellular microbes which encompass pathogen-containing vacuoles (PCVs) as well as viral replication compartments (20, 25, 36, 43-45), resulting in the innate immune detection and cell-autonomous clearing of PCV-resident pathogens (22, 25, 38, 46). After IFN- γ induction, mGBP2 molecules assemble in vesicle like structures (VLS) in the cytoplasm and, after *T. gondii* entry into the host cell, mGBP2 attacks the PV membrane (PVM) in orchestrated, supramolecular complexes, ultimately promoting the destruction of PVMs (36, 38). This stage is followed by recruitment of mGBP2 to the plasma membrane of the intracellular parasite, which in turn likely leads to its destruction (38). Multiparameter fluorescence image spectroscopy (MFIS) revealed that

mGBP2, on a molecular level, can form homomultimers or heteromultimers with mGBP1, mGBP3 and, to a lesser extent, with mGBP5 within VLS and at the PVM (38). For PV membrane interactions, isoprenylation / farnesylation and/or hydrophobic moieties in the C-terminus of the GBPs are essential (25, 31, 32, 38, 47). Eventually, especially mGBP2 and mGBP7, can be found localized at the parasite cell membrane and inside the targeted parasite (25, 38) probably also interfering with the integrity of *T. gondii* membranes. Importantly, inactivation of mGBP1, mGBP2, or mGBP7 in mice has been shown to result in a vastly impaired resistance to infections with type II *T. gondii* (25, 36).

Galectins are a family of lectins characterized by a carbohydrate recognition domain (CRD) with β -galactoside-binding ability (48). Galectins are nucleocytoplasmic, can also be exported from cells through a poorly defined unconventional secretion pathway (49) and can bind directly to bacteria thereby mediating antibacterial effects and modifying inflammatory signaling events (50). Presently, 15 galectin members have been identified in mammals (51). Immunomodulatory effects of galectins include recruitment of immune cells to the site of infection, promotion of neutrophil function, and stimulation of the bactericidal activity of infected neutrophils and macrophages (48). Previously it has been shown that Gal3 can bind *T. gondii* glycosphosphatidylinositols and might be a co-receptor presenting GPIs to TLRs on macrophages (52). Cytoplasmic galectins fulfill functions in several cellular programs including cell growth and apoptosis (53). Through binding to multivalent carbohydrates, galectins can multimerize and thereby cluster these surface glycoconjugates resulting in changes in cell migration, receptor endocytosis and various other cellular events (54, 55). Glycans are largely restricted to the luminal face but absent from the cytosolic face of intracellular vesicles. If vesicular damage occurs, luminal glycans are exposed to the cytosolic milieu, where they can be recognized by galectins (56, 57). Furthermore, vacuolar instability of bacterial PCVs is recognized by host β -galactoside-binding proteins of the galectin family (50, 58). Intracellular galectins can serve as danger receptors and can promote autophagy of the invading pathogen (59).

The aim of this study was to explore the molecular interaction(s) which enable mGBP2 to control *T. gondii* replication. By employing immunoprecipitation (IP) followed by mass spectrometry (MS) analysis, we have identified novel interaction partners of mGBP2 such as galectin-9 (Gal9) and cytoskeleton-associated protein 4 (Ckap4). Furthermore, we demonstrate that the inactivation of either Gal9 or Ckap4 impairs the cell autonomous IFN- γ induced immune control of *T. gondii* replication.

Results

Identification of Gal9 and Ckap4 as mGBP2 interaction partners by IP and MS

Previously, we could show that mGBP2 interacts with itself and other mGBP family members forming homo- and heteromultimers, respectively, in VLS and at the *T. gondii* PVM (38). For a profound understanding of the functions of mGBP2 in *T. gondii* infection, we searched for other interaction partners of mGBP2. To this end, mGBP2^{-/-} murine embryonic fibroblasts (MEFs)(36) expressing HA-tagged mGBP2 were constructed, stimulated with IFN- γ , and either infected with the type II strain *T. gondii* ME49 or left uninfected. An HA-tag specific IP was performed (Fig.S1) followed by MS based quantitative protein analysis (Fig.1). mGBP2^{-/-} MEFs transduced with an empty expression vector served as negative controls.

Statistical analysis revealed that 148 proteins were co-purified with HA-mGBP2 from the uninfected and 294 proteins from the *T. gondii* infected cells. Besides the bait protein mGBP2, further members of the mGBP family were enriched as prey proteins. Among them, a clear co-purification of mGBP1 and mGBP5 which previously were independently identified by MFIS as mGBP2 interacting members, could be observed in uninfected and infected HA-mGBP2 expressing MEFs (mGBP1 enrichment factor > 185 in both experimental conditions, mGBP5 enrichment factor 4.8 from uninfected and 15.1 from infected cells) (Fig.1), verifying the reliability of the co-IP and MS analysis. Furthermore, Ckap4 showed up to be specifically enriched in mGBP2 immunoprecipitation experiments.

Members of the galectin family were significantly enriched in IPs from *T. gondii* infected cells. From the list of potential mGBP2 interactors, Gal9 was analyzed further since Gal9 was found significantly enriched in IP samples from type II *T. gondii* ME49 infected cells (Fig.1) and interestingly a role for several other galectin family members had already been proposed in microbial infections (51). Furthermore, Ckap4 showed a high enrichment factor (uninfected: 9.2 / *T. gondii* infected: 14) comparable to the known mGBP2 interacting proteins mGBP1 and mGBP5 (Fig.1).

To validate and characterize the interactions of Gal9 and Ckap4 with mGBP2 in detail, mCherry-fused proteins of Gal9 or Ckap4 were co-expressed with full-length mGBP2 or with mGBP2 truncation mutants containing the GTP-binding domain only (G), the G-domain and the middle domain (GM) or the C-terminal effector domain (E) (31, 36). These cell lines were infected with *T. gondii* followed by co-IP. Using this approach, we could prove the interaction of mGBP2 with

Gal9 and Ckap4 and could determine a crucial function of the E-domain of mGBP2 for the interaction with Gal9 and Ckap4 (Fig.2).

Subcellular localization of Gal9 and Ckap4 and recruitment to the T. gondii PV

In a next step, the colocalization of mGBP2 and Gal9 or Ckap4 at the PVM of *T. gondii* was determined by confocal (Fig.S2) and Stimulated Emission Depletion (STED) microscopy (Fig.3). Doubly transduced mGBP2^{-/-} MEFs coexpressing GFP-mGBP2 with either mCherry-Gal9 or mCherry-Ckap4 were prestimulated with IFN- γ and infected with *T. gondii* ME49 (Fig.3, Fig.S2). GFP-mGBP2 and mCherry-mGBP2 coexpressing MEFs served as a positive control in STED microscopy colocalization experiments (Fig.S3). A distinctive colocalization or close association between mGBP2 and Gal9 and between mGBP2 and Ckap4 could be observed at the PVM already 2 h after infection. Interestingly, mCherry-Gal9 partly colocalized in GFP-mGBP2 containing VLS in uninfected MEFs, whereas a colocalization of mCherry-Ckap4 and GFP-mGBP2 containing VLS could hardly be visualized by confocal microscopy (Fig.S4). Furthermore, 5-6 h after infection, different stages of recruitment and accumulation of mGBP2 and its interaction partners at the PVM were observed (Fig.S2). Eventually, Gal9 and mGBP2 could be found to be located together within the intermembranous space of the PV and eventually within the cytosol of the parasite (Fig.S2). These results indicate that Gal9 and Ckap4 are interacting and acting together with mGBP2 and suggest that Gal9 and Ckap4 are intricately involved into the cell autonomous immunity mediated by mGBP2.

Recruitment of mGBP2 to the T. gondii PV in Gal9 or Ckap4 deficient cells.

Next, the roles of Gal9 and Ckap4 for the recruitment of mGBP2 to the *T. gondii* PV was analyzed. For this purpose, the *gal9* gene as well as the *ckap4* gene were inactivated applying CRISPR/Cas9 gene editing. The inactivation of Gal9 and Ckap4 in different clonal NIH 3T3 fibroblast cell lines was confirmed by sequencing of the targeted gene loci and, additionally, either by Surveyor nuclease assay and WB for Gal9 (Fig.S5) or by WB analysis with a specific antibody against Ckap4 (Fig.S6). No difference in *T. gondii* infection rates could be detected between WT or Gal9- or Ckap4 inactivated fibroblast cell lines (Fig.S7). Interestingly, a significant reduction in the recruitment efficiency of mGBP2 2 h

after infection was observed in the absence of Gal9 or Ckap4 in the targeted fibroblast cell lines (Fig.4). Recruitment of mGBP2 could be restored after reconstitution of the fibroblasts with either mCherry-Gal9 or mCherry-Ckap4 (Fig.4). Interestingly, the recruitment of Gal9 was also reduced in mGBP2 deficient MEFs, whereas the co-localization of Ckap4 at the *T. gondii* PVM was not affected by the lack of mGBP2 (Fig.4, Suppl. Fig.8). These data indicate that the recruitment of mGBP2 to the *T. gondii* PVM is more efficient in the presence of Gal9 and Ckap4.

T. gondii replication control by Gal9 and Ckap4

The course of *T. gondii* infection in cells deficient for Gal9 and Ckap4 was investigated in the next step. To address the question whether Gal9 and / or Ckap4 contribute to cell autonomous immunity against *T. gondii*, Gal9- and Ckap4-deficient fibroblast cell lines were stimulated with IFN- γ and infected with *T. gondii* (ME49) and the replication of the parasite was monitored. First, it was determined whether the inactivation of Gal9 or Ckap4 leads to increased or decreased infection rates in the targeted cell lines compared to wildtype cells. However, the number of *T. gondii* parasites detected in the gene edited cell lines compared to wildtype fibroblasts did not differ 2 h post infection (Fig.S7). Interestingly, a significant increase in *T. gondii* replication could be observed in fibroblasts harboring an inactivation of Gal9 or Ckap4 as determined by quantification of the number of *T. gondii* rosettes and single parasites 22 h after infection (Fig.5, Fig.S9). As a specific control and to verify the specificity of the CRISPR/Cas9 gene inactivation of Gal9 and Ckap4, Gal9- and Ckap4- deficient cell clones were reconstituted by transduction with mCherry-Gal9 and mCherry-Ckap4 expression constructs, respectively. Importantly, Toxoplasma replication in the reconstituted fibroblasts was inhibited comparably to WT cells (Fig.6, Fig.S9). These results clearly indicate an important role for Gal9 and Ckap4 in controlling the intracellular replication of the parasite and suggest that mGBP2, Gal9 and Ckap4 function as supramolecular complexes in the defense against intracellular pathogens.

Discussion

mGBP2 provides cell-autonomous immunity to the intracellular PV resident parasite *T. gondii* (31, 36, 38, 44, 60). Although it has been shown that mGBPs translocate to PVs, form supramolecular complexes, attack the membrane of the parasite itself, and, consecutively, restrict replication of the intracellular pathogen, the molecular mechanisms by which mGBPs control the parasite inside the host cell remain largely elusive. Here we propose two novel interaction partners of mGBP2, Gal9 and Ckap4, which play a critical role with respect to the recruitment and the antiparasitic function of mGBP2 in the context of cell-autonomous defense against *T. gondii* (for a schematic overview see Fig.S10). Previously, it was shown that Gal8 colocalizes with mGBP2 positive *Salmonella* spp. in unprimed bone marrow derived macrophages (BMDMs) (61). It was proposed that Gal8 monitors endosomal and lysosomal integrity and detects bacterial invasion of the cytosol by *Listeria* spp. or *Shigella* spp., serving as a versatile receptor for vesicle-damaging pathogens (61). Interestingly, it was also reported that Gal3, as a member of the Galectin family, and mGBP2 form protein complexes, which associate with vacuoles harboring secretion system-competent *Legionella pneumophila* and *Yersinia pseudotuberculosis* bacteria in response to vacuolar damage (62). In this study we show that Gal9 interacts and colocalizes with mGBP2 in IFN- γ stimulated MEFs and NIH 3T3 cells in VLS. Based on its structural organization, Gal9 belongs to the tandem-repeat type galectins containing two distinct but homologous CRDs (N-CRD and C-CRD) covalently connected by a linker peptide (48, 59). Recruitment of Gal9 to the *T. gondii* PV has not been described previously. Furthermore, Gal9 could not only be found at the PVM but also within the intermembranous space between the PVM and the *T. gondii* plasma membrane as well as in the cytosol of the parasites together with mGBP2 approx. 5-6 h post-infection. This indicates that Gal9 and mGBP2 might recruit to the PVM of *T. gondii* in preformed complexes. Mechanistically, a series of events involving mGBP/IRG mediated PVM indentation, PVM vesiculation/disruption, and parasite plasma membrane stripping and denudation, leading ultimately to degradation of the parasite, is proposed by us and others as hallmarks for intracellular *T. gondii* elimination (38, 63, 64). It can be suggested that poreforming or denuding activity of these IFN- γ induced GTPases allows Gal9 to transmigrate through the PVM and to recognize β -galactosides on the inner leaflets of the PVM and the parasite, labeling the parasite for further degradation processes. Parasite glycoconjugates play important roles in host cell invasion, and specific interactions between host galectins and parasite glycoconjugates are considered to be critical for pathogen recognition (55). Interestingly,

glycosylphosphatidylinositols (GPIs) of *T. gondii* (65) have been shown to be ligands of human Gal3 whereby both glycan and lipid moieties participate in the association of *T. gondii* GPIs with Gal3 (52). In this context, Gal3, -8, and -9 bind to host glycans in the luminal side of lysed phagosomes or permeated pathogen vacuoles (66). Intracellular Gal3 was characterized as a sensor of vacuolar rupture that occurs when bacteria such as *Shigella flexneri* or *Salmonella enterica* serovar Typhimurium actively enter the host cell cytosol (58, 67). Moreover, *S. flexneri* IpaH9.8 labels mGBP2 for degradation (68). Gal3, -8 and -9 were also shown to associate with Salmonella-containing vacuoles (SCV) (61, 69, 70). Here we show that Gal9 is required for the control of the parasite *T. gondii*.

Functionally, the recruitment of mGBP2 to the PVM was reduced in Gal9- inactivated cells and vice versa; the recruitment of Gal9 to the PVM was reduced in mGBP2 deficient cells. In accordance, significantly fewer Salmonella were targeted by Gal8 in Gbp^{chr3} KO BMDMs than in wild-type BMDMs (71). Also, the delivery of mGBP1 and mGBP2 to Legionella-containing vacuoles (LCV) or Yersinia-containing vacuoles is substantially diminished in Gal3- and, to a lesser extent, Gal8-deficient IFN- γ -primed iBMDM (62). In Salmonella-infected cells, SCV rupture and Gal3 recruitment was followed by a rapid and massive recruitment of hGBP1 (67). Interestingly, a specific polybasic protein motif (PBM: KMRRRK) was identified for hGBP1 which is sufficient to drive significant targeting of hGBP1 to *S. flexneri* recognizing exposed glycans from disrupted, bacteria containing, endosomes. Deletion of the PBM-motive leads to substantially reduced colocalization between Gal3-marked vacuoles and hGBP1 (72). mGBP2 lacks such a C-terminal PBM. Nevertheless, the interaction with Gal9 appears to be mostly dependent on the C-terminal domain of mGBP2. Rab GDP dissociation inhibitor α (RabGDI α) acts as a suppressor of the mGBP2-Irga6 axis, hereby the lipid-binding activity of RabGDI α is critical for IFN- γ -induced mGBP2-dependent cell-autonomous immunity against ME49 *T. gondii*. RabGDI α deficiency is accompanied by increased recruitment of mGBP2 and Irga6 to the parasite in MEFs and macrophages and results in enhanced IFN- γ -mediated *T. gondii* clearance *in vitro* and *in vivo* (73). These studies suggest on the one hand GBPs as escorts for distinct anti-pathogenic factors en route to PVs, on the other hand that the recruitment of galectins and GBPs to the PVs might be mutually dependent as part of a coordinated host defense program. This is supported by the significantly increased *T. gondii* proliferation in IFN- γ stimulated Gal9 knock out 3T3 fibroblasts 22h post-infection indicating an important role of Gal9 in cell-autonomous *T. gondii* elimination. In contrast, IFN- γ -primed Gal3^{-/-} BMDMs restricted Yersinia and Legionella bacterial growth, similar to WT BMDMs (62).

Thus, it might be speculated that the recognition of the lysed PV by Gal9 initiates the uptake of the parasite into autophagosomes or other degradation pathways as it was described for *Salmonella*, where GBPs promote LC3 recruitment via Gal8 (71). Interestingly, *T. gondii* infected Gal3^{-/-} mice develop reduced inflammatory responses in many organs (74) and succumb to an intraperitoneal infection with *T. gondii* associated with a deficient influx of neutrophils and macrophages into the peritoneal cavity (75). The function of Gal9 might be the non-redundant to Gal3 and Gal8 in parasitic degradation processes which will be addressed in the future in a Gal9-gene deficient mouse line.

Ckap4 and mGBP2 can colocalize at the PVM of *T. gondii* where Ckap4 binds to the C-terminal part of mGBP2. Ckap4, also known as p63, CLIMP-63, or ERGIC-63, is a 63 kDa, reversibly palmitoylated and phosphorylated, type II integral transmembrane (TM) protein. Ckap4 was originally identified as a resident of the endoplasmic reticulum (ER)/Golgi intermediate compartment (ERGIC) (76) linking the ER to microtubules (77). There is also steadily accumulating evidence for diverse roles for Ckap4 localized outside the ER, including data demonstrating functionality of Ckap4 as a cell surface receptor for Dickkopf1-cytoskeleton-associated protein 4 (DKK1) (78) and other ligands (79, 80) resulting in increased nuclear abundance of Ckap4 in response to the antiproliferative factor (APF) (81). Ckap4 must be palmitoylated to traffic to the cell surface (82).

After active invasion and sequestration in PVs, intracellular *T. gondii* parasites initiate the reorganization of the ER and recruit the microtubule organizing center and other factors of the host cell cytoskeleton to PVs (83) to obtain nutrition sources (84). Microtubules of the host, which are loaded with LDL cholesterol (85) form a connection with the PV and are anchored via the "Dense Granule" protein GRA7 of *T. gondii*, organizing a tubulo-vesicular network (TVN) (86). When localized to the ER, a major function of Ckap4 is to anchor rough ER to microtubules, organizing the overall structure of ER with respect to the microtubule network. Vacuole formation and trafficking within the cytoplasm of eukaryotic cells is dependent upon the cytoskeleton and cytoskeletal motors (87-89). Therefore, it might not be surprising that PCV are tightly associated with cytoskeletal components and that modulation of the cytoskeleton is a key process required for vacuole formation. Intimate interactions between *T. gondii* and host microtubules were shown to result in suppression of cell division and/or cause a mitotic defect, thus providing a larger space for parasite duplication (90). Also, interference with the cytoskeleton has been shown to be required for expansion of *Salmonella* and *Chlamydia* PCVs (91, 92). The regulation of the cytoskeleton by IFN- γ is of relevance in physiological and

pathophysiological processes, including cell migration, invasion, proliferation, defense against pathogens, and barrier function (93-98) and hGBP1 has been shown to be involved into the regulation of these cellular processes in response to IFN- γ (15, 17, 98-100). Interestingly, hGBP1 has been proposed to be involved in rearrangements of the cytoskeleton specifically mediating IFN- γ induced defense mechanisms, where actin-remodeling was dependent on the oligomerization and the GTPase activity of hGBP1 (101). Salmonella and Chlamydia PCVs are surrounded by a network of F-actin and other cytoskeletal components that provide structural support for the PCVs of both these pathogens as treatment with F-actin polymerisation inhibitors leads to the loss of integrity (92, 102, 103). Thus, Ckap4 might inhibit the establishment of the intracellular parasitic survival niche and this is involved into the replication control of *T. gondii*. The molecular function of Ckap4 in the mGBP2 complex and the putative ability of Ckap4 to associate with other members of the GBP family have to be addressed in the future. The findings of this study indicate that mGBP2 acts as a scaffold organizing the cell autonomous immunity in concert with Gal9 and Ckap4 and thus extends the understanding of molecular host responses in the control of intracellular parasites.

Materials and Methods

Expression Constructs.

The WT ORF of mGBP2 (NCBI accession numbers: mGBP-2, NM_010260.1) was cloned either into the pWPXL plasmid (Trono lab) as N-terminal GFP-fusion constructs or into the pWPI Plasmid (Trono lab) in frame with a N-terminal HA-tag. For the cloning of mCherry constructs (36), the pWPXL plasmid was modified by exchanging of the gene for GFP by the gene for mCherry (38). mRNA was isolated from freshly prepared WT MEFs prestimulated with IFN- γ and infected with *T. gondii*. The ORFs of Galectin 9 (NM_010708.2) and Ckap4 (NM_175451.1) were amplified by PCR and then cloned into the modified pWPXL plasmid as N-terminal mCherry-fusion constructs (38). The lentiviral envelope vector pLP/VSVG (Invitrogen) and the packaging vector psPAX2 (Trono lab) were used for the lentiviral genetic transfer.

Cell culture and transduction.

NIH 3T3 fibroblasts and MEFs were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen/Gibco) supplemented with 10% (v/v) heat-inactivated low endotoxin fetal bovine serum (FBS, Cambrex), 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine (Biochrom) and 0.05 mM β -mercaptoethanol (Invitrogen/Gibco). Human

foreskin fibroblasts (HS27, ATCC CRL-1634) were held in culture in Iscove's Modified Dulbecco's Medium (IMDM, Invitrogen/Gibco) with the same supplementations. 293FT cells were cultivated in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. All recombinant lentiviruses were produced by transient transfection of 293FT cells according to jetPRIME® transfection protocol (Polyplus, New York City) (38). NIH 3T3 cells and MEFs were seeded in 24 well plates (Corning Incorporated, Germany) and transduced with 600 µl of lentivirus with 25 µg Polybrene (Merck Millipore, Germany). After 5 h of incubation medium was changed. The transduction efficacy was analyzed by flowcytometry. Subsequently, only GFP or double GFP and mCherry positive cells were sorted and cultivated.

In Vitro Passage of Toxoplasma gondii.

Tachyzoites from *T. gondii* strain ME49 were maintained by serial passage in confluent monolayers of HS27 cells. After infection of fibroblasts, parasites were harvested and passaged as described previously (16).

Infection of murine MEFs with T. gondii.

Cells were stimulated with 200 U/mL IFN-γ (R&D Systems, Minneapolis, MN) 16 h before infection. For immunofluorescence, NIH 3T3 fibroblasts and MEFs were cultured in 24-well plates (Falcon, BD Biosciences, Germany) on cover slips (ø 13 mm, VWR International, Germany) and inoculated with freshly harvested *T. gondii* at a ratio of 50:1. To remove extracellular parasites, cells were washed with PBS.

Pull-Down and co-immunoprecipitation analysis of mGBP2

For pull down experiments, mGBP2^{-/-} MEFs reconstituted with HA-mGBP2 were used. mGBP2^{-/-} MEFs transduced with the empty pWPI vector were used as control cells. Cells were then stimulated with IFN-γ for 16 h, infected with the *T. gondii* strain ME49 for 2 h or left uninfected, subsequently lysed and postnuclear supernatants were incubated o/n with monoclonal mouse anti-HA- antibody coupled to agarose beads (clone HA-7, Sigma-Aldich, USA) at 4°C. IP probes were subjected to silver staining and to Western Blotting. Blots were stained with the mGBP2-specific antiserum (Eurogentec), mouse α-GFP (clones 7.1 and 13.1, Roche, Switzerland) and rabbit αHA (polyclonal, Sigma-Aldich, USA) antibodies. For IP experiments, postnuclear supernatants of mGBP2^{-/-} MEFs reconstituted either with WT GFP-mGBP2 or GFP-fused truncated mutants or just transduced with a GFP expressing vector, as an appropriate

control and coexpressing mCherry fused with Gal9 or Ckap4 or just the mCherry protein, were incubated o/n with RFP-Trap® or GFP-Trap® beads (ChromoTek GmbH, Germany) at 4°C. IP probes were subjected to Western Blotting. Blots were stained with the mouse α -GFP or α -mCherry (Clontech, EU) antibodies.

MS Analysis of interaction partners of mGBP2

A label-free mass spectrometry, based quantification approaches enabled by a highly reproducible and stable LC-MS/MS system was chosen for a quantitative analysis of the mGBP2 interactome. Six individual replicates of co-immunoprecipitation samples per group (HA-mGFP2, control-vector, HA-mGFP2 + ME49 and control-vector + ME49) were prepared from transfected IFN γ stimulated mGBP2^{-/-} MEFs (see above). Samples were prepared for mass spectrometric analysis essentially as described elsewhere (104). Briefly, proteins were stacked into an acrylamide gel (about 4 mm running distance), subjected to silver staining, de-stained, reduced and alkylated and digested with trypsin. Resulting peptides were extracted from the gel and approximately 500 ng peptides prepared in 0.1% trifluoroacetic acid for liquid chromatography and mass spectrometric analysis.

First, peptide samples were separated on an UltiMate 3000 RSLCnano chromatography system (Thermo Fisher Scientific). Peptides were initially trapped on a 2 cm long trap column (Acclaim PepMap100, 3 μ m C18 particle size, 100 Å pore size, 75 μ m inner diameter, Thermo Fisher Scientific) for 10 min at a flow rate of 6 μ l/min with 0.1 % (v/v) TFA as mobile phase. Subsequently, they were separated on a 25 cm long analytical column (Acclaim PepMapRSLC, 2 μ m C18 particle size, 100 Å pore size, 75 μ m inner diameter, Thermo Fisher Scientific) at 60°C using a 2 h gradient from 4 to 40% solvent B (solvent A: 0.1% (v/v) formic acid in water, solvent B: 0.1% (v/v) formic acid, 84% (v/v) acetonitrile in water) at a flow rate of 300 nl / min.

Separated Peptides were injected with distal coated SilicaTip emitters (New Objective, Woburn, MA, USA) into an Orbitrap Elite mass spectrometer via a nanosource electrospray interface. The mass spectrometer was operated in positive, data dependent mode with a spray voltage of 1.4 kV and capillary temperature of 275°C. First, a full scan from m/z 350 to 1700 was recorded with a resolution of 60000 (at 400 m/z) in the orbitrap analyser with a target for automatic gain control set to 1000000 and a maximal ion time of 200 ms. Then, up to 20 two- and threefold charged precursor ions with a minimal signal of 500 were isolated (isolation width 2) in the linear ion trap part of the instrument. Here, ions

were collected for a maximum of 50 ms with an automatic gain control set to 30000, fragmented by collision induced dissociation with a normalized collision energy of 35 and analysed at a resolution of 5,400 (at 400 m/z). Already fragmented precursors were excluded from fragmentation for the next 45 seconds.

Quantitative analysis of MS1 based precursor ions was carried out with Progenesis Q1 for proteomics (version 2.0, Nonlinear Dynamics, Newcastle upon Tyne, UK). The software first detects and aligns precursor ion signals (features) then associates information from MS2 based precursor identification and finally computes protein intensities on basis of associated features. Automatic processing was used without manual alignment adjustment. Here, one run of the control-vector + ME49 could not automatically aligned and was not further considered in the analysis. Searches were triggered by Proteome Discoverer (version 1.4.1.14, Thermo Scientific) and the percolator node was used for identification validation at 1% false discovery rate. Spectra were searched with the Mascot search engine (version MASCOT 2.4.1, Matrix Science, London, UK) considering cysteine carbamidomethylation as fixed and protein N-terminal acetylation and methionine oxidation as variable modification as well as tryptic cleavage specificity with a maximum of two missed cleavage sites. Mass precision was set to 10 ppm for precursor and 0.4 Da for fragment spectra. Database searches were carried out using the mus musculus (UP000000589) and toxoplasma gondii (UP000002226) reference proteome data sets downloaded on 18th January 2018 from UniProtKB including 52548 respectively 8404 entries. Only “high confident” peptide spectrum matches were used for further processing and peptide spectrum matches associated with proteins identified with at least two unique peptides. As we noticed a bias towards higher protein intensities in the mGBP2 overexpressing cells (arising from mGBP2 and associated proteins), no global normalization was performed but a normalization of protein intensities based on the sum of the Ig heavy chain proteins P18525, P18524. Calculated protein intensities were further processed within the Perseus (version 1.6.2.2, MPI for Biochemistry, Planegg, Germany) software environment. Here, Student’s t-tests were calculated on log2 transformed normalized intensities using the significance analysis of microarrays method for cutoff determination (S_0 set to 0.8, 5% false discovery rate, a minimum of 4 valid values per group). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE(105) partner repository with the dataset identifier PXD027029.

Immunofluorescence analysis.

Cells were fixed in 4% paraformaldehyde (PFA, Sigma-Aldrich, Germany) permeabilized with 0.02% saponin (Calbiochem-Merck) and blocked in 0.002% saponin with 2% goat serum (DaKoCytomation, Denmark). The outer membrane of *T. gondii* was visualized by anti-SAG1 (Abcam, UK) at a concentration of 1/700. As secondary reagents, 1/200 concentrated Alexa Fluor™ 633-conjugated goat anti-mouse IgG (H+L) (Invitrogen, ThermoFisher Scientific, USA) and Cy3-conjugated goat anti-mouse IgG plus IgM (Jackson ImmunoResearch Laboratories, UK) were used. Nuclei were counterstained with 1/2500 4',6-diamidino-2-phenylindole (DAPI, Invitrogen, ThermoFisher Scientific, USA). The cover slips were fixed in fluorescence mounting medium (Fluoromount-G, Southern Biotechnology Associates, Birmingham, AL). Fluorescence was visualized using a LSM780 confocal microscope (Zeiss, Germany). Image analysis and processing was performed with the ZEN (Zeiss, Switzerland) software.

Gated Stimulated Emission Depletion measurement

Gated Stimulated Emission Depletion (STED) measurements were performed using a TCS SP8 STED 3X (Leica, Wetzlar, Germany) equipped with an HC PL APO CS2 93x glycerol objective (Leica, Wetzlar, Germany, NA 1.3) at a scan speed of 1400 Hz. For the acquisition of the gated STED signal of the eGFPBoosterAtto488, 488 nm was used as the excitation laser line, 592 nm as the corresponding depletion laser line and the hybrid detector range was set from 498 nm to 580 nm with a 1 ns to 6 ns time gating. For the acquisition of the gated STED signal of the mRFPBoosterAtto647N, 633 nm was used as the excitation laser line, 755 nm as the corresponding pulsed depletion laser line and the hybrid detector range was set from 640 nm to 750 nm with a 0.8 ns to 6 ns time gating. Detection of the different signals was carried out in a frame sequential measurement setup. Deconvolved data of gated STED measurements were calculated using Huygens software (Huygens professional, Scientific Volume Imaging, Netherlands) with reduced signal to noise factor of 5 for eGFPBoosterAtto488 and 6 for mRFPBoosterAtto647N and a reduced iteration setting of 20 for both channels. Resulting deconvolved images were visually compared to the raw data to ensure the quality of the calculation. To correct for channel shift, a reference double staining of nuclear pores was acquired with similar settings and utilized as a reference for channel shift correction by the Fiji-plugin "multistackreg" (106).

CRISPR/Cas9 knock out strategy.

The Galectin 9 gene (lectin, galactose binding, soluble 9, Lgls9) as well the Ckap4 gene (P63; CLIMP-63; 5630400A09Rik) were disrupted by applying the CRISPR/Cas9 technology in NIH 3T3 fibroblasts (107). The knock out was performed by transfection of the pSpCas9(BB)-2A-GFP (PX458) plasmid (Addgene, USA), expressing a sgRNA (Ckap4: for CAC CGA TCC GCG CCG CCC GAC GGG T, rev AAA CAC CCG TCG GGC GGC GCG GAT C, Gal9: for CAC CGC GGG TTA ATG TAT GGA GAC T, rev AAA CAG TCT CCA TAC ATT AAC CCG C) corresponding to the sequence contained in the first exon of either Gal9 or Ckap4 gene. The sgRNA encoding plasmids were transfected into NIH 3T3 fibroblasts according to jetPRIME^R transfection protocol (Polyplus, New York City). The transfection efficacy was analyzed by flow cytometry. Subsequently, GFP positive cells were sorted and single cell colonies were cultivated. Mutated clones of the Gal9 gene were identified by Surveyor[®] Mutation Detection (Integrated DNA Technologies, Inc., USA) (for AAC TAG ATT GGG CCT GCC TC, rev AGA GAT CCC CCT GAC TCT GT, expected fragments, expected PCR fragment: 923 bp, expected digested fragments: 747, 176 bp). The knock out of both genes, respectively, was confirmed by PCR amplification, subcloning and sequencing of the corresponding gene stretches from different single cell colonies. Additionally, WB analysis was performed with a polyclonal rabbit anti-mouse antibody against Ckap4 (STJ110088, St John's Laboratory Ltd, UK) and a polyclonal rabbit anti-mouse antibody against Gal9 (ARP54821_P050, Aviva Systems Biology).

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Figures legends

FIGURE 1. Identification of mGBP2 interacting proteins using co-immunoprecipitation in combination with label-free quantitative mass spectrometry. An HA-mGBP2 fusion protein was expressed in mGBP2^{-/-} MEFs. mGBP2^{-/-} MEFs transduced with the empty expression vector were used as controls. MEFs were stimulated with IFN- γ (16h) and left uninfected or were infected with *T. gondii* ME49 for 2h. IPs were performed with an anti-HA antibody from cell lysates. Immunoprecipitated proteins were analyzed by label-free quantitative mass spectrometry. Five to six replicates were analyzed per condition and putative mGBP2 interacting proteins were identified from uninfected and *T. gondii* ME49 infected cells. Mean values of normalized intensities are shown in the bar plots $\pm$ standard deviations. In the table, details from the protein identification are shown: the confidence scores from the MASCOT search engine (108, 109), the number of identified unique peptides per protein as well as p-values from Student's t-tests. Furthermore, the enrichment ratios (intensity ratio between mGBP2 expressing cells and vector control) is given (a correction for multiple testing has been performed using the significance analysis of microarray approach, 5% false discovery rate, S_0 0.8, a minimum of 4 valid values per group).

FIGURE 2. Verification of Gal9 and Ckap4 as interaction partners of mGBP2 by IP analysis. mGBP2^{-/-} MEFs were reconstituted with GFP-mGBP2 WT or one of the indicated mGBP2 truncation mutants as well as one of the N-terminal mCherry fusion proteins mCherry-mGBP2, mCherry-Gal9 or mCherry-Ckap4. Cells were stimulated with IFN- γ for 16 h and infected with *T. gondii* ME49 for 4h. Cells were lysed and postnuclear supernatants were incubated o/n with either RFP-Trap® beads or with GFP-Trap® beads at 4°C. IP samples and appropriate cell lysate supernatants were subjected to Western Blotting. Blots were stained with α -GFP or α -mCherry antibodies. GTP-binding domain only (G), the G-domain and the middle domain (GM) or the C-terminal effector domain (E) (36).

FIGURE 3. Intracellular colocalization of mGBP2 interaction partners Gal9 and Ckap4 at the PVM of *T. gondii*.

Recruitment and colocalization of mGBP2, Gal9, and Ckap4 was analyzed after transduction of a GFP-mGBP2 fusion construct in mGBP2^{-/-} MEFs and additional transduction of either mCherry-Gal9 or mCherry-Ckap4. MEFs were seeded and incubated on glass slides, stimulated with IFN- γ for 16 h and subsequently infected with *T. gondii* ME49 for 2 h. After fixation, infected cells were treated with an α -RFP V_HH nanobody conjugated to eGFPBoosterAtto647N and with an α -GFP V_HH nanobody conjugated to eGFPBoosterAtto488 for enhancement of the immunofluorescence of mCherry and GFP, respectively. Glass slides were analyzed by STED microscopy. Bars 2 μ m. The graphs in the right panel depict a fluorescence intensity analysis of STED images on the far right with the ImageJ software (Fiji) for Atto488 and Atto647 fluorescence signals along the cross sections of PVMs as indicated.

FIGURE 4. Gal9 and Ckap4 influence the recruitment of mGBP2 to the PVM of *T. gondii*.

Recruitment of mGBP2 to the PVM was analyzed by transduction of a GFP-mGBP2 fusion construct in WT and independent NIH 3T3 cell line clones with verified CRISPR/Cas9 mediated inactivation of Gal9 or Ckap4 (see main text and Figs.S5/S6). Cells were stimulated with IFN- γ for 16 h and subsequently infected with *T. gondii* ME49 for 2 h. The recruitment rate of GFP-mGBP2 in WT cells was set as 100% and the recruitment rate of mGBP2 in the respective knock-out cells was calculated to this reference. The percentage values are plotted on the y-axis. Also, Gal9 and Ckap4 recruitment to the *T. gondii* PV was analyzed in mGBP2^{-/-} vs. WT MEFs transduced either with mCherry-Gal9 or mCherry-Ckap4. The rate of either Gal9 positive or Ckap4 positive PVMs in WT cells was set as 100% and related to the respective rates in mGBP2 knock out cells. The percentage values are plotted on the y-axis. After fixation, *T. gondii* were stained with an α -SAG1 antibody and the nuclei were labeled with DAPI. Glass slides were analyzed by confocal microscopy. In each cell line more than 100 PVs were counted. Mean values $\pm$ SD are shown.

FIGURE 5. Gal9 and Ckap4 are required for the intracellular control of replication of *T. gondii*.

WT and independent NIH 3T3 cell line clones with verified CRISPR/Cas9 mediated inactivation of Gal9 or Ckap4 (see main text and Figs.S5/S6) were stimulated with IFN- γ for 16 h and subsequently infected with *T. gondii* ME49. After fixation, *T. gondii* were stained with an α -SAG1 antibody and the cell nuclei were labeled with DAPI. Glass slides were analyzed by confocal microscopy. Bars, 5 μ m. The amounts of rosettes (replicative units) and single parasites inside the PV were

determined in WT, Gal9- and Ckap4- deficient NIH 3T3 fibroblasts 22 h after infection. In each cell line more than 100 PVs were counted. Mean values $\pm$ SD are shown (lower panel).

FIGURE 6. Gal9 and Ckap4 control the intracellular replication of *T. gondii*. WT and independent NIH 3T3 cell line clones with verified CRISPR/Cas9 mediated inactivation of Gal9 or Ckap4 (see main text and Figs.S5/S6) and NIH 3T3 cell line clones with verified CRISPR/Cas9 mediated inactivation of Gal9 or Ckap4 reconstituted with mCherry-Gal9 or mCherry-Ckap4 were stimulated with IFN- γ for 16 h and subsequently infected with *T. gondii* ME49 for 22 h. After fixation, *T. gondii* were stained with an α -SAG1 antibody and the cell nuclei were labeled with DAPI. Glass slides were analyzed by confocal microscopy. The amounts of rosettes (replicative units) and single parasites inside the PV were determined in WT, Gal9- and Ckap4- deficient and Gal9 or Ckap4 reconstituted NIH 3T3 fibroblasts 22 h after infection. In each cell line more than 100 PVs were counted. Mean values $\pm$ SD are shown.

FIGURE S1. Identification of mGBP2 interaction partners. mGBP2^{-/-} MEFs were reconstituted with HA-mGBP2 or a control vector and stimulated with IFN- γ for 16 h, infected for 2 h with *T. gondii* ME49 or left uninfected. Subsequently, cells were lysed and postnuclear supernatants were incubated o/n with α -HA antibody coupled agarose beads at 4°C for IP. One part of IP samples was separated via 10% SDS-PAGE and labelled using silver staining. Another part of the IP samples was subjected to Western Blotting and immune staining with an α -mGBP2 antiserum or an α -HA antibody. A third part of these IP samples was transferred to the MS analysis (see main text).

FIGURE S2. Localization of Gal9 and mGBP2 at *T. gondii*. Recruitment and colocalization of mGBP2 was analyzed in GFP-mGBP2 expressing mGBP2^{-/-} MEFs with additional transduction of either mCherry-Gal9. MEFs were stimulated with IFN- γ for 16 h and subsequently infected with *T. gondii* ME49 for 5 h. After fixation, *T. gondii* were stained with an α -SAG1 antibody and the cell nuclei were labeled with DAPI. Glass slides were analyzed by confocal microscopy. Bars 2 μ m.

FIGURE S3. Colocalization of mCherry-mGBP2 and GFP-mGBP2 at the PV membrane of *T. gondii*. Recruitment and colocalization of mGBP2 was analyzed in GFP-mGBP2 expressing mGBP2^{-/-} MEFs with additional transduction of mCherry-mGBP2 as control. MEFs were stimulated with IFN- γ for 16 h and subsequently infected with *T. gondii* ME49 for 2 h. After fixation, *T. gondii* were stained with an α -SAG1 antibody. Infected cells were treated with anti-RFP V_HH

nanobody conjugated to eGFPBoosterAtto647N and with anti-GFP V_HH nanobody conjugated to eGFPBoosterAtto488 for enhancement of immunofluorescence of mCherry and GFP respectively. Glass slides were analyzed by STED microscopy. Bars 2 μ m. The graphs depict a colocalization analysis of STED images with the ImageJ software (Fiji) for GFP and mCherry fluorescence.

FIGURE S4. Intracellular colocalization of mGBP2 with the interaction partners Gal9 and Ckap4. Colocalization of GFP-mGBP2 with mCherry-Gal9 or mCherry-Ckap4 was analyzed in mGBP2^{-/-} MEFs reconstituted with the indicated fusion proteins. Cells were stimulated with IFN- γ for 16 h. After fixation, cell nuclei were labeled with DAPI. Glass slides were analyzed by confocal microscopy. Bars 5 μ m. The right column depicts the results for a colocalization analysis using the Image Visualization and Analysis Software (Imaris) for GFP and mCherry fluorescence.

FIGURE S5. CRISPR/Cas9 gene editing of Gal9 and verification of Gal9 inactivation. (A) CRISPR/Cas9 and sgRNA specific for Gal9 were expressed in NIH 3T3 fibroblasts. Clones were picked and DNA from WT and three CRISPR/Cas9 Gal9 targeted cell clones of NIH 3T3 fibroblasts was isolated, PCR amplified (923 bp). PCR products were cloned into the pCR2.1 TA-cloning vector. The Gal9 mutations were verified by Sanger sequencing. Indels, AA sequence and premature stop codons (*) are indicated. (B) For independent mutation analysis, the Surveyor® Mutation Detection Kit for Standard Gel Electrophoresis was employed. Here, a mismatch-specific DNA endonuclease to scan mutations and polymorphisms in heteroduplex DNA is used. PCR amplicons from Gal9 mutant NIH 3T3 clones (test) and WT (reference) DNA were hybridized and the mixtures of hetero- and homo-duplexes were submitted to Surveyor Nuclease digestion. The reference DNA alone, treated similarly, served as a negative control. DNA fragments were analyzed by agarose gel electrophoresis. The formation of new cleavage products, due to the presence of one or more mismatches, is indicated by the presence of additional bands. The relative size of these cleavage products indicates the location of the mismatch or mismatches. (C) Postnuclear supernatants of NIH 3T3 cells from different CRISPR/Cas9 Gal9 sgRNA targeted clones and from WT cells were analyzed by WB. WT cells and one CRISPR/Cas9 Gal9 sgRNA targeted clone coexpressing GFP-mGBP2 and mCherry-Gal9 served as controls. Cells were stimulated with IFN- γ for 16 h. Blots were stained with α -Gal9 or α -mCherry antibodies.

FIGURE S6: CRISPR/Cas9 gene editing of Ckap4 and verification of Ckap4 inactivation. CRISPR/Cas9 and sgRNA specific for Ckap4 were expressed in NIH 3T3 fibroblasts. Postnuclear supernatants of NIH 3T3 cells from different CRISPR/Cas9 Ckap4 sgRNA targeted clones versus WT and CRISPR/Cas9 Gal9 sgRNA targeted clones were analyzed by WB. WT and one CRISPR/Cas9 Ckap4 sgRNA targeted clones coexpressing GFP-mGBP2 and mCherry-Ckap4 served as controls. Cells were stimulated with IFN- γ for 16 h. Blots were stained with α -Ckap4 or α -mCherry antibodies.

FIGURE S7. Inactivation of Gal9 or Ckap4 does not influence infection rates of *T. gondii*. The infection rates of *T. gondii* ME49 were analyzed in WT and independent NIH 3T3 cell line clones with verified Gal9 or Ckap4 inactivation. Cells were stimulated with IFN- γ for 16 h and subsequently infected with *T. gondii* ME49. After fixation, *T. gondii* were stained with the α -SAG1 antibody and the cell nuclei were labeled with DAPI. Glass slides were analyzed by confocal microscopy. Bars, 5 μ m. The amounts of parasites per cell were quantified 2 h after infection.

FIGURE S8. Influence of mGBP2 does on the recruitment of Gal9 and Ckap4 to the PVM of *T. gondii*. WT and mGBP2 deficient MEFs additionally transduced with either with mCherry-Gal9 or mCherry-Ckap4 fusion construct were stimulated with IFN- γ for 16 h and subsequently infected with *T. gondii* ME49 for 2 h. After fixation, *T. gondii* were stained with an α -SAG1 antibody and the cell nuclei were labeled with DAPI. Glass slides were analyzed by confocal microscopy. Bars, 5 μ m.

FIGURE S9. Gal9 and Ckap4 are required for the intracellular control of replication of *T. gondii*. WT and independent NIH 3T3 cell line clones with verified CRISPR/Cas9 mediated inactivation of Gal9 or Ckap4 (see main text and Figs.S5/S6) were stimulated with IFN- γ for 16 h and subsequently infected with *T. gondii* ME49 for 22 h. After fixation, *T. gondii* were stained with an α -SAG1 antibody and the cell nuclei were labeled with DAPI. Glass slides were analyzed by confocal microscopy. Bars, 5 μ m.

FIGURE S10. Schematic view of mGBPs, Gal9, and Ckap4 dynamics and multimerization in *T. gondii* infected cells. For details see Results and Discussion.

Gal9

Ckap4

mGBP2

mGBP1

mGBP5

accession	gene names	unique peptides	confidence score	uninfected			<i>T. gondii</i>		
				p-value	ratio mGBP2/control	significance mGBP2/control	p-value	ratio mGBP2/control	significance mGBP2/control
Q01514	mGBP1	19	1702.6	3.3E-08	186	+	2.8E-06	199.7	+
Q9Z0E6	mGBP2	55	4193.6	1.3E-07	66.8	+	1.5E-06	125.4	+
Q8CFB4	mGBP5	6	199.1	9.1E-03	4.8	+	6.3E-05	15.1	+
Q8BMK4	Ckap4	13	747.1	1.2E-02	9.2	+	1.0E-02	14	+
O08573	Gal9	2	79.9	1.3E-01	1.5		5.6E-03	3.8	+

Figure 1

IP RFP trap, WB α -mCherry

mCherry-Gal9

lysate, WB α -mCherry

mCherry-Gal9

mCherry-Gal9

GFP-mGBP2 WT
GFP-mGBP2 GM
GFP-mGBP2 ME
GFP-mGBP2 E

IP RFP trap, WB α -mCherry

mCherry-Ckap4

lysate, WB α -mCherry

mCherry-Ckap4

mCherry-Ckap4

GFP-mGBP2 WT
GFP-mGBP2 GM
GFP-mGBP2 ME
GFP-mGBP2 E

IP GFP trap, WB α -GFP

GFP-mGBP2 WT

lysate, WB α -GFP

GFP-mGBP2 WT

mCherry-Gal9 + GFP-mGBP2 WT

mCherry-Gal9 + GFP-mGBP2 GM
mCherry-Gal9 + GFP-mGBP2 ME
mCherry-Gal9 + GFP-mGBP2 E

IP GFP trap, WB α -GFP

GFP-mGBP2 WT

lysate, WB α -GFP

GFP-mGBP2 WT

mCherry-mGBP2 + GFP-mGBP2 WT

mCherry-mGBP2 + GFP-mGBP2 GM
mCherry-mGBP2 + GFP-mGBP2 ME
mCherry-mGBP2 + GFP-mGBP2 E

Figure 2

Figure 3

Figure 4

Figure 6