**Modulation of Host NF-κB Pathway by the *Toxoplasma gondii* Secreted Factor, GRA15**

By

Diana Lu

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University of California-Berkeley, 2007

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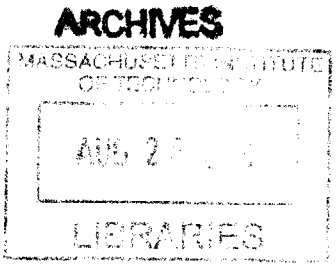




Signature of the Author _____

Department of Biology
September 2013

Certified by _____

Jeroen P. J. Saeij
Assistant Professor of Biology
Thesis advisor

Accepted by _____

Stephen Bell
Professor of Biology
Chair, Committee for Graduate Students

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## ABSTRACT

The apicomplexan protozoan *Toxoplasma gondii* is an obligate intracellular pathogen that infects all warm blooded animals, including nearly thirty percent of the human population worldwide. *Toxoplasma*'s success as a parasite owes largely to its ability to commandeer its host's immunologic and metabolic processes for its own benefit. During infection, *Toxoplasma* secretes a large number of proteins into the host cell. Many of these parasite factors modulate the signaling pathways of the host, including the pathway toward NF-κB activation. The NF-κB response to infection regulates the direction of host immunity, toward either the classical inflammatory or the alternative non-inflammatory pathway. Using forward genetic analysis, we have isolated the secreted protein GRA15 that is necessary and sufficient for host NF-κB activation. We find that GRA15 activates NF-κB nuclear translocation and transcriptional regulation in an IKK- and TRAF6-dependent manner. We additionally show that GRA15 may complex with TRAF3. Some *Toxoplasma* strains activate the host NF-κB pathway much more than others. A combination of factors, including differences in expression and sequence of GRA15, as well as other inhibitory parasite factors are responsible for conferring these strain differences.

Thesis supervisor: Jeroen P. J. Saeij
Title: Assistant Professor of Biology

## Dedication

For my grandfathers, Jun Yuan Zha and Yuan Kai Lu,
and for Wayne Hight, who was like a grandfather in their absence.
Rest in peace.

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Thank you to my thesis advisor, Dr. Jeroen P. J. Saeij for his brilliance, generosity, humor and patience both inside and outside the laboratory. You taught me how to perform quality research. You have shown me by example what it means to be a great scientist. You encouraged me to find and follow my dreams.

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Thank you to all my friends in Boston and beyond: We've known each other for so many years, and some of you know me better than I know myself. I don't say this often enough, but your loyalty, energy, humor and love have been my reward at the end of many, many long days. Thank you for listening. Thank you for nagging. Thank you for being there.

Thank you to my family. Virginia Sturdeyvant: you were a surrogate mother to me during my stay in California, and you continue to be a source of endless support and inspiration. My parents, Suping Lu and Yanyu Zha: I have always been humbled by your many sacrifices and proud of your hard-won accomplishments. You have shaped the person I am more than anyone else in the world. I would literally not be anywhere without you.

## Table of Contents

# Chapter I

# Introduction:

# *Toxoplasma gondii* and its interaction with the host NF-κB pathway

The interaction between autonomously living organisms and the infectious agents which parasitize them is one of the primary driving forces for the evolution and diversification of all life on earth. To better understand these host-pathogen relationships is to gain tremendous insight on selection, immunity, and the prevention and treatment of humanity's most notorious diseases. The intracellular protozoan *Toxoplasma gondii* is one of the most prevalent parasites of warm-blooded animals, and infects nearly one third of the human population worldwide. Here we introduce *Toxoplasma* and its modulation of the host NF-κB pathway, a master regulator of the inflammatory response to infection.

## I. Immunity

The immune system is a complex network of interconnected cellular processes that maintain the eukaryotic organism's capacity to function and proliferate in an unforgiving environment, recognizing foreign vs. self, aberrant vs. normal function and protecting the latter from the former. These cellular processes are regulated by networks of further interconnected signaling pathways, which often act in combination and in series to produce the specific, amplified barriers against organismal damage that we observe in nature.

The first line of defense against harmful stimuli is the innate immune system. Classical, or Type 1, activation of innate immunity occurs via the induction of inflammation and the complement cascade. In both cases, immunomodulatory cytokines are stimulated to be produced or activated, recruiting specialized white blood cells to the

site of infection. These cells remove infectious agents and cell debris, as well as activate the adaptive immune response, inducing T helper cell type 1 ($T_h1$) differentiation. An alternative, non-inflammatory innate immune response (Type 2) can also be activated. The Type II response induces T helper cell type 2 ($T_h2$) differentiation, leading to a humoral adaptive response (Murphy, 2011).

Nuclear Factor kappa B (NF-κB) is a family of primary transcription factors that controls the expression of an exceptionally large number of gene sets, including those encoding many anti-apoptotic, pro-inflammatory and immunoregulatory cytokines. Activation of the pathway leading to NF-κB nuclear translocation and transcriptional function is therefore one of the first and most decisive signals in the innate immune response to infection and injury (reviewed in (Beyaert, 2004; Gilmore, 2006)).

**The NF-κB pathway**

The NF-κB pathway is conserved in nearly all multicellular eukaryotic organisms (Figure 1). NF-κB is expressed in almost all cell types and participates in the stress response to a number of stimuli such as oxidative stress, irradiation, injury and antigen invasion (Brasier, 2006; Gilmore, 2006; Perkins, 2007; Tian and Brasier, 2003). Disruption of the normal function of this pathway has been implicated in a number of diseases, including atherosclerosis (Monaco et al, 2004), cancer (reviewed in (Escárcega et al, 2007; Karin, 2008)) and even neurodegeneration (Merlo et al, 2003; Boersma et al, 2011).

The NF-κB family is comprised of the RelA (p65), c-Rel, RelB, p105/p50 and p100/p52 subunits. Each subunit contains a highly conserved Rel homology (RH) domain that confers DNA-binding and dimerization activity. These subunits function as transcription factors when dimerized. Aside from RelB, which can only form heterodimers, all other NF-κB family members can dimerize with themselves and other members (Basak, et al, 2008). NF-κB subunits are continually produced by the cell and remain sequestered in the cytoplasm as homo- or heterodimers. The p65/p50 heterodimer is the primary NF-κB complex in most cell types (reviewed in (Li and Verma, 2003; Vallabhapurapu and Karin, 2009)).

Once activated, dimers enter the nucleus and bind to the promoter regions of target genes at distinct, but related, DNA motifs called κB binding sites, which are 9-10 bp in length. Each dimer has specific affinity to different κB sites, and many promoters contain multiple sites. On DNA, dimers also interact with other DNA-binding proteins. All of these factors contribute to the fine-tuned regulation of the multiple discrete but overlapping gene sets that are controlled by NF-κB (reviewed in (Hoffman et al, 2006)). NF-κB activation in immune cells leads to the expression of many pro-inflammatory cytokines, including interleukin-2 (IL-2), IL-12, and interferon-γ (IFN-γ). Activation in T cells is required for $T_h1$ differentiation (reviewed in (Beyaert, 2004; Gilmore, 2006; Perkins, 2007; Vallabhapurapu and Karin, 2009)).

Unstimulated, the nuclear localization sequence (NLS) created by dimerization of these transcription factors is masked by the ankyrin repeat domains of p100 and p105, or by inhibitors of κB (I-κBs). The I-κBs, I-κB-α, I-κB-β, I-κB-ε, and B cell lymphoma protein 3 (Bcl-3); compose a family of ankyrin repeat proteins that are also continually

expressed and are constitutively bound to NF-κB dimers (Jacobs and Harrison, 1998). Upstream stimulation induces phosphorylation of the ankyrin repeats by the I-κB kinase (IKK) complex. Phosphorylated I-κB is K48-ubiquitinated by the proteosomal targeting protein, beta transducin repeat containing protein (β-TrCP). This leads to the degradation of I-κB or the cleavage of the inhibitory domains of p100 and p105, the latter of which creates p52 and p50, respectively. The uncovered NLS is then recognized by the nuclear import machinery (reviewed in (Karin and Ben-Neriah, 2000; Gilmore, 2006)).

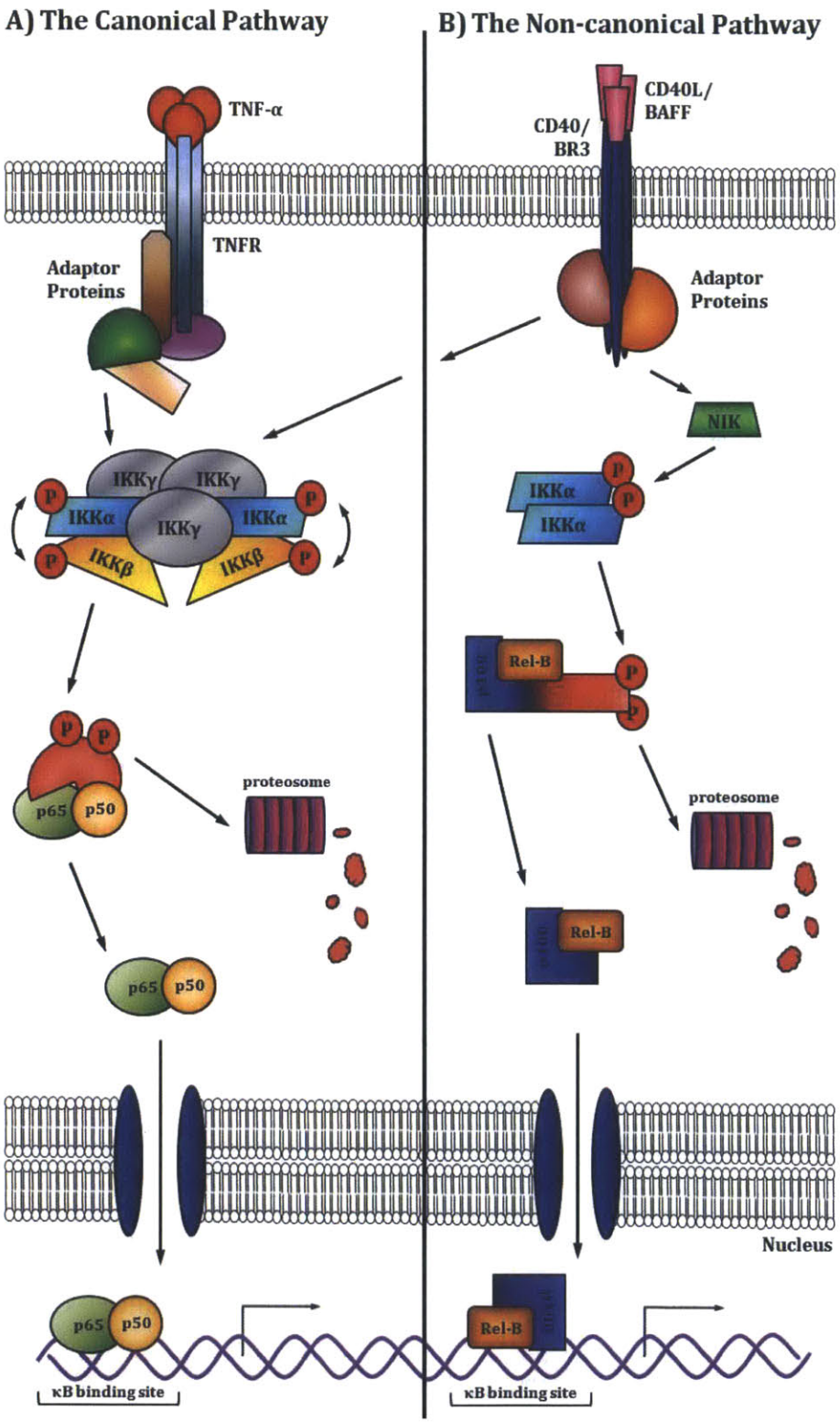


**Figure 1. The NF-κB Pathway**
A) The canonical pathway. Activation leads to the formation of the IKK-α, IKK-β and IKK-γ complex that induces the nuclear translocation of Rel subunits p65, p50 and c-Rel. B) The non-canonical pathway. Activation leads to the formation of the IKK-α dimer that induces the conversion of p100 to p52 and nuclear accumulation of the Rel-B and p100 dimer.

Many stimuli activate the pathway through IKK, a serine kinase complex (Figure 2). In the canonical pathway, IKK comprises the catalytically active subunits, IKK-α (Li et al, 1999a) and IKK-β (Li et al, 1999b); and a regulatory subunit, IKK-γ (Rothwarth et al, 1998). Commonly, extracellular stimuli, such as tumor necrosis factor alpha (TNF-α), bind to their partner receptors, which induces a conformational change in the receptor. Various adaptor proteins aggregate at the ligand-bound receptors, such as receptor interacting protein 1 (RIP1) (Ea et al, 2006). Adaptor complexes recruit IKK ubiquitinases and kinases. TNF-receptor (TNFR) associated factor 6 (TRAF6) and an E2 complex, which comprises ubiquitin conjugating enzyme 13 and ubiquitin conjugating enzyme variant 1a (Ubc13/Uev1a), have been observed to K63-polyubiquitinate IKK-γ (Deng et al, 2000) upon Toll-like receptor 4 (TLR4) activation. Likewise, the TNF-receptor activated kinase 1 (TAK1) (Wang et al, 2001) has been shown to phosphorylate IKK-α and IKK-β. There also exists a non-canonical pathway, in which NF-κB interacting kinase (NIK) (Regnier et al, 1997) specifically phosphorylates an IKK complex consisting of two IKK-α subunits. The homodimer phosphorylates p100/Rel B (Senftleben et al, 2001), which induces the proteosomal cleavage of the NLS-inhibitory domain of p100. This produces the transcriptionally active Rel B/p52 complex (Bonizzi et al, 2004).

Pathway control is more complex than the linear IKK to I-κB to NF-κB axis. NF-κB activity is also regulated by covalent modifications of the dimers after I-κB degradation, including ubiquitination, acetylation, methylation and prolyl isomerization. These can occur before translocation as well as within the nucleus (reviewed in (Gilmore, 2006; Karin 2008; Perkins, 2007)). Activation of NF-κB increases the expression of the

A20 deubiquitinase, also known as TNF-alpha induced protein 3 (TNFAIP3), and cylindromatosis protease (CYLD). These enzymes deubiquitinate activated IKK (reviewed in (Sun, 2008)). It is thought that I-κB-α, which has a strong nuclear export signal (NES), enters the nucleus, where it binds and exports the NF-κB dimer. I-κB-α expression is activated by NF-κB, creating a negative feedback loop. IKK-α has also been observed to enter the nucleus and bind to κB sites, where it promotes transcription by phosphorylating histone H3 (Anest et al, 2003; Yamamoto et al, 2003).

Many aspects of pathway regulation are still poorly understood. For example, the glutamate, leucine, lysine, serine-containing protein (ELKS) appears to be necessary for I-κB-α recruitment to the IKK complex in some cell types (Ducut Sigala et al, 2004). However, there has not yet been a thorough analysis of the identity and number of proteins comprising the IKK complex in all cell types.

It is also not fully understood how upstream factors activate IKK. In particular, it is unclear which kinases phosphorylate IKK in what cell types. NIK and TAK1 have been observed to phosphorylate IKK-α and IKK-β, respectively (Park et al, 2007; Wang et al, 2001). However, IKK-α and IKK-β can also self- and cross-phosphorylate (Häcker and Karin, 2006). IKK-upstream activation largely involves the aggregation of a number of receptor-interacting proteins and downstream adaptor proteins, such as myeloid differentiation primary response factor 88 (MyD88) and the IL-1 Receptor Associated Kinase 1 (IRAK1), respectively. The latter is capable of phosphorylating IKK, but the kinase function is not necessary for activation of NF-κB *in vivo*. Instead, mitogen activated protein/extracellular signal-related kinase (MAP/ERK) kinase 3 (MEKK3) has been implicated as the IKK kinase in IRAK1-mediated activation of NF-κB (Yao et al,

2007). The aggregation of the IKK-activating complex, which factors are necessary in which pathways and cell types, what factors interact directly with IKK and how each functions remain to be discovered (reviewed in (Ghosh and Karin 2002; Israël, 2010; Karin, 2008; Perkins, 2007)).

Finally, the effect that each different dimer complex contributes to a resulting expression profile is still not known for many physiological responses. The p65/p50 heterodimer is the primary complex activated by the canonical pathway. However, other dimers are found in various proportions in different cell types, and many genes have multiple different κB binding sites in their promoters. In addition, many genes also contain binding sites for other transcription factors in their promoters, and the interaction between NF-κB and other transcription factors, such as Bcl-3 (Bours et al, 1993), histone deacetylase 1 (HDAC1) and Creb-binding protein (CBP/p300) (Zhong et al, 2002), when bound to DNA has not been systematically characterized (reviewed in (Beyaert, 2004; Ghosh and Karin, 2002; Gilmore, 2006; Hoffman et al, 2006)).

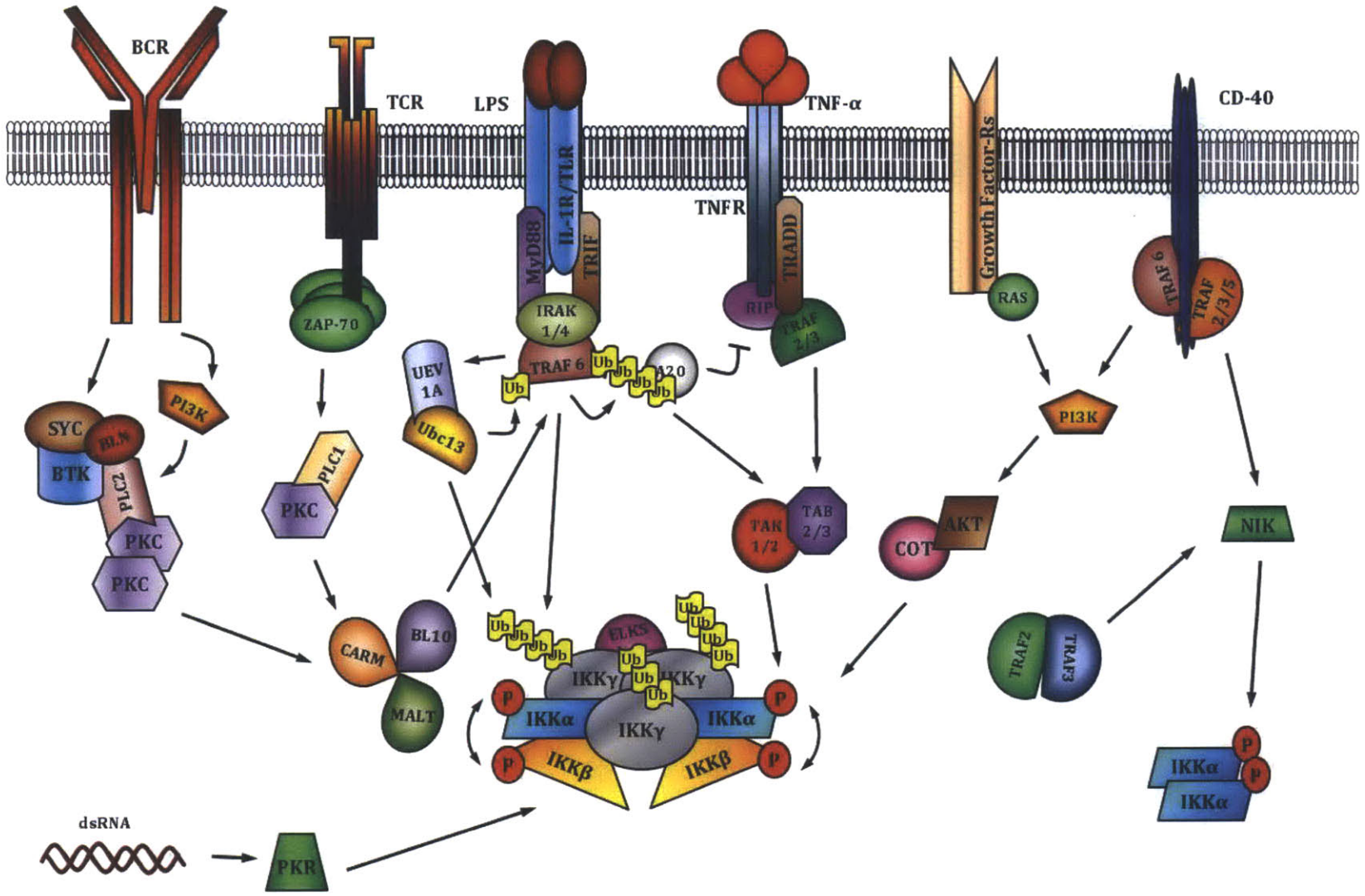


**Figure 2: Human proteins involved in NF-κB activation upstream of the IKK complex**
Perhaps not all, but many roads lead to IKK. Diagram of the multitude of signaling pathways which converge on the IKK complex.

**Pathogens and the host NF-κB pathway**

The study of the interaction between microbial pathogens and their hosts has enhanced our current knowledge of pathway components and their functions. Infection by foreign organisms stimulates NF-κB. The Pattern Recognition Receptors (PRRs) expressed by innate immune cells, which include the Toll-Like Receptors (TLRs) and the nucleotide-binding oligomerization domain (NOD)-Like Receptors (NLRs) recognize the protein or nucleotide products of microbial pathogens, such as bacterial lipopolysaccharides (LPS) and viral DNA or RNA. These are collectively called Pathogen Associated Molecular Patterns (PAMPs). Upon PAMP recognition, PRRs induce the activation of their associated signaling pathways, many of which converge on NF-κB to activate the inflammatory response (reviewed in (Fukata et al, 2009)).

It is therefore unsurprising that many pathogens—viral, bacterial and protozoan alike—have been reported to modulate the host NF-κB response to optimize for survival in the host ((reviewed in (Rahman and McFadden, 2011; Tato and Hunter, 2002)). Numerous viral, bacterial and protozoan parasites have evolved intricate mechanisms to inhibit or constitutively activate NF-κB function at various regulatory points along the pathway.

Expectedly, most infectious agents inhibit activation and thereby evade the inflammatory response and the aggregation and activation of immune cells. For example, the *Yersinia* outer protein J (YopJ) inhibits the activation of the IKK complex (Zhou et al, 2005; Mukherjee et al, 2006), while the infected cell protein 27 (ICP27) of Herpes

simplex virus (HSV) blocks the phosphorylation and ubiquitinylation I-κB-α (Kim, et al, 2008).

Alternatively, many infectious agents have been shown to activate NF-κB. The inflammatory response induces increased permeability of endothelial junctions, allowing the parasite to cross tissue barriers with greater ease. Inflammation also involves the recruitment of innate immune cells to the site of infection, which surrounds the parasite with more cell targets to infect. NF-κB target genes also include those encoding for many anti-apoptotic factors. Activation of NF-κB thus promotes survival of the infected cell, allowing the parasite to proliferate more and increases cell migration, resulting in further dissemination of the pathogen in the host. As a result of constitutive NF-κB activation, the host organism is often burdened by a constant pro-inflammatory immune response by parasites. This can lead to severe inflammatory disease outcomes, such as encephalitis and cancer.

Famously, the Tax1 protein of Human T-lymphotrophic virus 1 (HTLV1) induces the aggregation of IKK-γ, which leads to the constitutive activation of the IKK complex (Huang et al, 2009; Sun and Yamaoka, 2005; Wu and Sun 2007). Epstein-Barr virus (EBV), the causative agent of mononucleosis, also activates the phosphorylation of the kinase-active IKKs by inducing the aggregation of upstream receptor-associated adaptor proteins, such as the TRAFs and RIP1. The aggregation of these adaptor proteins recruits and activates kinases that have been observed to phosphorylate the IKK complex (de Oliveira, 2010).

Less is understood about the interaction between protozoan parasites and NF-κB, but precedents include *Theileria annulata*, which constitutively activates the IKK

complex (Heussler et al, 2002), and *Leishmania amazonensis*, which has been shown to activate then inhibit NF-κB signaling through an unknown mechanism in dendritic cells and macrophages (Ben-Othmann et al, 2008; Jayakumar et al, 2008).

*Toxoplasma gondii* is another interesting protozoan parasite with host NF-κB-modulatory capabilities. At the time that this thesis project began, it was yet unclear whether *Toxoplasma* activated or inhibited host NF-κB, as evidence existed to support both claims. The work presented in the subsequent chapters of this thesis definitively characterizes the manner of NF-κB modification and delineates the mechanism by which that process occurs.

## II. *Toxoplasma gondii*

*Toxoplasma gondii* is an obligate intracellular protozoan in the phylum Apicomplexa. This large and diverse protist group comprises many genera of intracellular parasites such as coccidia and plasmodia. *Toxoplasma* is a very genetically tractable representative of its phylum, which makes it an ideal model organism for the study of other apicomplexans such as *Plasmodium falciparum*, the causative agent of malaria (Kim and Weiss, 2004).

*Toxoplasma* is globally distributed, and can invade any warm blooded organism, including humans. The parasite has established a chronic infection in more than 25% of the human population worldwide (Joynson, 2005). Infection of immunocompetent

humans is usually asymptomatic, but infection by *Toxoplasma* strains endemic to South America often causes severe inflammatory symptoms in the brain and eyes (Gilbert et al, 2008).

In Europe and North America, infection can still cause disease in immuno-compromised and immuno-naïve individuals, Acquired immunodeficiency syndrome (AIDS) patients, transplant recipients who require immunosuppressive drugs and children who were infected in-utero are especially vulnerable (Mitchell et al, 1990). The disparity in infection pathology between South America and Europe and North America appears to be largely related to genetic differences of the parasite (Grigg et al, 2001), which will be discussed in greater detail below.

In addition, premature abortion due to complications resulting from *Toxoplasma* infection in pregnant livestock incurs major economic loss in the agricultural sector (Dubey and Jones, 2008). Insight on the nature of this parasite's proliferative success would not only be a worthy scientific achievement, but would additionally have useful economic and medical implications throughout the world.

## Life Cycle

*Toxoplasma gondii* can only reproduce sexually in felines, the definitive host. However, it can parasitize any warm-blooded animal and reproduce asexually in the intermediate host indefinitely. Infection occurs via ingestion of tissue cysts or sporocysts. In the feline intestinal lumen, the parasite differentiates into micro- and macro-gametes,

and sexual recombination between different strains is possible. More often than not, however, reproduction occurs asexually in a wide range of intermediate hosts (Figure 3).

Infection often begins with the entrance of free parasite into the innate immune cells of the host, such as dendritic cells or macrophages. *Toxoplasma* uses these cells to travel to other parts of the host's body, where it establishes chronic infection in muscle or nerve tissue, cell types with slower turnover rates than those of endothelial and hematopoietic cells (reviewed in (Innes, 2010)). Here, the free, fast-growing life cycle stage, the tachyzoite, converts to its slow-growing, encysted stage, the bradyzoite. The latter reside in parasitically dormant, immunologically isolated cysts containing thousands of parasites. *Toxoplasma* can persist in this encysted stage for the life of the host (reviewed in (Sibley, 2003)). However, encystation is a reversible process that is both genetically programmed and the result of immune pressure on the parasite (Laliberte and Carruthers, 2008). Only cysts, and not the rapidly dividing tachyzoites, are orally infections. Therefore, *Toxoplasma* must keep its host alive until cysts have been formed for asexual transmission between intermediate hosts. For this reason, activation of the NF-κB signaling is a good strategy for *Toxoplasma* to employ. Balance of immune regulation during infection is extremely important to the survival of both host and parasite. Too much inflammatory activation and the parasite will be eliminated entirely, while too little will kill the parasite before cysts can form.

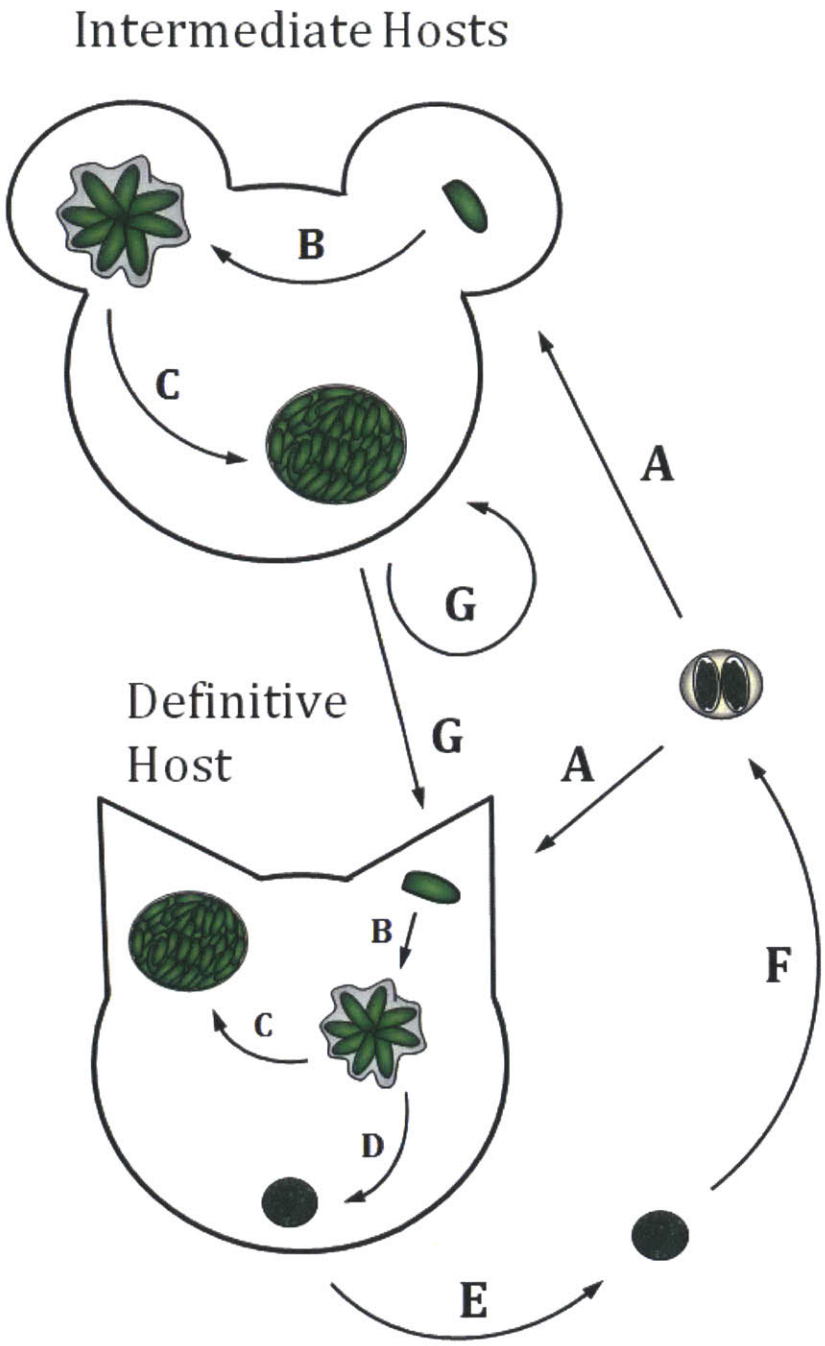


**Figure 3. The *Toxoplasma gondii* life cycle.**
Infection via ingestion of oocysts (A) or carnivorism of tissue cysts (G) leads to mitotic proliferation of free parasite in the host (B). Immune pressure induces a life stage switch, from fast-growing free parasite to slow-growing cyst (C). (D) The sexual life cycle of the parasite occurs in the definitive host, producing immature oocysts. These are excreted in the feces (E) and undergo sporogony outside the host to become mature oocysts (F).

## Invasion and infection

Invasion of the host cell occurs in three stages: 1) attachment of the parasite to the surface of the host cell, 2) export of parasite factors into the host cytoplasm and membrane, and 3) entrance of the parasite into the cell through a controlled process of membrane invagination and engulfment.

Successful invasion and establishment of infection is dependent upon the function of a large number of secreted proteins. These proteins are derived from *Toxoplasma*'s three secretory organelles, the micronemes, rhoptries and dense granules (Figure 5). Each organelle expels its contents at precise intervals for its corresponding stage(s) of infection.

First, microneme proteins (MICs) mediate attachment to and penetration of the host membrane. Second, a large number of proteins from the rhoptries (ROPs), as well as some proteins from the dense granules (GRAs), are injected into the host cell. These localize to host membrane, cytoplasm, or organelles and perform a number of functions, including host cell modulation. In stage three, parasite factors in the host membrane at the site of invasion form a growing barrier that excludes host proteins. The host factors are subsequently replaced by *Toxoplasma*-derived proteins (reviewed in (Carruthers and Sibley, 1997; Carruthers 1999; Opitz and Soldati, 2003)). Once the parasite has been fully internalized, the membrane pinches off to create an environmentally isolated parasitophorous vacuole (PV), in which the tachyzoite proliferates (Figure 4).

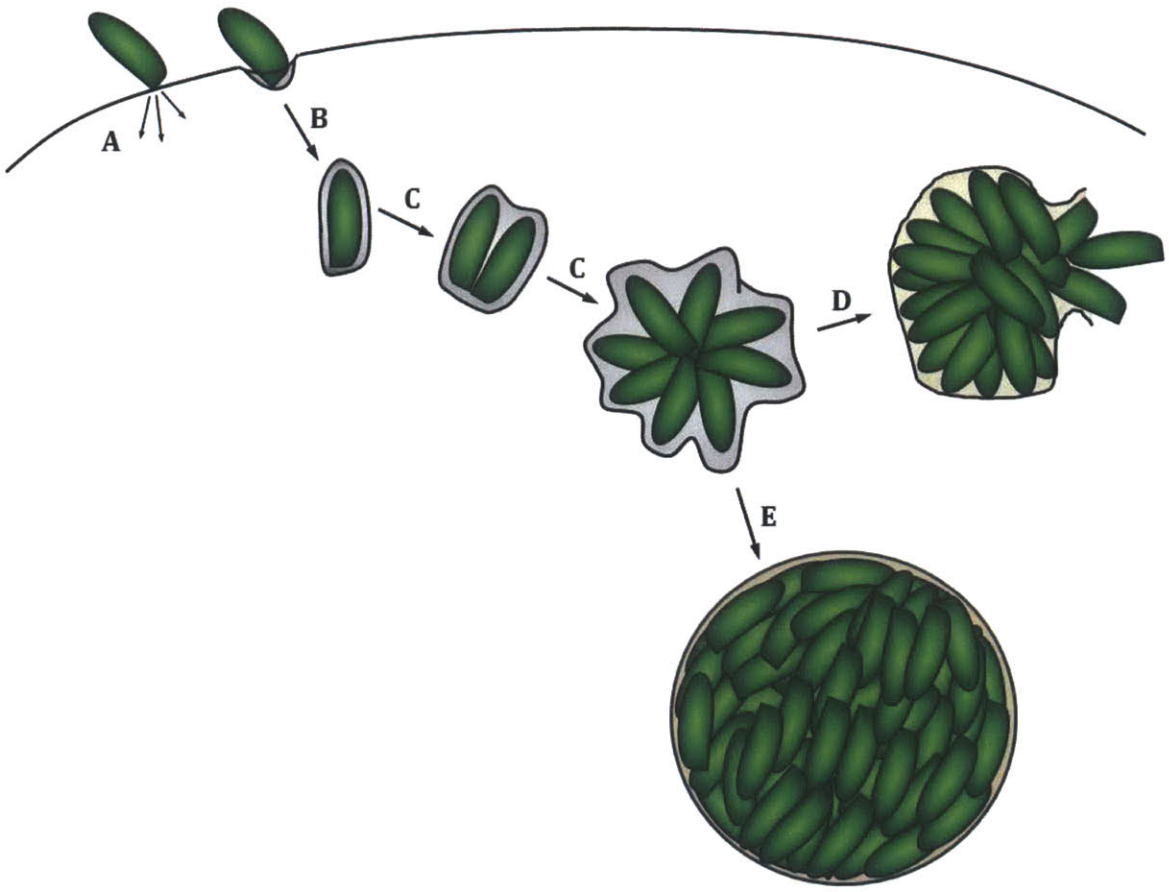


**Figure 4. *Toxoplasma gondii* infection**
A) Infection begins with attachment of the parasite to the host cell membrane. B) The host membrane invaginates, forming a parasitophorous vacuolar membrane around the parasite as it enters the cell. In the vacuole, the parasite proliferates (C) until the vacuole and host cell burst and the parasite can egress from the cell (D). Alternatively, immune pressure can induce a life stage switch, forming a dormant tissue cyst (E).

Many secreted ROPs contain a kinase domain. These proteins have been observed to directly manipulate host signaling factors to optimize cellular functions for parasite survival. For example, the virulence factors ROP5 and ROP18, which are injected into the host upon invasion, act in concert to inhibit IFN-γ mediated parasite clearance in murine macrophages (Behnke et al, 2012; Niedelman et al, 2012). ROP16, the parasite factor responsible for activating STAT3/6 signaling, directly phosphorylates STAT6 (Saeij et al, 2007; Ong et al, 2010).

GRAs are continually secreted by the parasite. Many GRAs are observed to localize to the PV membrane (PVM) or the intravacuolar space (IVS) (Hsiao et al, 2013). Most of the characterized GRAs function in maintaining the structural integrity of the IVS, or in regulating the exchange of nutrients and other cellular material between the PV and the extra-vacuolar environment (Leriche and Dubrumetz, 1990; Nam, 2009). However, recent work has identified GRA proteins that are secreted into the host cell and function to modify host signaling pathways (Bougdour et al, 2013). *Plasmodium* Export Elements (PEXEL), an protein sequence motif found in the N-terminal domain (NTD) of many GRAs, appear to be responsible for their PVM or extravacuolar space (EVS) localization (Hsiao et al, 2013).

At the time our studies began, our working hypothesis was that NF-κB modulation of *Toxoplasma gondii* was mediated by one or more of these secreted proteins. We therefore sought to identify the responsible factor(s) and to characterize its mechanism of action.

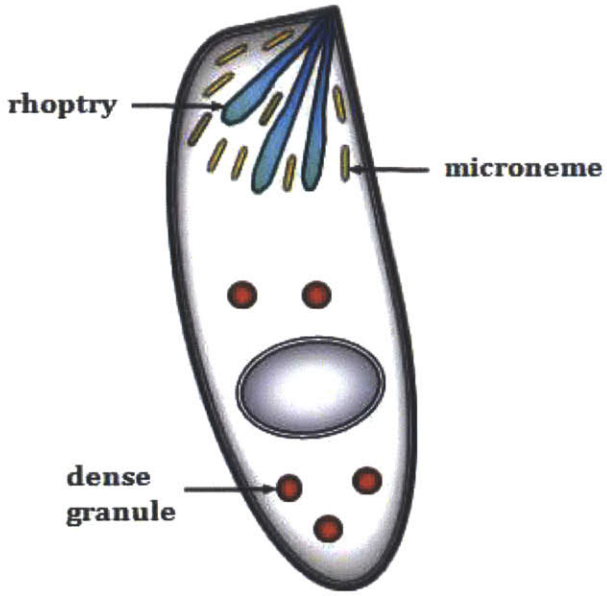


**Figure 5. *Toxoplasma gondii* secretory organelles**
A) The secretory organelles of *Toxoplasma gondii*, the micronemes, rhoptries, and dense granules. These organelles and their respective protein components will be discussed further in the text.

### *Toxoplasma* and the host NF-κB pathway

NF-κB signaling is extremely important to the host immune response against *Toxoplasma* infection. Live infection or injection with dead parasite activates the host immune system through recognition of *Toxoplasma* PAMPs. This results in NF-κB activation and the inflammatory response. Indeed, deletion of most NF-κB subunits in mice has a striking negative impact on host survival after *Toxoplasma* infection (Mason, 2004).

For years, the mechanism of NF-κB modulation by *Toxoplasma gondii* has been in dispute. Active infection has long been known to modulate NF-κB-induced immune signaling, though different studies have reported disparate observations. Several groups have demonstrated that infection inhibits the pathway, while others observe activation. Neither side of the debate could provide more compelling evidence than the other.

Infection of mouse fibroblasts in culture leads to constitutive p65 accumulation in the host nucleus. Interestingly, phosphorylated I-κB-α was also shown to accumulate on the PVM, even during infection of IKK-$\alpha^{-/-}$/IKK-$\beta^{-/-}$ cells. Molestina et al reported accumulation of phospho-I-κB-α at the extravacuolar surface of the PVM. This accumulation was observed even in IKK-$\alpha^{-/-}$/IKK-$\beta^{-/-}$ mouse embryonic fibroblasts (MEFs), implicating the existence of a *Toxoplasma*-derived IKK (Tg-IKK) (Molestina et al, 2003; Molestina and Sinai, 2005a).

However, the gene encoding this putative Tg-IKK has never been identified. The amount of phospho-I-κB-α accumulation on the PVM also does not appear to be correlated with the amounts of total cellular phospho-I-κB-α, nuclear translocation of NF-κB, or NF-κB transcriptional activity in the infected cell. Nevertheless, Molestina et al show that the Tg-IKK may be responsible for the continuation of signaling up to 24 hours post infection. At the later times points, endogenous NF-κB activation by host IKK is expected to be abrogated by inhibitory factors (Molestina and Sinai, 2005b).

Others claim that *Toxoplasma* infection inhibits NF-κB activation. These groups report that the NF-κB pathway of infected mouse macrophages and human fibroblasts is no longer responsive to PAMPs, reactive oxygen species (ROS) or chemical stressors. Acute infection *ex vivo* was observed to decrease p65 nuclear accumulation and *in vitro*

DNA binding, as well as downstream IL-12 secretion (Butcher et al, 2001; Shapira et al, 2002; Kim et al, 2004). From these data, it would appear that there exists parasite-derived factor(s) which modulate the interaction between p65—and possibly other NF-κB subunits—and their inhibitory elements, the nuclear import machinery, and/or DNA. Again, no gene products mediating these effects have ever been identified.

NF-κB is a highly sensitive pathway, and many specific characteristics of activation remain unclear, as described above. Discrepancies in the observations of infection-stimulated activation could therefore be attributed to a number of factors. These include differences in host cell type and species, multiplicities of infection (MOI), time points of observation, and reagents used in the respective assays. For example, inhibition of PAMP-stimulated activation was observed during early infection (< 6 hr post infection), while the phosphorylated (phospho-) I-κB assays were conducted on cells that had been infected for 16-24 hr.

## III. Strain differences in NF-κB modification by *Toxoplasma*

There is evidence of disparities in NF-κB modulation between different strains of *Toxoplasma* (Dobbin et al, 2002; Robben et al, 2004). Though most research, including the studies summarized in the previous section, has been conducted using only one standard laboratory strain of *Toxoplasma*, recent work in the field has focused on the observation of infection phenotypes of other strains, including many genotypically and geographically novel isolates (Khan et al, 2011; Khan et al, 2012; Minot et al, 2012). Strain-dependent variation in many clinically relevant phenotypes can thus be

characterized. Further, genetic comparison of strains which differ in these phenotypes can quickly identify the parasite factors responsible (Saeij et al, 2007; Khan et al, 2012), many of which are thought to be host-modifying secreted factors.

**Canonical strains**

Historically, research in the field has been conducted using parasite strains isolated from infections in Europe and North America. Genotyping of these classical laboratory strains have shown that the vast majority of these strains fall into three distinct, clonal haplotypes, so-called types I, II and III. These are collectively referred to as the canonical lineages. This clonal population structure suggests that these haplotype strains were created recently (<10,000 years ago). It is hypothesized that they carry some particularly favorable combination of alleles, which confers a strong selective advantage over all other genotypes that have since arisen on these continents (Grigg et al, 2001; Sibley and Boothroyd, 1992).

Genetic variation among the canonical lineages is very small. Strains within the same lineage are nearly identical, with less than 1% variation across the entire genome, and variation between different lineages is still only 1-3% (Minot et al, 2012). Despite this, numerous strain-dependent differences in the progression and disease outcome of *Toxoplasma* infection have been reported. For example, type I strains are highly lethal in laboratory mouse strains: infection by even one parasite is invariably lethal (Khan et al, 2009). Type II strains are less virulent, with a 50% lethal dose ($LD_{50}$) > $10^2$ parasites, and type III strains are almost benign, with an $LD_{50}$ > $10^5$ parasites (Saeij et al, 2006).

Variation between strains also exists for other clinically relevant phenotypes, particularly the inflammatory and innate immune responses to infection. Type I and type III lineages constitutively activate the host factors Signal Transducer and Activation of Transcription (STAT) 3 and 6 (Saeij et al, 2005; Saeij et al, 2006). These transcription factors function to downregulate the inflammatory response to infection and drive T cell differentiation to the $T_h2$ fate (Figure 6). With respect to NF-κB signaling, we and others have observed that the type II lineage induces more nuclear translocation of p65 than types I and III (Dobbin et al, 2002; Robben et al, 2004). Additionally, phospho-I-κB-α accumulates on the membrane of type I PVs, but not on those of type II (Molestina and Sinai, 2005a). Compared with type I, type II infection also results in higher IFN-γ production, IL-12 secretion, and inducible Nitric Oxide Synthase (iNOS) activity, all of which are contributors to inflammatory signaling (Dobbin et al., 2002; Robben et al., 2004; Kim et al, 2006).

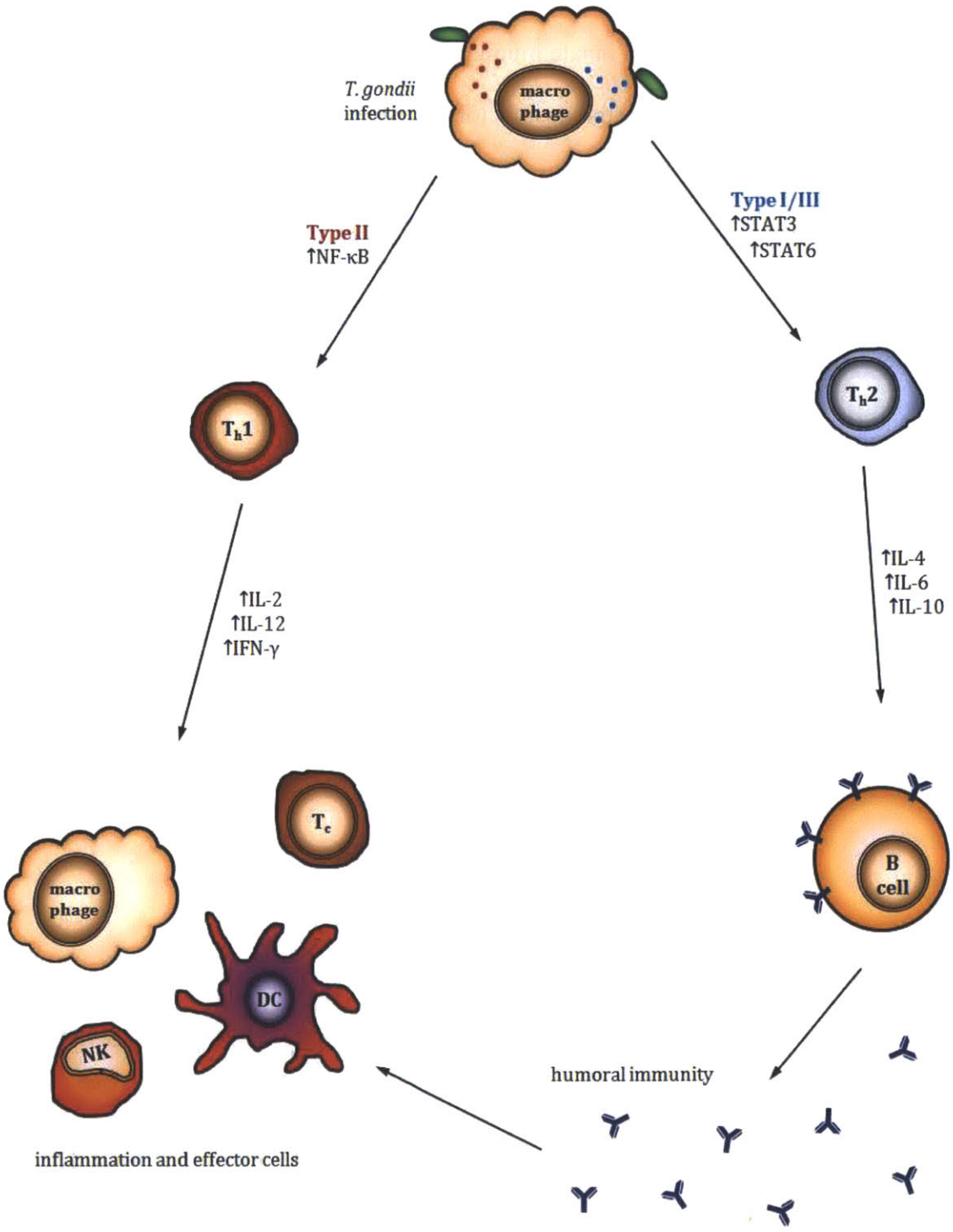


**Figure 6. Strain-dependent modulation of the host immune pathways by *Toxoplasma gondii*.**
Strain-specific secreted factors are responsible for differential modulation of the immune pathways of the host. Infection by type II strains induces NF-κB activation, leading to a type 1, inflammatory response while infection by type I and type III strains induces STAT3/6 activation, leading to the type 2 immune response.

The bimodal nature of some strain-specific disparities, as well as the lack of genetic variation between lineages, suggests that the differences in infection outcome and host-modulatory activity are the result of the combinatorial expression of only a handful of genetically encoded factors. If such is the case, it would be possible to tease out the parasite components responsible for the disease outcome of each strain by identifying the genes that encode them. Indeed, our group has previously applied this method successfully.

We infected samples of human foreskin fibroblasts (HFFs) with a *Toxoplasma* strain representing each of the canonical lineages. With microarray analysis, we generated a gene expression dataset for each sample of infected HFFs and identified host gene sets which were differentially modulated by infection with different strains. The majority of the strain disparities in host expression regulation seemed to be between the type II lineage and types I and III.

To identify parasite genetic factors involved, we also created expression profiles of HFFs infected with each of 19 filial 1 (F1) progeny from a cross between type II and type III lineage strains (Pfeffercorn and Kasper, 1974). These progeny contain random combinations of the type II and type III alleles and were used to correlate *Toxoplasma* genetic markers to strain-dependent host phenotypes. We used quantitative trait locus (QTL) analysis to identify genome regions with which each host expression phenotype associated. Next, transgenic analysis of polymorphic genes in these genome regions identified the specific gene products in *Toxoplasma* (Subramanian et al, 2005; Saeij et al, 2007).

The gene conferring constitutive STAT3 and STAT6 activation in types I and III was isolated in this manner. The gene product was identified as ROP16, a previously uncharacterized tyrosine kinase injected into the host cell from the rhoptries upon invasion (Saeij et al, 2007). Further biochemical characterization by other groups revealed that ROP16 directly phosphorylates STAT3 and STAT6 (Ong et al, 2010). The strain variance in ROP16 function was mediated by a single amino acid polymorphism that reduces its kinase activity in type II strains (Yamamoto et al, 2009).

We analyzed the expression patterns of HFF genes containing κB binding sites in their promoters in cell samples each infected with a strain representing the type I, II, or III lineages. Indeed, we identified the presence of a locus on chromosome ten that is polymorphic between type II and types I and III, which appears to modulate NF-κB transcriptional regulatory activity. In Chapter II, we discuss the results of our genetic analysis and determine the responsible gene product in this locus. In Chapter III, we present the biochemical assay performed to characterize the mechanism of its host-modulating activity. In Chapter IV, we analyze the strain differences in NF-κB activation among 29 strains of *Toxoplasma* representing the canonical lineages as well as atypical genotypes.

**Non-canonical strains**

As the *Toxoplasma gondii* field has grown, so has its geographical scope of study. In the last decade, more attention has been focused on genotyping and characterizing strains isolated from other areas of the world, especially South America. The rates of

*Toxoplasma* infection in Asia and Africa are similar to those found in Europe and North America (Minot et al, 2012). In contrast, many of the strains isolated from South American infection cases have atypical or unique allele combinations (Dardé, 2004; Khan et al., 2006; Pena et al., 2008). The population structure of *Toxoplasma* in this region is non-clonal, infection rates are higher and more cases of infection with multiple strains of different lineages have been diagnosed. Phylogenetic analysis of nearly 1000 strains identified 12 novel genetic lineages, so-called types IV-XIV (Khan et al., 2007; Khan et al., 2011; Su et al., 2012). However, this analysis only observed single nucleotide polymorphisms (SNPs) at 5 loci. SNP mapping of the entire genome showed that, for these non-clonal, non-canonical lineages, there is often sufficient divergence between strains that some cannot be easily categorized into any haplogroup. One possibility is that these unique strains are the progeny of very recent (<10,000 years ago) recombination events (Minot et al., 2012).

Additionally, infection cases in South America often present with drastically different and more malicious disease outcomes, even in immunocompetent patients, including encephalitis and death. The etiology of many of these pathologies appears to be overactivation of the inflammatory response to infection (Dardé et al., 1998; Grigg et al., 2001; Holland, 1999; Khan et al., 2006; McLeod et al., 2012a; Roberts and McLeod, 1999)). Even in Europe and North America, such unusual genotypes are often associated with patients with unusual clinical manifestations (Azjenberg, 2012). Therefore, these factors are likely to be responsible for the differences in NF-κB pathway activation, infection rates and disease outcomes of the South American strains.

The work discussed in later chapters seeks to determine whether this is due to the same gene products as those modulating infection with the canonical strains. If so, there may exist sequence polymorphisms that alter their function relative to the canonical gene products. It may also be that there are unidentified gene products active only in certain non-canonical genetic backgrounds. Possibly, there could be a combination of several factors.

Regardless, novel polymorphisms and allele combinations are useful in determining the mechanism of action of gene products involved in host modification, as well as in identifying new factors. Identification of the factors affecting the movement and proliferation of different strains of *Toxoplasma gondii* is crucial to disease management and spread of particularly virulent strains.

## IV. Summary

Here, we introduced the intracellular protozoan parasite, *Toxoplasma gondii*. We presented its process of invasion and infection, the resulting inflammatory and innate immune response of the host, and means by which the parasite evades that response to benefit its own survival. Particularly, we detailed the prior knowledge in the field regarding the interaction of *Toxoplasma* with the host NF-κB pathway. Finally, we discussed its global population structure and genotypic diversity. Chapter II will discuss the work performed to identify the *Toxoplasma* factor responsible for modulating the NF-κB activity of the host cell. In Chapter III, we characterize the mechanism of that

function. In Chapter IV, we observe the strain differences in function of this protein in canonical and non-canonical lineages. Infectious diseases are prevalent throughout world. Understanding the dynamics of host-pathogen interactions is important for our knowledge of molecular biology, evolution, and the immune system.

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# Chapter II

# Strain-Specific Activation of the NF-κB Pathway by GRA15, A Novel Dense Granule Protein

Author contributions: Identification of GRA15 genetic locus was performed by Jeroen Saeij. Co-infection assays were performed by Lindsay Julien, Diana Lu, and Lauren Rodda. Host expression profile analysis of cells infected by transgenic strains was performed by Lauren Rodda, Emily Rosowski, and Jeroen Saeij. Construction of overexpression strains was performed by Lauren Rodda, Emily Rosowski and Jeroen Saeij. Construction of knock-out strains was performed by Emily Rosowski. Construction of human overexpression cells was performed by Diana Lu. GRA15 localization assay was performed by Lindsay Julien, Diana Lu, and Lauren Rodda. Phospho-I-κB-α, type I inhibition, in vitro growth and in vitro IL-12 production assays were performed by Emily Rosowski. In vivo cytokine production and ROP16 assays were performed by Emily Rosowski and Kirk Jensen. GRA15 time course and NF-κB pathway activation assays were performed by Diana Lu. NF-κB subunit activation assay was performed by Rogier Gaiser and Diana Lu. GRA15 sequence analysis was performed by Diana Lu and Lauren Rodda.

# Strain-specific activation of the NF-κB pathway by GRA15, a novel *Toxoplasma gondii* dense granule protein

Emily E. Rosowski, Diana Lu, Lindsay Julien, Lauren Rodda, Rogier A. Gaiser, Kirk D.C. Jensen, Jeroen P.J. Saeij

Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139

**NF-κB is an integral component of the immune response to *Toxoplasma gondii*. Although evidence exists that *T. gondii* can directly modulate the NF-κB pathway, the parasite-derived effectors involved are unknown. We determined that type II strains of *T. gondii* activate more NF-κB than type I or type III strains, and using forward genetics we found that this difference is a result of the polymorphic protein GRA15, a novel dense granule protein which *T. gondii* secretes into the host cell upon invasion. A GRA15-deficient type II strain has a severe defect in both NF-κB nuclear translocation and NF-κB–mediated transcription. Furthermore, human cells expressing type II GRA15 also activate NF-κB, demonstrating that GRA15 alone is sufficient for NF-κB activation. Along with the rhoptry protein ROP16, GRA15 is responsible for a large part of the strain differences in the induction of IL-12 secretion by infected mouse macrophages. In vivo bioluminescent imaging showed that a GRA15-deficient type II strain grows faster compared with wild-type, most likely through its reduced induction of IFN-γ. These results show for the first time that a dense granule protein can modulate host signaling pathways, and dense granule proteins can therefore join rhoptry proteins in *T. gondii*'s host cell–modifying arsenal.**

CORRESPONDENCE
Jeroen Saeij:
jsaeij@mit.edu

Abbreviations used: BMM, BM-derived macrophage; DiRE, distant regulatory elements of coregulated genes; GSEA, gene set enrichment analysis; HFF, human foreskin fibroblast; IF, immunofluorescence; IKK, IκB kinase; MEF, mouse embryonic fibroblast; PV, parasitophorous vacuole; PVM, PV membrane; RACE, rapid amplification of cDNA ends; TFBS, transcription factor binding site.

*Toxoplasma gondii* is an obligate intracellular parasite capable of infecting a wide range of warm-blooded hosts, including humans. *T. gondii* establishes a lifelong chronic infection in the host by evading and subverting the immune system. *T. gondii* infection is usually asymptomatic in healthy humans but can lead to flu-like and neurological symptoms in immunosuppressed patients and the fetuses of pregnant women infected for the first time. The vast majority of *T. gondii* strains isolated from Europe and North America belong to three clonal lineages, types I, II, and III, which differ in many phenotypes, including virulence (Saeij et al., 2005). In mice, type I strains are categorically lethal, with an $LD_{100} = 1$, whereas type II or type III infections are not ($LD_{50} \approx 10^2$ and $\approx 10^5$, respectively; Sibley and Boothroyd, 1992; Saeij et al., 2006). Strain differences in the modulation of host immune signaling pathways are one way by which this diversity arises. For example, the strain-specific modulation of the STAT3/6 signaling pathway by the secreted kinase ROP16 accounts for some of the strain differences in virulence (Saeij et al., 2006). Evidence also exists for the strain-specific modulation of NF-κB (Robben et al., 2004), an important host signaling pathway in the regulation of inflammatory, immune, and antiapoptotic responses.

The NF-κB family of transcription factors is composed of five members: p50 (NF-κB1), p52 (NF-κB2), p65 (RelA), RelB, and c-Rel (Vallabhapurapu and Karin, 2009). In unstimulated cells, homo- or heterodimers of NF-κB are sequestered in the cytoplasm by members of the IκB (inhibitor of κB) family. Activation of NF-κB is initiated by the degradation of IκB proteins. This occurs via the activation of kinases called IκB kinases (IKKs), which phosphorylate two serine residues located in IκB regulatory domains, leading to their ubiqui-

E.E. Rosowski and D. Lu contributed equally to this paper.



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tination and subsequent proteasomal degradation. The NF-κB complex is then free to enter the nucleus where it can induce expression of specific genes that have NF-κB-binding sites in their promoters.

Many pathogens have developed strategies to modulate the host NF-κB pathway (Tato and Hunter, 2002). Several bacteria and viruses inhibit NF-κB activation and its resultant recruitment and activation of immune cells, resulting in enhanced survival of the pathogen. Other pathogens induce NF-κB activation, which inhibits apoptosis, an important defense against intracellular pathogens, and increases cell migration, thereby recruiting new cells to infect. Furthermore, NF-κB–mediated inflammation leads to tissue damage, allowing pathogens to cross tissue barriers. Thus, depending on the host, pathogen, and site of infection, an active NF-κB pathway can benefit either the host or the pathogen.

Mice deficient in some NF-κB family members have increased susceptibility to *T. gondii*, indicating the importance of this pathway in pathogen resistance (Mason et al., 2004). *C-Rel*$^{-/-}$ mice are highly susceptible to the acute stage of *T. gondii* i.p. infection, which can be rescued solely by treatment with IL-12, indicating that a major role of NF-κB in resistance to *T. gondii* is the induction of IL-12 secretion (Mason et al., 2004). IL-12 is a major mediator of the proinflammatory Th1 response development, and the major cause of chronic phase death in mice lacking RelB, p52, or the IκB protein Bcl-3 is also a deficient T cell response (Mason et al., 2004). Although it is clear that the NF-κB pathway is important for an adequate response to *T. gondii* infection, the mice used in these studies all lack a particular NF-κB subunit in every cell of their bodies, and it is currently unknown what the role of NF-κB is in specific cell types, such as those directly infected with *T. gondii*.

Evidence currently exists for both inhibition and activation of the NF-κB pathway in host cells by *T. gondii*. Less than 6 h after infection, a type I strain was shown to block the nuclear translocation of p65 and the in vitro binding of NF-κB subunits to DNA (Butcher et al., 2001; Shapira et al., 2002; Kim et al., 2004). Induction of IL-12 in response to TNF or LPS stimulation was also reduced (Butcher et al., 2001; Kim et al., 2004). This inhibition of the NF-κB pathway was dependent on active invasion by live parasites (Butcher and Denkers, 2002). After >6 h of infection, inhibition of p65 nuclear translocation and in vitro DNA binding was no longer observed (Kim et al., 2004; Leng et al., 2009). However, chromatin immunoprecipitation experiments showed that the in vivo binding of p65 to the TNF promoter was blocked even at late time points (Leng et al., 2009). Other groups, however, have shown that NF-κB is activated by a type I strain of *T. gondii*, and that this activation is necessary for the inhibition of apoptosis (Molestina et al., 2003; Payne et al., 2003; Molestina and Sinai, 2005b). A consistent observation has been the phosphorylation and ubiquitination of IκBα upon *T. gondii* infection, although it is unclear what effect this has on the nuclear translocation of NF-κB and transcription of downstream genes (Butcher et al., 2001; Molestina et al., 2003; Shapira et al., 2005). All of these studies used a type I strain of *T. gondii*, suggesting that different observations might be the result of different cell types and/or host species. Strain differences have been observed in the manipulation of the host NF-κB pathway by *T. gondii*. Type II strains were shown to cause the translocation of NF-κB to the nucleus of mouse splenocytes and mouse BM-derived macrophages (BMMs), whereas type I strains did not (Dobbin et al., 2002; Robben et al., 2004). This strain difference was also shown to have downstream effects, as infection of BMM with type II parasites resulted in high levels of IL-12 secretion compared with infection with type I parasites (Robben et al., 2004). At present, the *T. gondii* factors involved in the modulation of the NF-κB pathway is not known.

In our experiments, infection with type II strains induces a high level of NF-κB activation, whereas infection with type I or III strains does not. Using F1 progeny from a type II × type III cross, we identify a type II gene responsible for NF-κB activation, *GRA15*. The protein product of *GRA15* is a novel dense granule protein that is necessary and sufficient for p65 nuclear translocation and NF-κB–mediated host cell transcription. We show that GRA15 activates the NF-κB pathway independent of MyD88 and TRIF but dependent on TRAF6 and the IKK complex. Although GRA15 does not affect overall virulence of parasites, it does have more subtle phenotypes in vivo, affecting both parasite growth and cytokine levels.

## RESULTS

### Host gene expression analysis shows strain-specific activation of the NF-κB pathway

We previously generated a large gene expression dataset from human foreskin fibroblasts (HFFs) infected with type I, II, or III *T. gondii* strains (Saeij et al., 2007). In our dataset analysis, we focused on the human genes that were differentially regulated by type II strain infection because published data indicated that type II strains might induce more NF-κB nuclear translocation than type I strains (Dobbin et al., 2002; Robben et al., 2004). If *T. gondii* strains differ in the activation of the NF-κB pathway, this should lead to differences in expression of genes with NF-κB transcription factor binding sites (TFBSs) in their promoters. 105 genes were found to be more than twofold up-regulated in type II infections compared with type I and type III infections (Fig. S1 A). Analysis of TFBS in the regulatory elements of these genes revealed enrichment of NF-κB TFBS in their promoters (Fig. S1 B), and a network analysis of molecular relationships between the products of these 105 genes resulted in high scores for two networks whose central factors were the transcription factor NF-κB (network 1) and IL1β/PTGS2(COX-2) (network 2). IL1β and COX-2 are also regulated by NF-κB (Newton et al., 1997; Vallabhapurapu and Karin, 2009). These data suggest that there is at least one polymorphic locus between type II and type I/III strains that modulates the NF-κB pathway.

We also looked for polymorphic loci between type II and type III strains that modulate host gene expression using

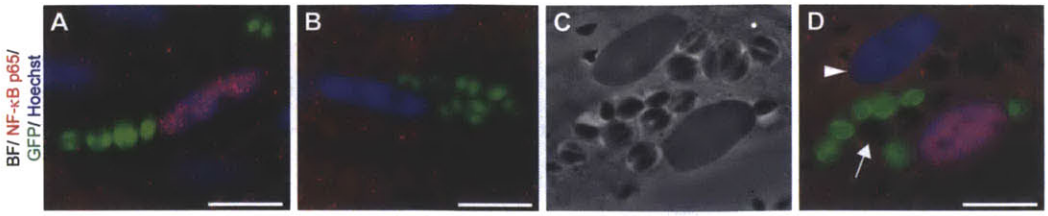



**Figure 1. *T. gondii* strains differ in the activation of NF-κB.** HFFs were infected with *T. gondii* strains for 18–24 h, fixed, and stained with α-NF-κB p65 (red) and Hoechst dye (blue). Shown are infection with a type II (GFP) strain (A) or a type III (GFP) strain (B), and HFFs coinfected with a type I (non-GFP, arrow) and a type II (GFP) strain of *T. gondii* (C, brightfield; D, IF). Cells infected with only type I parasites (arrowhead) do not contain nuclear NF-κB. This experiment has been repeated >10× with similar results. Bars, 5 μm.

quantitative trait locus analysis of human gene expression levels of cells infected with 19 different F1 progeny from II × III crosses. We identified 3,188 human cDNAs that were regulated by a specific *T. gondii* genomic locus (Saeij et al., 2007). 1,176 of these human cDNAs were regulated by a locus on chromosome VIIb. The *T. gondii* polymorphic ROP16 kinase resides on this chromosome and, via its strain-specific activation of STAT3/6, is responsible for the differential expression of many of the genes that are regulated by a chromosome VIIb locus (Saeij et al., 2007). Loci on chromosome X also influenced the expression of 563 human cDNAs. To discover if these cDNAs are regulated by a common transcription factor, we determined if any TFBSs were enriched in the promoters of genes that are differentially modulated by F1 progeny with a type II allele versus a type III allele at each chromosome X marker. At many markers, the expression of genes with NF-κB TFBS in their promoters was enriched in F1 progeny with a type II genotype, suggesting that a *T. gondii* factor responsible for strain differences in NF-κB activation resides on chromosome X (Fig. S1 C). Additionally, network analysis of molecular relationships between the 563 genes that were significantly influenced by a chromosome X locus and their gene products resulted in high scores for two networks, one of which had the transcription factor NF-κB as its central factor (Fig. S1 C). We therefore hypothesized that a polymorphic *T. gondii* locus on chromosome X contributes to differential regulation of the host NF-κB pathway by type II and type I/III strains.

### Toxoplasma type II parasites activate NF-κB

To investigate modulation of the NF-κB pathway by *T. gondii*, we infected HFFs with type I, II, or III *T. gondii* strains for 1–24 h and measured nuclear translocation of the NF-κB p65 subunit by immunofluorescence (IF). Starting after 4 h of infection and continuing until at least 24 h of infection, many cells infected with a type II strain contained high levels of p65 in their nucleus, whereas a type I or a type III strain did not induce translocation of high levels of p65 to the nucleus (Fig. 1 A and B; and not depicted). We have observed the translocation of p65 by infection with various type II strains, including ME49, Pru, DAG, or Beverley, and the absence of high levels of p65 translocation after infection with both RH or GT1 type I strains and CEP or VEG type III strains. Uninfected HFFs surrounding infected HFFs did not contain increased p65 in the nucleus, indicating that activation is not caused by a secreted host factor or a contaminant in the medium. Activation of p65 translocation after infection with type II strains was not inhibited by previous infection with type I or type III strains, demonstrating that this translocation is a result of specific activation by type II parasites rather than inhibition by type I/III parasites (Fig. 1, C and D; and not depicted). We have observed the activation of NF-κB p65 nuclear translocation by type II parasites in 293T cells, HeLa cells, mouse BMM, RAW264.7 murine macrophages, mouse embryonic fibroblasts (MEFs), and rat embryonic fibroblasts (see Fig. 3; and not depicted).

### Type I parasites do not inhibit NF-κB activation

Although we observed that a type I strain does not inhibit p65 nuclear translocation in a co-infection with a type II strain, previous studies have shown that in mouse macrophages, infection with type I parasites can inhibit the activation of NF-κB in response to LPS or TNF (Butcher et al., 2001; Shapira et al., 2002; Kim et al., 2004). To further investigate if type I parasites can inhibit NF-κB translocation, we infected BMM with type I parasites for 1 or 18 h, stimulated the cells with LPS or TNF, and measured the translocation of p65 to the nucleus by IF (Fig. 2 A). In uninfected cells, both LPS and TNF stimulation induced the translocation of p65 subunits to the nucleus. Prestimulation with LPS inhibited later LPS-induced translocation, as the activation of Toll-like receptor pathways induces negative-feedback mechanisms to inhibit further signaling (Lang and Mansell, 2007; Wang et al., 2009). However, preinfection with type I parasites did not inhibit LPS- or TNF-stimulated translocation at early or late time points after infection. In fact, preinfection with *T. gondii* led to higher levels of p65 translocation after LPS stimulation, perhaps as a result of increased TLR4 expression (Kim et al., 2004).

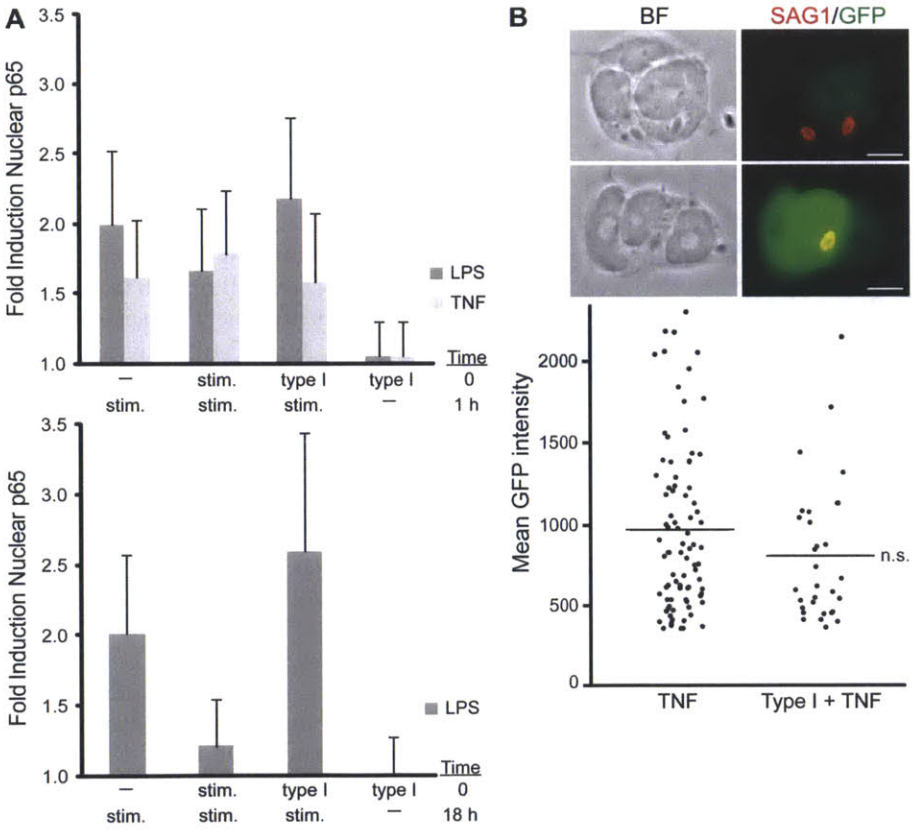
To test whether TNF-stimulated NF-κB–mediated transcription can be inhibited by type I parasites, we used a 293T NF-κB reporter cell line that expresses GFP upon NF-κB activation. Infection of this reporter cell line with type II parasites results in high levels of GFP in infected cells (unpublished data). We added type I parasites to these cells for 45 min, stimulated the cells with TNF, and measured GFP levels of

infected and uninfected cells by microscopy (Fig. 2 B). After 4 h of stimulation, both infected and uninfected cells had varying levels of GFP, with some cells containing almost no GFP and <50% of cells having a high level of GFP. However, the distribution of GFP intensity in the populations of infected and uninfected cells was not significantly different. Unstimulated cells, either infected or uninfected, had negligible levels of GFP (unpublished data). Type I parasites also did not inhibit NF-κB–mediated transcription of luciferase in a 293T NF-κB luciferase reporter cell line (unpublished data).

To investigate if type I preinfection might inhibit transcription of specific subsets of NF-κB–regulated genes, we infected HFFs with a type I strain for 18 h or left cells uninfected and subsequently stimulated the cells with TNF for 6 h. We then performed gene expression analysis using Affymetrix microarrays. Comparing the expression data to uninfected HFF expression data, the genes regulated by TNF stimulation alone and type I preinfection followed by TNF stimulation were not identical; however, genes with NF-κB TFBS in their promoters or belonging to an NF-κB–related pathway were equally enriched in those two samples (Fig. 2 C). We therefore conclude that type I parasites do not inhibit TNF- or LPS-stimulated NF-κB p65 translocation or TNF-stimulated NF-κB–mediated transcription, but they may be able to modulate other pathways or host cell transcription factors which are important for expression of a small subset of TNF stimulated genes.

### A *T. gondii* genomic locus on chromosome X mediates strain-specific activation of NF-κB

To find the *T. gondii* genomic regions mediating the type II versus type I/III strain-specific difference in activation of NF-κB, we infected HFFs with 27 F1 progeny derived from crosses

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| Transcription Factor Binding Sites | FDR q-value TNF | Type I + TNF |
|---|---|---|
| GGGNNTTTCC_V$NFKB_Q6_01 | 0.0134 | 0.0033 |
| NFKAPPAB_01 | 0.0174 | 0.0026 |
| NFKB_Q6_01 | 0.0199 | 0.0152 |
| CREL_01 | 0.0200 | 0.0907 |
| NFKB_Q6 | 0.0213 | 0.0229 |
| NFKAPPAB65_01 | 0.0236 | 0.1224 |
| NFKB_C | 0.0627 | 0.0041 |
| **Canonical Pathways** | **TNF** | **Type I + TNF** |
| ST_TUMOR_NECROSIS_FACTOR_PATHWAY | 0.0052 | 0.0025 |
| TNFR2PATHWAY | 0.0079 | 0.0473 |
| TOLL_LIKE_RECEPTOR_SIGNALING_PATHWAY | 0.0000 | 0.0056 |
| CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION | 0.0000 | 0.0000 |
| APOPTOSIS | 0.0000 | 0.0060 |

**Figure 2. Type I parasites do not inhibit NF-κB activation.** (A) C57BL/6 BMDM were untreated, stimulated with 100 ng/ml LPS or 20 ng/ml mouse TNF, or infected with type I parasites (MOI = 2) for 1 h (top) or 18 h (bottom). Cells were then restimulated for 30 min with LPS or 45 min with TNF, fixed, and stained with α-NF-κB p65 and Hoechst dye. The intensity of nuclear NF-κB p65 was quantitated in at least 10 cells per treatment. Values represent the fold induction of nuclear p65 levels over uninfected unstimulated cells. This experiment was done once in BMDM. A second experiment of HFFs infected with type I parasites and subsequently stimulated with TNF yielded similar results. Error bars represent standard deviation. (B) A 293T NF-κB GFP reporter cell line was plated on coverslips and infected with type I parasites (MOI = 1–2) for 45 min and then stimulated with 100 ng/ml human TNF for 4 h, fixed, and stained with α-SAG1 (red). The GFP intensity was quantitated for at least 30 infected cells and 30 uninfected cells (bottom; n.s. = not significant, two sample Student's *t* tests). Bars, 10 µm. This experiment was performed twice, with the same qualitative results. Similar results were also obtained with a 293T NF-κB luciferase reporter cell line. Horizontal bars represent the mean GFP intensity of cells. (C) Microarray analysis was done on HFFs preinfected with a type I strain (MOI = 7.5) for 18 h, or left uninfected, and subsequently stimulated with 20 ng/ml human TNF for 6 h. Genes were preranked for both samples by the difference in expression, as compared with uninfected untreated HFFs, and GSEA was used to determine whether genes with NF-κB TFBS in their promoters or belonging to NF-κB–related canonical pathways were enriched in either or both samples. False discovery rate q-values <0.25 were considered significant. One array per strain and treatment was done.

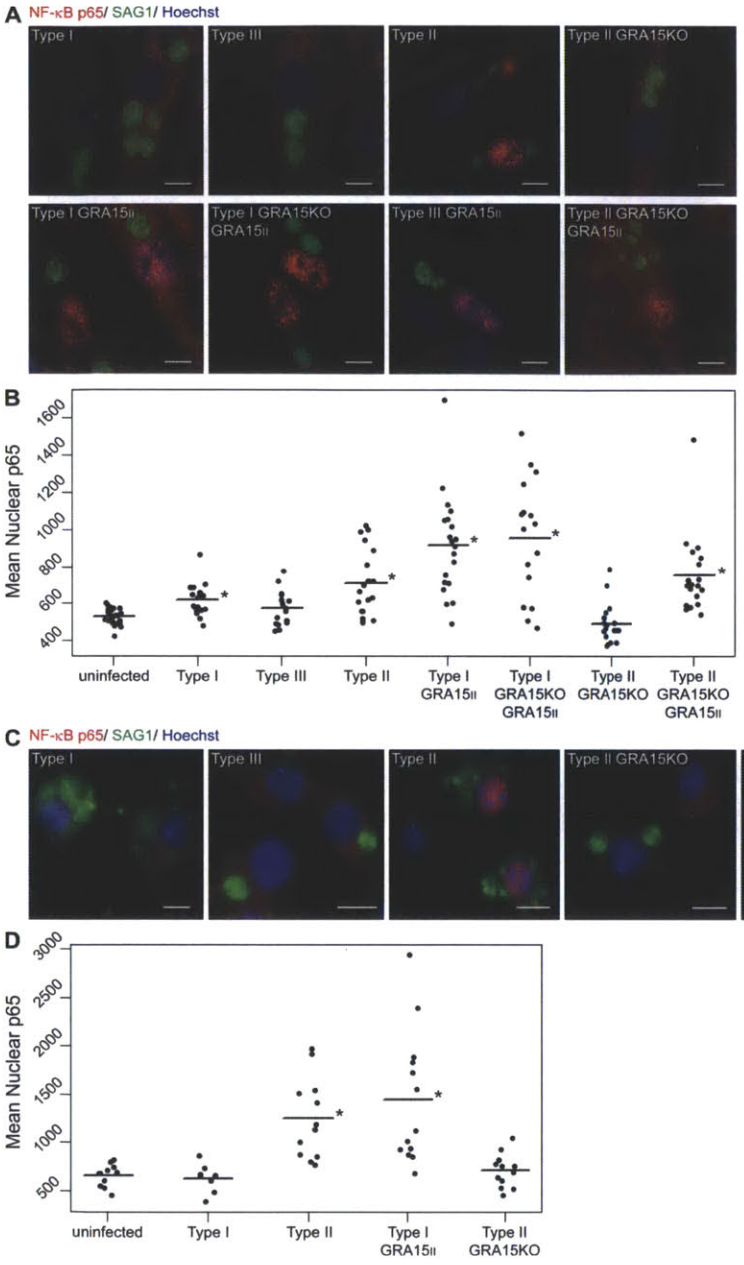


**Figure 3. GRA15 mediates NF-κB p65 translocation.** (A) HFFs were infected with *T. gondii* strains for 18 h, fixed, and stained with α-NF-κB p65 (red), α-SAG1 (green), and Hoechst dye (blue). Bars, 10 µm. (B) The amount of p65 in the nucleus was quantitated in at least 15 HFF cells for each strain. Asterisks indicate significantly higher levels of nuclear p65 compared with uninfected cells (*, $P < 0.0001$, two sample Student's *t* tests). (C) Mouse BMDMs (BALB/c) were infected with *T. gondii* strains for 24 h, fixed, and stained with α-NF-κB p65 (red), α-SAG1 (green), and Hoechst dye (blue). Bars, 10 µm. (D) The level of nuclear p65 in infected cells was quantitated in at least 12 infected cells per strain. Asterisks indicate significantly higher levels of nuclear p65 compared with uninfected cells (*, $P < 0.005$, two sample Student's *t* tests). One replicate experiment was done in both HFFs and mouse BMDM with similar qualitative results. Horizontal bars represent the mean nuclear p65 intensity over all cells.

between type II and type III strains and measured NF-κB activation by IF. Only HFFs infected with F1 progeny having type II alleles for the genetic markers *ROP2* and *GRA6* at the right end of *T. gondii* chromosome X contained nuclear NF-κB p65 (Fig. S2). Thus, the genomic region in the vicinity of genetic markers *ROP2-GRA6* harbors one or more genes involved in the activation of NF-κB. We developed new RFLP markers to more accurately define the place of recombination in progeny that are recombinant for chromosome X around *ROP2* and *GRA6* and therefore limit the genomic region involved in the activation of NF-κB and the number of possible candidate genes. With these new markers, genotyping of STE7 refined the 3′ boundary of the region and genotyping of S26 refined the 5′ boundary of the region (Fig. S2). The refined region between the markers *SAG2E* and *RC4* contains 45 predicted type II genes (ToxoDB.org, v6.0).

To identify the *T. gondii* gene responsible for NF-κB activation, we used a candidate gene approach. From the 45 predicted type II genes, the *SAG2CDXY* (*SRS49a/b/c/d*) locus was excluded, as a type II strain with this locus deleted still activates NF-κB (Saeij et al., 2008; unpublished data). Our first criterion for a protein able to interface with the host cell and modulate host cell signaling was the presence of a signal sequence. Of the 41 remaining genes, 17 are predicted to have a signal sequence. Of these 17 genes, four (*ROP8*, *ROP2A*, *GRA6*, and *63.m00001*) were consistently expressed in tachyzoites in infected macrophage cells, as determined by *T. gondii* microarrays (unpublished data). Our top candidate genes (*ROP2A*, *GRA6*, and *63.m00001*) were then tested by adding a type II C-terminal HA-tagged copy of the candidate gene, including at least 1,500 bp of the putative endogenous promoter, into a type I and/or III strain and assayed whether these transgenic type I/III strains activate NF-κB. Type I and III strains stably expressing a copy of *63.m00001* activated NF-κB in HFFs, whereas strains expressing a copy of *GRA6* or *ROP2* did not, indicating that *63.m00001*, hereafter referred to as *GRA15*, is the locus on chromosome X which mediates the strain-specific activation of NF-κB (Fig. 3 and Fig. S3).

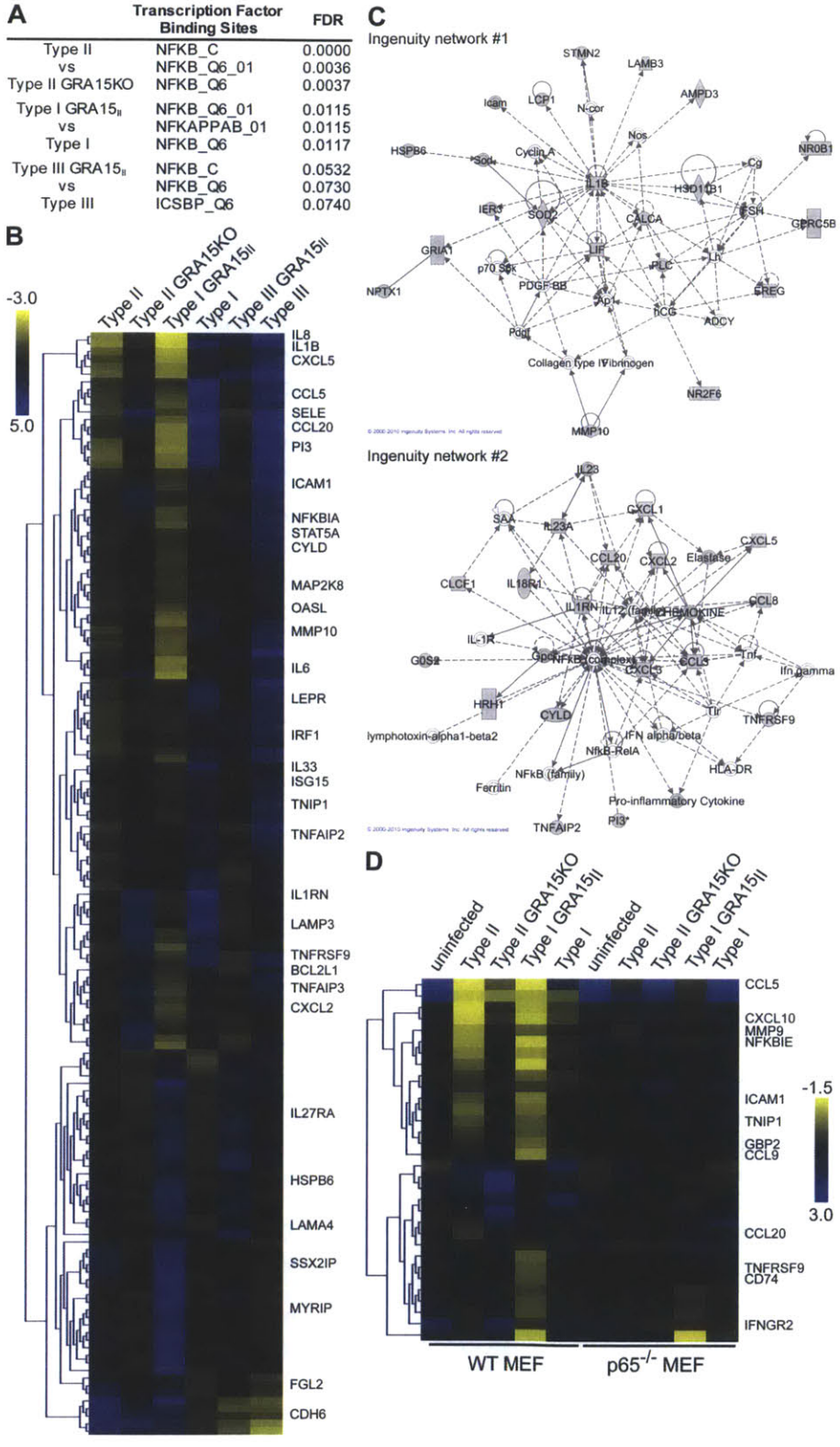


**Figure 4. GRA15 activates NF-κB–mediated transcription.** HFFs or MEFs were infected for 18–24 h with type II, type II *GRA15*KO, type I, type III, type I $GRA15_{II}$, or type III $GRA15_{II}$ *T. gondii* strains, and host cell gene expression was analyzed by Affymetrix microarrays. At least two arrays were done per strain in HFFs and one array was done per strain in MEFs. (A) The top three enriched known TFBSs from GSEAs comparing type II versus type II *GRA15*KO and type I/III $GRA15_{II}$ versus type I/III infections are shown. (B) For the 146 genes that are defined as core GRA15-regulated genes, mean $\log_2$ gene expression values were median centered, genes were clustered by hierarchical clustering, and a heat map is presented. The complete set of genes is listed in Supplemental data 2. (C) Ingenuity pathway analysis was done for these 146 genes. The top two scoring networks are shown. (D) In MEF arrays, 32 genes have the same expression level in WT and $p65^{-/-}$ cells and are regulated by GRA15. For these genes, $\log_2$ expression values were median centered, genes were clustered by hierarchical clustering, and a heat map is shown. The complete set of genes is listed in Supplemental data 2.

## GRA15 mediates NF-κB p65 translocation

We quantified the nuclear p65 IF signal of cells infected with type I, II, or III strains, confirming that a subset of type II–infected cells had a high level of nuclear p65 (intensity >800), resulting in a significant difference in the average nuclear p65 of type II–infected cells compared with type I or type III–infected cells (Fig. 3, A and B). Type I (but not type III)–infected cells did have significantly more nuclear NF-κB p65 than uninfected cells. Type I and type III strains engineered to stably express a type II HA-tagged copy of *GRA15* (type I $GRA15_{II}$ and type III $GRA15_{II}$) activated NF-κB (Fig. 3, A and B). As with type II–infected cells, a subset of type I $GRA15_{II}$–infected cells had very high levels of nuclear NF-κB, and the average nuclear p65 was significantly higher in these cells compared with type I–infected cells. A time-course experiment showed that this activation occurred starting 4 h after infection (Fig. S4).

To determine if type II *GRA15* was necessary for NF-κB p65 translocation in host cells, we generated type II *GRA15*KO strains (Fig. S5). Removal of the *GRA15* locus abolished p65 translocation by type II parasites in host cells, eliminating the subset of infected cells with a high level of nuclear p65 (Fig. 3, A and B). The level of nuclear p65 in cells infected with type II

*GRA15*KO parasites was not significantly different from the level in uninfected cells (Fig. 3 B). To confirm that *GRA15* is responsible for the NF-κB activation phenotype, we transfected $GRA15_{II}$ back into a type II *GRA15*KO strain. NF-κB p65 nuclear translocation was rescued in this type II *GRA15*KO $GRA15_{II}$ strain (Fig. 3, A and B). Additionally, a type I *GRA15*KO $GRA15_{II}$ strain activated translocation of p65, and infection with this strain or a type I $GRA15_{II}$ strain did not activate significantly different levels of nuclear p65, confirming that the type II copy of *GRA15* alone was sufficient for the nuclear translocation of p65 by type I strains of *T. gondii*. Similarly, in mouse BMM, infection with type II strains, but not type I/III strains, activated a high level of p65 nuclear translocation. This activation was also a result of the type II *GRA15* gene (Fig. 3, C and D).

To examine if p65 was the only NF-κB family subunit activated by GRA15, we infected HFFs with type I $GRA15_{II}$ parasites for 24 h and examined p50, p52, RelB, and c-Rel nuclear localization by IF (Fig. S6). Only p50 was specifically and significantly translocated to the nucleus by type I $GRA15_{II}$ strains (see Fig. 6 A). However, there was a significant increase in levels of nuclear c-Rel upon infection with both type I and type I $GRA15_{II}$ strains (see Fig. 6 D).

### GRA15 activates NF-κB–mediated transcription

To determine if the nuclear NF-κB p65 triggered by $GRA15_{II}$ is transcriptionally active, we infected HFFs with type II, type II *GRA15*KO, type I, type I $GRA15_{II}$, type III, or type III $GRA15_{II}$ *T. gondii* and hybridized RNA from the infections to Affymetrix human genome arrays to determine host cell gene expression. The expression of genes with NF-κB TFBS in their promoters and gene products belonging to an NF-κB–related pathway was enriched in type II versus type II *GRA15*KO infections and type I/III $GRA15_{II}$ versus type I/III infections (Fig. 4 A and Supplemental data 1). 146 transcripts were strongly regulated by GRA15, regardless of the *T. gondii* strain genetic background, and we defined these genes as core GRA15-regulated genes (Fig. 4 B). Network analysis of molecular relationships between these 146 genes and their gene products also demonstrated an enrichment of genes involved in cytokine and NF-κB signaling (Fig. 4 C).

Type I *T. gondii* parasites also activate NF-κB p65 translocation in HFFs, although at a much lower level than type II strains (Fig. 3). Microarray analysis confirmed that a type I strain can cause the activation of NF-κB–mediated transcription. The expression of genes with NF-κB TFBS in their promoters and gene products belonging to an NF-κB–related pathway was enriched in type I–infected HFFs over uninfected HFFs (Supplemental data 1). This activation is not dependent on *GRA15*; the expression of NF-κB regulated genes was enriched in a type I *GRA15*KO infection compared with uninfected cells, and not in a type I infection compared with a type I *GRA15*KO infection (unpublished data).

We also infected WT and $p65^{-/-}$ MEFs with type II, type II *GRA15*KO, type I, or type I $GRA15_{II}$ *T. gondii* strains and analyzed host cell gene expression by microarray (Fig. 4 D). In

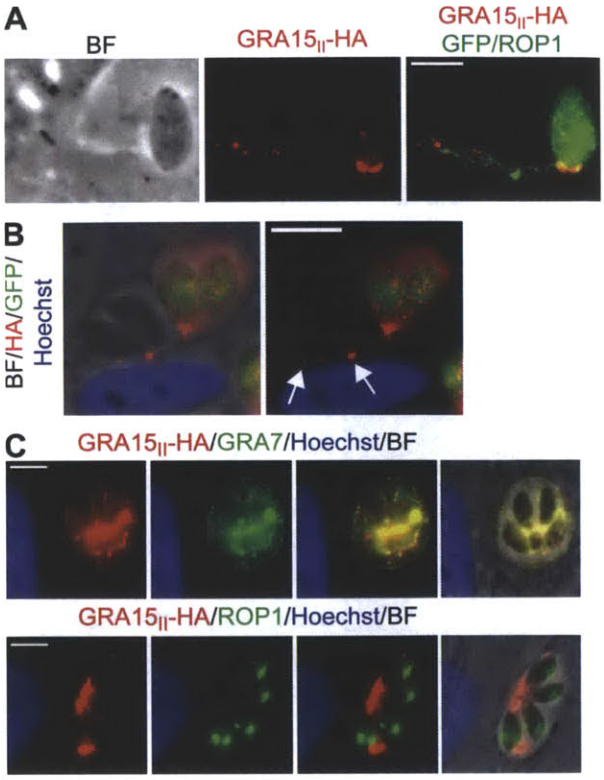


**Figure 5. GRA15 is a secreted dense granule protein.** (A) Parasites expressing GFP and an HA-tagged copy of $GRA15_{II}$ were added to HFFs for 5 min to allow attachment and evacuole formation. Cells were then fixed and stained with α-HA (red), α-ROP1 (green), and Hoechst dye (blue). The HA tag is present with ROP1 in evacuoles, indicating that GRA15 can be secreted into the host cell. This experiment was repeated once with cytochalasin D–treated parasites with the same results. (B) Co-infection of type I (non-GFP) and type I $GRA15_{II}$-HA (GFP) parasites, done once. Arrows indicate HA staining on the PVM of a non-GFP vacuole. (C) Co-staining of $GRA15_{II}$-HA with a dense granule marker, GRA7, shows colocalization of HA staining and GRA7 in both the dense granules and the PV. Conversely, costaining of $GRA15_{II}$-HA with a rhoptry marker, ROP1, shows almost no overlap between the HA tag and the rhoptries. Co-staining was done once, but the same $GRA15_{II}$-HA staining pattern has been observed in more than five independent experiments. Bars, 5 µm.

WT MEFs, we found 18 genes to be core GRA15-regulated genes (more than twofold different in type II vs. type II *GRA15*KO and type I $GRA15_{II}$ vs. type I infections). Of these 18 genes, only two are also GRA15 regulated in $p65^{-/-}$ host cells. This data indicates that the majority of host cell transcription induced by GRA15 was activated via the canonical p65/p50 NF-κB heterodimer; however, it is possible that a small subset of genes was activated by other NF-κB subunits, such as c-Rel/p50 dimers, or other transcription factors.

### GRA15 is a polymorphic secreted dense granule protein

The *GRA15* coding region is predicted to be 1,908 bp in type I and III strains, but only 1,653 bp in type II strains, as a result of either an insertion or deletion (indel; ToxoDB.org, v6.0). An intron is predicted in the type I and type III copies, very

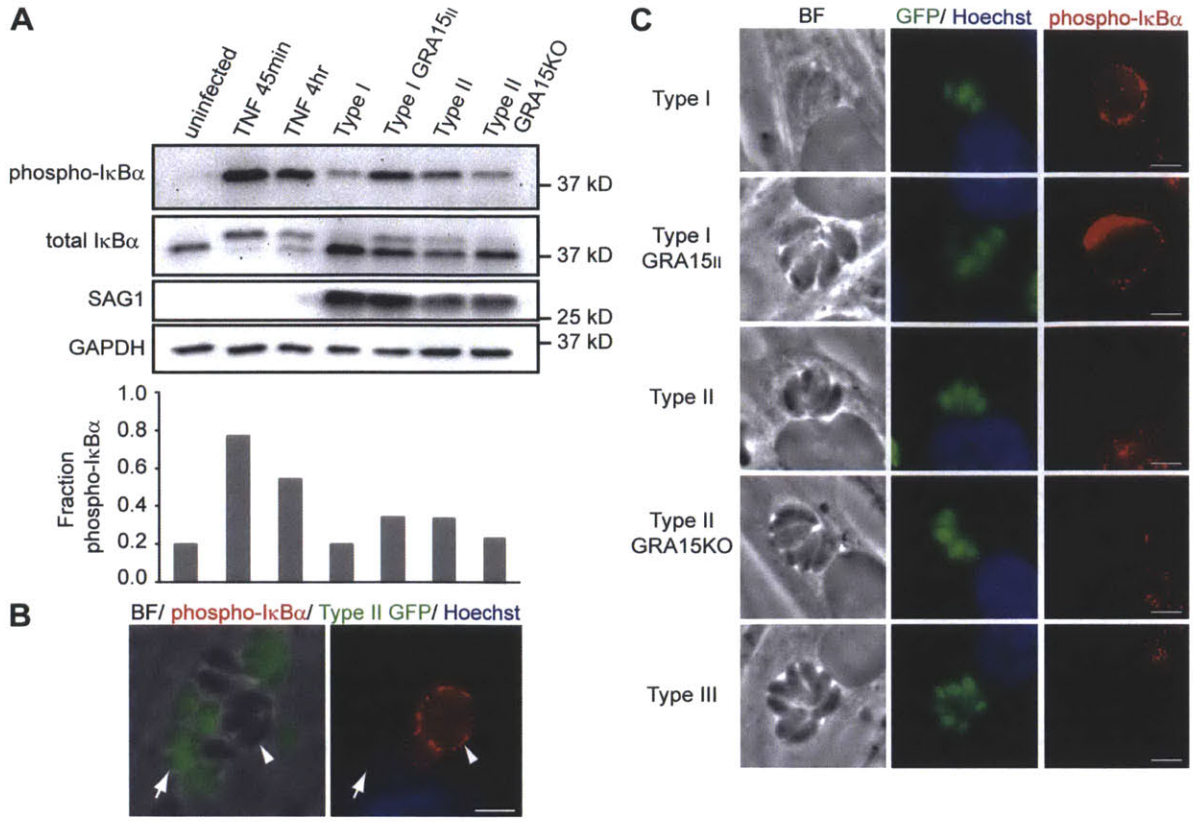


**Figure 6. GRA15 activity affects total levels phospho-IκBα but not PVM-associated phospho-IκBα.** (A) HFFs were infected with *T. gondii* strains for 24 h or stimulated with TNF for indicated times, and cell lysates were collected, run on an SDS-PAGE gel, and Western blotted for phospho-IκBα, total IκBα, SAG1 (parasite loading control), and GAPDH (host cell loading control). From the total IκBα blot, the fraction of phosphorylated IκBα was determined by comparing the intensity of the upper band (phosphorylated form) to the total intensity of the lower and upper band. This experiment was repeated once with type I and type I $GRA15_{II}$ strains only with similar results. (B) HFFs were coinfected with type I (non-GFP) and type II (GFP) parasites, fixed, and stained with α-phospho-IκBα (red) and Hoechst dye (blue). A type I PVM (non-GFP, arrowhead) and a type II PVM (GFP, arrow) are indicated. Bar, 5 µm. (C) HFFs were infected with *T. gondii* strains expressing GFP for 24 h, fixed, and stained with α-phospho-IκBα (red) and Hoechst dye (blue). This experiment has been done three times with similar results. Bars, 1 µm.

close to the indel. We sequenced the region around this indel of both type I and type II cDNA. Neither strain was found to have an intron in this region, and the indel was confirmed. To determine the full transcript of $GRA15_{II}$, we performed 5′ and 3′ rapid amplification of cDNA ends (RACE). Two $GRA15_{II}$ transcription start sites were found, one at −508 to −504 bp upstream of ATG and one at −277 bp upstream of ATG. Three polyadenylation sites were found, at +930, +992, and +1,144 bp downstream of the stop codon.

ToxoDB.org currently contains sequences from one strain for each of the three North American/European clonal lineages of Toxoplasma: GT1 (type I), ME49 (type II), and VEG (type III). The genomic sequences of strains within the same lineage are thought to be very similar; however, we sequenced the *GRA15* locus from RH (type I), Pru (type II), and CEP (type III) strains as well, three other strains which we have used in our experiments. Although Pru and CEP have an identical sequence to ME49 and VEG, respectively, the RH sequence contains a frameshift mutation at base 872. This frameshift leads to the mutation of a stretch of 22 amino acids (2 remain conserved), followed by a premature stop codon, truncating the protein to 312 amino acids, instead of 635 amino acids. The type I/III and II protein sequences differ most strikingly at the 84–amino acid indel near the C terminus of the protein. Besides this indel, five other amino acids are polymorphic between types I/III and II and one other single amino acid is inserted or deleted (Fig. S7).

We next looked by IF at the localization of GRA15 in the parasite and infected host cells. To determine if GRA15 was secreted into the host cell, we performed evacuole staining on a 5-min type I $GRA15_{II}$-HA infection of HFF cells using an antibody against the HA tag (Fig. 5 A). HA staining is clearly present in evacuoles, partially colocalizing with evacuoles containing rhoptry proteins, indicating that GRA15 is a secreted protein. Evacuoles containing GRA15 can also be seen after attachment of cytochalasin D–treated parasites (unpublished data). In parasitophorous vacuoles (PVs), costaining of the $GRA15_{II}$-HA protein with either a rhoptry marker, ROP1, or a dense granule marker, GRA7, showed colocalization of $GRA15_{II}$-HA with GRA7, with almost no overlap

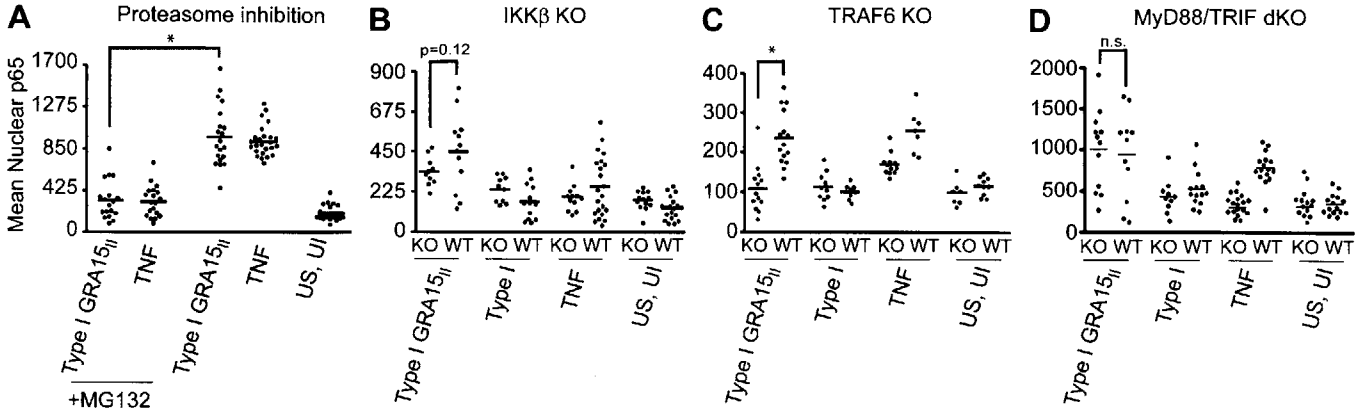


**Figure 7. GRA15 activity is dependent on IKK-β and TRAF6 and independent of MyD88 and TRIF.** Cells were infected with type I *GRA15*$_{II}$ or type I parasites for 4 h, stimulated with 20 ng/ml TNF for 1 h, or left unstimulated (US) and uninfected (UI). Cells were fixed and probed with an α-NF-κB p65 antibody and mean nuclear staining was measured. (A) HFF cells were preincubated with media containing 20 ng/ml MG-132 proteasome inhibitor before infection and TNF stimulation. (B–D) The activity of GRA15$_{II}$ in the absence of different components of the NF-κB pathway was assayed. (B) IKK-β$^{-/-}$ MEFs. (C) TRAF6$^{-/-}$ MEFs. (D) MyD88 $^{-/-}$/TRIF$^{-/-}$ BMM host cells. These experiments were repeated at least two times and quantification was performed on a representative experiment for each factor assayed. Asterisks (*) indicate data are significantly different (P-value < 0.05, Student's *t* test), and n.s. indicates data are not significantly different. Horizontal bars represent the mean nuclear p65 intensity over all cells.

between the HA tag and ROP1 (Fig. 5 C). GRA15 staining overlaps GRA7 staining in the dense granules and within the PV. GRA15 also localizes to the outside of the PV membrane (PVM), and in a co-infection of type I (non-GFP) and type I GRA15$_{II}$-HA (GFP) parasites, HA staining can be seen localized in the PVM and on the outside of the PVM of a parasite expressing GRA15$_{II}$-HA (GFP), as well as on the outside of the PVM of a parasite not expressing GRA15$_{II}$-HA (Fig. 5 B), which is consistent with dense granule localization. We therefore conclude that GRA15 is a dense granule protein.

### GRA15 affects total levels of the phospho-IκBα but does not affect PVM-associated phospho-IκBα

The nuclear translocation of NF-κB transcription factor subunits is dependent on the phosphorylation and degradation of an inhibitory protein, IκBα. We determined if GRA15 affected the overall levels of phospho-IκBα in infected cells by Western blotting and quantified the fraction of phosphorylated IκBα compared with the total level of IκBα (Fig. 6 A). Infection with a strain of *T. gondii* expressing GRA15$_{II}$ led to an increase in the fraction of total IκBα that was phosphorylated, although not to the extent of TNF-induced levels. This indicates that GRA15 activates NF-κB through the phosphorylation of IκBα.

Previously, a type I *T. gondii* protein extract was found to have IκBα-phosphorylating activity in vitro (Molestina et al., 2003). There is also evidence that this kinase activity can occur in vivo, as PVM-associated phospho-IκBα can still be observed in infected IKK-α/β double knockout MEFs (Molestina and Sinai, 2005a). However, in these IKK-α/β double knockout cells, after type I *T. gondii* infection NF-κB does not translocate to the nucleus or bind to DNA in vitro, and NF-κB– mediated gene expression is severely decreased (Molestina and Sinai, 2005b). To determine if the accumulation of phospho-IκBα on the PVM correlated with NF-κB activation, we infected HFFs with *T. gondii* strains and stained infected cells with a phospho-IκBα antibody. Although infection with type II parasites activated NF-κB to a much greater extent and led to higher levels of total phospho-IκBα compared with type I parasite infection, in a mixed infection of type I (non-GFP) and type II (GFP) *T. gondii*, phospho-IκBα accumulated almost exclusively on type I vacuoles (Fig. 6 B). We also found that the accumulation of phospho-IκBα at the PVM was independent of *GRA15*, as both type I and type I *GRA15*$_{II}$ PVMs accumulated phospho-IκBα, and neither type II nor type II *GRA15*KO PVMs accumulated visual levels of phospho-IκBα (Fig. 6 C). Phospho-IκBα also did not accumulate on type III PVMs (Fig. 6 C). We conclude that the accumulation of PVM-associated phospho-IκBα is specific to type I parasites and is not correlated with the overall level of NF-κB activation in the host cell.

### GRA15 is dependent on the IKK complex and TRAF6 but independent of MyD88 and TRIF

BLAST and Pfam searches for proteins with similar amino acid sequences or domains to GRA15$_{II}$ returned no significant results, providing no clues to the mechanism of GRA15 NF-κB activation (Altschul et al., 1990; Finn et al., 2008). To start to answer this question, we determined which components of the NF-κB signaling pathway were necessary for GRA15 activity. When NF-κB is activated, IκB proteins are phosphorylated and then degraded by the proteasome. We previously determined that GRA15 leads to the phosphorylation of IκBα (Fig. 6), and to determine if GRA15 activity is dependent on the proteasome, we pretreated cells with MG132, a proteasomal inhibitor. Our results show that activation of NF-κB by GRA15 required functional proteasomal degradation (Fig. 7 A). IκB proteins are normally

phosphorylated by the IKK complex, consisting of IKK-α, IKK-β, and IKK-γ (NEMO). IKK-γ is a regulatory subunit in the complex, whereas the β and α subunits are active kinases. IKK-β has greater kinase activity than IKK-α and is the principal kinase responsible for the phosphorylation of IκBα (Ghosh and Karin, 2002; Li and Verma, 2002). In WT MEFs, a type I $GRA15_{II}$ strain induces a 3.4-fold increase in nuclear p65 compared with uninfected cells. However, in IKK-$\beta^{-/-}$ MEFs, this increase is only 1.9-fold (Fig. 7 B). Many pathogens activate NF-κB via TLR agonists, and TLR signaling is mediated by the MyD88 and TRIF adaptor proteins. However, GRA15 is able to activate p65 nuclear translocation in MyD88/TRIF double knockout cells, indicating that it is not just a TLR ligand (Fig. 7 D). GRA15-mediated p65 activation is also not dependent on the RIP1 adaptor protein but is dependent on TRAF6 (Fig. 7 C and not depicted). Thus GRA15 appears to modulate NF-κB at a specific step in the pathway downstream of MyD88 and TRIF but upstream of, or in a complex with, TRAF6 and IKK proteins.

### GRA15 expressed in Hela cells is sufficient to activate NF-κB

We wanted to determine whether $GRA15_{II}$ alone is sufficient to activate p65 nuclear translocation or if other *T. gondii* secreted factors that are common to all type I, II, and III strains are also needed for this process. When we transiently transfected HeLa cells with a vector expressing the type II copy of GRA15 N-terminally fused with GFP, the nuclei of transfected GFP-positive cells contained p65, whereas the nuclei of nontransfected GFP-negative cells in the same culture did not (Fig. 8 A). The level of this nuclear localization is equivalent with activation by intracellular type I $GRA15_{II}$ parasites (Fig. 8 B). Expression of 55.m04955, an unrelated *T. gondii* protein, or Mob1A, a human protein present in the original vector, did not induce p65 translocation, indicating that this NF-κB activation is not the result of cell stress from protein overexpression (Fig. 8 A). $GRA15_{II}$ expression alone is therefore sufficient to recapitulate the induction of p65 nuclear translocation.

### GRA15 affects in vitro parasite growth

To assay the effect of GRA15 on in vitro parasite growth, we infected monolayers of fibroblasts with type I, type I $GRA15_{II}$, type II, or type II *GRA15*KO parasites, allowed the parasites to grow for 4–7 d, and then quantified the area of plaques formed on the monolayers. In HFF host cells, a type II GRA15KO strain formed significantly larger plaques than a type II strain ($P = 0.002$, Student's *t* test), and a type I strain formed significantly larger plaques than a type I $GRA15_{II}$ strain ($P = 0.024$, Student's *t* test; Fig. S8 A). However, in MEF host cells, type II and type II GRA15KO strains did not make significantly different sized plaques ($P = 0.841$, Student's *t* test), and the same was true of type I and type I $GRA15_{II}$ strains ($P = 0.371$, Student's *t* test; Fig. S8 A). This data indicates that GRA15 inhibits in vitro parasite growth in human fibroblasts but not mouse fibroblasts.

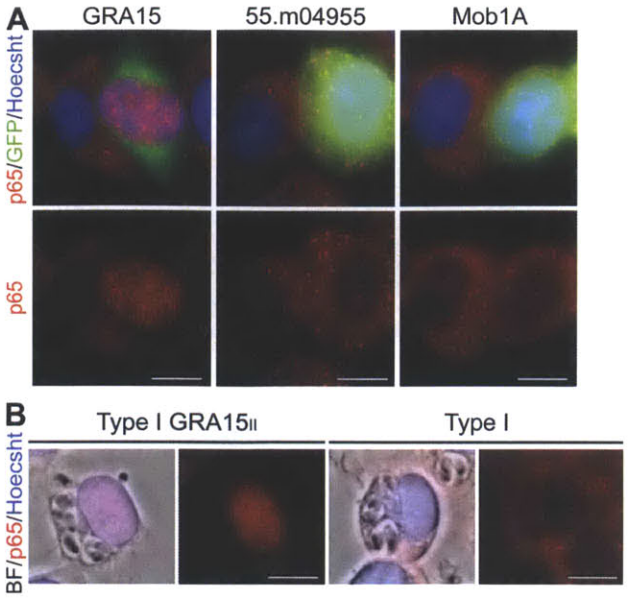


**Figure 8. GRA15 expression alone is sufficient to activate NF-κB in HeLa cells.** (A) HeLa cells were transfected with $GRA15_{II}$, an unrelated *T. gondii* gene (*55.m04955*), or an unrelated human gene (*Mob1A*) fused to *GFP*. Cells were then fixed and stained with α-NF-κB p65 (red) and Hoechst dye (blue). All cells expressing $GRA15_{II}$-GFP contain activated NF-κB p65, whereas cells expressing 55.m04955-GFP or Mob1A-GFP do not. Nontransfected non-GFP cells in the same culture also have no nuclear NF-κB p65. This experiment was repeated two more times with the same results. (B) HeLa cells were infected with type I $GRA15_{II}$ or type I parasites for 24 h, fixed, and stained with α-NF-κB p65 (red) and Hoechst dye (blue). Cells infected with a type I $GRA15_{II}$ strain contain comparable amounts of nuclear NF-κB p65 to transfected cells. This experiment was repeated a second time with similar results. Bars, 10 µm.

### GRA15 affects IL-12 production in vitro

In vitro infection of macrophages with different strains of *T. gondii* results in different levels of IL-12p40 secretion, with type II strains inducing much higher levels of IL-12p40, and some of this variation has been suggested to be a result of strain differences in NF-κB activation (Robben et al., 2004; Kim et al., 2006; Saeij et al., 2007). To examine the role of GRA15-mediated NF-κB activation in the induction of IL-12p40 secretion, mouse BMMs were infected with type I, type I $GRA15_{II}$, type II, or type II *GRA15*KO *T. gondii*, and levels of IL-12p40 in the supernatant were determined by cytokine ELISA (Fig. 9 A). As expected, type I–infected BMMs secrete a low level of IL-12p40 that is not significantly higher than the level secreted by uninfected cells, whereas type II induces a high level of IL-12p40 secretion. When *GRA15* is removed from the type II strain, IL-12p40 secretion decreases more than six-fold, implying a considerable role for this gene product in modulating host cell IL-12 signaling ($P = 0.001$, Student's *t* test). Similarly, the introduction of $GRA15_{II}$ in a type I strain leads to a significant increase in IL-12p40 secretion by BMM ($P = 0.008$, Student's *t* test). IL-12p70 secretion was also higher after type II infection, compared with a type I

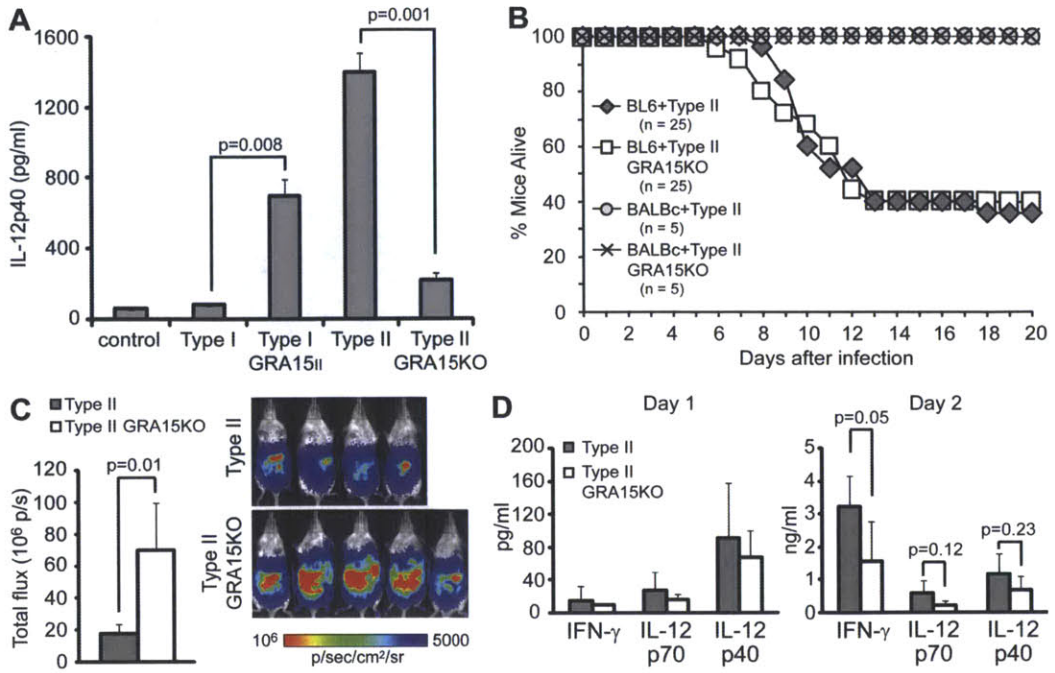


**Figure 9. GRA15$_{II}$ promotes IL-12 secretion in vitro and affects parasite growth and host cytokine production in vivo.** (A) BALB/c BMDMs were infected with *T. gondii* strains for 24 h, supernatants were collected, and IL-12p40 levels were determined by cytokine ELISA. These experiments were performed at least three times in BMDM using triplicate samples, as well as in RAW264.7 macrophages, all with similar results. (B–D) Mice were infected i.p. with tachyzoites of either a type II or a type II GRA15KO strain. (B) C57BL/6 or BALB/c mice were infected with 5,000 tachyzoites and survival of mice was monitored. In one experiment, five BALB/c mice were infected per strain, and in three separate experiments, a total of 25 C57BL/6 mice were infected per strain. (C) BALB/c mice were infected with parasites that express the enzyme luciferase. 5 d after infection, mice were i.p. injected with luciferin, anesthetized, and the flux (photons/sec/cm²/sr) was determined as a measure of parasite burden. Mice infected with a type II *GRA15*KO strain had significantly greater total flux (p/s) and, therefore, significantly greater parasite burden than mice infected with a type II strain. This burden difference 5 d after infection was observed in three independent experiments. (D) 1 or 2 d after infection, infected BALB/c mice were euthanized and an i.p. cavity wash was collected for IFN-γ, IL-12p70, and IL-12p40 cytokine ELISA. On day 2 after infection, mice infected with a type II *GRA15*KO strain had significantly lower levels of IFN-γ in the i.p. cavity than mice infected with a type II strain ($P = 0.05$, two-sample Student's *t* test). Five mice were infected per strain per day. Day 2 cytokine levels were measured in a separate experiment with similar results. Error bars represent standard deviation from one experiment.

infection, and partially dependent on the presence of a type II copy of *GRA15* (unpublished data).

The *T. gondii* polymorphic rhoptry kinase ROP16 also affects IL-12 secretion, and previous microarray analyses determined that the expression of many GRA15-regulated genes are also strongly affected by a locus on chromosome VIIb, where *ROP16* resides (Saeij et al., 2007). In these analyses, some genes, such as *SOCS-2* and *SOCS-3*, were regulated by loci on both chromosome VIIb and X (Fig. S9 A). We wondered if the effect of ROP16 on these genes was through modulation of the NF-κB pathway. We infected an NF-κB GFP reporter cell line with a type II strain or a type II transgenic strain expressing a type I copy of ROP16. A significantly greater percentage of type II–infected cells than type II *ROP16*$_I$–infected cells are GFP positive ($P = 1.5 \times 10^{-7}$, $\chi^2$ test), indicating that in a type II background, a type I copy of ROP16 significantly inhibits NF-κB activation (Fig. S9 B). In an NF-κB luciferase reporter cell line, infection with a type II ROP16$_I$ strain also induced significantly less luciferase activity than infection with a type II strain (unpublished data). Thus, both GRA15 and ROP16 affect NF-κB activity.

### GRA15 affects in vivo parasite growth and cytokine production

To assay the effect of GRA15 on parasite virulence, we infected C57BL/6 or BALB/c mice by i.p. injection with 5,000 tachyzoites of a type II or a type II *GRA15*KO strain and monitored mouse survival during the acute phase of infection (days 0–20; Fig. 9 B). C57BL/6 mice infected with either strain succumbed to infection at the same time, between days 4 and 18 after injection. Additionally, the same percentage of mice in each group survived, ~40%. BALB/c mice infected with either a type II or a type II *GRA15*KO strain did not die after infection.

To determine the effect of GRA15 on in vivo parasite burden, BALB/c mice infected i.p. with tachyzoites of either a type II or a type II *GRA15*KO strain that express the enzyme luciferase were imaged throughout infection. At day 5 after infection, mice infected with a type II *GRA15*KO strain had a significantly higher parasite burden than mice infected with a type II strain (Fig. 9 C; $P = 0.01$, Student's *t* test). Similarly, expression of GRA15$_{II}$ in a type I strain inhibited in vivo parasite growth (Fig. S8 B). Together, these experiments indicate that GRA15 inhibits in vivo parasite growth in both a type I and a type II background.

GRA15 affects IL-12 secretion by BMM in vitro, and we also assessed whether it affects cytokine secretion in vivo at the site of infection. BALB/c mice were infected i.p. with tachyzoites of either a type II or a type II *GRA15*KO strain. 1 or 2 d after infection, mice were sacrificed, the i.p. cavity was washed, and cytokine levels in the wash were determined by ELISA. On day 2 after infection, mice infected with a type II *GRA15*KO strain had significantly less IFN-γ in their i.p. cavities than mice infected with a type II strain (Fig. 9 D; $P = 0.05$, Student's *t* test). Although differences in IL-12p40 or p70 were not significant on either day 1 or day 2 because of large variations between mice, the average cytokine levels in type II *GRA15*KO–infected mice were consistently lower than cytokine levels in type II–infected mice. At these early time points, there was not a significant difference in parasite load between mice infected with either strain, as determined by imaging (unpublished data).

## DISCUSSION

The modulation of the NF-κB pathway by *T. gondii* has long been an area of debate, with some studies stating that *T. gondii* activates NF-κB and others that *T. gondii* inhibits NF-κB activation. In this study, we have conclusively shown that the three North American clonal lineages of *T. gondii* differ in their activation of the host NF-κB pathway; type II strains activate a high level of NF-κB p65 translocation, whereas type I and III strains do not (Fig. 3). Using F1 progeny from a type II × type III cross, we found that a locus on chromosome X is responsible for this polymorphic phenotype, and we identified the novel *T. gondii* factor GRA15 at this locus (Fig. S2). The type II copy of *GRA15* (*63.m00001*) is necessary in type II strains and sufficient in type I and III strains for NF-κB nuclear translocation and transcriptional activity (Figs. 3 and 4; and Supplemental data 1). We have observed this activation in a variety of human, mouse, and rat cell types, indicating that GRA15's activity is independent of host cell type and species. Additionally, when $GRA15_{II}$ is expressed in HeLa cells, it is sufficient to activate NF-κB.

It had been previously reported that infection with type I strains of *T. gondii* activates NF-κB in MEF and Henle 407 intestinal epithelial host cells (Molestina et al., 2003; Ju et al., 2009). Careful quantification of IF experiments showed that type I strains do slightly activate NF-κB p65 translocation, but the level of nuclear p65 is much higher in type II–infected cells than type I–infected cells (Fig. 3). It is possible that other groups have concluded that type I strains do not activate NF-κB because this activation is so low compared with that of LPS or TNF stimulation (Butcher et al., 2001; Shapira et al., 2002, 2005). Our IF experiments also showed that a type I strain activates c-Rel nuclear translocation, which might also affect host cell transcription (Fig. S6). By microarray analysis, some NF-κB–regulated genes are induced by type I infection, but a much greater number are induced by type II infection, and infection with a type I strain does not activate detectable GFP or luciferase transcription from an NF-κB reporter cell line (unpublished data), which is in concordance with another published study (Shapira et al., 2005). This low-level activation is also not dependent on GRA15, as there is no enrichment in NF-κB activation in a type I infection over a type I *GRA15*KO infection. Type III strains do not activate any p65 nuclear translocation or NF-κB–mediated transcription.

Our results agree with previous observations that phosphorylated IκBα accumulates on the PVM upon *T. gondii* type I infection (Butcher et al., 2001; Molestina et al., 2003; Shapira et al., 2005). However, levels of PVM-associated phospho-IκBα are not correlated with total levels of phospho-IκBα or the level of NF-κB transcriptional activity in the host cell. Phospho-IκBα is not observed on type II PVMs, and, although infection with *T. gondii* strains expressing $GRA15_{II}$ leads to higher total levels of phospho-IκBα, the presence of GRA15 does not affect PVM-associated phospho-IκBα (Fig. 6). A type I *T. gondii* protein capable of phosphorylating IκBα may play a role in low-level activation of NF-κB in type I strains. However, it is clear that the GRA15 protein in type II strains activates NF-κB to a much greater extent (Molestina and Sinai, 2005a).

In contrast, some groups have reported that infection with type I *T. gondii* strains inhibits NF-κB activation after stimulation with the cytokine TNF or the TLR ligand LPS (Butcher et al., 2001; Shapira et al., 2002; Kim et al., 2004). This inhibition has been observed after <6 h of infection in HFFs (Shapira et al., 2005), mouse BMM (Shapira et al., 2002; Kim et al., 2004), and thioglycolate-elicited cells (Butcher et al., 2001) and was not observed after 6 or 12 h of infection in mouse BMM (Kim et al., 2004; Leng et al., 2009). Our experiments confirm that type I *T. gondii* cannot inhibit LPS-stimulated NF-κB nuclear translocation or TNF-stimulated NF-κB transcriptional activity at a late time point in infection (Fig. 2, A and C). But, after a short infection (<5 h), we also do not observe inhibition of NF-κB p65 nuclear translocation or NF-κB–mediated transcription, contradicting previous studies (Fig. 2, A and B). It is true that some infected cells do not respond to LPS or TNF stimulation, and this observation may have lead to the conclusion that type I strains can inhibit NF-κB signaling. However, quantification of NF-κB p65 translocation and NF-κB reporter transcription in many uninfected and infected cells shows that preinfection with *T. gondii* does not alter the response of populations of cells to these stimuli.

The *GRA15* gene product is a novel *T. gondii* dense granule protein that is secreted into the host cell upon invasion (Fig. 5), representing the first identified Apicomplexan dense granule protein that can modulate host cell signaling pathways. Rhoptry proteins have already been identified as parasite factors that can alter host cell behavior, but our findings indicate that dense granule proteins should be viewed as candidate factors as well. It is still unclear why only the type II copy of *GRA15* activates NF-κB. Amino acid polymorphisms between the type II and type I/III copies of GRA15, including an insertion/deletion and several single amino acid changes, or the expression level of *GRA15* may be responsible for this polymorphic phenotype.

Although the GRA15 protein is secreted into the host cell upon parasite invasion, cells infected with type I $GRA15_{II}$ parasites did not have substantial p65 in their nuclei until ~4 h after infection (Fig. S4), and in our experiments NF-κB activation was usually assayed 18–25 h after infection. These slow kinetics are not unprecedented; Rac GTPase has been shown to initiate NF-κB nuclear translocation with a time course similar to that of GRA15 (Boyer et al., 2004). Additionally, the amount of GRA15 interacting with host cell proteins likely increases after PV formation as dense granule proteins are made and continuously secreted from intracellular parasites, and higher levels of GRA15 may be necessary to initiate NF-κB activation.

The precise mechanism by which GRA15 activates NF-κB has yet to be discovered. Our data suggests that GRA15 initiates canonical NF-κB activation, which preferentially induces the p65/p50 heterodimer (Hayden and Ghosh, 2004). A simple hypothesis is that GRA15 acts as a TLR ligand to activate the canonical NF-κB pathway; however, the activation of p65 translocation by GRA15 is not dependent on either MyD88 or TRIF, two proteins which are essential for TLR signaling. We did find that the activity of GRA15 is dependent on both IKK-β and TRAF6, suggesting that GRA15 acts either upstream of or in a complex with these proteins. A type I $GRA15_{II}$ strain activated more nuclear p65 translocation in WT MEFs than in IKK-$\beta^{-/-}$ or TRAF6$^{-/-}$ MEFs, although in IKK-$\beta^{-/-}$ cells this difference was not significant ($P = 0.12$). This is probably because IKK-α also has phosphorylating activity. Our microarray data further defines the placement of GRA15 in the NF-κB signaling pathway. GRA15 is able to constitutively activate NF-κB, but this activation leads to the expression of negative feedback regulators, such as the deubiquitinating enzymes A20 (*TNFAIP3*) and CYLD, which normally act to quickly down-regulate NF-κB signaling. Because TRAF6 and the IKK complex are both targets of these deubiquitinating enzymes, it is likely that GRA15 acts in concert with these proteins rather than upstream (Sun, 2008). We are currently looking for direct binding partners of GRA15 by coimmunoprecipitation.

We found that a type I copy of *ROP16* can inhibit NF-κB activation in a type II strain (Fig. S9 B). Why this inhibition occurs in a type II strain but not a type I strain is unknown, but the genetic backgrounds of type I and type II strains are very different and other polymorphic factors likely exist that affect STAT and/or NF-κB signaling pathways. How ROP16 inhibits NF-κB activation is also unclear, but it is likely to be through its activation of STAT6 and/or STAT3 (Ohmori and Hamilton, 2000; Nelson et al., 2003; Butcher et al., 2005; Hoentjen et al., 2005). This inhibition has significant consequences, for example, GRA15 and ROP16 have opposing effects on the expression levels of many genes, including IL-12, a particularly important cytokine in *T. gondii* infection (Fig. 9 A; Gazzinelli et al., 1994; Saeij et al., 2007). In fact, the single amino acid difference in ROP16 that causes it to be less active in type II strains (Yamamoto et al., 2009) may have been selected for in type II strains specifically to increase NF-κB activation by $GRA15_{II}$. However, GRA15 and ROP16 are expected to have additive or synergistic effects on the expression of other genes, such as the *SOCS* genes (Fig. S9 A). In any case, the modulation of host cell gene expression will depend upon the exact allelic combination of a variety of factors that *T. gondii* possesses, including *GRA15* and *ROP16*.

Early in infection (days 1–5), GRA15 affected both cytokine production and parasite growth in vivo. When a host is first infected by live parasites, type II strains expressing GRA15 activate NF-κB in host cells and induce IL-12 secretion, whereas infection with type II *GRA15*KO parasites or type I/III strains does not cause this early activation. IL-12 stimulates NK cells and T cells to secrete IFN-γ, and the observed effect of GRA15 on IFN-γ levels was likely via an effect on IL-12. Although levels of IL-12 in the i.p. cavity were not significantly different between mice infected with a type II or type II *GRA15*KO strain, the levels were consistently lower in type II *GRA15*KO–infected mice, and this difference may have been enough to lead to a significant difference in IFN-γ levels (Fig. 9 D). IFN-γ is the main mediator of host resistance to *T. gondii*, and differences in IFN-γ levels probably also explain the growth difference that we observed in vivo between a type II and type II *GRA15*KO strain at a slightly later time point 5 d after infection (Fig. 9 C). However, as the infection progresses and parasites lyse out of host cells, pathogen-associated molecular pattern proteins within the PV, such as profilin and cyclophilin, are released, and NF-κB will be activated via TLR signaling and CCR5 signaling by all strains. At this stage, IL-12 production, IFN-γ production, and parasite growth are then independent of the GRA15 locus, which might explain why a type II strain does not differ in overall virulence from a type II *GRA15*KO strain (Fig. 9 B). The location of GRA15 on chromosome X does indicate that it could represent a previously identified chromosome X virulence locus (Saeij et al., 2006).

Plaque assays showed that GRA15 also affects parasite growth in vitro (Fig. S8 A). However, this in vitro effect occurred specifically in human cells and not in mouse cells. The cause of this difference is currently unknown, but one possibility is that genes affecting amino acid levels, lipid levels, or levels of other nutrients are partially NF-κB regulated in human cells but not in mouse cells.

$GRA15_{II}$ may also have other effects in vivo that remain untested. As an intracellular pathogen, *T. gondii* must use host cells to traffic through the body of the host animal. NF-κB activation by $GRA15_{II}$ increases expression of many chemokines and adhesion molecules (our microarray data), and strain differences in NF-κB activation may therefore lead to differences in the ability to induce migration of host cells, as previously reported (Lambert et al., 2006, 2009). Infection of hosts with other pathogens is also highly relevant to disease outcome, and the activation of NF-κB by *T. gondii* might increase transcription of HIV retroviral sequences with NF-κB binding sites in their promoters (Gazzinelli et al., 1996). Lastly, NF-κB activation leads to a proinflammatory Th1-type immune response that may promote inflammatory disease manifestations such as

encephalitis and colitis, both of which have been observed mainly after type II strain infections (Hunter and Remington, 1994; Liesenfeld, 1999).

## MATERIALS AND METHODS

**Parasites and cells.** Parasites were maintained in vitro by serial passage on monolayers of HFFs at 37°C in 5% $CO_2$. RH or GT1 were used as representative type I strains, ME49 or Pru as representative type II strains, and CEP or VEG as representative type III strains. A Pru strain engineered to express firefly luciferase and GFP (Pru ΔHXGPRT A7; Kim et al., 2007), and CEP and RH strains engineered to express click beetle luciferase and GFP (CEP HXGPRT$^-$ C22 and RH 1–1; Boyle et al., 2007), have been described previously. Pru and RH strains expressing HXGPRT and generated from unsuccessful knockout transfections were used in assays comparing parasite growth. F1 progeny from type II × type III crosses were described previously (Sibley et al., 1992; Khan et al., 2005). A Pru strain expressing a type I copy of ROP16 was also described previously (Saeij et al., 2007). HFFs were grown in DME (Invitrogen) supplemented with 10% heat inactivated FBS (PAA), 2 mM L-glutamine, 50 μg/ml each of penicillin and streptomycin, and 20 μg/ml gentamycin. BMMs were obtained from female BALB/c, C57BL/6, or MyD88/TRIF double knockout (a gift from H. Ploegh, Whitehead Institute for Medical Research and Massachussetts Institute of Technology, Cambridge, MA) mice. BM was isolated by flushing hind tibias and femurs using a 27-gauge needle and/or by crushing the bones using a mortar and pestle, followed by passage over a cell strainer. Cells were suspended in DME supplemented with 20% L929 cell-conditioned medium, 10% heat-inactivated FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 1× MEM nonessential amino acids, and 50 μg/ml each of penicillin and streptomycin. 3–6 × $10^6$ cells were plated in 10 cm of nontissue culture–treated dishes (VWR) and incubated at 37°C, with 5% $CO_2$ in humidified air. After 6–7 d, cells were washed with PBS to remove nonadherent cells, harvested by dislodging with a cell scraper in PBS, and replated for the assay. 293T stable cell lines with four copies of the NF-κB consensus transcriptional response element driving the expression of GFP or GFP and luciferase (System Biosciences) were grown in DME supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 1× MEM nonessential amino acids, 10 mM HEPES, 50 μg/ml each of penicillin and streptomycin, and 20 μg/ml gentamycin. 293T cells were passed every 2–4 d using 0.05% trypsin-EDTA. WT MEFs were gifts from M. Karin (University of California, San Diego, School of Medicine, La Jolla, CA) and A. Sinai (University of Kentucky College of Medicine, Lexington, KY), IKK-$\beta^{-/-}$ MEFs were a gift from M. Karin (Li et al., 1999), NF-κBp65$^{-/-}$ MEFs were a gift from A. Sinai, and TRAF6$^{-/-}$ MEFs were provided by K. Fitzgerald (University of Massachussetts Medical School, Worcester, MA). MEFs were grown in DME supplemented with 10% nonheat inactivated FBS (PAA), 2 mM L-glutamine, 1 mM sodium pyruvate, 1× MEM nonessential amino acids, 10 mM Hepes, and 50 μg/ml each of penicillin and streptomycin. MEFs were passed using 0.05% trypsin-EDTA. All parasite strains and cell lines were routinely checked for Mycoplasma contamination and it was never detected.

**Reagents.** Antibodies against HA (3F10; Roche), mouse NF-κB p65 (sc-8008), human NF-κB p65 (sc-109), NF-κB p50 (sc-8414), NF-κB p52 (sc-7386), NF-κB RelB (sc-28689), NF-κB c-Rel (sc-71), *T. gondii* surface antigen (SAG)-1 (DG52; Burg et al., 1988), *T. gondii* dense granule protein GRA7 (Dunn et al., 2008), *T. gondii* rhoptry protein ROP1 (Tg49; Ossorio et al., 1992), phospho-IκBα (sc-8404), total IκBα (sc-847), and GAPDH (sc-32233) were used in the IF assay or in Western blotting. IF secondary antibodies were coupled with Alexa Fluor 488 or Alexa Fluor 594 (Invitrogen). Secondary antibodies used in Western blotting were conjugated to peroxidase (Kirkegaard & Perry Laboratories). Purified LPS (EMD), recombinant mouse TNF (AbD Serotec), and recombinant human TNF (Invitrogen) were used to stimulate cells. MG-132 (EMD) was used in proteasomal inhibition.

**IF.** Parasites were allowed to invade cells on coverslips and incubated for different time points. The cells were then fixed with 3% (vol/vol) formaldehyde in PBS for 20 min at room temperature, permeabilized with 100% ethanol and/or 0.2% (vol/vol) Triton X-100, and blocked in PBS with 3% (wt/vol) BSA and 5% (vol/vol) goat serum. Coverslips were incubated with primary antibody for 1 h at room temperature or overnight at 4°C, and fluorescent secondary antibodies and Hoechst dye were used for antigen and DNA visualization, respectively. Coverslips were mounted on a glass slide with Vectashield (Vector Laboratories), and photographs were taken using NIS-Elements software (Nikon) and a digital camera (CoolSNAP EZ; Roper Industries) connected to an inverted fluorescence microscope (model eclipse Ti-S; Nikon). Quantification of nuclear signal was performed by randomly selecting at least 10 infected cells per *T. gondii* strain and measuring the mean signal intensity per nucleus using the NIS-Elements software and Hoechst dye to define nuclei. For evacuole staining, this standard IF protocol was modified slightly. Parasites were added to HFFs on coverslips, spun down to bring them into contact with host cells, and allowed to attach to and invade host cells for 5 min at 37°C. Unattached parasites were washed off with PBS, and cells were fixed 3% (vol/vol) formaldehyde in PBS for 20 min at room temperature, blocked in PBS with 5% (vol/vol) fetal bovine serum and 5% (vol/vol) normal goat serum for 1–2 h at room temperature, and permeabilized by incubation in PBS with 0.2% (wt/vol) saponin at 37°C for 20 min. For proteasomal inhibition HFF monolayers were pretreated with 20 ng/ml of MG-132 for 1 h at 37°C. Cells were infected with parasites and spun down. 4 h after MG-132 addition, cells were washed with PBS and fresh media containing no inhibitor was added. The monolayer was incubated for one additional hour before fixation. To synchronize infection during time course assays, HFF monolayers were incubated on ice with cold media for 10 min before infection. For infection, supernatant from fully lysed parasite flasks was pelleted and washed three times with PBS and resuspended in cold media. After infection, monolayers were incubated on ice for 30 min, and then unattached parasites were washed off with cold PBS. Fresh prewarmed media was added and cells were incubated at 37°C to allow invasion and infection for the determined length of time.

**Generation of transgenic parasites.** The GRA15 coding region and putative promoter (1,940 bp upstream of the start codon) was amplified from type II *T. gondii* genomic DNA by PCR (forward, 5′-CCCAAGCTTGACTGCCACGTGTAGTATCC3′; reverse, 5′-TTA*CGCGTAGTCCGGGACGTCGTACGGGTA*TGGAGTTACCGCTGATTGTGT-3′). Sequence coding for an HA tag was included in the reverse primer (denoted with italics) to C-terminally tag the protein. $GRA15_{II}HA$ was then inserted into pCR8/GW (Invitrogen) by TOPO-TA cloning. Gateway cassette A was ligated into pTKO (gift from G. Zeiner, Stanford University School of Medicine, Stanford, California) at the EcoRV site, creating a Gateway destination vector (Invitrogen), pTKO-att (Fig. S10), and $GRA15_{II}HA$ was cloned into pTKO-att by LR recombination (Invitrogen). The pTKO-att-$GRA15_{II}HA$ vector was then linearized by digestion with XhoI (NEB). XhoI cuts off 244 bp of the putative promoter, leaving 1,696 bp intact upstream of the start codon. Linearized vector was transfected into RHΔHXGPRT and CEP HXGPRT$^-$ C22 parasites by electroporation. Electroporation was done in a 2-mm cuvette (Bio-Rad Laboratories) with 2 mM ATP (MP Biomedicals) and 5 mM GSH (EMD) in a Gene Pulser Xcell (Bio-Rad Laboratories), with the following settings: 25 μFD, 1.25 kV, ∞ Ω. Stable integrants were selected in media with 50 μg/ml mycophenolic acid (Axxora) and 50 μg/ml xanthine (Alfa Aesar) and cloned by limiting dilution. Expression of $GRA15_{II}$ was confirmed by IF for HA staining. Parasite strains already containing the HXGPRT gene (RH GRA15KO and Pru A7 GRA15KO) were cotransfected with 35 μg pTKO-att-$GRA15_{II}$ and 1 μg pTUB5-BLE (Soldati et al., 1995), containing the ble selectable marker. Stable integrants were selected extracellularly with 50 μg/ml Phleomycin (InvivoGen), and HA staining was confirmed by IF.

The ROP2 and GRA6 coding regions and putative promoters (at least 1,500 bp upstream of the start codon) were amplified from type II *T. gondii* genomic DNA by PCR (ROP2 forward, 5′-CACCGAGGTTGGAACTGTG-3′; ROP2 reverse, 5′-*CTTACGCGTAGTCCGGGACGTCGTACGGGTA*GATTGCCGTAACCGCCT-3′; GRA6 forward,

5′-CCCAAGCTTGAAGGACTGCGTTGAGTGTTTT-3′; and GRA6 reverse, 5′-GGAATTC*TTACGCGTAGTCCGGGACGTCGTAC-GGGTA*AAAAATCAAACTCATTCACACTTC-3′). Sequence coding for an HA tag was included in the reverse primers (denoted with italics) to C-terminally tag the proteins. ROP2$_{II}$HA was then inserted into pENTR/D (Invitrogen) and GRA6$_{II}$HA was inserted into pCR8/GW (Invitrogen) by TOPO cloning, and then cloned into pTKO-att (see Fig. S10) by LR recombination (Invitrogen). The pTKO-att-ROP2$_{II}$HA vector was then linearized by digestion with NotI (NEB). Linearized vector was transfected into parasites by electroporation. Electroporation and selection was done as in the previous paragraph, and HA staining was confirmed by IF.

**Generation of GRA15 knockout.** A targeting construct (Fig. S5 A) was engineered using a modified pTKO-att vector, pTKO2, and Multisite Gateway Pro 3-Fragment Recombination (Invitrogen). The hypoxanthine-xanthine-guanine ribosyl transferase (HXGPRT) selectable marker was removed from pTKO-att by Cre recombinase (NEB) to form pTKO2 (Fig. S10). 5′ and 3′ flanking regions of GRA15 were cloned from type I and type II genomic DNA. Primers contained att recombination sites (denoted in primer sequence with italics) and amplified 2,083 bp, 100 bp upstream of the GRA15 start codon and 2,071 bp, 34 bp downstream of the GRA15 stop codon (5′ forward, 5′-*GGGGACAAGTTTGTACAAAAAAGCAGGCTTA-A*GGGTCTGAACGTGTGCA-3′; 5′ reverse, 5′-*GGGGACAACTTT-GTATAGAAAAGTTGGGTG*ACCCGGCTTAAGTTGGTG-3′; 3′ forward, 5′-*GGGGACAACTTTGTATAATAAAGTTGC*ATGAC-CAAAAACCGATAA-3′; and 3′ reverse, 5′-*GGGGACCACTTTG-TACAAGAAAGCTGGGTA*CAAGTCGGCACATGCTTAGA-3′). These flanking regions were then cloned around the HXGPRT selectable marker flanked by 5′ and 3′ UTRs from DHFR, amplified from pTKO with primers containing att recombination sites (denoted in primer sequence with italics; DHFR::HPT forward, 5′-*GGGGACAACTTTTC-TATACAAAGTTGC*TCAGCACGAAACCTTGCAT-3′; and DHFR::HPT reverse, 5′-*GGGGACAACTTTATTATACAAAGTTGTGTGT-*CACTGTAGCCTGCC-3′). Before transfection, the knockout vector was linearized with the restriction enzyme NotI (NEB). RHΔHXGPRT, PruΔHXGPRT, and PruΔHXGPRT A7 parasites were transfected with the knockout construct by electroporation, as described in the previous section. Stable integrants were selected as in the previous section and cloned by limiting dilution. PCR with a forward primer upstream of the 5′ flanking region (P1, 5′-CATGGATGCTAATCGGCTTT-3′) and a reverse primer within the HXGPRT cassette (P2, 5′-GATCCAGACGTCTTCAATGC-3′; and P3, 5′-GGGGACAACTTTATTATACAAAGTTGTGTGT-CACTGTAGCCTGCC-3′) confirmed a disruption in the GRA15 locus. Additionally, PCR was done to confirm the inability to amplify GRA15 (P4, 5′-GATGATGGATCCATAATTCGGTGGCTTGGG-3′; and P5, 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTATCGGCACAT-GCTTAGAAG-3′; Fig. S5 B).

**Microarray.** For human arrays, HFFs were grown in a T25 to confluency. Parasite strains were syringe lysed and washed once with PBS. HFFs were infected with Pru ΔHXGPRT A7, Pru ΔHXGPRT, Pru A7 GRA15KO, Pru GRA15KO, RH 1–1, RH ΔHXGPRT, RH GRA15$_{II}$ (a transgenic RH strain expressing a type II copy of GRA15), RH GRA15KO, CEP HXGPRT C22, or CEP C22 GRA15$_{II}$ (a transgenic CEP strain expressing a type II copy of GRA15) at varying MOIs. For mouse arrays, WT or p65$^{-/-}$ MEFs were grown in a 12-well plate to confluency. Parasite strains were syringe lysed and washed twice with PBS. MEFs were infected with Pru ΔHXGPRT A7, Pru A7 GRA15KO, RH 1–1, or RH GRA15$_{II}$ at varying MOIs.

Plaque assays were done to assess viability of parasites and infections with similar MOIs were chosen. Some samples were also stimulated with TNF. At least two biological replicates were done for every sample, except RH GRA15KO infection, TNF stimulation, RH ΔHXGPRT preinfection followed by TNF stimulation, and all MEF samples. 18–24 h after infection or 6 h after stimulation, total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol and cleaned up using RNeasy Mini or MinElute kit (QIAGEN). RNA was labeled and hybridized to a human or mouse Affymetrix array (Human U133A 2.0 or Mouse 430A 2.0) according to the manufacturer's protocol. Probe intensities were measured with the Affymetrix GeneChip Scanner 7G and were processed into image analysis (.CEL) files with either GeneChip Operating Software or Expression Console Software (Affymetrix). Intensity values were normalized using the MAS5 algorithm such that the median intensity on the array was 500 using Expression Console software. The MAS5 algorithm gives a signal intensity value for every probe as well as a present, marginal, or absent call, based on mismatch probes. For all probes called present, signal intensity values <50 were increased to a minimum value of 50. For all probes called marginal or absent, the signal intensity value was set to 50. Expression data were clustered using MultiExperiment Viewer (Saeed et al., 2003, 2006). Microarray data will be uploaded to a public data depository.

Gene set enrichment analysis (GSEA) was used to find candidate transcription factors and canonical pathways that are modulated differently between *T. gondii* infections (Mootha et al., 2003; Subramanian et al., 2005). This program uses a priori–defined sets of genes and determines whether the members of these sets of genes are randomly distributed throughout a ranked list or primarily found at the top or bottom. As GSEA is generally used to generate hypotheses, gene sets enriched with a false discovery rate <0.25 were considered significant. Both transcription factor and canonical pathway gene sets from the Molecular Signatures Database were evaluated for enrichment (c2.cp.v2.5.symbols, c3.tft.v2.5.symbols.gmt; Subramanian et al., 2005). The gsea2 java release was run using all default settings. For analyses on RH GRA15KO, TNF, and RH ΔHXGPRT + TNF, and all MEF infections, for which we only did one array, MOI-matched arrays and the GSEA preranked function were used.

Distant regulatory elements of coregulated genes (DiRE; Gotea and Ovcharenko, 2008) and ingenuity pathway analysis (IPA) were also used. For every gene in a list, DiRE detects regulatory elements throughout the entire gene locus and looks for enrichment of TFBSs. IPA takes a list of genes and overlays them onto a global molecular network developed from information contained in the Ingenuity Pathways Knowledge Base. Networks of these genes are then algorithmically generated based on their connectivity. A network is a graphical representation of the molecular relationships between genes/gene products. Genes or gene products are represented as nodes, and the biological relationship between two nodes is represented as an edge. All edges are supported by at least one reference from the literature, from a textbook, or from canonical information stored in the Ingenuity Pathways Knowledge Base. The two networks with the highest score are indicated.

**5′ and 3′ RACE.** Total RNA was isolated from HFFs infected with Pru parasites using TRIzol reagent (Invitrogen) and cleaned up using the RNeasy Mini kit (QIAGEN). RACE-ready cDNA was synthesized using a GeneRacer kit with SuperScript III RT (Invitrogen). Nested PCR was done on the RACE-ready cDNA to determine both 5′ and 3′ transcript ends using gene-specific primers (5′ GRA15reverse, 5′-AGTCCTCCCCGTTTTCGGTCTGTT-3′; 5′ GRA15 nested reverse, 5′-GACTCTGAACGGGGACGGGTAGTC-3′; 3′ GRA15 forward, 5′-CTGTCCACTCAATAGACCCCGTTGT-3′; and 3′ GRA15 nested forward, 5′-AAGATGCCGTGCAAAGCCAACTTC-3′), provided GeneRacer 5′ and 3′ primers, and Phusion enzyme (Finnzymes). PCR products were cloned into the pCR4Blunt-TOPO vector (Invitrogen) and sequenced. Sequences were analyzed with Sequencher software (Gene Codes).

**Characterization of GRA15 sequence.** The coding sequence for GRA15 from types I (GT1), II (ME49), and III (VEG) was predicted from ToxoDB genomic sequence using ORF Finder (National Center for Biotechnology Information). GRA15 genomic DNA from additional strains (RH, Pru, CEP) was amplified by PCR and sequenced (forward, 5′-TCCGACT-CAGTGCGGGAAA-3′; and reverse, 5′-ATCCAGGTCCCCAAAGG-3′). To check for the presence of a predicted intron in the type I/III ORF,

type I cDNA was amplified by PCR and sequenced (forward, 5′-CACGTACACAACCCATCTCG-3′; and reverse, 5′-CGAATTCTCATGGAGTTACCGCTGATT-3′). 5′ and 3′ UTRs were determined by RACE, as described in the previous section. Amino acid alignments were done with ClustalW2 (EMBL-EBI). Similarity to known sequences was queried using BLAST (National Center for Biotechnology Information; Altschul et al., 1990) and PfamA (Finn et al., 2008).

**Western blot.** HFFs in a 6-well plate were infected with parasites (MOI = 5) for 24 h. Infected cells were washed with ice-cold PBS, lysed by addition of lysis buffer, boiled for 5 min, and subjected to 10% SDS-PAGE. Proteins were transferred to a polyvinylidene difluoride membrane, which was blocked in PBS/0.1% Tween-20/5% nonfat dry milk and incubated with primary and secondary antibodies. The blot was incubated with a luminal-based substrate (Immun-Star WesternC; Bio-Rad Laboratories) and chemiluminescence was detected using a charge-coupled device camera (Chemidoc XRS; Bio-Rad Laboratories). The bands were visualized using Quantity One 1-D analysis software and analyzed using ImageJ (National Institutes of Health).

**GRA15 expression in HeLa cells.** A type II copy of GRA15 or a type II copy of 55.m04955 was inserted into pIC242 (gift from I. Cheeseman, Whitehead Institute for Biomedical Research and Massachusetts Institute of Technology, Cambridge, MA), a Moloney Murine Leukemia Virus retroviral vector containing an N-terminal GFP protein fusion, by restriction/ligation. Amino acids 51–551 of $GRA15_{II}$ were included, as amino acids 1–50 were predicted to be a signal sequence by the signal peptide cleavage prediction server SignalP (Nielsen and Krogh, 1998; Bendtsen et al., 2004). These insertions replaced the original gene insert, Mob1A. Expression of GFP fusion proteins was promoted by the endogenous retroviral long terminal repeats. HeLa cells were then transiently transfected with expression vectors by lipofection using Fugene 6 Plus Transfection Reagent (Roche). Confluent cell cultures were split 1:10 into a 24-well plate (BD) containing glass coverslips. The cells were allowed to incubate at 37°C and 5% $CO_2$ for 4 h. After incubation, the medium was replaced with 1 ml of fresh supplemented DME, and liposomes were added dropwise to the cells. Liposomes were generated according to manufacturer protocol. In brief, 3 µl Fugene reagent was mixed into 20 µl of unsupplemented DME and allowed to stand at room temperature for 5 min. Next, 0.5 µg of appropriate plasmid DNA for each transfection was added, mixed, and incorporated into liposomes for 20 min before addition to cells. Cells were left in contact with liposome for 24 h until the cells were fixed and stained for NF-κB p65. This experiment was performed two times.

**In vitro cytokine ELISA.** BALB/c BMDMs were seeded ($10^5$ per well) in 96-well plates and left to adhere overnight at 37°C in 5% $CO_2$. Cells were infected with freshly lysed *T. gondii* tachyzoites at MOI = 20, 10, and 5, and supernatants (200 µl) were collected 24–48 h after infection and stored at −20°C if necessary. IL-12p40 levels were determined, for the cells infected with equal numbers of viable parasites as determined by plaque assay, using a commercially available ELISA kit (OptEIA Mouse IL-12 (p40) ELISA Set; BD) according to the manufacturer's instructions.

**Infection of mice.** Female BALB/c or C57BL/6 mice that were 6–10 wk old (The Jackson Laboratory) were used in all experiments. For i.p. infection, tachyzoites were grown in vitro and extracted from host cells by passage through a 27-gauge needle, washed three times in PBS, and quantified with a hemocytometer. Parasites were diluted in PBS, and mice were inoculated i.p. with tachyzoites of each strain (in 300 µl) using a 28-gauge needle. To image mice infected with a parasite strain that expressed the enzyme luciferase, mice were injected i.p. with 3 mg firefly D-luciferin dissolved in PBS, anesthetized with isoflurane, and imaged with an IVIS Spectrum-bioluminescent and fluorescent imaging system (Xenogen Corporation). Images were processed and analyzed with Living Image software. The MIT Committee on Animal Care approved all protocols. All mice were maintained in specific pathogen-free conditions, in accordance with institutional and federal regulations.

**i.p. wash and in vivo cytokine ELISA.** 1 or 2 d after i.p. infection, mice were sacrificed and the i.p. cavity was washed with 5 ml PBS. The i.p. wash was spun at 450 *g* for 5 min to pellet cells. Supernatant was collected and stored at −80°C if necessary. IFN-γ, IL-12p40, and IL-12p70 levels were determined using commercially available ELISA kits (ELISA Ready-SET-Go!; eBioscience) according to the manufacturer's instructions.

**Plaque assay.** For all assays comparing the effect of *T. gondii* on the host cell, cells were infected with different MOIs and a plaque assay was done to determine the viability of each strain. The infections with the closest MOIs were then used. For the plaque assay, 100 parasites per well were added to confluent HFFs in a 24-well plate and were incubated for 5–7 d at 37°C. The number of plaques was counted using a microscope. Plaque assays were also performed to assess the viability of parasites used to infect mice. To assay in vitro parasite growth, plaque size was measured using NIS-Elements software (Nikon) and a digital camera (CoolSNAP EZ; Roper Scientific) connected to an inverted fluorescence microscope (eclipse Ti-S; Nikon).

**Online supplemental material.** Fig. S1 shows that a type II locus on chromosome X strain-specifically induces expression of NF-κB–regulated genes. Fig. S2 shows mapping the strain-specific activation of NF-κB. Fig. S3 shows that type III strains complemented with *GRA6* or *ROP2* do not activate NF-κB. Fig. S4 shows time course of NF-κB activation by $GRA15_{II}$. Fig. S5 shows generation and confirmation of *GRA15*KO. Fig. S6 shows GRA15 activation of NF-κB family subunits. Fig. S7 shows GRA15 amino acid alignment. Fig. S8 shows that GRA15 affects in vitro parasite growth and inhibits in vivo parasite growth in a type I background. Fig. S9 shows that ROP16 can affect the expression of GRA15-regulated genes. Fig. S10 shows pTKO, pTKO-att, and pTKO2 vectors. Supplemental data 1 shows GSEA gene sets. Supplemental data 2 shows complete heat map gene lists. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20100717/DC1.

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## SUPPLEMENTAL MATERIAL

**Rosowski et al., http://www.jem.org/cgi/content/full/jem.20100717/DC1**

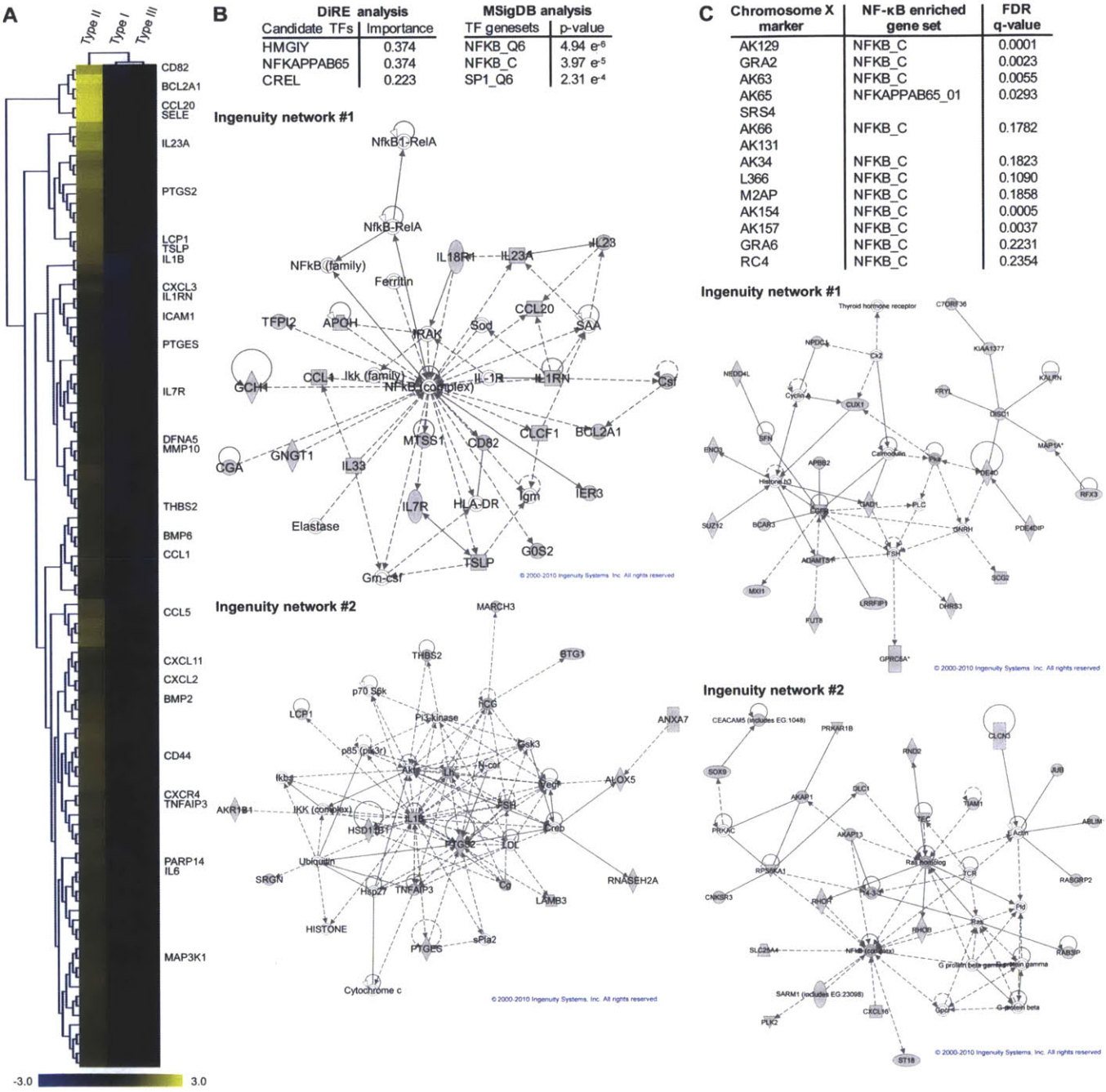



**Figure S1. A type II locus on chromosome X strain specifically induces expression of NF-κB–regulated genes.** (A and B) HFFs were infected with type I, II, or III *T. gondii* and host gene expression was determined by microarray analysis (three arrays per strain). (A) 105 genes are more than two-fold up-regulated in type II strains compared with type I/III strains. Mean $\log_2$ gene expression values were median centered, genes and strains were clustered by hierarchical clustering, and a heat map is was made, which is presented. The complete set of genes is listed in Supplemental data 2. (B) For this list of genes, the top three DiRE-enriched TFBS and molecular signatures database–enriched TFBS are shown and the top two ingenuity networks are shown. (C) HFFs were infected with F1 progeny from a II × III cross and host gene expression was determined by microarray analysis (one array per strain). Top: for each chromosome X marker, F1 progeny were split into two groups based on their genotype. TFBS enriched in genes differentially expressed by infection with progeny with a type II genotype were determined. NF-κB gene sets, among others, were found to be significant. Bottom: ingenuity pathway analysis was done using genes that had a sign LOD score for any chromosome X marker. The top two networks are shown.

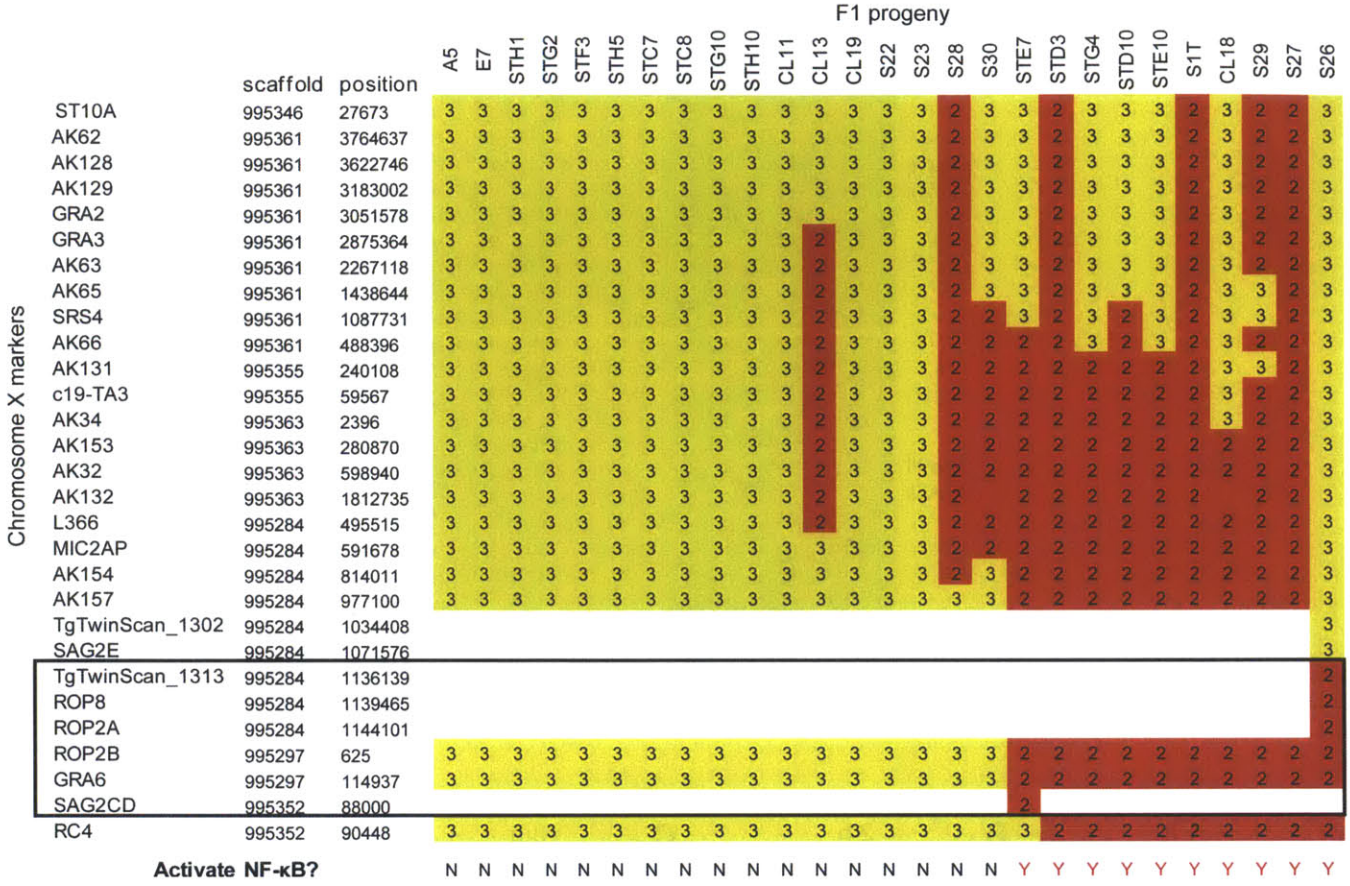



**Figure S2. Mapping the strain-specific activation of NF-κB.** The NF-κB activation phenotype of 27 F1 progeny derived from crosses between type II (NF-κB activating) and type III (NF-κB nonactivating) strains was determined by IFA. These progeny have also been genotyped at markers throughout the genome as either type II (red) or type III (yellow; Toxoplasma Genome Mapping Database). New RFLP markers were developed and used to further genotype progeny STE7 and S26. A black box outlines the minimal region in which the locus responsible for strain-specific NF-κB activation must reside.

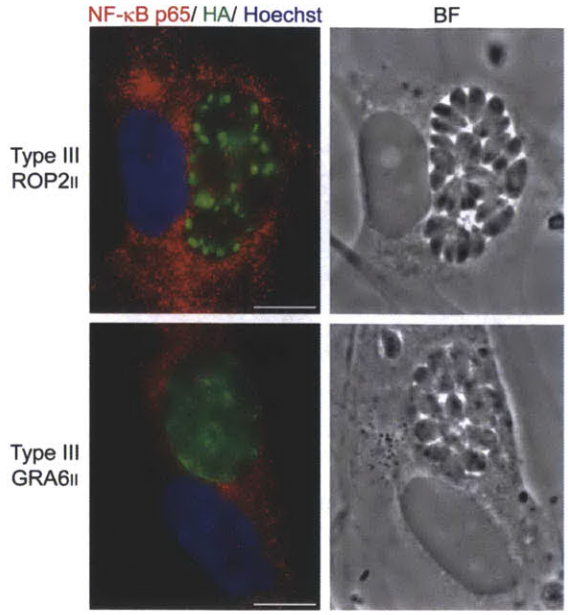



**Figure S3. Type III strains complemented with *GRA6* or *ROP2* do not activate NF-κB.** HFFs were infected with transgenic type III strains stably expressing an HA-tagged type II copy of either *ROP2* or *GRA6*. Cells were fixed 18–24 h later and stained with α-NF-κB p65 (red), α-HA (green), and Hoechst dye (blue). Bars, 10 µm. This experiment was done once.

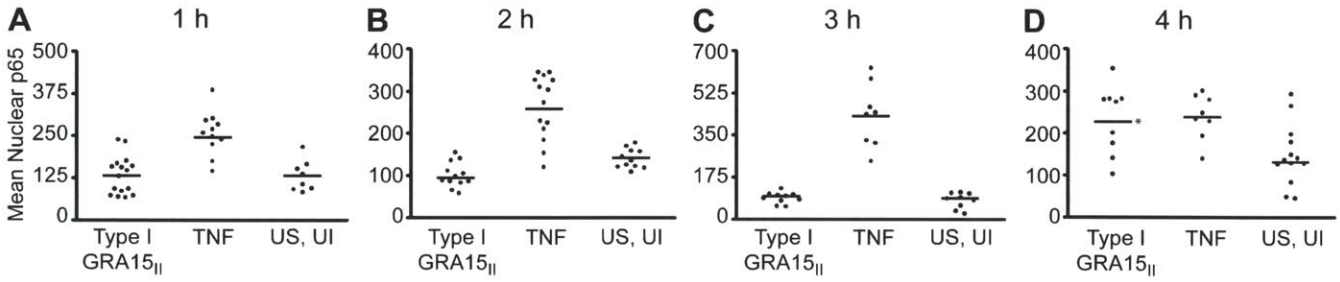



**Figure S4. Time course of NF-κB activation by GRA15$_{II}$.** HFF cells were infected with a type I *GRA15$_{II}$ T. gondii* strain, stimulated with TNF, or left unstimulated (US) and uninfected (UI). For infections, cells were infected on ice with fully lysed parasites. Cells and parasites were kept on ice for 30 min and then allowed to infect host cells at 37°C. Coverslips were fixed every hour for 4 h. A, B, C, and D correspond to 1-, 2-, 3-, and 4-h time points, respectively. The time course was repeated two times and quantification was performed on a representative experiment. Asterisk (*) indicates that mean nuclear p65 in infected cells was significantly different from in unstimulated and uninfected cells ($P < 0.05$, Student's *t* test). Horizontal bars represent the mean nuclear p65 intensity over all cells.

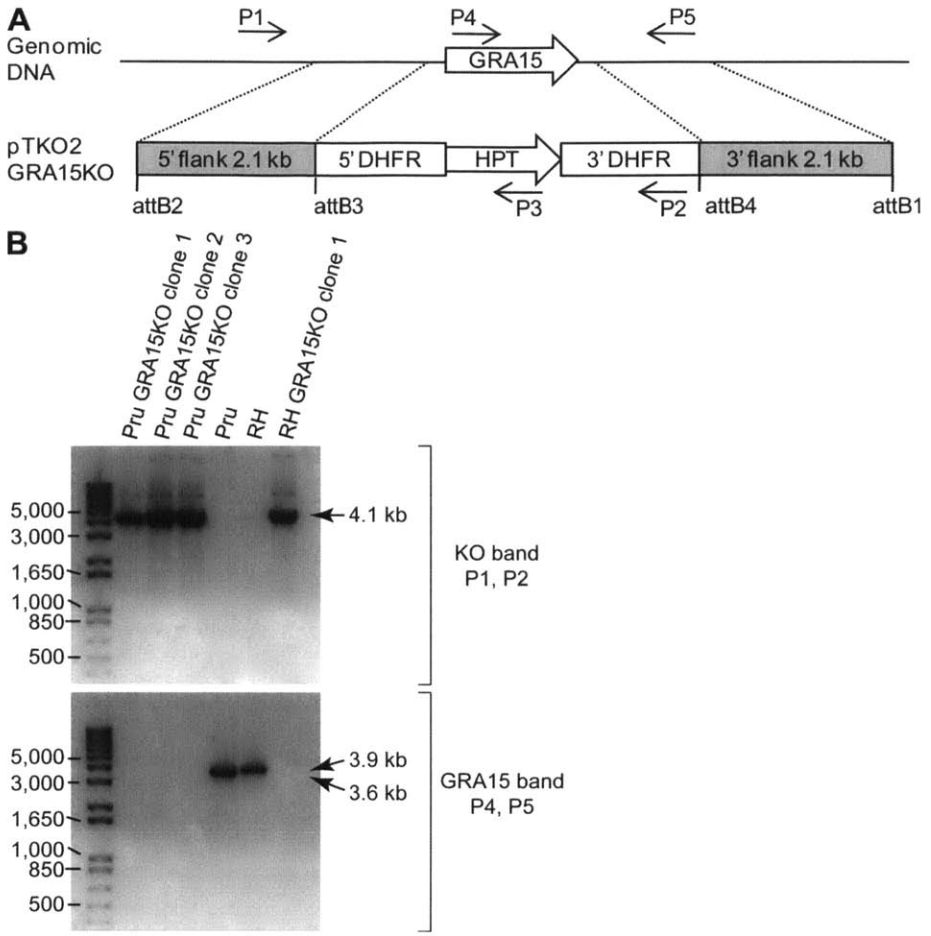



**Figure S5. Generation and confirmation of *GRA15*KO.** (A) Schematic of the GRA15 locus, not drawn to scale. Double homologous recombination between the knockout construct (pTKO2 GRA15KO) and genomic DNA replaces *GRA15* with the *HXGPRT* (*HPT*) gene, which was used for positive selection. Primers used in PCRs to confirm a knockout are shown (P1–P5). (B) After transfection of *T. gondii* strains with the GRA15KO vector, parasites were cloned by limiting dilution, and genomic DNA was isolated. This genomic DNA was then used as template in a PCR reaction to amplify a band that is only present in a successful knockout (top, P1 and P2 from Fig. 3, amplifies a 4.1-kb fragment). The intact *GRA15* locus was also amplified as a negative control (bottom, P4 and P5 from Fig. 3, amplifies a 3.9-kb fragment in type I/III strains and a 3.6-kb fragment in type II strains).

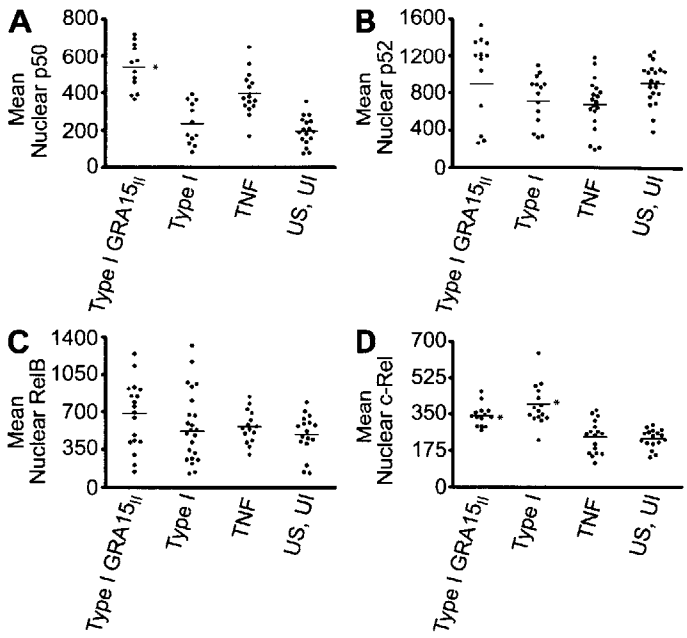
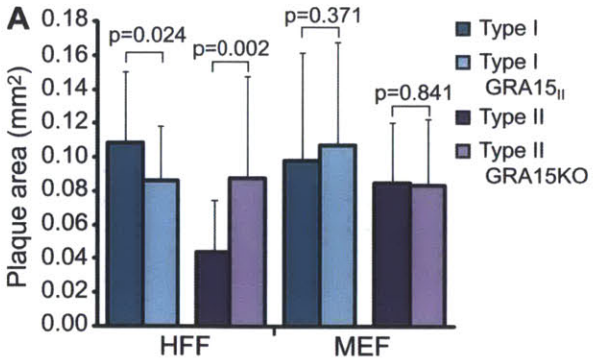



**Figure S6. GRA15 activation of NF-κB family subunits.** HFF cells were infected for 24 h with type I or type I *GRA15* $_{II}$ parasites, stimulated with TNF for 1 h, or left unstimulated (US) and uninfected (UI). Cells were fixed and probed with antibodies to different NF-κB subunits (A, p50; B, p52; C, RelB; D, c-Rel) and mean nuclear staining was quantified. These experiments were repeated three times and quantification was performed on a representative experiment for each factor assayed. Asterisks (*) indicate mean nuclear levels of the subunit are significantly different from levels in unstimulated and uninfected cells ($P < 0.05$). Horizontal bars represent the mean nuclear intensity over all cells.

```
TypeI_GT1  MVTTTTPTPPPGAPAVVPIFDVVYQLNPHVFRSRFSRRNRARRVVSSKSRSIIRWLGY
TypeI_RH   ................................................................
TypeII     ..........-.....................................A.............
TypeIII    ................................................................

TypeI_GT1  LTVLAAVILLGAYAVRRLSRDLSDSVRETRRGRRITGSVPPGTTRPRSESCTGTQVDG
TypeI_RH   ................................................................
TypeII     ................V...............................................
TypeIII    ................................................................

TypeI_GT1  GCGADTSTDGKSESEQTENGEDSRFSTRTPIHVTASTSPFATRKAAEERSSSPRDRKV
TypeI_RH   ................................................................
TypeII     ................................................................
TypeIII    ................................................................

TypeI_GT1  PEGAQLPTSSTPHAQRKDSGSDSRNPSTLIPSPGTNTFNMNFYIIGAGSSALDFIFPH
TypeI_RH   ................................................................
TypeII     ................................................................
TypeIII    ................................................................

TypeI_GT1  TPDAQATVVSPPRSAAAAPTVETVPRVRTYSTPTTLTLPTAPATATSNHMHASATPSP
TypeI_RH   ................................................................
TypeII     ................................................................
TypeIII    ........................L.......................V....

TypeI_GT1  PERPQNFRGGLMRQNGMVEGTSLTTTEAGMPAPLQSPQHIETEARLTYSNHLKSPHTP
TypeI_RH   RNVLKTSV.DSCGKTAWLR.HR----------------------------------------
TypeII     ................................................................
TypeIII    ..............................................................S..T

TypeI_GT1  ETPTVHSIDPVVGTSGHSVAVGSQSPAGGPPTDSRTPAALTPTSSSFSHADSLETSEH
TypeI_RH   ------------------------------------------------------------
TypeII     ................................................................
TypeIII    ................................................................

TypeI_GT1  PQSGPSLHPLISGIQDAVQSQLPLSQQETLPVVENATFFGPQQTPPWMDETAAAAIPL
TypeI_RH   ------------------------------------------------------------
TypeII     ................................................................
TypeIII    ................................................................

TypeI_GT1  APSQPGSRTQPISSPHTLLPLSGGVSAVPGPPRTENPRQPQVPGENSYYSVPTEPYPA
TypeI_RH   ------------------------------------------------------------
TypeII     ..................SR..............................------
TypeIII    ................................................................

TypeI_GT1  QDMSPLIRGTHSQTETVECGVNASSEGLAAGAPSSKSAENAQTGQGAGKSLLPVFLHP
TypeI_RH   ------------------------------------------------------------
TypeII     ------------------------------------------------------------
TypeIII    ................................................................

TypeI_GT1  QEQSPHSMPTLGAGRFGSGELQRTISDPGPQRAGATQADGIGAGGPRDTQSAVTP
TypeI_RH   ------------------------------------------------------------
TypeII     ----------------------.............A..........................
TypeIII    ................................................................
```

**Figure S7. GRA15 amino acid alignment.** The coding sequence of *GRA15* from types I (GT1), II (ME49), and III (VEG) was predicted by ToxoDB genomic sequence using ORF Finder (National Center for Biotechnology Information). *GRA15* was also sequenced in RH (I), Pru (II), and CEP (III). RH had a frameshift mutation at amino acid 290, resulting in the mutation of 20 amino acids and a premature stop codon at amino acid position 312. The type II sequence contains an 84–amino acid deletion of amino acids 519–602, as well as a single amino acid deletion and five single amino acid polymorphisms. Conserved (.) and missing (-) residues are indicated. The alignment was done by ClustalW2 (EMBL-EBI).




B

| Organ | Competitive Index Type I $GRA15_{II}$ vs. Type I |
|---|---|
| I.P. cavity | 0.22 ± 0.06* |
| lung | 0.096 ± 0.070* |
| spleen | 0.19 ± 0.02* |

**Figure S8. GRA15 affects in vitro parasite growth and inhibits in vivo parasite growth in a type I background.** (A) 100 parasites of a type I, type I *GRA15*$_{II}$, type II, or type II *GRA15*KO strain were added to confluent fibroblast monolayers (either HFFs or MEFs) in a 24-well plate and incubated at 37°C (4 d for type I infections, 6 d for type II infections in MEF host cells, and 7 d for type II infections in HFF host cells). The size of at least 15 plaques per strain per host cell was measured. This experiment was done three times in HFF host cells and twice in MEF host cells with similar results. P-values are from a two-sample Student's *t* test. Error bars represent standard deviation from one experiment. (B) One BALB/c mouse and one C57BL/6 mouse were coinfected with 500 type I $GFP^+$ parasites and 500 type I $GRA15_{II}$ $GFP^-$ parasites by i.p. injection. A plaque assay was done to confirm the ratio of $GFP^+$/$GFP^-$ parasites at injection. 5 d later, mice were sacrificed, an i.p. wash was done, and the spleen and a lung were isolated. The spleen and lung were ground through a cell strainer (70 µm and 100 µm, respectively) and then spun along with the i.p. wash to pellet parasites. Pellets were resuspended in fresh media, added to HFF monolayers in T175 flasks, and incubated at 37°C. 4–7 d later, T175s were scraped, parasites were pelleted and counted, and 2,000 parasites were added to HFF monolayers in T25 flasks. After 3–4 d at 37°C, $GFP^+$ and $GFP^-$ plaques were counted. Competitive index values were calculated and the means from two mice are presented. Asterisks indicate the CI is significantly different from 1 ($P < 0.05$, one sample Student's *t* test). This experiment was done once.

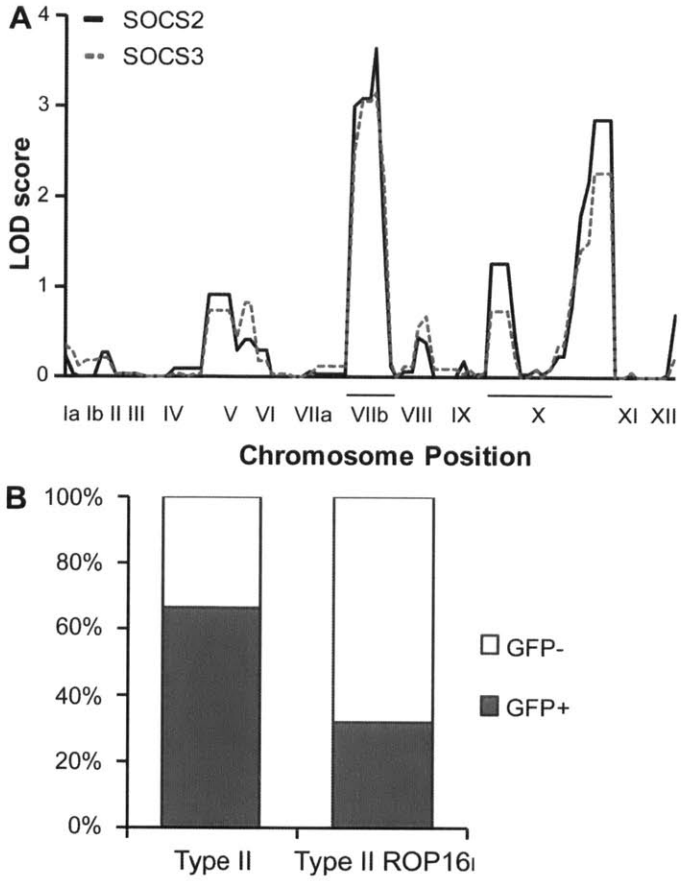



**Figure S9. ROP16 can affect the expression of GRA15-regulated genes.** (A) SOCS2 and SOCS3 map to loci on chromosome VIIb and X. Previously, microarrays and quantitative trait locus analysis were done on F1 progeny from a type II × type III cross to map the differential expression of host genes (Saeij et al., 2007). LOD scores for the SOCS2 and SOCS3 human genes are plotted for markers across the *T. gondii* genome. (B) ROP16 inhibits GRA15-activated NF-κB in a reporter cell line. An NF-κB GFP reporter cell line was infected with type II or type II $ROP16_I$ parasites. For each strain, the GFP level of at least 50 cells infected with only one vacuole containing at least two parasites was determined. The percentage of GFP-positive and GFP-negative host cells for each strain is shown. The relative percentages of GFP-positive and GFP-negative cells after infection with these two strains is significantly different ($P = 1.5 \times 10^{-7}$, $\chi^2$ test). This experiment has been done twice, and in two additional experiments similar results were obtained after infection of an NF-κB luciferase reporter cell line.

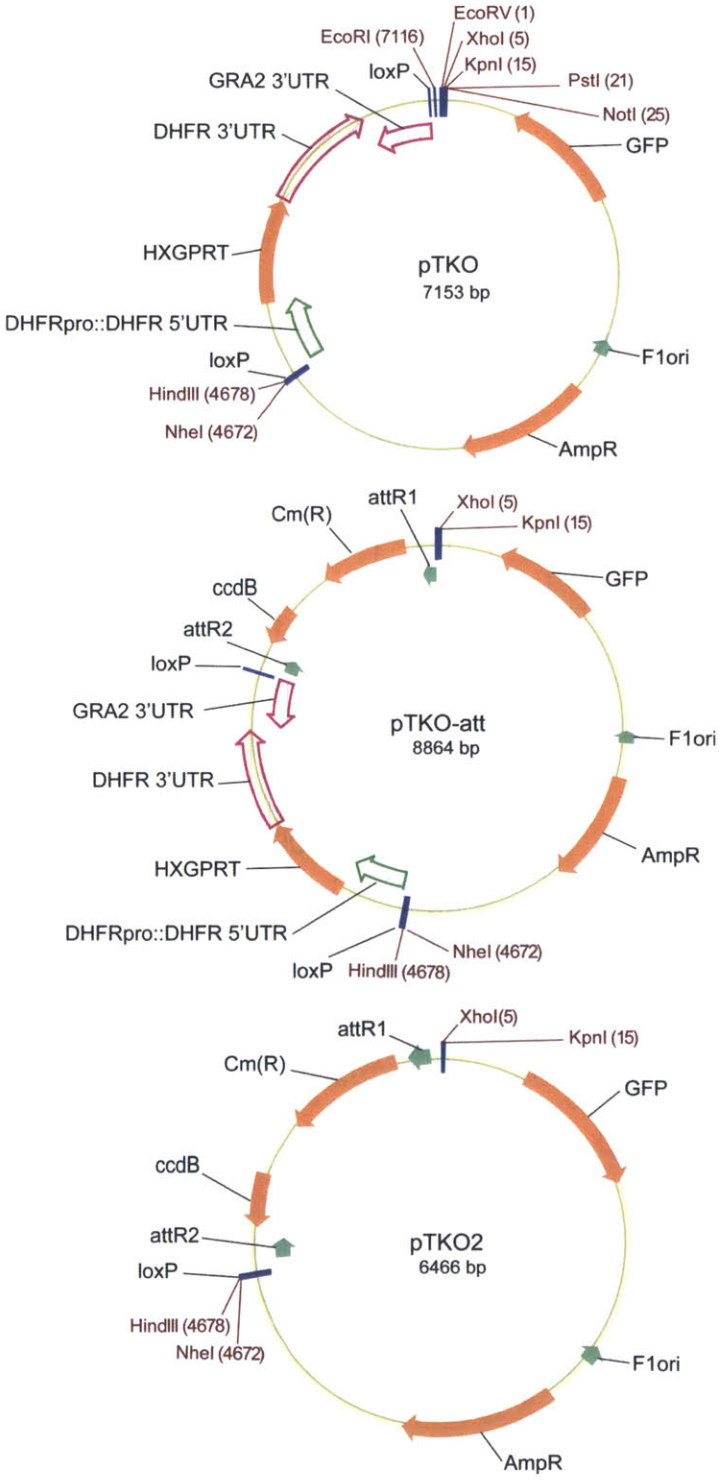



**Figure S10. pTKO, pTKO-att, and pTKO2 vectors.** pTKO contains the *HXGPRT* selectable marker, flanked by the *DHFR* promoter and UTRs. Gateway cassette A, containing the *ccdB* and *Cm(R)* genes flanked by attR1 and attR2 recombination sites, was ligated into pTKO at the EcoRV site, creating a Gateway destination vector, pTKO-att. The *HXGPRT* cassette was also removed from the pTKO-att vector by Cre recombination at loxP sites to form pTKO2.

**Excel files are also provided that contain the GSEA gene sets (Supplemental data 1) and complete heat map gene lists (Supplemental data 2).**

# Chapter II Addendum

## Results and Discussion

### IKK-α and IKK-β are partially redundant and functionally downstream of GRA15

In Chapter II, we find that GRA15$_{II}$ still retained some ability to activate NF-κB in IKK-$\beta^{-/-}$ mouse embryonic fibroblasts (MEFs). To confirm the results of Rosowski, et al, we assayed p65 nuclear density in infected IKK-$\alpha^{-/-}$ and IKK-$\alpha^{-/-}$/IKK-$\beta^{-/-}$ MEFs, as well as IKK-$\gamma^{-/-}$ rat embryonic fibroblasts (REFs) (Figure 1).

In IKK-$\alpha^{-/-}$ MEFs, the type I GRA15$_{II}$ strain activated nuclear p65 accumulation 3.4 times more than in non-infected, non-stimulated MEFs. However, type I infection also activated twice as much p65 translocation in uninfected, unstimulated cells. Therefore, the amount of GRA15$_{II}$-dependent activation in these cells is only 1.7 times higher than infection with a wildtype type I strain (Figure 1A). This is similar to the observation that infection with type I GRA15$_{II}$ induces 1.9 times more nuclear p65 than unstimulated IKK-$\beta^{-/-}$ MEFs. However, this is only partially GRA15$_{II}$-mediated, as type I GRA15$_{II}$ strain activated only 1.4 times the amount of p65 translocation as infection with wildtype type I strain (Figure 1B). In comparison, type I GRA15$_{II}$ strain induced 2.8 times as much nuclear p65 accumulation as wildtype type I strain(Figure 1A, B).

On the other hand, we see that in IKK-$\alpha^{-/-}$/IKK-$\beta^{-/-}$ MEFs, p65 activation is completely attenuated during infection with either strain, as well as with TNF-α stimulation (Figure 1C). In IKK-$\gamma^{-/-}$ REFs, the activation of NF-κB by GRA15$_{II}$ is similarly abolished. Type I GRA15$_{II}$ induced 1.2 times the p65 accumulation of wildtype strain infection and 1.6 times that of uninfected, unstimulated IKK-$\gamma^{-/-}$ REFs. In

comparison, GRA15$_{II}$ induces nuclear NF-κB accumulation at a rate 2.7 times higher than type I infection alone and 3.1 times higher than uninfected, unstimulated wildtype REFs (Figure 1D).

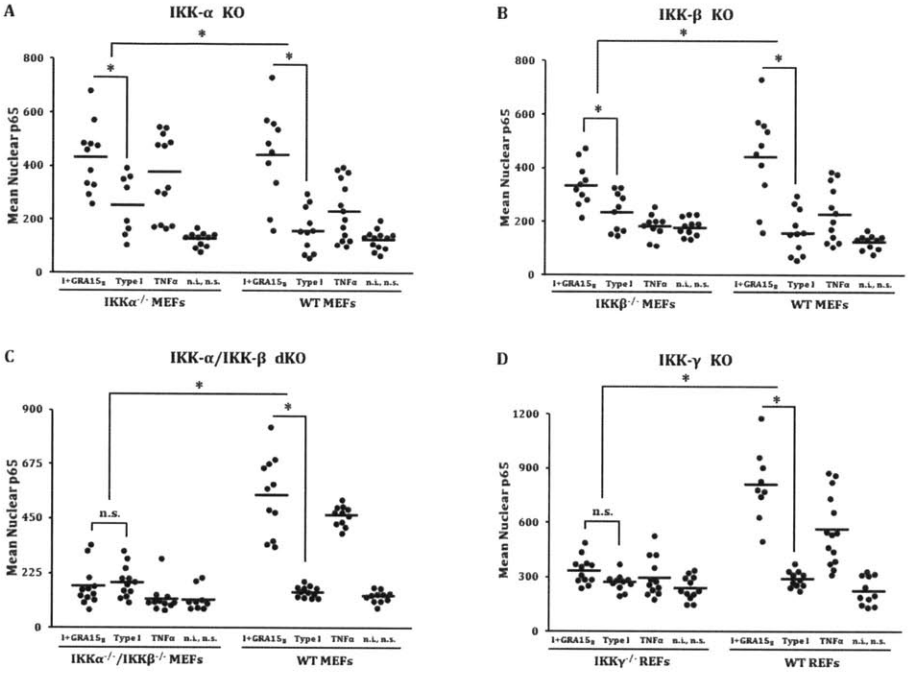



**Figure 1: GRA15 activity requires IKK-γ, while IKK-α and IKK-β are partially redundant.** Cells were infected with type I GRA15$_{II}$ or type I strain for 4 h, stimulated with 20 ng/ml human TNF-α for 1 h, or left uninfected and unstimulated (n.s., n.i.). Cells were then fixed and probed with an NF-κB p65 antibody and mean nuclear p65 density was measured. The activity of GRA15$_{II}$ in the absence of the three I-κB kinases was assayed in IKK-α$^{-/-}$ (A), IKK-β$^{-/-}$ (B), and IKK-α$^{-/-}$/IKK-β$^{-/-}$ (C) MEF, and IKK-γ$^{-/-}$ REF (D) host cultures. Each assay was repeated at least twice and quantification was performed on a representative experiment. Asterisks (*) indicate data are significantly different (*p-value* < *0.05*, Student's t test), and n.s. no significant difference. Horizontal bars represent the mean nuclear p65 intensity over all cells.

IKK-β and IKK-α kinase activity are both capable of phosphorylating I-κB-α, though IKK-β is a much more effective kinase (Li et al, 1999a; Li et al, 1999b). In the canonical pathway of NF-κB activation, IKK-γ acts as an adaptor protein to complex the two functional kinases in the IKK complex (reviewed in (Hacker and Karin, 2006)). In our observations of infection with Type I GRA15$_{II}$, as well as TNF-α stimulation in the

four knockout cell lines in Figure 1, we see that deletion of IKK-β has a greater effect than deletion of IKK-α on decrease in p65 accumulation. Taken together, these data confirm that $GRA15_{II}$ acts upstream of or on the canonical IKK complex, which is comprised IKK-α, IKK-β and the IKK-γ adaptor, and not the homodimeric IKK-α complex active in the non-canonical pathway.

**Overexpression of $GRA15_{II}$ activates c-Rel nuclear translocation**

We acquired new antibodies for p52, RelB and c-Rel (Figure 2) when those used in Rosowski et al expired. Bands representing p65 (65 kD) and c-Rel (75 kD) are present in the Western blot with their respective antibodies. Probing with the p50 antibody yielded two bands, the ~100 kD band representing the inactive p102 form and the proteosome-cleaved active product, p50. Probing with p52 antibody showed a band at ~100 kD, which is the inactive form of unprocessed p52. Probing with RelB antibody showed a band at ~62 kD, though this was not specific. However, we had tested two other RelB antibodies, neither of which yielded any signal in IF or Western blotting.

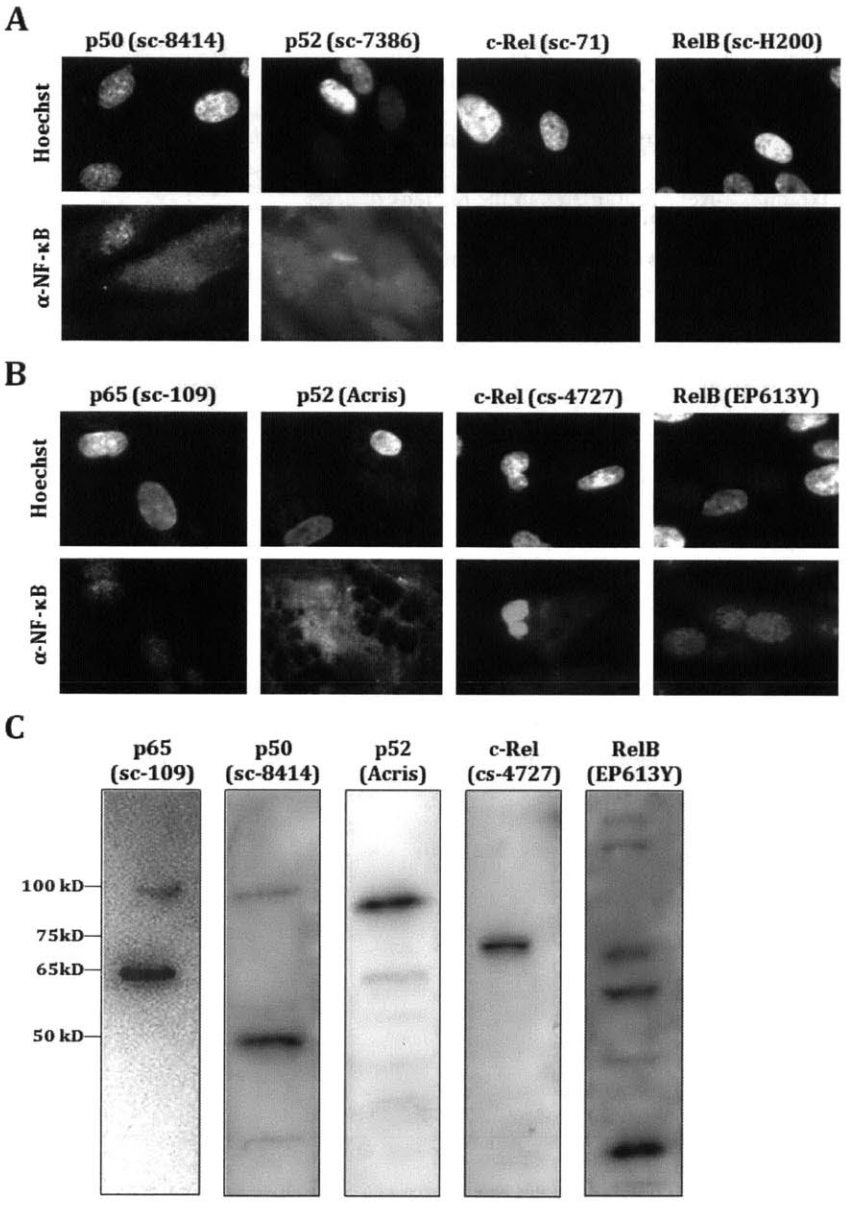



**Figure 2: NF-κB antibodies are specific to their corresponding antigens.**
A) IF re-probe of HFFs infected with Type I GRA15$_{II}$ with indicated antibodies was only successful for α-p50. B) IF assays of the same infection with a different antibody raised against the corresponding NF-κB subunit. Sc-107 was used as a positive control.
C) Western blots of HFF lysate using the indicated antibodies.

We repeated the IF assays performed in Figure S6 of Rosowski et al with the new antibodies (Figure 3). We obtained the same results for p52 and RelB as previously (Figure 3A, B, D), but we were surprised to find that type I GRA15$_{II}$ activated c-Rel highly in the new assay (Figure 3C). Like p65, c-Rel accumulates in the nucleus of cells infected by type I GRA15$_{II}$ but not in cells infected by wildtype type I. However, unlike p65, TNF-α stimulation does not significantly activate c-Rel nuclear translocation, suggesting that this observation is specific to the presence of GRA15$_{II}$.

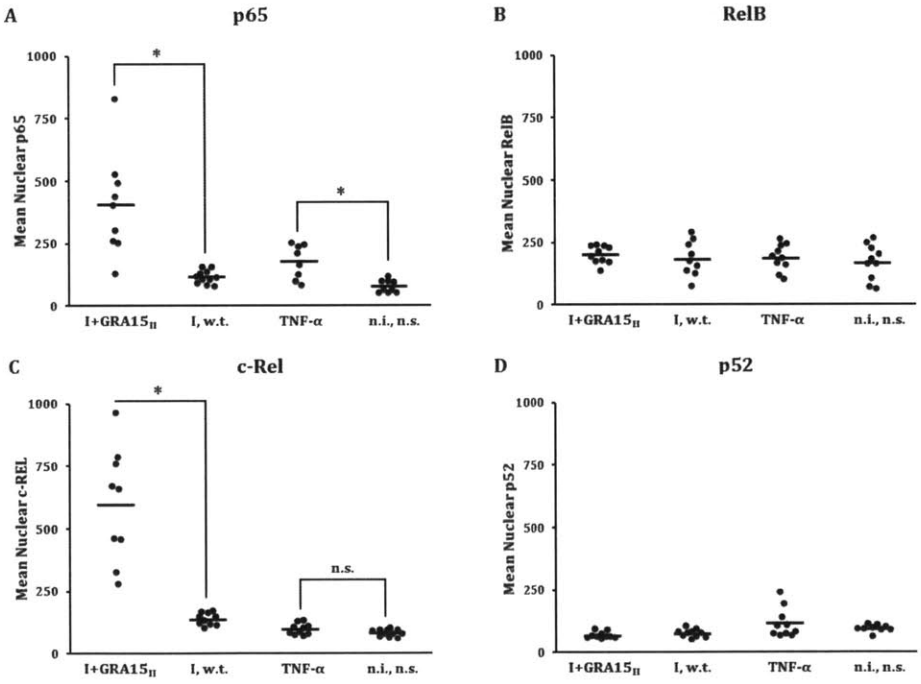



**Figure 3: GRA15 specifically activates p65 and c-REL nuclear translocation**. Cells were infected with type I GRA15$_{II}$ or type I strain for 24 h, stimulated with 20 ng/ml human TNF-α for 1 h, or left uninfected and unstimulated (n.i., n.s.). Cells were then fixed and probed with α-p65 (A), α-RelB (B), α-c-REL (C), or α-p52 (D) antibody and the mean nuclear density of each NF-κB subunit was measured. Each assay was repeated at least twice and quantifi cation was performed on a representative experiment. Asterisks (*) indicate data are significantly different (*p-value* < *0.05*, Student's t test), and n.s. no significant difference. Horizontal bars represent the mean nuclear p65 intensity over all cells.

## Conclusion

In addition to the results obtained in Chapter II, further mechanistic characterization revealed that GRA15 function requires IKK-γ. GRA15 also requires both IKK-α and IKK-β (Li et al 1999a; Li et al, 1999b), but their individual functions are partially redundant. Given that deletion of IKK-β and IKK-γ affect GRA15 function, and that the p65/p50 subunits are specifically activated, we conclude that GRA15 interacts with a signaling factor specific to the canonical NF-κB pathway (Li et al, 1999; reviewed in (Hacker and Karin, 2006)). We show that this activation occurs in all cell types assayed (primary human, rat, and mouse fibroblasts), indicating that GRA15 acts on a common evolutionarily conserved factor.

We update the results obtained in Figure S6D of Rowsowski et al. IF data using a new antibody against c-Rel (Cell Signaling, cs-4727) show that GRA15 from the type II strain activates c-Rel nuclear translocation in addition to that of p65 and p50. We believe these results to be more accurate because antibody cs-4727 was proven to be specific in IF and Western blotting tests.

The differential activation of specific NF-κB subunits and their distinct contributions to the regulation of gene set expression is only beginning to be characterized. However, it is safe to assume that there exist complex and fine-tuned mechanisms for the regulation of specific NF-κB subunits by different stimuli.

Activation of the Mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1) (Akagi et al, 1999) specifically activates c-Rel and not the other NF-

κB subunits (Gringuis et al, 2011). MALT-mediated activation of NF-κB has been shown to be mediated by the Ubc13/Uev1a, TRAF6, and TAK1 pathway toward IKK activation (Sun et al, 2004). Also, c-Rel expression is activated by p65, which amplifies and broadens the regulatory breadth of GRA15 on the host immune response. Increased c-Rel protein expression was not observed in HFFs (data not shown), but could be more pronounced in immune cells, where c-Rel performs a number of specific, regulatory functions (reviewed in (Hillard et al, 2002; Liou and Hsia, 2003)). Expression profile analysis of murine macrophages shows that c-Rel-induced gene sets are upregulated in cells infected by strains expressing $GRA15_{II}$ (Melo, 2013).

c-Rel and p65 are the primary transcriptionally active subunits of the NF-κB family involved in regulation of the immune response in cells, particularly those of the myeloid and lymphoid lineages. Both p65 and c-Rel regulate the expression of a large number of genes, including various cytokines and transcription factors that regulate the inflammatory response (Fullard et al, 2012). However, c-Rel activity specifically is crucial to determining the fate of B cell and T cell differentiation and macrophage and dendritic cell function (Liou and Hsia, 2012). Recently, c-Rel has been found to be involved in the production of the proliferation-inducing cytokine IL-21 by macrophages and dendritic cells (Chen et al, 2010), and is necessary to drive regulatory T cells to the $T_h1$ and $T_h17$ fates (Hilliard et al, 2002; Chen et al, 2011; Gringhuis et al, 2011). Thus, c-Rel activation in addition to p65 activation serves not only as a means of amplification, but also as another axis by which to control the immune response of the host against infection, one which ties the innate response to the adaptive.

Taken together, the data described in this chapter bring insight into the mechanism of function of GRA15, but the specifics thereof remain to be determined. Identification of the binding partner(s) of GRA15 in the host, and additional functional characterization of GRA15 will be presented in subsequent chapters.

## Materials and Methods

**Cells and Reagents.** IKK-$\alpha^{-/-}$, IKK-$\beta^{-/-}$, and IKK-$\alpha^{-/-}$/IKK-$\beta^{-/-}$ MEFs were generously provided by M. Karin. IKK-$\gamma^{-/-}$ REFs and wildtype REFs (5R line) were donated by S. Yamaoka. Antibodies against NF-κB2 p100/p52, RelB and c-Rel were obtained from Acris (AP02559PU-S), Millipore (EP613Y), and Cell Signaling (cs-4727), respectively.

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# Chapter III

# GRA15 co-immunoprecipitates with the TNF-receptor associated factor 3 (TRAF3)

## Summary

During the course of infection, *Toxoplasma* secretes hundreds of proteins into its host cell. These proteins function to improve the cellular milieu and are crucial to the parasite's survival and proliferation. Some of these proteins directly modulate the metabolic, cell cycle and immunologic signaling pathways of the host cell. The majority of the proteins which act directly on the host cell components have been reported to be rhoptry-derived kinases and phosphatases.

GRA15 is the first dense granule protein reported to have host-modulatory capabilities. This novel secreted factor directly and specifically acts on the host nuclear factor kappa B (NF-κB) pathway, a master regulatory pathway of the innate immune system. GRA15 is necessary and sufficient for the activation of host NF-κB. GRA15 function is dependent on the proteosomal degradation of inhibitor of kappa B (I- κB) kinase through the activation of the canonical I- κB kinase (IKK) complex.

Below, we present our work toward identifying the specific host protein(s) upon which GRA15 acts. We find that, though GRA15 is sufficient to activate host NF-κB nuclear translocation, the protein functions optimally in the presence of live *Toxoplasma* infection. The GRA15 amino acid sequence contains two TNF-receptor associated factor (TRAF) binding motifs (AAEE and SQQET). The TRAFs are a family of adaptor proteins which participate in NF-κB activation upstream of the IKK complex. These 4-5 AA binding motifs are found on all TRAF binding partners and have been shown to mediate their direct association with the TRAFs.

Deletion of TRAF6 was previously shown to decrease $GRA15_{II}$ activation of p65 nuclear localization during infection with $GRA15_{II}$-expressing parasite. We find evidence

of GRA15 interaction with TRAF3. In human cells infected with $GRA15_{II}$–expressing parasite, GRA15 co-immunoprecipitates with TRAF3, but TRAF3 did not consistently precipitate with GRA15 in the reciprocal experiment. Deletion of the TRAF binding motifs in $GRA15_{II}$ does not abrogate its ability to induce NF-κB activation in human cells. We conclude that GRA15 may function by complexing with TRAF3, which leads to IKK activation. However, this does not occur through direct interaction between the TRAF binding motifs of GRA15 and TRAF3.

## Introduction

*Toxoplasma gondii* is an extremely successful intracellular pathogen, in no small part due to its ability to evade immune detection by its host. Different strains of *Toxoplasma* have evolved lineage-specific methods of achieving this (Saeij et al, 2005). NF-κB is one of the master regulators of the immune response to *Toxoplasma gondii* infection. Previous work has shown that the canonical strains of *Toxoplasma* (types I, II, and III) differentially modulate the host NF-κB pathway, and that type II strains induce higher levels of activation than type I or type III (Dobbin et al, 2002; Robben et al, 2004).

We identified GRA15, a novel secreted protein, as the *Toxoplasma gondii* factor of type II strains ($GRA15_{II}$) that is necessary and sufficient to activate the NF-κB response of infected cells (Chapter II). GRA15 acts on the canonical pathway toward NF-κB activation upstream of or in parallel to the IKK complex and downstream of the myeloid differentiation primary response gene 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF) adaptor proteins. In the type II background, deletion of GRA15 results in subtle but clinically relevant consequences, including lowered interleukin 12 (IL-12) secretion by macrophages and slower *in vivo* parasite growth (Rosowski et al, 2011). Here we describe work toward characterizing the mechanism of GRA15 function.

The best characterized *Toxoplasma* host-modulatory factors are rhoptry-derived proteins (ROPs), many of which are kinases (reviewed in (Boothroyd and Dubremetz, 2008)). Many of these proteins, which are injected into the host cell upon invasion, directly interact with host signaling pathway components. For example, ROP16 is a

tyrosine kinase that activates host Signal Transducer and Activator of Transcription 3 and 6 (STAT3/6). This transcriptional regulatory pathway drives the innate immune response toward a type II, non-inflammatory fate. Rop16 is a tyrosine kinase that directly phosphorylates STAT3 and STAT6 (Yamamoto et al, 2009; Ong et al, 2010). Additionally, ROP5 and ROP18 are two proteins identified as virulence factors in mice. These kinases function coordinately to evade the pro-inflammatory Interferon-γ (IFN-γ) response of the host in the following scheme. ROP5 is a regulatory factor which directly interacts with ROP18. ROP18 is an active kinase that phosphorylates and inhibits the action of the Immune-related guanosine triphosphatases (GTPases) (IRGs). The IRGs comprise a class of murine immune effectors that normally aggregate on the *Toxoplasma* parasitophorous vacuole (PV) and target it for destruction (Zhao et al, 2009; Behnke et al, 2012; Niedelman et al, 2012).

Like ROPs, GRA15 is injected into the host cell upon invasion. However, GRA15 is also continually secreted by the dense granules for the duration of infection. GRAs have largely been characterized as proteins that function to maintain the metabolic and structural integrity of the PV and the intravacuolar network (IVN) (Leriche and Dubrumetz, 1990; Nam, 2009). $GRA15_{II}$ is sufficient to induce the nuclear translocation of p65 in human cells. However, during infection, the majority of this protein is observed to localize to the PV membrane (PVM). We therefore wondered whether association with the PVM or some other component of parasite infection was significant for its *in vivo* function. We conducted a cis-trans test, in which infection was decoupled from $GRA15_{II}$ expression. We found that the presence of parasite-derived $GRA15_{II}$ protein was sufficient to induce host NF-κB nuclear translocation. However, visibly higher levels of

activation were achieved when the host cell was infected with a type I strain, which does not activate NF-κB, prior to $GRA15_{II}$ exposure.

Because GRA15 is sufficient for host NF-κB activation, we hypothesized that this factor acts directly on the pathway by interacting with host protein(s). Therefore, our primary goal was to identify its host interacting partner(s). Furthermore, GRA15 has no predicted secondary or tertiary structure (analysis performed with BLAST, PHYRE, PROSITE and PPSEARCH algorithms). GRA15 functions upstream of or in parallel to the canonical IKK complex. This functionality has slow kinetics: only until four hours post infection do we observe full NF-κB activation in the host cell, and this activation is constitutive (Chapter II). From these observations and new evidence that some GRAs are constitutively secreted beyond the PVM (Hsiao et al, 2013), we hypothesize that upon invasion, very little GRA15 is secreted. Then, once the parasite invades, the dense granules secrete their contents constitutively so that they, including GRA15, slowly accumulate. Once there is enough GRA15 secreted into the host, full activation is achieved.

There exist a large number of adaptor proteins that can mediate NF-κB signaling upstream of IKK. There have also been many precedents for the direct modulation of these proteins by parasite-encoded factors (Chapter I, Figure 2). The Human T Lymphocyte Virus (HTLV) protein Tax1 has been observed to act as an adaptor that induces constitutive complexing of the IKKs (Huang et al, 2009). The Epstein Bar Virus (EBV) protein, latent membrane protein 1 (LMP1), initiates the aggregation of TNF-receptor associated factors 2 and 3 (TRAF2/3) independent of upstream signaling. This

leads to the phosphorylation of IKK-α homodimer and the activation of the non-canonical pathway (Huen et al, 1995; Eliopoulis et al, 1997).

Similar to LMP1, we identified two TRAF binding motifs (AAEE and SQQET) within the GRA15 primary sequence (Figure 1A). These motifs, (P/S/A/T)x(Q/E)E, PxQxxD, and PxExx(Aromatic/Acidic), where x is any amino acid, function as docking sites for the TRAFs on their interacting partners (Chung et al, 2007). We previously demonstrated that GRA15 partially requires functional TRAF6 to mediate p65 nuclear accumulation (Chapter II). We therefore hypothesized that one or more members of this family of signaling adaptors could be direct interaction partners of GRA15.

The TRAFs comprise a family of seven known receptor and adaptor-interacting proteins containing really interesting new gene (RING) domain, zinc finger, beta-transducin repeat, coiled-coil and C-terminal TRAF domain. Members of the TRAF family serve closely related but often non-redundant functions in the regulation of NF-κB activity (reviewed in (Chung et al, 2002). They function as adaptor proteins and ubiquitinases, intermediaries between upstream receptor-associated factors and IKK (Park et al, 1999; Ye et al, 1999; Chung et al, 2007).

The best characterized members of the TRAF family are TRAFs 2, 3 and 6. TRAFs 2 and 3 (TRAF2/3) mediate tumor necrosis factor receptor (TNFR)-activated NF-κB signaling (Figure 1B, middle). Upon binding ligand, TNFR recruits the TNFR associated death domain (TRADD), an adaptor protein that recognizes ligand-bound TNFR though the latter's cytosolic death domain (Hsu and Goeddel, 1995; Hsu et al, 1996). The TRAF binding motif of TRADD interacts with TRAF2/3 (Park et al, 2000), leading to their aggregation at the receptor base. This leads to the recruitment and

ubiquitination of receptor interacting protein 1 (RIP1) (Chen et al, 2006). RIP1 can incorporate a dimer comprising transforming growth factor beta (TGF-β)-activated kinase 1 and 2 (TAK1/2) and TAK1 binding protein 2 and 3 (TAB2/3) into the adaptor complex, where TAK1/2 phosphorylates IKK (Shibuya et al, 1996; Ninomiya-Tsuji et al, 1999; Kanayama et al, 2004).

TRAF2 and 3 can also complex independent of receptor stimulation (Park et al, 1999). Alone, TRAF2 binds and sequesters the IKK-α kinase NF-κB interacting kinase (NIK) in an inactive form. However, TRAF3 competitively inhibits formation of the TRAF2/NIK complex (Figure 1B, right), allowing free NIK to activate the non-canonical pathway (Vallabhapurapu et al, 2008).

TRAF6 mediates the Toll-like receptor 4 (TLR4) pathway toward NF-κB induction (Figure 1B, left). Activated TLR4 binds the receptor-associated factor MyD88, which leads to the recruitment of interleukin-1 receptor-associated kinases 1 and 4 (IRAK1/4) (Kawai et al, 2004). TRAF6 recruits the E2 ubiquitin ligase complex comprising ubiquitin conjugating enzyme 13 and ubiquitin conjugating enzyme variant 1a (Ubc13/Uev1a). Ubc13 K63-ubiquitinates TRAF6, activating its E3 ubiquitin ligase activity. TRAF6 then auto-ubiquitinates and recruits the TAB2/TAK1 complex to phosphorylate IKK (Deng et al, 2000). In addition, UEV1A/Ubc13 and TRAF6 ubiquitinate IKK-γ directly, is necessary for IKK activity in some cell types (Chen, 2005). The ubiquitin ligase p62 has also been shown to bind TRAF6 and RIP1 (Wooten et al, 2005).

**A**

MVTTTTPTPPPGAPAVVPIFDVVYQLNPHVFRSRFSRRNRARRVASSKSRSIIRWLGYLTVLAAVILLGAYAVRRVSRDL
SDSVRETRRGRRITGSVPPGTTRPRSESCTGTQVDGGCGADTSTDGKSESEQTENGEDSRFSTRTPIHVTASTSPFATRK
AAEERSSSPRDRKVPEGAQLPTSSTPHAQRKDSGSDSRNPSTLIPSPGTNTFNMNFYIIGAGSSALDFIFPHTPDAQATV
VSPPRSAAAAPTVETVPRVRTYSTPTTLTLPTAPATATSNHMHASATPSPPERPQNFRGGLMRQNGMVEGTSLTTTEAGM
PAPLQSPQHIETEARLTYSNHLKSPHTPETPTVHSIDPVVGTSGHSVAVGSQSPAGGPPTDSRTPAALTPTSSSFSHADS
LETSEHPQSGPSLHPLISGIQDAVQSQLPLSQQETLPVVENATFFGPQQTPPWMDETAAAAIPLAPSQPGSRTQPISSPH
TLLPLSGGVSAVPGPPRTENPRQPQVPGENSYYSVPTERTISDPGPQRAGATQADGIGAGGPRDTQSAVTP

**B**

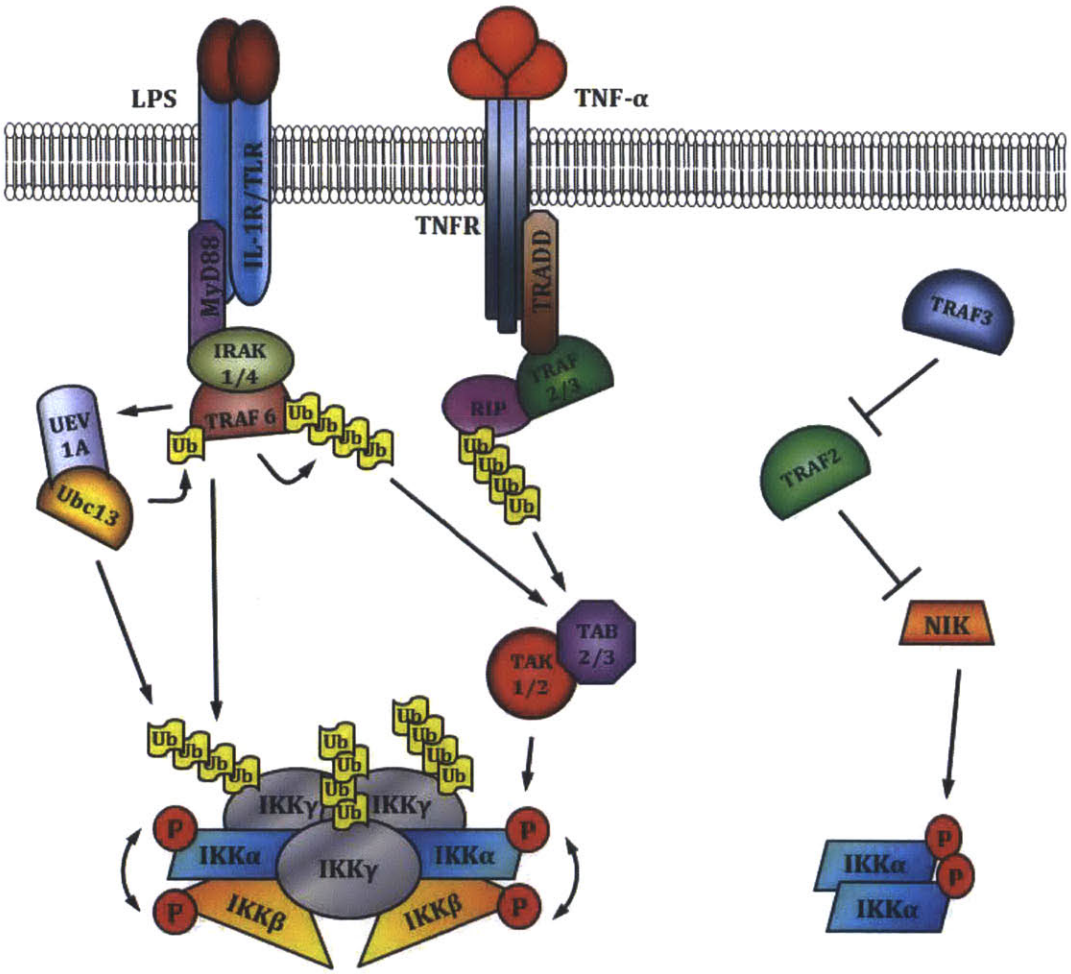


**Figure 1. TNF-receptor associated factors (TRAFs) and their relationship with GRA15 and NF-κB.** (A) The amino acid sequence of GRA15$_{II}$ (550 AA). Shown in red, AAEE and SQQET, are the two putative TRAF binding motifs. (B) Diagram of the functional relevance of TRAFs with respect to the NFkB activation. TRAFs 2 and 3 (TRAF2/3) complex with TNFR-associated adaptor proteins and activate the IKK kinase complex TAB/TAK. TRAF6 complexes with TLR4-associated adaptor proteins, where it recruits TAB/TAK and the E2 ubiquitin ligase complex Ubc13/UEV1A. Ubc13/UEV1A K63-ubiquitinates TRAF6, inducing TRAF6 auto-ubiquitination, as well as the K-63 ubiquitination of IKK-γ.

To determine whether GRA15 can complex with any of the TRAFs, we attempted to co-immunoprecipitate a number of TRAF family members with GRA15 from infected human foreskin fibroblasts (HFFs). We selected those TRAFs that have been observed to interact with IKK-upstream pathway components, TRAF2, 3, 5 and 6, as our primary candidates for co-immunoprecipitation experiments. The expressions of TRAFs 1 and 5 are cell-type dependent, and TRAFs 4 and 7 have never been observed to function in NF-κB activation. TRAF2, 3, and 6 represented the best candidate binding partners for GRA15 (Table 1).

**Table 1: All known TNF-receptor Associated Factors and their characteristics**

| | expression | NFkB association | # AA (kD) |
|---|---|---|---|
| TRAF1 | cell-type dependent | binds TRAF2, interacts with TNF-R associated adaptor proteins | 409 (40) |
| TRAF2 | all cell types | Binds TRAFs 1, 3, 5, interacts with TNF-R associated adaptor proteins and CD40-like receptors | 510 (52) |
| TRAF3 | all cell types | Binds TRAFs 1, 2, 5, interacts with TNF-R associated adaptor proteins and CD40-like receptors | 567 (60) |
| TRAF4 | variable | none observed | 470 (50) |
| TRAF5 | cell-type dependent | Binds TRAFs, 2, 3 interacts with TNF-R associated adaptor proteins | 558 (60) |
| TRAF6 | all cell types | interacts with TNF-R and IL1-R/TLR associated adaptor proteins and CD40-like receptors, recruits ubiquitin ligase complexes, ubiquitinates IKKγ | 530 (55) |
| TRAF7 | variable | none observed | 670 (70) |

We performed co-immunoprecipitations of the TRAFs and GRA15 using type I $GRA15_{II}$ infected human foreskin fibroblasts (HFFs). We analyzed the purified precipitates by Western blot and mass spectrometry. GRA15 consistently co-precipitated with TRAF3, but the reciprocal experiment did not detect specific association. Mass spectrometry (MS) analysis of the reciprocal experiment showed that TRAF6 was pulled down with GRA15, but this result was not consistently reproducible. No other interaction

between GRA15 and any other TRAFs was observed. One explanation for the inconsistency described could be that the antibody used to precipitate GRA15 in the reciprocal experiment interacts with GRA15 at the same site that TRAF3 binds.

To determine whether the TRAF binding motifs are necessary for GRA15 function, we created transgenic GRA15 clones in which the TRAF binding sites were deleted and expressed this mutant GRA15 in HeLa cells in the same manner as described in Chapter 2. These mutant proteins were still able to activate NF-κB nuclear translocation in HeLa cells.

From this, we determine that TRAF3 and GRA15 may complex, but that this interaction is not necessarily direct and is not mediated by the TRAF binding motifs of GRA15.

## Results and Discussion

### GRA15 functions optimally in the presence of infection

We conclude from Figure 5 of Rosowski et. al. that, when highly overexpressed, GRA15 is sufficient to activate NF-κB in the host cell. However during infection, GRA15 is localized to the PVM. We therefore wondered if the level of $GRA15_{II}$ expressed in parasites is also enough to activate NF-κB independent of infection, or if the presence of the PVM or some other component of infection was important for GRA15 function in the context of live infection.

To investigate these possibilities, we performed an infection assay in which the injection of parasite-derived GRA15 into the host cell was decoupled from live infection.

Cytochalasin D (CyD) is a helminth-derived inhibitor of actin polymerization (May et al, 1998). Polymerization of *Toxoplasma* actin is necessary for invasion of the host and formation of the PV. However, it is not necessary for attachment, injection of parasite factors, and infection after PV formation. In chapter II, we observed that GRA15, unlike most GRA15, is able to be secreted upon invasion. In the presence of CyD, therefore, *Toxoplasma* can excrete a small amount of GRA15 into the host without invading it. Also, if invasion occurs before treatment with CyD, the infection will persist in spite of the toxin. Because mammalian actin polymerization is also inhibited by CyD, we performed the experiment in cyD-resistant HeLa cells.

Using this system, we created three exposure scenarios. In the first, resistant HeLa cells were pre-infected with type I strain parasite, then either type I or type I $GRA15_{II}$ was allowed to inject GRA15 and other parasite factors into the host. In the second, type I or type I $GRA15_{II}$ was allowed to infect the resistant cells normally. In the third, no infection occurred, but type I or type I $GRA15_{II}$ was exposed to host in the presence of CyD (Figure 2).

In Figure 3A, we see that, even without pre-infection, injection of $GRA15_{II}$ is sufficient to induce nuclear accumulation of NF-κB p65. However, the amount of translocation is visibly higher when we perform the experiment with the type I pre-infected cells.

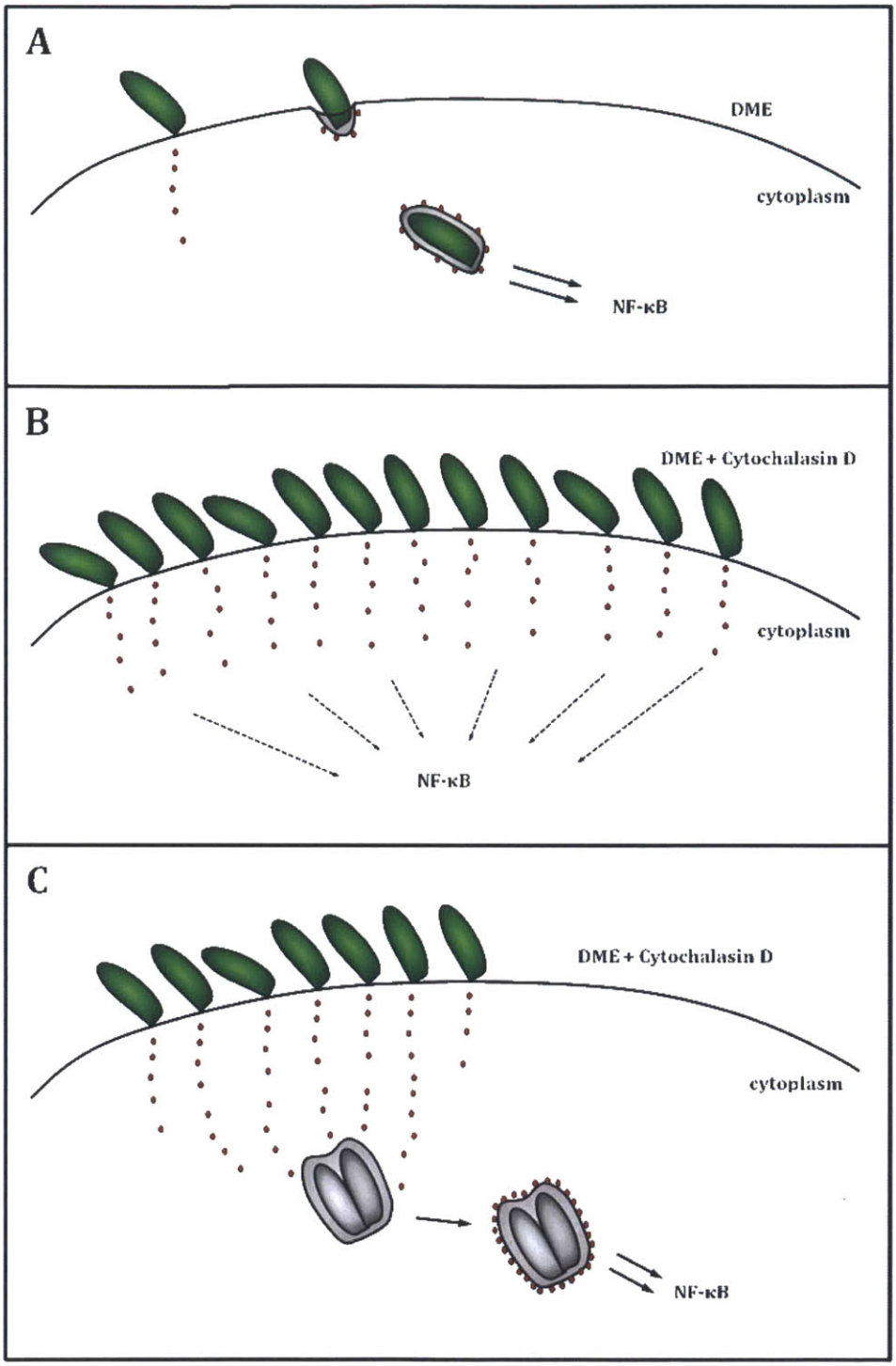


**Figure 2. Diagram of cytochalasin D infection assay.** Parasites shown in green represent Type I GRA15$_{II}$ strain. Parasites shown in grey represent Type I strain. Box A depicts standard attachment, secretion and invasion in cell culture, during which GRA15$_{II}$ is injected into the host cytoplasm, then localizes to the PVM of the infecting parasite. Box B depicts a scenario in which the infection culture is incubated with cytochalasin D. Without actin polymerization, *Toxoplasma* no longer has the ability to invade the host cell, while attachment and secretion functionality remain intact. In Box C, host cells previously infected with Type I strain are incubated with cytochalasin D and Type I GRA15$_{II}$ strain. Here, the latter strain is unable to invade the host, but injects GRA15$_{II}$, which localizes to the membrane of the Type I vacuole.

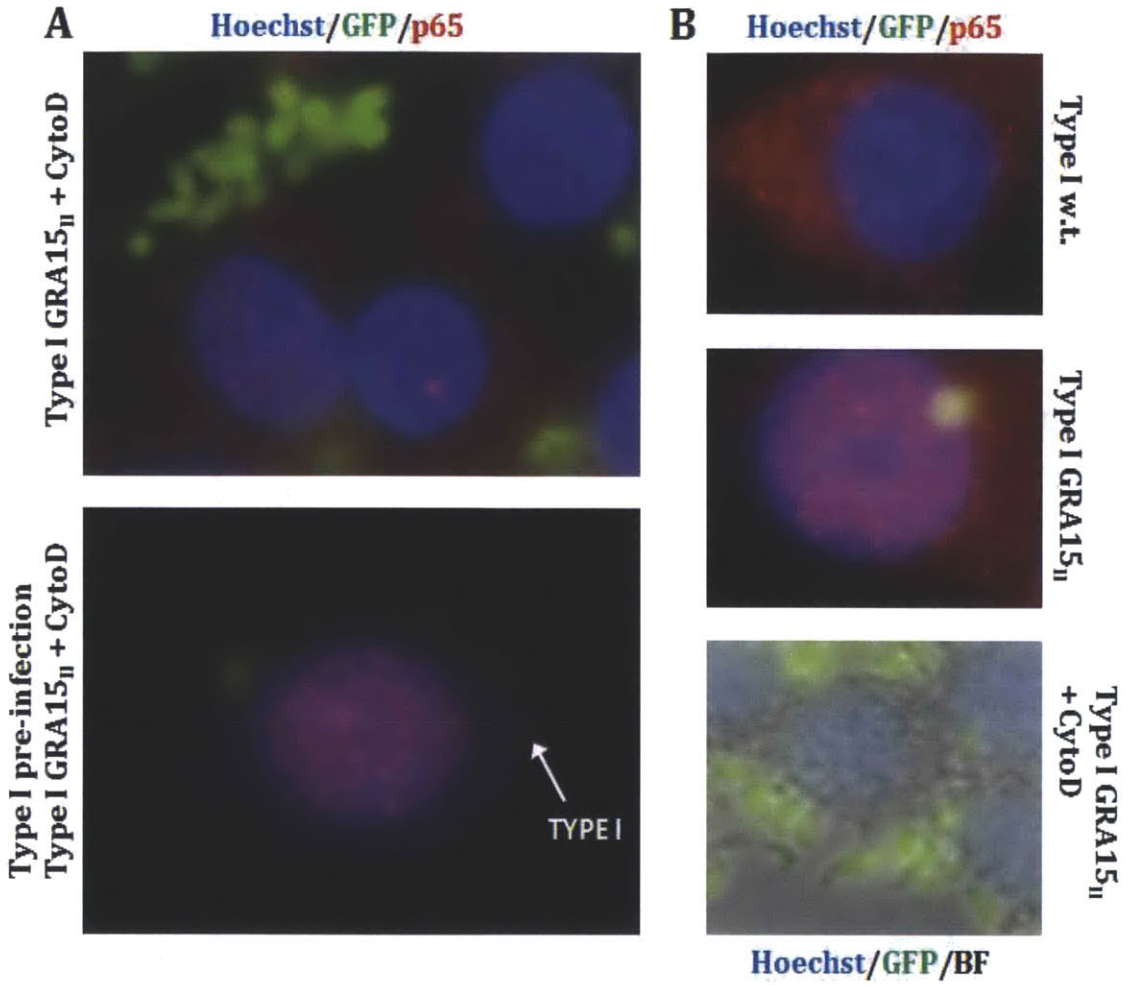


**Figure 3: GRA15 functions optimally in the presence of a PVM.** Cells were fixed and stained with α-p65 (red) and Hoechst nuclear stain (blue). (A) Cytochalasin D resistant HeLa cells were either infected (bottom) or uninfected (top) with Type I parasite for 8 h prior to treatment with cytochalasin D. Type I GRA15$_{II}$ strain expressing GFP treated with 100 µg/ml cytochalasin D for 4 h was then added to the HeLa cells for 4 hours to allow attachment, secretion, and full activation of NF-κB by GRA15$_{II}$. Arrow indicates nuclear staining of non-GFP Type I strain. (B) Untreated resistant HeLa cells were infected with wildtype Type I (top) or Type I GRA15$_{II}$ for 4 h (middle). (B, bottom) Merged bright field image of (A, top) showing parasites (green) attached to, but not infecting, HeLa cells (blue) in the presence of cytochalasin D. These assays were performed twice, in triplicate.

## Immunoprecipitation of GRA15

To identify GRA15 interacting partners, we performed co-immunoprecipitation experiments on infected human foreskin fibroblasts. For each experiment, we infected HFFs with either type I $GRA15_{II}$, wildtype type I as an infection control, or type I overexpressing another HA-tagged *Toxoplasma* secreted factor ($55_{III}HA$), as an HA and transgenic control. Samples was harvested 4 hours post-infection, a time point early enough for minimal inter-PV GRA15 accumulation but also late enough that full activation of NF-κB by GRA15 is observed (Rosowski et al, 2011). The infected cells were lysed and incubated with α-GRA15-bound beads. We then probed each sample for co-precipitating proteins via western blot.

First, we used an anti-haemagglutinin (HA) antibody to precipitate the C-terminally HA tagged $GRA15_{II}$ from the type I $GRA15_{II}$ strain. We immunoprecipitated TRAFs 2, 3, 5, and 6 from the infected HFFs, then immunoblotted with the anti-HA antibody to determine if GRA15 was present in each purified TRAF complex. In Figure 4A, we see the results of co-immunoprecipitation of GRA15 by anti-TRAF antibodies. SAG1 is a loading control and $55_{III}HA$ is an IP control. The TRAF5 immunoprecipitation was unsuccessful, as TRAF5 was not precipitated by its respective antibody. We see that that GRA15 only selectively co-precipitates with TRAF3. We also performed the reciprocal IP, in which $GRA15_{II}$ was precipitated with an antibody against the HA epitope bound to agarose beads. However, when we immunoblotted with antibody against TRAF3, we did not observe the interaction (Figure 4B). Probing this IP with antibodies against TRAFs 2, 5 and 6 were unsuccessful (data not shown).

Our second approach was to attempt to co-precipitate GRA15 interacting partners using a primary antibody raised against the GRA15 protein itself. We generated primary polyclonal antibodies which could be used to recognize and bind to endogenous GRA15 from all three canonical strains as well as the transgenically overexpressed protein from either parasite or cell lines. We chose two peptide sequences in the region of GRA15 which were conserved among the three canonical strains (Figure S1). Two peptides antigens were produced from these sequences by Yenzym Antibodies, LLC, each of which was used to inoculate two New Zealand White rabbits each. The resulting sera was purified with Thermos Life Sciences' Sulfolink antibody purification kit and tested for efficiency in immunoprecipitation, western blot, and immuno-fluorescence. One sample of purified sera, recognizing the C-terminal epitope, was observed to successfully probe for GRA15 in all tested assays and all tested strains (Figure S2). We therefore used this antibody, herein called α-GRA15, for all subsequent experimentation.

It is possible that the interaction of the HA antibody with GRA15 could disrupt binding. Because of this, we performed additional precipitation assays with α-GRA15. In these assays, HFFs were infected as previously described with wildtype type II, or one of two controls: wildtype RHΔ, a laboratory strain of type I in which GRA15 carries a missense mutation that deletes the epitope recognized by α-GRA15, or wildtype type III. Infected cells were lysed, and GRA15 was immunoprecipitated with the α-GRA15. Precipitate was then immunoblotted with α-TRAF3 antibody. In this assay, TRAF3 can be seen in non-specific complex with the precipitated beads in all three conditions, even the lysate in which there is no epitope for the GRA15 antibody to bind. Though it does

appear that TRAF3 is somewhat enriched, this is not conclusive enough to confirm interaction between these two factors (Figure 4C).

Though the interaction between GRA15 and the TRAFs could not be confirmed with these experiments, it is of note that the $GRA15_{III}$ precipitate appears to be largely degraded, while the same protein in type II is not. This observation may attest to the relative stabilities of the two alleles of the protein, lending a possible reason behind the strain dependency of its function. That is, perhaps $GRA15_{II}$ is more functional than $GRA15_{III}$ because the former protein is much more stable, due to polymorphisms between the alleles.

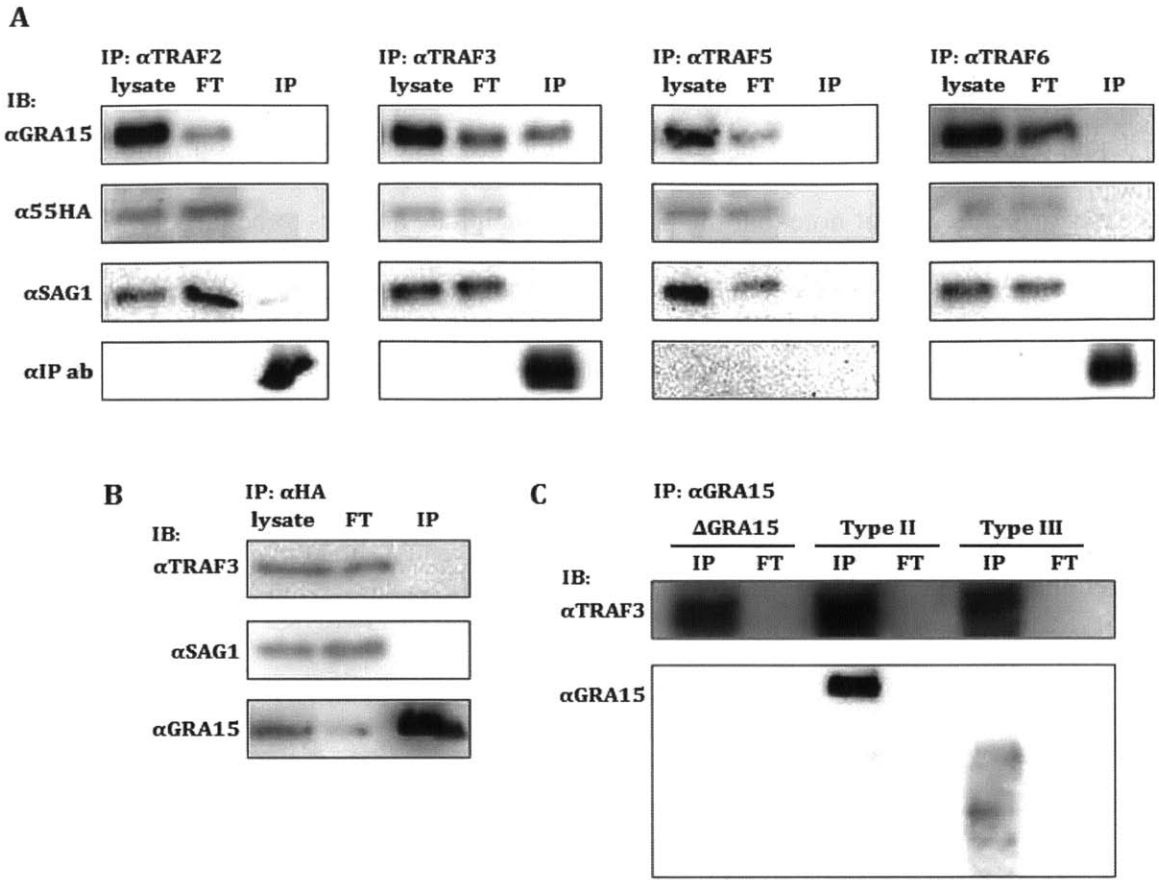


**Figure 4: GRA15 co-precipitates with TRAF3 but TRAF3 does not co-precipitate with GRA15.** HFFs were infected with Type I $GRA15_{II}$ or Type I $55_{III}$ (as an HA tag and transgenic overexpression control) 4 h prior to cell lysate collection. Lysates were incubated with designated antibody conjugated to agarose beads (IP) for 4 h at 4 C. Precipitated beads were collected and immunoblotted (IP lane) along with flow through (FT lane) and pre-IP lysate (lysate lane) with the designated antibody (IB) (A) Representative immunoblots of IPs with α-TRAF2, 3, 5, and 6 antibodies. From top to bottom panel, these are our antibody raised against amino acids 493-510 of GRA15 (αGRA15); the antibody against HA, to detect the transgenic overexpression control (α55HA); antibody against Toxoplasma surface antigen as a parasite loading control (αSAG1); and the antibody (αTRAF2, αTRAF3, αTRAF5, or αTRAF6, from left to right) used to perform the immunoprecipitation (αIP ab). $GRA15_{II}$ is co-precipitated only with TRAF3 and TRAF5 failed to precipitate. (B) α-HA immunoprecipitation of HA-tagged $GRA15_{II}$ does not co-precipitate with TRAF3. Each experiment was performed at least twice. (C) Immunoblot of IPs with α-GRA15. Lysate from HFFs infected with parasite expressing $GRA15_{II}$, $GRA15_{III}$, or no GRA15 expression. In GRA15II-infected cells, TRAF3 appears enriched but not specifically co-precipitated. Additionally, $GRA15_{III}$ appears to be degraded.

## IP/MS does not consistently show enrichment of human binding partners

We next attempted to identify GRA15 binding partners by immunoprecipitation followed by mass spectrometry. This also served as a less biased approach that above. We hoped it would yield potential interacting partners which we had not yet considered.

We scaled-up the immunoprecipitation against the HA epitope, which was performed as described above, ten-fold. We did not have enough α-GRA15 antibody for multiple replicates of IP/MS, so this assay was not performed. Immunoprecipitated samples were polyacrylamide gel electrophoresed (PAGE) on a 4 to 10 percent gradient gel and silver stained or Coomassie brilliant blue stained. The contents of each gel lane was individually processed, spec-ed, and analyzed by E. Spooner at the Whitehead Institute's spectrometry facility. Listed below is a representative table of the human proteins that were specifically enriched in the type I $GRA15_{II}$ infection but were not enriched in that of either the type I $55.m04955_{III}$ or the wildtype type I infection (Table 1). TRAF3 is not shown to be enriched, and none of the proteins in the list have been reported to have a specific functionality in regard to activating the NF-κB pathway. Peptides corresponding to the TRAF6 were occasionally enriched in the α-GRA15 sample. However, this result was not consistently observed. Also, it is of note that the *Toxoplasma* cyst matrix protein was the only protein to be consistently and selectively co-precipitated with GRA15 compared with the controls.

**Table 2: human proteins enriched in α-GRA15 IP of Type I GRA15$_{II}$ vs Type I strain-infected HFFs**

| Accession | Protein Name |
|---|---|
| gi\|154759259 | spectrin alpha chain, brain isoform 2 |
| gi\|157266300 | aminopeptidase N precursor |
| gi\|18426913 | drebrin isoform b |
| gi\|148839339 | nexilin isoform 1 |
| gi\|114155148 | tropomyosin alpha-3 chain isoform 5 |
| gi\|5031573 | actin-related protein 3 |
| gi\|63055057 | beta-actin-like protein 2 |
| gi\|112382250 | spectrin beta chain, brain 1 isoform 1 |
| gi\|7656991 | coronin-1C isoform 1 |
| gi\|150417973 | supervillin isoform 2 |
| gi\|20357552 | src substrate cortactin isoform a |
| gi\|27597085 | tropomyosin alpha-1 chain isoform 5 |
| gi\|61743954 | neuroblast differentiation-associated protein AHNAK isoform 1 |
| gi\|7705373 | LIM domain and actin-binding protein 1 isoform 2 |
| gi\|42734430 | polymerase I and transcript release factor |
| gi\|7657649 | tropomodulin-3 |
| gi\|219842212 | protein phosphatase 1 regulatory subunit 12A isoform a |
| gi\|63252904 | tropomyosin alpha-1 chain isoform 6 |
| gi\|33598968 | LIM domain only protein 7 isoform 1 |
| gi\|4504981 | galectin-1 |
| gi\|23238211 | actin-related protein 2/3 complex subunit 2 |
| gi\|50980301 | myosin phosphatase Rho-interacting protein isoform 2 |
| gi\|4757834 | BAG family molecular chaperone regulator 2 |
| gi\|5031571 | actin-related protein 2 isoform b |
| gi\|5453597 | F-actin-capping protein subunit alpha-1 |
| gi\|5031597 | actin-related protein 2/3 complex subunit 3 |
| gi\|194097350 | alpha-actinin-1 isoform a |
| gi\|4503057 | alpha-crystallin B chain |
| gi\|20357529 | guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2 |
| gi\|330864679 | F-actin-capping protein subunit beta isoform 2 |
| gi\|321400138 | CD44 antigen isoform 6 precursor |
| gi\|5031593 | actin-related protein 2/3 complex subunit 5 |
| gi\|224451080 | cytospin-A |
| gi\|117938759 | protein ALEX XLas |
| gi\|5031635 | cofilin-1 |
| gi\|13569956 | actin-related protein 2/3 complex subunit 5-like protein |
| gi\|4503483 | elongation factor 2 |
| gi\|51036603 | guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit γ-12 precursor |
| gi\|24797067 | HLA class I histocompatibility antigen, A-1 alpha chain precursor |
| gi\|282721075 | sushi repeat-containing protein SRPX isoform 2 |
| gi\|60097902 | filaggrin |
| gi\|167830475 | lactadherin isoform a preproprotein |
| gi\|24119203 | tropomyosin alpha-3 chain isoform 2 |

**TRAF Binding Site Deletion Mutants**

We assumed that if GRA15 were to directly bind to TRAF3, this should occur via one or both of the TRAF binding motifs in the GRA15 sequence (AAEE and SQQET). Using PCR splicing by overlapping extension (SOEing), we created three mutant clones of GRA15 in which one or both TRAF binding motifs were removed from the sequence. These mutants were inserted into the pIC242 vector and transiently expressed in HeLa cells. After 4 hour incubation, cells were fixed and GFP-positive cells were assayed for p65 nuclear localization (Fig. 5). All three TRAF binding motif deletion clones were able to activate nuclear localization of p65, with no visible difference from that of the wildtype $GRA15_{II}$. In contrast, in the *ΔAAEE* and *ΔSQQET* mutant $GRA15_{II}$ transfections, the adjacent cell, which is untransfected and does not express GFP, has visibly less nuclear p65 than the transfected, GFP-expressing cells (Fig 5).

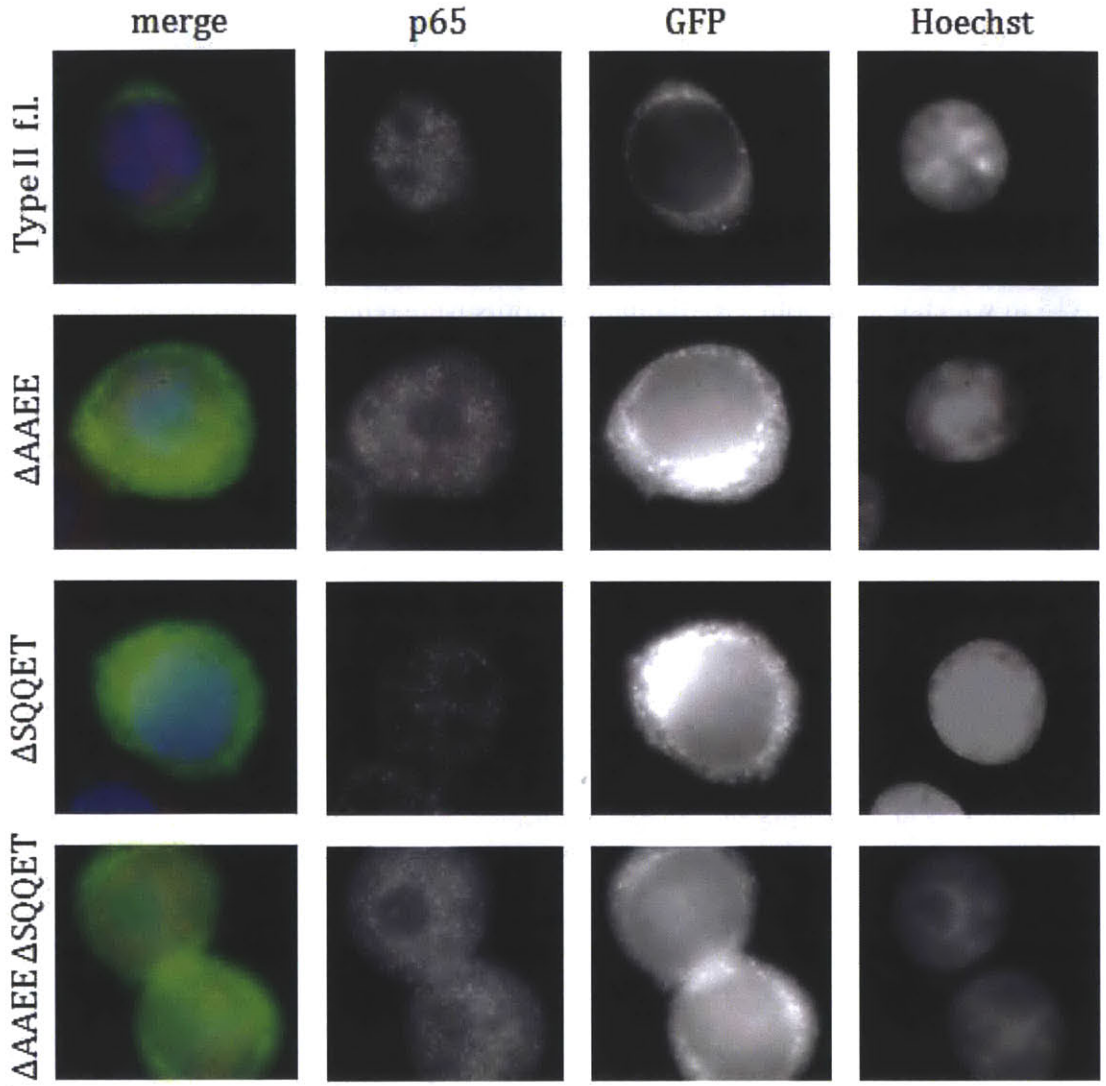


**Figure 5: TRAF binding motifs are not necessary for $GRA15_{II}$ function.** HeLa cells were transfected with full length (Type II f.l.) or mutant $GRA15_{II}$, in which one or both TRAF binding motifs have been deleted via splicing by overhanding extension (SOE-ing) PCR. All cells expressing $GRA15_{II}$-GFP, mutant or wild-type, contain activated p65 (red) in their nuclei (blue), whereas untransfected cells in the same cultures do not. This experiment was performed twice, with similar results.

## Conclusion

Interestingly, results of the of cytochalasin D experiment show that parasite-derived $GRA15_{II}$ is sufficient to activate p65 translocation, but the presence of infection increases its efficacy. Since a large amount of GRA15 protein is observed at the PVM, we can speculate that PVM association may be necessary for optimal GRA15 function. For example, because it is a highly unstructured protein, membrane association may stabilize or aggregate GRA15. Aggregation appears to be crucial for many upstream adaptor proteins in the activation of IKK, and this may also be true for GRA15.

Also, recent evidence shows that GRA15 contains a *Plasmodium* export element (PEXEL) within 150 base pairs of its signal peptide (SP). In *Toxoplasma,* the PEXEL motif tags the protein for export and PVM association, and is often found in secreted GRAs (Hsiao et al, 2013). GRA15 could similarly be secreted by the parasite and exported to the extra vacuolar space (EVS). It is likely that only a small amount of GRA15 is injected into the host upon invasion. However, because GRAs are continuously secreted, the protein can accumulate in the host over the course of infection. In contrast, transgenic expression driven by the lentiviral promoter induces constant, high levels of GRA15 production in HeLa cells. In this case, the main difference between the presence of infection and the lack thereof could simply be that a higher amount of GRA15 is present in host cells that have been pre-infected. It is possible that GRA15 must be present in the host cytoplasm in sufficient amount for it to function. This model also makes sense in the context of the slow kinetics of GRA15 activity observed in Chapter II.

From our immunoprecipitation assays we cannot definitively conclude that GRA15 directly interacts with any member of the TRAFs, though GRA15 consistently and specifically co-precipitates with TRAF3. It is possible that the observed co-precipitation of GRA15 with TRAF3 is relevant. It is possible that we are unable to see the interaction in the reciprocal experiment because the HA antibody used to precipitate GRA15, which is polyclonal, non-specifically binds to the site at which GRA15 interacts with TRAF3, inhibiting interaction. To determine if this is the case, we can add α-HA antibody to an α-TRAF3 immunoprecipitation and probe the purified complex with α-GRA15. If α-HA inhibits the interaction between TRAF3 and GRA15, then we would expect there to be less GRA15 with increasing α-HA addition.

We can attempt additional immunoprecipitation experiments and *in vitro* binding assays with TRAF3 and TRAF6 mammalian overexpression vectors (Sigma-Aldrich). If direct binding between overexpressed TRAF3 and overexpressed $GRA15_{II}$ in these assays is observed, we can observe direct interaction between the two proteins with *in vitro* binding assays (Zhou et al, 2005; Mukherjee et al, 2006) or fluorescent resonance emission tomography (FRET) analysis of GRA15 and TRAF3. In the latter case, we would need to conjugate both to fluorescent proteins and overexpress them in a cell line.

In our MS analyses, our samples were overwhelmed by host cytoskeletal components, and we were not able to detect an appreciative enrichment of likely GRA15 binding partners. However, TRAF6 was observed to be enriched in a few of our assays. This inconsistency could be because TRAF6 is expressed at much lower levels than cytomatrix proteins. In future experiments, we can perform comparative mass spectrometry analysis using stable isotope labeling by amino acids in cell culture

(SILAC). This would permit comparison two infection scenarios (e.g. type III vs. type II) in one sample, and can be much more sensitive method to screen possible protein-protein interactions than the traditional MS approach (Mann, 2006; Trinkle-Mulcahy, 2008).

Because we were not able to conclusively identify a host binding partner with classical biochemical methodology, we sought broader, less directed approaches. Many different stimuli can activate NF-κB, including TLR receptor binding, nuclear oligomerization domain (NOD)-like receptor (NLR) binding, DNA damage, etc. Almost all activation uses a variation of adaptor signaling that converges on an IKK complex, either the IKK-α, -β and -γ large heteromeric complex responsible for activating the canonical NF-κB pathway, or the IKK-α homodimer, which functions to activate the non-canonical pathway (Chapter 1, Figure 2).

As such, we identified twenty adaptor proteins from the literature (Chen and Baltimore, 1996; Burns et al, 2000; Gaide et al, 2001; Chung et al, 2002; Huang et al, 2003; Zhou et al, 2003; Briassouli et al, 2005; Bjørkøy et al, 2006; Daigler et al, 2008; Skaug et al, 2009; Vallabhapurapu and Karin, 2009). These proteins have been observed to participate in the activation or inhibition of the IKK complex, are expressed in HFFs, and are conserved at least among humans, rats, and mice. For the 19 candidates (Table 3) with constructs available from the Broad Institute's RNAi consortium (Root et al, 2006), we obtained lentivirus samples each containing one of 4 to 5 shRNA, as well as 293 cells infected with the corresponding constructs.

These reagents can be used to perform epistasis testing to refine the position in the NF-κB pathway in which GRA15 functions. We can infect the cell lines with a strain expressing $GRA15_{II}$ or transfect them with the GFP-tagged expression vector described

in chapter II. Then we can assay NF-κB activity through immunoblotting. Additionally, we can use the virus to co-transfect the shRNA and GRA15 expression vector into an NF-κB reporter cell line (Chapter II). This would allow screening of candidate genes in a high throughput, quantitative manner.

**Table 3: human NF-κB pathway genes selected for shRNA knockdown**

| NCBI Code | Gene Symbol | Gene Description |
|---|---|---|
| 8915 | BCL10 | CARD containing molecule enhancing NFκB |
| 330 | BIRC3 | TNFR2 TRAF signaling complex protein |
| 22900 | CARD8 | CARD inhibitor of NFκB activating ligands |
| 5610 | EIF2AK2 | double stranded RNA activated protein kinase |
| 23085 | ERC1 | RAB6 interacting protein 2 |
| 8517 | IKBKG | NFκB essential modulator |
| 10892 | MALT1 | MALT associated translocation |
| 9020 | MAP3K14 | serine/threonine protein kinase |
| 4215 | MAP3K3 | MAP/ERK kinase kinase 3 |
| 57162 | PELI1 | pellino protein |
| 8767 | RIPK2 | CARD-carrying kinase |
| 8878 | SQSTM1 | EBI3-associated protein p60 |
| 10010 | TANK | TRAF family member associated NFκB activator |
| 54472 | TOLLIP | Toll interacting protein |
| 7185 | TRAF1 | Epstein Bar Virus-induced protein 6 |
| 10758 | TRAF3IP2 | NFkB activating protein ACT1 |
| 7334 | UBE2N | bendless-like ubiquitin conjugating enzyme |

Though the specifics of GRA15 function remain to be discovered, the work described in this chapter begins to uncover the mechanism of action of the first characterized dense granule protein that can directly modulate signaling pathways of the host.

## Materials and Methods

**Cells and reagents.** Parasite and cell culture maintenance, wildtype and transgenic strains, cell cultures and expression plasmids have been previously described in Rosowski, et. al. Cytochalasin D resistant HeLa cells were a gift from D. Sibley. HeLa cells were provided by I. Cheeseman. FreeStyle max (293FS) cells, vesicular stomatitis virus glycoprotein VSV-G and p89 packaging plasmids were a gift from M. Hemann (Massachusetts Institute of Technology, Koch Institute for Integrative Cancer Research). Human shRNA plasmids and virus were produced by The RNAi Consortium (Broad Institute) based on sequences in the human open reading frame (ORF) eome library V8.1. Antibodies against HA, human NF-κB p65, *Toxoplasma gondii* surface antigen (SAG)-1, immunofluoresence (IF) secondary antibodies, and antibodies conjugated to horseradish peroxidase (HRP) for use in western blotting have also been previously described. Polyclonal rabbit antibodies against TRAF2 (sc-876), TRAF3 (sc-1828), and TRAF6 (sc-7221) were obtained from Santa Cruz Biosciences, and monoclonal rabbit antibody against TRAF5 (clone 55A219) was obtained from Cayman Chemicals.

**Cytochalasin D infection protocol.** Confluent HeLa cells were split 1:10 into a 24-well plate (BD) containing glass coverslips. The cells were allowed to incubate at 37 C and 5% $CO_2$ for 24 h in (Dulbecco modified eagle medium) DME supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM l-glutamine, 1 mM sodium pyruvate, 1× minimal essential medium (MEM) nonessential amino acids, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 50 μg/ml each of penicillin and streptomycin, and 20 μg/ml gentamycin. Following incubation, pre-infection wells were seeded with type I strain to allow invasion of the cells for 8 h. For the experimental wells, the medium

was replaced with 1 ml of fresh supplemented DME containing 100 ug/ml cytochalasin D. In control wells, the medium was replaced with 1 ml DME without cytochalasin D. All cells were incubated for 4 hours. During this time, 4 x $10^6$ type I $GRA15_{II}$ *Toxoplasma* were centrifuged and incubated with 5 ml DME with 100 ug/ml of cytochalasin D. Following incubation, HeLa cells were washed 3x in phosphate buffered saline (PBS), after which fresh medium containing cytochalasin D, and either $10^6$ of type I $GRA15_{II}$, $10^6$ of type I strain, or no parasite was administered to the appropriate wells. Infected cells were incubated for 4 h, then all coverslips were washed 3x in PBS and subjected to the immunofluoresence protocol as described in Rosowski, et. al, 2011.

**IF.** Cells were fixed with 3% (vol/vol) formaldehyde in phosphate buffered saline (PBS) for 20 min at room temperature, permeabilized with 100% ethanol, and blocked in PBS with 3% (wt/vol) bovine serum albumin (BSA) and 5% (vol/vol) goat serum. Coverslips were incubated with primary antibody for 1 h at room temperature or overnight at 4°C, and fluorescent secondary antibodies and Hoechst dye were used for antigen and DNA visualization, respectively. Coverslips were mounted on a glass slide with Vectashield (Vector Laboratories), and photographs were taken using NIS-Elements software (Nikon) and a digital camera (CoolSNAP EZ; Roper Industries) connected to an inverted fluorescence microscope (model eclipse Ti-S; Nikon). Quantification of nuclear signal was performed by randomly selecting at least 10 infected cells per *Toxoplasma gondii* strain and measuring the mean signal intensity per nucleus using the NIS-Elements software and Hoechst dye to define nuclei.

**Generation of α-GRA15 rabbit polyclonal antibodies.** Antibodies were produced by Yenzym Antibodies, L.L.C. with the Custom Peptide ShotGun™ Rabbit Antibody

Service: 1) Yen-de-Zyn™ Antigen Design Service; 2) Peptide synthesis with Antibody Production of GRA15 amino acids 493-510 (CGPPRTENPRQPQVPGENS-amide) and 120-139 (CDTSTDGKSESEQTENGEDSR-amide); 3) Peptide-keyhole limpet hemocyanin (KLH) or Peptide-THY conjugation; and 4) ShotGun™ Antibody Production (2 Rabbits per peptide). 5 ml pre-immune serum and 25 ml production antiserum per rabbit was tested by western blot and immunofluoresence for reactivity with $GRA15_{II}$ protein. All pre-immune sera were negative for reactivity against GRA15. Production antiserum sample 13207, corresponding to the N-terminal peptide, was reactive against GRA15 in IF, while sample 13309, corresponding to the C-terminal peptids, was reactive in IF, IP, and Western. After purification, the latter sample was used for all subsequent assays and herein called α-GRA15. Antibody from both reactive antiserum samples were purified as per the protocol in the Sulfolink Immobilization Kit for Peptides (Thermo Scientific). The concentration of purified antibody was determined by measuring absorbance at 280 nm using the Nanodrop 3300 spectrophotometer and corresponding analysis software (Thermo Scientific) and stored at 1 μg/μl in 1-30 μl aliquots at -80°C.

**Antibody conjugation and crosslinking.** Ten microliter per sample for western blot analysis and 60 μl per sample for mass spectrometry of Protein –A or –G Dynabeads (Invitrogen) were washed with HBS (10 mM HEPES-KOH pH 7.5, 150mM NaCl) and resuspended with antibody in HEPES buffered saline (HBS) (2 μg/sample for western blot and 15 μg/sample for mass spectrometry) at room temperature for 1 hr. Following coupling, antibodies were crosslinked to Dynabeads using 5 mM Bis(sulfosuccinimidyl)suberate (BS3) in conjugation buffer (20 mM Sodium Phosphate,

150 mM NaCl, pH 8.5) for 30 min at room temperature, with rotation, then washed 5x with HBS.

**Co-immunoprecipitation.** For western blot analysis immunoprecipitation samples, 3 T-25 flasks of fully lysed parasite were centrifuged at 7000 rpm and resuspended in 4 ml of fresh supplemented DME for each experimental condition. This medium was added to one T-25 flask of HFFs and parasites were allowed to invade cells for 4 hrs at 37°C and 0.5% $CO_2$. For mass spectrometry samples, six T-175 flasks of fully lysed parasite were used to infect two T-175 flasks of HFFs as described above for each experimental condition. Whole Cell Lysate was prepared in the following manner: cells were trypsinized in 0.25% trypsin for 5 min at 37°C then neutralized with DME containing fetal bovine serum (FBS). Cells were collected and centrifuged at 4,000 rpm at 4°C for 1 min. Cells were washed twice in cold PBS containing 1 mM phenylmethylsulfonyl fluoride (Thermo Scientific) and centrifuged at 16,000 g at 4°C for 1 min per wash. Cells were incubated in lysis buffer Lysis Buffer (50mM Hepes pH 7.5, 150 mM NaCl, 5 mM EDTA, 5 mM EDTA, pH 8.2, 0.5% Triton X-100, 10 mM β-glycerophosphate, 1 mM NaF, 1 mM dithiothreitol (DTT), 0.1 mM sodium orthovanadate ($Na_3VO_4$)) for 10 min at 4°C. Lysate was separated from membrane debris by centrifugation at 16,000 g for 10 min at 4°C, a small pellet should be seen. The soluble fraction was incubated with antibody-conjugated beads in HBS for 1 to 4 h at 4°C, then washed with NP-40 wash buffer (10mM HEPES-KOH, pH 7.5, 150 mM NaCl, 0.5% NP-40, 2.5 mM EGTA-KOH, 20 mM β-glycerophosphate) 3x for 10 min at 4°C. Beads were collected and processed for Western blotting or mass spectrometry.

**Western Blot:** Samples were boiled in 1% SDS buffer and electrophoresed by SDS-PAGE on a 4-10% gradient gel. Proteins were transferred to a polyvinylidene difluoride membrane, which was blocked in TBS/0.1% Tween-20/5% nonfat dry milk and incubated with primary and secondary antibodies. The blot was incubated with a luminal-based substrate (Immuno-Star Western C; Bio-Rad Laboratories) and chemiluminescence was detected using a charge-coupled device camera (Chemidoc XRS; Bio-Rad Laboratories) and Quantity One 1-D analysis software and analyzed using ImageJ (National Institutes of Health).

**Coomassie staining**: SDS-PAGE gels were immersed in staining solution (50% methanol, 10% glacial acetic acid, 2.5 mg/ml Coomassie Brilliant Blue R-250 in $ddH_2O$) for 1 hr on a rotating platform, then washed with $ddH_2O$. Destaining solution (15% ethanol, 5% glacial acetic acid in $ddH_2O$) was added to the gel and microwaved for 30 minutes in a container containing a sheet of Kimwipes™. The gel was replaced on the rotating platform until bands became visible (1 to 4 hrs).

**MS-compatible silver staining:** SDS-PAGE gel was fixed in 50% methanol + 5% glacial acetic acid for 20 min, washed in 50% methanol for 10 min, sensitized in 0.02% sodium thiosulfate for 1 min, and stained in 0.1% silver nitrate with 0.08% formalin (37% formaldehyde) for 20 min. Gel was developed in 2% sodium carbonate + 0,04% formalin until bands became visible, then stopped by incubation with 5% acetic acid for 10 min.

**Generation of TRAF binding site mutants.** TRAF binding motif deletion mutants of $GRA15_{II}$ were generated by PCR Splicing by Overlapping Extension (SOE). For mutant 1, *ΔAAEE*, amino acids 51-80 (forward, 5'–*ATAATTCGGTGGCTTGGGTATC*–3';

reverse, 5'–*GAAAAGCGTGAGTCCTCCCCTTCACTCTCAGACTTTCCATC* –3') and 85-550 (forward, 5'–*GATGGAAAGTCTGAGAGTGAAGGGGAGGACTCACGCTTTTC* – 3'; reverse, 5'–*TCATGGAGTTACCGCTGATTG* – 3' of $GRA15_{II}$ from the pIC242-$GRA15_{II}$ expression vector described in Chapter 1 were and amplified. For mutant 2, *ΔSQQET*, amino acids 51-430 (forward, 5'–*ATAATTCGGTGGCTTGGGTATC*–3'; reverse, 5'–*CGCATTTTCTACCACAGGTGATAGTGGAAGTTGGCTTTG* –3') and 436-550 (forward, 5'–*CAAAGCCAACTTCCACTATCACCTGTGGTAGAAAATGCG* – 3'; reverse, 5'–*TCATGGAGTTACCGCTGATTG* – 3') were and amplified. For mutant 3, *ΔAAEE/ΔSQQET*, amino acids 51-80 (forward, 5'–*ATAATTCGGTGGCTTGGGTATC*–3'; reverse, 5'–*GAAAAGCGTGAGTCCTCCCCTTCACTCTCAGACTTTCCATC* –3'), 85-430 (forward, 5'–*GATGGAAAGTCTGAGAGTGAAGGGGAGGACTCACGCTTTTC* – 3'; reverse, 5'–*CGCATTTTCTACCACAGGTGATAGTGGAAGTTGGCTTTG* –3'), and 436-550 (forward, 5'–*CAAAGCCAACTTCCACTATCACCTGTGGTAGAAAATGCG* – 3'; reverse, 5'–*TCATGGAGTTACCGCTGATTG* – 3') were and amplified. For SOEing, equal amounts of molecules of each fragment were calculated as follows:

[concentration of fragment #1]*[length of fragment #1 in bp]*[volume of fragment #1] = [concentration of fragment #2]*[length of fragment #2 in bp]*[volume of fragment #2]

Equal amounts of primers for amplifying amino acids 51-550 (forward, 5'–*ATAATTCGGTGGCTTGGGTATC*–3'; reverse, 5'–*TCATGGAGTTACCGCTGATTG* – 3') were then mixed with the template fragments, as well as Phusion polymerase, and placed into a Thermocycler under the following protocol: 1) 95°C for 5 min. 2) 95°C for 15 sec. 3) [lowest annealing temperature of all the primers] for 60 sec. 4) 68°C for [1 minute plus 1 minute for every 1kb of the total PCR product]. Repeat 2) – 4) 39 times.

Wildtype, *ΔAAEE*, *ΔSQQET*, or *ΔAAEE/ΔSQQET* copy of type II GRA15 was inserted into pIC242 retroviral expression vector described in Chapter 1. HeLa cells were then transiently transfected with expression vectors by lipofection using FuGene 6 Plus Transfection Reagent (Roche). Confluent cell cultures were split 1:10 into a 24-well plate (BD) containing glass coverslips. The cells were allowed to incubate at 37°C and 5% CO 2 for 4 h. After incubation, the medium was replaced with 1 ml of fresh supplemented DME, and liposomes were added dropwise to the cells. Liposomes were generated according to manufacturer protocol. In brief, 3 µl of FuGene reagent was mixed into 20 µl of unsupplemented DME and allowed to stand at room temperature for 5 min. Next, 0.5 µg of appropriate plasmid DNA for each transfection was added, mixed, and incorporated into liposomes for 20 min before addition to cells. Cells were left in contact with liposome for 24 h, at which time they were fixed and stained for NF-κB p65.

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**Figure S1: α-GRA15 antibodies were raised against two polypeptide sequences conserved in the three canonical strains.** Amino acid sequence alignment of GRA15 from types III and I (566 AA) and from type II (550 AA) strains. (red) Amino acids 120-139 and 493-510 were selected for peptide synthesis (CDTSTDGKSESEQTENGEDSR-aminde and CGPPRTENPRQPQVPGENS-amide, respectively). KLH conjucation and antibody production using these peptides was performed as per the procedures of Yenzym Antibodies, L.L.C. (Yen-de-Zyn Antigen Design Service™ and ShotGun™ Antibody Production).

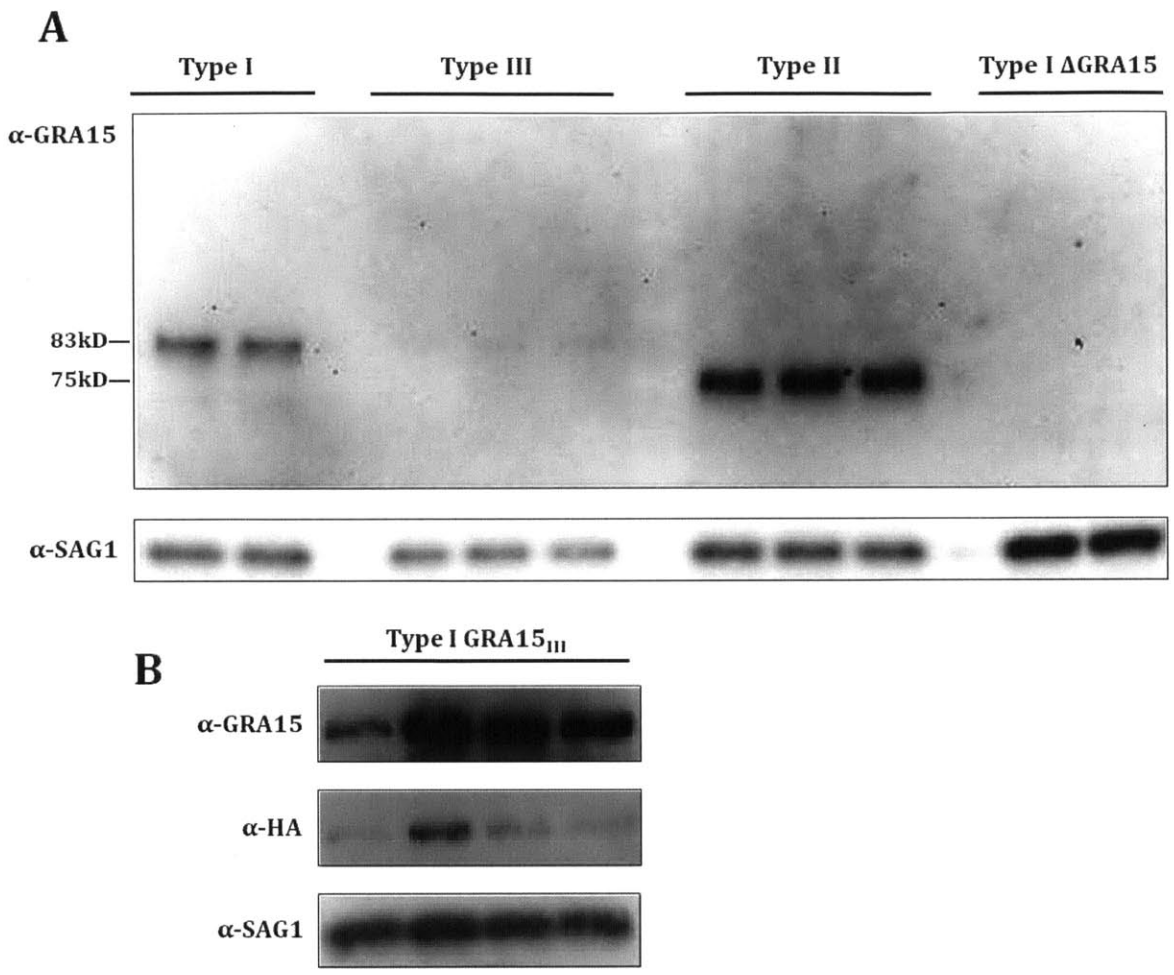



**Figure S2: Western blots confirming specificity of α-GRA15 for $GRA15_{II}$ and $GRA15_{I/III}$.** A) Lysate was obtained from type I (containing a full-length endogenous copy of $GRA15_{I}$), type III and type II strains, as well as from a type I strain not expressing GRA15 (type I ΔGRA15). Endogenous GRA15 protein was probed with purified α-GRA15 polyclonal rabbit antibody (α-GRA15). α-GRA15 recognizes both the $GRA15_{II}$ (75 kD) and $GRA15_{I/III}$ (83 kD) alleles. B) Lysate was obtained from four different clones of type I ΔGRA15 transgenically expressing the type III GRA15 allele (type I $GRA15_{III}$). Transgenic GRA15 was probed with α-GRA15 and compared to probe with α-HA. The parasite Surface Antigen 1 (SAG1) was also probed as a loading control (α-SAG1) in (A) and (B). α-GRA15 is a more sensitive antibody than α-HA.

# Chapter IV

# GRA15 functionality is expression- and sequence-dependent

Author contributions: IF assays of p65 nuclear translocation in non-canonical strains (Figure 1) was performed by Cynthia Cordeiro. RNA-seq and reporter assays of non-canonical strains (Figure S1), as well as control Western for c-Rel activation assay (Figure 4A) was performed by Mariane Melo. GRA15 sequencing in 29 strains was performed by Lindsay Julien, Diana Lu, Mariane Melo, Lauren Rodda, Emily Rosowski, and Ninghan Yang. Phylogenetic analysis was performed by Mariane Melo (Figure 2, S2). Transgenic parasites were created by Diana Lu. GRA15 protein expression assay, p65 and c-Rel assays of transgenic parasites were designed and performed by Diana Lu.

**Summary**

The intracellular protozoan *Toxoplasma gondii* has been shown to directly modulate the NF-κB response of its host. Strain-specific variation of this phenomenon has been observed among the three clonal lineages, the canonical types I, II, and III. The secreted dense granule protein GRA15 has been shown to be necessary and sufficient for the high degree of activation of the canonical pathway in type II strains. Recently, many other strains with unique, non-clonal genotypes have been isolated throughout the world, particular in South America. These non-canonical strains also appear to modulate host NF-κB pathway in a strain-dependent manner.

Here, we describe our analysis of 29 strains of *Toxoplasma gondii*, a collection comprising the three canonical lineages as well as more than twenty non-canonical strains with unique genotypes. We assay the strain differences in p65 nuclear translocation and p65-induced gene expression of infected cells, as well as the sequence and expression differences of GRA15 which may account for the former.

We show that, with few exceptions, only infection by strains similar to type II activates p65 nuclear translocation in the host, and this activity is largely attributed to GRA15. Surprisingly, some strains carrying the type I/III allele could also activate nuclear translocation of the p65 subunit. GRA15 from non-activating strains was able to activate p65 nuclear accumulation when overexpressed in the type I background. However, these transgenic strains were not observed to activate the transcription of NF-κB target genes as significantly. Only the type II and type II-like strains were able to highly induce gene expression in an NF-κB reporter cell line. Furthermore, only the type

II GRA15 allele was able to activate c-Rel, another NF-κB family subunit involved in regulating T cell differentiation and macrophage activation.

Our data suggest that a combination of sequence, expression and background genotype account for the observed strain differences in host NF-κB modulation by the parasite *Toxoplasma gondii*.

## Introduction

The intracellular pathogen *Toxoplasma gondii*, a haploid protozoan of the phylum Apicomplexa, infects nearly one third of the human population worldwide (Hill and Dubey, 2002). This global population structure is geographically divided. In Europe and North America, the vast majority of isolates belong to one of three clonal lineages, the canonical types I, II, and III. In South America, isolates are predominantly non-clonal, with unique, previous uncharacterized genotypes. Phylogenetic classification of these non-canonical strains broadly clustered each into a canonical lineage or one of 12 novel haplotypes (Khan et al, 2011a; Khan et al, 2011b; Su et al, 2012). However, many of these strains have such divergent allele combinations that they cannot be definitively grouped into any lineage (Minot et al, 2012).

This geographic segregation has also been noted for differences in infection pathology. In Europe and North America, infection of immunocompetent hosts is generally asymptomatic. Human toxoplasmosis is observed almost exclusively in immuno-compromised populations: the developing fetus, the acquired immune deficiency syndrome (AIDS) patient, and individuals taking immunosuppressants after receiving organ transplantation. In South America, the rates and severity of disease outcome is much higher. Cases of ocular and cephalic inflammatory disorders are reported more frequently, even in immunocompetent patients, often resulting in permanent injury and sometimes death (Khan et al, 2006; Pena et al, 2008).

More pronounced disease outcomes appear to be linked to the non-canonical genotypes. The rare patients presenting severe symptoms in Europe were infected by atypical strains (Ajzenberg, 2012). Type II and type XII, which is type II-like, are more

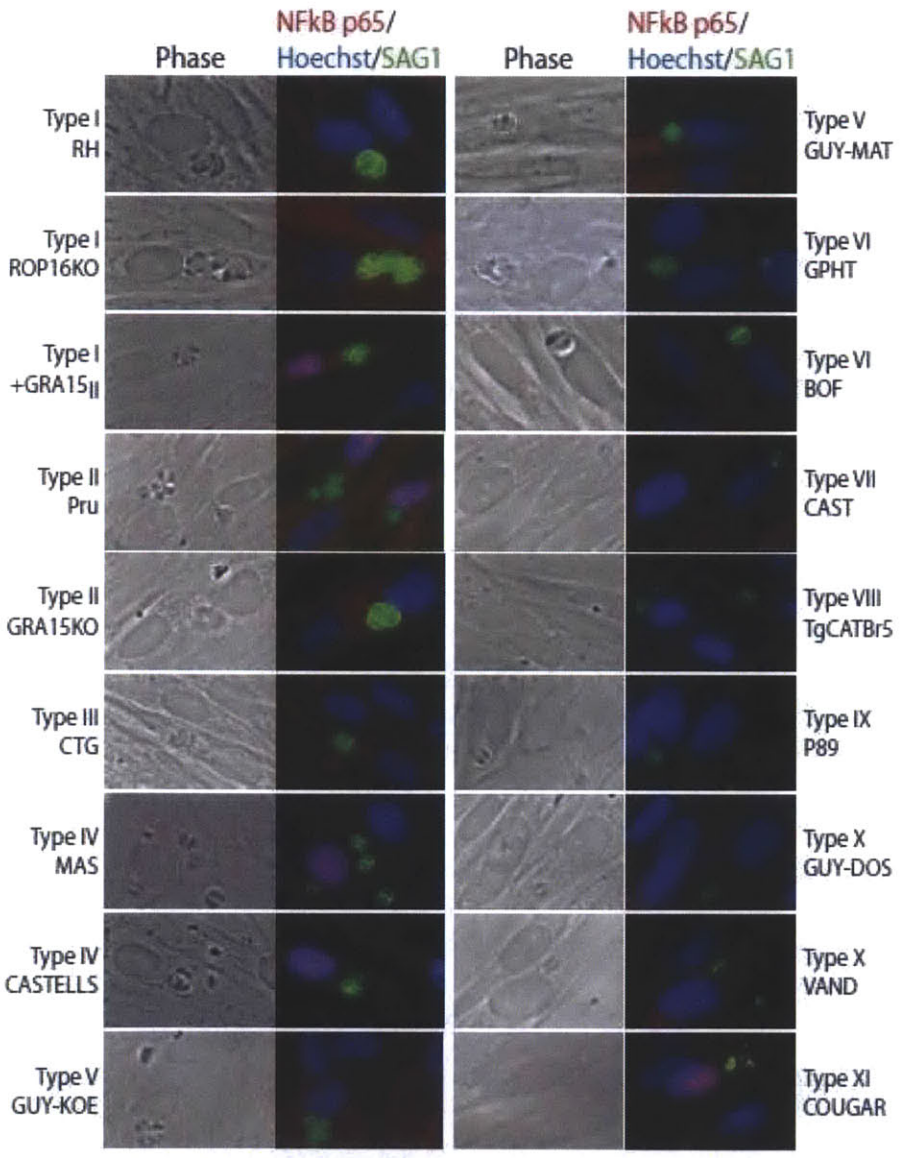
prevalent in North America than in Europe. This may account for the higher rate of inflammatory toxoplasmosis in North America (McLeod et al, 2012; Su et al, 2012).

We previously showed that type II strains strongly activate the host NF-κB pathway, the master regulator of the inflammatory response. This is induced by the secreted parasite factor, GRA15. GRA15 is a novel secreted dense granule protein. The allele found in type II strains ($GRA15_{II}$) is 550 AA and the allele found in type I and type III ($GRA15_{I/III}$) is 636 AA. The type II allele is necessary and sufficient for the nuclear translocation of p65 in host cells. In human and mouse cells, we observe induction of NF-κB-regulated gene expression, yielding inflammatory immune responses such as increased Interleukin-12 (IL-12) induction (Rosowski et al, 2011). A few non-canonical strains (MAS, CASTELLS, and COUGAR) have also been observed to activate p65 translocation in human foreskin fibroblasts (HFFs) (Fig. 1). Little else is known about these strains. The modulation of NF-κB by non-canonical strains likely contributes significantly to the pathology of infection by these strains, and this could be the result of strain differences in GRA15. Alternatively, non-canonical strains may employ an altogether different mode of NF-κB modulation.

Here we describe our work in characterizing the strain differences of GRA15 among 29 *Toxoplasma* isolates representing the three canonical lineages and a broad range of unique haplotypes (Melo et al, 2013). Sequence analysis reveals that the majority of these strains contain a copy of GRA15 that is similar to $GRA15_{I/III}$. A few non-canonical strains contained a copy of GRA15 that were more similar to $GRA15_{II}$, but contained the 84 AA insertion present in $GRA15_{I/III}$. We called these the type II-like GRA15s. Infection of a reporter cell line containing four κB binding sites showed that

only the strains containing type II and type II-like GRA15 were able to activate the expression of NF-κB-induced genes. This correlated with the amount of GRA15 expression in these strains.

**Figure 1: Activation of NFkB by Non-canonical Toxoplasma Strains**




*Courtesy of C. Cordeiro.* **HFFs were incubated with the indicated strains representing all 11 *Toxoplasma* haplotypes for 24 h. Cells were subsequently fixed and probed with a-p65 antibody. Only infection with types II, IV, and XI caused p65 accumulation in the host nucleus.**

A few non-canonical strains containing the allele found in types I and III ($GRA15_{I/III}$) were able to activate p65 translocation (Figure 1, 2). GRA15 expression was markedly higher in these strains than strains that did not activate p65. We created several transgenic strains overexpressing $GRA15_{I/III}$ in a type I background. All strains tested were able to activate p65 translocation to the nucleus. However, c-Rel translocation was only observed in cells infected with strains expressing $GRA15_{II}$.

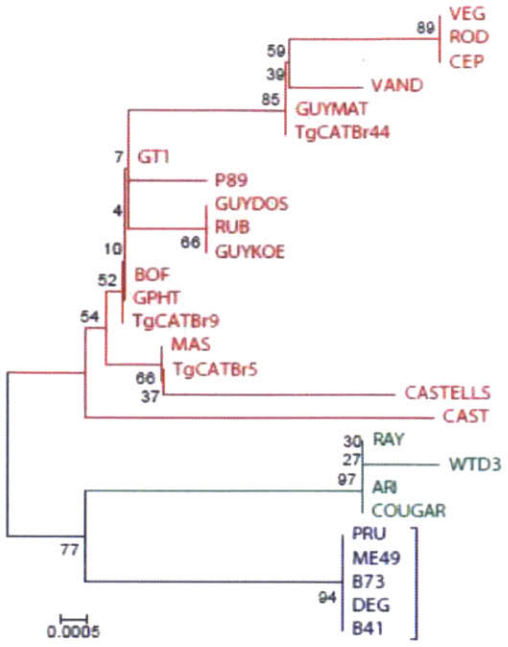
We speculate that GRA15 contributes significantly to the induction of the NF-κB response by the non-canonical strains. The variation of activity among the different genotypes appears to be due to a combination of factors, including GRA15 expression variation, sequence polymorphisms, and possibly other parasite factors affecting GRA15 function.

## Results and Discussion

### GRA15 sequence in most non-canonical strains is type I/III-like

We sequenced the GRA15 coding region of 28 *Toxoplasma* strains representing the 15 canonical and non-canonical lineages (Figure S2). B41 and B73 contain the $GRA15_{II}$ allele (550 AA), here represented by ME49, DEG and PRU. All other strains contained an allele (636 AA) without the 84 amino acid deletion (519-602 AA) seen in $GRA15_{II}$.

A




B

| Strain | Lineage | p65 | reporter | GRA15 exp |
|---|---|---|---|---|
| VEG | I/III | NO | LOW | LOW |
| ROD | I/III | NO | N.D. | LOW |
| CEP | I/III | NO | LOW | LOW |
| VAND | I/III | NO | HIGH | HIGH |
| GUYMAT | I/III | NO | LOW | LOW |
| TgCATBr44 | I/III | NO | N.D. | LOW |
| GT1 | I/III | NO | LOW | LOW |
| P89 | I/III | NO | LOW | LOW |
| GUYDOS | I/III | NO | LOW | LOW |
| RUB | I/III | NO | MED | LOW |
| GUYKOE | I/III | NO | HIGH | HIGH |
| BOF | I/III | NO | HIGH | HIGH |
| GPHT | I/III | NO | LOW | LOW |
| TgCATBr9 | I/III | NO | N.D. | LOW |
| MAS | I/III | YES | LOW | HIGH |
| TgCATBr5 | I/III | NO | LOW | LOW |
| CASTELLS | I/III | YES | MED | HIGH |
| CAST | I/III | NO | LOW | LOW |
| RAY | II-like | NO | HIGH | HIGH |
| WTD3 | II-like | NO | HIGH | HIGH |
| ARI | II-like | N.D. | N.D. | N.D. |
| COUGAR | II-like | YES | MED | HIGH |
| PRU | II | YES | HIGH | HIGH |
| ME49 | II | YES | HIGH | HIGH |
| B73 | II | YES | HIGH | HIGH |
| DEG | II | YES | HIGH | HIGH |
| B41 | II | YES | HIGH | HIGH |

**Figure 2: GRA15 sequence and expression compared with NF-κB activation in 27 strains.** A) Phylogenetic analysis of GRA15 sequence comparing 27 strains of Toxoplasma gondii lineage (type II in blue, type II-like in green, type I/III in red). B) table of strains and their predicted GRA15 lineage (type II in blue, type II-like in green, type I/III in red), p65 nuclear translocation phenotype, NF-κB reporter activation phenotype, and protein/RNA expression profile. Type II-like phenotypes are shown in blue. Intermediate phenotype is shown in green.

Of the type I/III alleles, phylogenetic analysis shows strains COUGAR, ARI, RAY and WTD3 in a cluster that is evolutionarily close to type II, while all other strains cluster with the type I (GT1) and type III (VEG, ROD, CEP) to varying degrees (Melo et al, 2013; Figure 2A). Additionally, we see greater strain differences among the type I alleles. $GRA15_{RH}$ has a frameshift mutation at amino acid 290 that mutates the next 20 amino acids and truncates the protein by 254 amino acids.

**Differences in GRA15 expression do not fully account for the variation in transcription of** NF-κB**-regulated genes in the infected cell.**

We quantified the expression of GRA15 protein in 22 of the 29 strains (Fig. S2C). Others in the Saeij lab determined the level of GRA15 mRNA in 25 strains, obtained by

high-throughput total mRNA sequencing. For each of these strains, they also determined the amount of NF-κB-induced gene expression activated by GRA15 in a 293T firefly luciferase reporter cell line (Fig. S2; Melo et al, 2013).

Both mRNA and protein expression were high for all the $GRA15_{II}$. This correlated with high reporter luciferase expression. WTD3, RAY, BOF, VAND and GUYKOE also were able to activate reporter expression. This was correlated with mRNA expression but not as much with protein expression or sequence. However, CASTELLS and COUGAR, two strains expressing the type I/III allele which could induce visible p65 translocation the strains which could visibly induce p65 in HFFs also had high protein expression. $GRA15_{RH}$, which has a truncation mutation, likewise has no protein or luciferase reads. Even more surprisingly, MAS, which activates p65 nuclear translocation, has a type I/III allele that is lowly expressed and does not activate reporter expression. These results suggest that, though high expression of GRA15 is able to activate the NF-κB pathway, the correlation is not consistent and other parasite factors may be involved.

**Overexpression of $GRA15_{I/III}$ in a type I background activates host NF-κB p65.**

Some strains containing $GRA15_{I/III}$ also activated p65 translocation, so the possibility existed that $GRA15_{I/III}$ was mediating this phenomenon. We therefore created transgenic strains overexpressing HA-tagged copies of $GRA15_{I/III}$. We amplified GRA15 from the non-activating strains GT1, representing type I (Yang et al, 2013; data not shown), CEP, representing type III, and CASTELLS, a non-canonical strain. Types I and III do not appreciably activate host p65 translocation. However, infection by CASTELLS was able to induce p65 nuclear translocation weakly (Figure 1)

We infected human fibroblasts with these transgenic strains and quantified the amount of nuclear NF-κB p65 induced by infection with each overexpression strain (Figure 3A). We also quantified the average amount of GRA15-HA signal in the area of the parasitophorous vacuole of the infected cells to compare the level of expression among the transgenic strains. Wildtype type I was used as an infection control. Because it did not express transgenic HA-tagged protein, no HA staining was observed around the PVM.

When overexpressed in a type I background, $GRA15_{I/III}$ was able to activate p65 translocation, whether the corresponding wildtype strain does or does not. However, there was less nuclear p65 in the cells infected with type I expressing $GRA15_{I/III}$ than in the strain expressing $GRA15_{II}$, even though GRA15 was more abundant in the area of the PV of these infecting strains (Figure 3B).

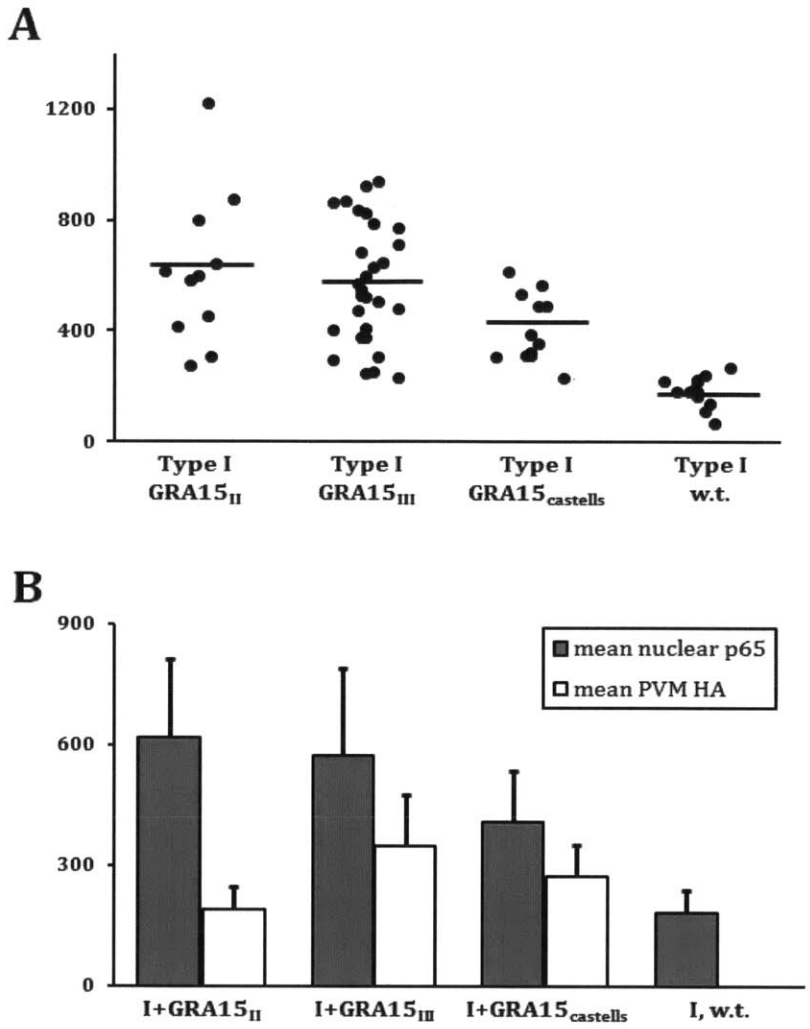



**Figure 3: $GRA15_{I/III}$ activates NF-κB when overexpressed in a Type I background.** A) HFFs were infected with wildtype type I strain (I, w.t.) or type I transgenically overexpressing GRA15 from CEP (type III), ME49 (type II)or castelles (non-canonical). Cells were then fixed and probed with α-p65 antibody and the mean nuclear density of infected cells was measured. B) mean nuclear p65 density from (A) plotted with mean transgenic GRA15-HA density on the PVM.

## Overexpression of $GRA15_{II}$ differentially activates c-Rel nuclear translocation

We previously observed that the transgenic type I $GRA15_{II}$ strain was also able to activate c-Rel nuclear accumulation. We thus tested whether any allele of GRA15 could also activate c-Rel. In Figure 4, we see that infection with type I $GRA15_{III}$ can induce a

significant increase in nuclear p65 accumulation with respect to wildtype type I. However, c-Rel is only very slightly activated by type I GRA15$_{III}$, and much more so by wildtype type II or type I GRA15$_{II}$, and this result was not due to differences in protein expression among the strains assayed. We conclude from this that only the type II allele of GRA15 activates c-Rel highly.

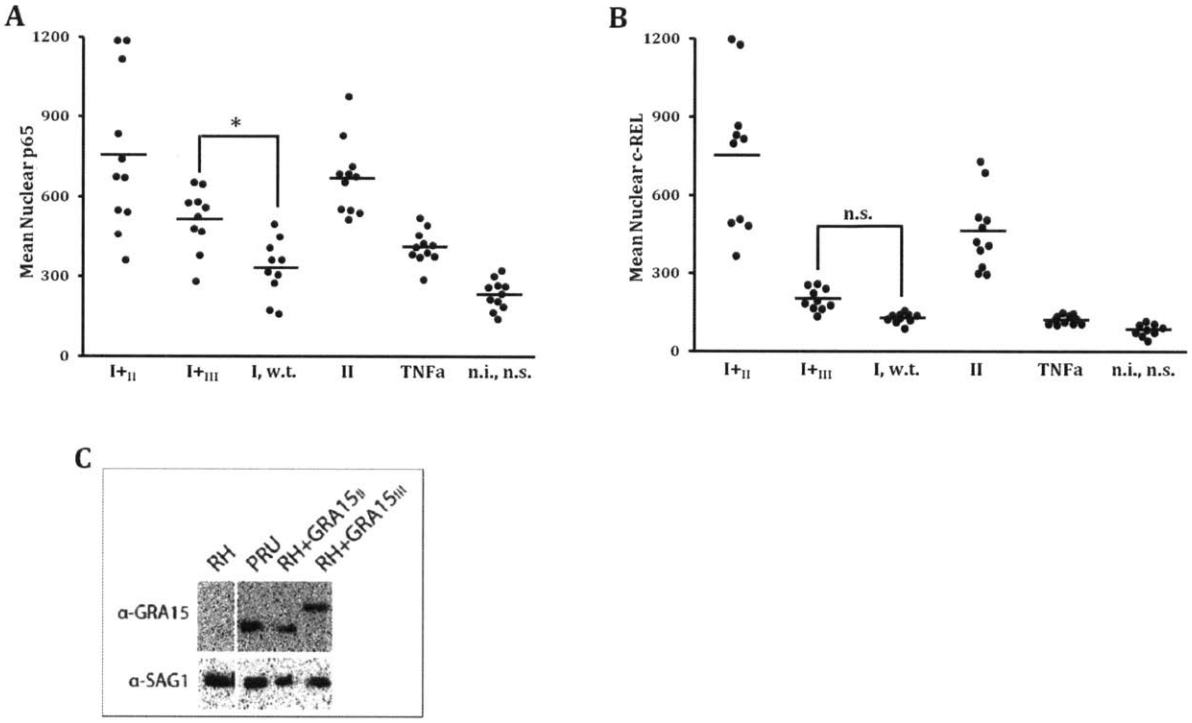



**Figure 4: C-REL nuclear translocation is specifically activated by GRA15$_{II}$.** (A) and (B) Cells were infected with type I GRA15$_{II}$ (I+$_{II}$), type I GRA15$_{III}$ (I+$_{III}$), type I (I, w.t.), type II (II) strain for 24 h, stimulated with 20 ng/ml human TNF-α for 1 h, or left uninfected and unstimulated (n.i., n.s.). Cells were then fixed and probed with α-p65 (A) or α-c-REL (B) antibody and the mean nuclear density of each NF-κB subunit was measured. Each assay was repeated at least twice and quantification was performed on a representative experiment. Horizontal bars represent the mean nuclear p65 intensity over all cells. C) *Courtesy of M. Melo: Western blot on lysates of the indicated strains using an antibody against GRA15 that detects the type II GRA15 (550.AA) and the type III GRA15 (636.AA). SAG1 antibody was used as a parasite loading control.*

## Conclusion

Here we characterize the etiology of strain variation of NF-κB activation in 29 strains of *Toxoplasma gondii,* including South American isolates which cause severe

disease outcomes in infected individuals. Because GRA15 modulated the strain difference in the canonical strains, it seemed likely that this would be true among the unique strains. We first characterized the protein sequence.

We sequenced the coding region of GRA15 from 28 *Toxoplasma* strains representing the canonical lineages, types I, II and III and unique strains dispersed among the 15 or more haplotypes. Three strains (B41. B73 and DEG) were determined to be type II and carried an allele of GRA15 that is identical to that of type II, which includes the 84 AA deletion producing the 550 AA protein, instead of the 566 AA $GRA15_{I/III}$Type II allele and lacks the N-terminal PEXEL motif. The latter polymorphism will be described in more detail below. Strains RAY, ARI, WTD3 and COUGAR were determined to be type II-like through phylogenetic tree construction (Melo, 2013), and all other strains carried the type I/III like allele. In previous IF assays, we observed high p65 nuclear translocation following infection with type II strains and weaker activation following infection with the non-canonical strains CASTELLS, COUGAR, and MAS (Figure 1). Interestingly, of these strains, only COUGAR is type II-like though it contains a GRA15 that is 636 AA.

We next created transgenic type I strains overexpressing type I/III-like GRA15, to determine to test the contribution of sequence polymorphisms to GRA15 function. A type I strain overexpressing $GRA15_{I/III}$ was observed to induce p65 nuclear translocation in the host. However, NF-κB induction was lower than that of strains overexpressing $GRA15_{II}$, even though expression of the transgenic protein was higher (Figure 3).

Since strains carrying $GRA15_{I/III}$ were also observed to induce p65 activation in the host, we wondered if GRA15 function was expression-dependent and these strains

had higher expression than the non-activators, or if some other parasite factor was modulating p65 activity in these strains. We determined each strain's ability to induce expression of firefly luciferase in an NF-κB reporter cell line containing four κB binding sites before the minimal luciferase reporter. We compared the relative luciferase activity of each strain to its expression level of GRA15 mRNA and protein.

Interestingly, only the strains carrying type II and type II-like alleles were able to induce high levels of NF-κB transcriptional activity. Transcript levels of these strains were also high. However, level of GRA15 mRNA expression was not completely correlated with activation. $GRA15_{WTD3}$, a type II-like allele, was the most highly expressed transcript, yet other strains with the same allele were able to induce higher luciferase activity. Further, $GRA15_{II}$ expression from all type II strains was less than WTD3 and each activated similar or higher levels of luciferase activity.

Comparing protein expression to transcript expression, we see that only type II GRA15 protein is expressed highly while none of the other strains expresses much protein by comparison. $GRA15_{CASTELLS}$ and $GRA15_{COUGAR}$ proteins levels were relatively high compared to other non-type II strains. Though the corresponding strains only activated moderate luciferase production in the reporter cell line, the high GRA15 protein expression could account for the low level of p65 nuclear translocation previously observed in IF. Surprisingly, MAS infection activated relatively low levels of luciferase expression and both $GRA15_{MAS}$ transcript and protein levels were relatively low. The lack of consistently direct correlation between expression and GRA15 activity suggests that other *Toxoplasma* factors may contribute to host NF-κB regulation by these strains.

ROP16 has previously been described to antagonize the function of GRA15 (Jensen et al, 2011). ROP38 has also been identified as a factor which inhibits NF-κB activation by GRA15 in the type III lineage (Melo et al, 2013). Strain differences in this and other *Toxoplasma* factors may also contribute to establishing a more or less permissive milieu for GRA15 function. To test this, we can transfect GRA15 from different strains into the directly into the reporter cell line. This will allow us to compare the functionality of GRA15 from different strains independent of other parasite factors.

Surprisingly, overexpression of $GRA15_{I/III}$ in a type I background was also observed to induce nuclear translocation of p65 but not c-Rel. Only the type II allele of GRA15, endogenously or when overexpressed, activated c-Rel. c-Rel and p65 are the primary transcriptionally active members of the NF-κB family and serve overlapping but distinct functions in the regulation of host immunity (Fullard et al, 2012). In particular, c-Rel appears to play a more significant role in mediating the connection between innate and adaptive responses (Liou and Hsia, 2012). The selective activation of c-Rel by type II strains could at least partially explain why these strains are able to induce far more reporter expression than other strains which activate p65 in IF. Furthermore, it can serve as a pathway to long-term immune activation of the host, which is important to maintaining Toxoplasma's ability to sustain a chronic, encysted infection. To determine the distinct functions of p65 and c-Rel activation, we can assay the expression profiles of $c\text{-}Rel^{-/-}$ host cells in comparison to $p65^{-/-}$ and wildtype cells We can also compare the *in vivo* consequences of infection between type I strains overexpressing different alleles of GRA15. In any case, this result demonstrates that there are striking functional disparities between GRA15 from type II versus that from other strains.

Though the GRA15 has relatively few polymorphisms, the only—and probably the most significant—one that is shared between all the type I/III alleles that is dissimilar in the type II copy is the deletion of 84 C-terminal amino acids in GRA15II that is present in all other strains. We speculated that the strain variation between type II and the other strains of GRA15 are due to this sequence difference.

We can further explore the questions presented above using the reagents generated by additional work completed for this dissertation. We have created numerous vectors for the expression of GRA15 in both *Toxoplasma gondii* and mammalian cells, including vectors expressing chimeras of type II amine-terminal domain (NTD) with type III carboxy-terminal domain (CTD), and type III NTD with type II CTD, respectively, in both a type I background as well as in the mammalian expression vector, pIC242. We have also generated expression vectors containing various lengths of truncated protein to assay the functionality of different regions of the protein.

We can delete GRA15 from non-canonical strains that activate NF-κB. This would allow us to determine if GRA15 is indeed the sole necessary factor in NF-κB activation during infection by these strains. To see what other properties GRA15 has, for example how it affects other signaling pathways and immune or cell cycle processes of the host, or vice versa, we can make different recombinant parasites, expressing all the possible combinations of knockout, wild type, and overexpression complements of GRA15 and some other factor involved in host modification, such as ROP16 and ROP38. Though many questions remain unanswered, we conclude from our analysis that the strain-specificity of GRA15 function is the result of a combination of factors: different expression levels, sequence polymorphisms which confer functional variation.

## Materials and Methods

**Cells and reagents.** Parasite and cell culture maintenance, wildtype and transgenic strains, cell cultures and expression plasmids have been previously described in Rosowski, et. al. All tissue culture reagents were obtained from Gibco, unless otherwise indicated. Poly(dA:dT) was obtained from Invitrogen. All oligonucleotide primers were obtained from Integrated DNA Technologies. Antibodies against HA, human NF- κB p65, IF secondary antibodies, and antibodies conjugated to HRP for use in western blotting have also been previously described (See Chapters 2 and 3). Antibodies against NF-κB2 p100/p52, RelB and c-Rel were obtained from Acris (AP02559PU-S), Millipore (EP613Y), and Cell Signaling (cs-4727), respectively. DNA sequences of GRA15 were obtained by Sanger sequencing performed by GeneWiz, Inc. and the DNA sequencing core facility at the Koch institute for Cancer Research. Multiple sequence alignment was generated by ClustalX.

**Parasites.** The following *Toxoplasma* strains, with the animal and country it was originally isolated from in brackets, were used in this study: ARI (human, USA), B41 (bear, USA), B73 (bear, USA), BOF (human, Belgium), CAST (human, USA), CASTELLS (sheep, Uruguay), CEP (cat, USA), COUGAR (Cougar, Canada), DEG (human, France), FOU (human, France), GPHT (human, France), GT1 (goat, USA), GUY-DOS (human, French Guiana), GUY-KOE (human, French Guiana), GUY-MAT (human, French Guiana), (USA); MAS (human, France), ME49 (sheep, USA), P89 (pig, USA), PRU (human, France), RAY (human, USA), RH-ERP and RH-JSR (human, USA), ROD (human, USA), RUB (human, French Guiana), TgCatBr5 (cat, Brazil),

TgCatBr9 (cat, Brazil), TgCatBr44 (cat, Brazil), VAND (human, French Guiana), VEG (human, USA), WTD3 (white-tailed deer, USA) (Khan et al., 2011b). All parasite strains and cell lines were routinely checked for *Mycoplasma* contamination and it was never detected.

**Generation of transgenic parasites.** The GRA15 coding region and putative promoter (1,940 bp upstream of the start codon) was amplified from type I, type III, type IV, and type XI *T. gondii* genomic DNA by PCR (forward, 5'–*CCCAAGCTTGACTGCCACGTGTAGTATCC*–3'; reverse, 5'–*TTACGCGTAGTCCGGGACGTCGTACGGGTA TGGAGTTACCGCTGATTGTGT*–3'). Sequence coding for an HA tag (CGCGTAGTCCGGGACGTCGTACGGGTA) was included in the reverse primer to C-terminally tag the protein. GRA15-HA was inserted into pCR8/GW (Invitrogen) by TOPO-TA cloning, then cloned into pTKO-att by LR recombination (Invitrogen). The pTKO-att-GRA15-HA vector was then linearized by digestion with XhoI (NEB), which cuts off 244 bp of the putative promoter, leaving 1,696 bp intact upstream of the start codon. Linearized vector was transfected into RH ΔHXGPRT parasites by electroporation in a 2-mm cuvette (Bio-Rad Laboratories) with 2 mM ATP (MP Biomedicals) and 5 mM GSH (EMD) in a Gene Pulser Xcell (Bio-Rad Laboratories), with the following settings: 25 FD, 1.25 kV, ∞Ω. Stable integrants were selected in media with 50 μg/ml mycophenolic acid (Axxora) and 50 μg/ml xanthine (Alfa Aesar) and cloned by limiting dilution. Expression of GRA15 was confirmed by IF for HA staining.

**High-throughput RNA sequencing.** The RNA was processed for high-throughput sequencing according to standard Illumina protocols. Briefly, after mRNA pull down from total RNA using Dynabeads mRNA Purification Kit (Invitrogen), mRNA was fragmented into 200-400 base pair-long fragments and reverse transcribed to into cDNA. Illumina sequencing adapters were added to each end, and samples were barcoded and multiplexed in a single lane (four samples per lane) on an Illumina sequencing high-throughput flow cell. On average 68 million 40 bp reads were obtained from each library. Reads in fastq format were mapped to either mouse (build 37.2) or *Toxoplasma gondii* (strain ME49, version 8.0) genomes using Bowtie 2.0.0 and Tophat 2.0.4. (Trapnell et al., 2012). Transcripts from each sample were assembled, merged, and their expression values in FPKM were calculated using the Cufflinks package, version 2.0.2. (Trapnell et al., 2012). Detailed methodology can be found at supplemental material, and settings used are included in Table S1A-B

**Western Blot:** Samples were boiled in 1% SDS buffer and electrophoresed by SDS-PAGE on a 4-10% gradient gel. Proteins were transferred to a polyvinylidene difluoride membrane, which was blocked in TBS/0.1% Tween-20/5% nonfat dry milk and incubated with primary and secondary antibodies. The blot was incubated with a luminal-based substrate (Immun-Star WesternC; Bio-Rad Laboratories) and chemiluminescence was detected using a charge-coupled device camera (Chemidoc XRS; Bio-Rad Laboratories) and Quantity One 1-D analysis software and analyzed using ImageJ (National Institutes of Health).

**IF.** Cells were fixed with 3% (vol/vol) formaldehyde in PBS for 20 min at room temperature, permeabilized with 100% ethanol, and blocked in PBS with 3% (wt/vol) BSA and 5% (vol/vol) goat serum. Coverslips were incubated with primary antibody for 1 h at room temperature or overnight at 4°C, and fluorescent secondary antibodies and Hoechst dye were used for antigen and DNA visualization, respectively. Coverslips were mounted on a glass slide with Vectashield (Vector Laboratories), and photographs were taken using NIS-Elements software (Nikon) and a digital camera (CoolSNAP EZ; Roper Industries) connected to an inverted fl fluorescence microscope (model eclipse Ti-S; Nikon). Quantification of nuclear signal was performed by randomly selecting at least 10 infected cells per *Toxoplasma gondii* strain and measuring the mean signal intensity per nucleus and per PVM using the NIS-Elements software and Hoechst dye to define nuclei.

**Plaque assay.** For the assays comparing the NF-κB nuclear localization as well as reporter activation by infection with transgenic parasites, cells were infected with different MOIs and a plaque assay was done to determine the viability of each strain. The infections with the closest MOIs were then used. For the plaque assay, 100 parasites per well were added to confluent HFFs in a 24-well plate and were incubated for 5 d at 37°C in 5% $CO_2$. The number of plaques per sample was counted by sight, under 4x magnification.

**Luciferase assay.** Confluent 293T NF-κB reporter cells were split 1:3 into a 96-well plate. 4 hr before infection/stimulation. Cells were infected by each strain in triplicate and incubated for 16 h at 37°C in 5% $CO_2$. Infected cells were lysed with Cell Culture Lysis

Reagent (Promega) supplemented with 1 mM phenylmethylsulfonyl fluoride (Thermo Scientific) and cells were sheared by mechanical force from pipetting up and down a few times. The plate was frozen at -80°C for at least 5 days and then thawed to room temperature. Luciferase Assay Substrate in Luciferase Assay buffer was used to measure luciferase activity in each well using a Varioskan Flash plate reader (Thermo Scientific) with the following program: well loop comprising a dispense step and luminometric read step with a 2 s lag time and a 10 s read time. The mean luminometric intensity for each strain was calculated and graphed with standard deviation for that mean.

**GRA15 protein expression assay.** Fully lysed parasites were collected from T25 flasks (1 for type RH, 3 for all other strains). Parasites were centrifuged at 5000 rpm for 10 minutes and washed 3x with 5 ml PBS in 15 ml BD Falcon tubes. Cells were resuspended in 1 ml PBS and transferred to 1.5 ml eppendorf tubes. Cells were pelleted, lysed in 100 µl lysis buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 5 mM EDTA, 5 mM EDTA, pH 8.2, 0.5% Triton X-100, 10 mM β-glycerophosphate, 1 mM NaF, 1 mM dithiothreitol (DTT), 0.1 mM $Na_3VO_4$), and sonicated (1 pulse, 1 sec). A 10 µl aliquot of each sample was taken to determine protein concentration by Bradford assay (2 µl lysate in 2 ml Coomassie dye. Absorbance taken with spectrophotometer at 595 nm. Standard curve was calculated with 0, 1, 2, 5, and 10 µl PBS) Rest of lysates were frozen in liquid nitrogen and stored in -80°C for at least one day. Lysates were thawed and diluted to the concentration of the strain with the lowest concentration in lysis buffer. A 50 µl aliquot of each lysate was taken and mixed with 50 µl 2D SDS-PAGE buffer supplemented with 35 µl 1 M DTT. Samples were boiled for 10 minutes in parafilm-sealed eppendorf tubes to

prevent evaporation or water accumulation from hot water bath and centrifuged at 13,000 rpm at 4°C the placed on ice. Samples were loaded (5 μg total protein/lane) and run in triplicate on a 4-20% gradient SDS-PAGE gel. Western blot was performed with antibody against GRA15 and the loading controls GRA7 and SAG1. Mean densitometric units of chemiluminescence was calculated for each band using the Quantity One 1-D analysis software and ImageJ (National Institutes of Health)

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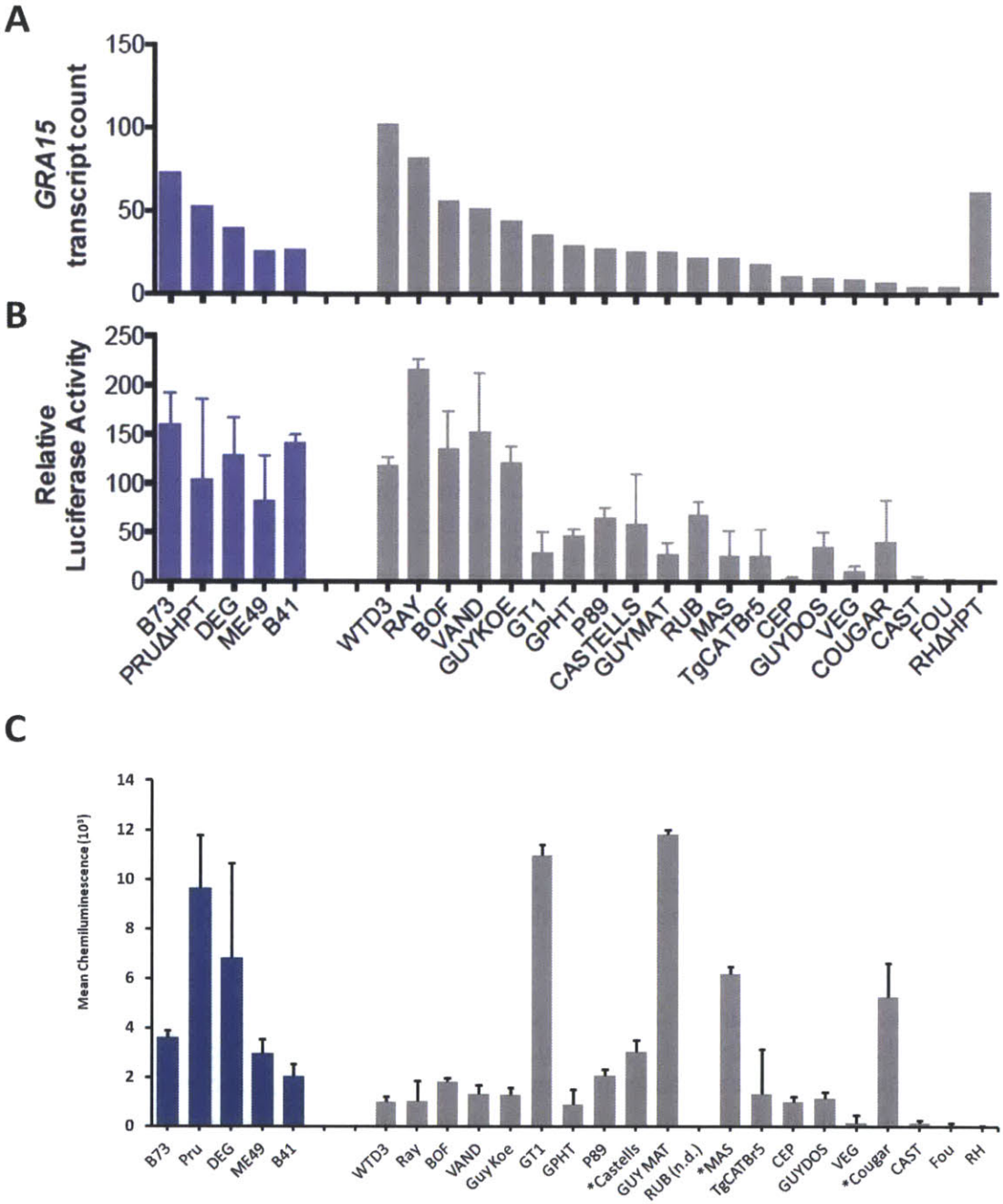


**Figure S1: NF-κB activation correlates with both GRA15 sequence and expression, but not entirely.** *Courtesy of M. Melo (Melo et al, 2013): A) GRA15 gene expression levels as determined by high-throughput RNA sequencing. B) Indicated Toxoplasma strains were used to infect HEK293 cells stably expressing firefly luciferase under the control of an NFκB promoter. 20 h post infection luciferase activity was measured in cell lysates. The relative luciferase activity was calculated by normalizing the raw luminescence values to the background.* C) Extracellular parasite of each indicated strain was lysed with IP buffer and centrifuged. Lysate supernatant was used to determine total protein concentration via Bradford assay and a normalized concentration of lysate was probed in triplicate with an α-GRA15 antibody in Western blot. This assay was performed twice. Relative densitometric read was calculated by normalizing the two assays' combined average horseradish peroxidase chemiluminescence of each strain to that of the negative control (RH) samples. No data for RUB (n.d.). *CASTELLS, COUGAR and MAS induce p65 nuclear translocation in infected HFFs (Figure 1)

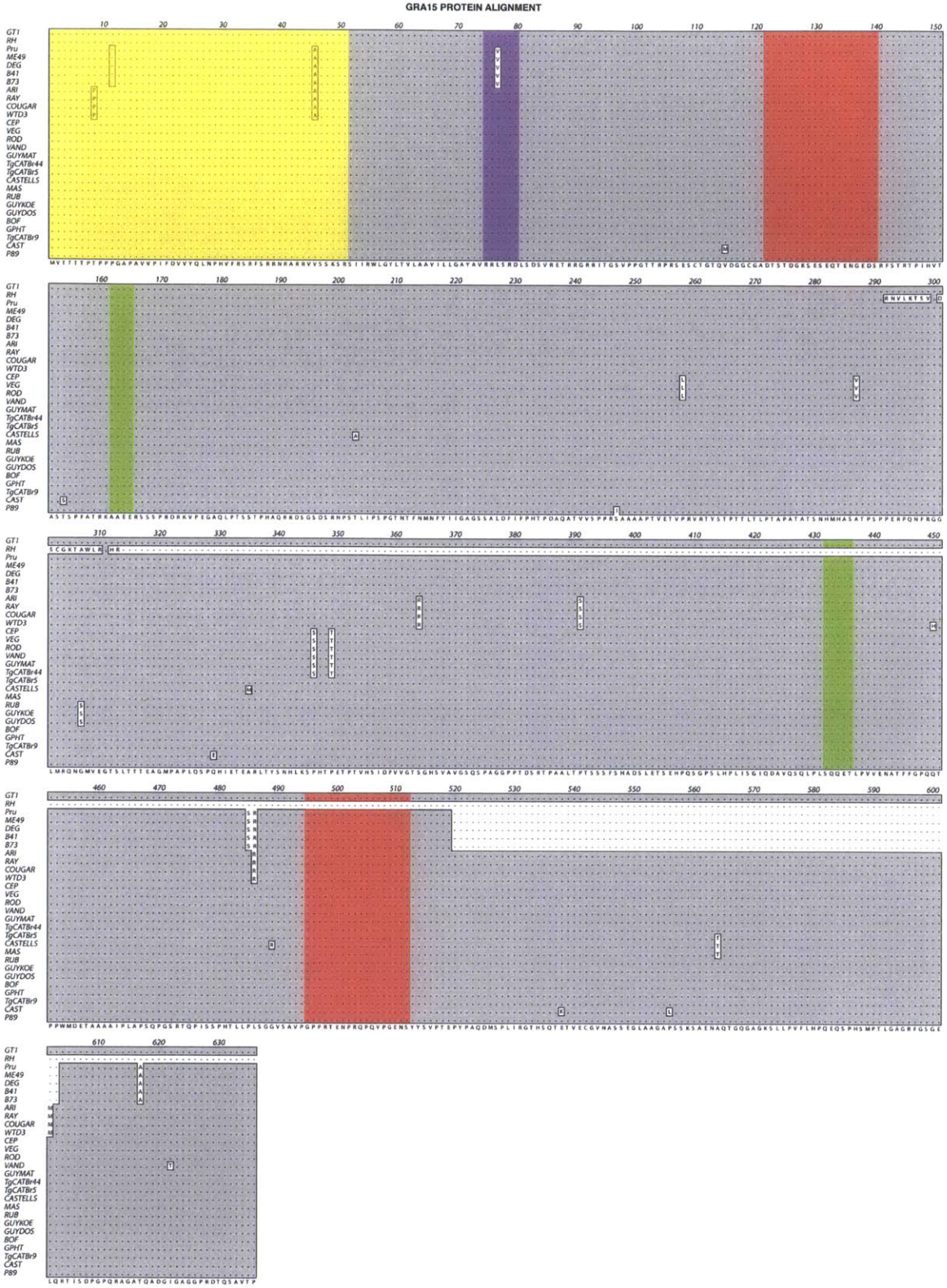


**Figure S2: GRA15 predicted protein sequence alignment in 28 *Toxoplasma* strains.** *Modified from Melo et al, 2013: The amino acid sequences were predicted from nucleotide sequences obtained by PCR sequencing, and the multiple sequence alignment was performed using the MacVector Software (vs12.6, Accelrys, Cary, NC, USA). Identical (.) and missing (-) aminoacids are indicated.* Signal peptide (yellow) was predicted using the SignalPPro algorithm. TRAF binding motifs (green), PEXEL sequence (purple), and α-GRA15 recognition peptides (red) are also indicated.

# Chapter V

# Discussion and Future Directions

Here we have identified GRA15 as the *Toxoplasma gondii* secreted factor responsible for host NF-κB activation. GRA15 is a secreted dense granule protein. In type II, GRA15 is a 550 AA protein that highly induces p65, p50 and c-Rel nuclear translocation. Subsequent NF-κB-regulated gene expression is also activation, resulting in higher levels of inflammatory stimulation *in vivo*. GRA15 is necessary and sufficient for NF-κB activation, though evidence suggests that components of infection, such as PVM association, may optimize its function. GRA15 function also requires functional host proteosome and IKK complex, and partially requires TRAF6 (Chapter II).

Additionally, GRA15 is continually expressed by the parasite and present in the intravacuolar space and PVM, and requires 4 hrs for full p65 activation. Compared to that of other NF-κB-activating stimuli such as Tumor Necrosis Factor-α (TNF-α), which takes only minutes, the time course of activation is very slow (Chapter II). Though GRA15 has no predicted secondary or tertiary structure, GRA15 amino acid sequence contains a *Plasmodium* export element (PEXEL) in its N-terminus, near the signal peptide (Chapter IV, Figure S1) (Marti et al, 2004). In *Toxoplasma*, the PEXEL motif, Rx(L/V)x(D/E) has recently been shown to target its containing protein for secretion to the PVM (Hsiao et al, 2013). GRA15 likely uses this sequence motif to be secreted to the PVM, where it can be shuttled out of the vacuole to interact with host elements.

We also identified two TRAF binding motifs in the primary sequence. This better fits a mechanistic model requiring continual secretion and accumulation of protein in the host, which may induce complexing of adaptor proteins such as the TRAFs. We observed interaction between $GRA15_{II}$ and TRAF3 in immunoprecipitation assays, but found that

this putative interaction was not mediated by either of the TRAF binding motifs (Chapter III).

All other strains, including those of the type I and type III canonical lineages, carry a 636 AA allele of GRA15, the $GRA15_{I/III}$ allele. Some non-canonical strains contain a 636 AA GRA15 which is more type II-like than others. In general, only strains expressing type II and type II-like GRA15 activate NF-κB. However, $GRA15_{I/III}$ can also induce host p65 nuclear accumulation when transgenically overexpressed in the type I background. Interestingly, only the strains expressing the type II or type II-like allele were able to activation high expression from an NF-κB-regulated reporter, and only strains expressing $GRA15_{II}$ activated nuclear accumulation of c-Rel in the host, though the type II-like GRA15 was not tested (Chapter IV).

From these data, we propose the following mechanistic model for GRA15 function: GRA15 is transcribed and translated. Following this, the signal peptide (SP) (AA 1-50) targets the protein for secretion via the dense granule. After SP removal, the NTD undergoes additional processing. Similar to the *Plasmodium* model, the GRA15 PEXEL is likely cleaved by an aspartic protease like plasmepsin in the ER. This cleavage is mediated by positions 1 and 3 (R and L/V) of the PEXEL motif has been observed to be necessary for PVM association in other GRA proteins (Hsiao et al, 2013). In *Plasmodium*, position 5 was necessary for PEXEL-containing proteins to be exported to the erythrocyte (Boddy et al, 2009), so perhaps GRA15 also uses this method to interact with export proteins at the PVM to exit the vacuole, where it interacts with host factors to activate the IKK complex.

This model is not without precedent. Another dense granule protein, GRA16, has been observed to be exported from the PV and into the host nucleus, where it interacts with the deubiquitinase HAUSP and the PP2A phosphatase to positively modulate cell cycle progression and the p53 tumor suppressor pathway (Bougdour et al, 2013). GRA16, also an unstructured protein, is continually exported through the PVM into the host cell, where it accumulates in the nucleus. We speculate that GRA15 functions similarly. Over the course of infection, GRA15 accumulates at the PVM or in the extra-vacuolar space (EVS). Here, it interacts with the canonical NF-κB pathway to induce the expression of gene sets activated by the transcription-regulatory subunits, p65 and c-Rel.

Strain-specific differences in GRA15 function appear to be mediated by a combination of factors, including mRNA and protein expression, protein sequence, and possibly other parasite factors. This has been shown to be the case for the rhoptry kinase, ROP16, in the activation of STAT6 (J. Saeij, unpublished observation).

$GRA15_{II}$ is universally more effective at activating p65 than $GRA15_{I/III}$, though the latter retains some function. We observe that higher mRNA and protein expression correlate with p65 nuclear localization and/or NF-κB-regulated gene expression. Strains that express $GRA15_{I/III}$ at high levels tend to induce high p65 nuclear accumulation, but not necessarily high reporter activity. To determine the relationship is causal, we could transfect GRA15 expression plasmids directly into mammalian cells. This allows us to assay GRA15's ability to activate p65 compared with reporter expression independent of other parasite factors.

In contrast, only type II and type II-like GRA15 are able to activate high NF-κB transcriptional regulation and c-Rel nuclear accumulation. Intrinsic differences in GRA15

protein sequence could contribute to this. To test this, we can compare the activities of $GRA15_{II}$ and $GRA15_{I/III}$ when they are expressed alone in the host cell.

The functionally relevant difference between $GRA15_{II}$ and $GRA15_{I/III}$ could simply be that the type II allele contains an 84 AA deletion, making it shorter and therefore easier to travel through protein channels of the PV and into the host cytoplasm via its PEXEL motif. To determine if protein secretion and/or PVM association is necessary for GRA15 function, we can assay the effect of PEXEL deletion from the protein sequence.

We do not directly observe GRA15 to be present by itself in the host cytosol, or in any organelles, however, it is still possible that GRA15 be present and functional in these regions, though the quantities thereof are too small for detection with standard immunofluorescence techniques. There could also be so much more GRA15 in and around the PVM that the signal from extra-PVM protein is too weak in comparison, and cannot be detected in the presence of the PV. One method to get around this problem is by conjugating the GRA15 protein with a cleavable beta-lactamase reporter (Lodoen et al, 2010) and assaying the enzymatic activity thereof in an inducible system. With these methods, future experimentation will uncover the mechanism of action of the first characterized dense granule protein that can directly modulate signaling pathways of the host.

Further, the type I allele, $GRA15_{GT1}$, does not activate p65 nuclear translocation. However, when transgenically overexpressed in another type I (RH) background (Yang et al, 2013), this copy induces p65 nearly as well as $GRA15_{II}$. This suggests that other parasite factors may also play a role. For example, there may be inhibitory factors in

these strains that are differentially regulated, which create a more or less permissible background for GRA15 to function. We already know of two parasite factors, ROP16 and ROP38, which inhibit the activation of p65 nuclear translocation by GRA15 (Jensen et al, 2011; Melo et al 2013). Because we observe that some strains induce p65 accumulation in the nucleus yet do not activate NF-κB -regulated gene expression, there may be additional unknown factors that inhibit NF-κB function in the nucleus. Factors such as GRA16 could be involved in the activation state of NF-κB subunits in the nucleus, which is dependent on ubiquitination, phosphorylation and other post-translational modifications (Hoffman and Ghosh, 2006). Another possibility is that there are factors modulating the interaction between the transcriptionally active subunits and DNA or the chromatin scaffold.

To identify other proteins that may be involved in NF-κB activation, we can perform a mutagenesis screen on the type I GRA15$_{II}$ strain. Since the transgenic protein is expressed in multiple copies, any mutant phenotype is more likely to be due to dysfunction in endogenous parasite factors, which are present in only one copy in the haploid genome, and we can therefore select for mutants that induce less reporter expression and sequence their genomes to identify the gene(s) responsible.

The host interacting partner of GRA15 remains to be confirmed, and we can employ a number of possible strategies for this process (Chapter III, conclusions). We previously attempted to precipitate GRA15 from a stable cell line. This approach was not successful, likely because NF-κB is constitutively activated in these transgenic cells (data not shown). To avoid this issue, we can express GRA15 in human cells using an inducible promoter and attempt to co-precipitate binding partners.

From our data, the TRAFs remain interesting candidates. One possibility is that GRA15 aggregation itself induces the recruitment of adaptor complexes that function in IKK activation. Being a highly unstructured protein, GRA15 could act as a scaffold for other proteins to bind. In this case, once a sufficient amount of GRA15 has accumulated in the host or on the PVM, it could mimic stimulated receptor and bound receptor-interacting proteins. Another possibility is that is at GRA15 is ubiquitinated by the TRAFs, and ubiquitination recruits IKK-activating elements, such as the TAB/TAK complex.

For *Toxoplasma* to be able to establish lifelong chronic infections in such a broad range of intermediate hosts, it must be able to adapt to many different host environments (Sibley, 2002). There is great variation in the susceptibility of different hosts to *Toxoplasma*, and to different strains of the parasite (Dardé et al, 1998; Saeij et al, 2005; Ajzenberg, 2012). It is therefore unsurprising that different strains have developed unique methods of host immuno-modulation, possibly as a means of evolving particular affinity to preferred hosts. As one of the primary regulators of the response toward pathogen infection, the level of NF-κB activity in the host determines the persistence or obliteration of the infecting parasite.

NF-κB activation promotes host survival. Activation of the inflammatory response induces the parasite to convert to the encysted life stage. The cyst is the only form that transmits infection between intermediate hosts. Activation of NF-κB therefore increases the parasite's chance of transmission to other hosts over a broad geographic area, assuming the host is motile. However, overactivation also leads to inflammatory pathologies resulting in deleterious consequences for both pathogen and host. Decreasing

NF-κB activation allows the parasite to evade the inflammatory response of the host, but too little immune activity leads to over-proliferation of the parasite and premature host death, preventing the parasite from infecting new organisms (Mason et al, 2004).

$GRA15_{II}$ highly activates host NF-κB in type II infections. These strains may have evolved this method of inflammatory activation to establish infection in hosts that normally do not respond to infection with strong stimulation of the type I innate immune response. However, when type II strain infects other hosts, the same adaptation may cause severe disease due to overactive inflammation (Ajzenberg, 2012).

Perhaps non-type II strains have retained kinase activity in ROP16 and evolved decreased GRA15 functionality in order to maximize their potential for survival in hosts that already respond to infection with high levels of inflammation. In these hosts, it is more useful for the parasite to control inflammation to protect the host and itself with it, from the resulting damage.

Optimization of NF-κB pathway control is extremely important for the persistence of *Toxoplasma* in different host environments. Understanding the dynamics of this process will undoubtedly lead to many useful clinical ramifications.

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# Appendix:
# List of Abbreviations

| | |
|---|---|
| β-TrCP: | beta Transducin repeat containing protein |
| A20: | TNF alpha induced protein 3 |
| AIDS: | acquired immunodeficiency syndrome |
| Bcl: | B cell lymphoma protein |
| bp: | base pair |
| BSA: | bovine serum albumin |
| CBP: | Creb-binding protein |
| CTD: | carboxyl-terminal domain |
| CYLD: | cylindromatosis protease |
| DME: | Dulbecco modified eagle medium |
| EBV: | Epstein-Barr virus |
| ELKS: | glutamate, leucine, lysine, serine-containing protein |
| ERK: | extracellular signal-related kinase |
| F1: | filial 1 progeny |
| FBS: | fetal bovine serum |
| FRET: | fluorescent resonance emission tomography |
| GRA: | dense granule protein |
| GTPase: | guanosine triphosphatase |
| HA: | haemagluttinin |
| HAUSP: | Herpesvirus-Associated Ubiquitin-Specific Protease |
| HBS: | HEPES buffered saline |
| HDAC: | histone deacetylase |
| HEPES: | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| HFF: | human foreskin fibroblast |
| HRP: | horseradish peroxidase |
| HSV: | Herpes simplex virus |
| HTLV: | human T-lymphotrophic virus |
| I-κB: | inhibitor of kappa B |
| ICP: | infected cell protein |
| IF: | immunofluoresence |
| IFN: | interferon |
| IKK: | I-κB kinase |
| IL: | interleukin |
| IL1-R: | interleukin 1 receptor |
| iNOS: | inducible nitric oxide synthase |
| IP: | immunoprecipitation |
| IRAK: | IL-1 receptor-associated kinase |
| IRG: | immune-related GTPase |
| IVN: | intravacuolar network |
| KLH: | keyhole limpet hemocyanin |
| LMP1: | latent membrane protein 1 |
| $LD_{50}$: | lethal dose, 50% |
| LPS: | lipopolysaccharides |
| MALT1: | mucosa-associated lymphoid tissue lymphoma translocation protein 1 |
| MAP: | mitogen activated protein |
| MS: | mass spectrometry |

MEF: mouse embryonic fibroblast
MEKK: MAP/ERK kinase
MEM: minimal essential medium
MIC: microneme protein
MOI: multiplicity of infection
MyD88: myeloid differentiation primary response factor (88)
NF-κB: nuclear factor kappa B
NIK: NF-κB interacting kinase
NLS: nuclear localization sequence
NLR: NOD-like receptor
NOD: nucleotide-binding oligomerization domain
NTD: amine-terminal domain
ORF: open reading frame
PAGE: polyacrylamide gel electrophoresis
PAMP: pathogen-associated molecular pattern
PBS: phosphate buffered saline
PEXEL: *Plasmodium* export element
PP2A: protein phosphatase 2A
PRR: pattern recognition receptor
PV: parasitophorous vacuole
PVM: PV membrane
QTL: quantitative trait locus
REF: rat embryonic fibroblast
RING: really interesting new gene
RIP: receptor interacting protein
RNAi: RNA interference
ROP: rhoptry protein
ROS: reactive oxygen species
shRNA: short hairpin RNA
SNP: single nucleotide polymorphism
SOEing: splicing by overlapping extension
STAT: signal transducer and activator of transcription
SAG: surface antigen
$T_h1$: T helper cell type 1
$T_h2$: T helper cell type 2
TAB: TAK1 binding protein
TAK: TGF-β activated kinase
TBS: tris buffered saline
TRIF: TIR-domain-containing adapter-inducing interferon-β
Tg-IKK: *Toxoplasma gondii*-derived IKK
TGF-β: transforming growth factor beta
TIR: Toll/Interleukin receptor
TLR: Toll-like receptor
TNF: tumor necrosis factor
TNFAIP: TNF alpha induced protein
TNFR: TNF receptor

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|---|---|
| TRADD: | TNFR-associated death domain |
| TRAF: | TNFR associated factor |
| Ubc13: | ubiquitin conjugating enzyme 13 |
| Uev1a: | ubiquitin conjugating enzyme variant 1a |
| VSV-G: | vesicular stomatitis virus glycoprotein |
| Yop: | *Yersinia* outer protein |