Human guanylate binding proteins: Generation of tools, and their role during *Toxoplasma gondii* infection

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Declaration

I, Ashleigh Christina Johnston, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Guanylate binding proteins (GBPs) are large GTPases that are substantially upregulated by interferons during infection. The human genome consists of seven GBP family members with high sequence identity. GBPs have been implicated to confer host resistance to a number of pathogens across several species. In mice, specific GBP family members are responsible for host defence mechanisms, including the induction of inflammasome responses during bacterial infections, and the disruption of pathogen vacuoles leading to effective protection against the parasite *Toxoplasma gondii,* and the bacteria *Salmonella enterica* typhimurium and *Chlamydia trachomatis*. *Toxoplasma* is an apicomplexan intracellular parasite that resides within a parasitophorous vacuole (PV), and can cause severe complications and even death in humans and other animals. The aim of this project was to analyse the characteristics and roles of individual human GBP family members in cells at steady state and in *Toxoplasma* infected cells. The first step was to develop tools to study the proteins, including producing and characterising specific antibodies, establishing cell overexpression systems and characterising cells deficient in certain GBPs. Using these tools, the subcellular localisations of GBP1 and GBP4 were determined to the cytoplasm and nucleus respectively. It was concluded that during type I and II *Toxoplasma* infection GBP1 and 4 are not recruited to the PV like in the mouse. Despite this, in human epithelial cells, GBP1 plays an important and specific role in the restriction of *Toxoplasma* replication. It was deduced that GBP4 protein levels are dramatically reduced during infection with the type I, but not the type II strain of *Toxoplasma*. GBP4 protein levels could be stabilised during type I *Toxoplasma* infection with an inhibitor of cysteine, serine and threonine proteases. Using an antibody specific for GBP1 and 2, a large dataset of potential interaction partners in a *Toxoplasma* strain-specific fashion was generated. The tools produced, specifically the GBP-specific antibodies, provide a valuable resource that can be used by other lab members and collaborators to more fully understand the functions of these interesting and important large GTPases.

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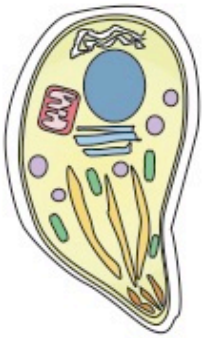

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Abbreviations

AIM2	Absent in melanoma 2 protein
ALR	Absent in melanoma 2-like receptor
Atg3	Autophagy protein 3
Atg5	Autophagy protein 5
Bcl-2	B cell lymphoma 2
BID	BH3 interacting-domain death agonist
BMDM	Bone marrow-derived macrophage
DAMPs	Danger-associated molecular patterns
DC	Dendritic cell
DMEM	Dulbecco's modified Eagle medium
ELISA	Enzyme-linked immunosorbent assay
FBS	Foetal bovine serum
GAP	GTPase activating protein
GBP	Guanylate Binding Protein
GKS	Glycine-Leucine-Serine
GRA15	Dense granule protein 15
GTP	Guanosine triphosphate
HAP1	Human haploid cell
HFF	Human foreskin fibroblasts
HUVEC	Human umbilical vein endothelial cell
IDO	Indolamine-2,3-dioxygenase
IFN	Interferon
IFNγ	Interferon-gamma
IL-12	Interleukin-12
IL-1β	Interleukin-1-beta
iPSCs	Induced pluripotent stem cells
IRF1	Interferon regulating factor 1
IRG	Immunity related GTPases
IRGM	Immunity related GTPase family M
LPS	Lipopolysaccharide
MEF	Mouse embryonic fibroblast

mGBP	Murine guanylate binding protein
MOI	Multiplicity of infection
MVP	Major vault protein
Mx	Myxovirus resistant protein
NK	Natural killer
NKG2D	Natural-killer group 2 member D protein
NLR	Nucleotide-binding domain, leucine rich repeat containing receptor
NO	Nitric oxide
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCV	Pathogen-containing vacuole
PFA	Paraformaldehyde
PIKE	PI-3Kinase Enhancer
PMA	Phorbol myristate
Pru	Prugniaud
PV	Parasitophorous vacuole
RFLP	Restriction fragment length polymorphism
ROP16	Rhoptry protein 16
ROP18	Rhoptry protein 18
RPMI	Roswell Park Memorial Institute
RRBP1	Ribosome binding protein 1
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TAE	Tris-acetate-EDTA
TLR	Toll-like receptor
TMEM109	Transmembrane protein 109
TMEM214	Transmembrane protein 214
TMEM33	Transmembrane protein 33
TNFα	Tumour necrosis factor-alpha
VMA21	Vacuolar ATPase assembly integral membrane protein 21

Chapter 1. Introduction

1.1 IFNγ driven host response

Interferons are cytokines made in and secreted from host cells in response to a vast array of pathogens. They play a vital role in inhibiting the replication and spread of viral, bacterial and parasitic pathogens (de Veer et al., 2001). It is now also clear that they play much wider roles encompassing the regulation of cell proliferation, differentiation, survival and death (Samarajiwa et al., 2009).

There are 3 types of interferons, types I, II and III. All three types are responsible for activating multiple signal pathways that in turn lead to transcriptional activation of many interferon regulated genes (Samarajiwa et al., 2009). As a result, mice with an interrupted receptor for type I or type II interferons are exceptionally susceptible to viral infections and exhibit multiple defects in host defence (de Veer et al., 2001).

There is much debate and uncertainty over how many genes are upregulated by interferons, with estimates ranging from 500 genes (Boehm et al., 1997) to >1300 genes by IFNγ alone (MacMicking, 2004). But it is certain that of the genes upregulated by these cytokines, only a tiny fraction of their protein products have been identified and characterised.

1.2 Guanylate Binding Proteins

In 1983, Cheng et al. treated human fibroblasts with interferons and analysed the proteins that were synthesised. They identified a protein that could bind guanylate agarose columns. This 67kDa protein was induced in both human and mouse fibroblasts, and could not be detected in unstimulated cells (Cheng et al., 1983). The same group went on to identify at least two genes encoding the 67kDa IFNγ-induced protein. Furthermore they show that one of these 'Guanylate Binding Proteins' (GBPs) had the capacity to bind GMP as well as GDP and GTP, likely due to a missing N(T)KXD consensus motif that would confer guanine specificity (Cheng et al., 1991). As a result, GBP1 was identified as a GTPase that converts GTP to GMP, a unique characteristic among GTPases.

1.2.1 GBP discovery

As characterisation of GBP1 continued, sequence analysis of GBP1 determined that it could be isoprenylated (Nantais et al., 1996). Isoprenylation of a protein involves the attachment of a farnesyl or geranyl-geranyl lipid moiety to a C-terminal CaaX motif. This addition is important in targeting proteins to intracellular membranes or for facilitating protein/protein interactions. In the case of GBP1, a farnesyl modification was identified when a decrease in incorporation of [^{3}H]-mevalonate was observed upon addition of a farnesyl transferase inhibitor (Nantais et al., 1996). Isoprenylation of GBP1 enables its localisation to the Golgi apparatus, but only in the presence of IFNγ and when aluminum fluoride is added to mimic the GTP bound state (Modiano et al., 2005).

During the study mapping GBP1 to chromosome 1, a large number of bands were recognised on a Southern blot using a GBP1 cDNA probe - indicating that there were other closely related genes to be discovered (Strehlow et al., 1994), and so began the search for more GBP family members.

GBPs 2, 3 and 4 were identified (Strehlow et al., 1994, Luan et al., 2002, Olszewski et al., 2006) and found to have a strikingly high level of identity to GBP1 (77%, 88% and 56% respectively). GBP2 was shown to have an isoprenylation site, although this time it was a geranyl-geranyl motif (Britzen-Laurent et al., 2010). A further protein with very high homology to the known GBPs was discovered, and was defined as GBP5. This family member had the ability to be transcribed into at least 3 splice variants, from which 2 different proteins were produced. GBP5 was also found to be isoprenylated with a geranyl-geranyl motif (Vestal and Jeyaratnam, 2011). Interestingly, one of the GBP5 splice variants is tumour-specific and is truncated by 97 amino acids at the C-terminus, losing its isoprenylation motif (Fellenberg et al., 2004, Wehner and Herrmann, 2010). In 2006 all of the human GBPs currently known were finally identified, defining seven GBPs and one pseudogene to be present (Olszewski et al., 2006). All seven members are highly similar and are located on chromosome 1 (Boehm et al., 1997), however they are induced differentially. GBPs 1, 2 and 3 are upregulated in endothelial cells by IFNγ, tumour necrosis factor-alpha (TNFα) and interleukin-1-Beta (IL-1β), whereas GBPs 4 and 5 are upregulated only by IFNγ. These cells did not express GBPs 6 or 7 (Guenzi et al., 2001, Tripal et al., 2007). The same study looked at the subcellular

localisation of GBPs 1-5 by overexpression of fluorescently tagged proteins. GBPs 1, 3 and 5 were shown to have a cytoplasmic localisation, and GBPs 2 and 4 appeared nucleocytoplasmic. Although the GBPs show such a remarkably high identity to one another, these differences in induction and localisation indicate that they might have different cellular functions (Tripal et al., 2007).

1.2.2 GBP structure and biophysical properties

The biochemical and biophysical properties of GBP1 have been much studied over the last 3 decades. In 2004, the residues that were crucial for nucleotide binding and for cooperative GTP hydrolysis were determined (Praefcke et al., 2004). On this basis, point mutants were produced that could mimic GBP1 in either its nucleotide free form (K51A) or in its GTP bound form (R48A) (Praefcke et al., 2004). An external GTPase activating protein (GAP) is not required, as GBP1 contains an internal GAP (Abdullah et al., 2009). Consequently, as the concentration of GBP1 increases, a higher specific GTP hydrolysis activity occurs, with the protein forming homo-oligomers which in turn leads to catalytic activation of the GTPase (Kunzelmann et al., 2005). The G-domain of GBP1 can cleave GDP directly, which leads to a conformational change, positioning the protein in order to cleave GDP (Ghosh et al., 2006) thus allowing the unique ability to hydrolyse GTP to GMP.

The crystal structure of full length GBP1 was solved in 2000 (Prakash et al., 2000). It was seen that the N-terminal 278 residues consisted of a modified G-domain (Figure 1). The C-terminal was found to form an extended helical domain that also has unique features, comprising two three-helix bundles, the core of which was formed by hydrophobic residues that are connected by only one hydrogen bond and instead is stabilised by water-mediated contacts (Prakash et al., 2000). The structure clearly implicated GBP1 as part of the same large-GTPase group that includes Mx-proteins and dynamin, and from this it was suggested GBP1 had the ability to form oligomers.

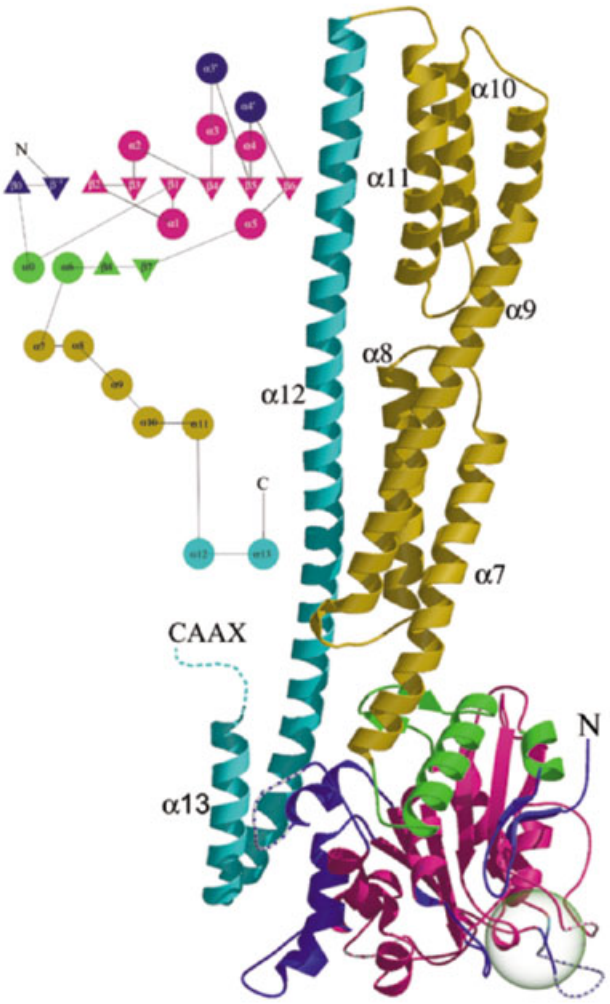


Figure 1 Tertiary Structure of GBP1

Structure of human guanylate binding protein 1 representing a unique class of GTP-binding proteins, (Prakash et al., 2000), *Nature* 403, 567-571.

Oligomerisation of proteins can regulate their subcellular localisation. Britzen-Laurent et al. showed that GBPs do homodimerise *in vivo* and that membrane association of the isoprenylated family members is a dependent factor (Britzen-Laurent et al., 2010). The K51A mutant, which represents nucleotide free GBP1, was not able to form dimers *in vitro*, indicating that GTP binding is required for this characteristic. The dimerisation and substrate binding occurs quickly with a rapid burst of phosphate ions before a steady state stage is reached. It is thought that the irreversible dissociation of the substrate acts as a rate-limiting step (Kunzelmann et al., 2005). Furthermore, GBP1 is not able to produce GMP if dimerisation does not take place, showing the allosteric step required is triggered

by dimerisation (Abdullah et al., 2010). Moreover, GBPs can heterodimerise *in vivo* in a hierarchical manner, resulting in the potential for membrane association of non-prenylated GBPs (Britzen-Laurent et al., 2010). The identification and characterisation of 11 murine GBPs (mGBPs) and 2 pseudogenes (Staeheli et al., 1984, Vestal et al., 1998, Han et al., 1998, Anderson et al., 1999b, Nguyen et al., 2002, Olszewski et al., 2006, Degrandi et al., 2007, Konermann et al., 2007) demonstrated that there was a high level of similarity between the human and the murine GBPs and showed that the proteins are well conserved across species (Degrandi et al., 2007). The mGBPs are found in clusters over two chromosomes, chromosome 3 containing mGBPs 1, 2, 3, 5, 7 and chromosome 5 containing mGBPs 4, 6, 8, 9, 10 and 11 (Kresse et al., 2008) (Figure 2).

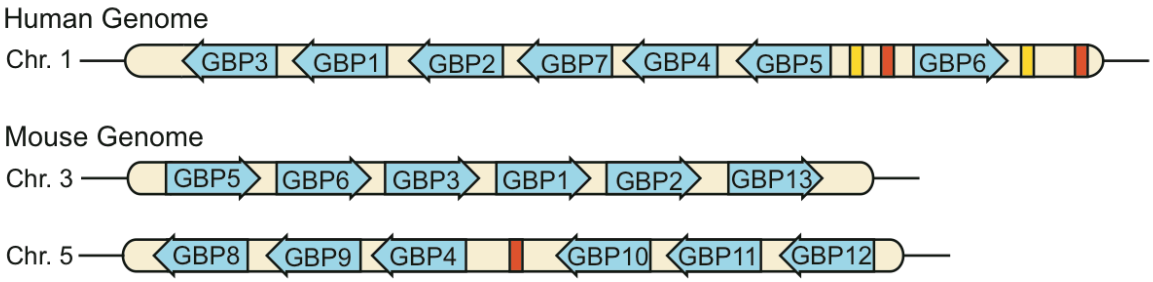


Figure 2 Genomic arrangements of GBPs

Schematic representation of the genomic arrangement of human and mouse GBPs. Red box denotes presumed pseudogenes. Yellow box denotes genes unrelated to GBPs. Adapted from (Shenoy et al., 2007), Immunobiology Volume 212, Issues 9–10.

1.2.3 GBPs as host defence proteins

The fact that these proteins are well conserved and are so highly upregulated in response to inflammatory cytokines would hint at a function involved in host response to pathogens. In both human and mouse, there is evidence that GBPs play an anti-viral role. Expression of GBP1 resulted in lower titres and reduced viral progeny of vesicular-stomatitis-virus and encephalomyocarditis virus in human cells (Anderson et al., 1999a). Restriction of the same two viruses was shown by mGBP2, the closest ortholog to human GBP1 (Carter et al., 2005). In 2007, Degrandi et al. characterised the molecular functions of mGBPs 1-10 (Degrandi et

al., 2007). C57Bl/6 mice were infected with either *Listeria monocytogenes* or *Toxoplasma gondii* before levels of mGBP induction were measured by either real time-PCR or mRNA expression patterns as detected on a gene chip array. All mGBPs were induced *in vivo* by these infections. Furthermore, the study defined the subcellular localisation of these GTPases during infection with *Toxoplasma gondii*. Interestingly, they showed that the parasitophorous vacuoles (PVs) of a strain of *Toxoplasma* considered to be non-virulent in mice were decorated with mGBPs 1, 2, 3, 6, 7 and 9 from just 30 minutes post infection in IFNγ-stimulated cells. Furthermore, infection with a virulent strain of *Toxoplasma* resulted in barely any mGBP decoration of the PVs (Degrandi et al., 2007). Human GBPs were also recruited to the PV, although in much lower amounts (Ohshima et al., 2014). The recruitment of GBPs to the PV of *Toxoplasma* in a virulence-strain dependent manner strongly suggested that these GTPases are an important set of host defence molecules.

1.3 *Toxoplasma gondii*

1.3.1 *Toxoplasma* characteristics and lifecycle

Toxoplasma gondii is an obligate intracellular parasite that resides within a parasitophorous vacuole and infects any nucleated cell type. While the definitive host is the feline, in which the sexual stage of the life cycle occurs, any warm-blooded animal can become an intermediate host. A succinct review of the first 100 years of *Toxoplasma* research was produced by Jitender Dubey in 2008 (Dubey, 2008).

Toxoplasma was first isolated in 1908 from the rodent *Ctenodactylus gundi* (Nicolle and Manceaux, 1908), where it was recognised as a new organism and named for it's shape (toxo – toxon Greek for arc) and the creature from which it was isolated (gundi) (Nicolle and Manceaux, 1909). While *Toxoplasma* was recognised within many animal samples over several decades it was not successfully isolated in a viable form until 1937, after which the isolates from humans and animals were proven to be the same species (Sabin and Olitsky, 1937). Studies on worldwide isolates of *Toxoplasma* found genetic variability (Pfefferkorn and Pfefferkorn, 1980, Dardé et al., 1987, Howe and Sibley, 1995,

Tibayrenc et al., 1991, Sibley and Boothroyd, 1992), with different strains demonstrating different levels of virulence in mice (Dubey, 2002, Lehmann et al., 2006). Molecular epidemiological and population biology studies first inclined that there were only three clonal lineages that result in the differing virulence and pathogenicity phenotypes (Sibley et al., 2009).

The parasites can be grouped into 'virulent' (type I), and 'avirulent' (types II and III). The virulent *Toxoplasma* are highly pathogenic and display very limited genetic diversity. The avirulent strains are further grouped into two distinct clonal lineages (Darde, 1996). These type II and III strains are the strains that have been isolated from AIDS patients and widespread outbreaks, and from agricultural animals (Belfort-Neto et al., 2007, Ferreira et al., 2008, Miller et al., 2004, Conrad et al., 2005, Demar et al., 2007, Demar et al., 2008). While the large majority of North American strains fall into these three recently derived lineages, the strains from South America have been shown to be more genetically diverse, falling into distinct genotypes (Sibley et al., 2009). These distinguished groups are shaped by biological geographic factors, population sweeps and infrequent sexual recombination.

There are several methods by which *Toxoplasma* has been genotyped. They include multi-locus enzyme electrophoresis, which was used in early studies on strain variation. This method was used to define several polymorphic enzymes and thus characterise isolates into three major zymodemes (Darde et al., 1992). Later, techniques focussed on identifying microsatellite markers. These are short, repeated segments of DNA that are frequently located in regions of non-coding DNA and was a successful method to use for high-throughput analyses (Ajzenberg et al., 2002b, Ajzenberg et al., 2002a). Restriction fragment length polymorphism (RFLP) was widely used as the method to genotype specific genetic loci, allowing for high-throughput analysis via PCR, restriction digest and gel electrophoresis, and was used to group types I, II and III (Sibley and Boothroyd, 1992). Direct sequencing of genomic regions using single nucleotide polymorphisms, insertions and deletions eventually revealed complete genetic diversity; for example highlighting the high diversity in the *GRA6* locus (Sibley et al., 2009). This is the method that provides the best approach for detecting polymorphisms in new isolates. With direct sequencing it could then be shown that the predominant clonal lineages varied by only 1-2% at the nucleotide level (Grigg et al., 2001) and

subsequent coalescence analysis supports a model by which the so-thought three linages evolve from a common ancestor in the last 10,000 years (Su et al., 2003), corresponding to the same time-frame when animals underwent domestication.

It was hypothesised that the divergence of isolates from South America was due not to differences in host range, but instead was due to geographical variations and recombinants of the genotypes prevalent in the north (Khan et al., 2006, Ferreira et al., 2008). However, when isolates from various regions were analysed in comparison to previously characterised North American samples there were in fact four ancestral lineages reconstructed. This shows that while South and North American *Toxoplasma* share a common ancestry, they are reproductively isolated (Khan et al., 2007); perhaps as a result of cats migrating over the Panamanian land-bridge (Johnson et al., 2006, Khan et al., 2007).

The lifecycle of *Toxoplasma* takes place in a number of hosts. The sexual stage of the lifecycle occurs in the intestine enterocytes of felines. Felines ingest an infected animal containing dormant parasite cysts and upon reaching the epithelium villi shizogonic and gametogonic stages develop within vacuoles situated alongside the brush border of the cell near the nucleus. These two stages are observed together in no particular sequence or preferred location along the entire villus epithelium (Hutchison et al., 1971). After a short incubation period of as little as 5 to 10 days, the felines release numerous diploid oocysts into the environment via their faeces (Dubey et al., 1972). The next stage of the *Toxoplasma* lifecycle takes place inside an intermediate host, which could be any warm-blooded animal. Ingested oocysts will develop into fast replicating haploid tachyzoites that multiply intracellularly by endodyogeny and disseminate throughout its host (Goldman et al., 1958, Frenkel and Dubey, 1973). Host death would occur if this stage continued indefinitely. Therefore, upon immune pressure from the host, the parasite slows its replication and develops into a haploid bradyzoite that will persist as tissue cysts in immune-privileged areas, like deep tissue and in the brain (including astrocytes, microglia and neurons), for the rest of the host's life (Jacobs et al., 1960a, Halonen et al., 1996, Fischer et al., 1997, Halonen et al., 1998, Freund et al., 2001, Schluter et al., 2001). Humans can become host to *Toxoplasma* in a number of different ways. One route is via the ingestion of undercooked meat that contains tissue cysts (Jacobs et al., 1960b, Desmonts et al., 1965). Humans can ingest oocysts that have been shed from

felines (Hutchison, 1965), by directly handling the faeces, from contaminated fruits and vegetables that are unwashed, or from a contaminated water supply (Benenson et al., 1982, Bowie et al., 1997, de Moura et al., 2006, Teutsch et al., 1979) (Figure 3).

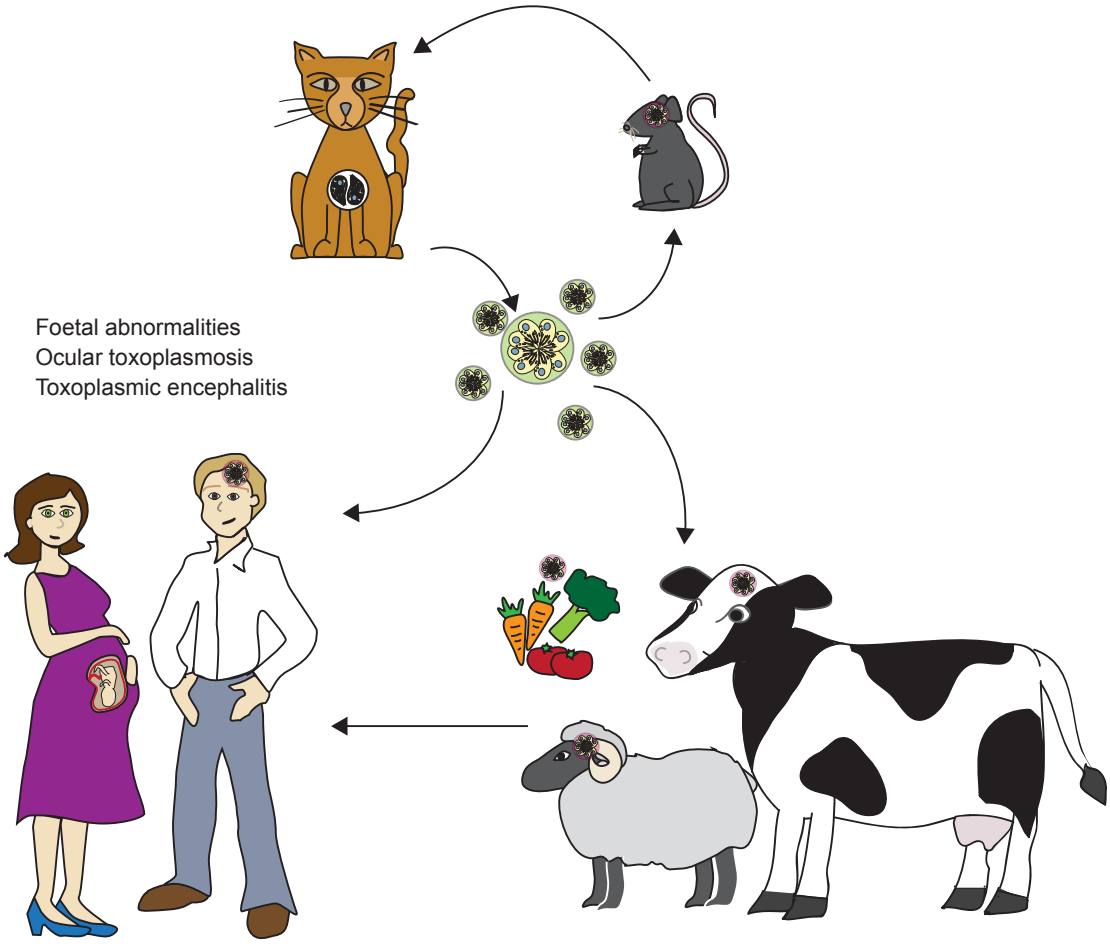


Figure 3 Lifecycle and hosts of *Toxoplasma*

Felines are the definitive host of *Toxoplasma*, and from cats parasite oocysts are shed in the faeces. These oocysts are ingested by intermediate hosts, in which the parasite develops first into tachyzoites, then dormant bradyzoites within cysts in immune-privileged sites. While most infections persist without the host being aware, complications can occur in immunocompromised hosts and pregnant females.

Once a human becomes infected, they can spend their whole life completely unaware of their *Toxoplasma* companion as the immune system keeps the parasite at bay. This results in a chronic infection that is not cleared (Johnson, 1992, Weiss et al., 2009). However, problems can arise under specific circumstances. If a woman becomes infected with the parasite during pregnancy, congenital

transmission and disease characterised by encephalomyelitis can occur that may lead to either abortion or severe foetal abnormality (Wolf et al., 1939, Sabin and Ruchman, 1942, Couvreur and Desmonts, 1962, Havelaar et al., 2007). Furthermore, a host who is immunocompromised, for example an AIDS patient or a patient undergoing immunosuppressive therapy, can die from toxoplasmosis of the brain when a lift of immune pressure causes *Toxoplasma* to revert back to its fast-replicating tachyzoite stage (Luft et al., 1983, Israelski et al., 1988). Before the AIDS epidemic neurological toxoplasmosis was rare, however during this time the level of acquired infection became apparent. Without treatment, *Toxoplasma* reactivation was fatal and even with treatment still carried a 10-30% mortality rate (Luft et al., 1983, Luft and Remington, 1992). Complications also arise in the form of ocular disease caused by *Toxoplasma*-driven inflammation and formation of lesions in the eye (Wilder, 1952, Grigg et al., 2001, Park and Nam, 2013). Although these occurrences of eye disease and severe ocular inflammation are relatively regularly observed outcomes of an infection (Janku, 1923, Wilder, 1952), studies within Brazil have shown an increased prevalence of ocular toxoplasmosis in this location. This variation in disease phenotype in acquired infection would suggest that in humans some strains may be more pathogenic that others (Silveira et al., 1988, Glasner et al., 1992).

Toxoplasma can cause severe disease and death in humans, but it is also a large problem in the animal world. This parasite has been responsible for innumerable abortions in sheep and thus has a high economic impact (Hartley and Marshall, 1957, Beverley, 1961, Dubey and Welcome, 1988). *Toxoplasma* demonstrated its parasitic success by causing widespread fatality in sea otters, fur seals and endangered monk seals when oocysts contaminated marine waters (Holshuh et al., 1985, Honnold et al., 2005, Conrad et al., 2005). For these reasons, it is important that this parasite is studied and understood further, to find effective treatments and vaccines to control it. Further, understanding the effects different strains produce will allow a more targeted approach for therapy.

The *Toxoplasma* strains that exist are the same species, however the polymorphic effector genes allow for varied responses. Virulence factors, including rhoptry protein 16 (ROP 16) and dense granule protein 15 (GRA 15) play key strain-dependent roles in host response to infection. While both type I (RH) and II (Prugniaud) parasites harbour GRA15 genes, the polymorphic nature of these

genes allows for different effector mechanisms, with type II able to manipulate the NFKB signalling pathway much more significantly than type I (Rosowski and Saeij, 2012). This same effector in type I is responsible for inhibiting interferon regulating factor 3, a proinflammatory transcription factor, (Rosowski and Saeij, 2012) as well as protecting the PV from host protection mechanisms (Virreira Winter et al., 2011) (discussed further later) while playing little effect in these manners in type II parasites. The ROP16 kinase in type I *Toxoplasma* plays an important role by phosphorylating STAT1 into a non-transcriptionally active form, successfully down-regulating IFNγ-signalling pathways. However, while type II does not show this effect, the same phenotype is produced when a type I copy of the gene is ectopically expressed (Rosowski and Saeij, 2012). The need to understand how different strains affect pathology will be important in understanding how to recognise and control infection.

1.3.2 Host responses against *Toxoplasma*

The immune response against *Toxoplasma* has been widely studied with many different aspects being highlighted as important for infection control. Dupont et al. elegantly reviewed the immune reactions to this parasite, emphasising the varied models by which a host controls this infection (Dupont et al., 2012). *Toxoplasma* is capable of infecting any nucleated cell type, with a slight preference for macrophages reported (Zhao et al., 2014). The active invasion of *Toxoplasma* into a cell requires the secretion of three waves of proteins. These proteins are made up of micronemes, dense granules and rhoptry proteins, and can modify the host cell behaviour and can inhibit immune responses (Lim et al., 2012). The parasite moves into the cell surrounded by host lipid membrane, which it can then modify to become it's safe-haven, the PV. *Toxoplasma* appears to hide from the immune system within the PV, so questions are raised as to how the host immune system interacts with the parasite.

Monocytes, neutrophils, dendritic cells (DCs) have all been implicated in orchestrating protection against *Toxoplasma*. It is true that one of the critical functions of these cells in the response to *Toxoplasma* is the production of interleukin-12 (IL-12). This in turn stimulates the production of IFNγ from natural killer (NK) cells and T cells (Gazzinelli et al., 1993, Gazzinelli et al., 1994, Hunter et

al., 1994). It is acknowledged that IFNγ is the main mediator of resistance to *Toxoplasma* since it is responsible for promoting a large array of killing mechanisms (Suzuki et al., 1988). A TH1 immune response ensues, which is characteristic of many intracellular pathogen infections. Mice that are deficient in either IL-12 or IFNγ cannot control infection and succumb shortly after challenge (Suzuki et al., 1988, Hunter et al., 1993, Gazzinelli et al., 1994). The role of toll-like receptors (TLRs) is important in host defence, as they are responsible for the initial sensing of parasite products. A range of TLRs, including TLR2, TLR4 and TLR11, respond to *Toxoplasma* profilin or to glycosylphophatidylinositols that are found on the parasite surface (Yarovinsky et al., 2005, Debierre-Grockiego et al., 2007). TLRs are also responsible for sensing the insult on the host that occurs as the tachyzoites translocate from the gut (Benson et al., 2009).

As IL-12 is a vital first step in the attack against *Toxoplasma,* numerous studies have been conducted to identify the primary cell types that are responsible for the release of this cytokine *in vivo*. Many sources have been identified, including neutrophils, inflammatory monocytes, macrophages and DCs (Gazzinelli et al., 1996, Bliss et al., 1999, Bliss et al., 2000, Scanga et al., 2002, Mordue and Sibley, 2003, Whitmarsh et al., 2011). $CD8^+$ DCs were defined as the prominent cell type releasing IL-12 *in vivo* (Reis e Sousa et al., 1997). Furthermore, in mice deficient in the transcription factor Batf3, a lack of $CD8^+$ DCs resulted in a severe IL-12 defect and reduced $CD8^+$ T cell responses; a consequence of which was increased parasite load and death of the host (Mashayekhi et al., 2011). Monocytes also play a role in resistance, showing an increased susceptibility to infection when the recruitment chemokine receptor, CCR2, is lacking in mice (Robben et al., 2005, Benevides et al., 2008, Dunay et al., 2010). One reason for their importance could be the role monocytes play in the production of nitric oxide (NO) (Dunay et al., 2010). This direct control mechanism has been shown to decrease parasite replication, with NO-deficient mice dying during *Toxoplasma* challenge (Benevides et al., 2008). While the function of NO in parasite control has to be fully elucidated, there is evidence that NO could stimulate early egress of tachyzoites from macrophages and by inhibiting reactivation of parasites to tachyzoites in the brain (Gazzinelli et al., 1993, Khan et al., 1997, Schluter et al., 1999, Yan et al., 2015). NK cells play an important role in the production of IFNγ (Denkers et al., 1993, Sher et al., 1993, Johnson et al., 1993, Hunter et al., 1994) and also promote the

adaptive immune responses with $CD8^+$ T cells via interactions with natural-killer group 2, member D protein (NKG2D) that is expressed on both cell types (Guan et al., 2007).

Adaptive immune responses are an important part of the defence of a host against *Toxoplasma*. Mice deficient in B cells or $CD4^+$ T cells survive the acute phase of infection and have normal IFNγ responses, but ultimately succumb to the disease as a result of increased parasite burden in the central nervous system (Denkers et al., 1997, Kang et al., 2000, Johnson and Sayles, 2002). $CD8^+$ T cells play a necessary role in both the acute and chronic infection. Mice lacking Batf3 transcription factor have severely depleted $CD8^+$ are significantly more susceptible to *Toxoplasma* and do not survive the acute phase of infection (Mashayekhi et al., 2011). Furthermore, when $CD8^+$ T cells are depleted in mice that are chronically infected, the parasite cysts are significantly increased in size, with an upset in the amount of cytokines being produced, as well as a loss of vaccine-induced immunity (Gazzinelli et al., 1991, Guiton et al., 2009). Therefore, in the host defence mechanism against *Toxoplasma,* both the innate and adaptive immune responses are crucial, with IL-12 and $CD8^+$ cells proving vital for host survival.

1.3.3 IFNγ effector mechanisms against *Toxoplasma*

There are a number of *Toxoplasma* killing mechanisms that are driven by the cytokine IFNγ. One of the mechanisms is the upregulation of indolamine-2,3-dioxygenase (IDO) 1 and 2. This enzyme is responsible for catalysing the degradation of tryptophan (Murray et al., 1989, Prendergast, 2008) in many cell types, including fibroblasts, macrophages and brain cells (Pfefferkorn, 1984, Murray et al., 1989, Daubener et al., 1996, Daubener et al., 2001). As *Toxoplasma* is an auxotroph for tryptophan, the IFNγ-meditated reduction of the amino acid results in inhibition of parasite growth. Mice that have been treated with IDO 1 and 2 inhibitors during a *Toxoplasma* challenge have much increased susceptibility to disease and significantly increased parasite burdens (Divanovic et al., 2012).

In mice, IFNγ is also responsible for the upregulation of a family of p47 large GTPases, the immunity related GTPases (IRGs). Mice that are deficient in Irgm3 exhibit normal IFNγ responses, but soon die due to increased parasite burden

(Taylor et al., 2000). As studies progressed, it became clear that other family members are involved in host response to *Toxoplasma*, and a similar susceptibility was seen in mice lacking Irgm1, Irgd, Irga6 or Irgb6 (Collazo et al., 2001, Zhao et al., 2009b, Fentress et al., 2010, Pawlowski et al., 2011). IRGs are actively co-localised to the PV of *Toxoplasma*, resulting in the PV forming a tight-fitting morphology around the parasite. After this recruitment, the PV membrane blebs and ruffles and finally lyses, and is stripped away (Martens et al., 2005, Ling et al., 2006, Hunn et al., 2008, Zhao et al., 2009a, Zhao et al., 2009b, Zhao et al., 2010, Khaminets et al., 2010, Steinfeldt et al., 2010). The result of this action is that the parasite is no longer in its safe-haven, and is free in the cytoplasm where it undergoes permeabilisation and is killed (Melzer et al., 2008).

Guanylate binding proteins also make up an important arm of the IFNγ host response against *Toxoplasma*. As mentioned earlier, mGBPs are recruited around the PV of type II 'avirulent' *Toxoplasma*, and can be manipulated to be inactive by the type I 'virulent' strain. It was hypothesised after this observation that GBPs were important anti-microbial effectors mechanisms.

As IRGs had been shown to have such an important function during *Toxoplasma* infection, it seemed likely that GBPs, which surrounded the PV in a similar manner, were also vital to host response. Mice deficient in the cluster of GBPs found on chromosome 3 ($GBP^{chr3-/-}$) were engineered by targeted chromosome deletion and infected with *Toxoplasma*. These mice showed significantly increased susceptibility to infection, attributed to a much increased parasite load throughout the animal (Yamamoto et al., 2012). Furthermore, it was identified that in bone marrow derived macrophages (BMDMs) from $GBP^{chr3-/-}$ mice, the recruitment of the IRGs is negatively affected. The result of the loss of recruitment of these effector molecules is a lack of blebbing and ruffling of the *Toxoplasma* PV in $GBP^{chr3-/-}$ BMDMs. When the GBPs were complemented back into the macrophages, IFNγ-meditated protection was restored (Yamamoto et al., 2012).

1.3.4 *Toxoplasma* and GBPs

In 2011, Virreira Winter et al. successfully confirmed that IFNγ-dependent relocalisation of mGBPs around the *Toxoplasma* PV correlated with the virulence type of the parasite. They identified three specific parasitic factors that determined the difference in virulence types between parasite strains. These were ROP16, rhoptry protein 18 (ROP18) and GRA15. Therefore, it was suggested that the virulence of *Toxoplasma* depended on the recognition of GBPs. A mass spectrometric analysis of mGBP1 isolated from an infected cell culture identified mGBPs 2, 4 and 5 as its binding partners, indicating that these large GTPases are brought the to PV as part of a large multimeric structure (Virreira Winter et al., 2011). How either GBPs or IRGs can recognise the PV of *Toxoplasma* is an interesting question. The parasite survives inside the cell protected by a vacuole composed of host cell membrane that keeps it largely invisible to host defence mechanisms. So how do these large GTPases recruit to the PV so efficiently? In 2013, it was shown that the targeting of these GTPases requires the formation of higher-order protein oligomers, and that this was regulated by the immunity related GTPase family M (IRGM) (Haldar et al., 2013). Irgm1 and Irgm3 proteins do not localise to the pathogen vacuoles, but instead reside on self-organelles. As a result, the organelles are guarded against an association with GBPs and IRGs. By inference, GBPs and IRGs were located upon the entities in the cell that are missing the ‘self’ IRGM proteins (Haldar et al., 2013).

The recruitment of mGBPs and mouse IRGs to the vacuole of *Toxoplasma* is dependent on the autophagy protein 5 (Atg5). Atg5 is part of a complex that facilitates the transfer of an ubiquitin-like protein from autophagy-related protein 8 (Atg8) to the lipid phosphatidylethanolamine, and a role in the activation of the GTPase activity of large GTPases is hypothesised (Haldar et al., 2013). Further investigation of the function of autophagy proteins in cell-autonomous responses against *Toxoplasma* showed that, in mouse, IFNγ-dependent suppression and GTPase recruitment was significantly reduced when Atg7 or Atg16L1 were not present (Ohshima et al., 2014). However, interestingly, when either Atg16L1 or all of the GBPs were knocked out in human cells, the IFNγ-dependent response was not affected (Ohshima et al., 2014). Deposition of mGBPs is shown not only to be dependent on autophagy proteins, but also on wider ubiquitination systems, with

the E3 ligase TRAF6 regulating association of mGBPs with the PV (Haldar et al., 2015).

Specifically, mouse GBP1 is implicated in contribution to cell-autonomous immunity against *Toxoplasma* (Selleck et al., 2013). The ability of this family member to recruit around the PV is mediated by ROP18, the pseudogene ROP5 or an active threonine kinase. When these virulence factors were knocked out, parasite clearance in BMDMs was possible in the presence of IFNγ. Mice deficient in mGBP1 showed an increased susceptibility even to type II 'avirulent' *Toxoplasma*. However, this susceptibility was reversed when the infecting strain was a ΔROP18 mutant (Selleck et al., 2013). Mouse GBP2 also plays a role in defence against *Toxoplasma*, with BMDMs from mGBP2-deficient mice being unable to control the replication of the parasite (Degrandi et al., 2013).

1.4 GBPs, bacteria and the inflammasome

As the roles played by the GBPs against *Toxoplasma gondii* are becoming clearer, so are the roles played against other pathogens. *Toxoplasma* is an intracellular pathogen that resides inside a vacuole, as is *Chlamydia trachomatis*. When HeLa cells were infected with *C. trachomatis*, it was observed that GBPs 1 and 2 localised to the vacuole, or inclusion, of this bacteria. Moreover, when these same GBPs were knocked down using silencing RNA, the inclusion sizes were significantly larger, indicating enhanced bacterial replication. The opposite result was observed when GBP1 or 2 were overexpressed in the same cells (Tietzel et al., 2009, Al-Zeer et al., 2013). A strain of *C. trachomatis* that was hyper-virulent, due to presence of a putative cytotoxin gene, was not affected by GBP overexpression (Tietzel et al., 2009). The inclusions decorated with GBPs were targeted for autophagic destruction (Al-Zeer et al., 2013), thereby implicating GBPs in functions related to autophagy. It was shown that Atg5 and 3 play vital roles in successful decoration of the pathogen vacuole with GBPs during a *Toxoplasma* infection, and the same is true for C. *trachomatis* (Haldar et al., 2014).

The mechanisms by which GBPs exert their functions have only been begun to unravel over the last four or so years. Inflammasomes are complexes responsible for sensing and altering the immune system to danger such as

infection or tissue damage. They are activated by nucleotide-binding domain, leucine rich repeat containing receptor (NLR) proteins and absent in melanoma 20-like receptor (ALR) proteins to activate the cleavage of caspase-1, which in turn upregulates IL-1β and IL-18 secretion as well as cleavage of gasdermin protein to induce pyroptosis (Guo et al., 2015). Shenoy et al. were able to demonstrate that GBP5 was a non-NLR or -ALR protein that could stimulate inflammasome assembly. Furthermore, in response to lipopolysaccharide (LPS), *Listeria monocytogenes* or *Salmonella enterica* typhimurium GBP5 selectively promoted NLRP3 inflammasome responses. BMDM from mice that were deficient in GBP5 had significant defects in their ability to cleave caspase-1, IL-1β and IL-18 *in vitro* indicating an important role in these events (Figure 4). *In vivo*, the GBP5-deficient mice had impaired defence and inflammasome assembly against a *L. monocytogenes* infection (Shenoy et al., 2012). Furthermore, mGBPs were implicated in the activation of the non-canonical inflammasome in caspase-11-dependent host defence against gram-negative bacteria (Pilla et al., 2014, Meunier et al., 2014) (Figure 4). *Legionella pneumophila* and *C. trachomatis* infection trigger pyroptosis in IFNγ-activated macrophages, however induction of this response is lost when $mGBP^{chr3-/-}$ macrophages are used (Pilla et al., 2014, Finethy et al., 2015). Additionally, the mGBP cluster on chromosome 3 is responsible for a pyroptosis reaction to the LPS from *L. pneumophila* or *S. enterica* typhimurium, suggesting a role for GBPs in cytosol sensing of bacteria (Pilla et al., 2014). GBPs are recruited to the pathogen-containing vacuoles of these bacteria and are necessary for the breakage of said vacuoles. The lysis of these safe-havens releases LPS in to the cytosol, where it is detected, allowing the host to mount a full response (Meunier et al., 2014). A second consequence of vacuole breakage is the recognition by galectin 8 of previously unexposed host glycans, which in turn leads to recruitment of nuclear dot protein 52 (NDP52) and the uptake of bacteria into autophagosomes (Thurston et al., 2012, Meunier et al., 2014).

The absent in melanoma 2 (AIM2) inflammasome response detects double-stranded DNA in the cytosol and mounts the appropriate host defence. The mGBPs 2 and 5 play a role in activation of this system during infection with the cytosolic bacteria *Francisella novicida* (Man et al., 2015, Meunier et al., 2015). After initial detection of the pathogen, IFNs upregulate interferon regulatory factor (IRF) 1, which in turn induces expression of mGBPs. Mouse GBPs 2 and 5 recruit to the

bacteria and promote bacteriolysis, DNA release, and therefore bacteria killing (Man et al., 2015, Meunier et al., 2015) (Figure 4).

The renewed interest in GBPs has shown them to be powerful responders to bacterial infections, reacting by inducing the inflammasome via a number of different pathways. It will be fascinating to see if this response is consistent for different pathogens, including *Toxoplasma gondii*.

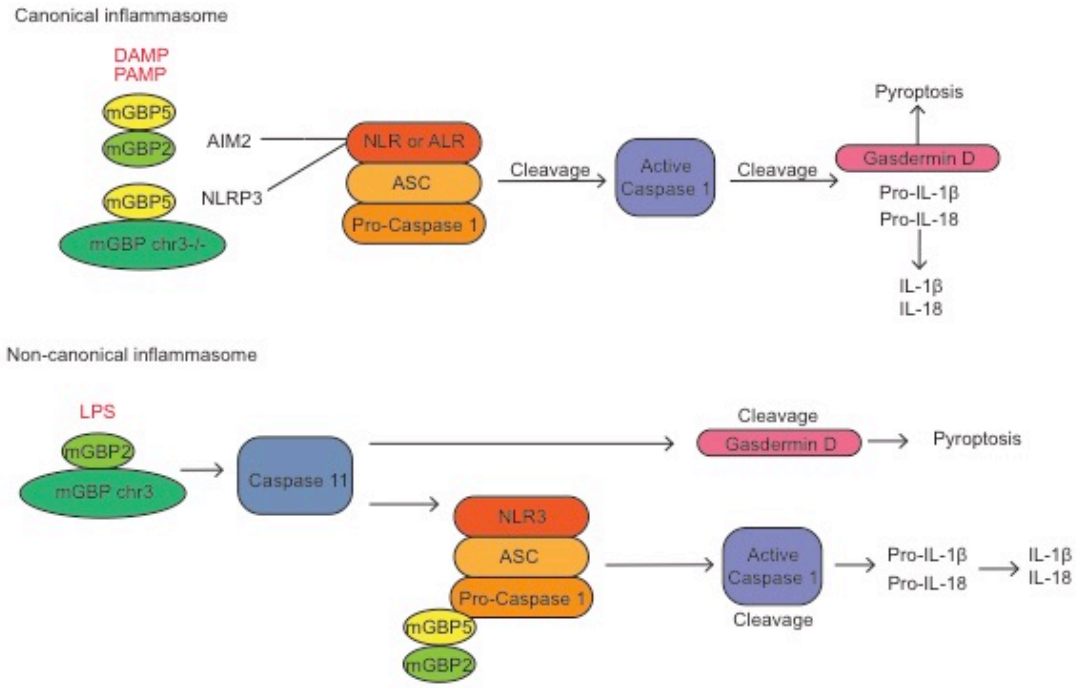


Figure 4 Mouse GBP dependent induction of inflammasome responses

Mouse GBPs have been implicated in both the canonical and non-canonical activation of inflammasomes. Adapted from (Kim et al., 2016), Nature Immunology 17, 481–489.

1.5 The aim of this thesis

As the roles that are dependent on GBPs become more unravelled and the mechanisms by which they work are becoming clearer, it is apparent they are a very important family of antimicrobial effectors. The mechanistic work so far regarding the inflammasome has been carried out in a murine species. Similarly, all but one study regarding GBPs and *Toxoplasma* infection focuses on mice (Ohshima et al., 2014). As GBPs have been shown to work so coherently with IRGs in a number of settings, it is important to move these studies into a human system. Humans only contain 2 IRG proteins; one is damaged in many ways and is

no longer IFN-inducible, and the other has only the G domain transcribed. As a result both versions are indicated to be non-functional in the conventional manner (Kim et al., 2012). While the GBPs are well conserved between species, there may be a vast difference in their modes of action as a result of the evolutionary pressures and the loss of a group of significant interaction partners.

The aim of this thesis is to study GBPs in a human system, while focussing on a *Toxoplasma gondii* infection model. The first step will be to develop the necessary tools for functional characterisation of GBPs, including producing antibodies and characterising mutant cells. As the GBP family members show such a high level of identity between family members, developing reagents that are specific for one family member is important to ensure the definite allocation of functions. Secondly, I will establish stable and reliable overexpression of these proteins in an appropriate cell type. While there have been overexpression models published in the literature, I, and others, have observed that no one has published systems that are consistently used. I will move to create these systems to enable the study of this group of large GTPases.

Using the reagents I will produce, I will study the subcellular localisation of individual GBPs both in a steady state cell and on the context of a *Toxoplasma* infection. I will determine whether or not GBPs recruit to the PV and how infection may change the location of individual family members. Furthermore, I will use these systems to study the effects of these proteins on the replication and survival of *Toxoplasma*. I will work to develop appropriate survival and invasion protocols in order to reliably assess the parasites viability.

I will begin to unravel the characteristics and functions of these intriguing proteins, but I also want to develop tools and protocols that will provide others, both within the lab and those who are collaborators, with a solid ground on which to further our knowledge of guanylate binding proteins.

Chapter 2. Materials & Methods

2.1 Tissue Culture

2.1.1 Cell culture

All cells were cultured at 37˚C in 5% CO_2. Human foreskin fibroblasts (HFFs) (ATCC #CRL-2429) were cultured in Dulbecco's Modified Eagle Medium (DMEM) + GlutaMax (Thermo Fisher Scientific #10566016) supplemented with 10% heat-inactivated foetal bovine serum (FBS) (Life Technologies #10500064). The cells were passed 1:10 when confluency was reached. A549 cells (ATCC #CCL-185) were cultured in DMEM + GlutaMax supplemented with 10% FBS and were passed 1:5 when 80% confluency was reached. Human umbilical vein endothelial cells (HUVECs) (Promocell #C12203) were cultured in M199 medium (Life Technologies #11043023) supplemented with 20% FBS, 10U/ml heparin (Sigma #H-3149) and 30mg/ml endothelial cell growth supplement (Upstate 02-102). HUVECS were allowed to reach 70% confluency before being passed 1:4, these cells were not used beyond passage 10. THP-1 monocytes (ATCC #TIB-202) were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium +L-glutamine (Gibco #11875093) supplemented with 10% FBS. These suspension cells were passed 1:4 when they reached $1X10^6$ cells/ml. To differentiate into macrophages, the cells with stimulated with 1μM phorbol myristate acetate (PMA) (Sigma #P8139) for 3 days. HEK293T cells, human kidney cells commonly used for retroviral vector production, (ATCC #CRL-3216) were cultured in DMEM + GlutaMax supplemented with 10% FBS, and were passed 1:5 when 70% confluency was reached. 3T3 fibroblasts (kind gift from Jonathan Howard) were cultured in DMEM +GlutaMax supplemented with 10% FBS and were passed 1:8 when 80% confluency was reached. When appropriate, the cells were stimulated for induction of protein expression by adding 1nM mifepristone hormone (Invitrogen #H110-1) and incubating for 24 hours at 37˚C in 5% CO_2. Where IFNγ stimulation was required, 10U/ml IFNγ (Promega #FHC24802) was added to the culture media and incubated for the appropriate time.

2.1.2 Culture of *Toxoplasma gondii*

Parasites expressing eGFP/luciferase or tdTomato, strains type I RH and type II Prugniaud (Pru) were maintained on a monolayer of HFFs in T25 culture flasks (Corning #CLS3289), with the cells cultured as described above. Once the *Toxoplasma* had exhausted the feeder cell supply, the monolayer was scraped and the parasites freed by syringe lysis using a 25-gauge needle, before 200µl type I or 500µl type II suspensions were reseeded onto fresh HFFs.

2.1.3 Infection of cells with *Toxoplasma gondii*

Cells were cultured as appropriate before the layer was scraped mechanically, with the resulting suspension was passed through a 25-gauge needle thereby liberating *Toxoplasma* from vacuoles by syringe-lysis. The *Toxoplasma* were counted on a haemocytometer and the appropriate multiplicity of infection (MOI) was seeded upon the cell monolayer. To ensure simultaneous invasion of cells, the cultures were then centrifuged for 5 minutes at 200xg at room temperature. The infection was allowed to persist for the appropriate length of time at 37˚C in 5% CO_2.

2.1.4 *Toxoplasma* irradiation

Where appropriate, the *Toxoplasma* were γ-irradiated in HFF cells with 15000 rad using a Gammacell 40 ^{137}Caesium Irradiator before following the infection protocol as described above.

2.2 Protein Biochemistry

2.2.1 Cell lysis

For adherent cells: the media was aspirated and the cell layer was washed 2 times by covering in ice-cold phosphate buffered saline (PBS) (Sigma #D8537). Ice-cold lysis buffer was added and the cell layer was scraped before the suspension was collected into an eppendorf tube and stored on ice. For suspension cells: the cells were centrifuged for 6 minutes at 300xg, the supernatant was discarded and the

cell pellet was resuspended in ice-cold PBS. The cells were centrifuged at 4˚C for 6 minutes at 300xg to wash them, and then the process was repeated. After the supernatant was aspirated following the second wash, ice-cold lysis buffer was added to the pellet, and it was resuspended by flicking the tube. The tubes were vortexed briefly to ensure lysis of the nuclei, before being incubated on an end-over shaker at 4˚C for 1 hour. The suspension was centrifuged at 12000xg for 15 minutes at 4˚C, after which the total soluble lysate was collected. The protein concentration was calculated using Bio Rad Protein assay dye reagent (Bio Rad #5000006). 100µl of reagent was added to 400µl PBS. 2.5µl of lysate was added to the mixture, followed by 500µl PBS. The mixture was incubated at room temperature for 10 minutes after which the absorbance (A) was quantified by a spectrometer at OD595. The protein concentration in mg/ml was calculated using the equation (A*14.44)/2.5.

2.2.2 SDS-PAGE and Immunoblot

Protein expression was analysed by separation with sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) followed by identification using antibodies in immunoblot. Cell lysates were produced as described. 10µg of protein denatured in Laemmli sample buffer containing dithiothreitol as reducing agent (NEB) was loaded into a precast 4-12% Bis/Tris NuPage gel (Thermo Fisher Scientific #NW0412) to be run on the Invitrogen Gel System, with a lane dedicated to the pre-stained protein standard ladder Novex Sharp (Thermo Fisher Scientific #LC5800). The gels were run using 200V until the dye front reached the bottom of the gel. The gel was transferred onto nitrocellulose membranes using the iBlot dry blotting system as described by the manufacturer (Thermo Fisher Scientific #IB21001) before staining with Ponceau-S solution (Sigma Aldrich #P7170-1L) to visualise protein. The membranes were blocked in Blotto solution (5% non fat milk in PBS + 0.05% Tween 20 with 0.02% sodium azide as preservative) for 1 hour with shaking at room temperature before probing with a primary antibody for the appropriate time on an orbital shaker. All commercial antibodies were diluted in buffers to manufacturer specification. The polyclonal anti GBP and 2 antibodies were diluted in 5% non fat milk in PBS + 0.05% Tween 20 with 0.02% sodium azide

as preservative, the polyclonal anti-GBP4 antibody was diluted in 1% BSA in PBS + 0.05% Tween 20 with 0.02% sodium azide, the monoclonal antibodies were not diluted. The membranes were washed 3 times for 5 minutes each in PBS 0.05% Tween 20 before being probed with the appropriate secondary antibody on an orbital shaker. After the membranes were washed 3 times for 5 minutes with PBS 0.5% Tween 20, they were incubated with Immobilion Western Chemiluminescent HRP substrate (Merck Millipore #WBKLS0100) for 5 minutes at room temperature, with shaking. The membranes were then exposed to x-ray film before developing the film and visualisation.

2.2.3 Immunoprecipitation

Cell lysates were prepared as stated, using buffer containing 0.5% NP-40 detergent. An appropriate volume of primary antibody was added to 0.5mg of the protein sample, which was mixed well and incubated for 30 minutes at 4˚C on an end-over shaker. At the same time, 50µl of Protein-G-sepharose beads (AbCam #ab193259) were prewashed in dolphin tubes (Sigma Aldrich, #Z717533) by centrifugation for 2 minutes at 1800xg 3 times using lysis buffer. The cell lysate was added to the beads and mixed well. The suspension was incubated for 1 hour at 4˚C on an end-over shaker. Centrifugation of the suspension at 4˚C for 2 minutes at 1800xg followed, the supernatant was removed and the beads were washed 3 times in lysis buffer by centrifugation. The washed beads were resuspended in 1x-SDS sample buffer and the sample was boiled for 5 minutes to remove the sample from the beads. The beads were pelleted by centrifugation in a microfuge at full speed (1200xg) for 30 seconds, and the supernatant was loaded onto a gel for SDS-PAGE and analysis.

2.2.4 Immunofluorescence

Cells were cultured on 9mm glass coverslips (Gerhard Menzel GMBH #LDRND9/1.5). When the cells reached confluency the media was aspirated and the cells were washed twice in PBS. The coverslips were incubated with 3% paraformaldehyde (PFA) for 15 minutes at room temperature, following which they

were washed once with Perm Quench buffer. The wash was replaced with fresh Perm Quench and the coverslips were incubated for 10 minutes at room temperature. The Perm Quench was aspirated and PGAS buffer was added, with the cells incubating for at least 5 minutes. The primary antibody was diluted at an appropriate concentration in PGAS. The coverslips were placed cell side down onto a 50µl drop of antibody solution and were incubated for 1 hour at room temperature in a humidified chamber. The coverslips were washed 3 times in PGAS before the incubation process was repeated, in the dark, with the appropriate fluorescent secondary antibody diluted in PGAS. The coverslips were subjected to 3 washes in PGAS, followed by 3 washes in PBS. The final PBS wash contained 1:10,000 dilution of Hoechst DNA stain (Thermo Fisher Scientific #H3570). The coverslips were rinsed twice in dH_2O before being mounted onto glass slides (Thermo Scientific Superfrost Plus #J1800AMNZ) using Mowiol-488 polymer (Sigma Aldrich #81381). The slides were incubated overnight in the dark at room temperature to ensure adhesion before being visualised on a bright-field or confocal microscope. Buffer recipes can be found in Appendix.

2.2.5 Confocal microscopy and image analysis

Slides were viewed using a Zeiss 510 Inverted Microscope with a x100 lens, and were analysed using Zen Blue software or were imaged on a Leica SP5-invert Confocal microscope using x100 objective and analysed using LAS-AF software. Further image formatting was done using Image J software.

2.2.6 Subcellular fractionation

Subcellular fractionation was carried out using the Qiagen Qproteome Cell Compartment Kit, #37502. This is a proprietary kit in which the buffer components are kept confidential. The protocol was followed as per manufacturer instructions. Briefly, $5x10^6$ cells were harvested, washed twice in ice-cold PBS by centrifugation for 5 minutes at 300xg. The cells were lysed in CE1 buffer by incubating on an end-over shaker at 4˚C for 10 minutes. The lysate was centrifuged at 4˚C for 10 minutes at 1000xg, with the resulting supernatant containing the cytosolic protein fraction.

This fraction was stored on ice, and the pellet was resuspended in CE2 buffer by pipetting up and down. The suspension was incubated for 30 minutes on the end-over shaker at 4˚C before centrifuging at 6000xg for 10 minutes at 4˚C. The resulting supernatant contains the membranous protein fraction; this was aspirated and stored on ice. The pellet was resuspended in 7µl manufacturers benzonase nuclease mixed with 13µl distilled water, and incubated for 25 minutes at room temperature. CE3 buffer was added to the mixture, and the suspension was incubated for 10 minutes at 4˚C on an end-over shaker. The sample was then pelleted by centrifugation at 6800xg at 4˚C for 10 minutes, with the resulting supernatant forming the nuclear protein fraction. This fraction was removed and stored on ice. The pellet comprises the cytoskeletal proteins, and was resuspended in buffer CE4. The samples were then precipitated with ice-cold acetone, before being resuspended in distilled water and quantified using the Bio Rad protein assay dye as described above.

2.2.7 Protein degradation inhibition

Cells were grown to 90% confluency, and were treated with the appropriate degradation inhibitor for 60 minutes. Type II *Toxoplasma* were used to infect the cells at an MOI of 3, with the infection being allowed to persist for 2 hours before the cells were harvested and lysed as described above for protein expression analysis. See Table 1 for information on usage and source of each component.

Table 1 Protein degradation inhibitors

Inhibitor	Manufacturer	Usage
MG132	Cambridge BioScience # CAY10012628-5	10µM
Leupeptin	Enzo Life Sciences #ALX-260-009	10µg/ml
Calpastatin	Cambridge BioScience #16501-100-CAY	10µM
Lactacystin	Cayman #19680219	40nM
Z-VAD-FMK	Invivogen #ABE266	4µg/ml

2.3 Overexpression and cell cloning

2.3.1 Cell transfection with TransIT 2020

TransIT 2020 lipid transfection reagent was purchased from Mirus (#MIR5404) and the protocol was carried out as per manufacturers instructions. Briefly, adherent cells were grown in 6 well plates, and were allowed to reach 80% confluency. Suspension cells were also plated in 6 well plates, and used at a density of $5x10^5$/ml. The reagent was warmed to room temperature before mixing gently by vortexing. 2.5µg DNA was added to 250µl of Opti-MEM serum free medium (Thermo Fisher Scientific #31985062) and mixed gently by pipetting. Then, 7.5µl of the TransIT reagent was added, mixing gently with pipetting, before incubating at room temperature for 15-30 minutes. The transfection mixture was added dropwise into the wells of cells and the plate was rocked gently. The cells were incubated at 37˚C in 5% CO_2 for 24 hours before being analysed for protein expression.

2.3.2 Cell transfection with FuGeneHD

FuGeneHD lipid transfection reagent was purchased from Promega, #E2311. Adherent cells were allowed to grow to 80% confluency in a 24-well plate. The reagent was warmed to room temperature, before vortexing gently to mix. 2µg of DNA was added to 100µl Opti-MEM serum free media, after which 6µl of the transfection reagent was added and was mixed gently by pipetting. The mixture was incubated at room temperature for up to 15 minutes, after which the mixture was added dropwise on top of the cells. The plate was rocked gently and was incubated for 24 hours at 37˚C in 5% CO_2 before the cells were analysed for protein expression.

2.3.3 Generation of TAP, mCherry and GFP tagged constructs for GBP overexpression

Coding sequences of hGBP3, hGBP4, hGBP5, hGBP6 and hGBP7 were amplified by PCR, using Fermentas PCR Master Mix (Thermo Fisher Scientific Inc)

according to manufacturer's protocol (Table 3). Primers were purchased from Sigma-Aldrich and were used to integrate appropriate sites for recognition by restriction endonucleases as well as to introduce HA, FLAG, mCherry or GFP epitope tags. The two restriction sites are not complementary to provide control over the inserted PCR product's orientation and to prevent re-ligation of the vector to form transformable circular DNA. To enable a higher level of efficiency of linear DNA cutting by the enzymes, short overhangs were placed 5' to the restriction sites. All primers with their tags and restriction sites are listed in the Appendix.

Both the PCR product and appropriate vectors were double digested for 1 hour at 37˚C as indicated in Appendix, Table 10. Successful digestion was verified by electrophoresis on a 1% agarose gel containing Gel Red (Biotium, #41003-BT) in 1X Tris-Acetate-EDTA (TAE) buffer at 100V until migration was complete. The samples were visualised under UV light. The bands were excised and the DNA was extracted using Qiagen Quick Gel Extraction kit (#28704). C1 vectors and the insert were ligated using a fast ligation protocol (Thermo Fisher Scientific #K1422), to the values as indicated in Table 10 with an incubation of 15 minutes at room temperature.

For each transformation, 3µl of the ligated vector preparation was added to One Shot Mach-1 *E. coli* competent cells (Life Technologies, # C862003). After 30 minutes incubation on ice, the cell suspension was subjected to heat shock at 42˚C for 30 seconds, before being returned to ice for 2 minutes. Following this, cells were recovered in 250µl SOC and shaken for 1 hour at 37˚C. The transformed cells were spread on agar plates containing the appropriate antibiotic selection and allowed to form colonies overnight at 37˚C. DNA constructs were harvested from transformed Mach-1 cells using the Qiagen Quicklyse Miniprep kit, # 27405, and DNA was quantified on a NanoDrop spectrophotometer.

2.3.4 GeneSwitch driven overexpression of GBPs

DNA from GBP image clones was amplified by PCR from the C1 and pcDNA3 constructs to include appropriate restriction sites for cloning into the pGene vector primers and restriction sites can be found in the Appendix. The plasmid and GBP DNA underwent restriction digest before ligation using a fast ligation protocol

(Thermo Fisher Scientific #K1422). The product was run on an electrophoresis gel, with the bands being visualised using UV light before excision. The constructs were extracted as above. 3T3 fibroblasts that stably express the pSwitch plasmid were obtained as a kind gift from Jonathan Howard. These cells were grown to 70% confluency before being transfected with the individual pGene-GBP constructs using the TransIT 2020 transfection reagent as described. 24 hours after transfection the media was replaced with complete media, and selection with 50µg/ml zeocin (Invivogen #ant-zn-1) began in order to produce stable clones. To induce protein overexpression, mifepristone was added and incubated with cells for 24 hours before experimentation or protein analysis.

2.3.5 Vaccinia virus driven overexpression of GFP-GBP4

GFP-GBP4 was amplified from the pGene-GFP-GBP4 plasmid including restriction sites for AscI and PacI, primer sequences are included in the Appendix. The product was run on an electrophoresis gel as described, before the correct bands were visualised using UV light, and excised and extracted as above.

The pJS4 plasmid for this system was a kind gift from Jason Mercer (UCL). The plasmid and DNA underwent a restriction digest with AscI and PacI enzymes (Thermo Fisher Scientific #FD1894/ #FD2204) using the Thermo Fast Digest system (#FD1894/ FD2204). The vector and insert were ligated using a fast ligation protocol before transformation into competent cells as described above. THP-1 monocytes and HUVECs were grown to $5x10^5$ cells/ml or 80% confluency respectively before being infected with Vaccinia virus by Jason Mercer. One hour post infection, the cells were transfected with the pJS4-GFPGBP4 construct using the TransIT 2020 reagent as described. The cells were incubated at 37˚C in 5% CO_2, with cells being lysed for protein expression analysis at specific time points.

2.3.6 Inducible lentiviral driven overexpression of GFP-GBP4

GFP-GBP4 was amplified from the pGene-GFP-GBP4 plasmid including restriction sites for MluI and NotI, primer sequences and PCR protocol are included in the Appendix. The product was run on an electrophoresis gel as described before the

correct bands were visualised using UV light, and excised and extracted as above. The pLVX-Tight-Puro, Tet-On-Adv, ∆8.9 and VSV-G plasmids were a kind gift from Annemarthe van der Veen and Caetano Reis e Sousa. A pLVX-mCherry plasmid was received as a kind gift from Rhiannon White. The pLVX-Tight-Puro plasmid and GFP-GBP4 DNA underwent restriction digest with the appropriate enzymes using the Thermo Fast Digest system (#FD0564/ # FD0593). The vector and insert were ligated using a fast ligation protocol (Thermo Fisher Scientific #K1422) before transformation into competent cells as described.

HEK293T wells were grown to 70% confluency before transfection with the VSV-G and ∆8.9 packaging plasmids along with either Tet-On-Adv, pLVX-mCherry or PLVX-TP-GFPGBP4 plasmids. Supernatant from the cells containing the viruses were collected at both 24 and 48 hours post-transfection. The samples underwent ultra-centrifugation for 90 minutes at 4˚C at 20,000g before being flash frozen and stored at -80˚C. THP-1 monocytes were grown to $5x10^5$ cells/ml before transduction with the viruses. The pLVX-mCherry or pLVX-TP-GFPGBP4 viruses were added to the cells, along with the Tet-On-Adv virus and 8µg/ml Polybrene. The cultures were centrifuged at room temperature for 90 minutes at 1200xg to ensure infection of cells, before being incubated at 37˚C in 5% CO_2. The media was changed on the cells 6 hours after transduction and replaced with complete media. After 48 hours the cells were selected on 4µg/ml Puromycin (Thermo Fisher Scientific #A1113802) and 500µg/ml G418 (Thermo Fisher Scientific #11811098). To induce the expression of the protein of interest, the cells were treated with 1µg/ml doxycycline (Sigma # D9891) and the cells were studied from 4 to 24 hours to analyse protein expression.

2.4 Antibody production

2.4.1 Antigenic peptide design for antibody production

In order to produce rabbit polyclonal peptide antibodies against all 7 hGBPs, I first aligned the protein sequences before selecting a unique sequence for each hGBP. The specific peptide sequences and alignment across the family can be found in Figure 5. The peptides were ordered from BioMatik Corporation, Canada. The sequences were initially chosen from homologs to mGBP peptides (Degrandi et al.,

2007) and were then further designed according to specificity ensuring there was as little similarity across the selected sequences as possible. Immunogenicity was also checked using software available online (Thermo Scientific, Pierce Antibodies, Antigen Profiler Peptide Tool). BioMatik conjugated the peptides to keyhole limpet haemocyanin in order to improve immunogenicity. The company Antibody Production Services proceeded to inject these immunogens into rabbits. A pre-bleed and subsequent test bleeds at 3 intervals were taken before the final bleed.

For the production of mouse monoclonal antibodies, the peptide sequences for GBP2 and 4 were elongated (Figure 5). Covalab Biotechnology Company produced the peptides, before immunising four mice per peptide. The preimmune sera were tested for background reactivity before immunisation. Hybridomas were produced up to 192 days post-inoculation; the supernatant was preliminary tested by enzyme-linked immunosorbent assay (ELISA) for reactivity against the peptide by the company. After reactive candidates were identified, I tested the supernatants by immunoblotting for specificity against lysates of mouse fibroblasts that inducibly overexpress TAP-tagged GBP family members. Three mice underwent fusion for the anti-GBP2 antibody. Of these, one mouse yielded no reactive hybridomas while the second and third mice produced a total of two reactive hybridomas. These were cloned to produce a total of four reactive antibodies (Table 3 and 4). In this project, monoclonal anti-GBP2 clone 9E12c1 was used. Two mice underwent fusion to produce hybridomas for monoclonal anti-GBP4 antibody production. From these, one of the fusion events produced no reactive hybridomas. The second fusion event yielded two reactive hybridomas, which were cloned and then subcloned to produce four reactive antibodies (Tables 5 and 6). In this project, monoclonal anti-GBP4 clone 9A12c6d3 was used.

2.5 *Toxoplasma*/ Host interaction assays

2.5.1 FACS for invasion assay

Cells were grown to 80% confluency and were infected with irradiated *Toxoplasma* as described above. After 1 hour of infection, the cells were incubated with 2x 0.5% Trypsin +EDTA (Life Technologies #15400-054) at 37˚C to allow the cells to detach from the culture plate. The reaction was quenched with warmed DMEM media

containing 10% FBS, and the cells were transferred into a 15ml Falcon tube to be pelleted by centrifugation at 300xg for 5 minutes. The media was aspirated and the cells were washed twice in ice cold PBS by centrifugation at 37˚C at 300xg. After the supernatant was aspirated following the final wash, the cells were resuspended in ice-cold 4% PFA and transferred to a well of a round-bottomed 96-well plate to be incubated on ice for 20 minutes. PBA was added on top and the plate was centrifuged at 450xg for 3 minutes to pellet the cells. The supernatant was removed by gently inverting the plate, after which the cells were resuspended in PBA and transferred to a FACS tube ready for analysis on a BD LSR-II flow cytometer.

2.5.2 Plaque assay

A549 cells were allowed to grow to confluency in a 24-well plate before being infected with either 300 type I or 600 type II *Toxoplasma*. The infection was allowed to persist for the desired length of time, before the cell layer was scraped and the parasites released by syringe lysis using a 25-gauge needle. This suspension was then plated onto unstimulated, confluent HFFs in 1:2 serial dilutions. The infection persisted for 4 days at 37˚C in 5% CO_2 after which plaques were counted using a microscope.

2.5.3 Assessing parasite vacuolar replication

Cells were grown on glass coverslips until confluency. They were infected with eGFP expressing *Toxoplasma* at an MOI of 0.5, and the infection was allowed to persist for the desired length of time. The cells on coverslips were washed extensively to ensure all parasites that had not invaded were removed, after which they were fixed, permeabilised and blocked as above. The coverslips were washed 3 times in PGAS, followed by 3 washes in PBS, with 1:10,000 Hoechst DNA stain contained in the last wash. They were then mounted on glass slides as described above. The slides were viewed on a bright-field microscope and the number of parasites per vacuole were counted using x63 lens. A minimum of 100 vacuoles was counted, with each sample produced in triplicate.

2.5.4 Statistical analysis

Parasite replication assays were carried out in triplicate and at least twice. For these two independent variables (time and parasite number) are compared and so a 2-way ANOVA statistical analysis was carried out to determine significance. Plaque assays were carried out in triplicated and at least twice. The plaque assay in Figure 15 was normalised to 100% growth in wild type unstimulated cells, thus in this experiment, a paired t-test was used to calculate significance. In plaque assays comparing growth between cell types as well as IFNγ-stimulation, experiments, two independent variables are compared (IFNγ-stimulation and cell type) and so a 2-way ANOVA was carried out to determine significance. When calculating the statistical significance between the densities of bands on an immunoblot or the intensity of fluorescence staining in immunofluorescence images an unpaired t-test was utilised due to the comparison of two independent groups.

Chapter 3. Generation and characterisation of molecular tools and techniques to study hGBPs

3.1 Results

3.1.1 Generation and characterisation of molecular tools and techniques to study hGBPs

3.1.1.1 Antibodies

As the seven GBPs have such a high level of identity across the family, obtaining antibodies that are specific is not trivial. Consequently, there is a lack of commercial antibodies available, with no guarantee of specificity for individual family members. To ensure I had antibodies that were directed to specific regions of the protein, thereby increasing the probability of specificity for each GBP, it was decided to produce antibodies with which to carry out this project. There are a number of options when producing bespoke antibodies. While it was necessary to obtain the best tools possible, cost considerations had to be taken into account. Therefore, I proceeded with a mixture of polyclonal and monoclonal antibody production. Unique peptide sequences were chosen for each family member, ensuring that each one had a high immunogenic capacity using an online software tool from Thermo Scientific (https://www.thermofisher.com/uk/en/home/life-science/antibodies/custom-antibodies/custom-antibody-production/antigen-profiler-antigen-preparation.html#). The peptides designed for the polyclonal antibody production ranged from 13 to 16 amino acids in length and were selected from the C-terminal of the protein sequence. This is because the C-terminus has the highest level of sequence variability, giving an increased likelihood of making antibodies that are family member specific (Figure 5). One rabbit per human GBP was inoculated with the appropriate peptide before test-bleeds were harvested at three time points after infection, prior to the final bleed. The sera from these bleeds were screened for reactivity in immunoblot against mouse fibroblasts that inducibly overexpressed the respective human GBP. Table 2 demonstrates the candidate polyclonal antibodies and their reactivity in immunoblot.

Furthermore, two of the unique peptide sequences were elongated in order to produce monoclonal antibodies. From the literature, it had been reported that GBPs 2 and 4 could be found in the nucleus under certain conditions (Tripal et al., 2007). With the exception of nuclear trafficking Ran proteins, human GTPases have had limited function reported in the nucleus, so these two candidates appeared very interesting. On the basis of this, monoclonal antibodies were produced to GBP2 and GBP4 (Figure 5). Tables 3-6 demonstrate the candidates for GBP2 and GBP4 monoclonal antibody production. Four mice per GBP were inoculated with specific antibody. The company determined levels of antibody via ELISA and the animals with high antibody titre underwent fusion. The resulting hybridoma supernatants were analysed by the company via ELISA against the inoculating peptide. After reactive candidates were identified, I tested the supernatants by immunoblotting for specificity against lysates of mouse fibroblasts that inducibly overexpress TAP-tagged GBP family members. Three mice underwent fusion in order to produce the anti-GBP2 antibody. Of these, one mouse yielded no reactive hybridomas while the second and third mice produced a total of two reactive hybridomas. These were cloned to produce a total of four reactive antibodies (Table 3 and 4). In this project, monoclonal anti-GBP2 clone 9E12c1 was used. Two mice underwent fusion to produce hybridomas for monoclonal anti-GBP4 antibody production. From these, one of the fusion events produced no reactive hybridomas. The second fusion event yielded two reactive hybridomas, which were cloned and then sub-cloned to produce four reactive antibodies (Tables 5 and 6). In this project, monoclonal anti-GBP4 clone 9A12c6d3 was used.

A polyclonal antibody had been made previously using full-length recombinant GBP1. However, it had never been properly characterised in regard to specificity and cross-reactivity to other family members, and therefore was not being used either reliably or to its full potential. This antibody was added to the repertoire requiring testing.

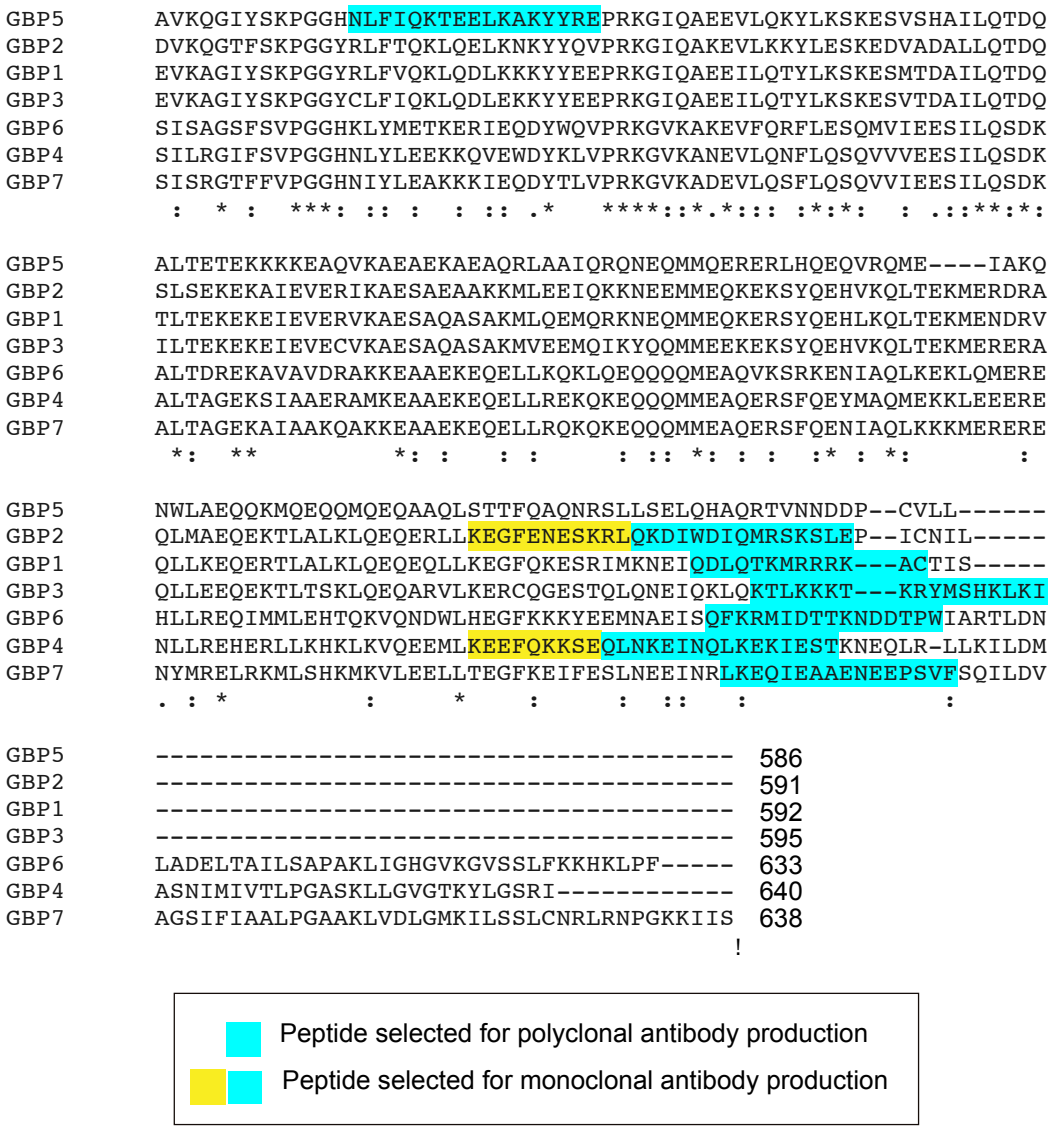


Figure 5 Alignment of GBP C-termini and peptides for antibody production

Sequences were aligned using Clustal Omega Multiple Sequence Alignment tool. Blue indicates peptides for polyclonal antibody production. The yellow section represents the additional amino acids that were selected in addition to the blue peptide sequence; this extended peptide was used for monoclonal antibody production for GBP2 and GBP4.

Table 2 Screening of polyclonal antibody sera for specific bands in immunoblot

GBP	Test bleed 1	Test bleed 2	Test bleed 3	Final bleed
1	✓	✓	✓	✓
2	✓	✓	✓	✓
3			✓	
4	✓	✓	✓	✓
5	✓			
6				
7	✓	✓	✓	✓

One rabbit per human GBP family member was inoculated with the appropriate peptide. Test bleeds were taken at 3 intervals before a final bleed. Where ticks are shown, a band for the individual GBP was observed in immunoblot. Where blank, no signal was observed.

Table 3 Candidates for monoclonal GBP2 antibody production from fusion of mouse 'R'

	Monoclonal human GBP2 4 mice immunised; V, B, R, N		
	Fusion of mouse R Day 66		
Hybridoma	Signal observed by ELISA	Signal observed in IB after ELISA	ID of clones with signal observed in IB
1D3			
1A5			
1B5			
1A7			
1E9	✓		
1E10			
1G11			
2G2			
2F3			
2H3			
2D6			
2C8	✓		
2A10			
2F10			
2B11			
2A12			
2C12			
3B2			
3G3			
3C5			
3D7			
3C9			
3G9			
3D10			
3C11			
3H11			

Continued…

	Monoclonal human GBP2 4 mice immunised; V, B, R, N		
	Fusion of mouse R Day 66		
Hybridoma	Signal observed by ELISA	Signal observed in IB after ELISA	ID of clones with signal observed in IB
4C1			
4G1			
4B2			
4B3			
4A4			
4C8			
5H2			
5B4			
5E4			
5B5			
5G5			
5E7			
5G10			
5G11			
5C12			
6F2			
6D3			
6H4			
6C5			
6E5			
6F6	✓		
6D7			
6F8			

A tick represents a signal in ELISA or a specific band in immunoblot. All candidates that demonstrated a signal in ELISA underwent testing in IB. Where blank, no signal was observed when screened in ELISA and/or immunoblot.

Table 4 Candidates for monoclonal GBP2 antibody production from fusion of mice 'V' and 'N'

	Monoclonal human GBP2 4 mice immunised; V, B, R, N		
	Fusion of mice V and N after Day 192		
Hybridoma	Signal observed by ELISA	Signal observed in IB after ELISA	ID of clones with signal observed in IB
1A6	✓	✓	1A6d6 1A6g2
1G12	✓		
2F4			
2H11	✓		
3H1	✓		
3F2	✓		
3B7			
5H12			
6A1	✓		
6B6	✓		
6H6	✓		
6D9	✓		
6G12			
7D1	✓		
7A5			
7A4	✓		
7G7			
7A12			
7G12			
8H1	✓		
8G9			
9F1			
9E3	✓		

Continued…

	Monoclonal human GBP2 4 mice immunised; V, B, R, N		
	Fusion of mice V and N after Day 192		
Hybridoma	Signal observed by ELISA	Signal observed in IB after ELISA	ID of clones with signal observed in IB
9B8			
9H9	✓		
9E12	✓	✓	9E12b2 9E12c1
10F1			
10H3	✓		
10C4			
10C5			
10G8			
11F6	✓		
13A3			
13D3			
13B10			
13D11	✓		
13C12			

A tick represents a signal in ELISA or a specific band in immunoblot. All candidates that demonstrated a signal in ELISA underwent testing in IB. Where blank, no signal was observed when screened in ELISA and/or immunoblot.

Table 5 Candidates for monoclonal GBP4 antibody production from fusion of mouse 'N'

	Monoclonal human GBP4 4 mice immunised; V, B, R, N		
	Fusion of mouse N Day 129		
Hybridoma	Signal observed by ELISA	Signal observed in IB after ELISA	ID of clones with signal observed in IB
1C2			
1D8			
4A4			
4H12			
6B9			
7E1			
9H6			
9C12			

A tick represents a signal in ELISA or a specific band in immunoblot. All candidates that demonstrated a signal in ELISA underwent testing in IB. Where blank, no signal was observed when screened in ELISA and/or immunoblot.

Table 6 Candidates for monoclonal GBP4 antibody production from fusion of mouse 'R'

	Monoclonal human GBP4 4 mice immunised; V, B, R, N			
	Fusion of mouse R Day 173			
Hybridoma	Signal observed by ELISA	Signal observed in IB after ELISA	ID of clones/ sub-clones with signal observed in IB	
2A8				
3F9				
4A7				
4E9	✓			
5E1	✓	✓	5E1d1 5E1g1	
5F9				
5C10	✓			
6C6				
6D10				
6H11				
9D5	✓			
9A12	✓	✓	9A12c6	9A12c6f8 9A12c6d3
10D2	✓			
10D3				
10H8				

A tick represents a specific band in immunoblot. All candidates that demonstrated a signal in ELISA underwent testing in IB. Where blank, no signal was observed when screened in ELISA and/or immunoblot.

All candidate antibodies were tested to ensure a band of the correct size could be detected in other cells via immunoblot using HUVEC lysates, with the best candidates taken forward for full characterisation. First, a polyclonal antibody produced against the GBP1 peptide was characterised. I, and others, have developed mouse 3T3 fibroblasts that inducibly overexpress tagged individual family members, which will be described later in this chapter. Using lysates from these cells I showed that the polyclonal antibody was specific for this GBP family member in immunoblotting (Figure 6A). Furthermore, a band of the correct size was detectable in immunoblots of A549 cell lysates that was subsequently lost when probing lysates from A549 cells deficient in GBP1 (made by Barbara Clough) (Figure 12). Using lysates from the same GBP-overexpressing 3T3 fibroblast cells, it was shown that this antibody reacts with both GBPs 1 and 2 in immunoprecipitation, but does not cross react with GBP3 (Figure 6B). Immunoprecipitation characterisation was limited to GBP1, 2 and 3 due to the highest level of identity being present between these family members. It was shown that the same antibody was specific to GBP1 in immunofluorescence by lack of staining in A549 cells that were deficient in GBP1 protein expression as compared to wild type cells (Figure 6C).

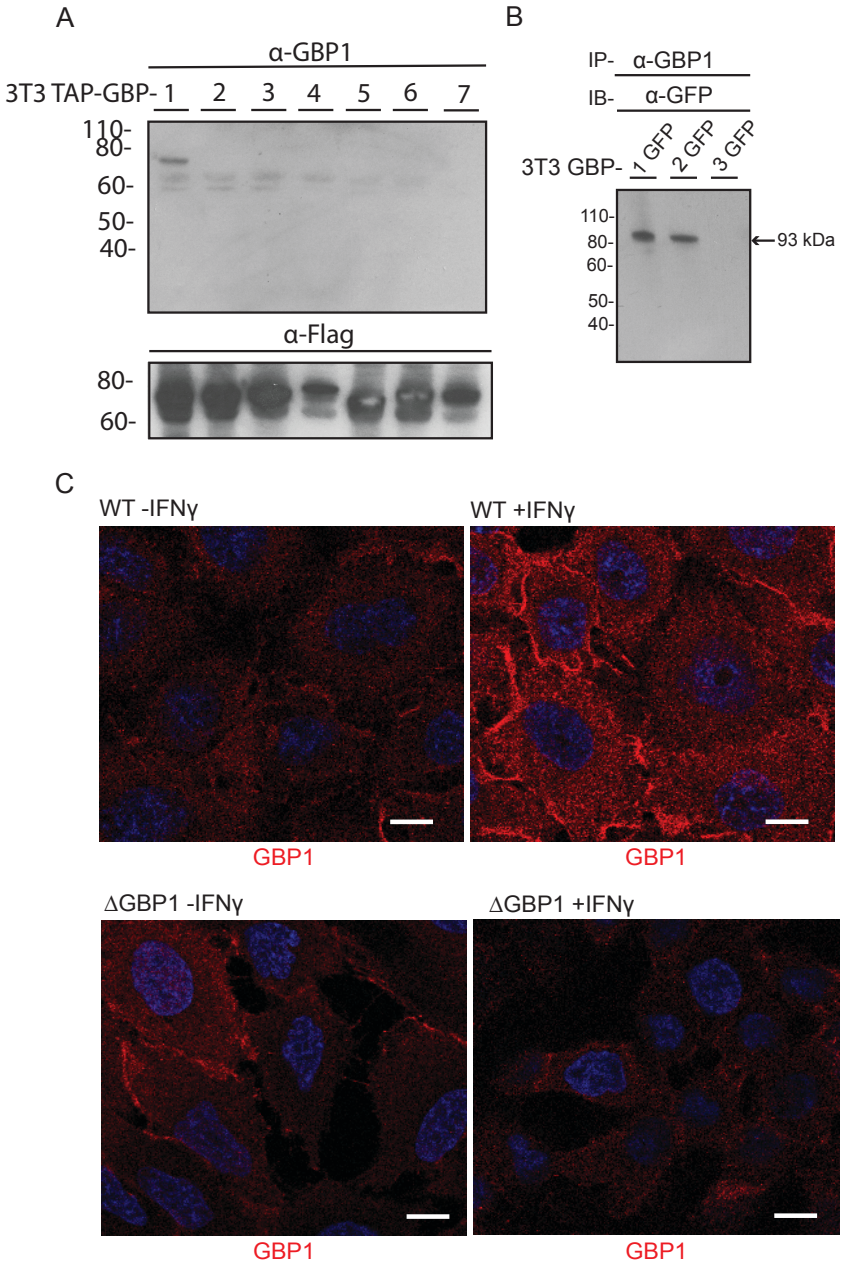


Figure 6 Characterisation of anti-GBP1 polyclonal antibody

Immunoblot showing anti-GBP1 antibody reactivity in 3T3 fibroblasts overexpressing individual TAP-tagged GBP family members. 10µg of protein was loaded into each lane for SDS-PAGE. The same membrane was probed with anti-Flag to show TAP (HA-Flag) was present (A). Immunoblot showing the immunoprecipitation capacity of anti-GBP1 antibody in 3T3 fibroblasts over expressing GFP-tagged GBP1, 2 or 3. 40µl of antibody was used to immunoprecipitate from 500µg of total lysate. 35µl of immunoprecipitated end product was loaded in each lane for SDS-PAGE (B). Immunofluorescence images showing staining with anti-GBP1 in A549 WT cells as compared to A549 ΔGBP1 cells. Scale bars 10µM. Cells were stimulated or not for 18 hours with 10U/ml IFNγ (C). Each representative of 3 independent experiments.

As mentioned above, it was previously reported that GBP2 and 4 could be found in the nucleus (Tripal et al., 2007), therefore these family members were used in both polyclonal and monoclonal antibody production. In the process of choosing suitable hybridoma clones to take forward, a monoclonal antibody specific to GBP2 was identified by immunoblot (Figure 7A). For the polyclonal antibody, a number of rabbits were immunised with peptide against GBP2, however none of the resulting candidates obtained showed any signal in an immunoblot. Once candidates had been selected, further characterisation showed that both monoclonal and polyclonal antibodies had a capacity for immunoprecipitation, however neither antibody was specific. The anti-GBP2 polyclonal antibody also has the capacity to immunoprecipitate GBP1 protein (Figure 7B) and the monoclonal antibody retrieves at least GBP1 and GBP3 in an unspecific manner (Figure 7C).

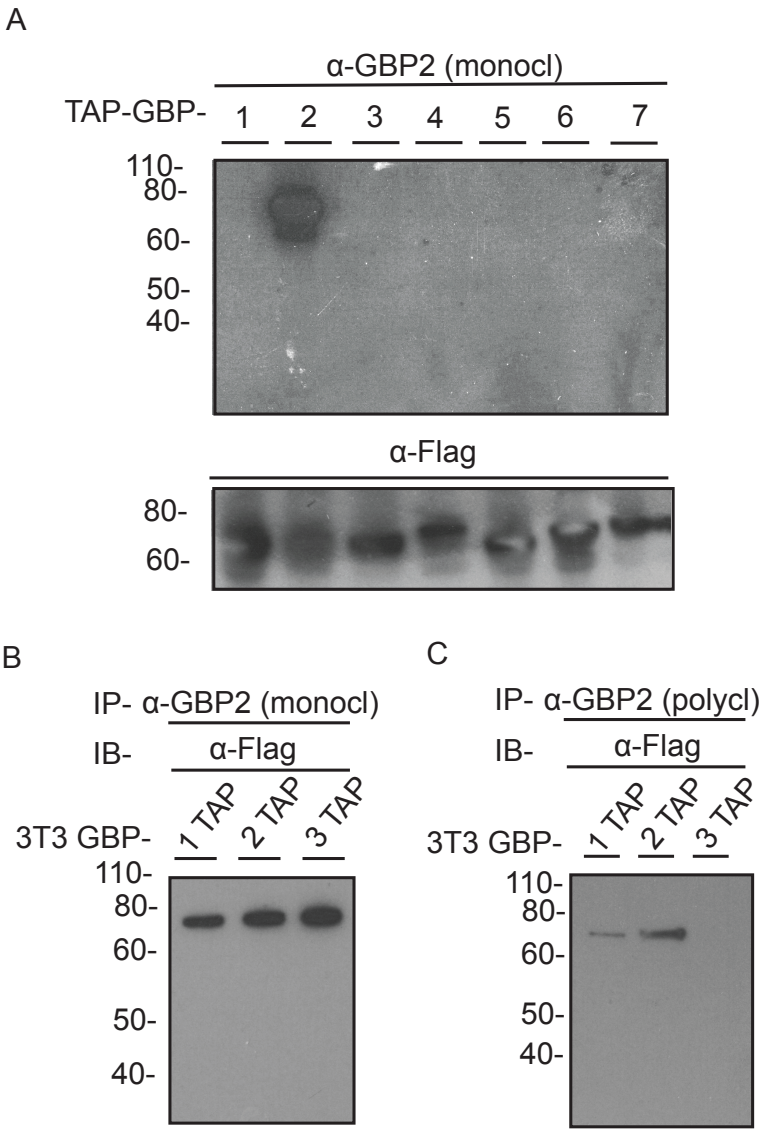


Figure 7 GBP2 antibody characterisation

Immunoblot showing reactivity of the monoclonal anti-GBP2 antibody in 3T3 mouse fibroblasts overexpressing individual TAP-tagged human GBPs. 10µg of total protein was loaded into each lane for SDS-PAGE. The same membrane was probed with anti-Flag to show TAP (HA-Flag) was present (A). Immunoblot showing the immunoprecipitation capacity of monoclonal anti-GBP2 antibody (B) and the polyclonal anti-GBP2 antibody (C) in 3T3 fibroblasts overexpressing TAP-tagged GBP1, 2 or 3. 35µl of immunoprecipitated end product was loaded in each lane for SDS-PAGE.10µl of antibody was used to IP from 500µg of total lysate in (B). 40µl of antibody was used to IP from 500µg of total lysate in (C). Each representative of 3 independent experiments.

When characterising the anti-GBP4 antibodies, a rabbit polyclonal antibody proved specific in immunoblots (Figure 8A). The monoclonal antibodies that were raised against GBP4 were tested in immunoblots against lysates from 3T3 fibroblasts

overexpressing individual GBPs. None were found to produce any signal in this application. However, upon further testing, it was shown that an anti-GBP4 monoclonal antibody could specifically retrieve GBP4 by immunoprecipitation (Figure 8B).

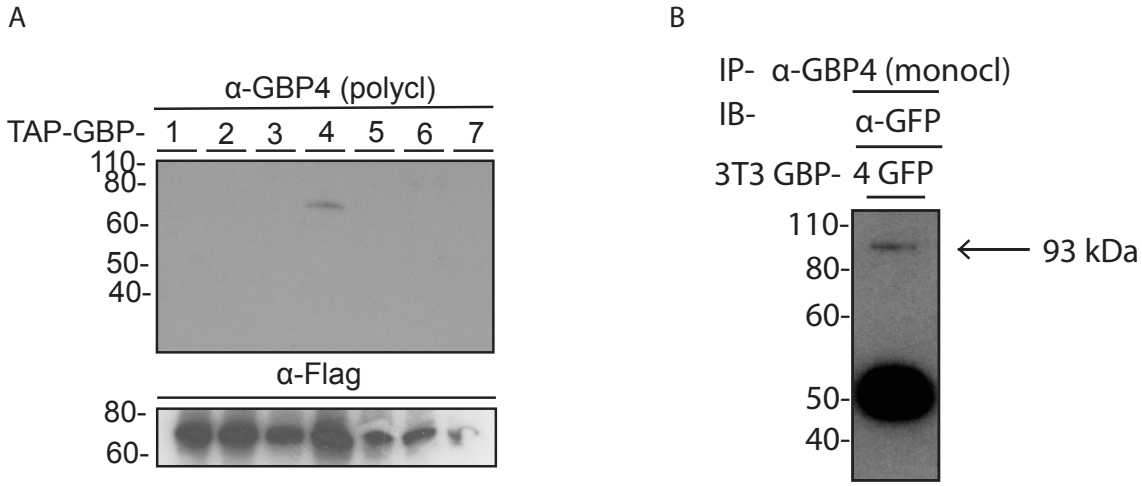


Figure 8 GBP4 antibody characterisation

Immunoblot showing reactivity of anti-GBP4 polyclonal antibody in 3T3 fibroblasts overexpressing individual TAP-tagged GBPs. 10µg of total protein was loaded into each lane for SDS-PAGE. The same membrane was probed with anti-Flag to show TAP (HA-Flag) was present (A). Immunoblot showing immunoprecipitation capacity of anti-GBP4 monoclonal antibody in 3T3 fibroblasts overexpressing GFP-tagged GBP4. 65µl of antibody was used to IP from 500µg of total lysate. 35µl of immunoprecipitated end product was loaded in each lane for SDS-PAGE (B). Each representative of 2 independent experiments.

A polyclonal antibody against GBP1 had previously been produced in rabbit by Sebastian Virreira Winter and Eva Frickel by using full-length recombinant protein. Due to the very high levels of identity between the seven GBP family members, it was important to fully characterise this antibody and determine its specificity. Lysates from 3T3 fibroblasts overexpressing individual GBP family members were probed with this recombinant protein antibody. It became clear that although the antibody recognised GBP1 protein, it was cross-reactive with GBPs 2, 3 and 6 as well (Figure 9). Therefore it has been labelled as an anti-pan-GBP antibody, and will be referred to as such from this point onwards.

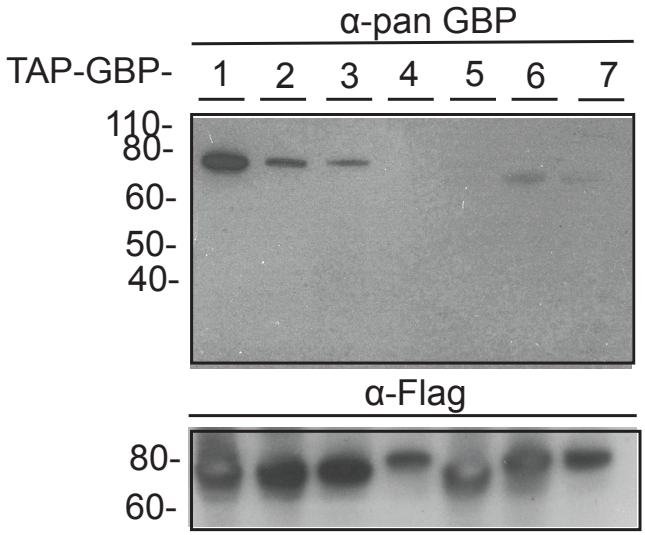


Figure 9 pan-GBP antibody characterisation

Immunoblot showing the reactivity of the anti-pan-GBP in 3T3 fibroblasts overexpressing individual TAP-tagged GBPs. 10µg of protein loaded into each lane for SDS-PAGE. The same membrane was probed to show that TAP (HA-Flag) was present. Representative of 3 independent experiments.

3.1.1 Overexpression of human GBPs in mammalian cells

In order to assess the function of the GBPs in the context of a *Toxoplasma gondii* infection, I planned to overexpress each family member in a variety of human cells. These cells would be infected with *Toxoplasma* before evaluating how parasite survival and replication would be affected. Within the literature, a number of techniques for overexpression of human GBPs have been demonstrated (Modiano et al., 2005, Pammer et al., 2006, Duan et al., 2006, Tripal et al., 2007, Schnoor et al., 2009, Tietzel et al., 2009, Al-Zeer et al., 2013, Forster et al., 2014, Ostler et al., 2014, Blondel et al., 2015, Li et al., 2015). At the same time, there are variations within even single laboratories in the methods used to overexpress this family of proteins. Therefore, to establish this technique, a number of transfection methods were employed in order to find the most efficient and least toxic approach.

First, I used a lipid based transfection method to transiently overexpress human GBPs. All seven of the individual GBP family members were cloned into C1 and pcDNA3 vector plasmids with either a mCherry- or GFP-N-terminus tag. This was a large undertaking that was done in collaboration with a lab at Duke

University, and with another member of the Frickel lab. GBP1 and 2, with both GFP and mCherry tags were produced by the Coers lab. GBPs 3-6 with a GFP tag were produced by Barbara Clough. I produced GFP-GBP7 and mCherry-tagged GBPs 3-7. These were then employed to transiently transfect a variety of human cell types with Mirus TransIT 2020 lipid transfection reagents. It became apparent that after transfection, the cells began to look very sickly. While fluorescent cells were observed (data not shown), after only a few hours of this transfection the cells died. Hence, these results imply that the cells did not tolerate the overexpression of these proteins. With an expression window of less than 12 hours before the cells succumbed, it was not possible to carry out an infection and viability assay with *Toxoplasma gondii*. Hence, transient overexpression was not a feasible method with which to study GBPs.

Following this, a 'Tet-On' inducible lentiviral method of transduction technique was employed. THP-1 monocytes would be stably transduced before differentiation into macrophages. The expression of tagged-GBPs is then inducible with the addition of doxycycline. Lentiviral transduction generally allows for a higher efficiency due to the advantage of it being able to transfect both dividing and non-dividing cells. Furthermore, with an inducible system, the transduced cells only have the protein expression upregulated when doxycycline treatment is applied. In order to stop the virus from recombining inside the cell, the genes necessary to package and assemble the virus are contained in separate vectors. To test the system, I cloned GBP4 into the pLVX-Tight-Puro-Vector. The pLVX-Tight-Puro plasmid containing GBP4 was transfected using TransIT 2020 into HEK293T cells along with the two plasmids containing the packaging components. At the same time, a separate population of HEK293T cells were transduced with the Tet-On-Adv, the lentiviral vector for producing a doxycycline-activated transactivator for this system, containing plasmid and the separate packaging plasmids. A control lentivirus was produced also by using a pLVX-mCherry vector transfected with the packaging plasmids. This would result in a non-inducible overexpression and will demonstrate whether the production of virus was effective by analysing the resulting transduction. The lentivirus produced was harvested at both 24 and 48 hours before being pooled, filtered, centrifuged and flash frozen, ready for transduction into THP-1 monocytes.

To transduce THP-1 monocytes, cells were incubated either with the mCherry-lentivirus or with a combination of the Tet-On-Adv and pLVX-GBP4 viral stocks. Polybrene was added to aid the reaction as it is a cationic polymer that can neutralise charge repulsion between the virions and sialic acid- allowing greater adsorption on to the cell surface (Hunn et al., 2008). The transduction was allowed to persist for 6 hours after which the media was changed and selection with G418 and Puromycin for doubly transduced cells began. The mCherry-expressing cells were viewed 24 hours post-transduction, with a high level of fluorescence being observed. Before inducing expression of GBP4, the THP-1 monocytes were differentiated into macrophages. They were then incubated with doxycycline for 24 hours to allow the protein to accumulate. After approximately 12 hours, the cells looked unhealthy and began to detach from the wells. By 24 hours the cells had almost completely ceded to the consequences of transduction of GBPs, when compared with control-transduced cells.

Another classically used viral-mediated method of transient protein overexpression employs Vaccinia virus (Falkner et al., 1992). This system has been used in cases of toxic protein expression as it can lead to very rapid production of the protein of choice due to its capacity to transcribe DNA in the cytoplasm. Conveniently, Vaccinia virus is able to infect any cell type. If GBPs could be overexpressed very quickly without a gradual accumulation of protein, the cells could be used for a short infection time point experiment before the cells were affected by the potential toxicity. Again, GBP4 was used to test the system, this time with a GFP-tag at the N-terminal. GFP-GBP4 was cloned into the pJS4 vector under a Vaccinia e/l promoter and this was transfected into unstimulated A549 cells, THP-1 monocytes and HUVECs that had been infected with Vaccinia virus 1 hour prior by Dr Jason Mercer (UCL). Lysates were made of each cell type at time points ranging from 4 to 24 hours post transfection. A very low expression was seen at time points after 4 hours post transduction in THP-1 monocytes, which could be due to the low transfection capacity of these cells. At 4 hours post transduction expression of GFP was visible in HUVECs. The HUVECs were visibly unhealthy from 8 hours post transduction, and the majority were non viable by the 12 hour post transduction time point. Immunoblotting of these lysates with the anti-GBP4 polyclonal peptide antibody demonstrated that GBP4 protein could be detected from 6 hours in HUVECs, and this increased significantly until 12 hours, after which

expression was lost- likely due to the cells being dead. What was interesting was the lack of GFP-tagged GBP4 expression in these cells. Potentially, the presence of the Vaccinia virus in the cell could itself begin to induce endogenous GBP4 expression in HUVECs. However, when the blots were reprobed, GFP could be detected in samples from 8 to 10 hours post infection suggesting that, in fact, the tag was being cleaved from the N-terminus of GBP4. It was decided that this was not an appropriate method to overexpress GBPs due to the fact Vaccinia may be inducing its own GBP response. Although I decided not to continue with this Vaccina driven method, I learned that tags may not be stable when expressed at the N-terminus of GBP4 and any constructs designed in the future must have tags inserted C-terminus instead.

While it was becoming increasingly clear that overexpression of GBPs was not a suitable method to study their function, it was possible that some inducible methods of overexpression could be used as a tool to characterise the bespoke antibodies. GeneSwitch is a system sold by Invitrogen that allows for mifepristone inducible expression of protein of choice. A hormone expression system is a more desirable one than a doxycycline inducible system, as antibiotics may affect responses of cells and thus potentially affect the *Toxoplasma* infection. The basis of the system is that a 'pSwitch' regulatory plasmid is stably transfected into the cell line of choice. After this, the 'pGene' plasmid containing the gene of interest is stably transfected into the cells with pSwitch. The pSwitch plasmid is responsible for expressing low levels of the GeneSwitch protein. This protein is dimerised upon treatment with mifepristone, following which it binds with the promoter in the pGene plasmid- thus inducing expressing of the protein of choice.

It was attempted to stably transfect HFFs with the first plasmid, pSwitch. However it was never possible to introduce this plasmid into the HFFs, therefore using this system in human cells was not continued. Correspondence with another lab revealed they too had problems with this system (Marianne Schmidt, personal communication). Shortly after beginning this endeavour, Invitrogen discontinued the system.

Mouse 3T3 fibroblasts that were stably expressing the pSwitch plasmid were obtained as a kind gift from Jonathan Howard (Hunn et al., 2008). GFP- and TAP-tagged GBPs were cloned into the pGene vector as a joint effort between myself, Barbara Clough and the Coers lab at Duke University. GFP- or TAP-GBP1

and 2 were cloned by our collaborators at Duke. Barbara Clough produced GFP-tagged GBPs 3-6. I produced GFP-tagged GBP6 and TAP-tagged GBPs 3-7. Sharing the work equally, Barbara Clough and I used these constructs to transfect 3T3 cells before selecting for successfully transfected cells with antibiotics. The cells were incubated with mifepristone for 24 hours before lysing and assessing GBP expression with the appropriate antibodies. It was clear that these cells could withstand the toxicity of GBP plasmid transfection and protein overexpression, with sustained and robust expression possible. Perhaps as the cells are from mouse origin, the interacting partners or pathways responsible for toxicity in human cells may be missing or incompatible. It should also be noted that these cells were not IFNγ inducible, therefore could not be a valid tool for studying GBP function.

3.1.1.1 Cell Characterisation

It is important that the study of human GBPs is carried out in an appropriate cell type. This means that the protein must be expressed, as well as demonstrating they are IFNγ sensitive within the particular cell type. For this project, GBPs were characterised first in A549 lung epithelial cells. These cells are robust cells that are widely accepted for biochemical analysis experiments, and have been utilised in a number of immune response studies, particularly in cancer studies (Brichory et al., 2001, Hartman et al., 2007, Hawdon et al., 2010, Li et al., 2012, Guitierrez et al., 2016). A549s were plated and allowed to adhere before stimulating with IFNγ overnight. Following this stimulation the cells were lysed and run on SDS-PAGE before being transferred to nitrocellulose membrane. The membranes were probed with the anti -pan-GBP, -GBP1, -GBP2 and -GBP4 antibodies. It was clear that all of the tested GBPs were substantially expressed in these A549 cells (Figure 10 A, B & C). They all demonstrated sensitivity to IFNγ, however interestingly GBP1 expression was also detected at basal level (Figure 10A), a result that will be confirmed by qPCR.

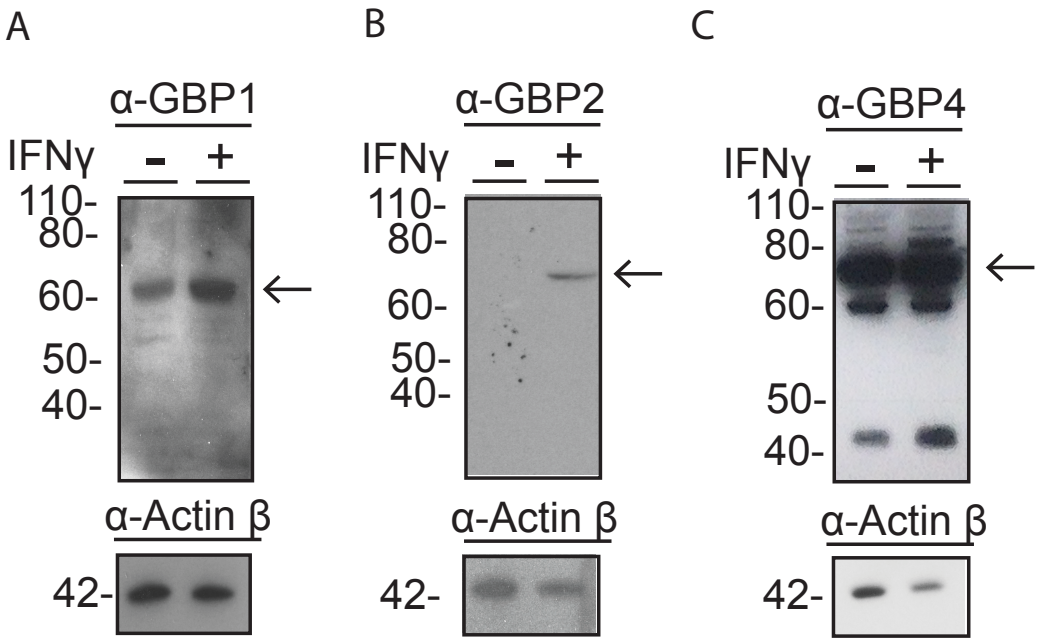


Figure 10 Expression of GBP1, 2 and 4 in A549 cells

Immunoblots showing levels of protein expression of GBP1 (A), GBP2 (B) and GBP4 (C) in A549 cells. GBP1 and GBP4 polyclonal antibodies were used, and GBP2 monoclonal antibody was used. 10µg of protein was added to each lane for SDS-PAGE. Cells were stimulated or not for 18 hours with 10U/ml IFNγ. Arrows represent GBP band. Each representative of 3 independent experiments.

THP-1 macrophages were characterised for GBP expression next. It is widely reported in the literature that macrophages are the frontline of defence to *Toxoplasma* (Dupont et al., 2012). The THP-1 cells were differentiated into monocytes before stimulating overnight with IFNγ. The macrophages were lysed and the lysates run on SDS-PAGE before being transferred to nitrocellulose membrane. These membranes, as above, were probed with anti –pan-GBP, -GBP1, -GBP2 and –GBP4 antibodies. Again, in this cell type GBPs were all well expressed in the cells induced with IFNγ (Figure 11 A, B & C). From the results of the characterisation of GBPs in A549 and THP-1 cells signified it was clear that these were suitable cells in which to study GBPs both in steady state and in *Toxoplasma* infected cells.

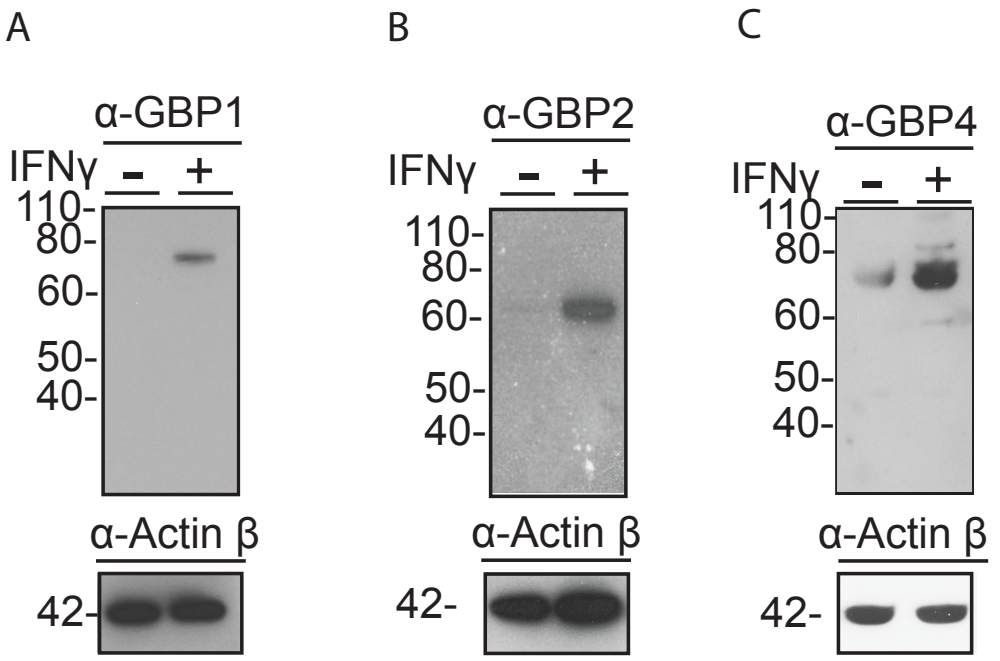


Figure 11 Expression of GBP1, 2 and 4 in THP-1 macrophages

Immunoblots showing the protein expression levels of GBP1 (A), GBP2 (B) and GBP4 (C) in THP-1 macrophages. GBP1 and GBP4 polyclonal antibodies were used, and GBP2 monoclonal antibody was used. 10µg of protein was added to each lane for SDS-PAGE. Cells were stimulated or not for 18 hours with 10U/ml IFNγ. Each representative of 3 independent experiments.

Production of knock out cells using the CRISPR-Cas9 genome editing technique spells great advances in cell biology. It is important however, that the cells are well characterised to ensure they are reliable. GBP1 knock out cells, using CRISPR-Cas9, were generated in A549 cells by Barbara Clough. Before they were implemented in studies using *Toxoplasma*, I needed to be sure that GBP1 protein was truly knocked out. First, a lack of GBP1 protein expression was confirmed. This was done by immunoblot using the specific anti-GBP1 polyclonal peptide antibody. It is clear that GBP1 protein cannot be detected in these cells (Figure 12A). The peptide immunogen used for developing this antibody is in a region from the C-terminal of GBP1 (Figure 5). The guide RNA used to make these A549 ΔGBP1 cells was targeted to a region towards the N-terminal part of the protein, indicating that a mutation has been made and therefore the protein cannot be translated. The same membrane was reprobed with the anti-pan-GBP antibody to

ensure that the expression of other family members was not affected (Figure 12B). To confirm the mutation was in the correct position sequencing on the A549 ΔGBP1 cells was carried out by Barbara Clough, with the disruption of the DNA sequence being exactly where predicted within the guide RNA region. Furthermore, the cells were plated on coverslips and fixed in order to carry out immunofluorescence staining with the anti-GBP1 antibody. It was clear that the A549 ΔGBP1 cells had significantly lower staining than their wild-type counterpart (Figure 6). The addition of these cells to the repertoire of GBP tools is very valuable due to the fact they can be utilised to monitor GBP1 expression during a *Toxoplasma* infection.

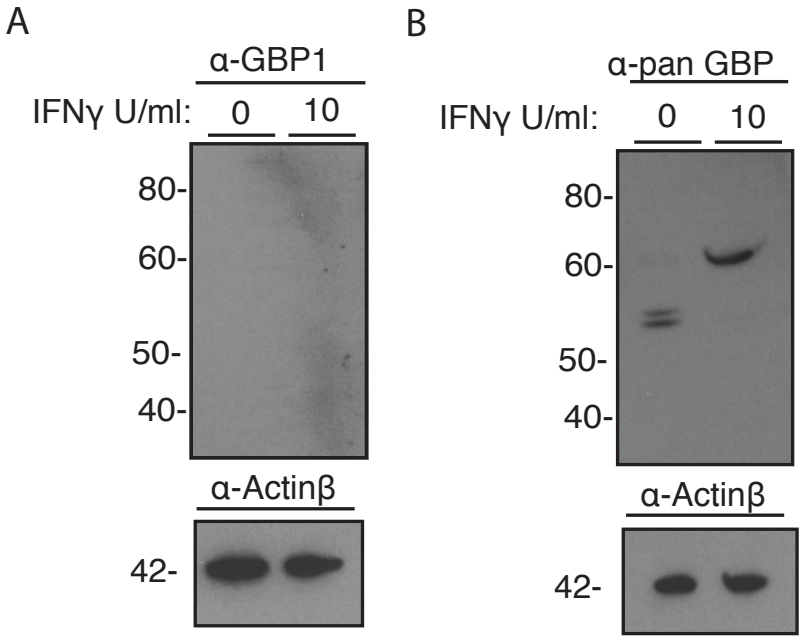


Figure 12 Characterisation of ΔGBP1 cells

Immunoblot showing lysates from A549 cells that had GBP1 targeted for disruption by CRISPR, probed with the anti-GBP1 polyclonal peptide antibody. 10μg of protein was added to each lane for SDS-PAGE. (A). Immunoblot showing lysates from A549 cells that had GBP1 targeted for disruption by CRISPR, probed with the anti-pan-GBP polyclonal antibody against recombinant GBP1 whole protein. 10 μg of protein was added to each lane for SDS-PAGE (B). Cells were stimulated or not for 18 hours with 10U/ml IFNγ. Each representative of 3 independent experiments. All samples from same experiment on the same day.

3.1.2 Optimisation of techniques to evaluate *Toxoplasma* infection characteristics

A large aim within this project is to determine whether or not GBPs play a role during infection with the parasite *Toxoplasma gondii*. In order to answer this question I require reliable methods to assess the survival and viability of parasites under varying conditions. In order to carry this out, I required techniques to determine how well the parasites were able to invade, replicate and form plaques.

To observe the efficiency of parasite invasion, a FACS based approach can be utilised. I used γ-irradiated td-Tomato parasites, which are invasion-competent but replication-deficient *Toxoplasma*, ensuring accurate assessment of parasite burden per cell without the confounding factor of parasite replication. These *Toxoplasma* were allowed to incubate with cells overnight before the cells were thoroughly washed and fixed. To analyse, cell reads were taken for the same length of time while gating focused on cells displaying a red fluorescence indicating the presence of an intracellular td-Tomato parasite. Since it was important to show that *Toxoplasma* has a capacity for invading A549 cells this method was employed. It was demonstrated that almost 50% of A549 cells were infected by this parasite, with a slight reduction of invasion efficiency seen when the cells were stimulated with IFNγ overnight prior to infection (Figure 13).

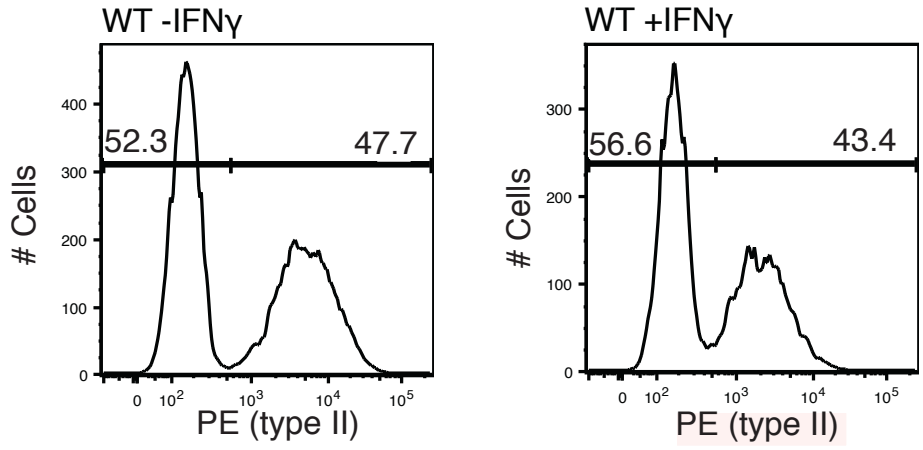


Figure 13 Invasion capacity of *Toxoplasma* in A549 cells

Graph displaying percentages of A549 cells that have been invaded by td-tomato type II *Toxoplasma* via fluorescence in the PE channel. An isotype control was carried out to ensure the correct peaks were observed. Cells were stimulated or not for 18 hours with 10U/ml IFNγ. MOI 1. Graphs representative of 3 independent experiments.

To determine how well a parasite can replicate within a cell, immunofluorescence microscopy was employed. *Toxoplasma gondii* expressing GFP were seeded onto A549 cells on glass coverslips and the infection was allowed to persist for a specific number of hours. The cells were then washed thoroughly and fixed before being mounted onto slides ready for analysis under the microscope. At least 100 vacuoles were identified per sample at random, and within each of the vacuoles the number of parasites were counted. By counting 1, 2, 4 or 8 parasites per vacuole, it was possible to calculate the number of times a parasite had replicated within the cell. It was necessary to show that *Toxoplasma* replicates, and can be controlled by IFNγ, in A549 cells in the way that has been widely described in the literature. Therefore, using this method, an IFNγ mediated control of *Toxoplasma* replication could be observed and calculated. I was able to show a significant reduction in parasite replication when the A549s were stimulated overnight with IFNγ prior to infection, this was apparent at 12 hours post-infection and became more striking by 18 and 24 hours post infection (Figure 14).

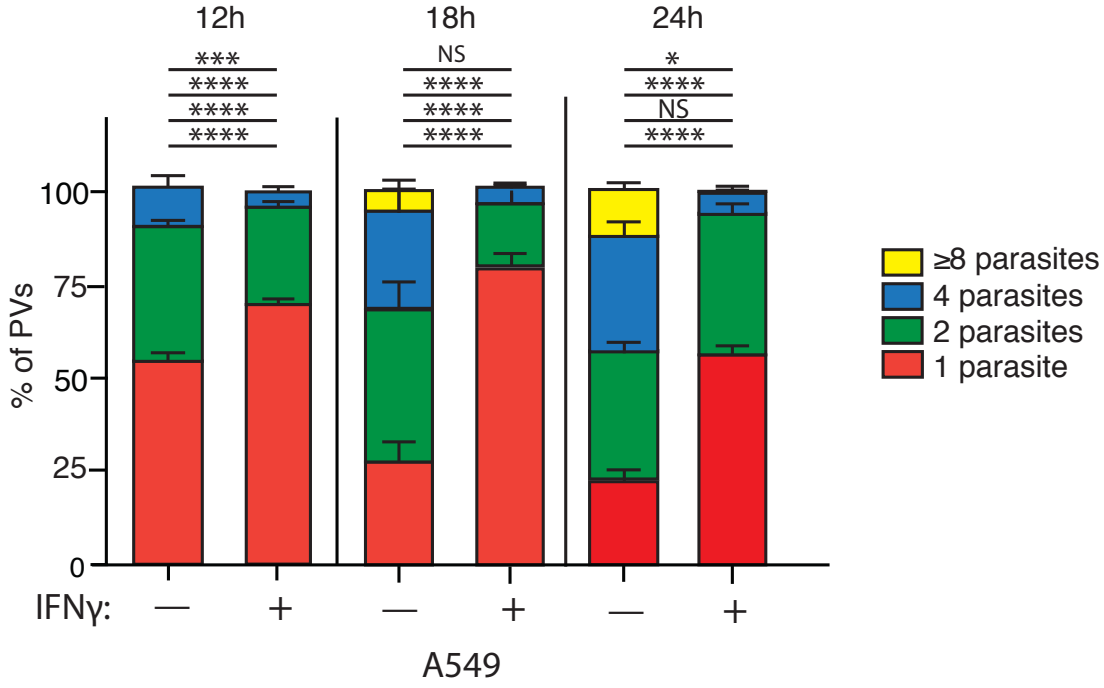


Figure 14 Replication of *Toxoplasma* in A549 cells

Graphical representation of replication of vacuolar type II *Toxoplasma gondii* in A549 cells. Cells were stimulated or not with 10U/ml IFNγ for 18 hours. N=3 independent experiments each performed in triplicate. Significance was determined using 2-way ANOVA, *p<0.01, ****p<0.00001.

A plaque assay is a widely accepted technique with which to determine the fitness of *Toxoplasma gondii*. The principal of this method is to seed parasites on a confluent layer of fibroblasts and count the resulting plaques formed after 3-5 days of infection. This method works well in contact inhibited cells such as HFFs. However there is a caveat in the fact that many other cell types do not stay adherent when infected with *Toxoplasma gondii*, as well as the fact that few cells are truly contact inhibited in growth. When a plaque assay was attempted with A549 cells, it was observed that after 24 hours of infection the cells began to lift meaning plaques were uncountable. In order to overcome this challenge, an indirect plaque assay was designed. This technique involved incubating parasites inside A549 cells for specific amounts of time before lysing the parasites from the cells and seeding the resulting numbers on a confluent monolayer of unstimulated HFFs in a dilution series. Plaques were then counted after the usual 3-5 days of growth in the HFFs. This method made it possible to move a plaque assay in to numerous cell types. When the plaques were counted after 3-5 days in unstimulated HFFs, *Toxoplasma* that had been incubated for 24 hours in pre-stimulated A549 cells showed an IFNγ-dependent restriction, demonstrating that this indeed was a representative way of determining parasite fitness (Figure 15).

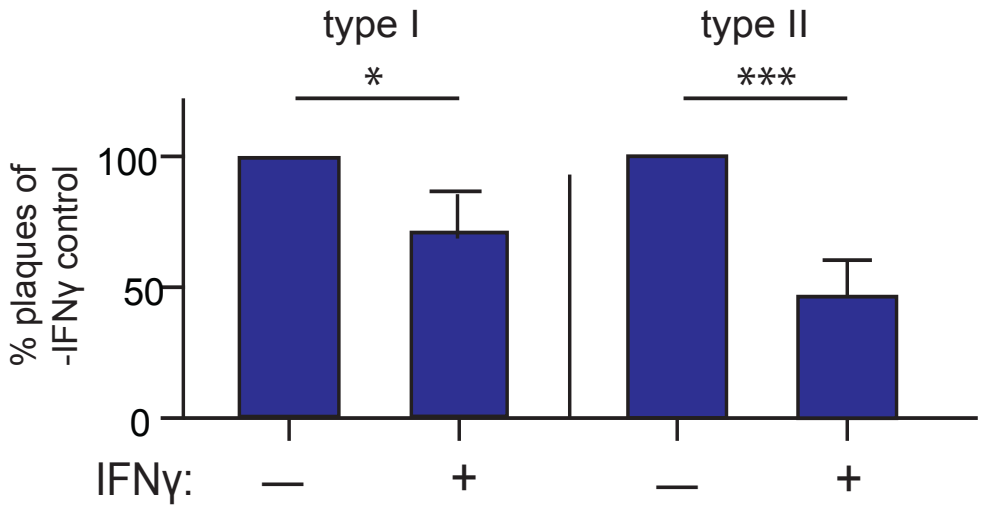


Figure 15 Indirect plaque assay showing *Toxoplasma* survival in A549 cells

Graph showing IFNγ-dependent restriction of type I and type II *Toxoplasma* as determined by indirect plaque assay after 24 hours of infection in A549 cells. Cells were stimulated or not with 10U/ml IFNγ for 18 hours. 300 type I parasites or 600 type II parasite were used. Values were normalised to 100% growth in unstimulated cells. N=3 independent experiments, each carried out in triplicate. Significance was determined using paired t-test, *p<0.01, ***p<0.0001.

3.2 Discussion

3.2.1 Tool characterisation

GBP-specific antibodies

One of the most important parts of a successful study is the quality of the tools at your disposal. In order to identify the characteristics of individual GBP family members, I began by ensuring I had the most reliable and well-characterised tools possible. The first step was to design polyclonal and monoclonal antibodies, which I fully analysed to be certain about the results they produced. Considering that GBPs were discovered over 30 years ago, the question arises why better tools are not more widely and commercially available. This is possibly due to the fact that the proteins have such a high level of identity and similarity that producing antibodies that are specific for individual family members is very difficult. The most widely used antibody directed at human GBP is one that recognises the family members 1 to 5.

A number of other groups have published articles using antibodies against both mouse and human GBPs that were produced in their respective labs. In 2002 Lubeseder-Martellato et al. produced a monoclonal antibody in rat against recombinant human GBP1 that was affinity purified against recombinant human GBP1-His_6 peptide. Two clones were produced and implemented in immunoblot, each reacted with both human GBP1 and GBP2 as determined by probing lysates from *Escherichia coli* that express recombinant His_6-tagged GBP1 or GBP2 (Lubeseder-Martellato et al., 2002). In 2007, Degrandi et al. produced polyclonal antibodies against mouse GBPs 1, 2, 3, 4, 5 and 7. This was done by immunising rabbits with peptides from each protein before the rabbit sera was texted against lysates of 293T cells that were transfected with each mouse GBP (1-10). Each serum was then affinity purified against the specific peptide sequence against which they were produced (Degrandi et al., 2007). It was from these sequences that I took inspiration when first identifying the specific peptides against which I would raise antibodies. Shenoy et al. produced a monoclonal antibody against full-length recombinant mouse GBP5, as well as polyclonal antibodies against peptide sequences from human GBPs 1 to 6. These affinity purified polyclonal antibodies were screened in turn against *E.coli* lysates expressing recombinant His-$_6$-tagged

individual human GBP family members to ensure there was no cross-reactivity (Shenoy et al., 2012). These other labs have shown it is possible to produce the tools required to study GBPs even though the difficulties I have outlined in this project exist. A monoclonal antibody is preferable in terms of longevity as the hybridomas can be brought back into culture to obtain more supernatant. However, Degrandi et al. and Shenoy et al., and now I have shown that polyclonal antibodies can be just as useful for making important discoveries about these proteins.

The aim within this project was to establish antibodies with which I could carry out immunofluorescence, immunoblotting and immunoprecipitation. I successfully obtained antibodies against GBP1, GBP2 and GBP4. The polyclonal anti-GBP1 antibody is proficient in immunoblotting, immunofluorescence and immunoprecipitation, however retrieves GBP2 also during immunoprecipitation. For GBP2, monoclonal and polyclonal antibodies were produced. The monoclonal antibody performs excellently in immunoprecipitation and immunoblotting. The polyclonal anti-GBP2 antibody was not functional for immunoblotting or immunofluorescence, but did efficiently retrieve GBP2 during immunoprecipitation. Monoclonal and polyclonal antibodies against GBP4 were produced, with the polyclonal antibody performing effectively in immunoblotting while the monoclonal antibody could be utilised in immunoprecipitation. More antibodies have been produced and characterised in part, however they have not been fully optimised and as such provide a valuable repertoire of tools that can potentially be utilised in the future (Appendix).

Many candidate antibodies that were produced recognised bands of 65-67kDa on an immunoblot, however on closer inspection they were not specific for individual family members. I was not surprised by this outcome. When protein family members show such a high level of identity, the cross reactivity of an epitope is expected. There was most cross-reactivity between GBPs 1 and 3 as these GBPs exhibit identity levels of 88% (Olszewski et al., 2006). In light of this, having produced a GBP1-specific antibody is highly valuable. The lack of specificity for one GBP family member was the biggest hurdle that I came upon while characterising these antibodies. However, this does not mean that they will not prove useful in the future. As mentioned above, most human GBP studies have published using a commercial antibody for GBP family members 1-5. Therefore, while we want to differentiate out the functions of the specific GBPs, we can use

combinations of these broad-range antibodies to determine overall expression of this family of large GTPases. A large problem I encountered through the process was the lack of signal altogether when testing for effectiveness. While I used software to choose the most immunogenic peptide sequence, as well as the company conjugating keyhole limpet haemocyanin to the peptide, in many of the animal subjects there was no apparent response. This could be due to an inaccuracy of the software predictive abilities. Perhaps the chosen immunogens were insufficient to produce an intense immune response to result in highly avid antibodies.

Polyclonal antibodies are relatively inexpensive to produce, but result in a pool of antibodies that potentially recognise a number of variations of the original immunogen peptide sequence. While this means there is a higher possibility of recognising the protein across a number of techniques, it increases the likelihood of an unspecific or weak reaction. Monoclonal antibodies are more costly to produce, and although the affinity and specificity is potentially higher for polyclonal antibodies, there is a higher chance that, as only one antigen sequence is recognised, this may not be a good candidate for target recognition. The monoclonal and polyclonal antibodies in this project were produced from very similar, but not identical, sequences. This fact together with the different natures of poly- and monoclonal antibodies means there is a slight discrepancy between the applications that were successful for the different candidates. The anti-GBP2 monoclonal antibody performs very effectively in immunoblotting, however the polyclonal counterpart is completely ineffective in this regard. Perhaps the elongation of the peptide sequence in the designing of the monoclonal antibody was necessary for the recognition of an epitope that produces an efficient immune response. Alternatively, the other components present in the polyclonal antibody may dilute the effect of the antibody and a pure, concentrated product from the hybridomas is required. With regards to the anti-GBP4 antibodies, it is likely that the polyclonal antibody is unable to immunoprecipitate due to a lack of affinity. The process of immunoprecipitation requires a binding strength sufficient to maintain the antibody-epitope interaction throughout the experimental process. It is likely that the monoclonal antibody clone I chose to progress with reacted to an epitope that maintained a much stronger immune response, and thus allowed for higher affinity antibodies.

It is becoming more apparent that each GBP family member seems to play a unique role, and for this reason, having the ability to separate out each GBP is becoming much more vital. Therefore it was imperative that I had a good tool with which to characterise the antibody candidates. This characterisation of the antibodies was only possible due the fact that a cell line that overexpressed individual GBP family members was made. The Coers lab at Duke University generously supplied the cloned constructs for GFP- and mCherry-tagged GBP1 and GBP2 expression. I cloned the constructs to produce the GFP-GBP7 cell line, as well as the mCherry-tagged GBP 3-7 constructs. Barbara Clough produced the constructs for GFP-tagged GBP 3-6 expression. Then sharing the work equally, Barbara Clough and I produced mouse fibroblasts that were transfected to stably express the components that allowed the inducible overexpression of individual, tagged human GBPs. As a result, each antibody could be tested against a range of lysates to show exactly which family members were being recognised. Furthermore, as mGBPs are not present without IFNγ stimulation, there was no risk of a false positive occurring in the form of cross-reaction with the mouse GBP counterparts. Another method by which to screen each antibody for specificity would be to use lysates of *E.coli* that express recombinant protein for each individual GBP. This method has been used successfully in the past and would be useful to implement as a second protocol to confirm our findings.

Further to the antibody production and characterisation of all human GBP family members, I chose to focus on GBP1 and GBP4. For GBP1, the antibody as described above was specific to GBP1 and proficient in IB and IF. Additionally, ΔGBP1 cells had been developed in the meantime (Barbara Clough, Frickel lab). For GBP4 I employed my specific polyclonal antibody to confirm that the protein was indeed a nuclear GTPase. Using these antibodies and other tools, I was able to outline a number of characteristics of GBPs in steady state and *Toxoplasma* infected cells. Repertoires of other antibodies, including a characterised antibody against GBP2, remain for others to continue this work.

Heterologous GBP expression

Protein overexpression is a technique that is classically used to study the function of said proteins during infection. Therefore, I wanted to develop stable overexpression systems in an appropriate cell type, with the intention of

investigating how this would affect the growth and survival of *Toxoplasma*. Unfortunately I was unable to produce human cells that reliably overexpressed any GBP protein. It was obvious that the cells were not able to withstand the introduction of additional GBPs, and I could not maintain the overexpressing cells. GBP1 has been implicated in basic cellular homeostatic responses, including actin remodelling (Ostler et al., 2014) and regulation of the epithelial barrier function (Schnoor et al., 2009). Perhaps when overexpression of GBP occurs, there is a basic cellular imbalance that causes this inability to retain the cells in a healthy state.

As more literature regarding the function of GBPs is emerging it is clear that, in mice at least, these large GTPases are playing an important role in the induction of inflammasome immune responses. This is potentially a logical explanation as to why the overexpression of GBP protein would result in the cells dying. The inflammasome response is a multi-protein innate immune complex is partly responsible for the inflammatory reaction against microbial pathogens (Guo et al., 2015). It assembles in the cytosol and the activation is reliant on signals in the form of danger-associated molecular patterns (DAMPs) or pathogen-associated molecular patterns (PAMPs) that are a consequence of invading pathogens (Martinon et al., 2002, Chen and Nunez, 2010, Lamkanfi and Dixit, 2014). The protein forming the scaffold of the complex defines the inflammasome type (Guo et al., 2015). The majority of inflammasomes are formed with Nucleotide-binding domain, leucine rich repeat containing receptor (NLRs). However non-NLR proteins, including absent in melanoma 2 (AIM2) and pyrin, can also establish an inflammasome (Takeuchi and Akira, 2010, Guo et al., 2015).

In 2012, Shenoy et al. identified human GBP5 as a non-NLR promoter of NLRP3 inflammasome pathway in response to *L. monocytogenes*, *S. enterica* typhimurium or to their lipopolysaccharides (LPS). This response leads to release of the proinflammatory cytokines IL-β and IL-18 (Shenoy et al., 2012), which are upstream in the process of pyroptosis- a type of inflammatory cell death (Lamkanfi and Kanneganti, 2012, Strowig et al., 2012). More recently, it has been shown that during *L. pneumophila* or *S. enterica* typhimurium infection in mice that the GBP proteins clustered on chromosome 3 are essential for the activation of a non-canonical inflammasome response and pyroptosis that is defined by activation of caspase-11 (Pilla et al., 2014, Meunier et al., 2014). This activation is dependent

on sensing the bacteria in the cytosol, and can be triggered by direct transfection of the LPS itself (Pilla et al., 2014). These bacterial pathogens are both contained in a vacuole, and in macrophages that lack the GBP$^{chr3-/-}$ family members there was a reduced staining for galectin-8, a marker for lysed vacuoles, compared to wild type. This indicated that these GTPases were also involved in the breakage of the pathogen vacuoles, allowing for inflammasome-activating sensing of the bacterial components (Meunier et al., 2014). The AIM2 inflammasome pathway is involved in induction of inflammation in response to the sensing of double-stranded DNA, and results in cell death by pyroptosis (Guo et al., 2015). Mouse GBP2 and GBP5 are implicated in the activation of this inflammasome type during infection with the cytosolic bacteria *Francisella novicida* by inducing bacterial lysis and release of DNA to the cytosol to be detected by the AIM2 complex (Meunier et al., 2015, Man et al., 2015). It would be very interesting to determine whether or not GBPs play a similar role during infection with *Toxoplasma* in both human and mouse species, especially as there has been no evidence published that the PV of this parasite is broken during infection in humans. While a secondary signal is also required for inflammasome induction, perhaps it is not a saturated response during upregulation of the endogenous protein, meaning that overexpressed GBP protein can further induce this defensive cell reaction.

These large GTPases have been strongly implicated, in response to pathogenic invaders, to activate a number of processes that result in host cell pyroptosis (Pilla et al., 2014, Meunier et al., 2014, Man et al., 2015, Meunier et al., 2015). Therefore, it would infer that the transfection of GBP DNA into a cell for heterologous overexpression is not tolerated as this protein family works to induce cell death.

Since starting this endeavour, communication with a number of other labs and individuals has revealed that they also are not able to reliably overexpress these proteins. While overexpression has been used in a number of GBP studies, often these expressions are not stable. Thus, there is too short a time window available after heterologous GBP protein expression meaning prolonged infection time points are not feasible. Moreover, if a cell is already compromised, there is no guarantee that the host/ pathogen relationship is occurring in the most biologically relevant manner. This further highlights that we need to invest time in developing other ways with which to determine GBP function.

CRISPR-mediated GBP knock out cells

To have a specific protein reliably knocked out in a cell is one of the most effective ways of analysing the role it plays in certain conditions. Therefore, I made sure to fully characterise cells that were made to be potentially deficient in GBP1 or GBP4 using the CRISPR-Cas9 genome editing technique (CRISPR targeting performed by Barbara Clough, Frickel lab). I successfully identified A549 cells that did not express GBP1 protein on an immunoblot. After these cells were sequenced to show that the GBP1 sequence was efficiently disrupted, they were to be used in experiments to determine the protein function during a *Toxoplasma* infection. Additionally, I characterised cells that were made to be deficient in GBP4 by immunoblot (CRISPR targeting performed by Joseph Wright, Frickel lab, data not shown). It is apparent that these cells have been manipulated, but rather than lacking GBP4 they contained a truncated version of the protein. It will be fascinating to work out if this truncated protein form is still active, and whether or not it affects the survival and growth of *Toxoplasma*.

The new development of being able to quickly and efficiently knock out proteins using CRISPR-Cas9 is an invaluable tool within the entire biological science field. This revolution has been important in this project with GBPs. Studies in the past have used knock down methods to determine a functional role of these proteins during infections with LPS, bacterial and viral pathogens (Shenoy et al., 2012, Pan et al., 2012, Al-Zeer et al., 2013). The method has proven useful and has led to breakthroughs that have been important to understanding roles that GBPs play. However, this is not a sustainable method of deleting a protein in order to understand how it functions over a long-term period. Now the advance of CRISPR-Cas9 offers a solution for this problem.

The progress made in the CRISPR field has been exponential. New plasmids to enable cloning of the Cas9 system are emerging constantly, resulting in more efficient ways to insert the guide RNAs and select for successful transfections. Importantly, characterisation of these cells needs to be stringent and complete. It is vital that the deletion of the protein is not only tested for using an immunoblot approach. A cut and nonhomologous repair of the DNA may occur, but there is no guarantee that the protein is not still transcribed in a truncated form. Depending on the location of the antibody's immunogen epitope, this truncated protein may or may not be detected by an antibody. Therefore the protein may not

be identified for example, but may still be fully or partly functional. For this reason, it is necessary to get the specific region of the DNA sequenced, and determine how much of the sequence has been disrupted. There is always the risk of off-target effects when manipulating the genome using a guide RNA that may locate to a gene that is not the desired one. For this reason, as well as sequencing the top predicted off-target regions, a second clone made using a different guide sequence should be used as comparison. In the case of GBP1, I ensured that there was no loss of staining with our pan-GBP antibody, indicating that other GBPs are still intact. Moreover, during the course of my project, I reinforced the results I acquired with a second clone (data not shown). This established that the effects I saw during infection with *Toxoplasma* were not due to an aberrant cell clone that produced results due to unknown genome manipulations. I was able to further confirm the knockout of GBP1 by complementing the protein back into the cell using a lipid transfection of the mCherry-GBP1 construct (produced by Coers lab). When the protein had been reintroduced, the cells responded like the wild type cells during *Toxoplasma* infection (see later, Chapter 4, Figure 20). Complementation is a quick and reliable method of confirming a phenotype is due to a real knockout rather than off-target effects. The next step with these cells is to sequence the regions of the other family members, to be sure that there is not disruption in any of those areas.

Research can only be as reliable as the tools we have. Therefore, the availability of characterised cells and antibodies is necessary to be sure of the outcomes of our experiments. There has been a renewed interest in studying this family of large GTPases lately, and to share our reagents with collaborators is a vital next step after the work that has been put in. Poor reagents can lead to a lack of data, and inconsistencies in observations. The analysis of these cells and antibodies engrossed a large part of my PhD project. As a result, I have an understanding of the difficulties in finding specific tools, and how to optimise processes to get the best from the product. I do believe that there needs to be more stringency in characterising commercially available tools, especially for targets that have a high level of similarity to something else. These commercial tools often have been produced with an immunogen that is not made publically known, and the analysis of these proteins for immunoblotting is frequently only the observation of a band of the correct size on a membrane which may or may not represent the correct protein. CRISPR-Cas9 presents a further opportunity for the

study of GBPs in this regard. The option of tagging the endogenous protein with an epitope tag is now a real possibility. To add a tag would mean that reliable immunoprecipitation and detection on an immunoblot or in immunofluorescence with very well established antibodies would become possible. This technique, while revolutionary cannot completely replace the need for antibodies as it is a time consuming process and is possible only with transfectable cells. Furthermore, even a small insert may affect the functions of the endogenous protein so caution should be taken.

3.2.2 Optimisation of experiments

Once reliable tools have been acquired, it becomes necessary to ensure that the techniques and methods being used are the most appropriate to answer the question being asked. I optimised an experiment to determine the invasive capacity of *Toxoplasma* in epithelial cells. Following this I determined both how to assess the replicative ability of the parasite in the vacuole and to assess the plaque forming ability within epithelial cells.

For the invasion assay I used γ-irradiated parasites; this is because they can invade effectively but have lost the ability to replicate as a result of DNA damage. Since epithelial cells are non-phagocytic, this means when I detect a *Toxoplasma* within a cell, I can be certain that it is the result of direct invasion. Next, I ensured that I could carry out an assay to calculate the replicative ability of vacuolar parasites in A549 cells using microscopy. This is a generally accepted mechanism with which to study parasite replication, however the protocol is usually carried out in fibroblasts. In this case, I confirmed that A549 epithelial cells restricted *Toxoplasma* in an IFNγ dependent manner, and optimised the concentration of cytokine to use to ensure that the results were consistent and reliable. When the concentration of IFNγ was too high, the infection could not successfully establish and so the results were not biologically accurate. I also ensured I chose the correct time points at which to count the parasites. It was difficult to ensure that it was possible to count the parasites across the different conditions over a time course. In the absence of IFNγ, the parasites replicated at much increased speed and often resulted in vacuoles so large it was impossible to

quantify the number of parasites within. Over time, I optimised the MOI of the infection alongside the appropriate time points to ensure that the counting was carried out in a consistent and reliable manner.

Assessing the ability of *Toxoplasma* to form plaques within a cell layer, and carrying out fitness assays is something that has been done extensively in the literature over the years. These assays have relied on the parasites being contained in fibroblasts. This is because these cells are heavily contact inhibited, and *Toxoplasma* is able to form very uniform, predictable and quantifiable plaques in these cells. This protocol is much more commonly implemented to assess parasite mutants against the wild type rather than to assess host cell factors. It has been used previously however to compare *Toxoplasma* growth in cells that have had host targets knocked down with siRNA, and those that had been stimulated by IFNγ with cells that are unstimulated (Virreira Winter et al., 2011, Niedelman et al., 2013). When it is necessary to move the parasites to another cell type, problems arise when trying to determine the pathogen replicative capacity. As the infection progresses in these other cell types, the cells begin to grown on top of each other and also many lose their adhesive capacities and lift from the culture dishes. As a result, it becomes impossible to accurately quantify how well the parasites have replicated. For this reason, I designed an experiment that could establish short-term survival of *Toxoplasma* in epithelial cells. This is important because it allows us to use pre-existing genetically modified non-fibroblast cells in *Toxoplasma* viability assays. It is time consuming to make genetically engineered cells in the first place, so where the mutant cell has already been produced, it would be undesirable to remake these genotypes in fibroblasts. Moreover there is also the fact that fibroblasts may not be the biologically relevant cell type to study. By incubating the parasites in the epithelial cells for a maximum of 24 hours before syringe lysing them out and plating them on the fibroblasts, the gradual lifting of the cells is avoided. This protocol will allow researchers to move their experiments between cell types, and not be restricted to fibroblasts.

The next step with these methods is to produce a high-throughput method that would be useful, for example, in screening a large number of parasite mutants or different host cell CRISPR knock out clones. One could possibly determine invasion, host cell killing and/or parasite replicative defects. This could be done by utilising FACS, and to determine how to accurately view the number of parasites

within a cell, and how many times they have replicated. This is possible due the fact that one can expand on the method I used to assess the percentage of infected cells by gating on fluorescent parasites inside the cells. As the number of parasites inside the cells increases, the mean fluorescence intensity would also be expected to increase, hence allowing quantification of replicative ability.

The efforts taken to overexpress these large GTPases have proven that this is not a viable or reliable method with which to study them. The fact that the cells are sickly means that results could be attributed to this, rather than directly because of the presence of GBPs. The importance of consistent, reliable and well-characterised tools and experimental mechanisms needs to be stressed more within the science community, with emphasis on analysing antibody specificity and cell genetic features.

Chapter 4. The impact of human Guanylate Binding Proteins on *Toxoplasma gondii* infection

4.1 Results

4.1.1 GBP1

GBP1 has been the most intensely studied human guanylate binding protein family member. It has been implicated in protection against pathogens such as hepatitis C virus (Itsui et al., 2009), influenza A virus (Zhu et al., 2013), vesicular stomatitis virus (Anderson et al., 1999a) and encephalomyocarditis virus (Anderson et al., 1999a). GBP1 also interacts with actin (Ostler et al., 2014) and plays a role in endothelial cell proliferation (Guenzi et al., 2001). While this GBP family member is believed to be relevant in pathogenic infection responses, these studies have relied on overexpression of GBP1, and hence we still do not understand how the endogenous protein acts. Considering the importance of murine GBPs in the context of restricting *Toxoplasma* infection, I decided to determine whether GBP1 could recognise the pathogen, and therefore whether or not it plays a role in host response.

The subcellular localisation of a protein often gives clues to its function. The literature has previously defined GBP1 as a cytosolic protein as determined by heterologous overexpression of the protein with a GFP-tagged GBP1 (Tripal et al., 2007, Britzen-Laurent et al., 2010). Furthermore, it has been shown that GBP1 localises to the golgi when treated with aluminium fluoride (AlF_3), a reaction that causes the GTPase to mimic GTP-binding (Britzen-Laurent et al., 2010). To determine the localisation of endogenous GBP1 and to answer if this is affected by *Toxoplasma*, a subcellular fractionation technique followed by SDS-PAGE was utilised. THP-1 macrophages were stimulated or not with IFNγ overnight before being infected with either type I (RH) or type II (Pru) *Toxoplasma*. The infection was allowed to persist for 2 hours before the cells were lysed and subjected to subcellular fractionation. The resulting product was run on a gel, transferred by western blot before being probed with the anti-GBP1 peptide polyclonal antibody. GBP1 was confirmed to be a cytoplasmic protein, with the location remaining unchanged on infection (Figure 16).

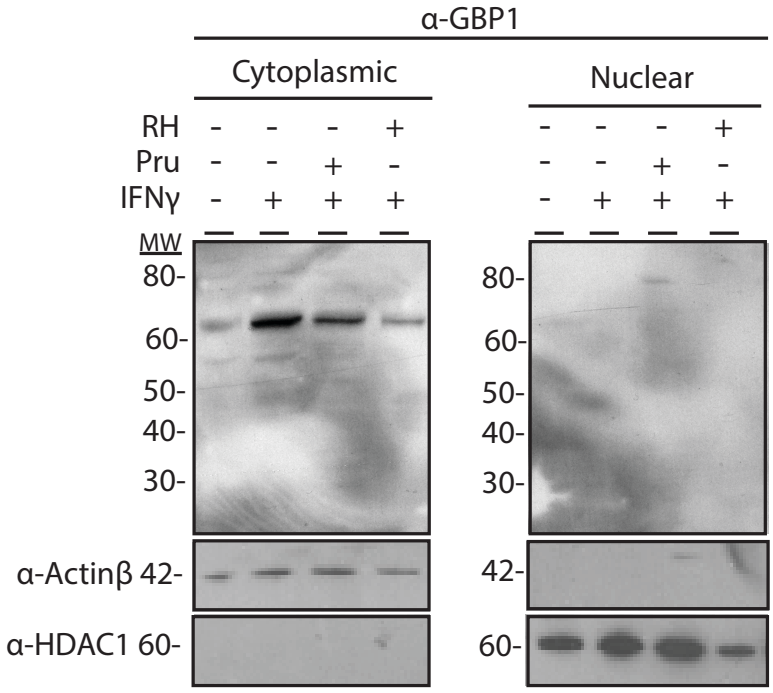


Figure 16 GBP1 is a cytoplasmically localised protein

Subcellular fractionation of THP-1 cells shows the location of GBP1 in steady state cells and in *Toxoplasma* infected cells, MOI 3. Cells were infected for 2 hours. 10µg of protein was added to each lane for SDS-PAGE. All samples were run on the same gel, blocks have been indicated for demonstration. Cells were stimulated or not with 10U/ml IFNγ for 18 hours. Representative of 3 independent experiments. Each panel indicates a separate gel, all samples from same experiment on the same day.

It is well documented that a selection of murine GBPs localise to the parasitophorous vacuole during a *Toxoplasma* infection (Degrandi et al., 2007, Virreira Winter et al., 2011, Selleck et al., 2013), Low levels of recruitment of human GBPs have also been reported (Ohshima et al., 2014). However, the immunofluorescence in the Ohshima study relied on an antibody that recognised GBPs 1-5, resulting in the recruitment status of individual family members being unknown still. To determine whether or not GBP1 was recruited to the PV during infection, A549 epithelial cells and THP-1 macrophages that had been stimulated or not with IFNγ, were seeded onto coverslips before infecting with type I or type II *Toxoplasma*. The infection was allowed to persist for 1 – 12 hours, with the cells being fixed at a number of time points, before being stained with the anti-GBP1 peptide polyclonal antibody and analysed by immunofluorescence microscopy. The

coverslips were viewed using a confocal microscope, and at no time point was recruitment of GBP1 to the PV observed in either cell type although a clear increase in cytoplasmic GBP1 expression was observed when cells were stimulated with IFNγ. Figures 17 and 18 show representative images of cells fixed at 1, 2 or 4 hours.

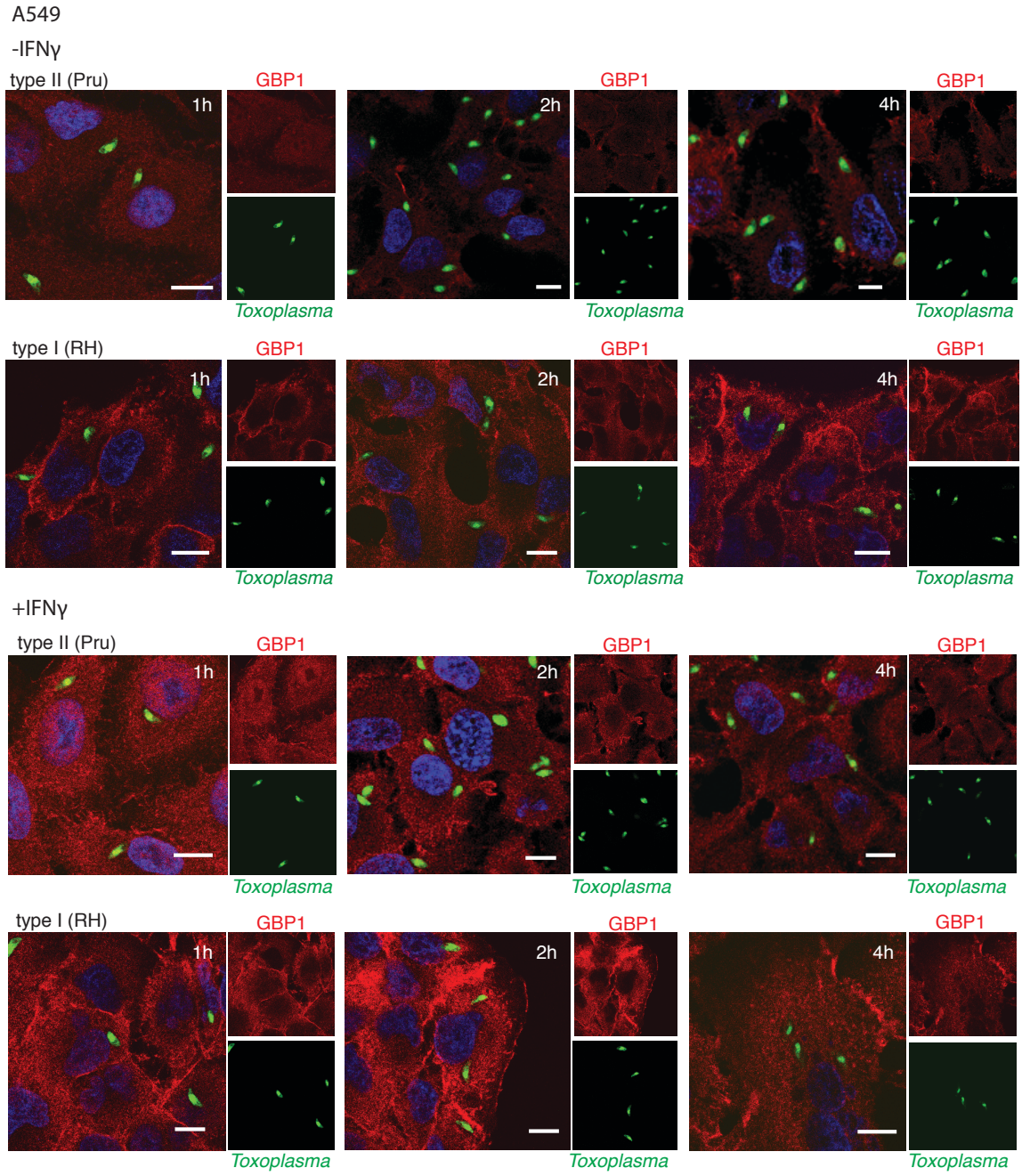


Figure 17 GBP1 does not recruit to the *Toxoplasma* parasitophorous vacuole in A549 cells

Confocal microscopy images showing the distribution of GBP1 in *Toxoplasma*-infected A549 cells over a time course. Images were viewed using 100X magnification. MOI 2 for type I, MOI 3 for type II. Cells were stimulated or not for 18 hours with 10U/ml IFNγ. All scale bars 10µm. Representative images from 4 experiments.

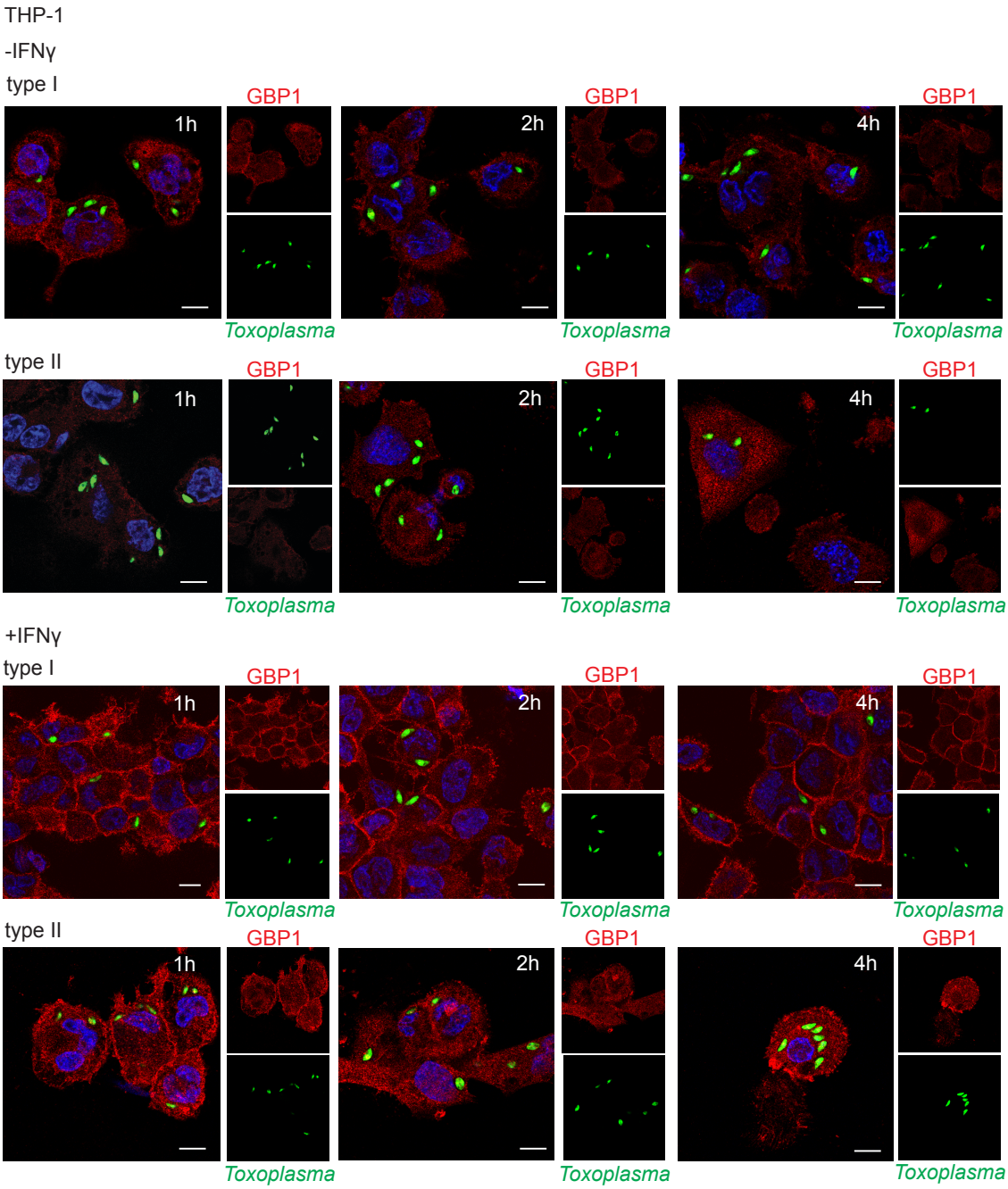


Figure 18 GBP1 does not recruit to the *Toxoplasma* parasitophorous vacuole in THP-1 macrophages

Confocal microscopy images showing the distribution of GBP1 in *Toxoplasma*-infected THP-1 macrophages over a time course. Images were viewed using 100X magnification. MOI 2 for type I, MOI 3 for type II. Cells were stimulated or not for 18 hours with 10U/ml IFNγ. All scale bars 10μm. Representative images from 3 experiments.

The recruitment of murine GBPs to the PV has always been thought to be necessary for their function in disruption of the parasitophorous membrane and subsequent parasite destruction (Yamamoto et al., 2012, Degrandi et al., 2013). In this study, it was clear that GBP1 did not recruit to the PV. Therefore, I sought to establish whether or not GBP1 still played a role in host restriction of *Toxoplasma gondii*. First, I assessed if the overall survival and viability of parasites was affected when in GBP1 deficient epithelial cells. A549 cells that had GBP1 knocked out by the CRISPR/Cas9 genome editing technique (made by Barbara Clough) were tested by indirect plaque assays alongside their wild type counterparts. Either type I or type II *Toxoplasma* were used to infect A549 cells that had or had not been stimulated overnight with IFNγ. The infection was allowed to persist for 6 hours before the cells were syringe lysed and the extracellular parasites were plated onto confluent, unstimulated HFFs. 3 to 5 days post infection, the plaques were counted and normalised to the number of plaques in wild type unstimulated A549s. The IFNγ stimulated cells overall produced a much-reduced number of plaques (Figure 19). Significantly, the A549 ΔGBP1 cells showed an increase of plaque numbers with type II parasite compared to the wild type cells. This increase in plaques was observed in both the IFNγ stimulated and unstimulated cells, perhaps due to the fact that GBP1 is already present and detectable at a basal level in these cells (Figure 19). There was no significant difference in the amount of plaques between wild type and ΔGBP1 A549 cell when type I parasites were used (Figure 19). These results suggest that indeed GBP1 plays a strain dependent role in the restriction of *Toxoplasma*, with type II parasites being more able to survive and replicate in the knock out cells. This result indicates that GBPs can have functions on vacuolar pathogens at a location away from the vacuole.

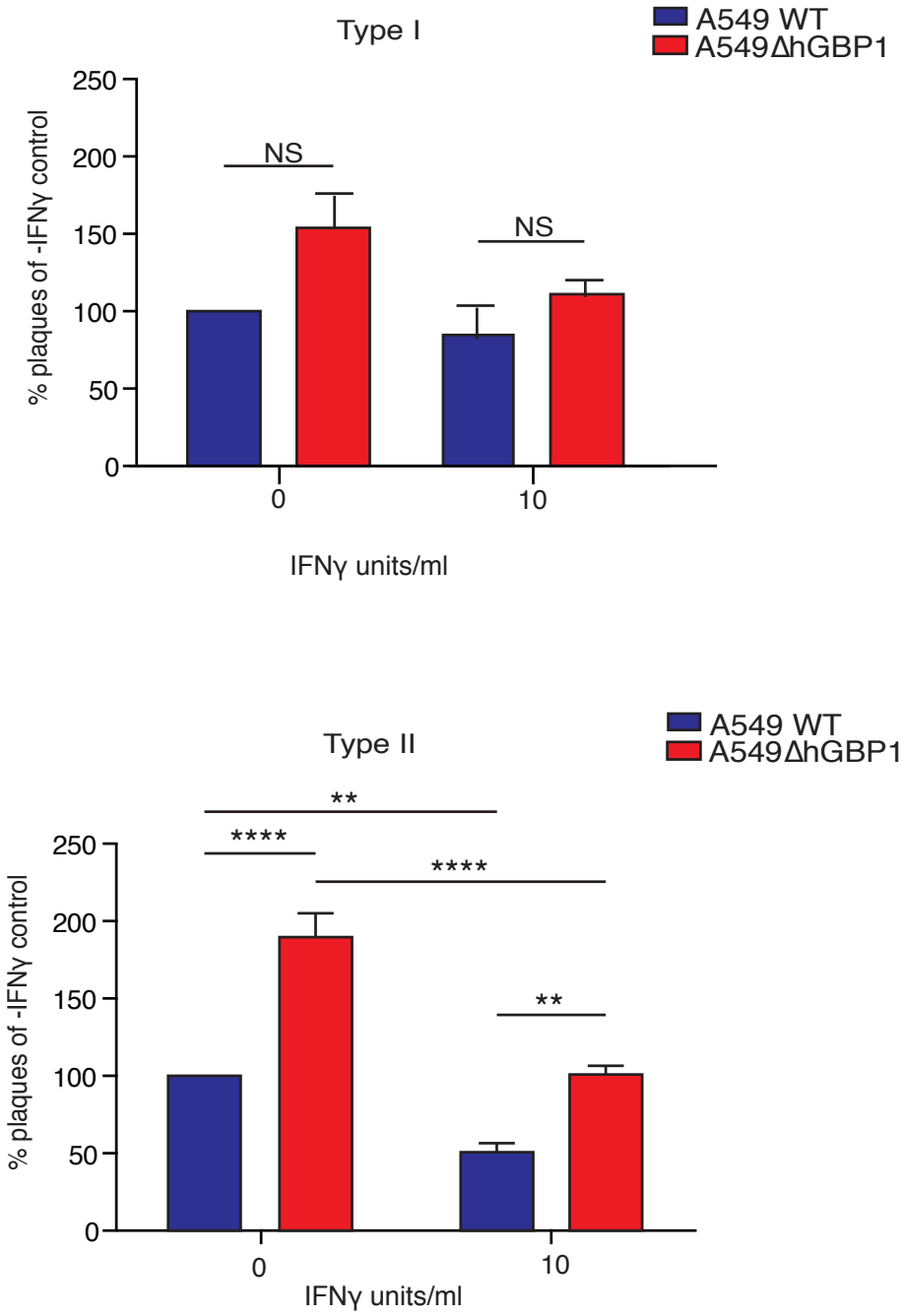


Figure 19 GBP1 can restrict the growth of type II *Toxoplasma* in A549 cells, but not type I

Percentage of *Toxoplasma* plaque growth in A549 wild type and ΔGBP1 cells compared to plaque growth in wild type unstimulated cells. A549 cells were stimulated or not for 18 hours with 10U/ml IFNγ, before *Toxoplasma* infection for 6 hours. 300 or 600 parasites per well were used for type I or type II parasites respectively, in a 24-well plate. Results were normalised to 100% in wild type unstimulated cells. N=3 independent experiments, carried out in triplicate. Significance calculated by 2-way ANOVA, **p<0.001, ****p<0.00001.

To ensure that the results were not an artefact due an aberrant clone produced by CRISPR, I complemented the knock out cells with GBP1 in order to rescue the wild type phenotype. A mCherry-tagged GBP1, or the empty mCherry-vector as a control, was transfected into the A549 ΔGBP1 cells using the FuGene lipid-based transfection method. The toxicity of the transfection seen in these cells was less than what was observed in wild type cells, likely resulting from a higher tolerance due to absence of endogenous GBP1 protein. The complemented cells were stimulated overnight or not, infected with type II *Toxoplasma* for 6 hours as previously described, before being syringe-lysed and plated on to confluent HFFs. The plaque numbers were compared to those produced in wild type cells, and again were normalised to parasite growth in the wild type unstimulated A549s. There was no significant difference between the number of plaques produced in wild type cells and those in the complemented knock out cells (Figure 20). Similarly, there was no statistically significant difference between the plaques produced in the ΔGBP1 cells compared to those that had been transfected with the mCherry control vector (Figure 20).

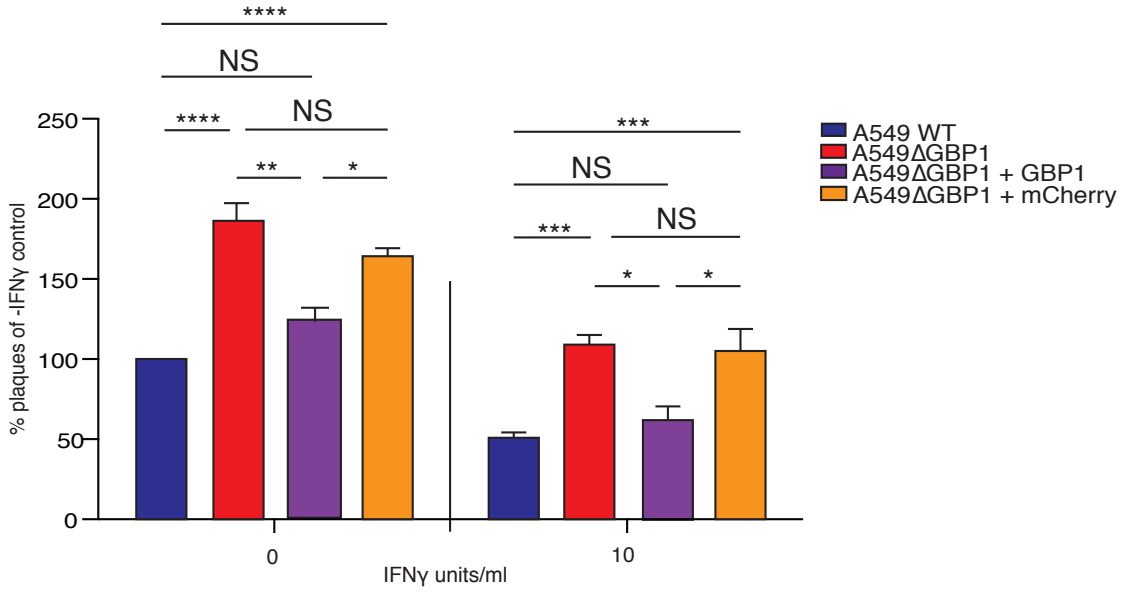


Figure 20 Complementation of GBP1 rescues control of *Toxoplasma* growth

A549 ΔGBP1 cells were transfected or not with mCherryGBP1 or mCherry before infecting with *Toxoplasma* for an indirect plaque assay and compared to plaque production in wild type A549 cells. Results were normalised to 100% wild type unstimulated cells. Cells were stimulated or not for 18 hours with 10U/ml IFNγ before infection with 600 *Toxoplasma* per well for 6 hours in a 24-well plate. N=3 independent experiments, carried out in triplicate. Significance calculated by 2-way ANOVA, *p<0.01, *p<0.001, ***p<0.0001, ****p<0.00001.

These results show that indeed GBP1 is responsible for at least part of the restriction of *Toxoplasma* type II in epithelial cells. This restriction occurs in a strain dependent manner and is independent of IFNγ stimulation.

Toxoplasma is an intracellular parasite with many complex stages of its life cycle. During the tachyzoite stage, in order to continue the infection it is necessary that the parasites are able to invade, replicate and egress. The restriction of *Toxoplasma* by GBP1 could be affecting any of these vital functions. As A549 cells do not have any phagocytic capacity, *Toxoplasma* relies completely on its own ability to invade this cell type. Therefore, I decided to first assess whether or not GBP1 played a role that was affecting the levels to which *Toxoplasma* could invade these cells. In order to do this, A549 ΔGBP1 cells, stimulated or not with IFNγ, were infected with γ-irradiated tdTomato-expressing *Toxoplasma* overnight before fixing the cells and analysing the populations using FACS. Cells that were fluorescing in the PE channel contained the intracellular tdTomato parasites. As the *Toxoplasma* had been γ-irradiated, they were rendered replication-deficient and therefore unable to distort the number of parasites known to have invaded. Wild type A549 cells were shown to be invaded by *Toxoplasma* at a rate of approximately 50%, as shown in Figure 13. When the percentage of A549 ΔGBP1 cells containing parasites were compared to wild type cells, there was no difference between the rates of invasion (Figure 21). This would suggest that the restriction of *Toxoplasma* by GBP1 is not by mediation of invasion of the parasites; hence GBP1 is able to affect the survival of *Toxoplasma* at a stage later on in the infection.

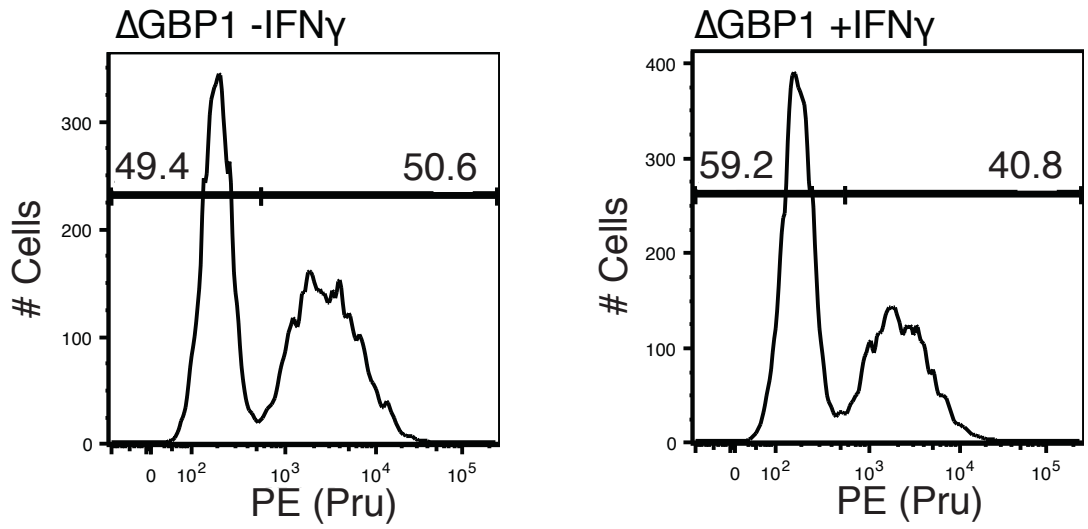


Figure 21 Invasion capacity of *Toxoplasma* in the absence of GBP1

Graph showing the percentage of A549 ΔGBP1 cell populations infected with irradiated tdTomato expressing type II (Pru) *Toxoplasma* that fluoresce in the PE channel. An isotype control was used to ensure the correct peak was observed. MOI 1. Cells were stimulated or not for 18 hours with 10U/ml IFNγ. Representative of 3 experiments.

In light of GBP1 not playing a role during the invasion of *Toxoplasma* invasion of A549 cells, I determined how the parasites were able to replicate in absence of GBP1. In order to do this, eGFP-expressing type-II *Toxoplasma* were seeded on to confluent A549 ΔGBP1 cells plated onto glass coverslips. These cells had been stimulated or not overnight with IFNγ. The infection was allowed to persist up to 24 hours, with the cells on coverslips being fixed and permeabilised ready for staining for immunofluorescence microscopy over a time course. The cells on the coverslips were stained with Hoechst to identify the nuclei before being mounted onto glass slides. Using a bright-field microscope, vacuoles inside the cells were located and the number of parasites within each one was counted. A minimum of 100 vacuoles was counted for each condition. The capacity of *Toxoplasma* to replicate in A549 wild type cells has already been determined in Figure 14. These data have been shown again to directly compare replication rates with those seen in A549 ΔGBP1 cells. The number of replication cycles (measured by counting parasites per vacuole) that had occurred was calculated at 12, 18 and 24 hours post infection. A clear restriction of growth by IFNγ is seen at all time

points as expected (Figure 22). At 12 hours, a significant difference between the replication in the wild type versus the ΔGBP1 cells was already seen. In the ΔGBP1 cells, there were significantly more vacuoles containing 2 parasites, indicating more *Toxoplasma* had undergone one replication cycle (Figure 22). By 18 hours post infection, ΔGBP1 cells contained significantly more vacuoles that harboured 2 or 4 parasites, indicating 1 or 2 replication cycles (Figure 22). At the final time point of 24 hours post infection, the IFNγ stimulated ΔGBP1 cells contained significantly more vacuoles that had 8 or more parasites within, indicating 3 or more replication cycles (Figure 22). By this time point and without IFNγ, the parasite replication is well progressed and there are numerous *Toxoplasma* in each cell. These results show that GBP1 plays a role in restricting the onset of replication of *Toxoplasma gondii* in A549 cells.

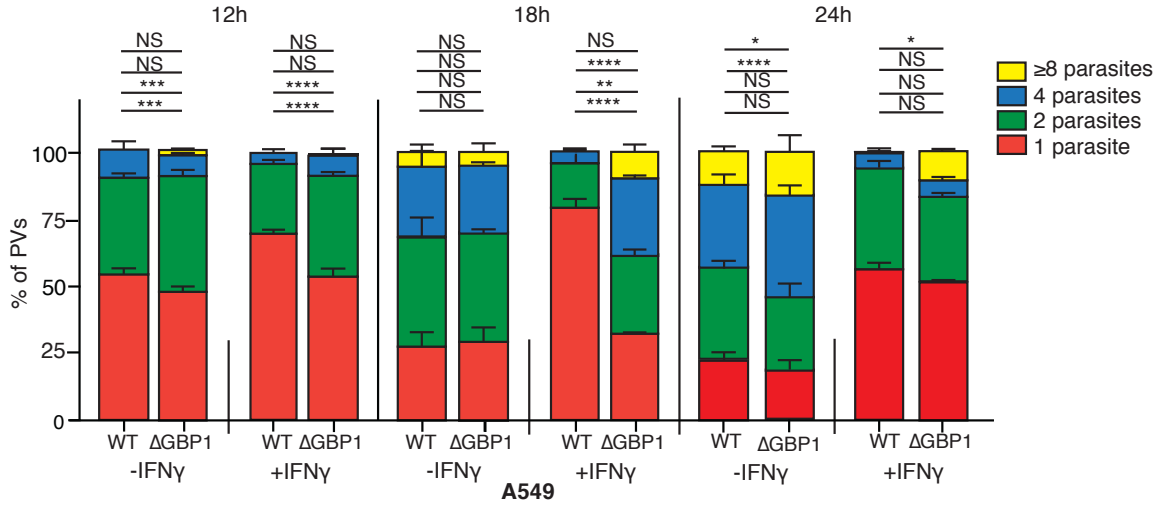


Figure 22 GBP1 restricts replication of *Toxoplasma* in A549 cells

Stacked graph demonstrating the percentage of PVs containing 1, 2, 4 or over 8 parasites in A549 wild type and ΔGBP1 cells. Cells were stimulated or not for 18 hours with 10U/ml IFNγ before infection with type II *Toxoplasma*, MOI 0.5. N=3 independent experiments, each carried out in triplicate. Significance calculated by 2-way ANOVA, *p<0.01, ***p<0.0001, ****p<0.00001.

4.1.2 GBP4

While there have been a number of studies focussing on GBP1, most of the other GBP family members are less well studied. One incompletely characterised family member is GBP4. A single study has used overexpressed fluorescent protein to

show that GBP4 has both cytoplasmic and nuclear localisation in HUVECs (Tripal et al., 2007). GTPases in the nucleus are not a common occurrence, with functions that usually equate to shuttling of other proteins (Moore, 1998). Interestingly, murine Mx1 protein is required to have a nuclear localisation in order to restrict influenza virus. Human Mx1 protein is solely cytoplasmic however and still exerts an effect against influenza (Zurcher et al., 1992). I first determined the subcellular localisation of GBP4 in a steady state cell. As the previous localisation study had been carried out using overexpressed tagged GBP4, it was important to confirm the location of the endogenous protein. First, I differentiated THP-1 monocytes into macrophages by stimulating them with 1μM PMA for 3 days. Following this, the cells were stimulated or not with IFNγ overnight. I carried out a subcellular fractionation, ran the resulting products on SDS-PAGE and transferred the gel onto a nitrocellulose membrane. This membrane was then probed with the anti-GBP4 peptide polyclonal antibody. Interestingly, GBP4 was found in the nuclear fraction, with none visibly present in the cytoplasm (Figure 23).

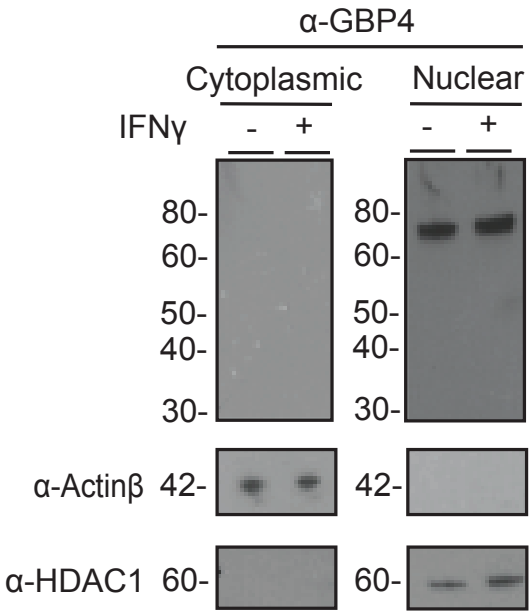


Figure 23 GBP4 is a nuclear protein in THP-1 macrophages

Immunoblot of THP-1 macrophages that have undergone subcellular fractionation and have been probed for GBP4 with the anti-GBP4 polyclonal peptide antibody. 10μg of protein was added to each lane for SDS-PAGE. SDS-PAGE was carried out on the same gel; blocks have been indicated for demonstration. Cells were stimulated or not for 18 hours with 10U/ml IFNγ. Representative of 3 independent experiments. For each respective antibody, all samples were exposed to the film for equal times before development.

To determine the localisation of GBP4 during *Toxoplasma* infection, another subcellular fractionation was carried out, but this time on *Toxoplasma*-infected THP-1 macrophages. As above, the macrophages were stimulated with IFNγ overnight before being infected with parasites for 2 hours. After this incubation, the cells were subjected to a subcellular fractionation before running on SDS-PAGE. The samples were then probed with the anti-GBP4 polyclonal antibody via immunoblot after transfer onto a nitrocellulose membrane. The results showed that as with the uninfected cells, GBP4 was present in the nucleus in the presence of type II *Toxoplasma* at an MOI of 3. However, when the cells were infected with type I parasites at an MOI of 3, GBP4 expression was completely lost. Furthermore, when the cells were infected with a combination of type I and II *Toxoplasma* (both MOI 3, resulting in overall MOI 6) GBP4 expression was partially decreased. In this double infection some expression can still be seen, implying there is no dominant effect by the type I parasites (Figure 24).

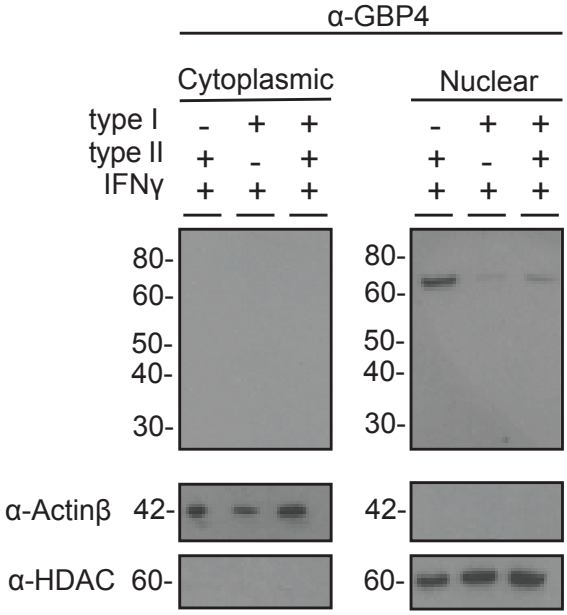


Figure 24 Type-I, but not type-II *Toxoplasma* alters protein expression of GBP4 in THP-1 macrophages

Immunoblot of THP-1 macrophages that were infected with *Toxoplasma* for 2 hours at an MOI of 3 before undergoing subcellular fractionation to be probed for GBP4. Cells were stimulated with 10U/ml IFNγ overnight. Representative of 2 independent experiments. 10μg of protein was added to each lane for SDS-PAGE. SDS-PAGE was carried out on the same gel; blocks have been indicated for demonstration For each respective antibody, all samples were exposed to the film for equal times before development.

The loss of GBP4 protein was very striking. Within the lab, degradation of the protein was not always observed. I determined whether the MOI of type I *Toxoplasma* used was the deciding factor for this phenotype. I infected THP-1 macrophages with increasing MOI of parasites before lysing the cells at 2 hours post infection and analysing protein expression by SDS-PAGE and immunoblot. It was clear that the degradation of GBP4 correlated with increased numbers of parasites during infection (Figure 25). Additionally, I analysed the band densities of GBP4 on the immunoblots using ImageJ software, and plotted the results as a percentage of the respective loading control band density in IFNγ-stimulated THP-1 macrophages (Figure 26). The graph shows very effectively the significant decrease of protein expression when THP-1 macrophages are infected with type I *Toxoplasma* parasites.

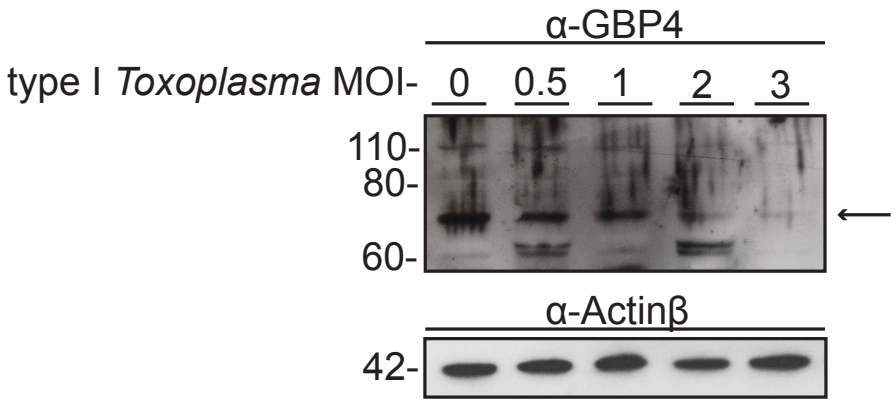


Figure 25 Effect of increasing type I *Toxoplasma* MOI on GBP4 protein expression

Immunoblot showing GBP4 protein expression in THP-1 macrophages infected for 2 hours with type I *Toxoplasma* using increasing MOIs. 10µg of protein was added to each lane for SDS-PAGE. Arrow indicates the GBP4 band. Cells were stimulated overnight with 10U/ml IFNγ. Representative of 2 independent experiments.

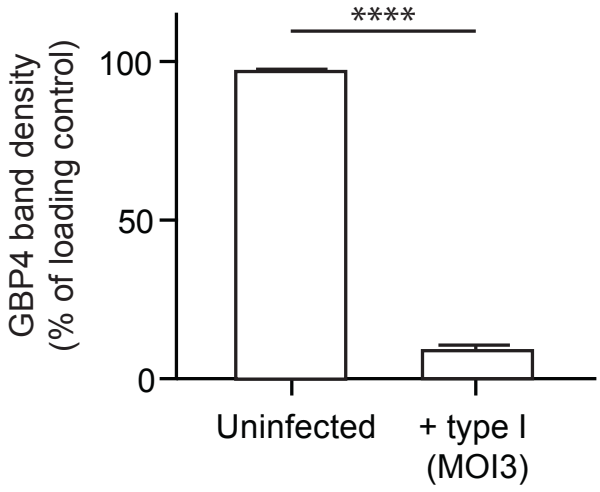


Figure 26 Density of GBP4 protein bands

Graph showing the density of GBP4 protein bands in immunoblot as compared to the respective loading control band. Bands show GBP4 expression in lysate from IFNγ-stimulated THP-1 macrophages infected or not for 2 hours with type I *Toxoplasma* at an MOI of 3. Inclusive of 3 independent experiments. Significance calculated with unpaired t-test, ****p<0.00001.

The fractionations were carried out at 2 hours post infection, so additionally I followed the expression of GBP4 by immunofluorescence to track protein loss. It would be interesting if any recruitment of GBP4 to the *Toxoplasma* vacuole were to be observed even though it appears, by immunoblot, to have a solely nuclear location in THP-1 macrophages. THP-1 monocytes were plated onto glass coverslips before differentiating with PMA to macrophages. When fully differentiated, cells were stimulated or not overnight with IFNγ before infecting with *Toxoplasma* at an MOI of 3. The cells on the coverslips were fixed and permeabilised periodically over a time course, after which they were stained with the anti-GBP4 polyclonal antibody. While there was a relatively high background level of staining, it was evident that GBP4 was a nuclear protein. It was also clear that, regardless of the time points investigated or parasite strain, the protein did not recruit to the *Toxoplasma* vacuole (Figure 27). During the infection with type I parasites, expression of the protein was efficiently depleted between 1 and 2 hours (Figure 27A). The GBP4 protein levels on infection with type II parasites did not change throughout the time course (Figure 27B). The loss across the population is graphically displayed after quantification of GBP4 staining in 100 cells (Figure 28). These results show that although GBP4 is found solely in the nucleus in THP-1

macrophages, type I *Toxoplasma gondii* have the ability to eliminate GBP4 protein staining very efficiently when using the C-terminal recognising polyclonal peptide anti-GBP4 antibody.

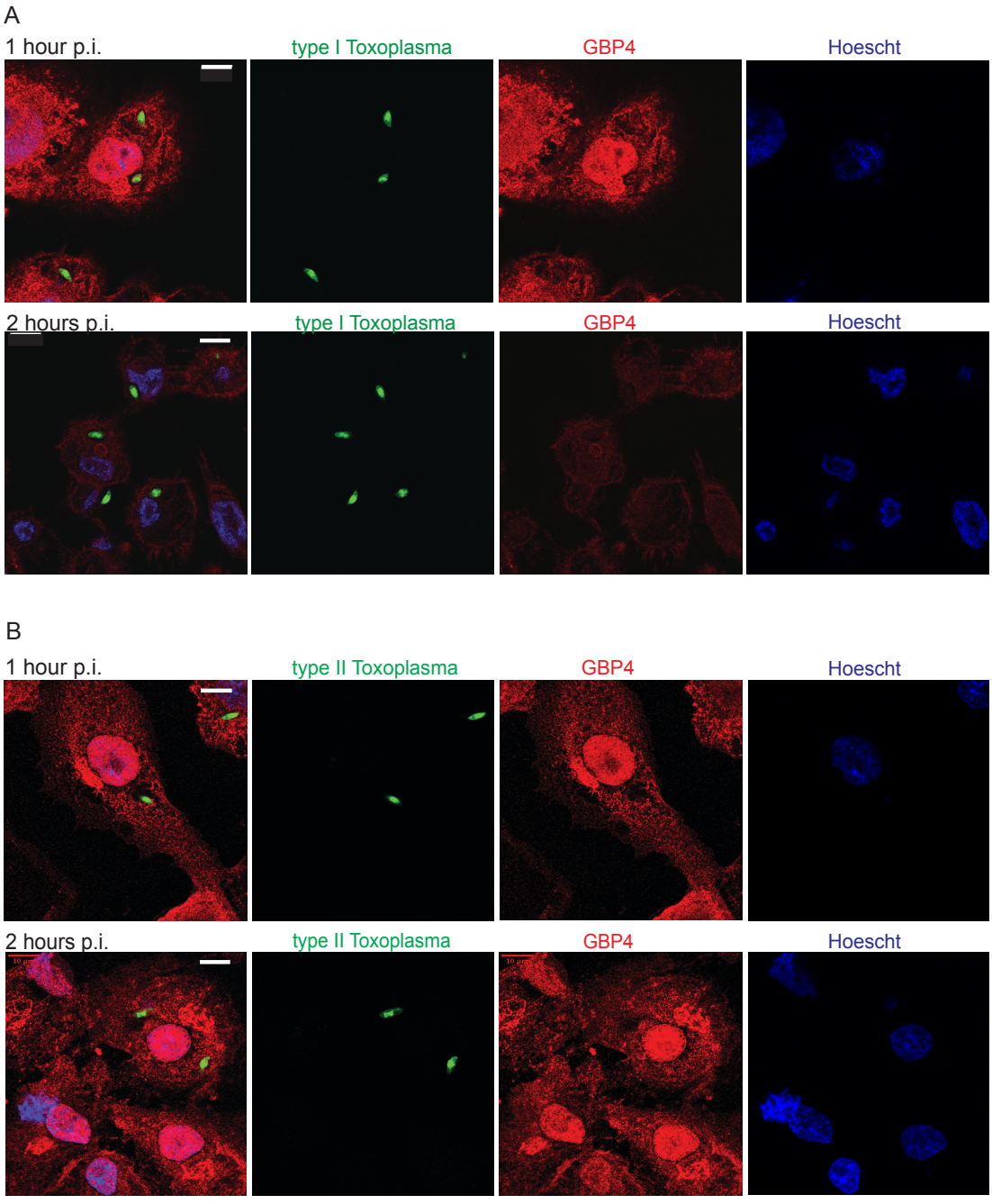


Figure 27 GBP4 is not recruited to the PV of *Toxoplasma*, and protein expression is lost during infection with type I parasites

Immunofluorescence showing the distribution of GBP4 in THP-1 cells at 1 and 2 hours post infection with type I (A) or type II (B) *Toxoplasma* at an MOI of 3. Cells were stimulated overnight with 10U/ml IFNγ. All scale bars 10μm. Representative images from 3 experiments.

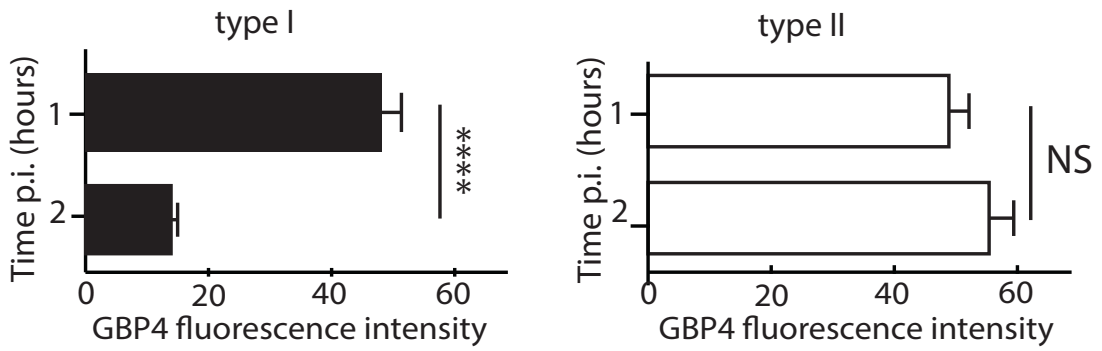


Figure 28 Staining of GBP4 protein is lost across the cell population upon infection with type I *Toxoplasma* but not type II

Fluorescence intensity of GBP4 staining in THP-1 cells infected with type I or type II *Toxoplasma*. Intensity was quantified using Image J in a minimum of 20 randomly selected cells. Cells were stimulated for 18 hours with 10units/ul IFNγ. Statistical analysis determined by unpaired t-test; ****p<0.00001. N=3 independent experiments

The loss of protein signal could be attributed to a number of factors. The GBP4 protein could be directed for degradation by the type I parasite. There could be inhibition of GBP4 transcription or translation. Furthermore, signal could be lost due to a modification or truncation of the protein C-terminus, rendering the antibody unable to recognise the peptide region against which it was produced. I pursued the hypothesis of protein degradation first.

The degradation of intracellular proteins is a tightly regulated and varied process. Originally it was thought that all proteins were degraded via the lysosome- a membrane-bound organelle containing acidic, digestive enzymes capable of proteolysis (Ciechanover, 2005). However, as it was discovered that lysosome deficient cells could degrade proteins in an ATP-dependent manner, it became clear that the process was much more complex and diverse (Ciechanover, 2005). The discovery of ubiquitin highlighted that different mechanisms were present and paved the way to determine the different mechanisms of protein degradation. The ubiquitin activating enzyme, E1, activates ubiquitin to be transferred to an ubiquitin-conjugating enzyme, E2. From here, the ubiquitin is transferred to a protein substrate that is bound by the ubiquitin ligase E3. Multiple conjugations of ubiquitin molecules suffice as the degradation signal for protein destruction via the ubiquitin-proteasome proteolytic system (Ciechanover, 2005). The 26S proteasome is a protease complex made up of the 19S and 20S proteasome, with the 20S forming

a catalytic core. It is this complex that is responsible for degrading the proteins conjugated to ubiquitin (Eytan et al., 1989). Furthermore, a number of other proteolytic enzymes and systems exist, with one example being the calpain family. Calpains are a Ca2+ dependent family of cysteine proteases that reside in the cytosol (Khorchid and Ikura, 2002). While the exact mechanism of how calpains dictate substrate specificity is not entirely clear, it is thought that amino acid preferences and secondary structures, alongside a potential PEST sequence, help define targets (Tompa et al., 2004). Another such example of a proteolytic system is the caspase family of cysteine proteases. This family gains catalytic ability following a long line of signalling events leading to dimerisation and often cleavage to induce activity (McIlwain et al., 2015). Caspase family members are activated differently, which results in a variation of their final functions. All of these proteolytic pathways lead to degradation, destruction or turnover of intracellular proteins.

As GBP4 protein expression is lost during infection with type I *Toxoplasma*, I sought to determine how GBP4 is degraded in a steady state cell, as well as how this happens in response to the parasite. A vast repertoire of protein-degradation inhibitors is commercially available, and from these I took a selection with which to test GBP4 degradation.

THP-1 macrophages were plated in 6-well plates before being stimulated overnight with IFNγ. Following this, the cells were treated as appropriate with the individual inhibitors. The macrophages were then infected with type I *Toxoplasma* at MOI 3 for 2 hours, after which cell lysates were run on SDS-PAGE. The resulting gels were transferred via Western blot before the membranes were probed with the anti-GBP4 peptide antibody. It was clear that when MG132 proteasome inhibitor was used in the uninfected samples that GBP4 could be greatly accumulated (Figure 29). This indicated that the GBP4 is routinely degraded via the 26S proteasome. However, the GBP4 could not be accumulated in the type I infected samples, suggesting that however GBP4 is being degraded in response to the parasite is through an alternative route. Interestingly, in the type I infected samples that were treated with leupeptin, GBP4 protein could once again be detected on the immunoblot (Figure 29).

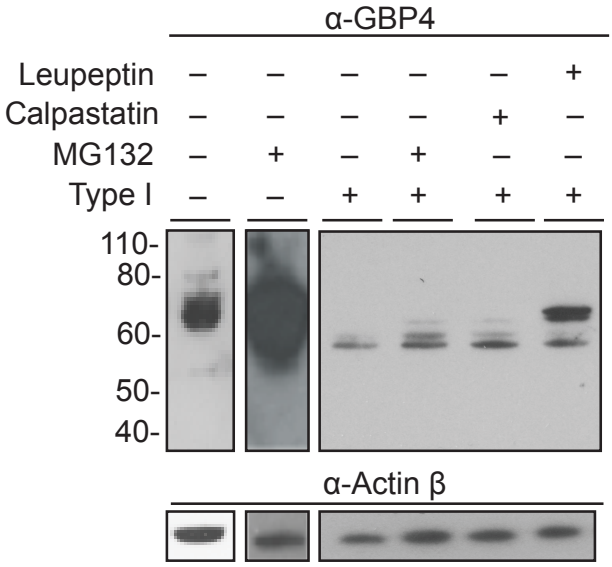


Figure 29 GBP4 protein can be accumulated by using protein degradation inhibitors

Immunoblot showing the levels of GBP4 protein with and without protein degradation inhibitors in THP1 macrophages that were uninfected or infected for 2 hours with type I *Toxoplasma,* MOI 3. 10µg of protein was loaded in to each lane for SDS-PAGE. Cells were stimulated for 18 hours with 10U/ml IFNγ. Cells were treated with the appropriate inhibitor for 1 hour before infection. Representative of 2 independent experiments; excluding the calpastatin lane which is N=1. Each panel was run on a separate gel, all samples from same experiment. For each respective antibody, all samples were exposed to the film for equal times before development.

This implies that the *Toxoplasma* driven degradation of GBP4 is mediated by cysteine, serine and/or threonine peptidases.

These results show that GBP4 does not recruit to the *Toxoplasma* PV during infection, and that type I *Toxoplasma* has the ability to direct host or parasite cysteine, serine and/or threonine proteases to degrade GBP4 protein in a rapid and efficient manner.

4.2 Discussion

Subcellular localisation of human GBPs

GBP1 and 4 do not localise to the PV of *Toxoplasma*, yet GBP1 at least still plays an important role in restricting the growth of the parasite (Johnston et al., 2016). Interestingly, GBP4 is a nuclear protein whose expression is lost upon infection

with the type I *Toxoplasma* strain, RH. The consequences of this is as yet unclear, however, it would suggest that not only are these proteins central in host protection, but the parasite has adapted in such a way to begin to avoid their effects.

The fact that these proteins are not localised to the PV shows that the mechanisms by which they operate vary extensively from their mouse counterparts. The recruitment of mGBPs to *Toxoplasma* was the first indicator that they played a role in this parasite infection (Degrandi et al., 2007), with approximately 50% of parasite vacuoles coated with members of the GTPase families. This localisation to the PV has been a guide for elucidating whether or not to pursue pathogen viability assays in bacterial models in murine cells (Haldar et al., 2014, Pilla et al., 2014, Meunier et al., 2014, Man et al., 2015). The fact that I did not find a localisation of GBPs to the vacuole would have implied they were not relevant during this infection. I wonder if other research groups have observed the same phenomenon and decided that they could not be important? In 2014 Ohshima et al. showed that human GBPs did recruit to the vacuole of *Toxoplasma*. This occurred at 6 hours post infection with a ME49 type II strain of the parasite in human haploid (HAP1) cells that are derived from a leukaemia cell line (Ohshima et al., 2014), with only a low percentage (approximately 6%) of PVs positive for GBP recruitment. While these results do not fall in line with what we have observed, in this study a commercial antibody against GBPs 1-5 was used. It is additionally noteworthy that the reported 6% recruitment is much less than the usual 30-60% recruitment of GBPs observed to pathogen vacuoles in murine cells (Degrandi et al., 2007, Virreira Winter et al., 2011, Haldar et al., 2013). However, it could be that family members 2, 3 and 5 have some level of recruitment to the vacuole. When staining with the anti-pan-GBP antibody that I characterised, I do not see any recruitment of protein against either the type I or the type II parasites. Furthermore, the accumulation of protein at the parasite did not match the phenotype seen with localisation of GBPs in murine cells. In the mouse, a solid ring of protein is acquired around the PV membrane, however in the Ohshima study there was a staining covering the whole parasite. This difference could arise as a result of a different response mechanism across the two host species. There is a chance however that the difference is due to an artefact or to unspecific staining of a dead parasite. A further explanation could be that these proteins act in a cell specific manner, a factor that investigators must take in to consideration in the future. If these

experiments were to be repeated in the future, using an anti-GRA antibody would prove the *Toxoplasma* was indeed within a vacuole if dense granule staining co-localised with the parasite, and would at least reinforce that the parasite has successfully invaded.

As it appears there is very limited recruitment of human GBPs to the PV of *Toxoplasma*, the question arises whether this is because they are fulfilling an entirely different role to mGBPs. Alternatively, would GBPs demonstrate the same responses if the same components were present in human cells as in mouse? There are many studies that outline the recruitment of mGBPs to the vacuole as being potentially dependent on glycine-lysine-serine (GKS)-containing IRGs (Yamamoto et al., 2012, Haldar et al., 2015). Humans however possess only 2 GKS-IRGs, and these are not IFNγ-sensitive (Bekpen et al., 2005). This theory was tested using the antibodies produced in this project, when I collaborated with Jörn Coers' laboratory at Duke University, who transfected mouse embryonic fibroblasts (MEFs) with human GBP1. These cells were then infected with *Toxoplasma* before staining with my polyclonal anti-GBP1 antibody for immunofluorescence microscopy. Interestingly, localisation of the protein to the PV was seen. This could imply that the lack of recruitment of GBPs to the PV of *Toxoplasma* is a result of human cells being deficient in IRGs (Johnston et al., 2016).

Our study continued to determine whether or not GBP1 recruited to *Salmonella enterica* typhimurium or *Chlamydia trachomatis* (Johnston et al., 2016). Furthermore, we looked to see if GBP1 was playing a role in host defence against these pathogens. It soon was clear that GBP1 did not recruit to the pathogen containing vacuoles (PCVs) of these intracellular bacteria, as elucidated by immunofluorescence microscopy (Johnston et al., 2016). These results are in contrast to work published previously, which state that GBP1 and GBP2 can recruit to the chlamydial vacuole (Tietzel et al., 2009, Al-Zeer et al., 2013). One of these studies relied on overexpression of the GBPs, which may have resulted in an artefact. Both of these studies focussed on cells that were not epithelial cells, it may be that GBP1 is functioning in a cell-specific manner. We concluded that GBP1 does not recruit to the PCVs of intracellular pathogens in epithelial cells.

As these proteins were not localised as expected, I moved to determine where they were located within the cell. Previous literature had signified, in an IFNγ stimulated cell, GBP1 was found in the cytosol, with a GTPase activity-dependent

Golgi association (Modiano et al., 2005, Tripal et al., 2007). The Tripal study also showed GBP4 to be found both in the nucleus and in the cytoplasm (Tripal et al., 2007). These findings were elucidated using an overexpressed GFP tagged version of the protein in HUVECs. Therefore, the reason that we do not see GBP4 in a cytoplasmic localisation could be a caveat due to the overexpression system, or a cell-specific phenotype. It would be interesting to see how these findings compare to the subcellular localisation of not only mGBP4, but all mGBPs in the uninfected cell, but there are very few results outlining the location of murine GBPs when vacuolar recruitment is not involved (Degrandi et al., 2007). I believe it is important to determine where these proteins are in the cell, as not only may there be an observable difference during an infection, it gives clues to the function they might be carrying out. This differential location in uninfected cells is another arm of evidence that GBPs are playing distinct and non-redundant roles within the cell.

GBP1 in host defence to *Toxoplasma gondii*

As GBP1 was not recruited to the PV during *Toxoplasma* infection, I initially questioned whether or not this protein was involved in the host defence to *Toxoplasma* at all. As I began to carry out the *Toxoplasma* growth assays in A549 ΔGBP1 versus wild type cells, it became clear that indeed GBP1 had the ability to restrict the growth and survival of this parasite. By complementing the protein back into the ΔGBP1 cells, I could restore the phenotype seen in wild type cells. This step proved that GBP1 was the effector of this restriction as opposed to a secondary effect introduced during the genetic modification of the cells. Even more so, the effects of GBP1 could be seen in a manner that was independent of IFNγ. This was fascinating as mGBPs are not present without the stimulation of IFNγ. However, it is not the case in human A549 cells, with GBPs showing expression at basal level, as demonstrated by immunoblot. This result outlines another important difference between mouse and human GBPs. Additionally, it has been demonstrated that IFNγ as being necessary for oligomerisation qualities (Britzen-Laurent et al., 2013). Furthermore, the association of GBP1 to the Golgi is IFNγ dependent (Modiano et al., 2005, Tripal et al., 2007). While it is true that GBPs are effectors of the IFNγ mediated resistance against a number of pathogens this project shows GBP1 can function in an IFNγ independent manner, implying that the

mechanism by which this GTPase family member operates does not require localisation to the Golgi or oligomerisation with itself or other GBPs.

After determining that GBP1 contributed to host defence of *Toxoplasma*, I defined at which stage of infection it was carrying out its effect. I first investigated whether the invasion rate of *Toxoplasma* into epithelial cells was altered in GBP1 deficient cells. As it was apparent that infection rates were not altered, I next determined how the initial replication of the parasite in the cell might be affected. This was done using fluorescent microscopy, taking advantage of *Toxoplasma* that heterologously express GFP. By counting how many times the parasite had replicated inside the vacuole it was clear even by 6 hours post infection that replication was enhanced when GBP1 was absent. This too was IFNγ independent, indicating this effect was the same one that resulted in the difference in ability to form plaques. This IFNγ-independent method of action opens another line of study to investigate how else these proteins can exert their functionality.

I was interested to observe that the restriction effects of GBP1 against *Toxoplasma* were exerted in a strain-dependent manner. Traditionally type I parasites are classified as virulent, while type II are avirulent. However, this is in the context of mice, as the nomenclature is resultant of the fact that one type I parasite is sufficient to kill a mouse, while thousands of the type II will be tolerated before the mouse succumbs to infection. In humans, there is only recently literature describing that the parasites interact differently with the host cell in a strain-dependent manner. It has been shown that human gene expression and host transcriptional responses to *Toxoplasma* can occur in a strain dependent manner (Saeij et al., 2006, Ong et al., 2011). Additionally, a study demonstrating that *Toxoplasma* is restricted by a non-canonical autophagy pathway, as well as my published work show that type I parasites largely evade specific host defence responses (Selleck et al., 2015, Johnston et al., 2016). This is an exciting area of study that should be investigated further. Amongst other things the specific virulence factors dictating these responses could be determined.

The fact that GBP1 can restrict pathogens falls in line with what is already published in the literature, with an effect against vesicular-stomatitis virus, encephalomyocarditis virus and *C. trachomatis* shown (Anderson et al., 1999a, Tietzel et al., 2009, Al-Zeer et al., 2013). However, the work published before my study regarding human GBPs and *Toxoplasma* present that they are not involved

during infection (Ohshima et al., 2014). When HAP1 cells had the complete GBP cluster on chromosome 1 removed using the CRISPR-Cas9 technique, *Toxoplasma* counts did not vary from counts in the wild type cells after 24 hours of infection with a type II strain (Ohshima et al., 2014). This could be another cell type specific phenotype that is not seen in epithelial cells. Alternatively, some residual GBP6 or 7 may be present and active in the cells, as the expression for these have not been elucidated. When I immunoblotted lysates from these cells, I noted that the levels of GBPs present in the wild type HAP1 cells, even after IFNγ stimulation, are very low (data not shown). HAP1 cells are derived from a cell line, KBM-7, that has been derived from leukaemia cells and contain only one copy of most human chromosomes (Kotecki et al., 1999, Carette et al., 2009). For this reason, they are favourable for use in genetic studies and screens, as it is easier to target a gene for full knock out when only a single chromosome needs to be effectively manipulated. However, these cells are not very biologically relevant in studies with infection models. As a result, it is a possibility that there is no difference seen after GBP deletion due to an insufficient GBP response in the wild type cells. It remains to be seen when the system used in my project is transferred to another cell type, whether a striking GBP1-dependent restriction of parasite growth is still present.

To determine whether or not GBP1 plays a role in the restriction of other pathogens, I worked with collaborators to determine the effect of this protein on *S. enterica* typhimurium and *C. trachomatis.* Both of these bacterial pathogens are intracellular, and furthermore reside within a PCV inside the cell. In this regard, they are comparable to *Toxoplasma gondii*. When pathogen viability assays were carried out after infection of epithelial cells that had either GBP1 intact or deleted, we saw no difference in bacteria growth for either *S. enterica* typhimurium or *C. trachomatis* (Johnston et al., 2016). This result suggests that GBP1 can act in a host defence role in a pathogen-specific manner. While other studies have been published demonstrating GBP1 restricts *C. trachomatis* growth, the work was carried out in either in HeLa cells or macrophages (Tietzel et al., 2009, Al-Zeer et al., 2013). Again, this suggests that GBPs may be functioning in a cell-specific manner, which needs to be taken into consideration by others looking to study this protein family.

GBP4 characteristics during *Toxoplasma gondii* infection

I initially chose GBP4 as a prioritised candidate for study due to the fact it was reported to have a cyto-nuclear localisation in the literature (Tripal et al., 2007). My results have shown that in THP-1 macrophages the localisation of GBP4 is solely nuclear. As the work done by Tripal et al. was performed in endothelial cells, we come again to hypothesise that GBPs are functioning in a cell-specific manner (Tripal et al., 2007). It is possible however, that the slight difference in subcellular localisation was an artefact due to the fact that overexpressed GFP-tagged GBP4 was used to determine the subcellular localisation. GTPases in the nucleus are most often found to be Ran proteins, which are responsible for trafficking RNA and proteins between the cytosol and nucleus (Moore, 1998). There are a few other GTPases in the nucleus that are carrying out functions in that location, for example PI-3Kinase Enhancer (PIKE) which interacts with nuclear PI3K to stimulate its lipid kinase activity (Ye et al., 2000). The most relevant nuclear GTPase to this study is the myxovirus resistant protein (Mx)-1 that is found in mouse. Mx proteins are part of the same large dynamin-like family of interferon-inducible GTPases as GBPs and IRGs. They are known to have important anti-viral properties, and are well-conserved between a number of species (Verhelst et al., 2013), with MxA and MxB present in humans and two versions of the MxA protein in mouse, Mx1 and Mx2 (Haller et al., 2015). These GTPases play vital roles against a broad range of both DNA and RNA viruses including influenza A and hepatitis B (Verhelst et al., 2013, Haller et al., 2015). As a result, Mx proteins can interact with the target viruses in both the nucleus and the cytoplasm. Interestingly, rodent Mx1 requires nuclear localisation to exert its functions (Zurcher et al., 1992). Conversely, the human Mx proteins are found solely in the cytoplasm, however can still exert full anti-viral mechanisms by inhibiting viral mRNA synthesis (Haller et al., 2015). This comparison is intriguing, as even within a family of highly identical proteins, Mx proteins seem to be playing different and necessary functions in murine versus human cells. This seems to also be the case with GBPs. The fact that, with GBP4, another member of this large-interferon inducible family has been located to the nucleus is striking, as so few GTPases are found in this cell compartment. Furthermore, GBPs and Mx proteins are GTPases that are highly conserved yet operating with diverse mechanisms across species.

The loss of GBP4 expression upon infection with type I, yet not type II *Toxoplasma* was intriguing, as no parasite virulence factors in humans have been defined yet. Only examples of *Toxoplasma* proteins manipulating mouse cell components have been described (Fentress et al., 2010, Steinfeldt et al., 2010, Virreira Winter et al., 2011, Jensen et al., 2013, Franco et al., 2014, Bougdour et al., 2014). The mechanism by which the parasite is manipulating GBP4 could be occurring at a transcriptional, translational or protein degradation level. By using inhibitors of protein degradation, I was able to accumulate the protein during infection. I ensured to use inhibitors that target varying aspects of protein degradation, to highlight the process by which this is happening. MG-132 is used to inhibit the 26S proteasome, as well as leupeptin that can inhibit cysteine/ serine/ threonine peptidases. Via these different reagents I was able to highlight while the protein is degraded in a 26S proteasome dependent manner in an uninfected cell, type I parasite infection results in GBP4 being degraded by cysteine, serine and/or threonine peptidases. Interestingly, it has been shown previously that type I *Toxoplasma* also effects protein levels in MEFs by a mechanism that can be inhibited when leupeptin is added to the culture (Carmen and Sinai, 2011). This study showed that degradation of BH3 interacting-domain death agonist (BID), a pro-apoptotic member of the B-cell lymphoma-2 (Bcl-2) family, is manipulated by the type I parasite in a leupeptin dependent manner (Carmen and Sinai, 2011). While the *Toxoplasma* virulence factor responsible for this host-protein manipulation has not been identified, it appears that type I parasites potentially possess a component that can modify both mouse and human cell proteins. Other inhibitors such as calpastatin to target the non-lysosomal calpain proteases, lactacystin for 20S proteasome inhibition and Z-VAD-FMK that is a specific caspase inhibitor were also preliminarily tested (data not shown). While I saw no accumulation of protein during infection when these inhibitors were used, a more thorough investigation is required.

For this reason, I conclude that the parasite is acting on the protein product. In order to formally rule out an effect on GBP4 transcript, however, it would be prudent to conduct a qPCR analysis on samples taken over a time course during *Toxoplasma* infection. A potential effect on protein translation could be analysed by the observing stability of GBP4 protein, with and without the protein translation inhibitor cycloheximide. By using this method in infected and uninfected cells one

can determine the half-life of the protein, and can demonstrate whether the parasite has an effect on protein translation or degradation. It is furthermore a possibility that modification or truncation of GBP4 results in the antibody being unable to recognise its target. Due to the fact I can inhibit the loss of protein recognition, it is unlikely that this is the case. However, the way to fully identify whether an epitope-masking post-translational modification or a truncation had occurred would be to utilise an antibody targeted to an epitope in a completely separate, N-terminal location. As no other specific GBP4 antibodies have been identified this not a possible course of action at the moment. Alternatively, if the endogenous protein was tagged using CRISPR-Cas9 at the N-terminus it may be possible to indisputably confirm this.

The disappearance of GBP4 in a parasite strain-specific fashion indicates it might be playing an important function during *Toxoplasma* infection. Whether or not the loss of expression is a host or *Toxoplasma* driven phenomenon is unclear. However, it is unlikely to be a host-driven mechanism due to the fact that the observation occurs with the type I parasite as opposed to the type II. This is due to the fact that traditionally type I is the 'virulent' parasite strain, and that some studies in human have already demonstrated that this may be true to a certain respect in man as well (Saeij et al., 2006, Ong et al., 2011, Selleck et al., 2015, Johnston et al., 2016). Cysteine, serine and threonine peptidases are activated by the cleavage of a pro-domain from their active sites (Di Cera, 2009, Verma et al., 2016). In the case of cysteine peptidases, a low pH or glycosaminoglycan polysaccharides can potentially disrupt salt bridges within this pro-domain and results in exposure of the active site (Verma et al., 2016). Many serine and threonine peptidases are activated in a calcium dependent manner, with an increasing calcium concentration resulting in an unmasked active site (Di Cera, 2009). Whether or not *Toxoplasma* can manipulate host factors to result in the activation of these proteases, or can release factors to induce exposure of the protease active site remains to be seen. Therefore it is likely that type I parasites actively function to circumvent control by GBP4.

The next step is to fully define the role that GBP4 plays against *Toxoplasma*. This can be achieved in the same way as with GBP1, using cells that have had the protein knocked-out using CRISPR-Cas9. Within the lab, preliminary results with A549 ΔGBP4 cells show that indeed the growth of *Toxoplasma* is enhanced when

GBP4 is not present (Joseph Wright, unpublished). While this result alone is exciting, it is interesting to note that this GBP4-mediated restriction is dependent on IFNγ stimulation. This difference in IFNγ dependence between GBP1 and GBP4 is another example of how the GBP family members appear to be playing their own unique roles and can function differently to each other. This IFNγ dependence could be due to a number of factors. Perhaps there is not a sufficient residual basal level of GBP4 in epithelial cells that is able to restrict pathogens. It may also be that the activation of the GTPase activity requires this cytokine, and in turn the activation is necessary for restriction. Alternatively, the mechanism by which GBP4 exerts its function maybe be dependent on an interaction partner or component that requires IFNγ stimulation. These partners could even be other GBPs, and as IFNγ is required for oligomerisation (Britzen-Laurent et al., 2010) it could be an assembly process that cannot occur. If an assembly process is required, there is a chance that other GBP family members can be found in the nucleus also. For example, overexpressed GBP2 has already been observed in the nucleus of endothelial cells (Tripal et al., 2007). I carried out subcellular fractionations followed by immunoblotting, and did not observe GBP2 in the nucleus of epithelial cells. However, this could be another cell specific phenomenon, or GBP2 may only be located in the nucleus under specific conditions. Alternatively, GBP4 could have a cytoplasmic location and role in specific conditions that may also require the GBP oligomerisation process. This project has resulted in an anti-GBP4 antibody that has the capacity for immunoprecipitation. This means it is possible to retrieve the protein from cells that were subjected to a number of different conditions, including stimulated and unstimulated, and infected and uninfected. Following isolation of protein interacting complexes, the identity of the samples could be determined by mass spectrometry in order to identify the candidate interaction partners and thus define the pathway by which GBP4 functions.

GBP4 is an interesting GBP candidate due to the fact that it is the least similar to the other GBP family members in terms of sequence, showing less than 60% similarity to all family members except GBPs 6 and 7 (Olszewski et al., 2006). Furthermore, when I analysed the sequence to determine if a nuclear localisation signal (NLS) was present, there were both monopartite and bipartite NLS motifs predicted, with relatively low prediction scores that suggest the protein is localised both in the nucleus and cytoplasm (Figure 30). Monopartite and

bipartite sequences are classical NLS motifs that have either one or two clusters of basic amino acids respectively (Lange et al., 2010). These NLS sequences are found towards the C-terminal end of the GTPase binding domain, and therefore would not be cleaved to induce relocalisation. Traditionally, a NLS motif would be determined and then mutated to observe the effects on protein expression and trafficking. Until we identify the site that is responsible for nuclear location, that step cannot be taken.

Predicted monopartite motif	
240	RHFFRKRKCFVF
242	FFRKRKCFV
Predicted bipartite motif	
196	RDFTLELKLDGNPITEDEYLENALKIPGKNPKI
223	PGKNPKIQNSNMPRECIRHFFRKRKFC

Figure 30 Predicted nuclear localisation signals within GBP4 amino acid sequence

Predicted monopartite and bipartite NLS signals within GBP4 protein, as determined using the cNLS Mapper tool (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi).

The sequence of GBP4 is additionally interesting as there is a 21 amino acid long extended N-terminal region that has two methionines, leading to a dispute as to which is the correct protein translation start site (Olszewski et al., 2006). Additionally, there is a chance that the N terminus is subject to modification and as a result might affect protein function. In this project, I was able to show that when GBP4 was tagged at the N-terminus, cleavage and accumulation of the tag occurred up to a few hours post-expression of the protein. While this is useful to know for the production of tools in the future, it was apparent that something interesting was occurring. This cleavage occurred in IFNγ-stimulated cells and during deliverance of GBP4 DNA into the cell by Vaccinia virus. It is a possibility that the truncation of GBP4 is required for the activation and anti-pathogen response. In order to address this question, it would be prudent to immunoprecipitate GBP4 before N terminal sequencing the to compare the N terminus protein of GBP4 in the nucleus. Once this has been identified, we would have a clue as to the N terminal start site and potential cleavage sites. If these sites

could be mutated, assays could be carried out to study the effect of the N terminus on GBP4 function. The problem lies in the fact that it has been proven unsuccessful to overexpress these GTPases in human cells. This presents a problem that is prudent for mutation of either the N terminus or the NLS motifs. Here again is a hurdle that could be solved by CRISPR-Cas9. This system allows not only for DNA to be mutated to delete translation of a protein, but can also introduce site-directed mutations that lead to insertion of a tag, or a single nucleotide change (Ran et al., 2013). Therefore, employing CRISPR-Cas9, one could generate clonal cell lines in which the endogenous GBP4 is mutated as described at specific regions.

The interaction between pathogens and their hosts is complicated and variable. There are many examples of pathogens that can manipulate host features that result in increased virulence and pathology and thus promote their own survival. Numerous bacteria that can cause disease in humans have been shown to covalently modify host response GTPases, including *Vibrio cholera, Escherichia coli, L. pneumophilia* and *Stapylococcus aureus* (Aktories, 2011), so the modification of GBP GTPases would be a plausible virulence strategy for *Toxoplasma*. Other bacterial pathogens have the ability to exert transcriptional control. *Listeria monocytogenes* interferes with the SUMOylation machinery that is responsible for transcriptional regulation (Cossart, 2011). Furthermore *Listeria* and other bacteria including *Mycobacterium tuberculosis*, *Shigella flexinuri* and *Heliobacter pylori* can epigenetically control expression of genes at the mRNA level, via the modification of histone expression and/ or chromatin structure (Hamon and Cossart, 2008, Cossart, 2011). *Toxoplasma* is also capable of manipulating a number of features in the murine host. The Rop18 kinase of *Toxoplasma* can directly phosphorylate IRGs so that there is reduced PV recruitment, with Rop16 hypothesised to work similarly on GBPs (Fentress et al., 2010, Steinfeldt et al., 2010, Virreira Winter et al., 2011). Rop16 kinase and Gra15 also are responsible for interrupting the Jak-STAT and NFκB pathways in order to promote pathogen survival (Jensen et al., 2013). Furthermore, *Toxoplasma* manipulates the cell to specifically induce the transcription factor c-myc. This results in the upregulation of c-myc dependent products that play roles in mechanisms including cell cycle progression, apoptosis, cell differentiation, and metabolism (Franco et al., 2014). Gra16 and Gra24 are secreted dense granule proteins that both contain nuclear localisation signals, and have been shown to alter host genome expression via the

regulation of p53 and p38α signalling pathways respectively (Bougdour et al., 2014). As GBP4 is found within the nucleus, it is possible that it is intercepting parasite responses that aim to modify cell genetic responses. Alternatively, perhaps GBP4 is involved in regulating transcription in response to infection, possibly due to specific interactions with important components to this pathway. Therefore *Toxoplasma* could be affecting transcriptional defence processes indirectly via GBP4.

GBPs that have been best characterised in the literature, GBP1 and 5 (Tietzel et al., 2009, Shenoy et al., 2012, Al-Zeer et al., 2013, Johnston et al., 2016) sit on an entirely different branch of the GBP phylogenetic tree to GBP4 (Figure 31). Therefore, the fact that the GBP4 sequence differs the most compared to these other family members would infer that it would be most likely to play a unique role in infection. GBP4 has not been intensively studied in the literature. There is one paper by Hu et al. that focuses on mouse GBP4 as a negative regulator of Sendai virus-induced IFN-alpha (IFNα). This downregulation of IFN occurs as a result of IFN-regulatory factor 7 (IRF7) inhibition. Sendai virus was used to infect cells that had mGBP4 silenced, as well as wild type cells; after which the supernatant from these cultures was added to cells infected with VSV. The VSV was observed to replicate less efficiently when supernatant from the mGBP4 silenced culture was used (Hu et al., 2011). This suggests that a higher level of IFNα is secreted from cells in response to Sendai virus when mGBP4 is not functional. Perhaps mGBP4 is targeted specifically by Sendai virus, and manipulated to enhance viral survival via negative regulation of IFN. Alternatively, perhaps it is playing a vastly different role to other mGBP family members, and does not restrict viral replication in cells. It would be interesting to see if mouse GBP4 reacts in a similar manner across different viral infections, and to investigate whether human GBP4 shows a similar phenotype during viral infection.

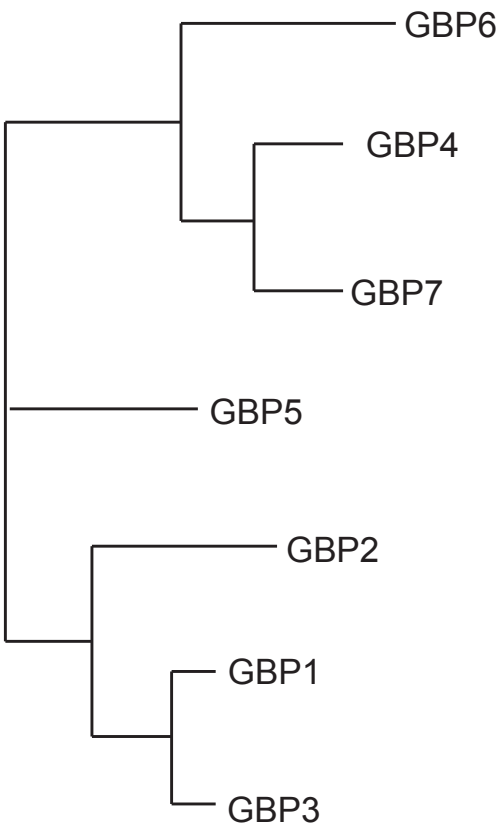


Figure 31 The phylogenetic tree showing distribution of GBP family members

The phylogenetic tree, created using amino acid sequences for GBP family members, with the ClustalW Phylogeny software.

Murine versus human cells as models of infection

Mouse models are an invaluable tool and have been used to study innumerable processes that have benefitted medical research. It is possible to engineer genome modifications, and interbreed them to become genetically identical to limit variability in results. From mice, it is straightforward to obtain primary cells that in most cases will provide a biologically relevant result. These are features that are obviously impossible or near impossible in the process of studying human biology. While it is possible to obtain primary cells, the range is limited and often difficult to acquire, especially if a large number of cells are required over a long time period. For this reason, human cell lines that have been adapted for *in vitro* culture are generally utilised. I ensured the cell lines I worked with expressed GBPs and were IFNγ-responsive. However, these lines are often adapted from a cancerous sample, or

are immortalised with cancer or viral genes to survive in a culture dish; both A549 and THP-1 cells are adapted from cancerous samples. A wide variety of cell types exist, including endothelial cells, epithelial cells, fibroblasts and monocyte cells. These cell lines are not always the most biologically relevant to an infection and can represent only single cell results rather than a whole animal response. Therefore, many studies will still move to identify pathways and component functions in the mouse, or other animal models, before describing a comparison in human cells. While this is a successful strategy in many studies, this project has highlighted that the animal model is not always going to show characteristics that are reproducible in, or representative of, human responses.

For many years, the host response to *Toxoplasma* by interferon-inducible GTPases, the IRGs and GBPs, have been intensively studied. As mentioned previously, the human genome comprises only 2 copies of GKS-IRG genes, and these are not IFNγ-sensitive (Bekpen et al., 2005). As the GBP family is much better conserved, it seemed prudent to understand GBP responses in mice so that there was a likely chance to determine responses that were also replicated in a human host. While looking to determine whether or not GBP1 or 4 played a role in defence against *Toxoplasma* infection, the first priority was to determine whether or not these proteins localised to the PV. As it quickly became clear that this was not the case, I wondered if GBPs could play a role at all. This is because mouse GBPs play a specific role in disrupting the PV, and hence leading to death of the parasite (Yamamoto et al., 2012, Meunier et al., 2014, Pilla et al., 2014). Furthermore, mGBPs have been shown to interact extensively with IRGs in order to carry out their defensive capacities (Haldar et al., 2013). However, it is now clear that in stark contrast to in mice, human GBPs play an important role in a location away from the parasite itself and in the absence of canonical IRG GTPases.

The subcellular locations of human GBPs have been described in the literature previously (Modiano et al., 2005, Tripal et al., 2007) and have been more specifically described in my Results chapter 2. No report has located murine GBPs to the nucleus. While it may be the case that the finding is yet to occur, it would be interesting that human and mouse GBPs have potentially evolutionarily diverged so that the roles they play are totally separate. GBPs 1 and 5 have also been reported to have a localisation to the golgi (Modiano et al., 2005, Tripal et al., 2007), indicating there may be a role of these GTPases related to golgi-mediated protein

modification or vesicular trafficking. Whether or not GBP1 and 5 are fulfilling the same function at the golgi is unknown. However, seeing these two family members specifically localise at the organelle could imply that the GBPs that are not localised to the golgi upon IFNγ stimulation may be performing a different role.

Determining the subcellular localisation of proteins gives key clues as to the functions they carry out, and can elude to potential interaction partners. We are speculating that GBP4 could play a role in transcription, while GBP1 and 5 may be involved in post-transcriptional protein modifications considering their association with the golgi apparatus (Modiano et al., 2005, Tripal et al., 2007). I have postulated that mouse and human GBPs have very different characteristics, but perhaps mouse GBPs are actually capable of carrying out the same functions as their human counterparts. Mouse GBPs are not all found at the vacuole during *Toxoplasma* infection. The murine GBPs 8, 10 and 11 are found distributed in vesicles throughout the cytoplasm, and do not relocalise to the PV upon infection with the parasite (Degrandi et al., 2007). This could mean that the PV-absent family members are not functional during infection, or alternatively they could be playing a separate role altogether maybe in line with human GBPs. This project underlines that it is important to look at the functions of all GBPs at a location away from the pathogen vacuole.

In functionality experiments in the mouse, it is now known that the mGBP cluster located on chromosome 3 is responsible for the activation of the inflammasome, in turn leading to activation of caspase-11 (Pilla et al., 2014, Meunier et al., 2014). Humans do not possess caspase-11 and instead have the homologs caspase-4 and -5. It will be interesting to see how these studies progress, and if it can be shown that GBPs are capable of activating human caspase-4 and -5 to cause cell death. A relatively high level of GBP conservation exists across species (Li et al., 2009), and already GBPs have been implicated in inflammasome activation in zebrafish (Tyrkalska et al., 2016). It will be exciting to see if GBPs across multiple species are able to induce inflammasome activation in response to infection.

The human versus mouse host reactions to *Toxoplasma* has recently been appreciated to be different not only on the level of parasite restriction, but also for parasite recognition by the host cell. It is known that in mice, host cell invasion by the parasite is not required for IL-12 release and instead *Toxoplasma* profilin, a

soluble endotoxin, is required for simulating high levels of this cytokine from DCs (Yarovinsky et al., 2005). This stimulation of DCs requires recognition of the profilin by TLR11 and 12, with mice deficient in these genes succumbing quickly to *Toxoplasma* infection (Yarovinsky et al., 2005, Koblansky et al., 2013). What is interesting is that humans lack a functional TLR11 gene and are completely deficient in the TLR12 gene (Roach et al., 2005), but still mount an effective response to the infection and do not succumb to it. As a result, when this same mechanism of IL-12 was investigated in DCs derived from primary peripheral blood monocytes a completely different mechanism was observed. Instead of responding to profilin, the response was dependent on the phagocytosis of live tachyzoite parasites, while actively invading live parasites were not sufficient (Tosh et al., 2016). In mouse, $CD8\alpha^+$ DCs are principally responsible for IL-12 release in the spleen (Reis e Sousa et al., 1997, Mashayekhi et al., 2011), with $CD11b^+$ $CD8\alpha^-$ being the main producer of IL-12 at the site of infection (Goldszmid et al., 2012). However, Tosh et al. show that the human counterpart of $CD11b^+$ $CD8\alpha^-$ DCs, the $CD1c^+$ DCs, produce some IL-12, and the human equivalent of $CD8\alpha^+$ DCs are unresponsive. Instead, $CD16^+$ monocytes are a main producer of this vital cytokine; further highlighting differences in host responses dependent on species (Tosh et al., 2016). Findings like these suggest that when studying host defence mechanisms against *Toxoplasma* using a mouse model may not provide results that are transferrable to human.

Mice are a natural intermediate host for *Toxoplasma*, so it is logical that these two species have evolved alongside each other, resulting in a specialised host-parasite relationship (Gazzinelli et al., 2014). Selective pressure has led to a significant alteration in the immune system. It is hypothesised that this coevolution has led to the emergence of highly specialised mechanisms of defence including the varying TLR responses and the maintenance of IRG function (Gazzinelli et al., 2014). Perhaps in the absence of this selection pressure, human GBPs have developed an entirely different set of qualities against this pathogen and others. Investigators should bear this in mind when studying the roles of these large GTPases, particularly with *Toxoplasma* infection.

As it is becoming clear that mouse models will not provide all the answers regarding human responses to this parasite, the scientific community is requiring a more biologically relevant tool with which to study human host defence. As I

mentioned above, human cell lines can only provide an approximation of true situations during an *in vivo* infection. Macrophages would be the cell of choice with which to study host response to *Toxoplasma*, as these cells are probably preferentially infected by the parasite (Zhao et al., 2014). A number of human macrophage cell lines exist, including THP-1 cells, AML-193, U-937 and HL-60 cells. All of these cell lines were derived from patients with either monocytic leukaemia or histiocytic, non-Hodgkin lymphoma. THP-1s are the most commonly used human macrophage line, however these cells, as with the other cell lines, require activation to differentiate from monocytes to macrophages. This means they are already in an activated immune state that may affect the process of infection by *Toxoplasma* or other pathogens. DCs play such an important role during *Toxoplasma* infection that they are another cell type that would be very interesting to be able to study *in vitro.* While THP-1 monocytes can be carefully cultured to differentiate to DCs (Berges et al., 2005) this again is a very artificial system. To study human DCs, it is required to isolate them from human blood samples, which of course presents a large limiting factor to biochemical analysis.

An additional problem arises when we consider that these cell lines may not have all of the immunoregulatory pathways intact. The most extreme example of cells used for immune studies that are arguably not biologically relevant is perhaps the use of HeLa cells. HeLa cells were isolated in 1951 from a cervical cancer sample, which upon inspection contained 76-80 heavily mutated chromosomes, compared to a normal 46 chromosomes. Additionally, almost 2000 genes are expressed more highly than the physiological range of 16 human tissue samples, with these genes duplicated up to six times (Landry et al., 2013). This is due to the effects of the human papilloma virus that was the origin of the cancer, as a result of its ability to inactivate p53. Because of this aberrant chromosome number and irregular genetic makeup, the cells may possess an unbalanced set of immune modulatory molecules. However, while this is one of the most severe examples, the cells used in this project are also not without caveats. THP-1 cells have been analysed to have better response mechanisms to stimuli other than human-macrophage cell lines, for example U937 cells, however they respond much less than macrophages that have been differentiated from primary peripheral blood monocytes (Sharif et al., 2007, Chanput et al., 2010, Chanput et al., 2014). There is evidence too that the TLR expression levels in THP-1 cells can be variable

dependent on the concentration of PMA induction used during the macrophage-differentiation step. This means there may be caveats in the analysis of a response to a pathogen that relies on a specific TLR expression pattern. A549 are epithelial cells that were isolated from lung adenocarcinoma. Upon analysis they were shown to have 13 abnormal chromosomes with half of the abnormalities deriving from deletions and amplifications of genes (Peng et al., 2010). While I ensured that the GBPs I was interested in were present in these cells, there is a possibility that interaction partners or up- and down-stream effectors are not present or functional. This would result in non-biologically relevant results, with the potential of missing a GBP function altogether. For these reasons, it is important that we begin to move our studies into cells that are more likely to mimic natural responses.

To this end, I began working with induced pluripotent stem cells (iPSCs). These are cells that are taken from a human sample and reprogrammed to become pluripotent progenitors of monocytes and other cell types. These cells can provide researchers with primary cells that can survive in tissue culture that have not been adapted from cancerous tissue, or that have been adapted over such a long time that they do not recognise as natural cells. The iPSCs that I handled were re-programed at the Sanger Institute in Cambridge to become monocyte precursors. I obtained these cells from the Sanger and became competent in their culture and maintenance. During my PhD I focussed on learning to maintain and culture these iPSCs, with the aim to carry out CRISPR-Cas9 GBP deletion on the monocytes progenitors. Once this would be achieved, it would be possible to differentiate the cells to primary macrophages that were deficient in specific GBPs. Other lab members can now employ CRISPR gene manipulation before differentiating them into primary macrophages. This would provide a very biologically relevant tool with which to study human responses to infection- a tool that would potentially revolutionise our understanding of cell autonomous reaction to pathogens. To repeat the experiments in these cells would give the most biologically relevant insight into how GBPs function *in vivo*, particularly in the context of a *Toxoplasma* infection.

Chapter 5. Results gathered to be used for continuation of GBP work in the Frickel Lab

5.1 Results

5.1.1 Protein interaction partners of GBP2

As I began to unravel the characteristics of human Guanylate Binding Proteins and their functions during infection with *Toxoplasma*, I used the antibodies I generated to identify specific GBP interaction partners. I obtained data that could not be fully investigated at the time. However, I will discuss the raw data produced in this chapter. Based on my findings and analysis, additional work remains that can be mined to continue this line of research. A number of projects will be able to begin and to be continued within the Frickel lab.

Mass spectrometry of immunoprecipitated GBP1 and 2 in collaboration with Bram Snijders within the Francis Crick Institute led to the generation of the data set of potential GBP interacting partners. The anti-GBP2 peptide polyclonal antibody produced in rabbits was very effective at retrieving GBP2 from A549 lysates. In this application, the antibody cross-reacted with GBP1, therefore immunoprecipitating this family member also (Chapter 3, Figure 7). A549 cells were stimulated overnight with IFNγ before being infected or not with type I or type II *Toxoplasma*. The infection was allowed to persist for 2 hours before the cells were lysed using lysis buffer containing the mild detergent 0.5% NP-40. This was to ensure that interactions between proteins were maintained. The lysates were subjected to a pre-clearing step, during which they were incubated with the preimmune serum that was obtained during the antibody production process, followed by an incubation of this mixture with Protein Sepharose G beads. This stage limits the number of non-specific interactions with the beads. After this step, the anti-GBP2 antibody was allowed to mix with the pre-cleared cell lysate before the complex was added to fresh Protein Sepharose G beads. The beads were then spun down from the suspension, isolating GBP2, GBP1 and their potential interaction partners. The protein complexes were removed from the beads by boiling in SDS loading buffer and the resulting product was run on SDS-PAGE. The gel was only permitted to

run for a very short distance to ensure a dense concentration of protein that would be then analysed by mass spectrometry.

At this stage, the samples were given to Bram Snijders and Vesela Encheva who ran the experiment and gave me back a data-set containing gene identities of the proteins isolated. Briefly, they carried out the analysis by excising the protein piece and destained it using 50% acetonitrile, 50mM ammonium bicarbonate, reduced with 10mM dithiothretiol, alkylated with 55mM iodoacetamine. The proteins were then digested with 6ng/ml trypsin overnight at 37˚C before the peptides were extracted in formic acid and 1% acetonitrile. These peptides were loaded on to an Easy Spray column (Thermo Fisher). Reverse phase chromatography was performed using the RSLC nano U3000 (Thermo Fisher). The in-gel digested samples were run on a linear gradient of 80 % acetonitrile, 0.1 % formic acid from 2 to 40% over 35 minutes. The Q exactive was operated in a data dependent mode on the most abundant ions. They created the list of 908 proteins by searching the parent ion and tandem mass spectra against *Homo sapiens* and *Toxoplasma gondii* databases. Furthermore the Max Quant software added a list of 247 common laboratory contaminants to the database. The datasets were filtered on a posterior error probability to achieve a 1% false discovery rate on protein and peptide levels. Finally, they used the Max Quant matching function to increase overlap between experiments.

I received the information in the form of a Perseus software file. Importantly, we retrieved GBP1 and GBP2 proteins in the experiment demonstrating that the immunoprecipitation protocol had worked. Using this software, I analysed the potential protein interactions that had the lowest false discovery rates and which occurred in a *Toxoplasma* infection specific manner. I was interested in proteins that were identified during infection with the parasite as compared to the uninfected control (Table 7).

Other members of the lab can further mine this dataset. The next step with this information would be to carry out another immunoprecipitation with the GBP2 antibody before probing an immunoblot with an antibody against one of potential protein partners. It is true that GBP1 was retrieved with this antibody and therefore the list contains potential interaction partners of this protein too.

Based upon this list, there a large number of possible mechanisms by which GBP1/2 exert their function in terms of a *Toxoplasma* infection. Mouse GBPs have

been implicated in both canonical and non-canonical inflammasome induction. One wonders whether or not human GBPs can play a similar role. One of the potential interaction partners that I identified from the mass spectrometry run was transmembrane protein 214 (TMEM214), which is a known interactor of caspase-4, the human equivalent of mouse caspase-11 (Li et al., 2013). This candidate was found in a type II infection-dependent manner. This is interesting as GBP1 can restrict type II parasites, but not type I. TMEM214 acts an anchor for procaspase-4 at the endoplasmic reticulum outer membrane (Li et al., 2013). As GBP1 is isoprenylated and membrane targeted, there is a possibility that it interacts with TMEM214 to ensure cleavage of procaspase-4 to caspase-4.

The data obtained from the mass spectrometry analysis leaves room to speculate that metabolic functions could be driven by GBP1/2. This is because proteins like Lon protease homolog, Vacuolar ATPase assembly integral membrane protein (VMA21) and major vault protein (MVP) were co-immunoprecipitated from the sample in an infection dependent manner. VMA21 and MVP were retrieved from samples in a strain-independent manner. This would suggest that they do not play a role in the GBP1 strain-dependent restriction of *Toxoplasma*. Instead a more general role could be carried out, or potentially these are protein interaction partners with GBP2 and result in an un-yet identified host defence function. VMA21 is of particular interest as it is an assembly chaperone of the mammalian proton pump complex in the endoplasmic reticulum. When levels of VMA21 are manipulated, autophagy is blocked leading to a deficient mTORC1 pathway, resulting in ineffective autolysosomes that can cause vacuoles in the cell (Ramachandran et al., 2013). Lon protease homolog is a mitochondrial protease that plays a vital function in the degradation of damaged, oxidised and mis-folded proteins. Additionally, some proteins may be degraded by Lon protease homolog in normal conditions (Quiros et al., 2015). This protease has also been implicated in regulation of mitochondrial gene expression and in chaperoning membrane complex components (Bota and Davies, 2002). MVP is highly conserved between species and has functions that are not yet fully understood. However, it is known that MVP is involved in a number of cellular signal transduction pathways, including STAT1 and MAPK activation, and is thought to result in a cell survival response (Berger et al., 2009). Rab-18 was also retrieved. This protein is found in endosomes and lipid droplets, and is implicated in membrane trafficking between

the endoplasmic reticulum and the golgi apparatus (Dejgaard et al., 2008). As GBP1 and 2 are isoprenylated for targeting to membranes and to enhance protein-protein interactions, it would be logical that they could be involved in trafficking other proteins to specific areas in the cell.

Table 8 displays potential interaction partners of GBP1/2 that were retrieved in all conditions (unless otherwise stated). GBP1/2, or any GBP, may play a role in the cell that is independent of infection. In this case, the relevant interaction partner(s) would be retrieved in all conditions. While this would be a more difficult route to follow up, it is an interesting line of investigation that could be considered in the future. A large number of candidates that suggest a function in translation were identified (Table 2), with almost the complete ribosomal complex being retrieved. Ribosomes are abundant in cells, thus this result could potentially be an artefact, but it is worth taking note of this finding. Furthermore, a significant selection of initiation of translation factors was isolated. These proteins bind to the ribosome complex and trigger the beginning of the biosynthesis. There is a possibility that GBPs interact with these proteins to induce the synthesis of a certain product to mediate host defense. Elongation factors were also identified as potential interaction partners (Table 8). These are a set of proteins that enable the elongation from the first binding site on the ribosome, to the last.

These candidates present a large number of opportunities to determine interaction partners of GBP1/2 that may result in identifying a mechanistic pathway for these proteins. It appears that these proteins could play a role in translation, metabolic pathways and/or inflammasome induction.

Table 7 Protein interaction candidates for GBP1 and 2 identified in a *Toxoplasma* strain-dependent manner

Table showing possible protein interaction partners of GBP1/2 as identified by immunoprecipitation of GBP1/2 from A549 cells followed by mass spectrometry analysis. The candidates common to both type I and type II infection highlighted in blue. Cells were stimulated for 18 hours with 10U/ml IFNγ. Infection persisted for 2 hours, MOI 3.

Type I infected samples	Type II infected samples
Copine-7, 5, 8, 6, 4, 9, 2, 3	CDGSH iron sulphur domain containing protein 1
Cytosolic purine-5-nucleotidase	Copine-7, 5, 8, 6, 4, 9, 2, 3
DNA-directed RNA polymerases I, II, III subunit RPABC3	Cytosol aminopeptidase
Dynactin subunit 5	Cytosolic purine 5-nucleotidase
Major vault protein	DNA-directed RNA polymerases I, II, III subunit RPABC3
Myosin-10	Dilicholphosphate mannosyltransferase subunit 3
Nucleosome assembly protein-1 like 1	Dynactin subunit 5
Peptidoglycan recognition protein 1	Lon protease homolog
Proteolipid protein 2	Major vault protein
Putative cytochrome b c1 complex subunit Rieske-like protein 1	Mitsugumin
Ras-related protein R Ras 2	Myoferlin
Replication protein A 14kDa subunit	Nucleosome assembly protein-1 like 1
RRBP1	Paladin
TMEM 109	Protein furry homolog like
TMEM 214	Proteolipid protein 2
TMEM33	Pru 116kDa U5 small nuclear ribonucleoprotein component
Translational activator GCN1	Ras-related R-Ras 2
Vacuolar protein sorting associated protein 35	Ras-related protein Rab-18
VMA21	Replication protein A 14kDa
	Transgelin
	Translational activator GCN1
	Translocating chain-associated membrane protein-1
	UP-regulated during skeletal muscle growth protein 5
	UPD-glucose glycoprotein glucosyltransferase
	Vacuolar protein sorting-associated protein 35
	Vitamin-K dependent protein-S
	VMA21

Common to both infections

Table 8 Protein interaction candidates of GBP1 and 2 that implicate roles in translation.

Table showing possible protein interaction partners of GBP1/2 as identified by immunoprecipitation of GBP1/2 from A549 cells followed by mass spectrometry analysis. Candidates that are implicated in translation have been listed. The candidates are common to uninfected, and type I and type II infected samples, excepting candidate highlighted in yellow that was detected in the uninfected sample only. Cells were stimulated for 18 hours with 10U/ml IFNγ. Infection persisted for 2 hours, MOI 3.

Ribosomal proteins	Initiation of translation factors	Elongation factors
40S S27	1A	Elongation factor 1-gamma
40S S3	2 subunit 1	Transcription elongation factor B polypeptide 1
40S S23	3 subunit A	Transcription elongation factor B polypeptide 2
40S 19	3 subunit B	Elongation factor 1-delta
40S S4	3 subunit C	Eukaryotic translation elongation factor 1 epsilon-1
40S SA	3 subunit D	Elongation factor 2
40S S24	3 subunit E	Elongation factor 1-beta
40S S17	3subunit F	Elongation factor Tu, mitochondrial
40S S12	3 subunit H	Elongation factor 1- alpha 1
40S S9	3 subunit I	
40S S5	3 subunit J	
40S S10	3 subunit K	
40S S20	3 subunit L	
40S S7	3 subunit M	
40S S8	4 gamma 1	
40S S16	4H	
40S S14	5A-1	
40S S18	5B	
40S S13	6	
40S S11		
40S S25		
40S S21		
60S P0 (acidic)		
60S L35		
60S L16		
60S P1 (acidic)		
60S L12		
60S L22		
60S L23		
60S L11		

Identified in uninfected group only

5.2 Discussion

Throughout this project, I have continuously developed tools that I could use to characterise GBPs, but also that other people could use. I have not only designed and characterised antibodies and cells, I have optimised experiments and collated a large dataset that can be mined to identify protein interaction partners of GBP1 and 2. The antibodies I have designed and characterised, with the cells that are being developed in the lab, are invaluable tools that can be used to determine GBP function in a wide array of pathogen types.

During, this project I have highlighted that there are relatively basic questions regarding the GBP family members that still need to be addressed. While the GTPase activity of GBP1 has been extensively studied *in vitro* (Praefcke et al., 1999, Praefcke et al., 2004, Kunzelmann et al., 2005, Ghosh et al., 2006, Abdullah et al., 2009, Vopel et al., 2010, Abdullah et al., 2010, Wehner et al., 2012), the properties and characteristics of the other GBPs are widely unknown. The GTPase activity of GBP4 and other family members has not been elucidated. The requirements for the activation of GTPase activity or activation of other cellular components still remain largely unknown. There could be underlying differences here that could account for the differences observed between the characteristics of and the roles played by both human GBP family members, and GBPs across species. It is apparent also that some functions of GBP1 occur in a GTPase independent manner. The inhibition of endothelial cell invasion and proliferation occurs regardless of the GTPase nucleotide bound state (Guenzi et al., 2001, Guenzi et al., 2003). The fact that GBP1 can restrict type II *Toxoplasma* in an IFNγ-independent manner would suggest that this mechanism too occurs by GTPase-independent means. Therefore it is still important to take into account GBP function when IFNγ is not present.

On a similar note, there are many basic aspects of protein turnover and principles that have not been investigated. For GBP4, parasite infection clearly impacts on protein levels observed. The synthesis and degradation rates, and the routes of degradation are generally unknown. Using the protein synthesis inhibitor cycloheximide coupled with protein expression quantification, characterisation of protein production can be achieved. The samples will be incubated with inhibitor

before washing the cycloheximide off and tracking the rate that protein expression can once again be detected in an immunoblot. This will be possible using the antibodies that I have produced throughout the project. Conversely, the route of degradation for each GBP can be determined by using inhibitors of specific degradation pathways. The rate of degradation can be calculated by incubating the sample with a protease inhibitor, after which this inhibitor will be washed off and protein expression will be quantified by immunoblot. Cycloheximide must be used in these experiments too to ensure the degraded protein is not being replaced by freshly synthesised product.

The antibodies I have designed and characterised, with the cells that are being developed in the lab, are important tools that can be used to determine GBP function in a wide array of pathogen types. To show that GBP1 is able to restrict *Toxoplasma* replication in a location away from the parasitophorous vacuole changes the way we should think about these proteins. While the discovery is groundbreaking, there is no further clarification as to the mechanism by which GBP1 conducts this function. The above results demonstrating potential interaction partners of GBP1 present a very real possibility to narrow down the area in which this GBP family member, at least, exerts its function. The mass spectrometry data that I have collated and prioritised can be mined in many different ways to discover interacting partners and hopefully identify mechanisms by which this GBP family member functions. GBP1 has been implicated in cell remodelling and endothelial cell proliferation (Schnoor et al., 2009, Ostler et al., 2014) in humans, as well as in inflammatory responses (Shenoy et al., 2012, Pilla et al., 2014, Meunier et al., 2014, Meunier et al., 2015, Man et al., 2015). However metabolic or translational functions would be completely novel. With the discovery of 'off-site' GBP responses, this opens the possibility for never before considered roles of this protein family that do not involve the pathogen vacuole.

I have shown in this dataset that there are a vast number of potential mechanisms by which GBP1 could be functioning against *Toxoplasma* infection. These include inflammasome responses via the identification of TMEM214, an interaction partner of caspase-4 (Li et al., 2013). Also a role in trafficking of proteins is possible after the retrieval of proteins such as Rab-18, a protein required for trafficking between the endoplasmic reticulum and golgi apparatus. As GBP1 is isoprenylated (Nantais et al., 1996) and has been localised to the golgi (Modiano et

al., 2005, Tripal et al., 2007), this is one exciting prospect to follow, as there may be a role in regulating post-translational modification or packaging of proteins. Identifying translational-activator-GCN1 along with a vast array of ribosomal proteins and initiation of translation factors points to a function in mediating translation. Translational functions are further implicated with the identification of a large number of elongation factors, which are responsible for forming the initial peptide bond to the ribosome complex. If GBP1 truly does interact with components such as Lon protease homolog, VMA21 and MVP then a metabolic function can be implicated (Berger et al., 2009, Quiros et al., 2015, Ramachandran et al., 2013). VMA21 is implicated in autophagy and cell death, an important cell defence mechanism (Ramachandran et al., 2013). GBP1 can restrict *Toxoplasma* in a strain-dependent manner, however the interaction with VMA21 occurs in a strain-independent manner. This would suggest that if the interaction between GBP1 and VMA21 was real, it is not a dominant mechanism of protection against *Toxoplasma*. However, there could be a general function of GBP1 that is dependent on this interaction. Alternatively, as GBP2 is also a bait protein in this mass spectrometry experiment, VMA21 may interact with this family member. As the function of GBP2 against *Toxoplasma* is not yet characterised, there is a possibility that a role is fulfilled by this interaction. MVP is another protein interaction candidate that is immunoprecipitated in a strain-independent manner. This protein is implicated in cellular signal transduction pathways, thought to result in increased cell survival responses (Berger et al., 2009). As GBP expression has generally been associated with cell death responses it could be that GBP1 negatively regulated MVP, or vice versa. If GBP1/2 were proven to interact with Lon protease homolog, a function in degradation of misfolded or damaged proteins would be likely (Quiros et al., 2015). This again would be an interesting line of study to investigate, considering the relationship between GBP1 and the golgi, with potential for protein modification and regulation.

All of the above interactions can be confirmed or discounted by repeating the immunoprecipitation with the GBP antibodies before probing an immunoblot with antibodies against the candidate proteins. To distinguish between GBP1 and GBP2 specific antibodies, one could immunoprecipitate from A549 ΔGBP1 cells and verify which interactions are missing or still present. The missing interactions

would signify a GBP1-specific interaction partner, and remaining candidates would denote GBP2-specific proteins.

These results are definitely exciting, and open numerous doors in the challenge of outlining mechanistic function of these large GTPases.

Chapter 6. Conclusion

Overall this project has made progress in developing the tools that are necessary to fully elucidate the functions played by human guanylate binding proteins. I have shown that GBP1 works to restrict *Toxoplasma* during vacuolar replication. GBP4 is a nuclear GTPase that loses expression upon infection with type I *Toxoplasma* parasites. I have highlighted the importance of looking at functions of GBPs at a location away from the PV. This project underlines that breakthroughs made in mice are not always transferrable across species. While the mouse may not be the most suitable model to study GBPs with regards to their functions in humans, I have shown it is possible to move studies into a human system and that research in primary cells is becoming more accessible. I have demonstrated that GBPs are playing specific and relevant roles during infection, and have provided a great number of tools that will be used to further our understanding of these interesting and important GTPases. I firmly believe that research should be built on each other's successes, and that sharing reagents and knowledge is key - I hope the work done during my PhD will contribute to this.

Chapter 7. Appendix

Table 9 Primer lists for GBP cloning

Primer name	Vector	Restriction sites	Sequence
GBP3 For	C1 GFP/ mCherry	BamHI/ BspEI	GGATCCTCCGGAATGGCTCCAGAG ATCCACATGACA
GBP3 Rev		BamHI/ SacI	GGATCCCTCGAGTTAGATCTTTAAG CTTATATGCGACAT
GBP5 For	C1 GFP/ mCherry	BamHI/ BspEI	GGATCCTCCGGAATGCTTTAGAGAT CCACATGTCA
GBP5 Rev		BamHI/ SacI	GGATCCCTCGAGTTAGAGTAAAACA CATGGATCATCG
GBP6 For	C1 GFP/ mCherry	HindIII/ BspEI	AAGCTTTCCGGAATGGAATCTGGAC CCAAAATGT
GBP6 Rev		HindIII/ SacI	AAGCTTCTCGAGTTAAAAGGGGAGC TTATGCTTT
GBP 7 For	C1 GFP/ mCherry	BamHI/ BspEI	GGATCCTCCGGAATGGCATCAGAG ATCCACATG
GBP7 Rev		BamHI/ SacI	GGATCCCTCGAGTCAGCTTATAATT TTCTTACCAGGA
GBP4 For	pcDNA3 FlagHA	BamHI/ KpnI	GGATCCGGTACCATGGGTGAGAGA ACTCTTCACGCT
GBP4 Rev		BamHI/ XhoI	GGATCCGAGCTCTTAAATACGTGAG CCAAGATATTTTGT
GFP/mCherry GBP3 For	pGene	BamHI/ BspEI	GGATCCTCCGGAATGGCTCCAGAG ATCCACATGACA
GFP/mCherry GBP3 Rev		BamHI/ SacI	GGATCCCTCGAGTTAGATCTTTAAG CTTATATGCGACAT
GFP/mCherry GBP5 For	pGene	BamHI/ BspEI	GGATCCTCCGGAATGCTTTAGAGAT CCACATGTCA
GFP/mCherry GBP5 Rev		BamHI/ SacI	GGATCCCTCGAGTTAGAGTAAAACA CATGGATCATCG

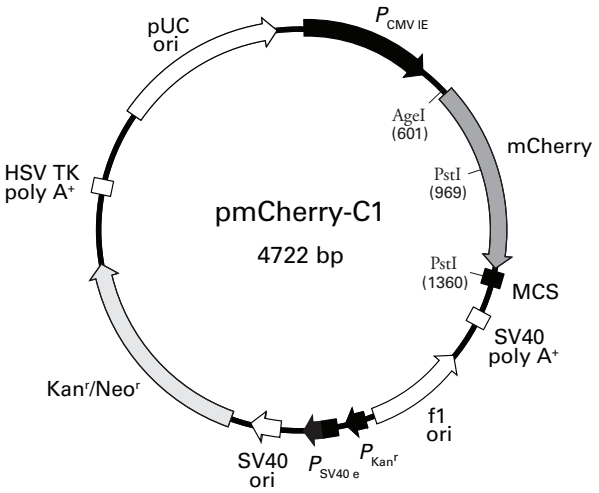
GFP/mCherry GBP6 For	pGene	HindIII/ BspEI	AAGCTTTCCGGAATGGAATCTGGACCCAAAATGT
GFP/mCherry GBP6 Rev		HindIII/ SacI	AAGCTTCTCGAGTTAAAAGGGGAGCTTATGCTTT
GFP/mCherry GBP7 For	pGene GFP	BamHI/ BspEI	GGATCCTCCGGAATGGCATCAGAGATCCACATG
GFP/mCherry GBP7 Rev		BamHI/ SacI	GGATCCCTCGAGTCAGCTTATAATTTTCTTACCAGGA
GFP GBP4 For	pGene	BamHI/ KpnI	GGATCCGGTACCATGGGTGAGAGAACTCTTCACGCT
GFP GBP4 Rev		BamHI/ XhoI	GGATCCGAGCTCTTAAATACGTGAGCCAAGATATTTTGT
GFP GBP4 For	pJS4	AscI	AAGCTTGGCGCGCCATGGTGAGCAAGGGCGAGGAGCT
GFP GBP4 Rev		PacI	AAGCTTTTAATTAATTAAATACGTGAGCCAAGATATT
GFP GBP4 For	pLVX-Tight-Puro	MluI	AAGCTTACGCGTATGGTGAGCAAGGGCGAGGAGCTG
GFP GBP4 Rev		NotI	AAGCTTGCGGCCGCTTAAATACGTGAGCCAAGATATT

Table 10 PCR Amplification of GBP for C1/ pcDNA3/ pGene vectors

Temperature (˚C)	Time	Cycles
95	2 minutes	1
95	30 seconds	35
Tm-5	30seconds	
72	1 minute/ kb	
72	5 minutes	1
4	Pause	1

Table 11 PCR amplification of GBP4 for pJS4 and pLVX-Tight-Puro

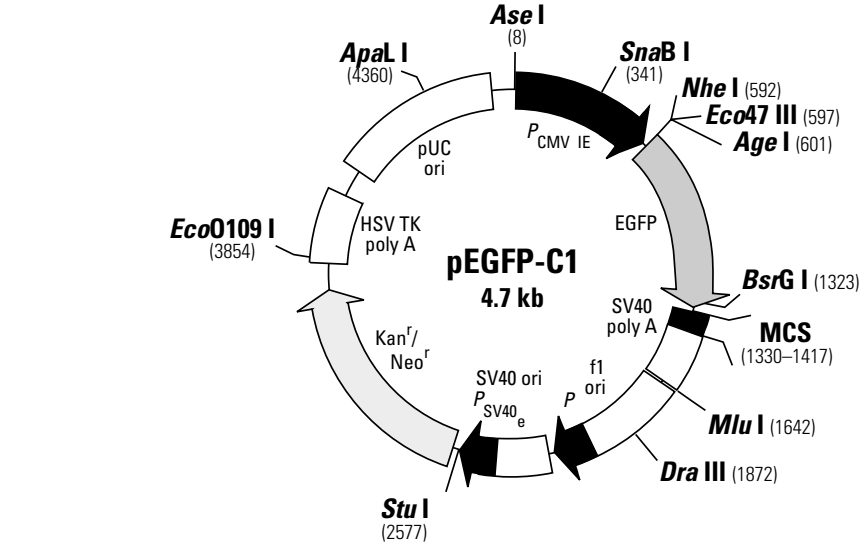
Temperature (˚C)	Time	Cycles
95	1 minute	1
95	15 seconds	35
60	15 seconds	
72	5 seconds	
72	5 minutes	1
4	pause	1




```
                                                                SacI
                                                        XhoI                                    SalI
End mCherry   BspEI                   BglII                         HindIII     EcoRI           AccI
1315 TAC AAG|TCC GGA CTC AGA TCT CGA GCT CAA GCT TCG AAT TCT GCA GTC

                KpnI                  XmaI
SalI                    SacII         SmaI
AccI  Asp718                  ApaI          BamHI                 XbaI*           BclI
1363 GAC GGT ACC GCG GGC CCG GGA TCC ACC GGA TCT AGA TAA CTG ATC ATA
                                                  STOP  STOP  STOP
```

Figure 32 mCherry-C1 vector map



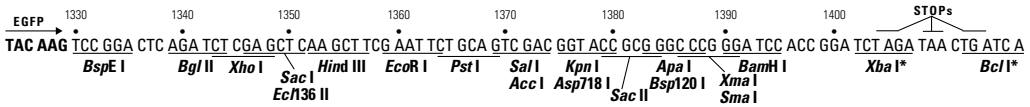


Figure 33 eGFP-C1 vector map

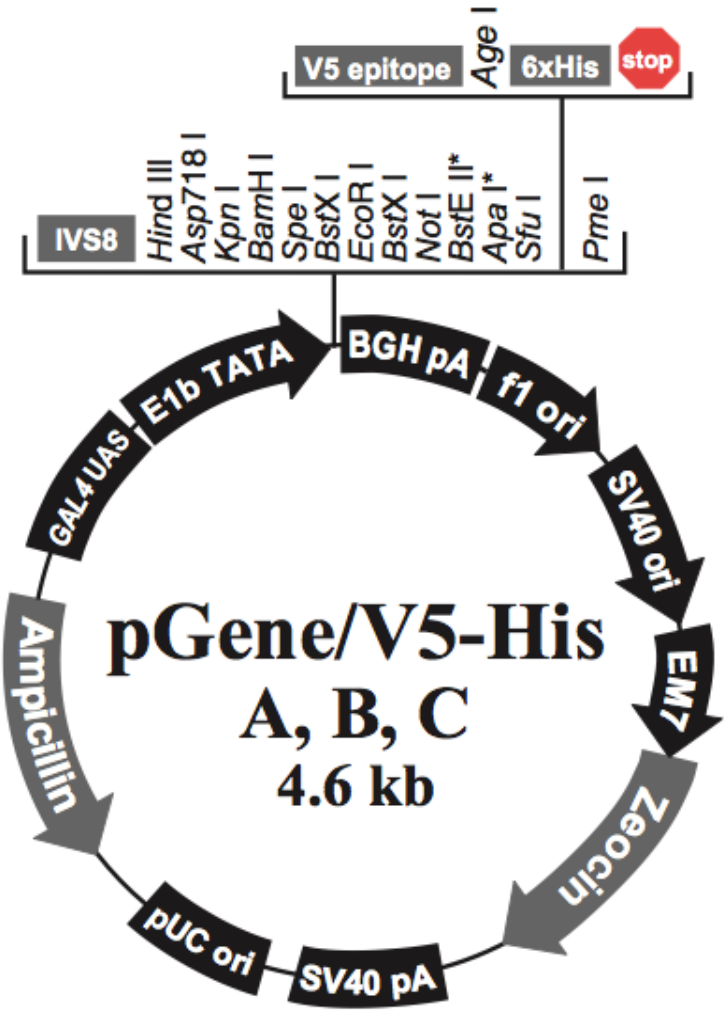


Figure 34 pGene vector map

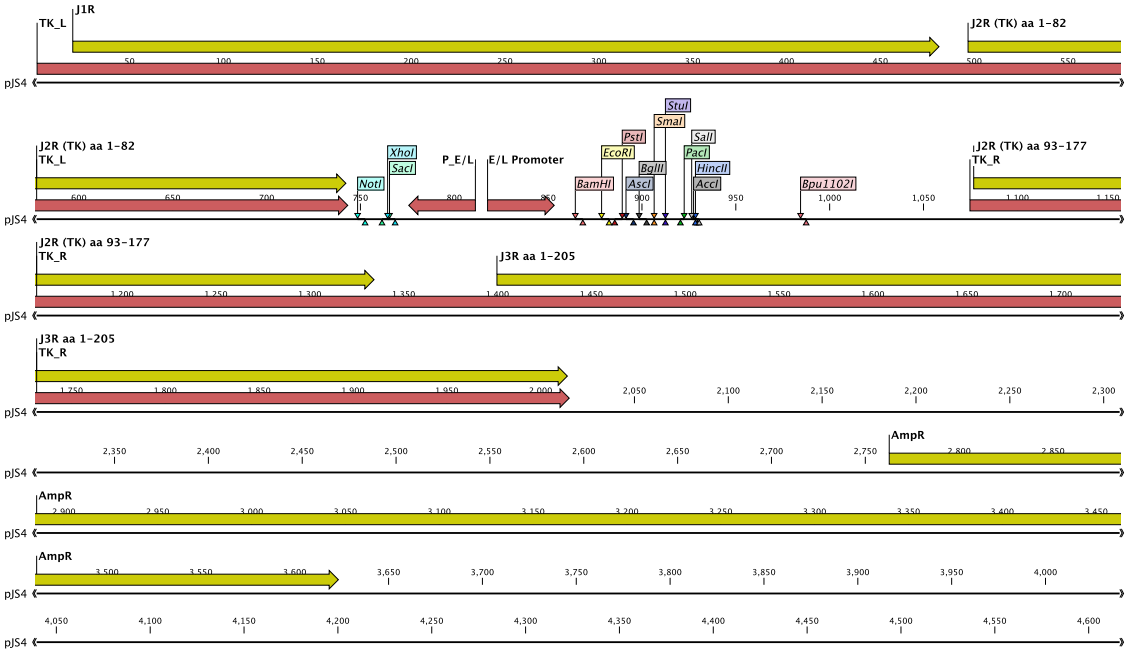
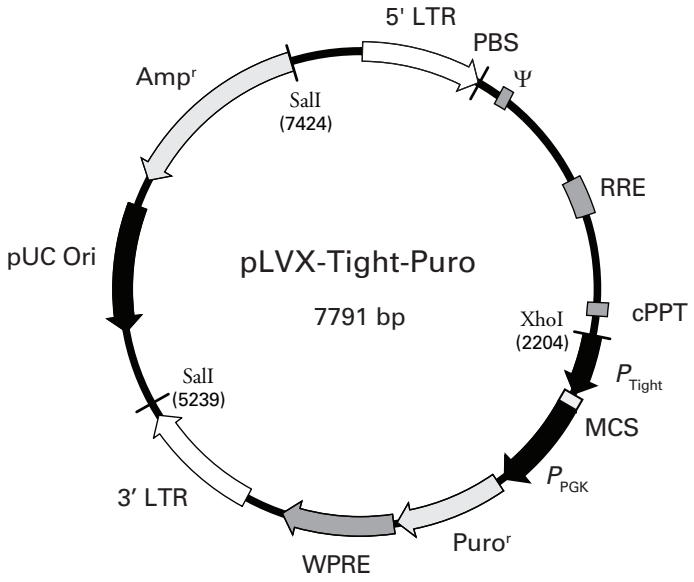


Figure 35 pJS4 vector map




```
                BamHI                                   MluI
                      NotI          XbaI*                       EcoRI
2521  TGGAGAAGGA TCCGCGGCCG CGCCGGCTCT AGATCGCGAA CGCGTGAATT CTACCGGGTA
      ACCTCTTCCT AGGCGCCGGC GCGGCCGAGA TCTAGCGCTT GCGCACTTAA GATGGCCCAT
```

Figure 36 pLVX-Tight-Puro vector map

Table 12 Peptide sequences for polyclonal antibody production

Human GBP	Peptide Sequence
1	QDLQTKMRRRKAC
2	QKDIWDIQMRSKSLE
3	KTLKKKTKRYMSHKLKI
4	QLNKEINQLKEKIEST
5	NLFIQKTEELKAKYYRE
6	QFKRMIDTTKNDDTPW
7	LKEQIEAAENEEPSVF

Table 13 Peptide sequences for monoclonal antibody production

Human GBP	Peptide Sequence
2	KEGFENESKRLQKDIWDIQMRSKSLE
4	KEEFQKKSEQLNKEINQLKEKIEST

Table 14 GBP antibodies produced

	Source	Raised against	Specificity	Concentration	Protocol	Rating	Specificity	Concentration	Rating	Specificity	Concentration	Rating
hGBP1	Rb polyclonal	Peptide	Specific	1:100	1h RT 5% Blotto	Excellent	hGBP1, 2	40ul/0.5mg	Very Good	ND	1:100	Very good
hGBP1	Rb polyclonal	Whole protein	hGBP1, 2, 3, 5	1:5000	1h RT 5% Blotto	Excellent				ND	1:1000	Very good
hGBP2	Rb polyclonal	Peptide	Specific	1:100	1h RT 5% Blotto	Excellent	hGBP1, 2	40ul/0.5mg	Very Good	ND	1:100	Very good
hGBP2 9E12	M monoclonal	Peptide	Specific	Pure supernatant	1h RT	Excellent	Specific	5ul/0.5mg	Very Good			
hGBP2 1A6	M monoclonal	Peptide	Specific	Pure supernatant	1h RT	Poor	Specific	5ul/0.5mg	Excellent			
hGBP3	Rb polyclonal	Peptide	Specific	1:100	1h RT 5% Blotto	Good						
hGBP4	Rb polyclonal	Peptide	Specific	1:100	1h RT PBS 0.05% Tween	Very Good	ND	40ul/0.5mg	Good	ND	1:100	Very good
hGBP4 5E1	M monoclonal	Peptide	Specific	Pure supernatant	1h RT	Doesn't work	ND	5ul/0.5mg	Excellent			
hGBP4 9A12	M monoclonal	Peptide	Specific	Pure supernatant	1h RT	Doesn't work	ND	5ul/0.5mg	Excellent			
hGBP5	Rb polyclonal	Peptide	ND	1:100	1h RT 5% Blotto	Poor						
hGBP6	Rb polyclonal	Peptide	ND	1:100	1h RT 5% Blotto	Poor						
hGBP7	Rb polyclonal	Peptide	ND	1:100	1h RT 5% Blotto	Poor						

Buffer recipes

Blotto
5% skim milk powder (Sigma # 70166)
0.05% Tween 20 (Sigma # P1379)
PBS

Perm/ Quench
50mM NH_4Cl
0.2% Saponin
PBS

PGAS
0.2% Gelatin, porcine (Sigma #G-7765)
0.02% Saponin
0.02% NaN_3
PBS

Chapter 8. Publications

Cellular Microbiology (2016) **18**(8), 1056–1064 doi:10.1111/cmi.12579
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Breaking Report

Human GBP1 does not localize to pathogen vacuoles but restricts *Toxoplasma gondii*

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Summary

Guanylate binding proteins (GBPs) are a family of large interferon-inducible GTPases that are transcriptionally upregulated upon infection with intracellular pathogens. Murine GBPs (mGBPs) including mGBP1 and 2 localize to and disrupt pathogen-containing vacuoles (PVs) resulting in the cell-autonomous clearing or innate immune detection of PV-resident pathogens. Human GBPs (hGBPs) are known to exert antiviral host defense and activate the NLRP3 inflammasome, but it is unclear whether hGBPs can directly recognize and control intravacuolar pathogens. Here, we report that endogenous or ectopically expressed hGBP1 fails to associate with PVs formed in human cells by the bacterial pathogens *Chlamydia trachomatis* or *Salmonella typhimurium* or the protozoan pathogen *Toxoplasma gondii*. While we find that hGBP1 expression has no discernible effect on intracellular replication of *C. trachomatis* and *S. typhimurium*, we observed enhanced early *Toxoplasma* replication in CRISPR hGBP1-deleted human epithelial cells. We thus identified a novel role for hGBP1 in cell-autonomous immunity that is independent of PV translocation, as observed for mGBPs. This study highlights fundamental differences between human and murine GBPs and underlines the need to study the functions of GBPs at cellular locations away from PVs.

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Introduction

The cytokine Interferon gamma (IFNγ) is an important mediator of host response against an array of intracellular pathogens (MacMicking, 2012). Such infections include the apicomplexan parasite *Toxoplasma gondii*, in which case IFNγ drives effector mechanisms to eliminate the fast replicating acute phase tachyzoite stage. The intravacuolar bacteria *Chlamydia trachomatis* and *Salmonella typhimurium* are likewise targeted by IFNγ-driven host responses in the acute stages of infection, resulting in *C. trachomatis* reticulate bodies forming an aberrant non-dividing form, and in *S. typhimurium* clearance (Kazar *et al.*, 1971; Pie *et al.*, 1997). In response to infection, IFNγ upregulates a vast number of proteins, with a family of large GTPases, the Guanylate Binding Proteins (GBPs), being among the most highly induced (Cheng *et al.*, 1983). GBPs have been studied *in vitro* or in murine models and are important in immune activation and restricting intracellular pathogens, including viruses, bacteria and protozoan parasites (MacMicking, 2012).

In mice, mGBPs accumulate around pathogen-containing vacuoles (PV) of intracellular pathogens such as *Toxoplasma* (Degrandi *et al.*, 2007; Virreira Winter *et al.*, 2011; Haldar *et al.*, 2013; Selleck *et al.*, 2013), *C. trachomatis* (Coers *et al.*, 2008; Haldar *et al.*, 2013), *Mycobacterium bovis* BCG (Kim *et al.*, 2011) and *S. typhimurium* (Meunier *et al.*, 2014). At PVs mGBPs act cooperatively to assemble host defense responses that include an oxidative burst, the delivery of antimicrobial peptides and the induction of autophagy (Kim *et al.*, 2011). Additionally, mGBPs promote the disintegration of *Salmonella*-containing vacuoles thereby exposing bacteria to the cytosol where they can activate the cytosolic LPS sensor caspase-11 (Meunier *et al.*, 2014). Rapid activation of caspase-11 in response to infections with *Legionella pneumophila* or *Chlamydia muridarum* requires additional lysis-independent function of mGBPs

that are poorly characterized (Pilla *et al*., 2014; Finethy *et al*., 2015). In addition to their association with PVs mGBPs also co-localize with the cytosolic bacterial pathogens *Listeria monocytogenes* and *Francisella novicida* (Kim *et al*., 2011; Man *et al*., 2015; Meunier *et al*., 2015). The association of mGBPs with *F. novicida* prompts bacteriolysis and the activation of the cytosolic DNA sensor AIM2 (Man *et al*., 2015; Meunier *et al*., 2015).

Human GBPs are much less well understood, and their functional significance remains largely unknown. hGBP5 promotes NLRP3 inflammasome assembly and consequently IL-1β production in response to LPS and nigericin (Shenoy *et al*., 2012). hGBP1 can mediate the inhibition of endothelial cell proliferation (Guenzi *et al*., 2001) and has been shown to exhibit anti-viral properties. When hGBP1 expression is silenced using small hairpin RNAs, Dengue virus burden increases (Pan *et al*., 2012). hGBP1 also mediates an antiviral effect against vesicular stomatitis virus and encephalomyocarditis virus when overexpressed in HeLa cells (Anderson *et al*., 1999). While these studies indicate that human GBPs have relevance during pathogenic infection, we do not understand which microbes they target and how the endogenous proteins act.

The structure and biochemical properties of GBPs have been studied in detail, with the structure of hGBP1 revealing a globular GTPase domain connected to an arm-like extension, and revealing the very fast GTP hydrolysis rate of 95 min^{-1} (Prakash *et al*., 2000). Some hGBPs also have the ability to perform two consecutive hydrolysis steps from GTP to GMP (Schwemmle and Staeheli, 1994). hGBP 1, 2 and 5 contain a 'CaaX' prenylation motif at their C-terminus, implying a capacity to target membranes.

In agreement with their biophysical properties and observations made for mGBPs, hGBPs have been postulated to recognize PVs formed by type II *Toxoplasma* vacuoles as well as *C. trachomatis* PVs, also known as inclusions (Tietzel *et al*., 2009; Al-Zeer *et al*., 2013; Ohshima *et al*., 2014). It has been reported that interference with hGBP1 and 2 expression in IFNγ-primed cells led to increased *C. trachomatis* inclusions size, indicating better growth of the bacteria in absence of these proteins (Tietzel *et al*., 2009). Many of these studies have relied on heterologously expressed proteins or an antibody recognizing several GBP family members. A recent study on *Toxoplasma* postulates no effect on parasite restriction by hGBPs (Ohshima *et al*., 2014). It thus remains unclear which, if any, hGBPs target *Toxoplasma* PVs, and whether endogenous hGBPs can target and restrict *C. trachomatis*.

Here, we demonstrate that hGBP1, in contrast to its closest murine orthologues mGBP1 and 2 fails to recognize PV formed by *C. trachomatis*, *S. typhimurium* or *Toxoplasma* in human epithelial cells. Our data indicate that hGBP1 is not essential for the execution of cell-autonomous control of the replication of these pathogens in unprimed and IFNγ-primed human epithelial cells. However, in a similar manner to mGBPs, hGBP1 is able to restrict *Toxoplasma* early after host cell infection. This restriction cannot be attributed to an invasion mechanism, but rather hGBP1 is responsible for delaying the onset of parasite replication. GBPs have only been reported to play a role in cell-autonomous control of infections if they accumulated around the pathogen-containing vacuole. Here we show that this is not the case for hGBP1. We thus define a novel role for hGBP1 with its capacity to restrict *Toxoplasma* in early infection without targeting to the PV.

Results

hGBP1 does not localize to intracellular pathogen vacuoles in epithelial cells

Several mGBPs recruit to PVs in an IFNγ-dependent manner, disrupt PV integrity and facilitate pathogen destruction. We explored whether hGBP1 was able to target intracellular vacuoles formed by the bacterial pathogens *C. trachomatis* and *S. typhimurium* or the parasite *Toxoplasma*. Human GBP1 is the closest orthologue of mGBP2 previously shown to associate with vacuolar *C. trachomatis*, *S. typhimurium* and *Toxoplasma* in murine fibroblasts, epithelial cells, macrophages and spleen tissue (Degrandi *et al*., 2007; Tietzel *et al*., 2009; Virreira Winter *et al*., 2011; Al-Zeer *et al*., 2013; Haldar *et al*., 2013; Selleck *et al*., 2013; Meunier *et al*., 2014). To this end, we produced a specific peptide antibody that could distinguish hGBP1 from all other hGBPs by immunoblot, and additionally a pan-hGBP antibody that recognized hGBPs 1, 2, 3 and 5 (Fig. S1A). While we could not overexpress hGBP4, this family member is least identical to hGBP1 so we are confident the antibody will not cross react. We show that A549 cells express GBPs including hGBP1 at steady state level and further upregulate the protein in response to IFNγ (Fig. S1B).

To further characterize this newly generated anti-hGBP1 antibody, we employed CRISPR/Cas9 technology to generate a lung epithelial A549-derived cell line lacking hGBP1 expression. A region 5′ to the GTPase domain of *Gbp1* was targeted for disruption, and deletion was confirmed by sequencing and immunoblot (Fig. S1C and D). The cells were further analysed by the pan-hGBP antibody, demonstrating that other hGBPs are still intact with normal protein expression (Fig. S1D). Using anti-hGBP1 for immunofluorescence staining we found that both naïve and IFNγ-primed and ΔhGBP1 cells exhibited low background immunofluorescence intensity. In contrast, parental A549 cells showed a robust increase in the anti-hGBP1 immunofluorescence signal when primed with

IFNγ (Fig. S1E), demonstrating that anti-hGBP1 specifically detects endogenous hGBP1 *in situ*.

Having confirmed the specificity of the anti-hGBP1 antibody we asked whether hGBP1 associated with PVs. To do so, we infected IFNγ-primed A549 cells with three representative vacuolar pathogens. Immunofluorescent microscopy showed that hGBP1 does not recruit around the PVs formed by *C. trachomatis* (Fig. 1A), *S. typhimurium* (Fig. 1B) or *Toxoplasma* (Fig. 1C). This lack of recruitment was observed at all time points tested (data not shown) and not affected by the absence of presence of IFNγ priming (Fig. S2A to C).

In order to associate with membranes hGBPs first transition into an active GTP-bound state and following hydrolysis hGBPs in the GDP-bound state form tetramers that can attach to membranes (Syguda *et al.*, 2012). We therefore considered that the anti-hGBP1 antibody failed to detect hGBP1 in the active, membrane-associated state. To test for this, we ectopically expressed hGBP1 N-terminally fused to mCherry. We observed that mCherry-hGBP1 heterologously expressed in mouse embryonic fibroblast (MEFs) localized to *Toxoplasma* PVs (Fig S3A). Staining with anti-hGBP1 overlapped with the mCherry-hGBP1 signal showing that anti-hGBP1 can detect hGBP1 in its active, membrane-associated state and indicating that hGBP1 fails to associate with PVs in human A549 cells. We stained IFNγ induced MEFs with anti-hGBP1 to ensure our antibody did not overlap with mGBPs at the PV (Fig S3B). Last, we expressed mCherry-hGBP1 in A549 cells and failed to observe any association of mCherry-hGBP1 *Toxoplasma* PVs (data not shown), further corroborating our finding that in striking contrast to mGBP1 and 2, hGBP1 is not recruited to intact vacuolar membranes of intracellular pathogens in infected human cells.

hGBP1 restricts Toxoplasma, *but not the intravacuolar bacterial pathogens* C. trachomatis and S. typhimurium

As the subcellular location of hGBP1 was not consistent with observations made with mGBPs and the roles they play during intracellular pathogen infection, we were interested to determine whether hGBP1 could still play a role in controlling these particular infections. We assessed the ability of the pathogens to replicate in the absence of hGBP1 in comparison to their wild-type cells. For *C. trachomatis* and *S. typhimurium* infections, inclusion forming unit (IFU) or colony forming unit (CFU) assays were employed, respectively, in the presence or absence of IFNγ. Priming cells with IFNγ restricted the replication and growth of both *C. trachomatis* and *S. typhimurium*

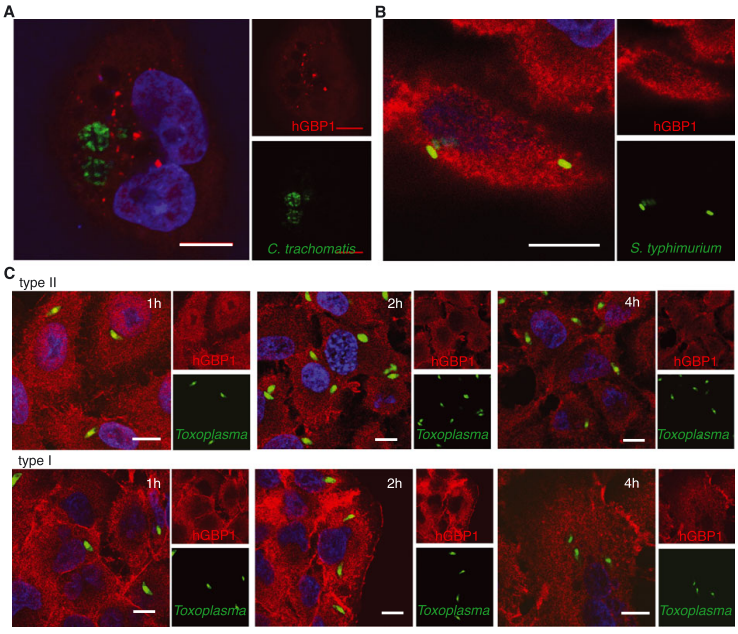

Fig. 1. hGBP1 does not localize to the intracellular pathogen vacuole.
A. Immunofluorescent confocal image of *C. trachomatis* vacuoles 20hpi in mCherry-hGBP1 expressing A549 cells primed with 200 U/ml IFNγ. $N = 2$.
B. Immunofluorescent confocal image of *S. typhimurium* vacuoles 4hpi in A549 cells primed with 50 U/ml IFNγ stained for endogenous hGBP1. $N = 3$.
C. Immunofluorescent confocal image of *Toxoplasma* vacuoles in A549 cells primed with 10 U/ml IFNγ at the indicated time points post infection. $N = 3$.
All scale bars 10 μm.

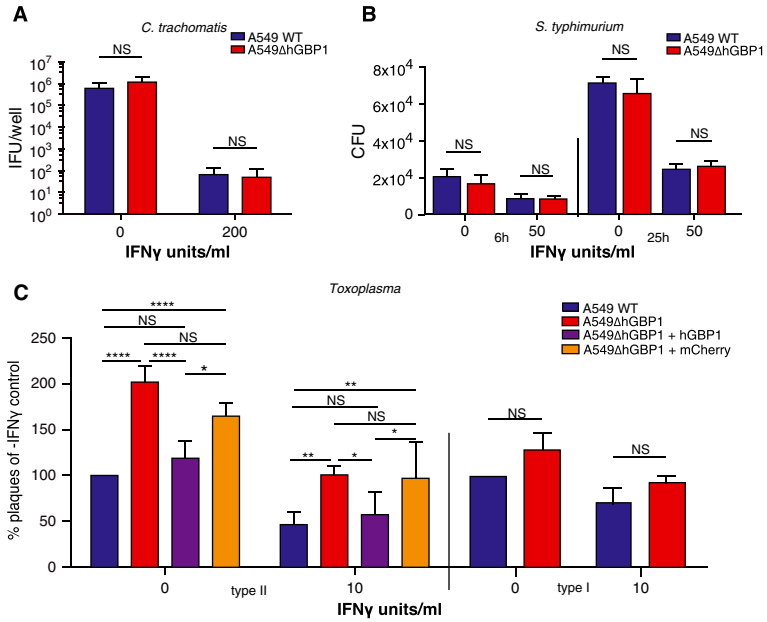

Fig. 2. hGBP1 restricts growth of *Toxoplasma gondii* but not *C. trachomatis* or *S. typhimurium*
A. *C. trachomatis* IFU assays carried out in A549 cells primed or not with 200 U/ml IFNγ, 40hpi. *N* = 2. Error bars represent standard error of the mean (STEM).
B. *S. typhimurium* CFU assays carried out in A549 cells primed or not with 50 U/ml IFNγ, 6 and 25hpi. *N* = 3. Significance was analysed by 2way ANOVA.
C. Plaque assays showing viability of *Toxoplasma* incubated for 6 h in WT, ΔhGBP1, ΔhGBP1 + hGBP1 or ΔhGBP1 + cherry A549 cells primed or not with 10 U/ml IFNγ before seeding onto HFF. Counts were taken 4 days after HFF infection. *N* = 5, Complementation *N* = 3. Significance was analysed by 2way ANOVA.

(Fig. 2A and B). However, the ability of bacteria to sustain themselves via replication in the presence or absence of immune pressure was the same regardless of the presence of hGBP1 (Fig. 2A and B). We then determined whether *Toxoplasma* replication was influenced by the absence of hGBP1. We compared the capacity of the parasite to replicate and form plaques in fibroblasts after an initial infection in A549 cells primed or not with IFNγ. Type I or type II *Toxoplasma* were incubated in A549 cells for 6 h, a time period too short for replication to occur, meaning potential differences in plaque number would be a result of parasite killing or early restriction. Twice as many type II *Toxoplasma* were able to survive when the infection took place in ΔhGBP1 cells, while type I parasites remained unaffected (Fig. 2C). Intriguingly, this was still true in cells that were not primed with IFNγ. An immunoblot confirmed the presence of hGBP1 even at basal steady state level (Fig. S1B). In accordance, the number of viable *Toxoplasma* parasites was largely reduced to levels seen in parental A549 cells when ΔhGBP1 A549 cells were complemented with hGBP1 (Fig. 2C). Thus hGBP1 does not restrict replication of the bacterial pathogens *C. trachomatis* and *S. typhimurium*, but does promote restriction of type II *Toxoplasma*.

hGBP1 mediates early replication of Toxoplasma *infection*

As we determined that *Toxoplasma* underwent early restriction by hGBP1, our next move was to identify the stage at which parasites were limited. We excluded differential invasion as a reason for the enhanced recovery of parasites from ΔhGBP1 cells. FACS analysis of A549 cells infected with fluorescent parasites showed that regardless of genotype of the cell, type II parasites invaded around 50% of A549 cells when an MOI of 4 was used (Fig. 3A). Immunofluorescence microscopy was used to ensure that individual cells of each genotype were infected with low and equivalent numbers of parasites (Fig. 3B). We used immunofluorescence microscopy to assess the early replication status of *Toxoplasma* in A549 cells. At 24 h post infection (hpi) it was evident that ΔhGBP1 cells contained more vacuoles filled with 2 or more parasites than co-isogenic wildtype cells did (Fig. 3C). By counting the number of parasites per vacuole over an infection time course, we quantified the replication efficiency of *Toxoplasma* (Fig. 3D). At 12hpi significantly more vacuoles contained parasites that had replicated once in the ΔhGBP1 cells as compared to the wild-type counterpart, regardless of IFNγ priming. By 18hpi, numbers of vacuoles

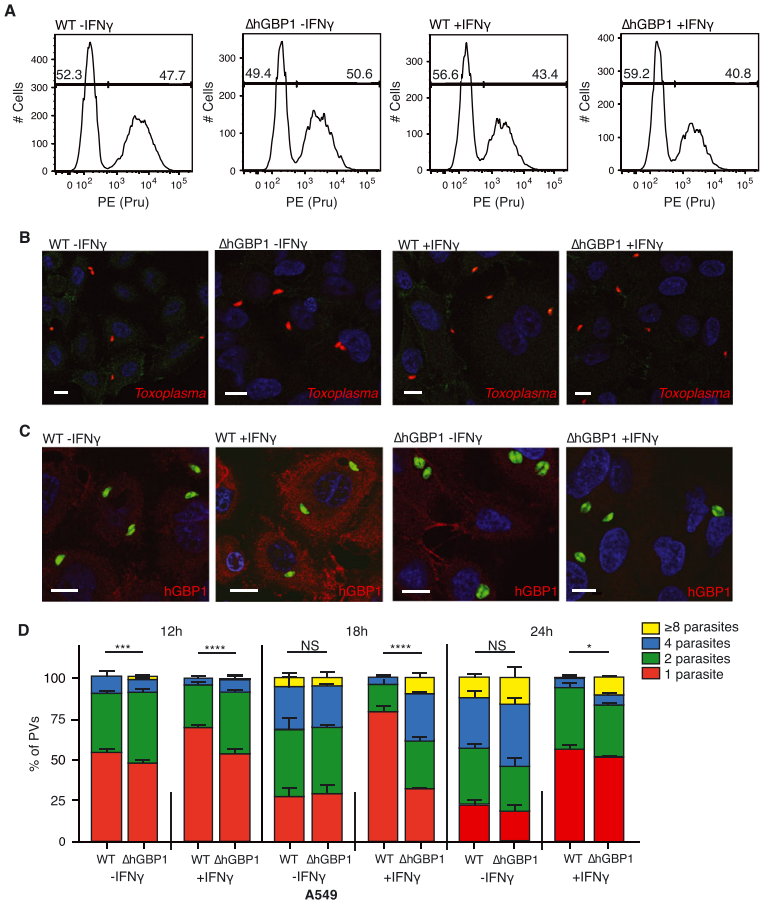

Fig. 3. hGBP1 mediates early replication of *Toxoplasma gondii*.
A. FACS analysis determined the number of A549 cells that had been invaded by *Toxoplasma* 1hpi. Cells had been primed or not with 10 U/ml IFNγ. Representative of 3 independent experiments.
B. Immunofluorescence confocal images taken at 1hpi show the same numbers of parasites enter individual cells.
C. Immunofluorescence confocal images of A549 cells primed or not with 10 U/ml IFNγ.
D. Replicating parasites were determined by counting the number of parasites within vacuoles at specified times post infection. $N = 2$. Significance was analysed using 2way ANOVA.
All scale bars 10 μm.

containing 2 or 4 parasites, indicating 1 or 2 replication cycles, respectively, were significantly increased in the IFNγ primed ΔhGBP1 (Fig. 3D). Finally, counts taken at 24 h show a significantly increased number of vacuoles that hold parasites that have undertaken 3 or more replication cycles in the IFNγ primed ΔhGBP1 A549 cells.

Our observations suggest that hGBP1 can delay the onset of parasite replication. We concluded that type II *Toxoplasma* is restricted early after invasion in A549 cells by a yet unknown mechanism driven by hGBP1 without the protein targeting to the vacuole.

Discussion

We find that hGBP1 mediates an initial attenuation of type II *Toxoplasma* early post-infection, without impacting subsequent replication of *Toxoplasma*, *C. trachomatis* or *S. typhimurium* in human epithelial cells. The observed early *Toxoplasma* attenuation is not because of a defect in parasite invasion in the presence versus absence of hGBP1. Rather, we hypothesize that hGBP1 either mediates early killing or slows down early replication of the parasite, as at 12, 18 and 24hpi the ΔhGBP1 cells

contain significantly more parasites that have undergone replication. To our knowledge this is the first report demonstrating that an IFN-inducible GTPase can restrict growth of an intracellular PV-resident pathogen independently of any detectable association of the GTPase with the PV itself.

Cell-autonomous restriction pathways for intracellular pathogens driven by host resistance proteins often see the protein localize to PVs. For example, ubiquitin-driven autophagy and galectin-mediated control of *Salmonella* relies on a pathogen-localization step of ubiquitin and galectin (Perrin *et al*., 2004; Thurston *et al*., 2012). Additionally in mouse, Immunity Related GTPases (IRGs) and GBPs mediate cell-autonomous killing by directly localizing and disrupting PVs (Martens *et al*., 2005; Ling *et al*., 2006). We clearly define that endogenous hGBP1 is not targeted to *C. trachomatis*, *S. typhimurium* or *Toxoplasma*. We do, however, show that hGBP1 possesses the ability to recruit to *Toxoplasma* PVs when heterologously expressed in mouse fibroblasts (Fig. S3A). These results suggest that human cells lack a cellular pathway for the delivery of GBPs to PVs that is present in mouse cells. In support of this model, we recently demonstrated that the translocation of mGBP1 and mGBP2 to PVs in mouse cells requires GKS proteins, a subset of IRG protein found in mouse but not in human cells (Haldar *et al*., 2015). Therefore, it appears likely that the deficiency of hGBP1 recruitment to PVs in human cells is due in part to the absence of GKS encoding genes from the human genome.

Conflicting reports attributed hGBPs to have a function in intracellular *C. trachomatis*, but not *Toxoplasma* control in non-hematopoietic cells (Tietzel *et al*., 2009; Ohshima *et al*., 2014). Both reports find hGBPs localized to PVs 24hpi. Reduction of bacterial inclusions upon ectopic expression of hGBP1 and 2 was observed in HeLa cells. Curiously the demonstrated localization of hGBP1 is observed for wild type, GTPase-deficient and helical-domain-only protein. We, and others, have previously shown that GTPase-activity deficient mouse GBP1 does not recruit to PVs (Kim *et al*., 2011; Virreira Winter *et al*., 2011). It is conceivable that detecting overexpressed hGBP1 with antibody staining presents with different results than detection of endogenous hGBP1. Equally, overexpressed hGBP1 might exert a different effect than endogenous hGBP1 and HeLa cells may present with a different restriction pathway than other epithelial cells.

Ohshima et al. successfully knocked out the entire GBP locus in haploid fibroblast-like human cancer cells, with the resulting cells showing no defect in IFNγ-induced cell-autonomous control of *Toxoplasma* at 24hpi. The early restriction of the parasite by hGBP1 we observe may have been missed or may not exist in fibroblasts. They also demonstrate that a low percentage of *Toxoplasma* PVs were decorated with hGBPs at 6hpi. The staining was carried out using an antibody against hGBP1-5, so it is conceivable that the protein present at that location comprises another family member(s). In contrast to our findings, hGBP1 has been shown to localize to the bacterial inclusion in THP1 macrophages and restrict *C. trachomatis* as demonstrated by shRNA knockdown (Al-Zeer *et al*., 2013). It remains to be investigated if human GBPs can localize to PVs of other pathogens in macrophages. Combined with our results, this leads us to speculate that hGBP1 may restrict select pathogens in a cell type- and localization-dependent manner.

hGBPs are highly upregulated in all stages of infection with intracellular pathogens and are often found in transcriptional analysis of patient samples. Combined with knowledge acquired from murine studies, it is almost certain that hGBPs have an impact on the control of these infections. We find that hGBP1 plays a role in mediating early restriction of *Toxoplasma* soon after infection without directly localizing to the pathogen. Previous definitions of how this host restriction factor family is acting against vacuolar intracellular pathogens were reliant on the protein being present at the vacuole. Clearly, we have to rethink this rather simplistic assessment as a proxy for potential functions especially for the human GBPs. Early restriction of pathogens directly after invasion is the first step a cell takes to combat the invading foreign agent. These early restriction mechanisms have to act in a rapid and precise manner in order to start the cascade of intracellular defense mechanisms. Because hGBP1 is expressed at steady state level and we observe its defense activity even in absense of interferon priming, it most likely interacts with and directs specific cellular machineries that do not require induction. Future work will elucidate the mechanism by which hGBP1 can mediate early restriction of *Toxoplasma*.

Experimental procedures

Cell culture

A549 lung epithelial cells were grown in DMEM with glutamine (Life Technologies) supplemented with 10% FBS and cultured at 37 °C in 5% CO_2. Where appropriate, cells were stimulated overnight by addition of human IFNγ (R&D Systems) to growth media. Human foreskin fibroblasts (HFF) were maintained in DMEM with glutamine supplemented with 1% FBS and cultured at 37 °C in 5% CO_2.

Bacteria culture and infection

C. trachomatis serovar L2 434/Bu containing a GFP expression vector (Wang *et al*., 2011) was propagated in Vero cells. Elementary Bodies (EBs) were purified by sequential density gradients (Saka *et al*., 2011), and MOI was determined for purified EBs through infection of confluent Vero cells. For infections,

purified EBs were diluted in cell culture medium (DMEM + FBS), and then added to tissue culture dishes containing cells. Infection was facilitated by spinning for 30 min at 1560 ×g at 10 °C. Finally, infection was allowed to continue to the desired time point at 37 °C and 5% CO2. *S. typhimurium* WT strain 22023S (received from David Holden) was cultured in Luria-Bertani (LB) broth and grown overnight at 37 °C in a shaking incubator. To allow invasive properties of *S. typhimurium*, a further culture was produced in anaerobic conditions, gently shaking for 2 h before infection. An optical density reader was used to measure the absorbance at 600 nm wavelength (OD600). The multiplicity of infection (MOI) was calculated using 1.0164/OD600 = μl containing 1×10^6 bacteria and the bacteria were spun at 1000 rpm for 5 min to synchronously infect cells. At 1hpi, cells were washed with PBS and new DMEM medium was added containing 100 μg/ml gentamycin and 10 mM HEPES. At 2hpi cells were washed twice and 10 μg/ml gentamycin was added into new DMEM medium.

Parasite culture and infection

Toxoplasma expressing luciferase/eGFP or tdTomato (RH type I or Prugniaud type II) were maintained *in vitro* by serial passage on monolayers of HFF cells. For infection, *Toxoplasma* were syringe lysed from HFF cultures and added to cell cultures at an MOI 2 for type I parasites, and MOI 5 for type II parasites. The cultures were centrifuged at 1000 rpm for 5 min to synchronize infection, before being maintained at 37 °C in 5% CO_2. Where appropriate, parasites were irradiated in HFF cells with 15 000rads.

Antibodies

A unique sequence for hGBP1 was selected (QDLQTKMRRRKAC) and the peptide conjugated to keyhole limpet haemocynin was ordered from BioMatik Corporation, Canada. Rabbits were inoculated with these peptides, and final bleeds taken after 11 weeks (APS, Cambridge).

Fixed immunofluorescent microscopy

For *Toxoplasma* and *S. typhimurium* infections, A549 cells were plated on coverslips (Thermo Fisher) and cultured and infected as above. The cells were fixed in 3% paraformaldehyde (PFA) and permeabilized in Perm Quench (see Supporting Information) before incubating sequentially with primary and secondary antibodies diluted in PGAS (see Supporting Information) for 1 h at room temperature. Coverslips with cells were washed in PBS, with the final wash containing 1 μg/ml Hoechst. Coverslips were mounted on glass slides with Mowiol 4-88. Slides were viewed on a Zeiss Axioplan II Epifluorescence microscope or on an SP5-inverted Confocal microscope and analysed using LAS-AF software. Images were further formatted using ImageJ software. For Chlamydial microscopy, A549 and MEF cells ectopically overexpressing mCherry-hGBP1 were infected, as above. At 20hpi, cells were fixed with 4% PFA, before being stained with Hoechst in PBS + 300 mM Glycine. After thorough washing in PBS, slides were mounted with Mowiol containing 0.01% p-phenylenediamine (PPD). Slides were visualized using a Zeiss 510 Inverted Confocal Microscope, and analysed using Zen Blue software.

Pathogen viability assays

An IFU assay was used to assess *C. trachomatis* replication. WT and hGBP1-deficient A549 cells were plated to confluence in 12-well plates and stimulated for approximately 16 h with 200 U/ml human IFNγ. Cells were infected with *C. trachomatis*, as above, at an MOI of 1. At 40hpi, bacteria were harvested by lysing cells in water for 10 min at 37 °C with frequent mixing, and 5X sucrose-phosphate-glutamic acid buffer (SPG) was added to a final concentration of 1X. Bacteria-containing lysates were then added to cell culture medium (DMEM + 10% FBS), and 10-fold serial dilutions were performed. These dilutions were used to infect confluent Vero cells in black-walled 96-well plates, as above. At 24hpi, infected Vero cells were fixed and permeabilized for 5 min on ice with ice-cold Methanol, and blocked with 2% BSA in PBS. Wells were stained with a mouse monoclonal anti-*Chlamydia* LPS antibody, followed by goat anti-mouse IgG AlexaFluor 488 and Hoechst. For each well, 10 images were taken on a Zeiss Obzerver.Z1 scope using a 20X objective, and the number of *Chlamydia* inclusions/field was enumerated and averaged across the 10 images. The number of infectious units/well of A549 cells was calculated, taking into account the well area represented by each field and accounting for dilution. For each condition, infections were performed in triplicate (3 wells of A549 cells) for a single experiment. *S. typhimurium* CFU assays were performed by infecting cells for desired period of time before washing twice with ice-cold PBS and lysing with 0.5% Sodium deoxycholate (NaDOC) (Sigma-Aldrich) in PBS. Each sample was mechanically scraped and pipetted up and down. The lysates were diluted appropriately in LB and spread on a pre-warmed 10 cm^2 agar plate, incubated overnight at 37 °C followed by colony counting. Parasite viability was assessed by indirect plaque assay. A549 cells were infected with 300 type I parasites or 600 type II parasites in 24 well plates for the desired length of time before scraping and syringe lysing the cell layer to release the *Toxoplasma*. This suspension was then plated onto confluent HFF in a 24 well plate in serial dilutions of 1:2. This infection was allowed to persist at 37 °C at 5% CO_2 for 4 days, after which plaques were counted using a microscope.

Parasite invasion assay using Flow cytometry

A549 cells were infected with irradiated type II Pru parasites for 1 h before washing twice in PBS. Cells were lifted with 2X Trypsin, before quenching in DMEM. The cell pellet was washed twice in PBA (see Supporting Information) before fixing with 4% PFA for 20 min on ice. PBA was added to quench reaction before suspension was centrifuged at 1500 rpm for 3 min at 4 °C, after which cell pellet was resuspended in PBA and analysed on a BD LSR-II. Results were analysed using FlowJo software.

Overexpression of mCherry-hGBP1

The mCherry or mCherry-hGBP1 plasmids were transfected into A549 ΔhGBP1 or SV40 T-antigen immortalized Mouse Embryonic Fibroblasts using lipid transfection.

CRISPR/Cas9 mediated deletion of Gbp1

The guide RNA sequence hGBP1 guide1, 5′ cattacacagcctatggtgg 3′ to human *Gbp1* was selected using the optimized CRISPR design site crispr.mit.edu. Oligonucleotides were synthesized (Sigma-Aldrich) and annealed and cloned into the CRISPR vector p48139 containing a puromycin selection cassette (Ran et al. 2013. pSpCas9 (BB)-2A-Puro (pX459) was a gift from Feng Zhang. Addgene plasmid no 48139), using the BbsI restriction site, according to the Addgene CRISPR Genome Engineering Toolbox (Zhang Lab) www.addgene.org/crispr/zhang/. Transfection of A549 cells with hGBP1 guide RNA-containing p48139 plasmid was performed using FuGENE6 reagent (Promega) according to the manufacturer's instructions. Selection using 2 μg/ml puromycin was commenced 30–36 h post transfection and continued for 48 h. The puromycin was then removed and the cells allowed to recover before selecting individual clones. To confirm disruption of hGBP1, clones were cultured and either lysed with cell lysis buffer containing 1% Triton X100 (Sigma) for SDS-PAGE and immunoblotting with hGBP1-specific antibody or lysed with DNAreleasy (Anachem Ltd) for subsequent DNA sequence analysis.

Author Contributions

ACJ, AP, BC, JC and EMF designed experiments, ACJ, AP, BC and MS performed experiments, BC and SVW generated reagents, ACJ, AP, BC, JC and EMF wrote the manuscript, and JC and EMF supervised the study.

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Supporting information

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

Fig. S1. hGBP1 peptide antibody is specific for use in immunoblots and immunofluorescence.
Fig. S2. hGBP1 does not localise to the intracellular pathogen vacuole.
Fig. S3. hGBP1 specifically recognises hGBP1 targeted to type II *Toxoplasma* PVs in mouse embryonic fibroblasts.

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