Identification and characterization of *Toxoplasma gondii* effectors

that modulate innate immune pathways

by

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BSc Biology
Imperial College, London, 2008

SUBMITTED TO THE DEPARTMENT OF MICROBIOLOGY IN PARTIAL FULFILMENT
OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY IN MICROBIOLOGY
AT THE
MASSACHUSETTS INSTITUTE OF TECHNOLOGY

FEBRUARY 2015

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Abstract

Toxoplasma gondii is an obligate intracellular pathogen that is able to chronically infect all warm blooded animals including an estimated one third of the human population. Because only the chronic parasite stage can be transmitted to new hosts, *Toxoplasma* has to balance evading the immune response, to escape detection and destruction, and activating the immune response, to enable host survival and establishment of a chronic infection. A crucial component of host innate immunity is the NF-κB pathway that leads to secretion of the cytokine IFN-γ, which is essential for activation of host toxoplasmacidal mechanisms. To achieve this balance between immune evasion and activation, *Toxoplasma* modulates host cell processes by secreting proteins into the host cell. These *Toxoplasma* proteins are secreted from specialized secretory organelles known as rhoptries and dense granules and are called ROPs and GRAs respectively. Using comparative genomics of *Toxoplasma* strains that differ in modulation of host cell processes, we identified *Toxoplasma* dense granule proteins GRA2 and GRA15 that determine strain differences in IFN-γ evasion and NF-κB activation responses respectively. We determined that *Toxoplasma* GRA15, which activates NF-κB, likely interacts with TRAF2, an upstream NF-κB adaptor. In addition, we characterized GRA15 regions required for NF-κB activation through GRA15 structure function analysis. We identified another *Toxoplasma* effector ROP38 that repressed NF-κB activation and mice challenged with *Toxoplasma* ROP38 knockout strains showed increased weight loss during acute infection and increased numbers of brain cysts, which is the parasite stage crucial in establishment of chronic infection. Our identification and characterization of *Toxoplasma* effectors and their mechanisms of action provide insight into interactions between *Toxoplasma* and host innate immune pathways, and might aid in design of drugs to counter this highly successful pathogen.

Thesis Advisor: Jeroen Saeij, Associate Professor of Biology

Acknowledgements

First and foremost, I would like to thank my thesis advisor and mentor, Jeroen. You have been instrumental in my development as a scientist, and your advice has truly been formative during my graduate school years. I've learnt tremendously from you with your high standards for science. You are also someone who really cares, able to reach that fine balance like *Toxoplasma*, and that has made all the difference when the going in science gets tough. Thank you for your guidance, and I hope to bring the same scientific rigor and excitement as a true Saeij-entist to my future endeavors.

Next, I would like to thank my thesis committee members. To Dennis, thank you for your support across graduate school and joy in TAing for 7.26. To Gerry, thank you for your feedback over the years and giving insights to the various projects. To Marc-Jan, it has been enjoyable to collaborate and meet you at conferences, thank you for being on my defense committee.

The Saeij lab has been an amazing and wonderful place to do science. To current members of the Saeij lab: Ana - for scientific and life musings, a steadfast companion over failed experiments and frustrations; Dan – your advice on biochemistry, food, life, etc etc; Kim – your liveliness really brightens up lab; Ben – your humor cracks me up while discussing science; Eleni – keen to learn and running about constantly. To past members of the Saeij lab: Lindsay – your presence in lab will always be sorely missed; Emily – pushing me to excel in scientific thinking, and showing me the ropes; Wendy – marathon plaque assay skills I still marvel at; Musa – my baymate for years who can, without fail, make me laugh and cry at the same time; Kirk – your weather plots have excited and inspired me; Mariane – scientific discussions and collaborations will always go better with wine. I have also enjoyed interacting with many others – Erwan, Renee, Diana, Joris, Ailan, Darlene and Judy and learnt much from UROPs – Lauren, Kenneth, Dordy, Stephanie, Quynh, Yaning, Natalle, Brittany, Monique, Kiva, Deepshika. You all were my second family away from home, and I will always treasure these memories in my heart.

To my Microbiology classmates who are a great bunch – Mark, Chris, Andres, Anna and Nicole; Bonnie Lee who worries about the rest so we worry about science. To my friends whom I've hung out at MIT over the many years: my roommate Mingjuan – for listening, cooking, taking care of me when I fell sick; Ye Hongye – reminding me life exists outside lab and pulling through very difficult times together; Wang Jia – for always being a good sport; Yoong Keok – quirky jokes aside, your help has always been appreciated; my A*STAR batchmates Richie and Shengyong – jio-ing for great makan places. To everyone who has touched my life in Boston one way or the other, thank you.

To all my friends in Singapore and elsewhere in the world, several of whom understand this journey: Pamela, Phyllis, Trisna, Yihlin, Peigeok, Shuying, Hui Hui, Hock Boon, Yoke Pean – here's to many more years ahead. To Benjamin Lim – your unwavering support and endless patience, I look forward to further adventures and exploration down the journey of life. Last but not least, my family: Grandpa, Grandma, Papa, Mama, Jie and Ningxin. All your support these years has encouraged me to spread my wings and pursue my dreams. Every single one of you made this wholly possible.

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Chapter One - Introduction

Introduction

Infectious diseases account for up to one fifth of mortality and morbidity worldwide, with even higher numbers in developing regions (www.who.int). A small number of infectious diseases, mainly HIV/AIDS, tuberculosis and malaria, account for the majority of reported deaths and morbidity. However, microbial pathogenesis leading to severe host morbidity and mortality can be an evolutionary dead-end because this would reduce the chances of transmission to other hosts. Rather, the evolutionary success of a pathogen is often defined by its ability to achieve high rates of transmission in the natural environment. In evolutionary terms, the eukaryotic intracellular pathogen, *Toxoplasma gondii*, is exceedingly successful, as it is estimated to chronically infect about a third of the human population (Sibley, Ajioka 2008), and has the remarkable ability to infect all warm blooded animals.

Though most chronically infected patients are asymptomatic, *Toxoplasma gondii* is a clinically important pathogen. In immunocompromised patients, such as HIV/AIDS patients, there can be development of brain encephalitis. In addition, when pregnant women become acutely infected for the first time, *Toxoplasma* infection can lead to severe fetal malformations and abortions (McLeod et al. 2012). Ocular disease can also result due to *Toxoplasma* infection even in immunocompetent humans. In Brazil, up to 18% of individuals have ocular toxoplasmosis (Roberts, McLeod 1999), and 30-50% of posterior uveitis can be attributed to toxoplasmic retinochoroiditis in the USA (Shobab et al. 2013). Furthermore, toxoplasmosis is the third leading cause of food-borne infections requiring hospitalization in the USA (Mead et al. 1999).

In addition to its clinical relevance, *Toxoplasma* has been used as a eukaryotic protozoan model system for other related, less experimentally tractable pathogens, most notably *Plasmodium* spp., the causative agent of malaria. Both *Toxoplasma* and *Plasmodium* are unicellular pathogens belonging to the phylum Apicomplexa, named for the presence of the apical complex involved in attachment and invasion (Dubremetz et al. 1998). Other related apicomplexan parasites include *Cryptosporidium*, one of the leading causes of human food-borne diarrhea (McDonald et al. 2013), and *Eimeria*, which infects livestock and causes economically significant disease (Chapman et al. 2013).

Toxoplasma life cycle and transmission

Toxoplasma gondii has a complex life cycle, mediated by three different invasive forms in various hosts (**Figure 1**) (Sibley, Ajioka 2008). The sexual cycle occurs exclusively in felines, where ingestion of the infectious form of the parasite present in the environment or in contaminated meat, causes *Toxoplasma* to differentiate into micro- and macro-gametes that fuse and develop into oocysts, which are subsequently shed in feline feces (Dubey 2009). These oocysts then sporulate and mature, and are transmitted to intermediate hosts via contaminated food or water. After ingestion by intermediate hosts, oocysts develop into tachyzoites, the rapidly replicating form, which invade and replicate within intestinal enterocytes. During the lytic replication cycle of tachyzoites, tachyzoites are able to invade most nucleated cells, form a non-fusogenic vacuole known as the parasitophorous vacuole (PV) and replicate within the PV (Black, Boothroyd 2000). There is subsequent egress and invasion of neighboring cells. Tachyzoites cross the intestinal epithelial barriers and reach the lamina propria, where they are recognized by innate immune cells, mainly resident macrophages and dendritic cells. There is

subsequent rapid spread to the draining lymph nodes and eventually throughout the body of the host (Hunter, Sibley 2012). After dissemination throughout the host, tachyzoites eventually differentiate into bradyzoites, the dormant form. Tissue cysts containing hundreds of bradyzoites are formed within the central nervous system and skeletal muscle, enabling lifelong chronic infection of the host (Dubey, Lindsay & Speer 1998). Continual propagation outside the sexual cycle can occur via horizontal transmission when tissue cysts are ingested by other intermediate hosts (Sibley, Ajioka 2008).

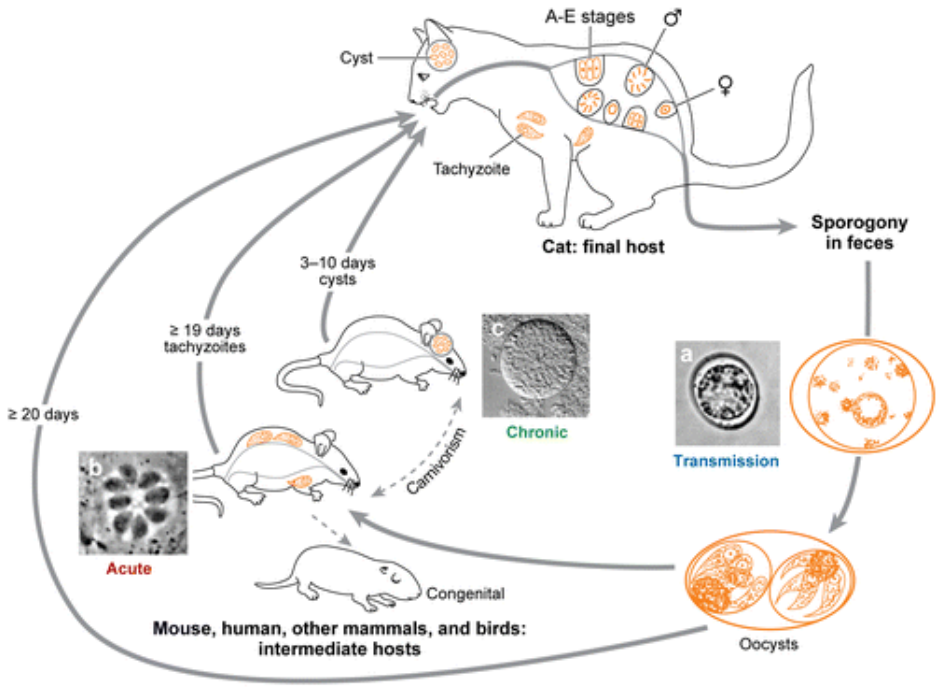

Figure 1. Life cycle and transmission of *Toxoplasma gondii*. The sexual cycle of *Toxoplasma* occurs exclusively in felines, where sexual development and meiosis occurs within the small intestine to form micro- and macrogametes after mitotic replication (A-E stages). Infectious oocysts are shed in feces from felines into the environment, and ingestion of contaminated food or water causes acute infection in all warm-blooded animals, where the asexual cycle occurs. Acute infection is mediated by tachyzoites (rapidly replicating) but these tachyzoites eventually convert to bradyzoites (dormant) which form cysts in muscles and brains, enabling chronic lifelong infection. Horizontal transmission between intermediate hosts can occur when predators ingest cyst containing tissues. Reproduced from (Sibley, Ajioka 2008).

It is important to understand tachyzoite to bradyzoite stage conversion, as this conversion is crucial in establishing chronic infections, allowing successful transmission of the parasite. Moreover, conversion from bradyzoite back to tachyzoite form, also known as reactivation, is often responsible for life threatening pathology (Skariah, McIntyre & Mordue 2010). One of the earliest known inducers of bradyzoite formation is tachyzoite exposure to alkaline conditions (Weiss et al. 1995), or heat shock (Soete, Camus & Dubremetz 1994). Immune cytokines are important *in vivo* inducers of bradyzoite conversion as they can lead to production of free radicals, such as nitric oxide, that are involved in bradyzoite development in macrophages (Bohne, Heesemann & Gross 1994). Thus, activation of the immune response leads to parasite clearance, but also induces bradyzoite differentiation and cyst formation, which are responsible for chronic infections.

Specialized Toxoplasma subcellular organelles

Toxoplasma has subcellular polarity, with an apical end containing specialized secretory organelles such as micronemes, rhoptries and dense granules (**Figure 2**) (Boothroyd, Dubremetz 2008). Micronemal proteins (MICs) and rhoptry neck proteins (RONs) mediate attachment and invasion, and are involved in the formation of the moving junction, a ring-like structure that represents the contact point between parasite and host membranes during invasion (Boothroyd, Dubremetz 2008). Parasite invasion is driven by *Toxoplasma*'s own actin myosin motor, independent of the host cytoskeleton (Sibley 2011). Rhoptry bulb proteins (ROPs) are released into the host cell upon invasion, while dense granule proteins (GRAs) are continually secreted into the parasitophorous vacuole (PV) during tachyzoite replication. The PV contains a network of membranous nanotubules connecting parasites and the parasitophorous vacuole membrane

(PVM), and this network is thought to participate in nutrient exchange between parasite and host cell or maintain structure during parasite replication (Travier et al. 2008). Dense granule proteins are involved in proper PV biogenesis, as dense granule protein GRA2 knockout parasites exhibit defects in the intravesicular network of the PV (Mercier et al. 2002). In addition, ROPs and GRAs are increasingly recognized as crucial mediators of the host response to *Toxoplasma* infection.

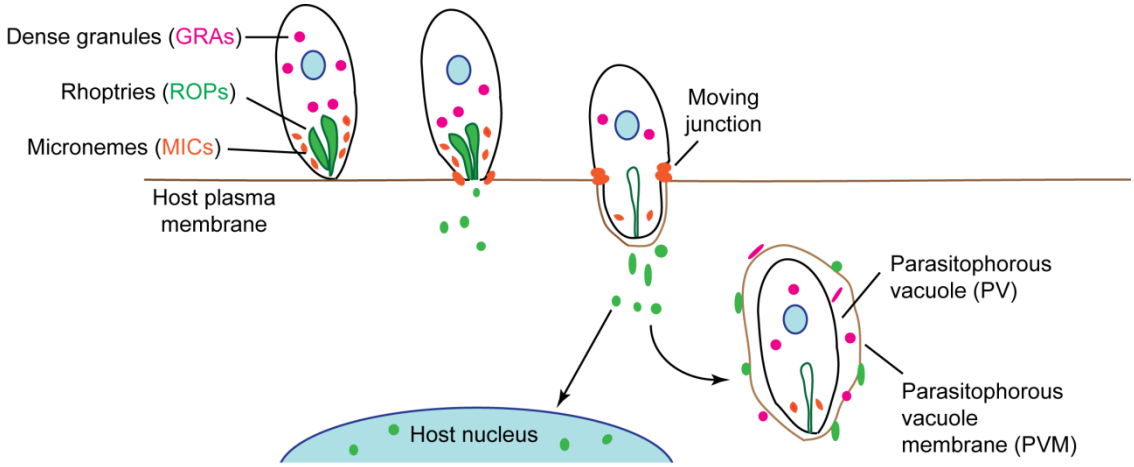

Figure 2. *Toxoplasma* specialized subcellular organelles. Micronemal (MICs; orange) and rhoptry neck and bulb (RONs and ROPs; green) proteins are secreted during attachment and invasion. MICs and RONs are involved in the formation of the moving junction, enabling a non-fusogenic parasitophorous vacuole (PV) to form. ROPs are also secreted during invasion, while dense granule proteins (GRAs; pink) are usually secreted into the PV throughout parasite replication. ROPs and GRAs are involved in the modulation of numerous host cell processes, and several have been identified to localize to the host nucleus. (Picture courtesy of Emily Rosowski)

Toxoplasma population structure and genetic diversity

Understanding the genetic diversity and population structure of a pathogen allows us to understand host selective pressures and how pathogens counteract these host responses (Homolka et al. 2010). A well characterized example is the Gram negative bacterium *Vibrio cholera*, the etiological agent responsible for an epidemic outbreak in Haiti in 2010 (Robins, Mekalanos 2014). Mobile genetic elements can be acquired, lost or altered in *V.cholera*, which contain genes encoding the cholera toxin, responsible for acute diarrhea symptoms in patients, or genes conferring antibiotic resistance (Chin et al. 2011).

It is important to understand the population structure to appreciate the genetic diversity present in *Toxoplasma*. *Toxoplasma* was initially identified as having a clonal population, consisting of three clonal lineages known as types I, II and III, in North America and Europe (Howe, Sibley 1995). However, recent analysis has shown that type II dominates in Europe, types II, III and 12, another clonal lineage, dominate in North America, while type I strains are more frequently isolated in Asia (Shwab et al. 2014). Despite limited genetic heterogeneity between these clonal lineages, they differ dramatically in a number of phenotypes such as virulence in mice, *in vitro* growth, migratory capacity *in vivo* and cytokine induction (Saeij, Boyle & Boothroyd 2005). In susceptible laboratory inbred mice, type I strains are lethal with one parasite while types II and III are less virulent, with LD_{50} (lethal dose required to kill 50% of mice infected) of ~10^3 and ~10^5 parasites respectively (Howe, Sibley 1995, Su et al. 2002). Sexual crosses between these three clonal lineages has enabled a forward genetic approach to identify important *Toxoplasma* genes involved in the modulation of host signaling pathways and virulence in mice (Saeij et al. 2006, Taylor et al. 2006).

In addition, the clonal population structure described for North America and Europe does not hold for other places such as South America. Genome wide SNP analysis across 26 strains encompassing global isolates, including South American strains, showed that there was significant diversity and genetic admixture in *Toxoplasma* population structure (Minot et al. 2012), and is unlikely to fit a previously proposed model that *Toxoplasma* belongs to 14 haplogroups derived from 6 ancestral strains (Su et al. 2012). This strain diversity in *Toxoplasma* is clinically important because atypical *Toxoplasma* isolates with different allele combinations are often associated with infected patients exhibiting unusual clinical symptoms, suggesting that different parasite genotypes are associated with different clinical outcomes (Boothroyd, Grigg 2002). There is high prevalence of ocular toxoplasmosis in South America, where there are numerous atypical strains present (Holland 2003). In North America, atypical strains of *Toxoplasma*, classified as non-exclusive type II strains, are associated with more severe ocular symptoms at birth for congenital infections (McLeod et al. 2012). In Europe, there is increased association between atypical strain infection and chronically infected immunocompetent patients exhibiting ocular symptoms (Shobab et al. 2013). Thus, investigating genetic differences between *Toxoplasma* strains would enable better understanding of disease outcome variation during acute and chronic *Toxoplasma* infection.

The immune response to *Toxoplasma*

Immune responses can be classified as innate, the first line of defense against pathogens but without immunological memory upon pathogen re-exposure, or adaptive, which creates immunological memory after an initial response to a specific pathogen (Janeway, Medzhitov 2002). Innate immunity is essential in pathogen recognition and initial clearance, and it also shapes the adaptive immune response for targeted pathogen elimination. Thus, pathogens have to overcome this multifaceted host immune response to successfully colonize their preferred niche (Deitsch, Lukehart & Stringer 2009).

Innate immune recognition

Innate immune responses start with pathogen recognition, usually through pathogen associated molecular patterns (PAMPs), which are conserved, essential structures found in pathogens (Janeway, Medzhitov 2002). Sensing of these PAMPs occur through pattern recognition receptors (PRRs), of which Toll-like receptors (TLRs) are a well characterized subset (Medzhitov 2009). Myeloid differentiation primary response 88 (Myd88) is a TLR adaptor protein (**Figure 3**), and transduces activating signals upon TLR recognition to downstream intermediates, culminating in activation of specific signaling pathways such as mitogen activated protein kinases (MAPKs) (Krishna, Narang 2008) and NF-κB (Medzhitov et al. 1998).

Toxoplasma is recognized in mice by TLR11 and TLR12, which bind to the *Toxoplasma* protein profilin, an essential parasite protein involved in parasite motility and host cell invasion (Yarovinsky et al. 2005, Plattner et al. 2008, Raetz et al. 2013). UNC93 homologue B1 (UNC93B1), a protein involved in the trafficking of endosomal TLRs, was shown to interact

directly with TLR11 (Pifer et al. 2011), and UNC93B1 knockout mice are hypersensitive to *Toxoplasma* infection (Melo et al. 2010). However, TLR11 is a human pseudogene and TLR12 is absent in humans, yet immunocompetent humans are relatively resistant to *Toxoplasma*, and the receptor used by humans to sense the parasite is currently unknown.

Innate immune signaling pathways

MAPK pathways are signaling pathways downstream of PRRs, involved in diverse host responses ranging from innate immunity to apoptosis, which play important roles during infection. MAPKs are a family of protein kinases with the following three subfamilies most extensively studied: extracellular signal regulated kinases 1/2 (ERK 1/2), JUN N-terminal kinases (JNKs) and p38 kinases (Krishna, Narang 2008). MAPK pathways have a core three tiered signaling module, consisting of three evolutionarily conserved, sequentially acting kinases – a MAPK, a MAPK kinase (MAPKK) and a MAPKK kinase (MAPKKK). Activation of MAPKs occurs through a phosphorylation cascade, and MAPKs phosphorylate target substrates on serine or threonine residues which are followed by prolines (Zhang, Kaplan 2000). All three branches of the MAPK pathway have been shown to be transiently activated during *Toxoplasma* infection in dendritic cells and macrophages (Valere et al. 2003) and *Toxoplasma* infection of macrophages induces p38 dependent cytokine secretion (Mason et al. 2004).

The NF-κB pathway is a crucial pathway regulating gene expression in numerous host cellular processes, ranging from cell death and proliferation to innate and adaptive immune responses (Hayden, Ghosh 2008). The NF-κB transcription factor family contains several members, such as p65 (RelA), RelB, c-Rel, p50 and p52, and they can homodimerize or heterodimerize to mediate

gene transcription, but are usually inactive under normal physiological conditions. The NF-κB pathway can be further divided into the classical/canonical and the alternative pathway (**Figure 3**). For classical NF-κB activation, stimulation activates multiple adaptors, such as the tumor necrosis factor receptor associated factors (TRAFs) and downstream kinases like TAK1, to transduce the signal further to converge on the Iκ-B kinase β (IKKβ) complex. Activated IKKβ phosphorylates inhibitory protein Iκ-Bα, which is ubiquitylated and targeted for proteasomal degradation. This unmasks the nuclear localization domains found on p65/p50 heterodimers and nuclear translocation occurs, causing downstream transcription of NF-κB dependent genes (**Figure 3, left panel**) (Lawrence 2009). For alternative NF-κB activation, stimulation activates adaptors like TRAF2 and TRAF3 to cause activation of IKKα and downstream phosphorylation of p100. This then causes p100 processing into p52, and enables subsequent heterodimerization into p52/RelB subunits, which then translocates to the nucleus (**Figure 3, right panel**) (Rahman, McFadden 2011).

NF-κB has been implicated in *Toxoplasma* pathogenesis, as c-Rel or RelB knockout mice showed increased susceptibility during acute *Toxoplasma* infection. Both RelB and c-Rel deletions led to defective IFN-γ serum levels upon infection, though there were no differences observed in IL-12 levels (Caamano et al. 1999, Mason, Liou & Hunter 2004). However, the generation of specific Th1 effector cells, which are part of adaptive immunity, did not seem impaired in *Toxoplasma* infected c-Rel knockout mice (Mason, Liou & Hunter 2004).

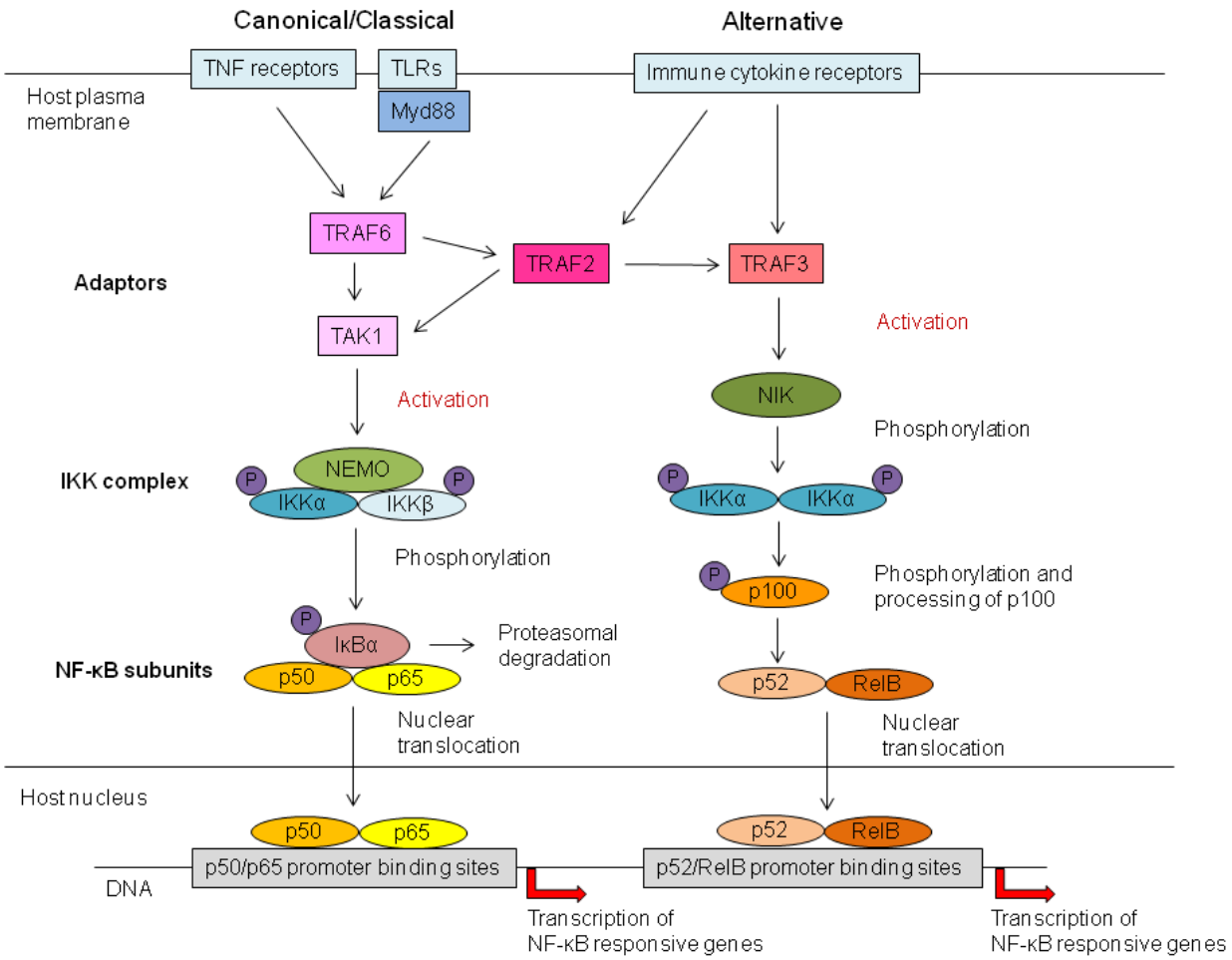

Figure 3. The canonical and alternative NF-κB pathways. The canonical NF-κB pathway can be activated by TLR recognition, which depends on adaptor Myd88 to transduce the signal downstream to signaling adaptors such as TRAF2 and TRAF6. The TRAFs in turn recruit TAK1, a downstream kinase, and causes IKKβ to be phosphorylated. IKKβ then phosphorylates inhibitory protein Iκ-Bα and releases p50/p65(RelA) to translocate to the nucleus. The alternative NF-κB pathway has TRAF2 and TRAF3 as upstream adaptors, where TRAF3 is constitutively bound to NIK, a downstream kinase. Upon activating stimulus, TRAF3 is degraded by the proteasome, and NIK phosphorylates IKKα. IKKα then phosphorylates and processes p100 into p52, enables heterodimerization of p52/RelB and subsequent p52/RelB nuclear translocation. Modified from (Rahman, McFadden 2011, Hacker, Tseng & Karin 2011).

Effector mechanisms involved in Toxoplasma killing

One of the major NF-κB and MAPK dependent cytokines essential for host resistance to *Toxoplasma* is IL-12. Mice infected with virulent *Toxoplasma* strains and subsequently treated with IL-12 show significantly increased survival times (Hunter et al. 1995). Antigen presenting cells (APCs) such as dendritic cells and macrophages, are sources of IL-12, which in turn trigger natural killer (NK) cell and T cell secretion of cytokine IFN-γ (**Figure 4**). Neutrophils are also an important source of IFN-γ, but their secretion of IFN-γ is independent of TLR11 and IL-12 (Sturge et al. 2013).

IFN-γ, in turn, is the major cytokine responsible for host resistance to *Toxoplasma* infection (Suzuki et al. 1988). At least two main IFN-γ dependent cell autonomous resistance mechanisms in mice have been identified - firstly, production of nitric oxide, which inhibit the parasite metabolic enzymes (Adams et al. 1990); secondly, immunity related GTPases (IRGs), which coat and disrupt the parasitophorous vacuole membrane, eventually leading to lysosome mediated degradation (Khaminets et al. 2010). Other IFN-γ dependent mechanisms have also been shown in human cells, where IFN-γ induces indoleamine 2,3 dioxygenase, which leads to tryptophan starvation and subsequent inhibition of parasite growth (Pfefferkorn, Eckel & Rebhun 1986). However, though IFN-γ activates downstream toxoplasmacidal mechanisms, nitric oxide (induced by IFN-γ) is also a signal that induces tachyzoite-to-bradyzoite conversion, which eventually leads to chronic *Toxoplasma* infection (Bohne, Heesemann & Gross 1994).

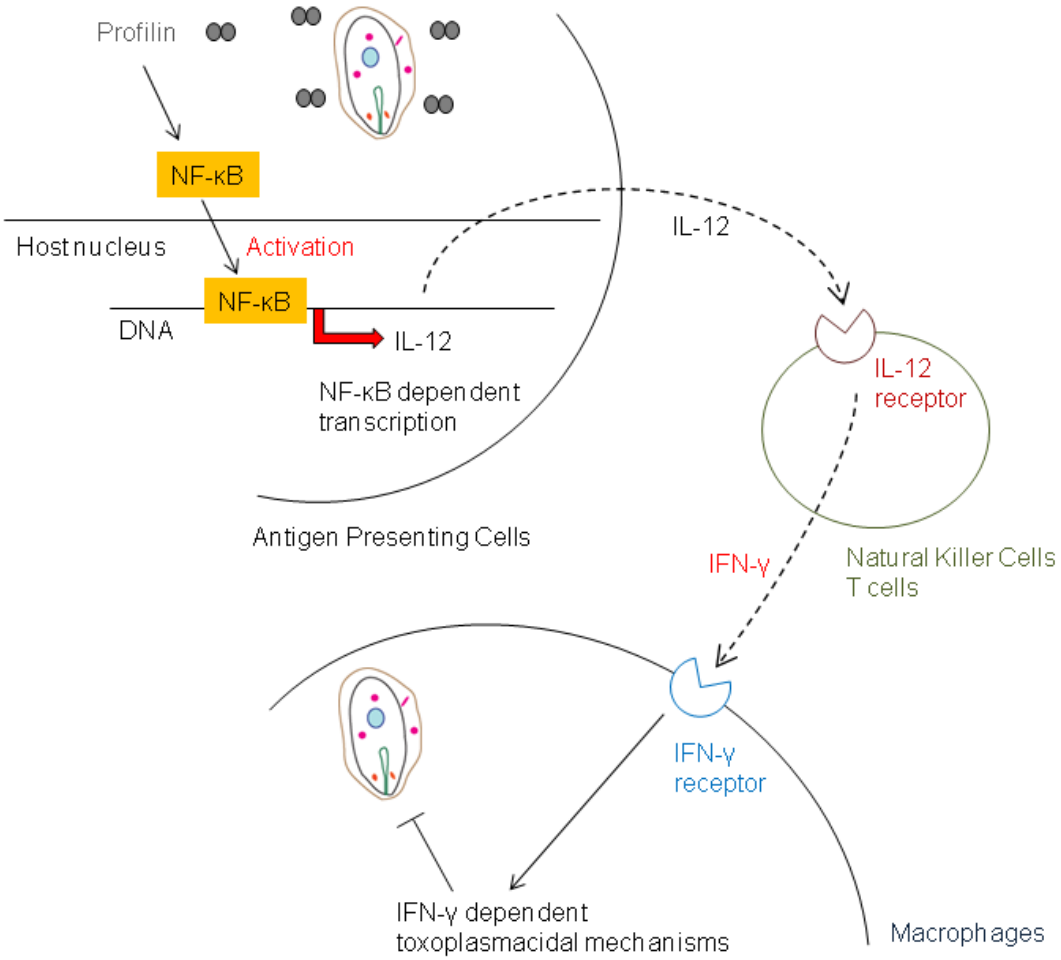

Figure 4. The innate immune response to *Toxoplasma*. Pathogen associated molecular patterns (PAMPs) from *Toxoplasma*, such as profilin, are recognized by antigen presenting cells, leading to activation of downstream signaling pathways such as NF-κB. This in turn causes IL-12 secretion and activates natural killer cells and T cells to produce IFN-γ. IFN-γ is required for activation of downstream cell autonomous IFN-γ dependent toxoplasmacidal mechanisms and is essential for control of acute *Toxoplasma* infection. IFN-γ production by CD4+ and CD8+ T cells, part of adaptive immunity, is also essential for control of chronic *Toxoplasma* infection. Modified from (Yarovinsky, 2014).

Adaptive immune responses against Toxoplasma

Adaptive immunity is crucial for control of chronic *Toxoplasma* infections, and is required for development of immunological memory, which enables the host to respond more rapidly upon re-exposure to the same pathogen (Dupont, Christian & Hunter 2012). Adaptive immunity is mediated by immune cells known as T and B lymphocytes. T lymphocytes are divided into further subsets based on their surface receptors, known as CD8+ and CD4+ T cells (Zander, Butler 2013). The importance of these T lymphocytes in chronic *Toxoplasma* infections is highlighted by the reactivation of lesions in immunocompromised HIV patients with low CD4+ T cell counts (Dupont, Christian & Hunter 2012). Furthermore, *Toxoplasma* cysts are re-activated and fulminant parasitaemia occurs upon simultaneous CD4+ and CD8+ depletion in chronically infected mice, though CD8+ depletion and CD4+ depletion individually led to partial reactivation or no reactivation respectively (Gazzinelli et al. 1992). CD4+ T cell help is required for maintenance of CD8+ effector function during chronic infection (Lutjen et al. 2006). To control chronic *Toxoplasma* infection, CD8+ T cells secrete IFN-γ, which then activate IFN-γ dependent mechanisms (**Figure 4**) (Wang et al. 2004).

Pathogen effectors and the immune response

The way pathogens are able to overcome these innate and adaptive host immune responses, in order to successfully replicate and achieve transmission, is through pathogen effectors. Effectors are specialized proteins targeting numerous host pathways, such as mammalian intracellular signaling, vesicular trafficking and cytoskeletal pathways (Bhavsar, Guttman & Finlay 2007).

The presence of effectors is ubiquitous across diverse pathogens, and they are a unifying theme in host pathogen interactions.

Specific pathogen effectors targeting the MAPK and NF-κB innate pathways underline the importance of these two pathways in immunity (Rahman, McFadden 2011). *Yersinia* spp. contain YopJ, which acetylates upstream MAPK kinases (MAPKK6 and MEK2) on specific residues in the activation loop, likely inhibiting MAPK phosphorylation and subsequent activation (Shan, He & Sheen 2007). The same *Yersinia* YopJ effector also inhibits NF-κB activation through inhibition of phosphorylation of Iκ-B (Mukherjee et al. 2006). Another pathogen, *Shigella flexneri*, contains the effector OspF, a unique phosphatase that dephosphorylates and inactivates a specific MAPK, ERK1/2. This subsequently prevents histone phosphorylation and silences NF-κB dependent transcription, which leads to dampening of inflammatory responses (Bhavsar, Guttman & Finlay 2007, Ogawa et al. 2008).

Modulation of host cell processes by Toxoplasma effectors

Balancing the evasion and activation of the host immune response is crucial to successful chronic infection for *Toxoplasma*, as stimulation of proinflammatory responses could lead to parasite clearance and overt host immunopathology, whereas suppression of inflammatory responses could lead to uncontrolled parasite replication and consequent host death. Thus, *Toxoplasma gondii* uses effector proteins to modulate host signaling pathways, involved in immune responses and cell proliferation, in order to fine tune host responses and enable successful parasite replication and transmission.

The rhoptries and dense granules have been identified to contain numerous *Toxoplasma* effectors that are secreted into the host cell upon invasion and during replication. Examples of rhoptry proteins (ROPs) modulating host cell processes include the rhoptry kinase ROP16, identified from mapping quantitative trait loci of host gene expression using the type II by III *Toxoplasma* cross (Saeij et al. 2007). ROP16 from type I and III strains, but not type II strains, are able to constitutively activate transcription factors STAT3 and STAT6. Active ROP16 was subsequently shown to directly phosphorylate STAT3 and STAT6, and the STAT3 phosphorylation activity was abolished by a single ROP16 polymorphism (Yamamoto et al. 2009, Ong, Reese & Boothroyd 2010).

Another *Toxoplasma* rhoptry kinase, ROP18, was identified from mapping quantitative trait loci of mouse virulence using type I by III or type II by III *Toxoplasma* crosses (Saeij et al. 2006, Taylor et al. 2006). Type III strains have extremely low levels of ROP18 due to an insertion in the promoter sequence, and overexpression of ROP18 in type III strains increased their virulence significantly in mice (Taylor et al. 2006). ROP18 has been shown to phosphorylate the immunity related GTPases (IRGs), which mediate IFN-γ mediated killing of *Toxoplasma* (Fentress et al. 2010). Another *Toxoplasma* effector ROP5, a cluster of tandemly duplicated rhoptry pseudokinases, is crucial for virulence, where knocking out the ROP5 cluster in the virulent type I strain dramatically reduced its virulence in mice (Reese et al. 2011). ROP5 inhibits mouse IRG Irga6 oligomerization, where oligomerization is required for PV vesiculation leading to parasite killing (Niedelman et al. 2012). ROP18 together with ROP5, block the immunity related GTPases (IRGs) and enable virulent *Toxoplasma* strains to evade the IFN-γ response (Niedelman et al. 2012).

In addition to ROP16 and ROP18, rhoptry kinase 38 (ROP38), has been implicated in repression of host transcriptional responses to infection and is able to alter the MAPK ERK1/2 in a *Toxoplasma* strain dependent manner (Peixoto et al. 2010). Overexpression of ROP38 in a type I background was sufficient to change the ERK signaling profile from type I to type III (Peixoto et al. 2010). However, the molecular mechanism of ROP38 leading to changes in host transcriptional responses and specific MAPK pathways remain unknown. In addition, ROP38 is strongly differentially expressed upon tachyzoite to bradyzoite conversion, and is part of a tandemly duplicated gene family, which consists of other rhoptry proteins such as ROP29 and ROP19 (Peixoto et al. 2010). However, the *in vivo* effects of ROP38 on cyst formation remain unclear.

Dense granule proteins (GRAs) are also increasingly recognized to be important in host cell modulation, where varied approaches have identified GRAs and the specific host pathways they interfere with. GRA24 was determined *in silico* to be a candidate *Toxoplasma* protein secreted into the host cell, and was shown biochemically to interact directly with host MAPK p38, leading to p38 autophosphorylation and translocation to the nucleus. Early gene expression in macrophages was strongly influenced by GRA24, as IL-12 cytokine secretion was promoted by GRA24 (Braun et al. 2013).

GRA15 was identified from mapping quantitative trait loci of NF-κB activation of type II and III *Toxoplasma* cross (Rosowski et al. 2011). *Toxoplasma* GRA15 polymorphisms and expression affect NF-κB activation levels upon parasite infection (Rosowski et al. 2011, Melo et al. 2013, Yang et al. 2013), with GRA15 from type II strains sufficient to cause NF-κB activation in RH (type I), a strain that does not activate NF-κB in host cells upon infection. Furthermore, GRA15

mediated NF-κB activation is independent of adaptor molecules Myd88 and TRIF, but dependent on TRAF6, an intermediate transducing protein (Rosowski et al. 2011). However, the exact mechanism of GRA15 leading to NF-κB activation remains unclear.

Thesis findings

In Chapter II, we note that phenotypic variation between different type I strains can have important consequences. For example, differences between type I strains in oral infectivity of cysts can lead to differences in transmission, while differences in growth rates can lead to differences in dissemination throughout the host during acute infection. However, the genetic basis for these type I strain differences is not well established. Therefore, we used a comparative genomics approach to identify genetic differences between different type I strains and identified *Toxoplasma* genes that contain polymorphisms or are differentially expressed between different type I strains. In addition, we examined the host response to different type I strains through host transcriptional profiling and observed significant differences in NF-κB activation. We showed that polymorphisms in GRA15 account for strain specific differences in NF-κB activation. We also find that polymorphisms in another dense granule protein GRA2 determined type I strain differences in evasion of IFN-γ mediated toxoplasmacidal mechanisms. These findings emphasize the role of dense granule proteins in modulating the host cell and addresses how infection with different *Toxoplasma* strains can lead to varying host responses.

In Chapter III, we note that GRA15 is a *Toxoplasma* effector that determines strain specific NF-κB activation, but the exact mechanism by which GRA15 activates the NF-κB pathway remains unclear. Even though type II, but not type III, strains activate NF-κB upon infection, we

established that both $GRA15_{II}$ and $GRA15_{III}$ are sufficient for NF-κB activation upon ectopic expression in human cells. Using a biochemical approach, we identified candidate host proteins that interact directly with $GRA15_{II}$ or $GRA15_{III}$ and validated the GRA15 interaction with TRAF2, an upstream NF-κB adaptor protein. In addition, using GRA15 truncation mutant analysis, we identified a particular N terminal region of GRA15 that is necessary for NF-κB activation. Understanding the molecular mechanism through which GRA15 acts would enable better understanding of strain specific NF-κB activation and its downstream effects on innate and adaptive immunity.

In Chapter IV, we note that ROP38 is strongly differentially expressed between *Toxoplasma* strains and exerts repressive effects on host transcription *in vitro*, but its effects on particular signaling pathways and its role during *in vivo* infection are not well established. We showed that ROP38 dampens the NF-κB activation response in type II infected host cells *in vitro*, though downstream NF-κB dependent responses, such as IL-12 secretion, are not affected by ROP38. We also observed that mice challenged with type II strains deleted for ROP38 had increased weight loss during acute infection and increased brain cyst burden upon establishment of chronic infection. Moreover, this increased weight loss and increased cyst burden observed was more pronounced in orally infected mice. Understanding how ROP38 represses host transcription and its effects on cyst burden *in vivo* would allow further insights into how *Toxoplasma* balances between immune evasion and activation to establish chronic infection and enable successful parasite transmission.

In Chapter V, we conclude with how these findings have furthered our understanding of *Toxoplasma*-host interactions and discuss experimental limitations and future directions.

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Chapter Two - Genetic basis for phenotypic differences between different *Toxoplasma gondii* type I strains

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Jeroen Saeij and Ninghan Yang analyzed the sequencing and parasite/host gene expression data. Ninghan Yang performed luciferase assays, immunoassays and p-IκBα immunofluorescence assays. Andrew Farrell, Gabor Marth and Marc-Jan Gubbels performed, processed and annotated the sequencing data between RH-ERP and GT1. Wendy Niedelman made the GRA2 complemented strains and performed plaque assays and IRG evasion. Mariane Melo contributed RNA-seq data for RH-ERP and GT1. Diana Lu and Lindsay Julien made the $GRA15_{GT1}$ complemented strain.

Originally published in BMC Genomics 14 (467). July 10, 2013.

Supplementary Data is available online at http://www.biomedcentral.com/1471-2164/14/467/additional.

Abstract

BACKGROUND: *Toxoplasma gondii* has a largely clonal population in North America and Europe, with types I, II and III clonal lineages accounting for the majority of strains isolated from patients. RH, a particular type I strain, is most frequently used to characterize *Toxoplasma* biology. However, compared to other type I strains, RH has unique characteristics such as faster growth, increased extracellular survival rate and inability to form orally infectious cysts. Thus, to identify candidate genes that could account for these parasite phenotypic differences, we determined genetic differences and differential parasite gene expression between RH and another type I strain, GT1. Moreover, as differences in host cell modulation could affect *Toxoplasma* replication in the host, we determined differentially modulated host processes among the type I strains through host transcriptional profiling.

RESULTS: Through whole genome sequencing, we identified 1,394 single nucleotide polymorphisms (SNPs) and insertions/deletions (indels) between RH and GT1. These SNPs/indels together with parasite gene expression differences between RH and GT1 were used to identify candidate genes that could account for type I phenotypic differences. A polymorphism in dense granule protein, GRA2, determined RH and GT1 differences in the evasion of the interferon gamma response. In addition, host transcriptional profiling identified that genes regulated by NF-κB, such as interleukin (IL)-12p40, were differentially modulated by the different type I strains. We subsequently showed that this difference in NF-κB activation was due to polymorphisms in GRA15. Furthermore, we observed that RH, but not other type I strains, recruited phosphorylated IκBα (a component of the NF-κB complex) to the

parasitophorous vacuole membrane and this recruitment of p- IκBα was partially dependent on GRA2.

CONCLUSIONS: We identified candidate parasite genes that could be responsible for phenotypic variation among the type I strains through comparative genomics and transcriptomics. We also identified differentially modulated host pathways among the type I strains, and these can serve as a guideline for future studies in examining the phenotypic differences among type I strains.

Background

Toxoplasma gondii is an obligate intracellular pathogen from the phylum Apicomplexa and is estimated to infect about one third of the world population (Hill, Dubey 2002). It usually establishes an asymptomatic, chronic infection, but immunodeficient individuals can develop severe disease such as encephalitis and retinitis. *Toxoplasma* has a relatively complex life cycle, containing both asexual and sexual stages. The sexual cycle occurs in felines, but because a single parasite can give rise to both micro and macro-gametes, usually self fertilization occurs (Wendte et al. 2010). Sexual recombination leading to new genotypes can only occur when felines are infected simultaneously with at least two different strains (Pfefferkorn, Pfefferkorn & Colby 1977). The low occurrence of concurrent infection events, together with horizontal transmission between intermediate hosts through consumption of infectious cysts, likely accounts for the highly clonal population structure observed in North America and Europe (Sibley, Ajioka 2008).

In North America, three clonal lineages known as types I, II and III were thought to predominate (Howe, Sibley 1995, Khan et al. 2011). However, recent SNP analysis at five loci in ~950 strains, representing worldwide diversity, clustered these strains into 15 haplogroups and showed a high prevalence of type 12 strains in North America (Su et al. 2012), while a large number of divergent strains are present in South America. Using genome-wide SNPs, it was shown that even within these haplogroups (except for haplogroups I, II, III and 6), there is often significant diversity and most strains appear to have formed through recent recombination events (Minot et al. 2012). Between lineage diversity is estimated to be 1 to 3% while diversity within the type I, II and III clonal lineage is ~0.01% (Boyle et al. 2006, Minot et al. 2012). There are also strong phenotypic differences between lineages (Saeij, Boyle & Boothroyd 2005), such as acute virulence in mice, with type I strains being uniformly lethal ($LD_{100} = 1$), while types II and III are less virulent, with $LD_{50} \geq 10^3$ and $LD_{50} \geq 10^5$, respectively (Saeij, Boyle & Boothroyd 2005). Another phenotype that has been correlated with virulence is *in vitro* growth rate, with type I parasites having a higher growth rate compared to types II and III (Radke et al. 2001).

RH is the most commonly used type I strain for characterization of numerous aspects of *Toxoplasma* biology, ranging from active invasion, replication, to host cell egress, and it has been used extensively for molecular genetic analyses. RH was initially isolated from a 1939 case of toxoplasmic encephalitis by Albert Sabin and was subsequently cloned by Elmer Pfefferkorn for *in vitro* culture in 1977 (Sabin 1941, Pfefferkorn, Pfefferkorn 1976). This cloned RH-ERP strain has likely undergone laboratory adaptation due to extensive *in vitro* lab passage, unlike RH-JSR, a non-cloned RH isolate that was propagated in mice and has undergone little serial passage in culture (Khan et al. 2009). There are several other isolates of the type I lineage

defined by PCR-RFLP at multiple marker alleles, such as GT1, an isolate from goat skeletal muscle (Sibley, Boothroyd 1992), from which the complete genome has been sequenced (www.toxodb.org) (Gajria et al. 2008).

Surprisingly, RH-ERP displays significant parasite phenotypic variation compared to GT1 and RH-JSR (Khan et al. 2009), with RH-ERP having increased extracellular survival times, higher *in vitro* growth rates and loss of ability to form orally infective cysts (Villard et al. 1997, Khan et al. 2009). Moreover, using *Toxoplasma* microarrays, it was observed that RH-ERP parasite gene expression is significantly different compared to RH-JSR and GT1 (Khan et al. 2009), with upregulation of certain ABC transporters in RH-ERP compared to RH-JSR or GT1. RH-ERP and GT1 also differ in the modulation of certain host processes; a serum response factor (SRF) reporter cell line is activated more by RH-ERP compared to GT1, and transcript levels of early growth response factor 2 (EGR2), a SRF target gene, are higher upon RH-ERP infection compared to GT1 (Wiley et al. 2011, Phelps, Sweeney & Blader 2008). In addition, there is increased immune related GTPase (IRG) coating of the parasitophorous vacuole membrane (PVM) of GT1 compared to RH-ERP in IFN-γ stimulated mouse embryonic fibroblasts (MEFs) and subsequent higher levels of GT1 killing compared to RH-ERP (Niedelman et al. 2012). Differential modulation of host pathways could affect host cell survival or nutritional availability and subsequent *Toxoplasma* replication within the host cell.

The genetic basis for phenotypic variation between RH-ERP and the other type I strains is currently unknown. Many of these phenotypic differences are important determinants of acute virulence and chronic infection, as increased growth rates can lead to higher parasite burdens *in vivo*, and cyst formation is essential for *Toxoplasma* transmission (Hill, Dubey 2002). Thus,

understanding the genetic basis for the enhanced growth rate of RH-ERP and its inability to form orally infectious cysts could provide important insights into *Toxoplasma* virulence determinants. To examine the potential genetic basis for phenotypic differences between RH-ERP and GT1, we compared the complete genome sequences of RH-ERP and GT1 and identified single nucleotide polymorphisms (SNPs) and insertions/deletions (indels) across the genome. We also compared differential parasite gene expression between RH-ERP and RH-JSR/GT1 through our own independent transcriptional profiling and previous studies (Bahl et al. 2010, Khan et al. 2009). Using our SNP/indel and differentially expressed gene list, we then identified a number of candidate genes that could be responsible for the phenotypic differences observed between RH-ERP and RH-JSR/GT1, including a dense granule protein, GRA2. We then attributed the differences between RH-ERP and GT1 in evasion of IFN-γ-mediated killing in MEFs to GRA2. To identify novel differences in host cell modulation among type I strains, we performed transcriptional profiling of human foreskin fibroblasts (HFFs) infected with RH-ERP, RH-JSR and GT1. Several host pathways were found to be differentially modulated, such as the NF-κB pathway, which is activated by GT1 but not by RH-ERP or RH-JSR. Other host phenotypes that were different across type I strains included IL-12p40 (a NF-κB dependent cytokine) secretion by infected macrophages and recruitment of p-IκBα to the PVM, which was partially dependent on GRA2. Our results show that genetic mutations accumulated over time due to continuous laboratory passaging, can lead to large phenotypic changes and the candidate genes identified can serve as a guideline for future studies in examining phenotypic differences among type I strains.

Results and Discussion

Identification of genetic differences between RH-ERP and GT1

To determine the genetic differences between RH-ERP and GT1, we sequenced RH-ERP using Illumina sequencing and downloaded the complete genome sequence of GT1 (**Table 1A** and www.toxodb.org). A list of single nucleotide polymorphisms (SNPs) and insertions/deletions (indels) between RH-ERP and GT1 was generated, and a total number of 1,394 SNPs and indels were identified. There were 230 SNPs/indels within predicted coding regions, 484 SNPs/indels within predicted genes but outside coding regions, and 680 SNPs/indels outside predicted genes. From the 230 SNP/indels within predicted coding regions, we further identified 133 SNPs leading to nonsynonymous amino acid changes, 43 SNPs leading to synonymous amino acid substitutions, and 54 indels within predicted coding regions (**Table 1B** and **Supplementary Table 1**).

We then determined whether there was functional enrichment in the genes containing the 1,394 SNPs/indels. Genes with nonsynonymous SNPs and indels within coding regions were enriched in 3'5' cyclic nucleotide phosphodiesterase, protein kinase, ATP binding and metal ion binding activities (p-value < 0.05). Genes with SNP/indels within 1000 bp upstream of predicted ATG start were enriched in nucleoside triphosphatase, nucleotide binding and ATPase activities (p-value < 0.05) (**Table 1C**). Interestingly, addition of a cGMP-specific phosphodiesterase inhibitor, Zaprinast, has been shown to induce *Toxoplasma* egress from host cells (Lourido, Tang & Sibley 2012). Moreover, deletion of phosphodiesterase δ in a related Apicomplexan, *Plasmodium berghei*, caused severe defects in formation of normal ookinetes and ookinete

gliding motility (Moon et al. 2009). Thus, it is possible that the phosphodiesterases that have nonsynonymous SNPs and/or indels could contribute to differences in growth rate of RH-ERP compared to GT1.

RH-ERP tachyzoites grow faster, have higher extracellular viability and loss in ability to form orally infectious cysts compared to GT1/RH-JSR tachyzoites (Khan et al. 2009, Villard et al. 1997). Thus, these phenotypic differences are likely due to parasite genes that are polymorphic and/or differentially expressed between RH-ERP and GT1. We used our SNP/indel data and *Toxoplasma* gene expression data to identify candidate genes, focusing on genes that have non-synonymous, non-conservative SNPs and/or are differentially expressed between RH-ERP and RH-JSR/GT1.

Table 1 Genetic differences between RH-ERP and GT1

A. Summary of comparative genome sequencing between RH-ERP and GT1

Description	RH-ERP (parental)	RH-ERP F-P2 (mutant)
Total base pairs sequenced	6.1×10^9	6.5×10^9
Total paired end reads	4.0×10^7	4.4×10^7
Total reads aligned	3.7×10^7	3.9×10^7
Total reads aligned (%)	90.1	90.5
Human reads (%)	32.2	49.4
Toxoplasma gondii reads (%)	57.9	44.1
GT1 genome covered (%)	96.5	96.9
Reads in unassembled contigs (%)	0.9	0.7
Total Shared Variation Called	1,394	

B. Genetic differences between RH-ERP and GT1

SNPs/indels within predicted coding regions	230	Nonsynonymous SNPs	133
		Synonymous SNPs	43
		Indels	54
SNPs/indels within predicted genes, outside coding regions	484	SNPs/indels in 1000 bp upstream of predicted ATG start (5′UTR)	SNPs/indels in 1000 bp downstream of predicted end codon (3′UTR)
SNPs/indels outside predicted gene	680	133	143

C. Functional enrichment in genetic differences between RH-ERP and GT1

Gene set	Enrichment in Gene Ontology function	P-value	Number of annotated genes with SNPs/indels	Percent of annotated genes with SNPs/indels in gene set (%)	Total number of genes annotated	Percent of annotated genes in genome (%)
Nonsynonymous SNPs and indels within predicted gene coding regions	3′5′-cyclic nucleotide phosphodiesterase	3.57^{03}	3	2.19	16	0.24
	Protein kinase	9.74^{03}	7	5.11	120	1.75
	ATP binding	0.02	11	8.03	270	3.93
SNPs/indels within 5′UTR of predicted genes (1000 bp upstream of ATG start)	Nucleoside triphosphatase	8.47^{03}	4	4.17	58	0.85
	Nucleotide binding	0.01	5	5.21	103	1.50
	ATPase activity	0.03	3	3.13	48	0.70

Table 1. Genetic differences between RH-ERP and GT1. (A) Details for genome sequencing of RH-ERP parental and RH-ERP F-P2 mutant, where common variants between these two strains were determined as SNPs/indels between RH-ERP and GT1. (B) Total number of single nucleotide polymorphisms (SNPs) and insertions/deletions (indels) identified between RH-ERP and GT1, with the number of SNPs/indels identified in each category as indicated. (C) Top three functional enrichment of nonsynonymous SNPs and indels present in gene coding regions, together with genes which contain SNP/indels 1000bp upstream of predicted ATG start, using hypergeometric enrichment.

Analysis of genes with polymorphisms in coding regions between RH-ERP and GT1

From the list of 133 nonsynonymous SNPs, we identified 104 SNPs which led to non-conservative amino acid changes. We then identified 33 SNPs in genes which had transcript levels expressed above background levels of 6.5 in RH-ERP tachyzoites through *Toxoplasma* array data (Khan et al. 2009, Bahl et al. 2010), and identified several candidate genes which could lead to differences in phenotypes between RH-ERP and GT1 (**Table 2A** and **Supplementary Table 1**). The gene encoding for dense granule protein GRA2 (TGGT1_083030) was found to have a glycine (GT1) to serine (RH-ERP) substitution, and was expressed highly in both RH-ERP and GT1 from array data. RHΔ*gra2* differs in several phenotypes compared to parental RH-ERP (Δ*hxgprt*), such as the disruption of the intravacuolar network within the PV, decreased virulence in mice and enhanced susceptibility to IRG-mediated killing (Mercier et al. 1998, Mercier et al. 2002, Niedelman et al. 2012).

Interestingly, an ATP-dependent RNA helicase (TGGT1_081400) contained one conservative and two non-conservative nonsynonymous SNPs, indicating possible positive selection in this gene and was highly expressed in RH-ERP and GT1 from array and RNAseq data (**Supplementary Table 1**). RNA helicases of the DEAD box family are involved in multiple aspects of RNA metabolism, ranging from formation of the exon junction complex, mRNA export and translation initiation (Linder, Jankowsky 2011). Interestingly, eIF4A, the model of DEAD helicases, has been shown to be downregulated at the transcript level in attenuated type I tachyzoites (through prolonged *in vitro* passage) and type II bradyzoites compared to virulent type I tachyzoites (Gastens, Fischer 2002).

Table 2 Top candidate genes that have nonsynonymous amino acid changes between RH-ERP and GT1

A. Candidate genes containing nonsynonymous SNPs between RH-ERP and GT1

ToxoDB GT1 ID	ToxoDB annotation	A.A. change (GT1 to RH-ERP)	RH-ERP expression (Microarray)	Possible involvement in Toxoplasmaprocess
TGGT1_083030	28 kDa antigen, putative (GRA2)	Gly to Ser	13.8	Growth
TGGT1_113990	SRS29C	Ser to Phe	13.5	Invasion
TGGT1_114020	SRS29A	Val to Met	12.7	Invasion
TGGT1_069190	DEAD/DEAH box helicase, putative	Ile to Asn	11.9	Transcriptional control
TGGT1_030300	acid phosphatase, putative (GAP50)	Ile to Asn	11.4	Gliding motility
TGGT1_020630	conserved hypothetical protein (contains glycosyltransferase 17 family)	Leu to Arg	11.1	Growth
TGGT1_066370	hypothetical protein (eukaryotic initiation factor 3)	Leu to Phe	10.7	Translation
TGGT1_081400	ATP-dependent RNA helicase, putative	Arg to Cys Ala to Asp	9.6	Transcriptional control
TGGT1_069890	apoptosis-regulating basic protein, putative	Leu to Phe	8.8	Growth
TGGT1_118630	U2 small nuclear ribonucleoprotein, putative	Thr to Ile	8.7	Growth
TGGT1_098160	Coronin, putative	Met to Val	8.6	Gliding motility
TGGT1_086050	sushi domain-containing protein (RON1)	Asp to Gly	7.9	Invasion
TGGT1_009970	CCR4-NOT transcription complex subunit, putative	Pro to Ser	7.6	Transcriptional control

B. Candidate extracellular viability genes containing nonsynonymous SNPs between RH-ERP and GT1

ToxoDB GT1 ID	ToxoDB annotation	A.A. change (GT1 to RH-ERP)	Fold change between extracellular and intracellular tachyzoites
TGGT1_057870	conserved hypothetical protein (contains RNA recognition motif)	Asp to His	2.6
TGGT1_021310	pinA, putative (contains forkhead associated domain)	Phe to Leu	2.0
TGGT1_104520	conserved hypothetical protein	Leu to Pro	2.0
TGGT1_016250	hypothetical protein (cyclic nucleotide-binding domain-containing protein)	Ile to Asn	2.0
TGGT1_065470	conserved hypothetical protein (contains oligomerization domain)	Ser to Cys	-2.3
TGGT1_098520	EF hand domain-containing protein, putative	Val to Phe	-2.6
TGGT1_087130	apyrase, putative	Pro to Leu	-3.2
TGGT1_026200	acylamino-acid-releasing enzyme, putative	Trp to STOP	-3.5

C. Candidate bradyzoite genes containing nonsynonymous SNPs between RH-ERP and GT1

ToxoDB GT1 ID	ToxoDB annotation	A.A. change (GT1 to RH-ERP)	Fold change between 8 day M4 in vitrobradyzoites and 2 day M4 tachyzoites	Fold change between 21 day M4in vivocysts and 2 day M4 tachyzoites
TGGT1_013440	conserved hypothetical protein	Gly to Ser	1.8	1.6
TGGT1_020630	conserved hypothetical protein (contains glycosyltransferase 17 family)	Leu to Arg	2.3	1.5

Table 2. **Top candidate genes that have nonsynonymous amino acid changes between RH-ERP and GT1.** (A) Candidate genes that have non-conservative, nonsynonymous amino acid changes between RH-ERP and GT1 and are expressed highly in RH-ERP as determined by *Toxoplasma* arrays. *Toxoplasma* array expression values are log2 transformed values, ranging from 6.5 (minimum) to 14.3 (maximum). (B) Candidate genes that are differentially expressed between intracellular and extracellular RH-ERP tachyzoites (Lescault et al. 2010). (C) Candidate genes that are differentially expressed between M4 *in vitro* tachyzoites, *in vitro* induced bradyzoites and *in vivo* cysts (Buchholz et al. 2011).

In addition, SRS29A and SRS29C both had a non-conservative, nonsynonymous amino acid substitution, and were highly expressed in both RH-ERP and GT1 in array data. SRS29A was also 1.7 fold more highly expressed in RH-ERP extracellular tachyzoites compared to RH-ERP intracellular tachyzoites (**Supplementary Table 1**). A recent study showed that RH-ERP overexpressing SRS29C was significantly attenuated in mouse virulence compared to parental RH-ERP strain, though this overexpressing strain did not have any significant differences from the parental strain with regards to invasion, attachment or growth *in vitro* (Wasmuth et al. 2012).

Another candidate gene identified was GAP50 (TGGT1_030300), with a isoleucine (GT1) to asparagine (RH-ERP) substitution and was highly expressed in both RH-ERP and GT1 in array data (**Table 2A**). GAP50 is the membrane anchor of the glideosome complex, which is required for gliding motility (Frenal et al. 2010). It also interacts with other components such as TgMyoA, TgMLC1 and TgGAP45, and requires N-glycosylation for proper localization to the inner membrane complex (Gaskins et al. 2004, Fauquenoy et al. 2011). Interestingly, a rhoptry neck protein, RON1 (TGGT1_086050), has an aspartic acid (GT1) to glycine (RH-ERP) substitution, and is 1.3 fold more highly expressed in RH-ERP compared to GT1in RNAseq data (**Supplementary Table 1**). RON1 has a distinct rhoptry neck localization (Bradley et al. 2005), and several rhoptry neck proteins constitute the moving junction, which is required for invasion of the host cell (Alexander et al. 2005). Thus, polymorphisms in these genes could account for differences in invasion, growth or extracellular viability between RH-ERP and GT1.

We also examined these 104 non-conserved, nonsynonymous SNPs for genes that are differentially expressed between RH-ERP intracellular and extracellular tachyzoites (Lescault et al. 2010), as RH-ERP has increased extracellular viability compared to GT1 (Khan et al. 2009).

We noted a predicted acylamino-acid releasing enzyme (TGGT1_026200) that contained a nonsense mutation leading to a premature stop codon, and had decreased expression in extracellular tachyzoites (**Table 2B**). In addition, a putative pinA gene (TGGT1_021310) with a phenylalanine (GT1) to leucine (RH-ERP) substitution had increased 2.0 fold expression in extracellular tachyzoites compared to intracellular tachyzoites. This parasite gene contains a forkhead associated domain that is involved in binding to phosphopeptides and forkhead-type transcription factors are involved in the regulation of cell cycle stage specific transcription in budding yeast (Simon et al. 2001). Thus, these genes could be involved in the difference in extracellular viability reported between RH-ERP and GT1.

RH-ERP also exhibits a loss in ability to form infective cysts, unlike GT1 (Villard et al. 1997). Thus, we also examined the 104 non-conserved, nonsynonymous SNPs for parasite genes which are differentially expressed between M4 (type II) tachyzoites, M4 *in vitro* bradyzoites and M4 *in vivo* cysts (Buchholz et al. 2011). Two parasite genes containing substitution polymorphisms between RH-ERP and GT1 were upregulated in both *in vitro* bradyzoites and *in vivo* cysts (**Table 2C**). One of the genes (TGGT1_020630) contains a glycosyltransferase domain that is involved in transferring N-acetylglucosamine to the core mannose of complex N-glycans. Interestingly, TGGT1_020630 was also identified to be consistently more highly expressed in RH-ERP compared to GT1 (**Table 3B**). Thus, this gene could potentially be involved in the loss of ability in RH-ERP to form orally infectious cysts, as bradyzoites contain numerous amylopectin granules and the tissue cyst wall consists of lectin binding sugars (Craver, Rooney & Knoll 2010, Coppin et al. 2003).

RH-ERP and GT1 GRA2 both complement Irgb6 coating but show differences in IRG mediated killing

As mentioned above, GRA2 was found to have a single polymorphism from glycine (GT1) to serine (RH-ERP) in our SNP analysis. Furthermore, previous work on *Toxoplasma* immune evasion mechanisms have shown that RH-ERP infected IFN-γ stimulated MEFs had decreased percentage of parasite vacuoles coated with Irgb6, an immunity related GTPase (IRG), compared to GT1 infected MEFs (Niedelman et al. 2012). In addition, RHΔ*gra2* infected IFN-γ stimulated MEFs had increased percentage of parasite vacuoles coated with Irgb6 compared to RH-ERP infected MEFs. Thus, we examined the possible effects of the GRA2 polymorphism on Irgb6 coating and IRG evasion through complementation of RHΔ*gra2* with either RH-ERP *GRA2* or GT1 *GRA2*.

As noted previously, there was a significant difference between RH-ERP and RHΔ*gra2* or GT1 in percentage of parasite vacuoles coated with Irgb6 (**Figure 1A** and **1B**, *p*-value = 0.0004 (RHΔ*gra2*) and *p*-value = 0.002 (GT1), Student's *t* test). Both RH-ERP GRA2 and GT1 GRA2 were able to complement Irgb6 coating, as both complemented strains had similar percentage parasite vacuoles coated with Irgb6 compared to RH-ERP. However, some *Toxoplasma* parasites are still able to survive, escape IRG coated vacuoles and invade a new cell, thus Irgb6 coating may not fully measure parasite killing (Virreira Winter et al. 2011). Therefore, to measure IFN-γ mediated killing in MEFs, the relative number of parasite plaques that form 4-7 days on IFN-γ stimulated MEFs compared to unstimulated MEFs were measured (also referred to as plaque loss). Surprisingly, unlike Irgb6 coating, there were differences between the two complemented strains in the plaque loss assay (**Figure 1B**), though both complemented strains expressed similar

levels of GRA2 (**Supplementary Figure 1**). The RH-ERP *GRA2* was able to complement the difference in plaque loss between RH-ERP and RHΔ*gra2*, with no significant difference in plaque loss between RH-ERP and RHΔ*gra2*+RH-ERP-GRA2 (*p*-value = 0.33, Student's *t* test). However, the GT1 *GRA2* was unable to complement the difference in plaque loss, and there was still a significant difference in plaque loss between RH-ERP and RHΔ*gra2*+GT1-GRA2 (*p*-value = 0.008, Student's *t* test), similar to the difference observed between RH-ERP and GT1 (*p*-value = 0.01, Student's *t* test) (**Figure 1B**).

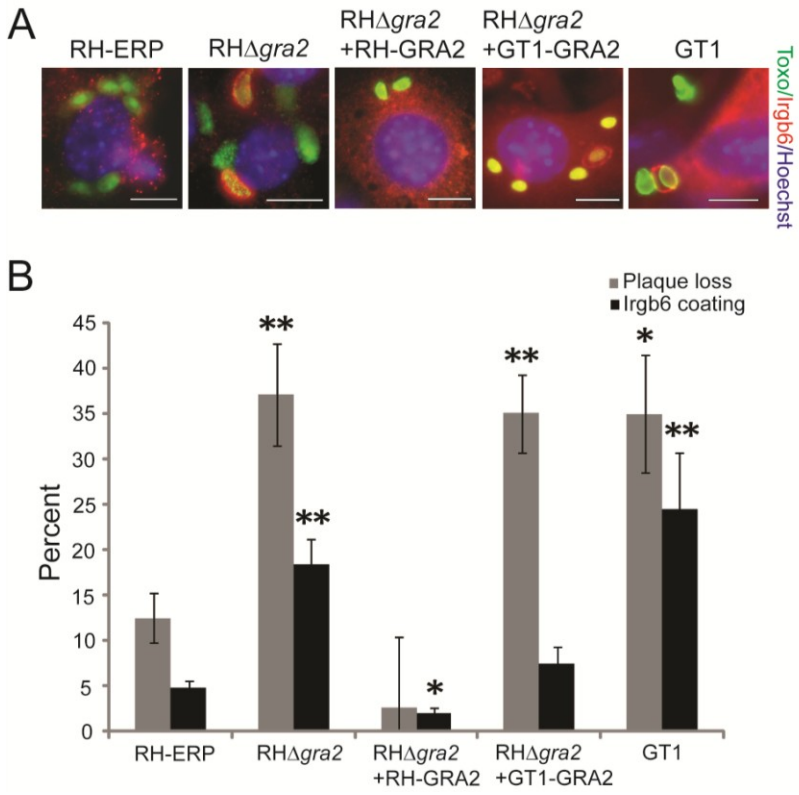


Figure 1. Differences in complementation of plaque loss and Irgb6 coating between RH-ERP and GT1 GRA2. (A) Mouse embryonic fibroblasts were stimulated with IFNγ for 24 hours, infected with RH-ERP, RHΔ*gra2*, RHΔ*gra2*+RH-ERP-GRA2, RHΔ*gra2*+GT1-GRA2 and GT1 for 1 hour and stained for Irgb6 (red), Hoechst (blue) and SAG1 (RH-ERP, RHΔ*gra2* and GT1) or TdTomato (complemented strains) (green). Scale bars represent 10µm. (B) Quantification of Irgb6 localization on the parasite containing vacuole and percentage plaque loss after 4-7 days on IFNγ stimulated MEFs compared to unstimulated MEFs. Mean + SEM, of at least 4 independent experiments, * p-value < 0.05 and ** p-value < 0.01, Student's *t*-test compared to RH-ERP infected MEFs.

It is possible that the difference in complementation observed between RH-ERP GRA2 and GT1 GRA2 in the plaque assay is due to *Toxoplasma* killing in MEFs involving other IRG family members in addition to Irgb6. The different IRGs are known to play individual, non-redundant roles during infection, as mice lacking different IRGs exhibit susceptibility to *Toxoplasma* infection at different stages of infection (Collazo et al. 2001). Furthermore, Irgb6 coating is likely dependent on proper parasitophorous vacuole biogenesis, which is disrupted in RHΔ*gra2* (Mercier et al. 2002), whereas plaque loss is likely not solely dependent on proper PV biogenesis.

Identification and analysis of differentially expressed parasite genes among type I strains

In addition to polymorphisms, phenotypic differences between RH-ERP and GT1 can also be attributed to differential expression of parasite genes among the different type I strains. This differential expression could be due to two main reasons (Cheung et al. 2010). Firstly, polymorphisms in the *cis*-regulatory regions, such as the promoter or untranslated regions (UTRs), could lead to differences in transcription initiation, transcript stability or transcript decay. Secondly, polymorphic or differentially expressed transcriptional regulators, such as the Apetala 2 (AP2) transcription factors (Behnke et al. 2010), could affect the expression levels of non-polymorphic genes. These transcriptional regulators can affect gene expression levels at different levels of RNA metabolism, such as controlling the formation of the exon junction complex, the export of mRNA through the nuclear complex and translation initiation (Linder, Jankowsky 2011, Behnke et al. 2010, Miller, Reese 2012).

Since sequence variation in promoter regions between strains could lead to differential gene expression, we determined whether any of the 1164 SNPs/indels we identified outside predicted gene coding regions were inside a promoter/5' UTR (defined as 1000 bp upstream of predicted ATG start codon) or 3' UTR (defined as 1000 bp downstream of predicted end codon). We found 133 SNPs/indels within the 5' UTR of 110 genes and 143 SNPs/indels within the 3' UTR of 106 genes. Of these 216 genes with 5' or 3' UTR SNPs/indels, microarray analyses showed that 43 genes were ≥1.5 fold differentially expressed between RH-ERP and GT1 (p-value = 0.07, hypergeometric test) (**Supplementary Table 1**).

We identified a putative dihydrolipoamid dehydrogenase (TGGT1_041490) with a 5' UTR polymorphism, that was expressed 1.7 fold higher in RH-ERP compared to GT1 using array data, and was also expressed higher than in other canonical strains such as PRU and VEG (ToxoDB.org). Moreover, this same putative dihydrolipoamid dehydrogenase was expressed approximately 11 fold higher in RH-ERP intracellular parasites compared to extracellular parasite (**Table 3A** and **Supplementary Table 1**), which could indicate a possible role in intracellular growth. Dihydrolipoamide dehydrogenase serves as a component of several multifunctional complexes, such as pyruvate dehydrogenase, the glycine cleavage system and branched chain amino acid dehydrogenase complexes (Schoneck et al. 1997). Thus, increased expression of this gene in RH-ERP could lead to differences in glycolytic metabolism, or resistance to reactive nitrogen intermediates. Interestingly, deletion of dihydrolipoamide dehydrogenase in an intracellular bacterial pathogen, *Mycobacterium tuberculosis*, causes a significant attenuation of virulence in mice (Venugopal et al. 2011).

Notably, there was a single ABC transporter (TGGT1_025370, also called $TgABCG_{107}$ (Ehrenman et al. 2010)) with a 5' UTR polymorphism, though it was only expressed 1.3 fold higher in RH-ERP compared to GT1 using array data. It was shown that cells transfected with $TgABCG_{107}$ accumulated larger amounts of cholesterol in an ATP-dependent manner compared to untransfected cells, indicating a role for $ABCG_{107}$ in lipid homeostasis in *Toxoplasma* (Ehrenman et al. 2010). In addition, we noted an ATP dependent helicase (TGGT1_113930) with a 5' UTR polymorphism that was expressed 1.4 fold higher in RH-ERP compared to GT1 using array data (**Supplementary Table 1**). Thus, overexpression of these ATP dependent candidate genes identified could contribute to the enhanced growth of RH-ERP.

Interestingly, we identified an AP-2 transcription factor (AP2VIIA2; TGGT1_072850) with a 3' UTR polymorphism, and is more highly expressed in M4 *in vitro* bradyzoites and *in vivo* cysts compared to *in vitro* tachyzoites (Buchholz et al. 2011) (**Supplementary Table 1**). 24 AP-2 transcription factors have cyclical expression profiles corresponding to the tachyzoite division cycle, whereas 11 AP-2 mRNAs are induced during *in vitro* bradyzoite differentiation (including AP2VIIA2) (Behnke et al. 2010). Therefore, this AP2 transcription factor could be involved in the regulation of genes required for bradyzoite differentiation and cyst formation.

Table 3 Top candidate genes that are differentially expressed between RH-ERP and GT1

A. Candidate genes containing 5′ and 3′UTR SNP/indels and are differentially expressed between RH-ERP and GT1

Position	ToxoDB GT1 ID	ToxoDB annotation	Fold change RH-ERP/ GT1 (Microarray)	Possible involvement in Toxoplasmaprocess
5′UTR	TGGT1_041490	dihydrolipoamid dehydrogenase, putative	1.7	Growth
5′UTR	TGGT1_027570	microneme protein, putative	1.6	Invasion
3′UTR	TGGT1_11488	tRNA splicing2′phosphotransferase, putative	1.6	Translation
5′UTR	TGGT1_090150	NBP2B protein, putative	1.6	Unknown
5′UTR	TGGT1_073790	transporter, major facilitator family protein	-5.1	Growth

B. Candidate genes consistently differentially expressed between RH-ERP and GT1

ToxoDB GT1 ID	ToxoDB annotation	Fold change RH-ERP/GT1 (Microarray)	Possible involvement in Toxoplasmaprocess
TGGT1_098460	ankyrin repeat-containing protein	12.1	Transcriptional control
TGGT1_073210	hypothetical protein	5.7	Unknown
TGGT1_020630	hypothetical protein (contains glycosyltransferase 17 family)	5.7	Unknown
TGGT1_051960	ABC transporter transmembrane region domain-containing protein	3.0	Growth
TGGT1_030200	Peptidyl-tRNA hydrolase PTH2 domain-containing protein	2.3	Growth
TGGT1_000480	GRA12 homologue	2.0	Growth
TGGT1_126470	hypothetical protein	-2.1	Unknown
TGGT1_048210	rhoptry kinase family protein ROP38 (ROP38)	-3.7	Host cell modulation
TGGT1_047990	rhoptry kinase family protein ROP29 (ROP29)	-4.0	Host cell modulation
TGGT1_081480	zinc finger (CCCH type) motif-containing protein	-8.0	Transcriptional control
TGGT1_021770	microneme protein, putative	-9.2	Invasion
TGGT1_126670	rhoptry protein ROP8 (ROP8)	-11.3	Host cell modulation

Table 3. Top candidate genes that are differentially expressed between RH-ERP and GT1. (A) Candidate genes that have a SNP/indel present in 1000bp region upstream of predicted ATG start or downstream of predicted end codon, and leading to expression differences of ≥1.5 fold in *Toxoplasma* arrays. (B) Candidate genes that are consistently differentially expressed between RH-ERP and GT1 using the three *Toxoplasma* array datasets, but not necessarily containing SNPs/indels in the 5' or 3' UTR regions.

Another reason for differential parasite gene expression could be polymorphic or differentially expressed trans-regulators, such as the AP2 transcription factors (Behnke et al. 2010) that could regulate the expression of many non-polymorphic genes. Alternatively, differential expression of genes could be under epigenetic control, such as post translational modification of histone proteins or arginine methylation (Saksouk et al. 2005, Dixon et al. 2010). Thus, we investigated genes that were differentially expressed between RH-ERP, RH-JSR and GT1, regardless of SNPs in the coding or regulatory regions (**Supplementary Table 2**). We used expression datasets available from a previous published study (Khan et al. 2009), another independent dataset with

parasite gene expression levels comparing RH-ERP and GT1 (Bahl et al. 2010), and our own *Toxoplasma* arrays measuring parasite gene expression levels comparing RH-ERP, RH-JSR and GT1 (GSE44191). We analyzed for transcripts that were ≥1.5 fold differentially expressed between RH-ERP and GT1 across these three datasets, to identify genes that were consistently up or downregulated between RH-ERP and GT1. We identified 13 transcripts that had consistently increased and 13 transcripts that had consistently decreased expression in RH-ERP compared to GT1 across all three independent datasets respectively.

From the 13 consistently upregulated transcripts in RH-ERP compared to GT1 (**Supplementary Table 2**), we identified a putative ankyrin repeat containing protein (TGGT1_098460). This gene was highly expressed in RH-ERP in both array and RNA-seq datasets, but not in GT1 (**Table 3B**), and had lower expression in canonical strains such as PRU and VEG (www.toxodb.org). Ankyrin repeats mediate molecular recognition via protein-protein interactions (Li, Mahajan & Tsai 2006), and proteins containing these repeats are involved in a large number of cellular functions, ranging from modulation of the NF-κB response to transcriptional regulation (Al-Khodor et al. 2010). Interestingly, TgANK-1, a parasite protein containing ankyrin repeats, is induced upon bradyzoite differentiation using RH parasites, and localizes to the parasite cytosol (Friesen et al. 2008). Another gene that was consistently upregulated was a GRA12 homologue, and GRA12 co-localizes with GRA2 and interacts with GRA2 or GRA2-associated proteins (Michelin et al. 2009). Therefore, this gene could be associated with the increased growth rate of RH-ERP compared to GT1.

Of the 13 transcripts with increased expression in GT1 compared to RH-ERP, three encode for known rhoptry proteins, ROP8 (TGGT1_126670), ROP29 (TGGT1_047990) and ROP38

(TGGT1_048210) (**Table 3C**). ROP29 and ROP38 are part of a repeated gene family, and overexpression studies of ROP38 in the RH-ERP background showed that ROP38 has major effects on host gene expression (Peixoto et al. 2010). Furthermore, as ROP8, ROP29 and ROP38 have predicted signal peptides, these rhoptry proteins might be involved in differential host modulation between RH-ERP and GT1. Several rhoptry proteins are known to be secreted into the host cells, where they play major roles in modulation of the host cell functions (Melo, Jensen & Saeij 2011).

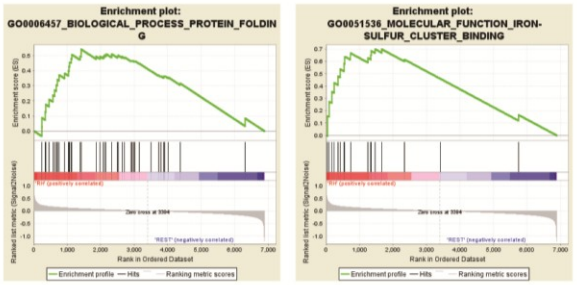
Differentially modulated parasite pathways between type I strains

In addition, we analyzed whether the differentially expressed genes between RH-ERP, RH-JSR and GT1 across the three independent datasets were enriched in annotated biological functions (Hunter et al. 2009). We focused on functional enrichment of parasite genes differentially expressed in RH-ERP compared to RH-JSR and GT1, using our own arrays and expression data from previous studies (Khan et al. 2009, Bahl et al. 2010). There were 15 Gene Ontology (GO) and 25 InterPro genesets that were significantly enriched in RH-ERP compared to RH-JSR/GT1 (false discovery rate (FDR) < 0.10). Parasite genes differentially expressed in RH-ERP compared to RH-JSR/GT1 were enriched in GO processes such as transcription, translation, protein folding and iron-sulfur cluster binding (**Figure 2A** and **Supplementary Table 3**). Similarly, parasite genes differentially expressed in RH-ERP compared to RH-JSR/GT1 were enriched in InterPro domains such as peptidyl-prolyl cis-trans isomerases, cyclophilin-like and DNA-dependent RNA polymerases (**Figure 2B** and **Supplementary Table 3**). This is in accord with observations that RH-ERP has a higher growth rate, likely requiring increased transcription and translation, and

increased extracellular viability (Khan et al. 2009), likely requiring expression of stress proteins to survive extracellular stress.

A GSEA Gene Ontology enrichment for differentially expressed parasite genes in RH-ERP compared to RH-JSR/GT1

Gene Ontology Pathway	FDR q-value
Protein folding	0.02
Structural constituent of ribosome	0.02
Iron sulfur cluster binding	0.03
Peptidyl prolyl cis-trans isomerase activity	0.03
Cellular components associated with the ribosome	0.03



B GSEA InterPro enrichment for differentially expressed parasite genes in RH-ERP compared to RH-JSR/GT1

InterPro Domain	FDR q-value
Cyclophilin-like	0.001
Cyclophilin type peptidyl prolyl cis-trans isomerase	0.001
Translation	0.02
Transcription, DNA-dependent	0.03
SNARE-like	0.03

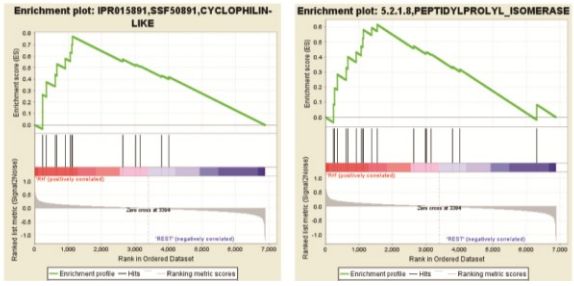
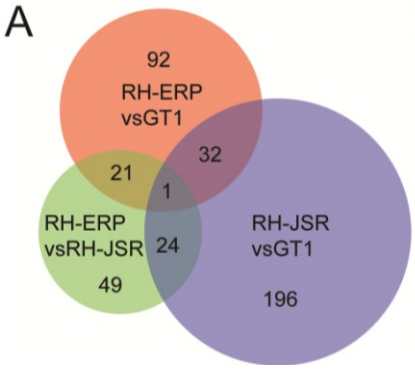


Figure 2. Differential parasite gene expression between type I strains. (A) The top five enriched Gene Ontology (GO) pathways using GSEA in differentially expressed parasite genes in RH-ERP compared to RH-JSR/GT1 and the corresponding GSEA diagrams, using *Toxoplasma* genesets with Gene Ontology annotations. The GSEA diagrams show the enrichment score (green line), which reflects the degree to which that particular gene set (header above) is overrepresented in the differentially expressed genes between RH-ERP and RH-JSR/GT1 (ranked by their differential expression values). The middle portion of the diagram shows where the members of the particular gene set appear in the ranked gene list. The bottom portion of the diagram shows the value of the ranking metric, which measures the correlation of a gene with upregulation (positive value) or downregulation (negative value) in RH-ERP compared to RH-JSR/GT1. (B) The top five enriched InterPro domain using GSEA in differentially expressed parasite genes in RH-ERP compared to RH-JSR/GT1 and the corresponding GSEA diagrams, using *Toxoplasma* genesets with InterPro annotations.

Differential modulation of host pathways between type I strains

Because some of the differentially expressed or polymorphic *Toxoplasma* genes, such as ROP38, might be involved in the modulation of host cell signaling pathways (Peixoto et al. 2010), we determined whether different type I strains differ in their ability to modulate the host cell response. To do this, we infected HFFs with RH-ERP, RH-JSR and GT1 for 24 hours and determined host gene expression profiles using microarrays. We identified 146, 95, and 253 host transcripts that were consistently up/downregulated ≥1.5 fold in every experiment comparing RH-ERP against GT1, RH-ERP against RH-JSR and RH-JSR against GT1 respectively (**Figure 3A** and **Supplementary Table 4**). As ROP38 was differentially expressed between RH-ERP and GT1, and has major effects on host gene expression, we wanted to determine the effects of ROP38 in differentially expressed host transcripts between RH-ERP and GT1. To do this, we used expression datasets available from a previous study that compared host gene expression after infection with RH-ERP or RH-ERP overexpressing ROP38 (Peixoto et al. 2010). From the 146 host transcripts that were consistently differentially expressed between RH-ERP and GT1, 14 were also ≥1.5 fold differentially expressed between RH and RH overexpressing ROP38 (**Supplementary Table 4**). Thus, these 14 genes which are differentially expressed between RH-ERP and GT1 could be due to ROP38 expression differences.



B

Consistently upregulated host genes in GT1 infected HFFs compared to RH-ERP

Transcription factor	Importance
NFKAPPAB	0.25
ISRE	0.20
STAT1	0.18
NFE2	0.15
IRF7	0.10

Figure 3. Differential host gene expression between type I strains. HFFs were infected for 24 hours with RH-ERP, RH-JSR and GT1 and host gene expression was analyzed using microarrays. Three arrays were done per strain for RH-ERP and RH-JSR, and two arrays were done for GT1. (A) Venn diagram showing the number of host genes that were consistently differentially expressed ≥1.5 fold or more for each pairwise comparison between type I strains. (B) Top five enriched known transcription factors from DiRE analysis of consistently upregulated host genes in GT1 infection compared to RH-ERP.

We subsequently determined if the promoters of the consistently differentially regulated host genes were enriched for specific transcription factor binding sites (TFBS) (Gotea, Ovcharenko 2008, Subramanian et al. 2005). We noted an NF-κB signature in the promoter of host genes that were upregulated ≥1.5 fold in GT1 compared to RH-ERP (**Figure 3B**), and also noted that type I interferon and STAT1 signatures were present in host genes upregulated in HFFs infected with GT1 compared to RH-ERP. In addition, we observed STAT6 enrichment in the promoter of host genes that were consistently upregulated in RH-JSR compared to RH-ERP (**Supplementary Table 5**).

NF-κB activation and induction of IL-12p40 secretion upon infection are differentially modulated by type I strains and are dependent on GRA15

Previous studies have shown that type II strains activate NF-κB to a much higher level compared to types I and III, and this difference is due to polymorphisms in the dense granule protein, GRA15 (Rosowski et al. 2011). It was noted that high levels of nuclear translocation of the p65 subunit of NF-κB were present in host cells infected with several type II strains (ME49, PRU, DAG and Beverly), while host cells infected with RH-ERP and GT1 had much lower levels of p65 translocation (Rosowski et al. 2011). Our host transcriptional profile analysis across the type I strains indicated that there is an enrichment in NF-κB binding sites in the promoters of host genes that are more highly induced by GT1 infection compared to RH-ERP (**Figure 3B**). To validate this analysis, HEK293T NF-κB reporter cells with NF-κB binding sites driving the expression of GFP and luciferase were infected with RH-ERP, RH-JSR and GT1 for 24 hours and assayed for NF-κB dependent luciferase activity (**Figure 4A**). In accord with the transcriptional profiling analysis, GT1 infection induced much higher NF-κB mediated luciferase activity compared to RH-ERP (p-value = 0.001, Student's t test), although this induction was still 2-3 fold lower compared to cells infected with Pru, a type II strain (data not shown).

We then investigated whether GRA15 could explain the differences in NF-κB activation between the type I strains, since our SNP analysis indicated an indel in GRA15 when comparing RH-ERP and GT1 (**Supplementary Table 1**). Moreover, RH-ERP was shown to contain a frameshift deletion compared to GT1, leading to a nonfunctional GRA15 in RH-ERP (Rosowski et al. 2011). Because RH-JSR also does not induce NF-κB activation, we sequenced *GRA15* from RH-JSR and, surprisingly, we found that RH-JSR contains a frameshift insertion at base 734. This

mutation is independent from the RH-ERP frameshift deletion at base 872. The RH-JSR insertion causes a mutation of a stretch of 34 amino acids followed by a premature stop codon (**Supplementary Figure 2**). Thus, both RH-ERP and RH-JSR contain truncated GRA15 proteins of 312 and 278 amino acids respectively, while GT1 has the full length protein of 635 amino acids. GRA15 was observed to affect parasite growth both *in vitro* and *in vivo*, as RH-ERP expressing $GRA15_{II}$ has reduced plaque size in HFFs and reduced parasite burden in mice (Rosowski et al. 2011). Thus, there could have been a selective pressure for a nonfunctional GRA15 in RH-ERP and RH-JSR, allowing for increased parasite burden in the host or faster replication *in vitro*.

Since RH-ERP provides a null genetic background with respect to GRA15, we generated transgenic RH-ERP parasites overexpressing GRA15 from GT1 and assayed for NF-κB mediated luciferase activity using the same NF-κB reporter cell line described above (**Figure 4A**). The NF-κB reporter luciferase activity after infection with RH-ERP+$GRA15_{GT1}$ was significantly higher than the activity after infection with RH-ERP (p-value = 0.0001, Student's t test). This data supports the hypothesis that differences in NF-κB activation among type I strains are due to GRA15. In addition, the NF-κB family of transcription factors consist of five members, p65, c-REL, REL-B, p50 and p52 (Hayden, Ghosh 2008), and the NF-κB reporter contains four repeated, canonical NF-κB binding sites which can be bound by the different NF-κB subunits. Previous studies showed p65 nuclear localization in host cells infected with type II strains (Rosowski et al. 2011) and we wanted to determine whether p65 nuclear translocation could be responsible for differential NF-κB activation observed in host cells infected by GT1 compared to RH-ERP. HFFs were infected with RH-ERP, RH-JSR, GT1 or transgenic RH-

ERP+GRA15$_{GT1}$, and nuclear localization of p65 was examined by immunofluorescence (**Figure 4B**). There was significant nuclear translocation of p65 in cells infected with transgenic RH-ERP+GRA15$_{GT1}$ parasites compared to uninfected cells (p-value = 0.02, Student's t test), but no significant p65 nuclear translocation was observed in cells infected with RH-ERP and GT1 (p-value > 0.05, Student's t test). It is possible that the GRA15 in GT1 activates a different NF-κB subunit from p65, such as c-REL or p50, but transgenic high overexpression of GRA15$_{GT1}$ in RH-ERP activates p65 in addition to other subunits. It is also possible that there are other GT1 genes that could have an inhibitory effect on p65 translocation.

GRA15 from type II strains (GRA15$_{II}$) was also shown to affect levels of IL-12p40, a NF-κB dependent cytokine, by infected macrophages *in vitro* (Rosowski et al. 2011), and regulation of IL12-p40 production has been linked to NF-κB activation (Robben et al. 2004). Thus, we investigated whether type I strains differ in their ability to induce secretion of IL-12p40 and CCL2, another NF-κB dependent cytokine, and examined whether GRA15 from GT1 played a role in these differences. GT1 infection of C57BL/6 bone marrow derived macrophages resulted in secretion of higher levels of both IL-12p40 and CCL2 in culture supernatants compared to RH-ERP infection (**Figures 4C** and **4D**, p-value = 7.5x10^{-5} (IL-12p40) and p-value = 0.0003 (CCL2), Student's t test). This phenotype can be partially attributed to GRA15, as macrophages infected with RH-ERP+GRA15$_{GT1}$ induced higher levels of IL-12p40 and CCL2 compared to RH-ERP (**Figure 4C** and **4D**, p-value = 0.0006 (IL-12p40) and p-value = 0.003 (CCL2), Student's t-test). However, GRA15$_{GT1}$ in the RH+GRA15$_{GT1}$ background was not sufficient to increase secretion of IL12-p40 by infected macrophages to levels comparable to those observed in macrophages infected with GT1. Moreover, infection with RH-JSR, which has a

nonfunctional GRA15, induced IL12-p40 to levels higher than infection with RH-ERP+GRA15$_{GT1}$ (p-value = 0.003 (IL12-p40) and p-value = 5.9x10^{-5} (CCL2), Student's t-test). Therefore, it is likely that other *Toxoplasma* genes contribute to induction of IL-12p40 and CCL2 production by infected macrophages, especially given that the regulation of IL12-p40 is more complex than sole control by NF-κB (Sanjabi et al. 2000).

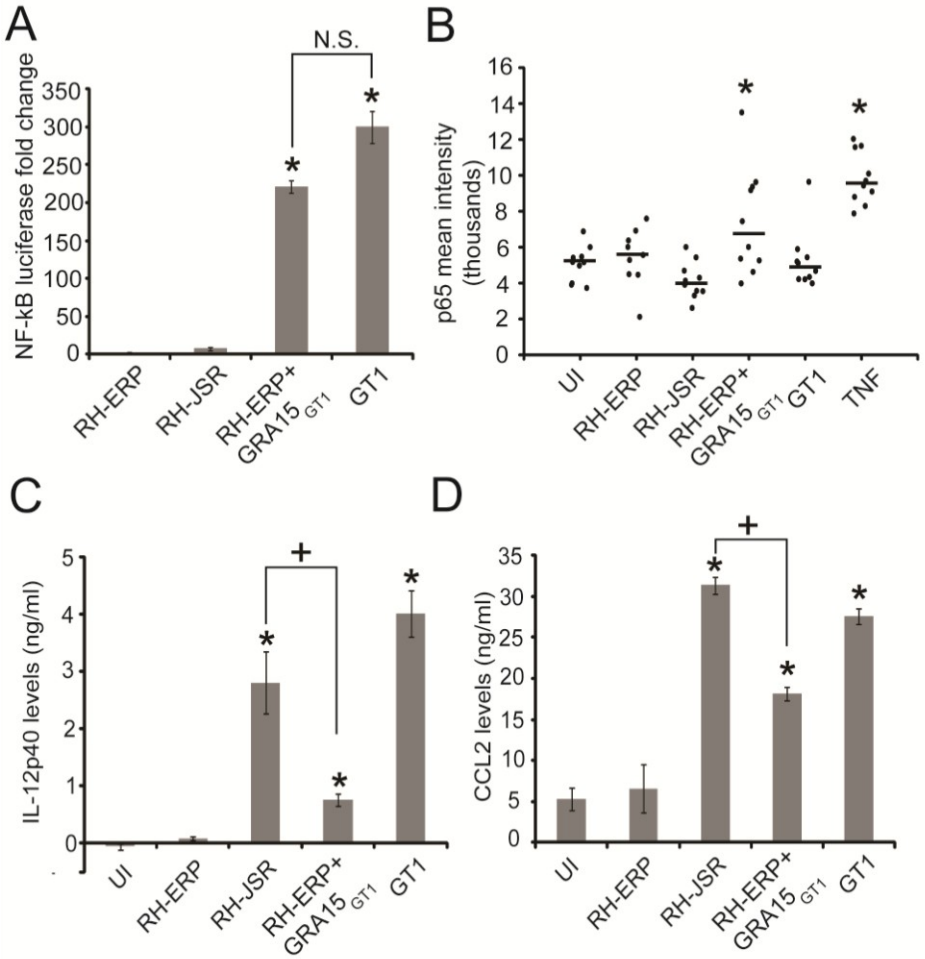


Figure 4. Type I strains differentially activate NF-κB nuclear translocation, and is dependent on GRA15. (A) HEK293T NF-κB reporter cells were infected with RH-ERP, RH-JSR, RH+GRA15$_{GT1}$ and GT1. For each strain, the luciferase reading after 24 hours infection was taken. The graph shows averages from three independent experiments, with levels representing fold change of NF-κB luciferase readings normalized to uninfected, unstimulated control cells, and the error bars represent the standard error. Mean + SEM, of three experiments, * p-value < 0.01, N.S. not significant, Student's t-test. (B) HFFs were infected for 24 h with RH-ERP, RH-JSR, GT1 and RH+GRA15$_{GT1}$ transgenic, uninfected cells were

stimulated with TNF-alpha for 1 h (TNF), or left unstimulated and uninfected (UI). Cells were fixed, probed with p65 antibody and mean nuclear staining was quantified, the error bars represent the standard deviation, * p-value < 0.05, Student's t-test. Quantification shown is representative of three independent experiments. (C) IL12-p40 and (D) CCL2 levels were measured in supernatants from C57BL/6 bone marrow derived macrophages infected for 24 hours with RH-ERP, RH-JSR, RH+GRA15$_{GT1}$ transgenic and GT1. The graphs shown are representative of three independent experiments for IL-12p40 and two independent experiments for CCL2, and the error bars represent the standard deviation, * p-value < 0.01, Student's t-test between strain and RH-ERP infected BMMs, + p-value < 0.01, Student's t-test between the two conditions indicated.

Localization of p-IκBα at the PVM is strain specific, independent of NF-κB activation and partially dependent on GRA2

In the canonical NF-κB activation pathway, IκBα normally inhibits NF-κB translocation and sequesters NF-κB in the cytoplasm. However, upon stimulation, IκBα is phosphorylated and subsequently targeted for proteasomal degradation via ubiquitination, exposing the nuclear localization signal in NF-κB and allowing nuclear localization to occur (Hayden, Ghosh 2008). It has been previously reported that phospho-IκBα (p-IκBα) is localized to the PVM in RH-ERP infected mouse embryonic fibroblasts (MEFs), and this has been linked to NF-κB activation (Molestina et al. 2003, Molestina, Sinai 2005). However, there was little or no p-IκBα localization at the PVM in cells infected with type II and type III strains (Rosowski et al. 2011). Moreover, localization of p-IκBα was observed using RH-ERP, and it is currently unknown whether the same phenomenon holds true for other type I strains.

Thus, to determine whether p-IκBα is also redirected to the PVM in cells infected with other type I strains, immunofluorescence (IF) with antibodies against p-IκBα was performed in HFFs infected with RH-ERP, RH-JSR and GT1. In agreement with previous studies, p-IκBα localized to the PVM of RH-ERP, but there was much less accumulation of p-IκBα at the PVM in HFFs

infected with other type I strains (**Figure 5A**). Quantification of the intensity of p-IκBα around the PVM confirmed that vacuoles containing RH-ERP recruited significantly more p-IκBα than vacuoles with RH-JSR or GT1 (**Figure 5B**, *p*-value = 0.009 (RH-JSR) and 0.008 (GT1), Student's *t*-test). It is unlikely that NF-κB activation levels are correlated with the level of p-IκBα recruitment around the PVM, given previous data that type II strains activate NF-κB in infected host cells but do not induce accumulation of p-IκBα to the PVM (Rosowski et al. 2011). This is further supported by the observed differences in type I strains, since neither RH-ERP nor RH-JSR activate NF-κB (**Figure 4A**) but RH-ERP induces strong accumulation of p-IκBα at the PVM. In addition, localization of p-IκBα was similar in HFFs infected with RH-ERP+GRA15$_{GT1}$ compared to RH-ERP infected HFFs (data not shown), even though RH-ERP+GRA15$_{GT1}$ activates NF-κB, which further supports that NF-κB activation is unrelated to accumulation of p-IκBα on the PVM.

To determine whether accumulation of p-IκBα at the PVM could be due to a soluble factor secreted by RH-ERP, immunofluorescence was performed in cells co-infected with both RH-ERP and GT1. Recruitment of p-IκBα to the PVM was observed only in vacuoles containing RH-ERP (GFP^{+}), whereas little or no p-IκBα was recruited to the GT1 PVM (**Figure 5C**), implying that p-IκBα translocation is not induced by soluble secreted parasite factors. We also tested the effect of ROP16, a known secreted rhoptry kinase, on p-IκBα recruitment, and saw no observable differences between RH-ERP and RHΔ*rop16* (**Supplementary Figure 3**). We also observed that p-IκBα colocalized with GRA7 at PVM extensions (**Figure 5D**) where another dense granule protein, GRA14, has been described to be trafficked to (Rome et al. 2008). Furthermore, as mentioned before, GRA2 is known to affect the vesicular network in the PV and

PVM (Coppens et al. 2006, Travier et al. 2008) and we identified that RH-ERP GRA2 differs in one amino acid from RH-JSR/GT1 GRA2 (**Supplementary Table 1**).

Thus, we examined whether dense granule proteins had an effect on p-IκBα recruitment. Even though there were no observable differences in PVM localization of p-IκBα between RH-ERP and RHΔ*gra15* (**Supplementary Figure 3**), there was observed reduction of p-IκBα accumulation at the PVM in cells infected with RHΔ*gra2* (**Figure 5B**). Since the vacuoles formed by the RHΔ*gra2* exhibit disruptions in the intravacuolar network (Mercier et al. 2002), lack of p-IκBα recruitment to the PVM could be due to disruption of parasitophorous vacuole biogenesis. Alternatively, as GRA2 has been found in complexes with other dense granule proteins (Braun et al. 2008), these GRA proteins could be directly interacting with host IκBα.

In addition, complementation of RHΔ*gra2* with either RH-ERP GRA2 or GT1 GRA2 partially restored the p-IκBα localization phenotype, with increased PVM recruitment of p-IκBα present in both complemented strains compared to RHΔ*gra2* (**Supplementary Figure 4A**). Quantification of the intensity of p-IκBα around the PVM confirmed that both complemented strains did not have significantly different PVM recruitment of p-IκBα compared to RH-ERP (**Supplementary Figure 4B**, p-value = 0.45 (complemented RH-ERP GRA2) and p-value = 0.58 (complemented GT1 GRA2), Student's t-test). This suggests that although GRA2 expression is necessary for full accumulation of p-IκBα to the PVM, it is likely due to proper parasitophorous vacuole biogenesis rather than the specific polymorphism present in GRA2 between RH-ERP and GT1.

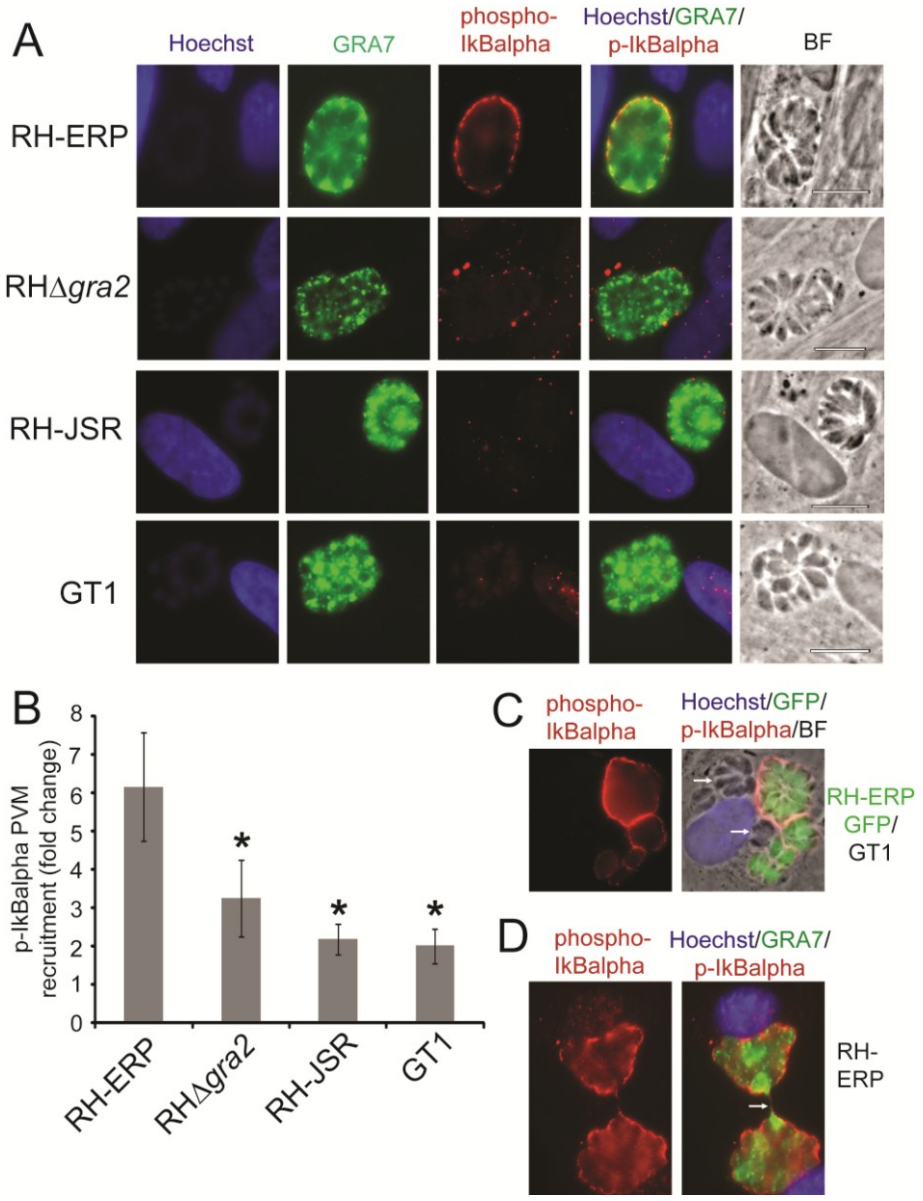


Figure 5. p-IκBα localizes to the parasitophorous vacuolar membrane of RH-ERP, but not RH-JSR or GT1, and is partially dependent on GRA2. (A) Human foreskin fibroblasts were infected with type I strains for 24 hours, fixed with formaldehyde and stained with p-IκBα (red), GRA7 (green) and Hoechst (blue). Pictures are representative of at least three experiments. (B) Quantification of p-IκBα recruited to the PVM of RH-ERP, RHΔ*gra2*, RH-JSR and GT1. The intensity of p-IκBα recruited to the PVM was quantified in at least 5 cells per condition. The graph shows the average from three independent experiments, with levels showing average p-IκBα recruitment quantification, and the error bars represent standard error. * p-value < 0.01, Student's t test. (C) HFFs were infected with RH-GFP and GT1 for 24 hours, fixed with formaldehyde and stained with p-IκBα (red), GRA7 (green) and Hoechst (blue). Arrows point to GT1 vacuoles. (D) Mouse embryonic fibroblasts were infected with RH-ERP for 30 hours, fixed with formaldehyde and stained with p-IκBα (red), GRA7 (green) and Hoechst (blue). Arrows point to the intervacuolar PVM extension.

Conclusions

In summary, through comparative genome and gene expression analysis, we identified a list of candidate genes that could be responsible for the phenotypic differences between different type I strains. We show that polymorphisms in *GRA2* and *GRA15* determine type I strain differences in survival in IFN-γ stimulated cells and activation of NF-κB, respectively. Future experiments will focus on the contribution of the individual genes to the increased growth rate, higher extracellular viability and loss of orally infectious cyst formation ability in RH-ERP. Thus, these identified parasite genes and differentially modulated host pathways could lead to design of new parasite targets relevant to *Toxoplasma* pathogenesis and chronic infection. However, it is likely that many of these phenotypes are affected by a combination of SNPs/indels and/or expression differences across multiple parasite genes, rather than being controlled by individual or a few genes.

Materials and Methods

Parasites and cells

Human foreskin fibroblasts (HFFs) were grown in DMEM (Invitrogen) supplemented with 10% heat inactivated FBS (PAA), 50 µg/ml each of penicillin and streptomycin, and 20 µg/ml gentamycin. A HEK293T stable reporter cell line with four copies of the NF-κB consensus transcriptional response element driving the expression of GFP and luciferase (System Biosciences) were grown in the same DMEM but supplemented with 1 mM sodium pyruvate, 100µM MEM nonessential amino acids and 10 mM HEPES. These NF-κB 293T reporter cells were passed every 2-4 days using 0.05% trypsin-EDTA (Rosowski et al. 2011). C57BL/6J mouse embryonic fibroblasts (MEFs) were a gift from A. Sinai (University of Kentucky College of Medicine, Lexington, KY) and grown in HFF media supplemented with 10 mM HEPES. Parasites were maintained *in vitro* by serial passage on monolayers of HFFs at 37ºC in 5% CO_2. RH-ERP is a clone of the original RH isolate, subjected to continuous passage *in vitro* until the time of present study. RH-JSR was a gift from David Sibley (Washington University in St. Louis, Saint Louis, MO) and is a noncloned line of the original RH isolate, subjected to propagation in mice and cryopreserved since 1988. GT1 was originally isolated from skeletal muscle of a goat in 1980, and was subject to passage in laboratory conditions (Khan et al. 2009).

Reagents

All tissue culture reagents were purchased from Gibco (Life Technologies Corporation, USA), unless otherwise stated. Antibodies against *Toxoplasma* dense granule protein GRA7 were kindly provided by John Boothroyd (Stanford University, Palo Alto, CA) (Dunn et al. 2008).

Anti-mouse p-IκBα (sc-8404), anti-human NF-κB p65 (sc-109) and anti-goat TGTP (sc-11079) antibodies were purchased from Santa Cruz Biotechnology (California, USA). Recombinant human TNFα was obtained from Invitrogen (Life Technologies Corporation, USA), and lipopolysaccharide was purchased from EMD Millipore (Merck KGaA, Darmstadt, Germany).

Generation of transgenic parasites

To generate RH-ERP (ΔHXGPRT) parasites expressing the GT1 allele of GRA15, the GRA15 coding region and putative promoter (1,940 bp upstream of the start codon) was amplified from GT1 genomic DNA by PCR (forward, 5'–CACCTTGACTGCCACGTGTAGTATCC–3', reverse, 5'-TTA*CGCGTAGTCCGGGACGTCGTACGGGTA*TGGAGTTACCGCTGATTGTGT–3'). Sequence coding for a C terminal HA tag was included in the reverse primer (denoted with italics). GRA15$_{GT1}$HA was then cloned into pENTRD/D-TOPO (Invitrogen), and into pTKO-att (Rosowski et al. 2011) through LR recombination (Invitrogen). The pTKO-att-GRA15$_{GT1}$HA vector was then linearized by digestion with XhoI (New England Biolabs, Inc.). Linearized vector was transfected into RH-ERPΔHXGPRT by electroporation as previously described (Rosowski et al. 2011). 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. Immunofluorescence was used to confirm expression of GRA15$_{GT1}$ via HA staining.

To generate RHΔ*gra2* parasites complemented with either RH-ERP GRA2 or GT1 GRA2, the GRA2 coding region and putative promoter (1,143 bp upstream of the start codon) was amplified from RH-ERP (ΔHXGPRT) and GT1 genomic DNA by PCR (forward, 5'-**GGGGACAACTTTTCTATACAAAGT**TGAGCATGTAGGTGGAACGC-3', reverse, *5′-*

TTA*CGCGTAGTCCGGGACGTCGTA*CGGGTACTGCGAAAAGTCTGGGAC-*3')*. Sequence coding for the attP4r recombination site was included in the forward primer and a C terminal HA tag was included in the reverse primer (attP4r in bold and HA tag in italics). A second PCR added the attP3 recombination site after the HA tag and the insert was cloned into pDONR 221 P4r-P3r (Invitrogen) using BP recombination (Invitrogen). The GRA2-HA insert was then flanked by the genomic DNA both upstream and downstream of the UPRT locus and inserted into the pTKO2 destination vector (Rosowski et al. 2011) by LR recombination (Invitrogen). pTKO2-GRA2HA was linearized by digestion with HindIII (New England Biolabs, Inc.) which does not cut within GRA2 and pTUB-CAT was digested with NotI (New England Biolabs, Inc.). pTKO2-GRA2HA and pTUB-CAT were co-transfected into RHΔ*gra2* as previously described (Rosowski et al. 2011). Stable integrants were selected by passage of 10^6 parasites every 2 days in 2 μM chloramphenicol and cloned by limiting dilution. Immunofluorescence and Western blot were used to confirm expression of RH-ERP GRA2 or GT1 GRA2 via HA staining.

Luciferase Assays

HEK293T NF-κB reporter cells were seeded at a density of 4 x 10^4 cells per well for 4 hours in a black 96 well clear bottom plate (Corning). Parasites were syringed lysed, washed once with PBS and three different multiplicity of infection (MOIs) per strain were used to infect reporter cells. As a positive control, recombinant human TNFα was used at 20ng/μl to stimulate uninfected cells at the same time as infection. After 24 hours of infection, uninfected, stimulated and infected cells were lysed using Cell Culture Lysis reagent, and luciferase activity in lysates was measured according to manufacturer's protocol (Promega). Data from cells infected with similar MOIs, as determined by plaque assay, were used.

In vitro cytokine ELISAs

C57BL/6 bone marrow-derived macrophages (BMMs) were isolated as described (Jensen et al. 2011), and plated in DMEM, supplemented with 20% L929 supernatants, two days before infection. Parasites were syringe lysed, washed once with PBS and three different MOIs per strain were used to infect uninfected macrophages. As a positive control, purified lipopolysaccharide (100ng/mL) was used to stimulate uninfected BMMs 3 hours before supernatants were collected. After 24 hours of infection, supernatants from uninfected, stimulated and infected cells were collected and stored at -80ºC until ELISAs were performed. IL-12/23p40 and CCL2/MCP-1 levels in culture supernatants were determined using commercially available ELISA kits (ELISA DuoSet®, R&D Biosystems), according to the manufacturer's instructions.

Immunofluorescence

Irgb6 staining in MEFs were performed as previously described (Niedelman et al. 2012). Percent Irgb6 coating was determined in a blind fashion by finding intracellular parasites and then scoring Irgb6 coating as positive or negative. For p65 and p-IκBα staining, HFFs were plated on coverslips in 24 well plates until confluent, and subsequently infected with parasites for different timepoints. HFFs were then fixed with 3% (vol/vol) formaldehyde in PBS for 20 min at room temperature, permeabilized with 0.2% (vol/vol) Triton X-100 in PBS, and blocked in PBS with 3% (wt/vol) BSA and 5% (vol/vol) goat serum. Coverslips were incubated with primary antibodies overnight at 4ºC, and fluorescent secondary antibodies, coupled with Alexa Fluor 488 or 594 (Invitrogen), 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 p65 nuclear localization was performed as previously described (Rosowski et al. 2011). Quantification of p-IκBα localized to the PVM was performed using NIS-Elements software (Nikon). Parasitophorous vacuoles (PVs) were chosen at random through GRA7 staining. The intensity of fluorescent p- IκBα was measured by drawing two lines at right angles across the long and short axes of the vacuoles, and intensity profiles were obtained for each line. The fold change intensity of each line was taken by dividing the highest peak value where each line crossed the margins of the PVM by the lowest value for each line (taken as background). The signal intensity for each vacuole was given as the mean of the two intensity fold changes obtained per vacuole.

Plaque Assays

Plaque assays were set up as previously described (Niedelman et al. 2012). Briefly, MEFs were seeded the day before, and stimulated with 1000 U/ml mouse IFNγ or left unstimulated before infection in a 24 well plate. Plaques were then counted in unstimulated and stimulated MEFs after incubation for 4-7 days at 37 °C.

SNP and INDEL list generation between RH-ERP and GT1

To reduce the possibility of sequence errors and cell line specific mutations, genomic DNA from two related RH-ERP lines, 2F-1-YFP2 parent and an F-P2 ENU mutagenized child, were prepared and sequenced (Farrell et al. 2012). Illumina sequencing produced 40,495,290 and 43,514,016 reads for the parent and F-P2 samples respectively. The FASTQ sequence traces were aligned to a FASTA reference containing both *Toxoplasma gondii* GT1 genomic reference v5.0 and the Human genome reference build 37. Reads were aligned to the GT1 genome as well as the Human genome to filter out any possible human contamination. MOSAIK v1.0 was used to perform the alignments using the standard parameters. Greater than 90% of the reads aligned for each sample, with rougly half of the reads filtered out as human contamination. Variants were called using the variant caller FreeBayes (E., G. 2012) using standard parameters, software version 0.7.2. Varations were then filtered to identify variants that were called in both parental and F-P2 samples at an allele balance greater than 75%.

RH-ERP and GT1 gene expression via RNA-seq

RNA was isolated from C57BL/6 bone marrow derived macrophages infected with RH-ERP and GT1. These were processed and sequenced as described previously (Minot et al. 2012). Reads were then mapped to the GT1 genome (ToxoDB.org).

Toxoplasma microarray analysis

For *Toxoplasma* arrays, image analysis files (.CEL) files from published microarray data from HFFs infected with RH-ERP, RH-JSR or GT1 were downloaded from the Gene Expression

Omnibus (GEO) database (Series GSE16115 and GSE22315). In addition, independent *Toxoplasma* arrays were performed on total RNA isolated from HFFs infected for 24 hours with RH-ERP, RH-JSR and GT1 (Series GSE44191). Each sample was hybridized to the *T. gondii* Affymetrix microarray (Bahl et al. 2010). The image analysis files from GSE16115, GSE22315 and GSE44191 were all processed together using the Expression Console Software, normalized using Robust Multi Array (RMA) algorithm, and all background values less than 6.5 were set to 6.5. These arrays were then imported into Multi-Experiment Viewer (MeV) (Saeed et al. 2003), all genes were median-centered and loaded into Genomica (Segal et al. 2004) as the array dataset.

To create Genomica and GSEA custom gene sets to determine biological function enrichment (Segal et al. 2004, Subramanian et al. 2005), we downloaded the Gene Ontology (GO) and InterPro (IP) annotations for *Toxoplasma* genes using the ME49 v8 reference genome (ToxoDB.org). To determine functional enrichment in groups of genes with similar annotations (gene sets), gene sets were loaded into Genomica (Segal et al. 2004) and selected as gene sets to analyze. The default parameters were used to run the hypergeometric enrichment analysis (the complete set of *Toxoplasma* gene IDs and their associated functional assignments in Genomica or GSEA format is available from the authors upon request).

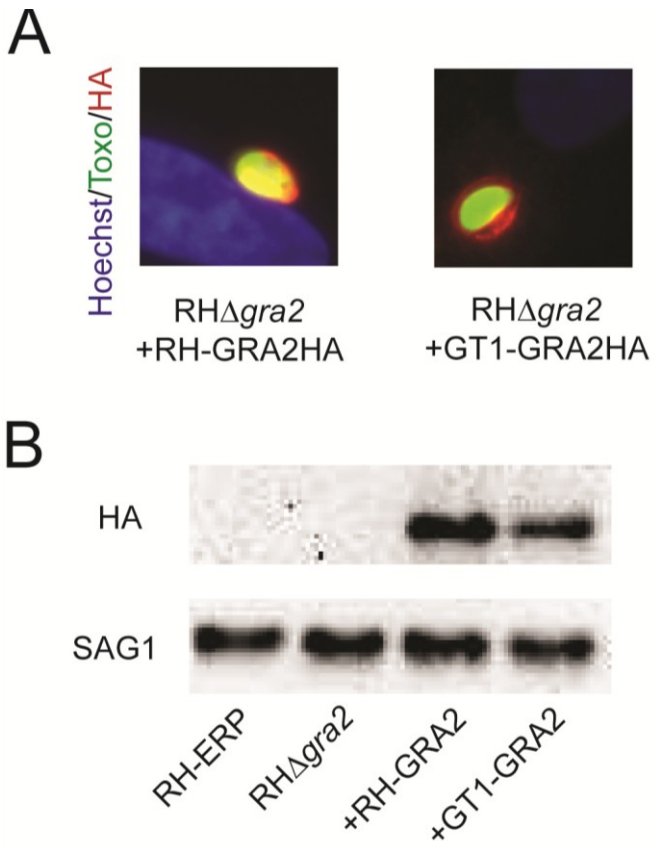
Human Microarray analysis

HFFs were grown in 6 well plates until confluency was reached. Parasites were syringe lysed and washed once with PBS. HFFs were infected with RH-ERP, RH-JSR and GT1 at three different MOIs for 24 hours. Plaque assays were done to assess viability of parasites and infections with

similar MOIs were chosen. Three biological replicates were done for RH-ERP and RH-JSR while two biological replicates were done for GT1. TRIzol reagent (Invitrogen) was used to isolate total RNA according to the manufacturer's instructions and RNA was cleaned up using MiniElute kit (Qiagen). RNA was labeled and hybridized to human Affymetrix arrays (Human U133A 2.0) according to manufacturer's protocol. Probe intensities were measured with the Affymetrix GeneChip Scanner 3000 7G and were processed into image analysis (.CEL) files with Expression Console Software (Affymetrix), which can be accessed through GEO (GSE44189). Intensity values were normalized using RMA through Expression Console, and all background values less than 6.5 were set to 6.5.

GSEA was used to find candidate transcription factors and canonical pathways that were modulated differently between the type I *Toxoplasma* strains (Subramanian et al. 2005). Both transcription factor and canonical pathway gene sets from the Molecular Signatures Database (MSigDB) were used to determine enrichment (c2.cp. symbols, c3.tft. symbols), using default parameters except the range of set size, which was changed to a minimum of 5 and maximum of 3000. Analysis of distant regulatory elements of coregulated genes (DiRE, http://dire.dcode.org) (Gotea, Ovcharenko 2008) was performed using a random set of 5000 background genes and using target elements of top 3 evolutionary conserved regions (ECRs) and promoter ECRs. For every gene in a list, DiRE detects regulatory elements throughout the entire gene locus and looks for enrichment of transcription factor binding sites.

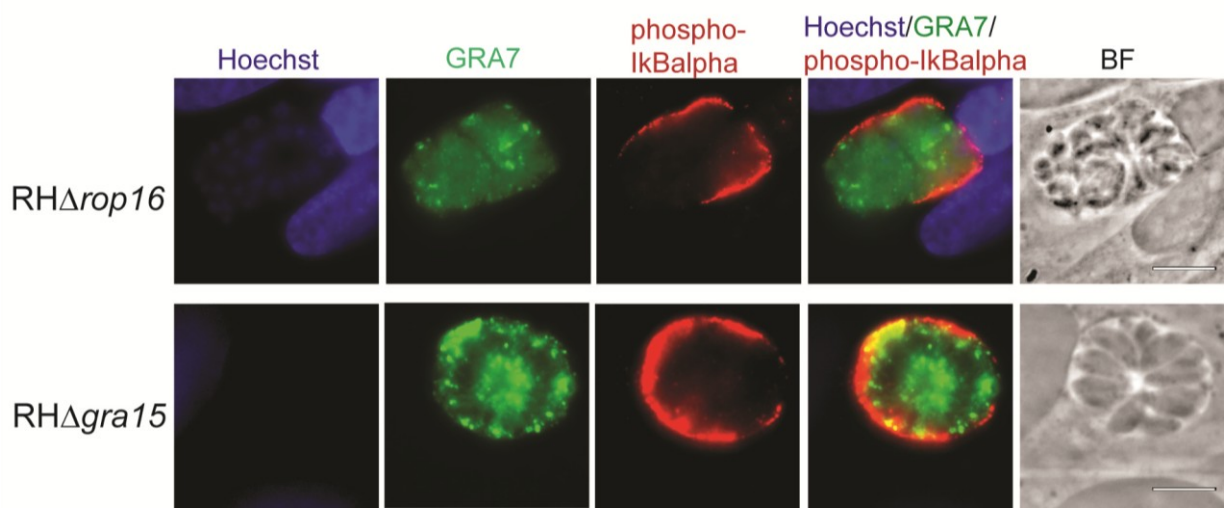
Supplemental Figures



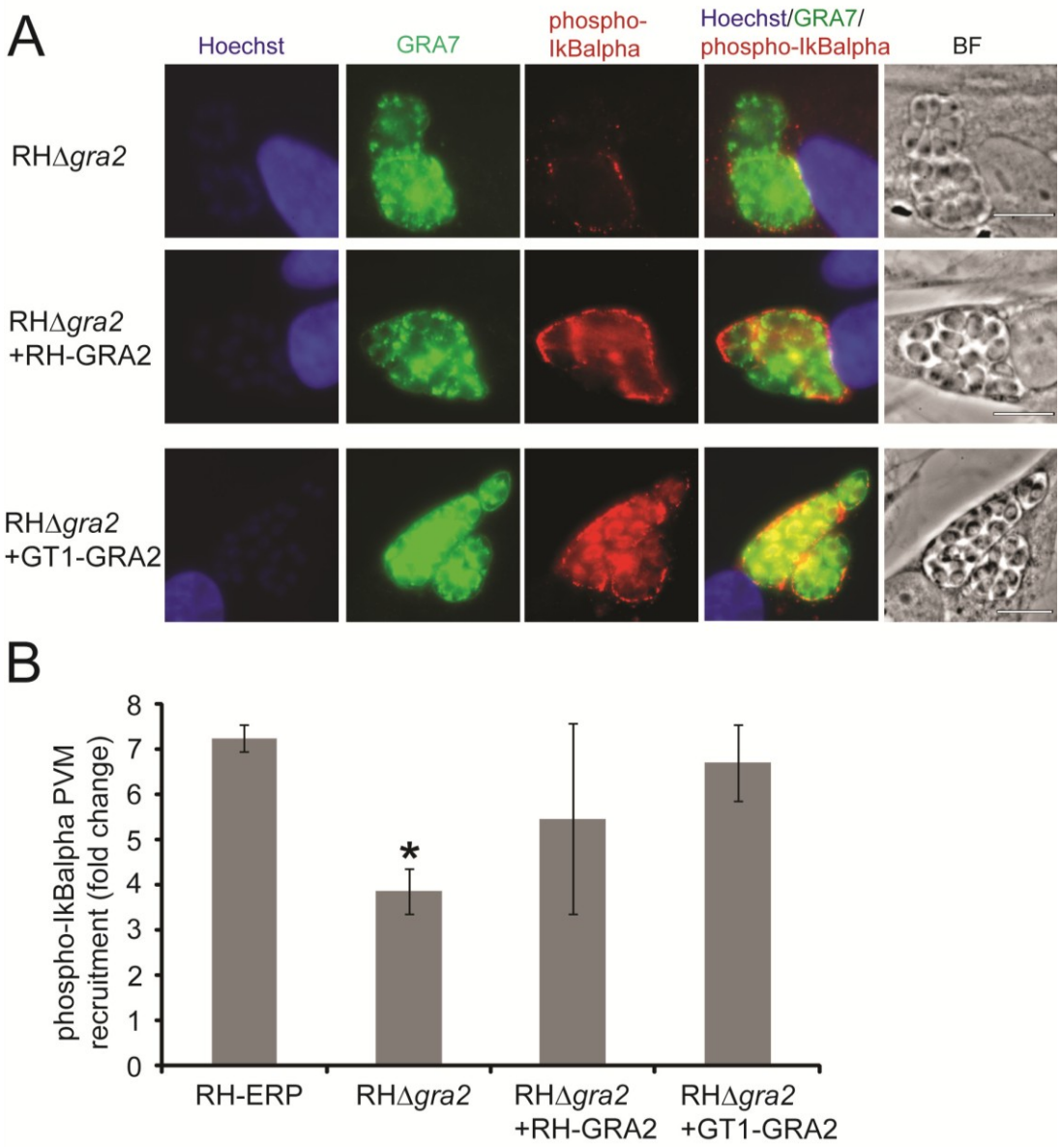
Supplementary Figure 1. Expression and localization of GRA2 in the RHΔ*gra2* complemented strains. (A) Immunofluorescence of HA (red) in RHΔ*gra2* complemented with RH-ERP-GRA2-HA or GT1-GRA2-HA parasites, co-stained with Hoechst (blue) and TdTomato (green). (B) Western blot for HA (top) and SAG1 (bottom) comparing HA expression of RHΔ*gra2* complemented with RH-ERP-GRA2-HA or GT1-GRA2-HA.

```
GT1_GRA15       MVTTTTPTPP PGAPAVVPIF DVVYQLNPHV FRSRFSRRNR ARRVVSSKSR SIIRWLGYLT
RH-ERP_GRA15    .......... .......... .......... .......... .......... ..........
RH-JSR_GRA15    .......... .......... .......... .......... .......... ..........

GT1_GRA15       VLAAVILLGA YAVRRLSRDL SDSVRETRRG RRITGSVPPG TTRPRSESCT GTQVDGGCGA
RH-ERP_GRA15    .......... .......... .......... .......... .......... ..........
RH-JSR_GRA15    .......... .......... .......... .......... .......... ..........

GT1_GRA15       DTSTDGKSES EQTENGEDSR FSTRTPIHVT ASTSPFATRK AAEERSSSPR DRKVPEGAQL
RH-ERP_GRA15    .......... .......... .......... .......... .......... ..........
RH-JSR_GRA15    .......... .......... .......... .......... .......... ..........

GT1_GRA15       PTSSTPHAQR KDSGSDSRNP STLIPSPGTN TFNMNFYIIG AGSSALDFIF PHTPDAQATV
RH-ERP_GRA15    .......... .......... .......... .......... .......... ..........
RH-JSR_GRA15    .......... .......... .......... .......... .......... ..........

GT1_GRA15       VSPPRSAAAA PTVETVPRVR TYSTPTTLTL PTAPATATSN HMHASATPSP PERPQNFR.G
RH-ERP_GRA15    .......... .......... .......... .......... .......... RNVLKTSVGD
RH-JSR_GRA15    ....PQRGGR TNCRNSSKGS HLLDTNNINP TNGTSDRH-- ---------- ----------

GT1_GRA15       LMRQNGMVE. TSLTTTEAGM PAPLQSPQHI ETEARLTYSN HLKSPHTPET PTVHSIDPVV
RH-ERP_GRA15    SCGKTAWLRG HR-------- ---------- ---------- ---------- ----------
RH-JSR_GRA15    ---------- ---------- ---------- ---------- ---------- ----------

GT1_GRA15       GTSGHSVAVG SQSPAGGPPT DSRTPAALTP TSSSFSHADS LETSEHPQSG PSLHPLISGI
RH-ERP_GRA15    ---------- ---------- ---------- ---------- ---------- ----------
RH-JSR_GRA15    ---------- ---------- ---------- ---------- ---------- ----------

GT1_GRA15       QDAVQSQLPL SQQETLPVVE NATFFGPQQT PPWMDETAAA AIPLAPSQPG SRTQPISSPH
RH-ERP_GRA15    ---------- ---------- ---------- ---------- ---------- ----------
RH-JSR_GRA15    ---------- ---------- ---------- ---------- ---------- ----------

GT1_GRA15       TLLPLSGGVS AVPGPPRTEN PRQPQVPGEN SYYSVPTEPY PAQDMSPLIR GTHSQTETVE
RH-ERP_GRA15    ---------- ---------- ---------- ---------- ---------- ----------
RH-JSR_GRA15    ---------- ---------- ---------- ---------- ---------- ----------

GT1_GRA15       CGVNASSEGL AAGAPSSKSA ENAQTGQGAG KSLLPVFLHP QEQSPHSMPT LGAGRFGSGE
RH-ERP_GRA15    ---------- ---------- ---------- ---------- ---------- ----------
RH-JSR_GRA15    ---------- ---------- ---------- ---------- ---------- ----------

GT1_GRA15       LQRTISDPGP QRAGATQADG IGAGGPRDTQ SAVTP-
RH-ERP_GRA15    ---------- ---------- ---------- ------
RH-JSR_GRA15    ---------- ---------- ---------- ------
```

Supplementary Figure 2. Differences in GRA15 sequence between RH-ERP, RH-JSR and GT1. RH-JSR and RH-ERP contain indels at position 734 and 872 respectively, which lead to independent frameshifts and early stop codons in the GRA15 protein. Dots represent consensus with the GT1 GRA15 sequence, dashes represent missing amino acids in RH-ERP or RH-JSR GRA15 compared to GT1 GRA15, red indicate amino acids different in RH-ERP from GT1 and purple indicate amino acids different in RH-JSR from GT1.



Supplementary Figure 3. No differences in PVM localization of p-IκBα present between RH-ERP, RHΔ*rop16* and RHΔ*gra15*. Human foreskin fibroblasts were infected with RH-ERP and knockout strains at intended MOI 1 for 30 hours, fixed with formaldehyde and stained with p-IκBα (red), GRA7 (green) and Hoechst (blue). Pictures are representative of at least two experiments.



Supplementary Figure 4. Partial restoration in PVM localization of p-IκBα present in RHΔGRA2 complemented with either RH-ERP GRA2 or GT1 GRA2. (A) Human foreskin fibroblasts were infected with RHΔ*gra2*, RHΔ*gra2* complemented with either RH-ERP GRA2 or GT1 GRA2 for 30 hours, fixed with methanol and stained with p-IκBα (red), GRA7 (green) and Hoechst (blue). Pictures are representative of three experiments. (B) Quantification of p-IκBα recruited to the PVM of RH-ERP, RHΔ*gra2* and RHΔ*gra2* complemented with either RH-ERP GRA2 or GT1 GRA2. The intensity of p-IκBα recruited to the PVM was quantified in at least 5 cells per condition. The graph shows the average from three independent experiments, with levels showing average p-IκBα recruitment quantification, and the error bars represent standard error. * p-value < 0.01, Student's t test.

Acknowledgements

This work was supported by National Institutes of Health grants AI080621 to JPJS and AI081220 to MJG and GTM. NY is supported by an A*STAR NSS (PhD) graduate scholarship, WN and DL are supported by the pre-doctoral grant in the Biological Sciences (5-T32-GM007287-33). MM was supported by the Knights Templar Eye Foundation postdoctoral fellowship. The authors wish to thank David Sibley for the gift of RH-JSR strain, the MIT BioMicroCentre and Manlin Luo for help with the microarrays and members of the Saeij lab for helpful comments and technical assistance.

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Chapter Two - Addendum

Results and discussion

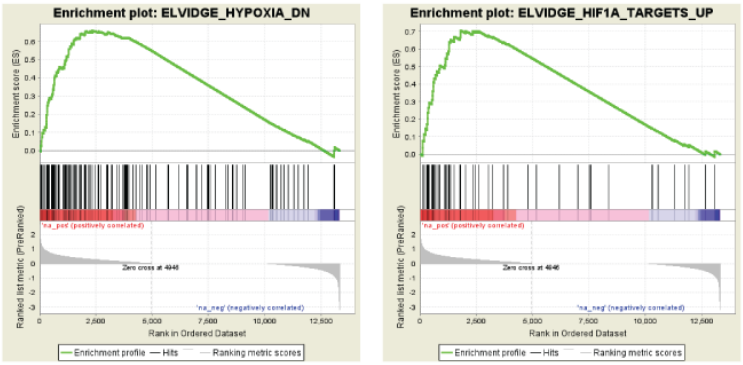
RH-ERP does not activate the hypoxia or serum response pathways

In Chapter II, we identified that the NF-κB transcription factor pathway is differentially regulated in HFFs infected with RH-ERP compared to infection with GT1. The same analyses also indicated that hypoxia inducible factor-1 (HIF-1) transcription factor pathway was enriched in host genes which were differentially expressed between HFFs infected with RH-ERP compared to infection with RH-JSR (**Figure 1A**). Indeed, it has been previously reported that *Toxoplasma* infection, specifically using the RH-ERP strain, causes HIF-1 activation upon HIF-1 reporter transient transfections of human and mouse cell lines (Spear et al. 2006). However, strain differences in activation of HIF-1 were not investigated during this study.

In addition, there was enrichment in the serum response factor (SRF) in host genes differentially expressed between HFFs infected with RH-ERP or RH-JSR (**Figure 1B**). Moreover, previous studies showed that *Toxoplasma* activates the serum response, upon SRF reporter transient transfections of mouse cell ines, which is dependent on active invasion by *Toxoplasma*. Interestingly, SRF activation was highest in RH-ERP infection, as compared to GT1 (another type I strain), or other canonical strains (type II or III strains) (Wiley et al. 2011).

A

GSEA enrichment of host genes differentially expressed between RH-ERP and RH-JSR infected HFFs



B

Consistently upregulated host genes in RH-ERP infected HFFs compared to RH-JSR

Transcription factor	Importance
BACH2	0.62
LBP1	0.19
NFKAPPAB	0.18
STAT6	0.15
TEL2	0.14
SRF	0.14

Figure 1. Differential host gene expression between host cells infected with RH-ERP or RH-JSR. (A) The GSEA diagrams show the enrichment score (green line), which reflects the degree to which that particular gene set (header above) is overrepresented in the differentially expressed host genes between host cells infected with RH-ERP or RH-JSR (ranked by their differential expression values). The middle portion of the diagram shows where the members of the particular gene set appear in the ranked gene list. The bottom portion of the diagram shows the value of the ranking metric, which measures the correlation of a gene with upregulation (positive value) or downregulation (negative value) in RH-ERP compared to RH-JSR. (B) Top six enriched known transcription factors from DiRE analysis of consistently upregulated host genes in RH-ERP infection compared to RH-JSR.

Both the hypoxia and serum response pathways are likely to be important in the host response to *Toxoplasma*. There is crosstalk between the hypoxia pathway and NF-κB, and HIF-1 has strong effects on inflammatory and immune responses (Cramer et al. 2003), while the serum response pathway regulates the levels of early response genes which are involved in host immune responses (Gomez-Martin et al. 2010). Thus, we wanted to validate our transcription factor

analysis by examining type I strain differences in activating HIF and SRF. Moreover, previous reports showed transcription factor activation through HIF and SRF reporter transient transfections, but did not show activation using stable reporter cell lines, which are integrated into the genome and influenced by higher order gene regulation (such as chromatin environment). Therefore, we constructed three different HEK293 reporter cell lines, namely HIF-1, SRF and CREB, with consensus transcription factor binding sites driving the expression of GFP and luciferase. To test the specificity of these cell lines, we treated these reporter cell lines with DMOG (dimethyloxallyl glycine), a stabilizer of HIF-1, PMA (phorbol myristate acetate), a serum response activator, and forskolin, which raises cAMP levels and activates CREB. Treatment with DMOG specifically activated the HIF-1 reporter cell line, whereas treatment with PMA activated both the SRF and CREB reporter cell line (**Figure 2A**).

We then infected the HIF-1 reporter cell line with RH-ERP, but unlike previous reports, we observed that RH-ERP infection did not cause HIF-1 activation even at high multiplicities of infection, though the HIF reporter was still functionally responsive with DMOG (**Figure 2B**). As we were not able to validate our transcriptional analysis that RH induces HIF-1, we did not investigate further into strain differences in HIF-1 activation. In addition, we wanted to investigate whether there were type I strain differences in the serum response, as noted in our transcriptional profiling analysis. However, we observed that despite a functional response with PMA, we did not observe SRF activation by RH-ERP with the SRF reporter cell line, and there were no differences upon infection with other type I strains (RH-JSR and GT1) (**Figure 2C**). It is possible that the difference in HIF and SRF activation due to RH-ERP infection between our observations and previous reports could be due to differences in binding site context, where

reporter cell lines have the binding sites integrated into the genome in comparison to transient transfections.

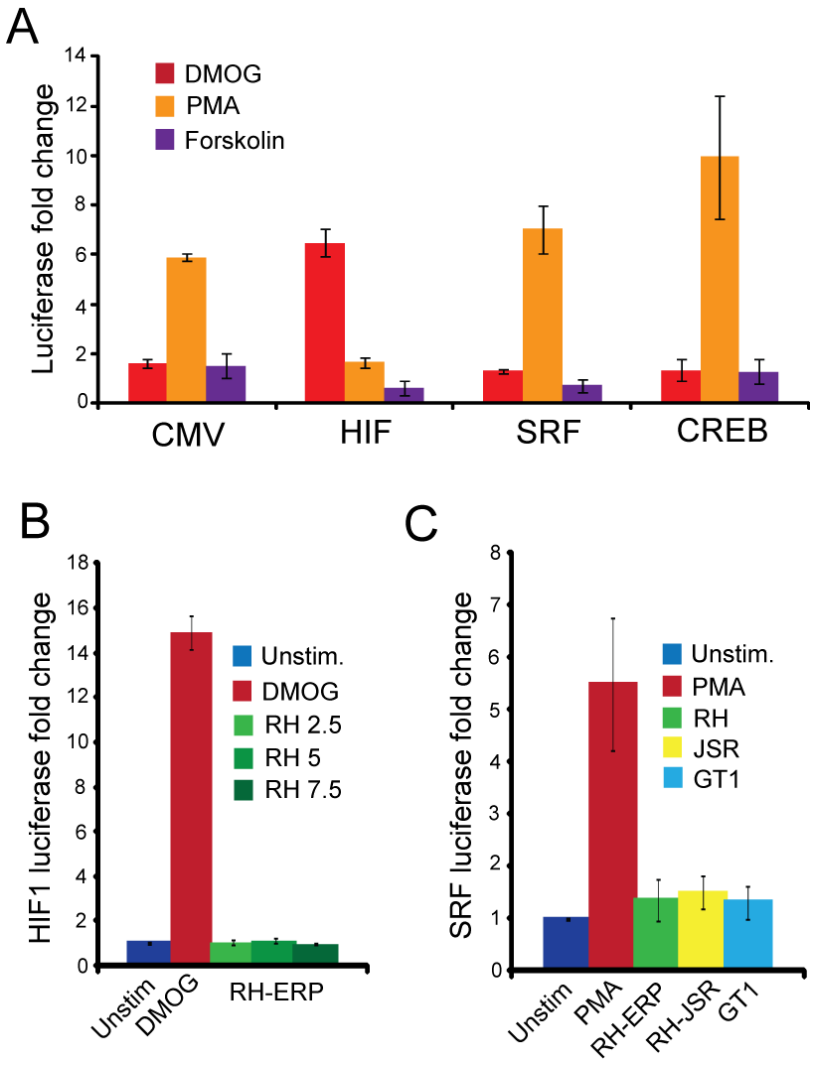


Figure 2. RH-ERP does not activate HIF or SRF reporter cell lines. (A) HEK293 HIF-1, CREB and SRF luciferase reporter cell lines were left unstimulated or stimulated with DMOG (1mM), PMA (10ng/mL) or forskolin (100µM). Cells were lysed 24 hours later and luciferase activity measured. Average luciferase induction was normalized to unstimulated cells, error bars represent standard deviation of technical replicates. (B) HEK293 HIF1 reporter were stimulated with DMOG or infected with RHΔ*hxgprt* at increasing MOIs. Cells were lysed 24 hours later and luciferase activity measured. Average luciferase induction was normalized to unstimulated cells, and results are from three independent experiments. Error bars represent standard deviation. (C) HEK293 SRF reporter cells were stimulated with PMA or infected with RHΔ*hxgprt*, RH-JSR, or GT1 at varying MOIs. Cells were lysed 24 hours later and luciferase activity measured. Average luciferase induction was normalized to unstimulated cells, and results are from at least four independent experiments. Error bars represent standard deviation.

Polymorphisms between RH-ERP and GT1 in ATP dependent RNA helicase (TGGT1_081400) do not affect parasite growth

From our nonsynonymous SNP analysis between RH-ERP and GT1 (Chapter II), we identified an ATP dependent RNA helicase which contains two non-conservative amino acid changes and is highly expressed in RH-ERP and GT1. Therefore, we wanted to investigate whether this particular ATP dependent RNA helicase could affect parasite growth, as RH-ERP grows faster than GT1 (Khan et al. 2009). We undertook allelic replacement of this ATP dependent RNA helicase from GT1 into an RH-ERP background, and confirmed the SNPs present between RH-ERP and GT1 (data not shown). We observed localization to the parasite cytosol of RH-ERP with either the endogenous copy of TGGT1_081400 or the GT1 version of TGGT1_081400 (**Figure 3A**). We then hypothesized that RH-ERP endotagged with the GT1 helicase would have decreased parasite growth compared to RH-ERP wild type, and assayed growth through parasites per vacuole counts and plaque size. We did not observe any significant differences in parasites per vacuole counts between the RH-ERP wild type strain and the GT1 endotagged mutant (**Figure 3B**). Moreover, there were no significant differences in plaque size between RH-ERP wild type and GT1 endotagged mutant (**Figure 3C**). It is likely that the growth difference between RH and GT1 is influenced by multiple polymorphic *Toxoplasma* genes with additive effects and the effect of one gene is difficult to observe. Moreover, there could be redundancy in function between *Toxoplasma* genes, as has been observed in the moving junction required for parasite invasion, where disruption of AMA1 leads to upregulation of AMA1 and RON5 homologues (Lamarque et al. 2014).

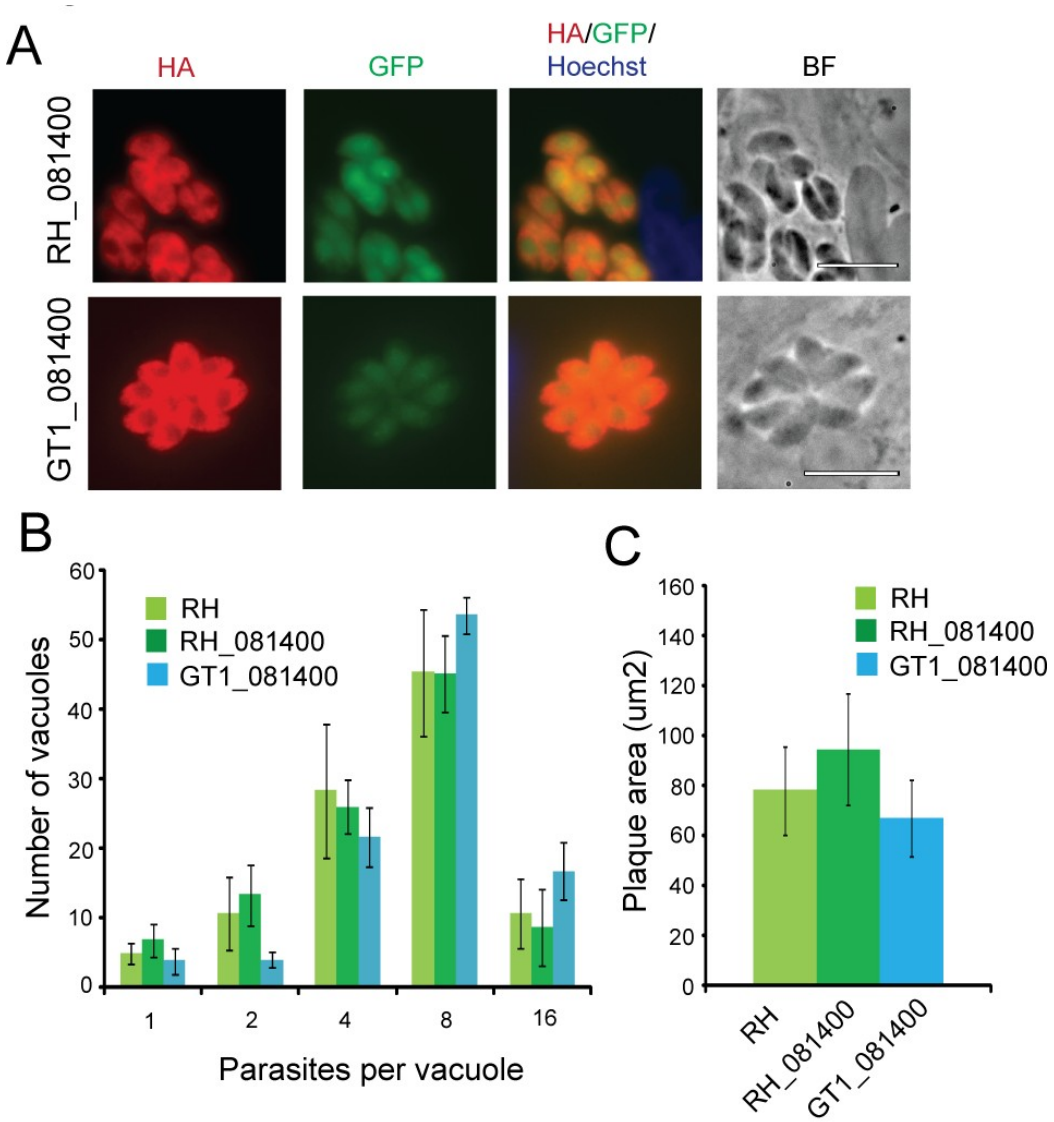


Figure 3. Polymorphic ATP helicase (TGGT1_081400) does not affect parasite growth in RH-ERP. (A) Human foreskin fibroblasts were infected with RHΔ*hxgprt*Δ*ku80* endogenously tagged with either RH ATP helicase-HA (RH_081400, top panel) or GT1 ATP helicase-HA (GT1_081400, bottom panel), for 24 hours, fixed with formaldehyde and stained with HA (red), Hoechst (blue) and GFP (green). (B) HFFs were infected with RHΔ*hxgprt*Δ*ku80* (RH), RH_081400 or GT1_081400, for 24 hours, fixed with formaldehyde. 100 vacuoles were counted per strain, and total number of parasites per vacuole counted. Results are from three independent experiments, and error bars represent SEM. (C) A hundred parasites (RH, RH_081400 and GT1_081400) were added to confluent HFFs in 24 well plates, and incubated at 37°C for 5 days. The size of at least 20 plaques per strain was measured, and results are averages from four independent experiments. Error bars represent SEM.

Materials and Methods

Reporter cell lines construction

HIF1 (TR026PA-1), SRF (TR029PA-1) and CREB (TR202PA-1) pGreenFire1 (pGF1) lentiviral reporter vectors containing a Neo selection cassette and a minimal CMV promoter followed by four tandem consensus transcription factor binding sites driving the expression of Firefly luciferase was purchased from System Biosciences. Each individual vector was co-transfected using FuGene 6 reagent (Roche) with vectors containing gag, pol and VSV-G proteins, according to manufacturer's instructions. Supernatant containing virus was collected three days after transfection, and added to HEK293 cells (ATCC) with polybrene (Sigma). Geneticin (Invitrogen) was used at 750 µg/ml to select HEK293 cells with the integrated constructs, and were subsequently cloned by limiting dilution. Dimethyloxallyl Glycine (DMOG, Enzol Life Sciences) was used at 1mM to test the HIF1 reporter, phorbol 12-myristate 13-acetate (PMA, Enzol Life Sciences) was used at 10ng/mL to test the SRF reporter, and forskolin (Cayman Chemical) was used at 100µM to test the CREB reporter.

Luciferase assay

HEK293 pGF1-HIF1, pGF1-SRF and pGF1-CREB cells were plated in 96 well plates (Corning) at 4*10^4 cells per well, and grown for 16 hours. Cells were then stimulated with either inducing chemicals for 24 hours or infected with RHΔ*hxgprt*, RH-JSR or GT1 at varying MOIs for 24 hours. Cells were lysed with 40µl of cell culture lysis reagent (Promega) containing 1x protease inhibitor (Roche), and stored at -80°C. 100µl of luciferase assay substrate (Promega) was added

per well, and luciferase readings read using Varioskan flash reader (Roche). Luciferase readings were normalized to unstimulated, uninfected cells.

Generation of ATP dependent RNA helicase endotagged parasites

Genomic sequences were obtained from the ToxoDB database. Primers were designed to amplify 1,593bp upstream of predicted ATG start site (5' – **CACC**CAGTTAACCGGTGGCAAAGT – 3'), and contained the 5'-CACC-3' sequence required to perform the directional TOPO cloning in pENTR/D-TOPO (Invitrogen, USA), together with the reverse primer (5' – TTA*CGCGTAGTCCGGGACGTCGTACGGGTA*CCACGCATCCATGCCCCCA – 3') containing the hemagglutinin (HA; in italics) sequence followed by the stop codon. These primers were used to amplify TGGT1_081400 from RHΔ*hxgprt* and GT1 genomic DNA, cloned into pTKOatt using Gateway Recombination Cloning Technology (Invitrogen, USA). The vectors were linearized with PpuMI, which cuts in a unique restriction site downstream of ATG site within the cloned fragment. Linearized vector was transfected into RHΔ*hxgprt*Δ*ku80* parasites by electroporation. Electroporation was performed in a 2 mm cuvette (Bio-Rad Laboratories, USA) with ATP (2mM, MP Biomedicals) and glutathione (5mM, EMD) in a Gene Pulser Xcell (Bio-Rad) at 25uFD, 1250V, ∞Ω. Stable integrants were selected in media containing mycophenolic acid (50µg/ml) and xanthine (50µg/ml).

Parasites per vacuole count

Parasites were added to confluent HFFs in 24 well plate coverslips at varying MOIs, and after 24 hour infection, fixed with 3% (v/v) formaldehyde in PBS for 20 minutes at room temperature, blocked with PBS with 3% (w/v) BSA and 5% (v/v) goat serum. Coverslips were incubated with

anti-HA (Roche, 1:500) at 4°C overnight, and fluorescent secondary and Hoechst were used for HA and DNA visualization respectively. 100 vacuoles per condition were chosen at random, and the number of parasites per vacuole recorded.

Plaque assay

HFFs were plated to confluency in 24 well plates, one hundred parasites per well were added and incubated for 5 days at 37°C. 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 fluorescent microscope (model eclipse Ti-S; Nikon).

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Chapter Three - Characterizing the mechanism of NF-κB activation by dense granule protein GRA15

Abstract

Toxoplasma gondii has effector proteins localized in specialized subcellular organelles, the rhoptries and dense granules, to modulate host cell processes. Dense granule protein GRA15 leads to activation of the nuclear factor kappa B (NF-κB) pathway, which plays an important role in innate immunity and inflammation. However, how GRA15 activates the NF-κB pathway and the GRA15 regions required for NF-κB activation remain unknown. We find that both type II and type III GRA15 are sufficient to activate NF-κB when ectopically expressed. Using co-immunoprecipitation, we identified candidate host proteins that interact with GRA15, including TNF receptor associated factor 2 (TRAF2), which is an adaptor protein functioning upstream of the NF-κB transcription factor. We also refined the GRA15 region necessary for NF-κB activation through truncation mutant function analysis. Understanding the mechanism for GRA15 dependent NF-κB activation will shed light on differential NF-κB activation by different *Toxoplasma* strains, which in turn leads to varying host immune responses and disease outcomes upon infection.

Introduction

During the *Toxoplasma* life cycle, it needs to maintain a balance between immune evasion, to enable dissemination, and immune activation, to induce chronic infections. To modulate the host immune response, *Toxoplasma* makes use of specialized effector proteins from subcellular organelles known as the rhoptries and dense granules. Rhoptry kinases/pseudokinases were first identified to play major roles in mouse virulence and modulation of host gene expression. However, dense granule proteins are increasingly characterized as key immune modulators, such as GRA15 activating the NF-κB pathway (Rosowski et al. 2011), GRA16 positively regulating the p53 cell cycle response (Bougdour et al. 2013) and GRA24 activating the p38 mitogen activated protein kinase (MAPK) pathway (Braun et al. 2013).

The p38 MAP kinase and NF-κB pathways have well established roles in inflammation and immunity. The p38 MAPK pathway is involved in inducing production of proinflammatory cytokines, such as tumor necrosis factor alpha (TNF-alpha) and IL-1 (Arthur, Ley 2013). The NF-κB pathway consists of the canonical and alternative pathways, with the former involved in innate, inflammatory and adaptive immune responses while the latter is involved in regulation of lymphoid organ development, B cell function and adaptive immunity (Hacker, Tseng & Karin 2011). Most activating stimuli act through the canonical pathway, leading to activation of NF-κB dimers such as c-REL, Rel-A (p65) or p50, whereas the alternative pathway is induced by a much smaller subset of stimuli, and leads to activation of p52/RelB NF-κB complexes (Hayden, Ghosh 2008).

The TNF receptor associated factor (TRAF) proteins are key intermediates in the NF-κB pathway (**Chapter I, Figure 3)**, and are typified by the C-terminal TRAF domain, which consists of a coiled-coil domain mediating protein-protein interactions, and the N-terminal zinc finger domains and RING finger motif (Xie 2013). There are six well characterized TRAF members, which can transduce activating signals towards either the canonical or the alternative NF-κB pathways, through the assembly of signaling complexes at the intracellular domains of transmembrane receptors (Hacker, Tseng & Karin 2011). Moreover, TRAFs belong to the family of E3 ubiquitin ligases that target downstream substrates for polyubiquitylation and control downstream protein-protein interactions (Deshaies, Joazeiro 2009).

The canonical NF-κB pathway is regulated by TRAF2 and TRAF6, whereas the alternative NF-κB pathway is regulated by TRAF2 and TRAF3. Upon classical activating stimuli such as tumor necrosis factor alpha (TNF-α), TRAF2, an E3 ubiquitin ligase, becomes polyubiquitinated and activates a downstream kinase, RIPK, to cause activation of the IKK complex (Verstrepen et al. 2008). Other canonical activating stimuli, such as lipopolysaccharide (LPS), causes oligomerization of TRAF6 and activation of its E3 ligase activity, which leads to recruitment and activation of TAK1, a downstream kinase. TAK1 then binds to the regulatory subunit of the IKK complex to activate IKKα/β, causing phosphorylation of IκBα, and allowing nuclear translocation of p65/p50 heterodimers to occur (Xie 2013).

The alternative NF-κB pathway is characterized by low steady state levels of NF-κB inducing kinase (NIK) in unstimulated cells, because NIK is constantly targeted for ubiquitination and proteasomal degradation involving a multi subunit ubiquitin ligase complex (Hacker, Tseng & Karin 2011). Within this multi subunit complex, TRAF2 and TRAF3 are molecular bridges that

bring cIAP, another E3 ubiquitin ligase, to directly ubiquitinate and target NIK for proteasomal degradation (Vallabhapurapu et al. 2008, Zarnegar et al. 2008b). Upon stimulation of the alternative pathway, the ubiquitin ligase complex is disrupted and TRAF2/TRAF3 are degraded by the proteasome, allowing NIK to accumulate and autophosphorylate, which leads to IKKα activation and p100 processing. p100 conversion to p52 enables heterodimerization of p52 and RelB, which then translocates to the nucleus to activate NF-κB dependent transcription (Sun 2011).

Given the crucial role NF-κB plays in inflammation and the immune response, many pathogen effector proteins target the NF-κB pathway (Rahman, McFadden 2011). One of the most well characterized pathogens that activates NF-κB is the Epstein Barr virus (EBV), which encodes the effector latent membrane protein 1 (LMP1) that persistently activates NF-κB (Mosialos et al. 1995). LMP1 is a six membrane spanning molecule that contains two C terminal activation (CTAR) domains, CTAR1 and CTAR2, which activate the alternative and canonical NF-κB pathways respectively. CTAR1 interacts with TRAF2 and TRAF3, to activate the alternative NF-κB pathway, and this alternative activation is dependent on the TRAF binding site in CTAR1 (Luftig et al. 2004). On the other hand, CTAR2 interacts with TRAF6, leading to activation of the canonical NF-κB pathway, dependent on eight residues within CTAR2 (Wu, Nakano & Wu 2006).

Like other pathogens, *Toxoplasma* encodes an effector protein, GRA15, which causes NF-κB activation. GRA15 was shown to account for differences in NF-κB activation between types I (RH and GT1), II and III strains upon infection (Rosowski et al. 2011, Yang et al. 2013). GRA15 was also shown to be responsible for human monocyte secretion of IL-1β (Gov et al. 2013),

which is a NF-κB dependent cytokine that activates downstream inflammatory genes (Dinarello 2011). GRA15 also determines the upregulation of cell surface receptor CD40 expression on mouse macrophages, which leads to IL-12 production and induction of the IFN-γ response (Morgado et al. 2014). Thus, strain differences in GRA15 can lead to differences in activation of innate immune responses in both humans and mice, which can lead to different adaptive immune responses important in controlling chronic *Toxoplasma* infection.

It is currently unclear if strain differences in activation of NF-κB are due to GRA15 amino acid differences or caused by differences in GRA15 expression levels (Melo et al. 2013). GRA15 dependent NF-κB activation is similar between Myd88/TRIF (adaptors directly downstream of Toll-like receptors (TLRs)) double knockout cells compared to wild type cells (Rosowski et al. 2011), thus it is unlikely that GRA15 acts as a TLR ligand. However, there was a significant decrease in GRA15 dependent NF-κB activation in TRAF6 knockout cells, an adaptor involved in canonical NF-κB activation, indicating that TRAFs are likely involved in the mechanism of NF-κB activation by GRA15. Thus, the exact mechanism of GRA15 dependent NF-κB activation remains unclear. We show that both type II and type III GRA15 are sufficient to cause NF-κB activation upon ectopic expression. Using co-immunoprecipitation, we identify candidate host proteins interacting with GRA15, including TRAF2, an upstream NF-κB adaptor protein. In addition, we identified particular regions of GRA15 that are important for NF-κB activation through structure-function mutant analysis.

Results

GRA15 ectopic expression is sufficient to drive NF-κB activation

Type III and GT1 (type I) GRA15 have 635 amino acids, while type II GRA15 has 550 amino acids due to a deletion at the C terminus (**Figure 1A**). To determine whether GRA15 (II) is sufficient to activate NF-κB upon ectopic expression in human cells, we engineered a stable inducible HEK293-derived (Trex-293) cell line expressing type II GRA15 (from residues 51 to 550 to exclude the signal peptide) with a C terminal HA-FLAG double epitope tag under the control of the tetracycline operator. Upon induction of GRA15 (II) through tetracycline addition, we observed that GRA15 accumulates mainly in the cytoplasm upon ectopic expression (**Figure 1B**). We then measured NF-κB activation by quantifying p65 nuclear translocation upon GRA15 (II) induction and saw a significant increase in p65 nuclear translocation compared to control, non-induced cells (**Figure 1C**, p-value = 5.06×10^{-18}). Thus, GRA15 (II) is sufficient for nuclear translocation of p65 upon ectopic expression.

To determine whether polymorphisms in GRA15 between type II and type III strains lead to differences in NF-κB activation (**Figure 1A**), we engineered GRA15 (III) overexpressing cells and observed similar cellular cytoplasmic localization to GRA15 (II) overexpressing cells (**Figure 1B**). Expression of GRA15 (III) induced a similar increase in p65 translocation compared to expression of GRA15 (II), which was significantly higher compared to control, non-induced cells (**Figure 1C**, p-value = 1.28×10^{-14}). Overexpression of an unrelated *Toxoplasma* protein (TgME49_247520 (II)), which also showed a cytoplasmic subcellular localization, did not lead to increased p65 nuclear translocation (**Figure 1C**, p-value = 0.98).

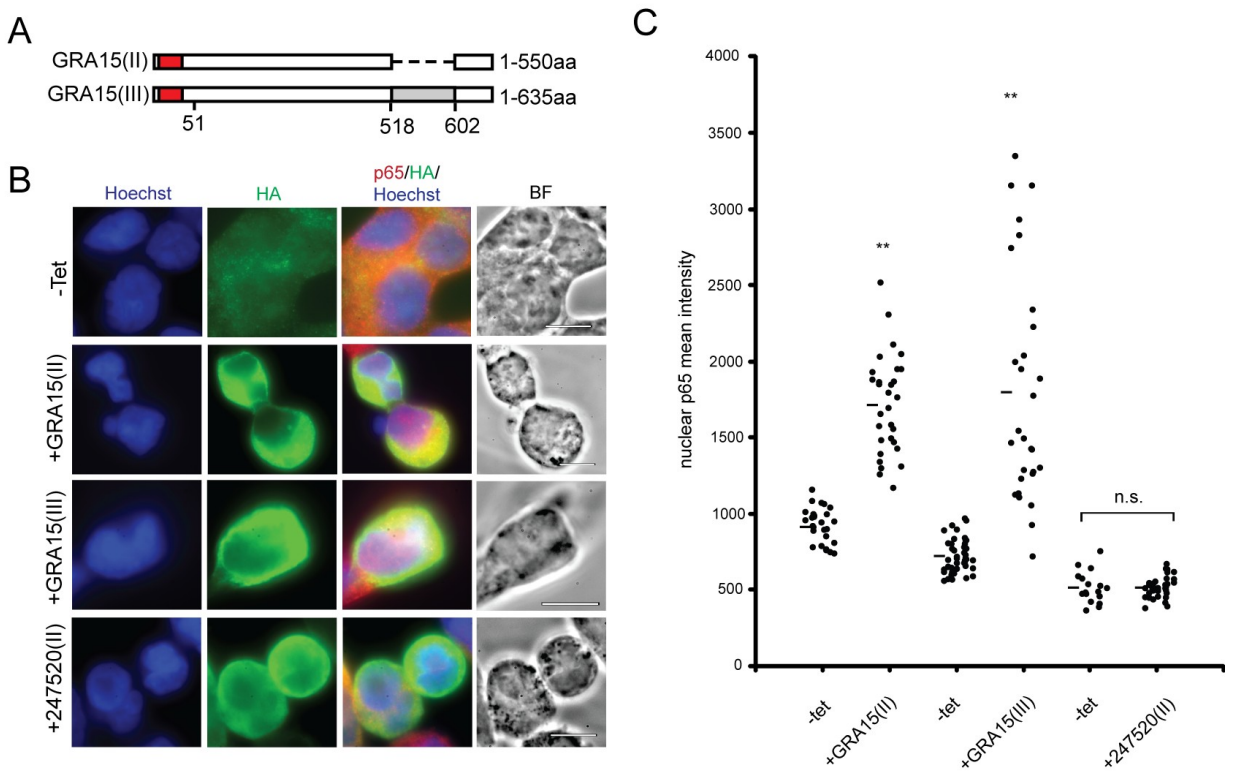

Figure 1. Ectopic expression of GRA15 is sufficient to activate NF-κB. (A) Schematic showing GRA15 (II) and GRA15 (III) protein sequence and alignment, red box indicates signal peptide. (B) TRex-293 overexpressing GRA15 (II) were induced with tetracycline (1µg/ml) for 24 hours, fixed with formaldehyde and stained for p65 (red), HA (green) and Hoechst (blue). Scale bar represents 10µm. (C) The nuclear amount of p65 was quantified in at least 20 cells per condition. Double asterisk indicates significantly higher levels of nuclear p65 compared to non-induced control cells of the same background (p-value < 0.05, n.s. = not significant, Student's t test).

We noted that the observed molecular weight of GRA15 (II) and GRA15 (III) (75kDa and 90kDa respectively) when ectopically expressed, was higher than the expected predicted sizes (57kDa and 67 kDa respectively; www.web.expasy.org) (**Figure 2A**). We also immunoblotted for the endogenous GRA15 in HFFs infected with types I, II and III strains, and observed a similar increase in GRA15 size compared to the expected GRA15 size (**Figure 2B**). Furthermore, as previously reported, RH and RH-JSR had indels leading to premature stop codons (**Chapter II**) and there was no detectable GRA15 in HFFs infected with RH or RH-JSR (**Figure 2B**, data not shown for RH-JSR).

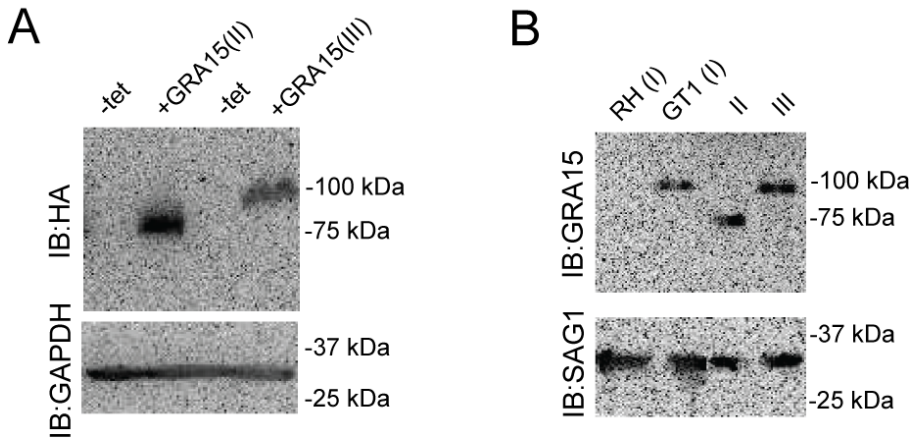

Figure 2. Molecular weight of GRA15 is similar between parasites and ectopic expression in cells. **(**A) Immunoblot on TRex-293 GRA15 (II) and TRex-293 GRA15 (III) overexpressing cell lysates induced with tetracycline for 24 hours, using an anti-HA antibody. GAPDH antibody was used as loading control. (B) Western blot on parasite lysates of indicated strains using an antibody against C terminus of endogenous GRA15, with SAG1 antibody as loading control.

Identification of GRA15 candidate host interacting proteins

Even though there is evidence that GRA15 requires TRAF6 to achieve full NF-κB activation (Rosowski et al. 2011), it is unclear whether GRA15 directly interacts with TRAF6 or acts indirectly through TRAF6 to cause NF-κB activation. Therefore, to determine potential host proteins that interact with GRA15 or are in the same complex with GRA15, we harvested whole cell lysates from TRex-293 GRA15 (II), GRA15 (III) and TgME49_247520 (II) 24 hours post induction with tetracycline and confirmed that all lysates had strong expression of each *Toxoplasma* protein (**Figure 3A**). These lysates were then subjected to immunoprecipitation using the HA antibody, and immunoprecipitates were probed with GAPDH, a likely unspecific cytosolic protein (**Figure 3B**). Immunoprecipitates were ran on SDS-PAGE, each lane encompassing the entire molecular weight range was excised and subjected to mass spectrometry (**Figure 3C**).

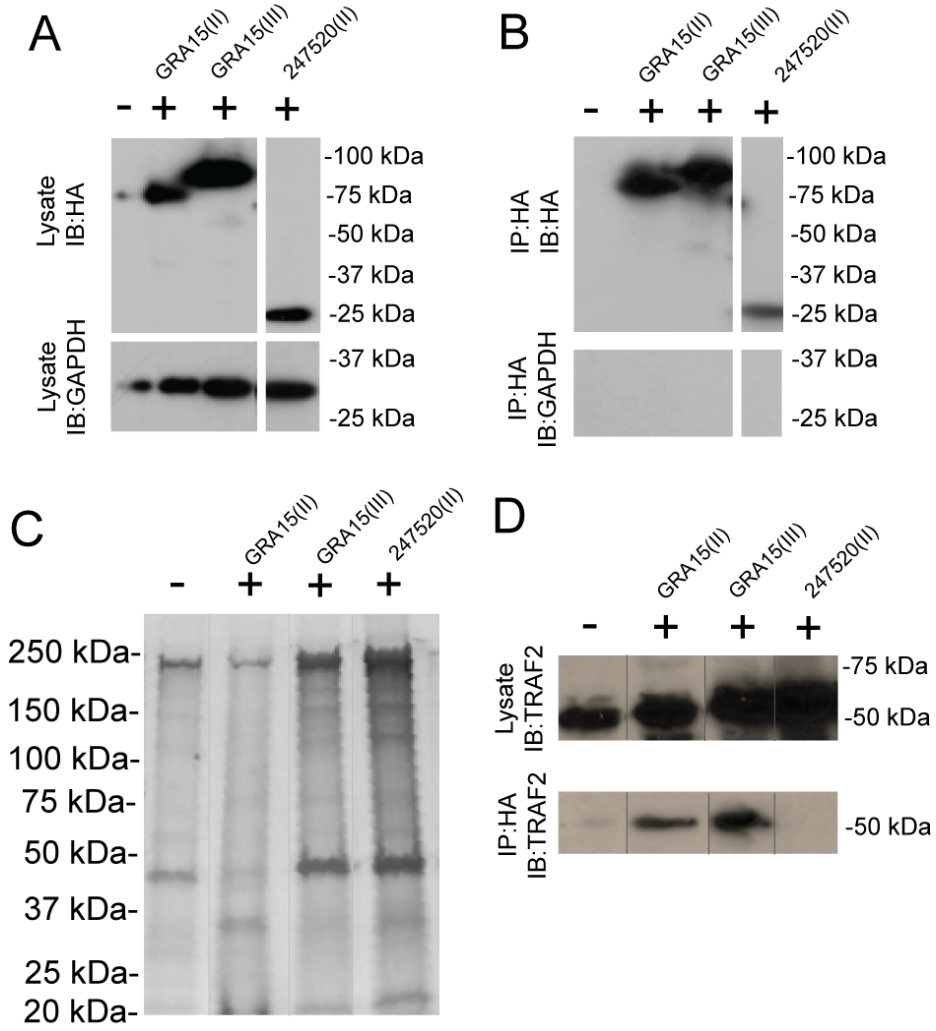

Figure 3. Identification of candidate host proteins interacting with GRA15. (A) Whole cell lysates of TRex-293 GRA15 (II), GRA15 (III) or TgME49_247520 (II) were induced with tetracycline (1µg/ml), harvested 24 hours post-induction and probed with anti-HA (top) or anti-GAPDH (bottom) as a loading control. (B) TRex-293 GRA15 (II), GRA15 (III) or TgME49_247520 (II) were immunoprecipitated with anti-HA antibodies and probed with anti-HA (top) or anti-GAPDH (bottom). (C) Immunoprecipitates for GRA15 (II), GRA15 (III) and TgME49_247520 (II) were run on a gradient 4-15% SDS PAGE gel, subject to colloidal Coomassie staining and lanes were excised for mass spectrometry. (D) TRex-293 GRA15 (II), GRA15 (III) and TgME49_247520 (II) whole cell lysates (top) and immunoprecipitates (bottom) were probed with anti-TRAF2, with expected size of 50 kDa. Lines indicate image of lanes spliced together due to technical limitations.

To control for potential spurious interactions detected through mass spectrometry, we refined the list of proteins to those identified only in GRA15 (II) or GRA15 (III) overexpressing conditions, and excluded proteins detected in immunoprecipitates from TgME49_247520 (II)

overexpressing conditions. We identified a list of candidate host proteins which could have direct interactions with GRA15 (II) and GRA15 (III) (**Table 1**). We observed that TNF-receptor associated factor 2 (TRAF2) was detected in GRA15 (II) and GRA15 (III) immunoprecipitates, but was absent in non-induced controls and TgME49_247520 (II) immunoprecipitates. To validate this observation, we immunoblotted whole cell lysates and immunoprecipitates of non-induced control cells, and cells overexpressing GRA15 (II), GRA15 (III) and TgME49_247520 (II) with antibodies against TRAF2. There was strong expression of TRAF2 in all of the cell lysates, but TRAF2 was only present in GRA15 (II) and GRA15 (III) immunoprecipitates, and absent in non-induced and TgME49_247520 (II) immunoprecipitates (**Figure 3D**). We also observed that the NF-κB p100 subunit, part of the alternative NF-κB pathway, and an E3 ubiquitin-protein ligase RING2 were present at low levels in GRA15 (II) immunoprecipitates, but absent in all other conditions (**Table 1**).

Protein name	Accession Number	Molecular Weight	Non-induced control	GRA15 (II)	GRA15 (III)	TgME49_247520(II)
histone H2B type 1-B	gi|10800140	14 kDa	0	281	103	0
coiled-coil domain-containing protein 86	gi|13129104	40 kDa	0	18	7	0
zinc finger protein 512B	gi|34013528 (+1)	97 kDa	0	13	28	0
TNF receptor-associated factor 2	gi|22027612 (+1)	56 kDa	0	9	8	0
HLA class I histocompatibility antigen, A-1 alpha chain precursor	gi|24797067	41 kDa	0	5	3	0
probable ATP-dependent RNA helicase DDX28	gi|256773275	60 kDa	0	5	7	0
E3 ubiquitin-protein ligase RING2	gi|6005747	38 kDa	0	5	0	0
nuclear factor NF-kappa-B p100 subunit isoform a	gi|117320531 (+2)	97 kDa	0	2	0	0
ubiquitin carboxyl-terminal hydrolase 46 isoform 2	gi|197116355 (+2)	42 kDa	0	0	0	4
YTH domain-containing family protein 2 isoform 2	gi|290543591	57 kDa	0	0	0	3
netrin-4 precursor	gi|93204871	70 kDa	0	0	0	3
ATP-dependent RNA helicase DDX55	gi|41327779	69 kDa	0	0	0	3
zinc finger RNA-binding protein	gi|34101286 (+1)	117 kDa	0	0	0	2

Table 1. Table of candidate GRA15 host interacting proteins through mass spectrometry analysis of immunoprecipitates. List of GRA15 host interacting candidates from immunoprecipitation of GRA15 (II), GRA15 (III) or TgME49_247520 (II). Mass spectrometry values are derived from total spectra.

GRA15 does not directly interact with TRAF3

Previous preliminary studies suggested that there may be a direct interaction between GRA15 and TRAF3 (Lu 2013). To determine whether GRA15-TRAF3 interaction is present in TRex-293 cells overexpressing GRA15 (II) cells, we transiently transfected TRAF3 into the Trex-293 expressing GRA15 (II) or TgME49_247520 (II) overexpressing cells, followed by induction of GRA15 (II) or TgME49_247520 (II), and immunoblotted for TRAF3 in the immunoprecipitates. Even though there was strong expression of TRAF3 upon transient transfection in cell lysates, there was no significant difference in enrichment of TRAF3 in immunoprecipitates which were non-induced or induced with GRA15 (II) or TgME49_247520 (II) (**Figure 4A and 4B**, top right panels).

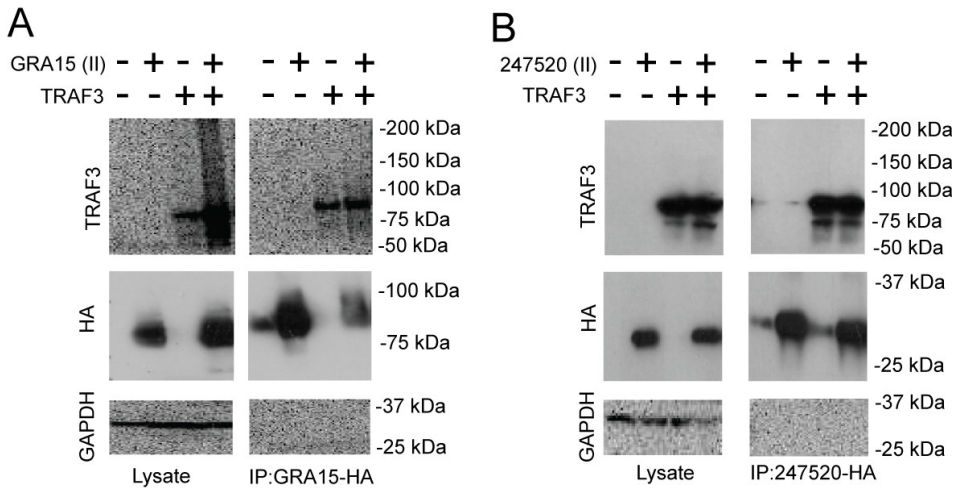

Figure 4. GRA15 does not have a direct interaction with TRAF3. (A) TRex-293 GRA15 (II) cells were transiently transfected with TRAF3 or left untransfected for 24 hours, then both transfected and untransfected cells were kept non-induced or induced with tetracycline (1µg/ml) for 24 hours, and cell lysates were subject to immunoprecipitation with beads conjugated to anti-HA antibodies. Left panel shows cell lysates and right panel shows immunoprecipitates. Immunoblots against anti-TRAF3 (top), anti-HA (middle) and GAPDH (bottom) as a loading control. (B) Cell lysates and immunoprecipitations were performed exactly as in (A) but with TRex-293 TgME49_247520 (II) cells. Left panel shows cell lysates and right panel shows immunoprecipitates. Immunoblots against anti-TRAF3 (top), anti-HA (middle) and GAPDH (bottom) as a loading control. Representative immunoblots shown for at least two experiments.

However, we observed that compared to non-induced, TRAF3 transfected cell lysates, in overexpressing GRA15 (II) cell lysates transfected with TRAF3, there were higher molecular weight species of TRAF3 (**Figure 4A**, top left panel). This was specific to GRA15 (II) overexpressing lysates, because no similar higher molecular weight species of TRAF3 were observed in cell lysates overexpressing TgME49_247520 (II) (**Figure 4B**, top left panel). This indicates that even though there is no likely direct interaction between GRA15 and TRAF3, overexpression of GRA15 could lead to post translational modification of TRAF3 indirectly.

Identification of GRA15 sequence necessary for NF-κB activation

GRA15 does not have any known homology to any known proteins (www.ncbi.nlm.nih.gov/BLAST). Therefore, to determine which region of GRA15 is required for GRA15 dependent NF-κB activation, we engineered GRA15 N terminal and C terminal truncation mutants. We expressed the full length GRA15 (II) without the signal peptide (schematic GRA15 (II) **Figure 5A**), C terminal truncations (51-479, 51-517 and 51-527) and N terminal truncations (80-550, 170-550 and 270-550), in a mammalian expression vector. We transiently transfected both full length and truncated GRA15 mutants in the HEK293 NF-κB reporter cell lines to determine differences in NF-κB activation. There were no significant differences in GRA15 dependent NF-κB activation with the GRA15 C terminal truncations, but all of the N terminal mutants had decreased NF-κB activation compared to full length GRA15 (**Figure 5A**). The N terminal mutant with the smallest region truncated is GRA15 (II) $_{80\text{-}550}$, indicating that residues 51 to 80 are necessary for GRA-15 dependent NF-κB activation.

To ensure that the decrease in NF-κB activation was not due to lack of protein expression, we immunoblotted for GRA15 upon transient transfection of HEK293 cells, and observed that the N terminal GRA15 mutants were strongly expressed, even more strongly than the full length GRA15 (**Figure 5B**). Thus, the decrease in NF-κB activation is specific to the missing sequences in the N terminal GRA15 sequences and not because of protein expression differences. We observed that even though GRA15 is a relatively disordered protein (Yang et al. 2005), amino acids 43 to 77 seem to have a more ordered protein structure (**Figure 5C**, double asterisks), which may indicate a role in function for amino acids 51-80 in GRA15.

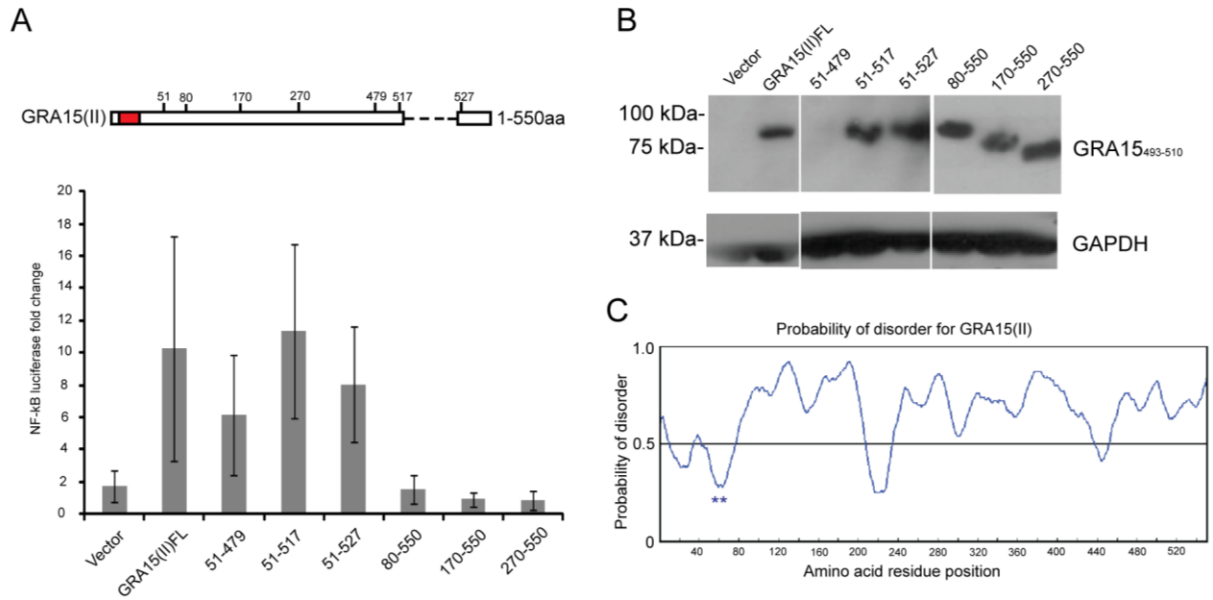

Figure 5. N terminus region of GRA15 (II) is required for full NF-κB activation. (A) Transient transfections of the NF-κB reporter cell line with GRA15 (II) N terminal or C terminal truncation mutants. These GRA15 (II) mutants were co-transfected with Renilla luciferase expressing vector, and conditions with comparable transfection efficiencies were used. Graph shows average firefly luciferase fold changes across three independent experiments, in comparison to untransfected control cells, and error bars represent SEM. (B) NF-κB reporter cell lines were transiently transfected with GRA15 N terminal or C terminal truncation mutants, and immunoblotted for GRA15 (raised against residues 493 to 510) (top panel) and GAPDH (bottom panel) for loading control. (C) Structural disorder predictor for GRA15 (II), with double asterisk indicating increased structural order across residues 43 to 77, including residues 51 to 80.

Discussion

We observed that upon ectopic expression of both type II or type III GRA15, there was significant NF-κB activation compared to non-induced controls. However, upon infection of human or murine cells, type II strains strongly activate NF-κB while type III strains induce very weak or no NF-κB activation (Rosowski et al. 2011). The difference between type II and III strain difference in NF-κB activation, despite both having a GRA15 able to cause NF-κB activation, could be due to lower expression of GRA15 in type III strains (Melo et al, 2013). However, cells infected with type III strains overexpressing GRA15 (II) do not activate NF-κB, while cells infected with type I (RH) overexpressing GRA15 (II) have significant NF-κB activation, though both transgenic strains express GRA15 (II) to similar levels (Melo et al. 2013). It is likely that parasite strains with a functional GRA15 that is highly expressed contain other *Toxoplasma* effectors that have an inhibitory effect, such as ROP38, which is highly expressed in type III strains and inhibits NF-κB (**Chapter IV**). The activity of rhoptry kinases, such as ROP16, can be observed within minutes of infection (Ong, Reese & Boothroyd 2010), whereas GRA15 dependent NF-κB activation is only observed 4 hours post infection. The inhibition of NF-κB dependent genes due to other early secreted *Toxoplasma* effectors could occur early before sufficient levels of GRA15 accumulate to cause NF-κB activation. Thus, this type II/III parasite strain difference in NF-κB activation upon infection could be due to other *Toxoplasma* effectors which are polymorphic or differentially expressed between type II and III strains.

We identified several host proteins which may directly interact with GRA15, or are present in a complex with GRA15. One of the most promising interaction partners was TRAF2, which co-

immunoprecipitated with both GRA15 (II) and GRA15 (III), but was absent in all other immunoprecipitations. TRAF2 and TRAF6 are both involved in the canonical NF-κB activation pathway (Xia, Chen 2005), whereas TRAF2 and TRAF3 are involved in non-redundant negative regulatory roles in the alternative NF-κB activation pathway (Vallabhapurapu et al. 2008, Zarnegar et al. 2008b). There is also cross regulation between the canonical and alternative NF-κB pathways, with the NF-κB inducing kinase (NIK), which mediates alternative NF-κB activation, reported to control the canonical NF-κB pathway (Zarnegar et al. 2008a). Another candidate that co-immunoprecipitated at low levels with GRA15 (II) was the NF-κB p100 subunit, though type I parasites expressing GRA15 (II) infected HFFs did not have significantly increased nuclear translocation of RelB or p52 levels compared to uninfected cells (Rosowski et al. 2011).

It is important to note the limitations of immunoprecipitating and identification of candidate host interacting proteins upon ectopic expression of *Toxoplasma* proteins. A possibility is that spurious interactions that are not physiologically relevant are identified due to protein expression at non-stoichiometric concentrations upon overexpression. Another limitation of this approach is that host interacting proteins identified in the HEK293 background may not be highly expressed in other cells types such as macrophages, which are more physiologically relevant during *Toxoplasma* infection. Therefore, to confirm these interactions reported by our mass spectrometry experiments, reciprocal IPs will have to be performed, where TRAF2 or p100 is pulled down with its corresponding antibody upon GRA15 overexpression, and immunoprecipitates are immunoblotted for GRA15 using antibodies against the HA epitope tag. To further confirm this in the parasite setting, GRA15 immunoprecipitations using macrophages

infected with RH overexpressing GRA15 (II) or type II parasites should also be performed, and GRA15 immunoprecipitates should be detected for TRAF2 to confirm this interaction.

GRA15 dependent NF-κB activation is present at 4 hours but not at earlier timepoints (Rosowski et al. 2011), which would be more consistent with the alternative NF-κB pathway, which requires minutes to hours due to protein processing and synthesis, while classical NF-κB activation occurs within minutes of activating stimuli (Hayden, Ghosh 2008). An earlier timepoint for harvesting the GRA15 cell lysates for immunoprecipitation, such as 4 hours compared to 24 hours, would be useful to determine earlier interacting proteins, which may be lost at later timepoints. Many of the interacting proteins included histone and nuclear proteins, such as zinc finger 512B and coiled coil domain 86 (**Table 1**), but given the cytoplasmic localization of GRA15, these interactions are likely to be non-specific. Thus, to increase specificity for further immunoprecipitations, it will be useful to fractionate cell lysates into nuclear and cytosolic fractions, and use the cytosolic fraction for downstream immunoprecipitations and mass spectrometry analysis.

We were not able to confirm a direct interaction between TRAF3 and GRA15, through immunoprecipitation of GRA15-HA in the TRex-293 cell background. Previous studies reported interaction using immunoprecipitation of TRAF3 and probing for GRA15, but the reciprocal immunoprecipitation using antibodies against GRA15 and probing for TRAF3 also did not show detectable interactions (Lu 2013). It is possible that the antibody used to immunoprecipitate GRA15 could occlude its interaction site with TRAF3, but another distinct possibility is that GRA15 has an indirect interaction with TRAF3, potentially through interacting with TRAF2. In cell lysates, but not immunoprecipitates, overexpressing GRA15 (II) and transfected with

TRAF3, we consistently observed a TRAF3 smear of increased molecular weight species (**Figure 4A**), a possible indication of polyubiquitination, which would target TRAF3 for proteasomal degradation (Hacker, Tseng & Karin 2011). The presence of TRAF3 smear of higher molecular weight species is specific to GRA15 (II) overexpression, because no TRAF3 smear was observed in cell lysates overexpressing TgME49_247520 (II). A possible model for GRA15 could be binding and activation of TRAF2 that goes on to ubiquitylate downstream adaptors such as TRAF3 or TRAF6. Proteasomal degradation of TRAF3, a negative regulator of NIK, could then lead to NIK activation and downstream activation of canonical or alternative NF-κB. However, to test this model, further experiments using the specific 26S proteasome inhibitor MG132 before induction of GRA15 (II) would be required.

We also identified a N terminus region of GRA15 (residues 51-80) required for NF-κB activation, through the use of GRA15 truncation mutants. We observed that this GRA15 region required for NF-κB activation has increased predicted structural order compared to the rest of GRA15, but it is unclear whether there is function associated with this increased order. Interestingly, upon further examination of GRA15 primary sequence, there are three possible TRAF 2/3/5 binding motifs ($AAEE_{160-163}$, $PSPGT_{204-208}$ and $SQQET_{430-434}$) and one possible TRAF 6 binding motif ($PGENSY_{506-511}$) which could function as docking sites for TRAFs (Wu 2007). Even though these putative motifs are not contained within residues 51-80, $PSPGT_{204-208}$ is present in residues 200 to 240, which also has increased structural order compared to the rest of GRA15 (**Figure 5C**). It is still unknown whether these putative TRAF motifs are involved in GRA15 dependent NF-κB activation, and these await structure function analysis through site directed mutagenesis to determine their necessity in NF-κB activation.

We have shown that GRA15 (II) and GRA15 (III) are sufficient for NF-κB activation upon ectopic expression, and our findings indicate a role for TRAF2 in the mechanism for GRA15 dependent NF-κB activation. We have also identified a region of GRA15 essential for NF-κB activation, and further experiments are required for a more complete understanding by which GRA15 induces signaling to cause NF-κB activation.

Materials and Methods

Plasmids

The vector pcDNA-LIC-HF was a gift from M.A. Hakimi and A. Boughdour. Primers were designed to amplify after the predicted signal peptide to the predicted stop codon. Forward primers to amplify GRA15 (5'-**TGGCTGGTGCTGGTGCCCAT**ATAATTCGGTGGCTTGGGTATCTT-3') together with reverse primers (5'- **GCTCCGGCTCCTGCCCCAGC**TGGAGTTACCGCTGATTGTGTG-3'), contained Ligation independent cloning (LIC) sequences (in bold), and were used to amplify GRA15 from PRU (II) and CEP (III) genomic DNA. Forward primers to amplify TgME49_247520 (5'- **TGGCTGGTGCTGGTGCCCAT**GGTTCACAAAATCCTGCTGG-3') together with reverse primers (5'-**GCTCCGGCTCCTGCCCCAGC**ATCTTTCTTGTGCCCTGTGTCCCGCGT-3') contained LIC sequences (in bold), and were used to amplify TgME49_247520 from PRU (II) genomic DNA. PCR products were treated with T4 DNA polymerase (using only TTP at 100mM). The pcDNA-LIC-HF vector was digested with SmaI, and treated with T4 DNA polymerase (using only ATP at 100mM) to generate long overhangs. The PCR fragment and vector were then annealed for 15 minutes at room temperature, generating expression vectors with *Toxoplasma* genes C terminally tagged with HA-FLAG.

The pCMV6-AC-TRAF3-GFP mammalian expression vector was purchased from Origene, expression of TRAF3-GFP C terminal fusion promoted by the CMV promoter. The vector pIC242 was a gift from I. Cheeseman. The GRA15 (II) full length (51-550aa) and truncation

mutants were amplified and inserted into pIC242 by restriction/ligation, expressing GRA15 (II) mutants as N terminal fusion GFP proteins. Expression of GFP fusion proteins was promoted by the endogenous retroviral long terminal repeats. GRA15 truncation mutations were confirmed by sequencing.

Inducible TRex-293 cell line construction

The TRex-293 cell line was a gift from J. Niles. TRex-293 cells were seeded at 75% confluency, and cotransfected with expression vectors and puromycin resistance vector (ratio of 10:1), using XtremeGENE 9 DNA transfection reagent (Roche). Cells were split 2 days post transfection, and subjected to puromycin (Calbiochem) selection at 1ug/ml. Foci were picked and expanded at least one week post selection, and positive foci were selected through HA expression using immunofluorescence and immunoblotting.

Immunofluorescence

TRex-293 cells overexpressing GRA15 (II), GRA15 (III) or TgME49_247520 (II) were plated at 80% confluency in 24 well coverslip plates, induced with tetracycline (Sigma-Aldrich) at 1µg/ml for 24 hours, fixed with formaldehyde for 20 minutes, blocked with blocked with PBS with 3% (w/v) BSA and 5% (v/v) goat serum. Coverslips were incubated with anti-HA (Roche, 1:500), anti-p65 (Santa Cruz, SC-109, 1:500) at 4°C overnight, and fluorescent secondary and Hoechst were used for HA, p65 and DNA visualization respectively. Quantification of p65 nuclear localization was performed by randomly selecting cells and measuring the average signal intensity per nucleus using the NIS-Elements software and Hoechst to define the nucleus.

Western blotting

$10^{\wedge 6}$ of TRex-293 overexpressing cell lines were lysed with lysis buffer, boiled for 5 minutes, iced for 5 minutes, and subjected 10% SDS-PAGE. Proteins were transferred to a PVDF membrane, blocked with PBS/0.1% Tween-20/5% nonfat dry milk, incubated with either anti-rabbit TRAF2 (Santa Cruz, sc-876, 1:250), anti-rabbit TRAF3 (Santa Cruz, sc-1828, 1:500), anti-rat HA (Roche, 1:500), anti-mouse FLAG (Sigma 3165, 1:1000), anti-rabbit GRA15 (Yenzyme, residues 493-510, 1µg/mL) or anti-mouse GAPDH (Santa Cruz, SC-32233, 1:500), overnight at 4°C and followed by secondary antibodies.

Transient transfections

TRex-293 GRA15 (II) and TRex-293 TgME49_247520 (II) were seeded at $10^{\wedge 6}$ cells per T25, and incubated overnight at 37°C. Cells were then transiently transfected with XtremeGene9 transfection reagent (Roche) and pCMV6-AC-TRAF3-GFP (Origene), incubated for 24 hours at 37°C before induction of protein expression with tetracycline for 24 hours at 1µg/ml. NF-κB reporter cell lines were seeded at $7.5*10^{\wedge 4}$ cells per well in 24 well plates, and incubated for 4 hours at 37°C. The cells were then transiently transfected with XtremeGENE 9 transfection reagent (Roche), together with pRL-TK (gift from E. Bell) and pIC242-GRA15 (II) (full length and mutants), and incubated for 24 hours at 37°C. Cells were lysed in 100µl Passive Lysis Buffer (Promega), and both firefly luciferase and Renilla luciferase activity were measured in lysates according to manufacturer's instructions. Data from conditions with comparable transfection efficiencies were used.

Antibody conjugation and crosslinking

10µl of Dynabeads G (Invitrogen) were washed with HBS (10 mM HEPES-KOH, pH 7.5, 150 mM NaCl) and resuspended with either anti FLAG M2 antibody (Sigma Aldrich, F1365) or anti HA antibody (Roche) (5µg for Western Blot, 30µg for mass spectrometry) at room temperature for 1 hour. Following conjugation, antibodies were crosslinked to Dynabeads G using BS3 (Bis (sulfosuccinimidyl) suberate) at 5 mM in conjugation buffer (20 mM sodium phosphate, 150 mM sodium chloride, pH 8.0) at room temperature for half an hour and quenched with Tris-Cl (50 mM, pH 7.5) at room temperature for 15 minutes. Crosslinked Dynabeads were then washed 3 times with HBS, and stored at 4°C until further use.

Co-immunoprecipitation

TRex-293 overexpressing GRA15 (II), GRA15 (III) or TgME49_247520 (II) cells were grown in T25s until 100% confluency, and induced with tetracycline (1µg/mL) for 24 hours. Cells were then scraped in ice-cold PBS and centrifuged at 1500rpm for 5 minutes, and washed twice with 1x PBS with 1mM PMSF (phenylmethylsulfonylfluoride, Thermo Scientific) through centrifuging at 13,000 rpm for 1 minute at 4°C. Cells were lysed in 500ul lysis buffer (300 mM NaCl, 50 mM HEPES pH 7.5, 0.5% NP-40) for 1 hour at 4°C, and soluble fraction obtained by centrifuging at 13,000 rpm for 15 minutes at 4°C. The soluble fraction was incubated with conjugated Dynabeads G:FLAG or Dynabeads G:HA beads overnight at 4°C, rotating, and washed 5 times with HBS (10 mM HEPES-KOH, pH 7.5, 150 mM NaCl). Beads were then boiled in sample buffer with DTT (dithiothreitol, 100 mM) for 5 minutes and subject to either

Western blotting or colloidal Coomassie (Invitrogen) staining for further mass spectrometry analysis.

Mass spectrometry

SDS Page gels were immersed in staining solution (20% methanol, 80% colloidal Coomassie) for 3 hours on a rotating platform, and destained using milliQ water overnight on rotating platform, until visible bands were seen. Each lane encompassing the entire molecular weight was then excised and subject to trypsin digest. These were then analyzed by reversed phase HPLC and a ThermoFisher Orbitrap linear ion trap mass spectrometer. Peptides were identified from the MS data using SEQUEST algorithms 44 that searched a species specific database generated from NCBI's non redundant database.

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Chapter Four – Rhoptry kinase ROP38 represses NF-κB and affects cyst burden *in vivo*

Abstract

Toxoplasma gondii modulates the host response through effector proteins found within specialized subcellular organelles known as the rhoptries. Rhoptry proteins (ROPs), in particular rhoptry kinases and pseudokinases, are crucial in mediating host responses. Rhoptry kinase ROP38 is involved in modulation of host gene expression, with a role in regulating the mitogen activated kinase (MAPK) response. However, it is not known whether ROP38 has effects on further downstream pathways such as the NF-κB pathway, a master regulator mediating innate immune responses. Though ROP38 is upregulated upon tachyzoite to bradyzoite conversion *in vitro*, it remains unclear whether ROP38 affects cyst formation *in vivo*, an important event in *Toxoplasma* chronic infection. We find that overexpression of ROP38 in a type II strain represses the NF-κB response, but does not affect NF-κB dependent secretion of cytokines such as IL-12p40 and CCL2. To investigate how ROP38 represses host transcriptional processes, we identified candidate host proteins interacting with ROP38, indicating a likely role for adaptor proteins upstream of MAPKs. In addition, mice challenged with ROP38 knockout parasites demonstrated increased weight loss and increased brain cyst burden, though serum cytokine levels were not affected, indicating ROP38 involvement in cyst formation *in vivo*. Elucidating the effects of ROP38 *in vivo* and molecular pathways affected by ROP38 reveal the likely role ROP38 plays in bradyzoite conversion and repression of host innate immune responses.

Introduction

Eukaryotic kinases are key mediators in host responses as they control protein phosphorylation events that regulate numerous signaling pathways. Kinases contain a conserved kinase domain that has a catalytic triad (KDD; lysine-aspartic acid-aspartic acid) required for transfer of γ-phosphate from ATP to donor proteins (Hanks, Hunter 1995). In eukaryotes, kinases usually target serine, tyrosine or threonine residues in target proteins for phosphorylation and this post translational modification can result in changes in conformation or activity of downstream targets, like activation of downstream kinases and transcription factors (Krishna, Narang 2008).

One of the most evolutionarily conserved signaling pathways regulated by eukaryotic kinases are the mitogen activated protein kinase (MAPK) pathways, involved in diverse host responses from innate immunity to apoptosis (Dong, Davis & Flavell 2002). MAPK pathways have a core three tiered signaling module, consisting of three sequentially acting kinases – a MAPK phosphorylated by MAPK kinase (MAPKK), in turn phosphorylated by MAPKK kinase (MAPKKK). MAPKs are a family of protein kinases with the following three subfamilies most extensively studied: extracellular signal regulated kinases 1/2 (ERK1/2), JUN N-terminal kinases (JNK) and p38 kinases (Krishna, Narang 2008). Activation of MAPKs occur through the phosphorylation cascade, but regulation is coupled to dephosphorylation reactions mediated by protein phosphatases (Keyse 2000).

Pseudokinases, on the other hand, have mutations in the conserved catalytic triad required for kinase activity and are unable to facilitate phosphate transfer. However, they are increasingly recognized to play important roles in acting as regulators of enzymatic processes and signaling

pathways (Boudeau et al. 2006). Moreover, certain classified pseudokinases with mutated critical residues in the catalytic triad can still retain phosphorylation ability, through substitution of catalytic residues in other domains within the protein (Min et al. 2004). There are several mechanisms of action proposed for pseudokinases, ranging from being signal integrators, which bring together different components of diverse signaling pathways, to enzymatic competitors, which bind to target substrates and prevent phosphorylation by other kinases (Reiterer, Eyers & Farhan 2014).

Toxoplasma gondii secretes effector proteins, which are crucial in modulating host immune responses, from specialized, subcellular organelles, namely the rhoptries and dense granules. Rhoptries are initially discharged into the host cell during invasion, making proteins contained in these organelles key candidates in modulating host cell signaling (Boothroyd, Dubremetz 2008). The rhoptries contain a large family of kinases and pseudokinases (ROPKs) (Talevich, Kannan 2013) and several ROPKs are involved in modulating host gene expression and virulence.

Toxoplasma rhoptry kinases modulating host responses include ROP16 which was first identified using strain differences in host gene expression (Saeij et al. 2007). ROP16 was then demonstrated to be an active kinase that directly phosphorylates STAT3 and STAT6, which are transcription factors that negatively regulate Th1 inflammatory responses (Yamamoto et al. 2009, Ong, Reese & Boothroyd 2010). In addition, the ROP5 loci, which is highly duplicated in *Toxoplasma* and annotated to be rhoptry pseudokinases, was identified to be responsible for type I and II virulence differences (Behnke et al. 2011). The ROP5 expanded loci enable evasion of the immunity related GTPases (IRG) coating response (Reese et al. 2011), and polymorphisms in the ROP5 surface binding to IRGs, which are also highly polymorphic, indicate strong co-

evolution between pathogen and host immune responses (Reese, Shah & Boothroyd 2014). Thus, rhoptry proteins have diverse functions in modulating host processes, and gene family expansions are increasingly recognized to play important roles in *Toxoplasma* virulence.

Rhoptry kinase ROP38 contains the predicted catalytic triad conserved in eukaryotic kinases and plays a role in modulating host gene expression, such as the MAPK pathways (Peixoto et al. 2010). Host cells infected with RH (type I) overexpressing ROP38 repressed host transcriptional processes and delayed the kinetics of ERK1/2 phosphorylation in host cells, when compared to RH infection (Peixoto et al. 2010). Moreover, ROP38 was identified to be strongly differentially expressed between type I strains (Yang et al. 2013) and most highly expressed in type III strains across different canonical and atypical strains (Peixoto et al. 2010, Melo et al. 2013). In addition, ROP38 has increased expression during tachyzoite to bradyzoite differentiation *in vitro* (www.toxodb.org), though ROP38 expression is decreased in cysts harvested *in vivo* from mice (Buchholz et al. 2011).

In addition, ROP38 is part of an expanded gene family that consists of other rhoptry kinases such as ROP29 and ROP19. Expanded gene families are also likely to have divergent functional roles due to more relaxed constraints (Zhang 2003), but current differences in function between ROP38, ROP29 and ROP19 are unknown. Indeed, expanded gene families in *Toxoplasma* have a significant enrichment of secreted proteins and genes involved in tachyzoite to bradyzoite conversion in these expanded gene loci (Adomako-Ankomah et al. 2014). Therefore, the ROP38 expansion, together with bradyzoite and strain specific expression, could indicate its importance in *Toxoplasma* pathogenesis.

Thus, we examined whether ROP38 affects other signaling pathways, such as NF-κB, which is differentially regulated between canonical *Toxoplasma* strains (**Chapter III**), and observed that NF-κB activation was repressed by ROP38. We also investigated possible mechanisms for ROP38 repression on host transcription through co-immunoprecipitation and identified candidate host interacting proteins that likely function upstream of the MAPK pathways. Previous studies reported that ROP38 does not have effects on virulence using the RH type I strain (Peixoto et al. 2010), which is defective in cyst formation (**Chapter II**). Given the differential expression of ROP38 between tachyzoites and bradyzoites (www.toxodb.org), we also examined differences in virulence and cyst burden using a type II strain that is more cystogenic. We observed that when ROP38 is absent, there is increased weight loss during acute infection and increased cyst burden upon the onset of chronic *Toxoplasma* infection.

Results

ROP38 is conserved between strains across different Toxoplasma haplogroups

To determine ROP38 polymorphisms, we amplified ROP38 from genomic DNA derived from the three canonical strains (types I, II and III) and atypical strains (FOU, CAST, MAS, CASTELLS, TgCatBr5, COUGAR and GUYMAT), and performed evolutionary analysis on the nucleotide sequence. We observed that ROP38 sequence is relatively conserved among the canonical and atypical strains with a low phylogenetic distance between strain specific ROP38 alleles, except for MAS that was more divergent, using ROP19 from type II as an outgroup (**Figure 1A**). Furthermore, there were similar rates of nonsynonymous and synonymous amino acid changes across the ROP38 protein coding sequence (**Supplementary Figure 1**), and conservation of sequence may indicate purifying selection present (Zhang 2003). This is in agreement with recently published data looking at the ROP38 sequences between 13 *Toxoplasma* isolates (canonical and atypicals strains including MAS and TgCatBr5), that reported low sequence variation (Xu et al. 2014).

It was previously reported that ROP38 is part of the same gene family as ROP29 and ROP19. To determine how similar in sequence these proteins are, we performed an evolutionary analysis on available sequences of ROP38, ROP29 and ROP19 from ME49 (type II), together with annotated active kinases from ME49 (type II) that were reported to cluster together with this gene family (www.toxodb.org, (Peixoto et al. 2010)). We observed that ROP29 was more divergent in sequence compared to ROP38 and ROP19 (**Figure 1B**), and members of this family may not be

as alike as previously thought. This could indicate a functional divergence between ROP38 and the other family members like ROP29.

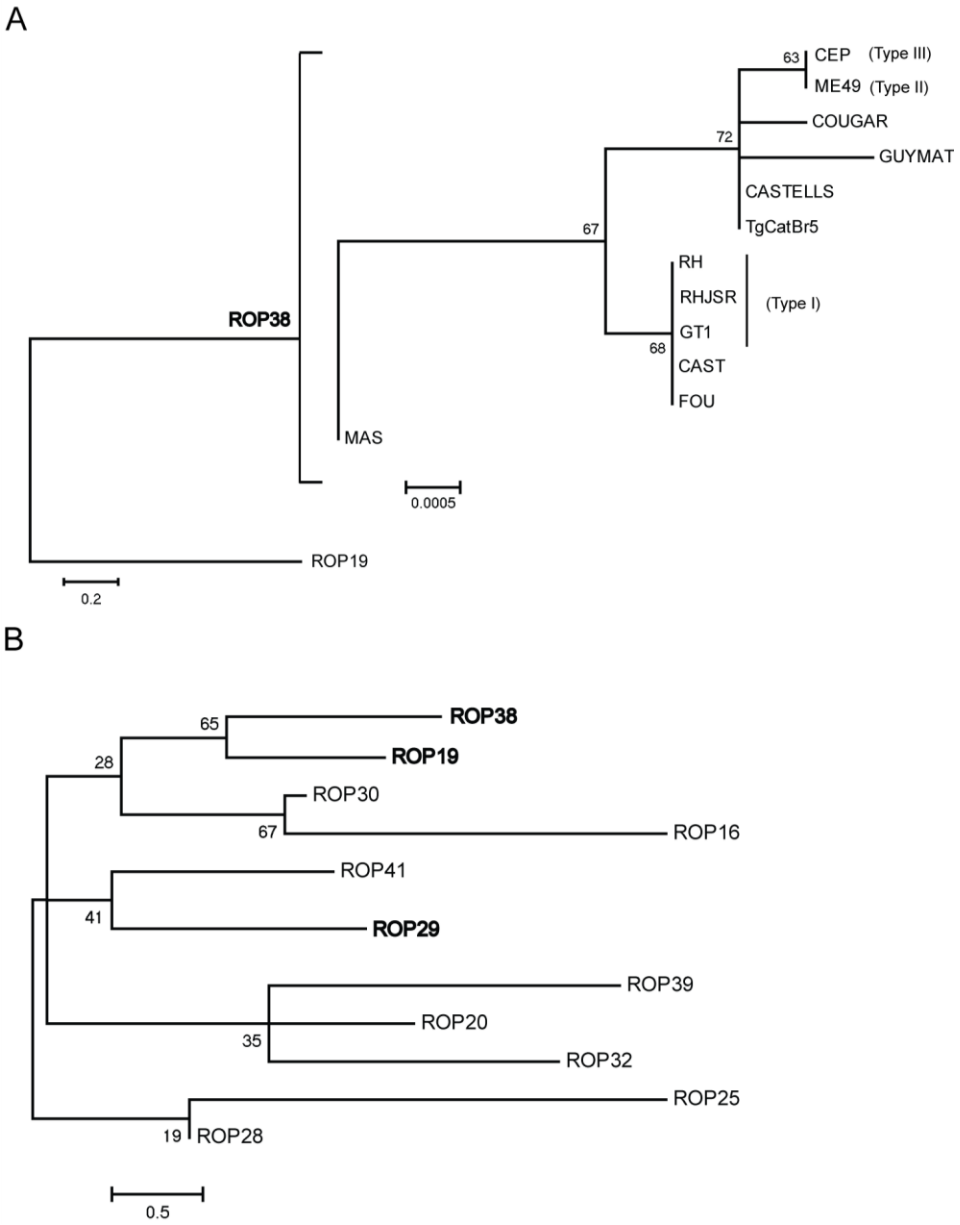


Figure 1. Evolutionary analysis of ROP38. (A) Evolutionary bootstrap tree of ROP38 from canonical and atypical strains, inferred from 500 replicates using the Maximum Likelihood method. The branch encompassing ROP38 alleles has been magnified, with a different phylogenetic distance indicated in diagram compared to the ROP19 outgroup. (B) Evolutionary bootstrap tree of ROP38 and related homologues, inferred from 500 replicates using the Maximum Likelihood method. All analyses were performed in the MEGA5 software.

ROP38 negatively regulates the NF-κB pathway

It has been reported the ROP38 has a repressive effect on general host transcriptional processes (Peixoto et al. 2010). We wanted to examine the effects ROP38 had on the NF-κB pathway, given the important role NF-κB plays in innate immunity. However, the ROP38 repressive effect on host transcription used RH (type I) parasites overexpressing ROP38 (Peixoto et al. 2010), which has a non-functional GRA15 (**Chapter II**) and does not activate the NF-κB pathway. Therefore, we overexpressed ROP38 in the type II strain, which strongly activates NF-κB upon infection, and observed that there was strong colocalization of ROP38-HA with ROP7, a previously identified rhoptry protein (**Figure 2A**).

To determine whether there were any differences observed in NF-κB activation upon ROP38 overexpression, we used a NF-κB reporter cell line, with NF-κB transcription factor binding sites driving the expression of luciferase upon activation of NF-κB. Upon infection of the NF-κB reporter cell line with type II and type II overexpressing ROP38, there was a significant decrease in NF-κB activation in the type II overexpressing ROP38 infected cells compared to the type II infected cells (**Figure 2B**, p-value = 0.0005, Student's t test). However, when we tested NF-κB activation through quantification of nuclear p65, we did not see any significant differences in p65 nuclear translocation between type II overexpressing ROP38 and type II infected cells (**Figure 2C**). We also wanted to test whether ROP38 could have effects on other transcription factors as well. The serum response factor (SRF) transcription factor has been reported to be activated in the three canonical strains (Wiley et al. 2011). Thus we infected a serum response factor (SRF) reporter cell line, but there was no significant decrease in SRF activation in the type

II overexpressing ROP38 infected cells compared to the type II infected cells (**Supplementary Figure 2**) (*p*-value = 0.17 , Student's *t* test).

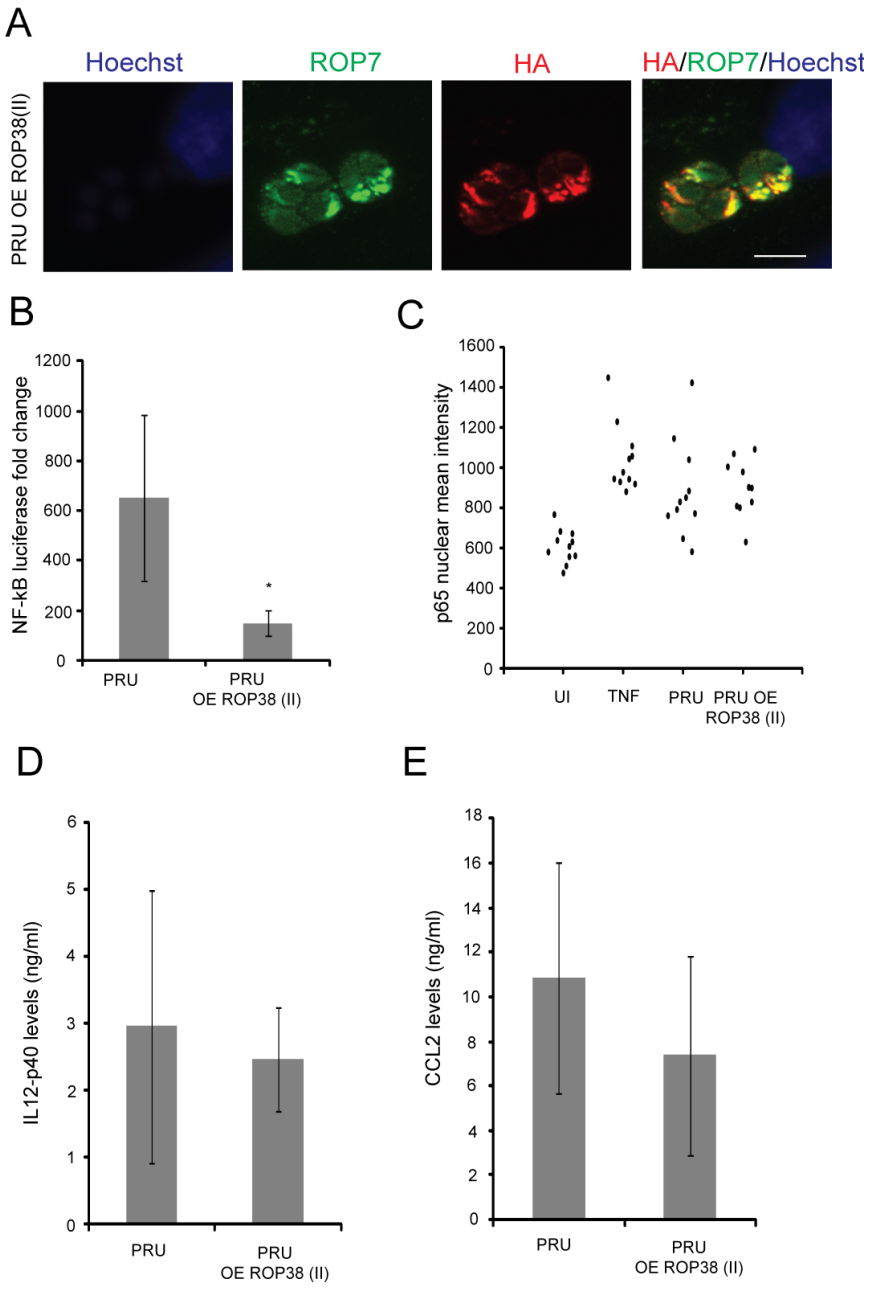


Figure 2. ROP38 has a repressive effect on NF-κB activation. (A) HFFs were infected with PRU overexpressing ROP38 (PRU OE ROP38 (II)) for 24 hours and co-stained with anti-HA (red) and anti-ROP7 (green). (B) An NF-κB reporter cell line was infected with PRU (II) and PRU OE ROP38 (II) strains, and luciferase activity was measured 24 hours post infection. Graph is the average of three independent experiments, and error bars represent standard deviation. Asterisk indicate significant

difference between the two conditions (p-value <0.05, Student's t test). (C) HFFs were infected with PRU (II) and PRU OE ROP38 (II) strains, and p65 nuclear intensity was quantified in uninfected (UI), tumor necrosis factor-alpha (TNF-α) stimulated and infected cells. At least 10 cells were quantified per condition. (D) IL12-p40 and (E) CCL2 levels were measured in supernatants from C57BL/6 bone marrow derived macrophages infected for 24 hours with PRU and PRU OE ROP38 (II) transgenic parasites. The graphs shown are representative of two independent experiments, and error bars represent standard deviation.

IL12-p40 is a NF-κB dependent cytokine, and has been implicated in resistance in mice to *Toxoplasma* (Gazzinelli et al. 1994). Thus, we wanted to investigate whether ROP38, with its inhibitory effect on NF-κB activation, has an effect on downstream NF-κB dependent cytokine levels, such as IL-12p40 and CCL2/MCP-1. C57BL/6 macrophages were infected with the type II and type II overexpressing ROP38 strains, and IL-12p40 and CCL2 levels were measured from supernatants. We observed no significant differences in either cytokine levels between cells infected with these two strains (**Figures 2D and 2E**).

Identification of candidate host proteins interacting with ROP38

ROP38 has a repressive effect on host transcriptional pathways, with the repression on NF-κB observed and delaying the kinetics of ERK1/2 phosphorylation (Peixoto et al. 2010). However, it is unclear whether ROP38 acts directly on MAPKs or indirectly through upstream adaptors to modulate these host signaling pathways. To determine the mechanism of action by ROP38, we engineered a stable inducible HEK293-derived cell line expressing type I ROP38 with a C terminal HA-FLAG double tag under the control of the tetracycline operator. In order to identify host proteins that directly interact with ROP38 or are in the same complex as ROP38, we harvested whole cell lysates from TRex-293 ROP38 (I) 24 hours post induction with tetracycline and confirmed that lysates had strong expression of ROP38, at the expected size of 60 kDa (**Supplementary Figure 3A**). These lysates were then subjected to immunoprecipitation using

the HA antibody, and immunoprecipitates were probed with HA or GAPDH (**Supplementary Figure 3B**). Immunoprecipitates were ran on SDS-PAGE, each lane encompassing the entire molecular weight range was excised and subjected to mass spectrometry.

To control for potential spurious interactions detected through mass spectrometry, we refined the list of proteins to those identified only in ROP38 (I) expressed conditions, and excluded proteins detected in immunoprecipitates from immunoprecipitates overexpressing TgME49_247520 (II), an unrelated *Toxoplasma* protein. We identified a list of candidate host proteins which could have direct interactions with ROP38 or are in the same complex interacting with ROP38 (**Table 1**). We observed that ROP38 immunoprecipitates yielded host protein adaptors upstream of MAPKs, including calmodulin dependent protein kinase type IV, MAPK kinase kinase kinase 4, receptor type tyrosine protein phosphatase gamma and G protein coupled receptor kinase 6.

Protein name	Accession Number	Molecular Weight	Non-induced control	ROP38 (I)	TgME49_247520(II)
tropomyosin beta chain isoform 1	gi|42476296	33 kDa	0	65	0
calcium/calmodulin-dependent protein kinase type IV	gi|4502557	52 kDa	0	5	0
chaperone activity of bc1 complex-like, mitochondrial	gi|34147522	72 kDa	0	5	0
mitogen-activated protein kinase kinase kinase kinase 4 isoform X29	gi|578805223	157 kDa	0	4	0
rho-related GTP-binding protein RhoB precursor	gi|4757764	22 kDa	0	4	0
receptor-type tyrosine-protein phosphatase gamma	gi|194097398	162 kDa	0	3	0
G protein-coupled receptor kinase 6 isoform B	gi|51896035	67 kDa	0	3	0
epidermal growth factor receptor kinase substrate 8 isoform X1	gi|530399079	93 kDa	0	2	0
GTPase HRas isoform 1	gi|194363762	21 kDa	0	2	0
E3 ubiquitin-protein ligase MIB1	gi|30348954	110 kDa	0	2	0
dephospho-CoA kinase domain-containing protein	gi|568815653	27 kDa	0	2	0

Table 1. Table of candidate ROP38 host interacting proteins through mass spectrometry analysis of immunoprecipitates. List of ROP38 host interacting candidates from immunoprecipitation of ROP38 (I) or TgME49_247520 (II). Mass spectrometry values are derived from total spectra.

Generation of ROP38 knockout in type II parasites

In order to investigate the role of ROP38 in modulating host transcription and parasite virulence *in vivo*, we targeted the ROP38 locus, together with the ROP29 and ROP19 expanded family loci nearby, for deletion from the type II strain (schematic shown in **Figure 3A**, primers used in knockout vector and diagnostic PCRs in **Supplementary tables 1 and 2**). We were able to successfully obtain 5' knockout bands from type II transfected populations and clone 1, but were unable to obtain it from clone 2, and checked for genomic DNA quality by 5' control bands (**Figure 3B**, top and middle panels). The 5' knockout band amplified from outside of the 5' region flanking ROP38 to the *hxgprt* gene only present in the knockout vector and indicated that integration of the knockout vector successfully occurred at the 5' flanking region of ROP38, through homologous recombination. We then determined the loss of ROP38A using allele specific primers, and were unable to amplify ROP38A from clone 1, though we could amplify ROP38A from parentals, transfected populations and clone 2 (**Figure 3B**, bottom panel).

It was reported that three copies of ROP38 exists in type II strains (Adomako-Ankomah et al. 2014). Thus, we wanted to determine whether ROP38B/C were deleted using allele non specific primers and were able to amplify ROP38B/C from transfected populations, clone 1 and clone 2. Moreover, we were still able to amplify ROP29 from the transfected populations, clone 1 and clone 2 (**Figure 3C**, top and middle panels). In addition, though we were able to obtain 3' control bands (**Figure 3C**, bottom panel), we were unable to obtain 3' knockout bands for all the clones isolated. Thus, it is likely that we obtained a partial knockout with ROP38A deleted, but other ROP38 copies and other gene loci such as ROP29 and ROP19 are still present and intact. We have attempted to knockout ROP38 together with other related loci such as ROP29 and

ROP19 twice, but we were not able to successfully obtain clones that have all ROP38, ROP29 and ROP19 copies deleted and obtained diagnostic PCR results similar to that shown in Figure 3 (data not shown).

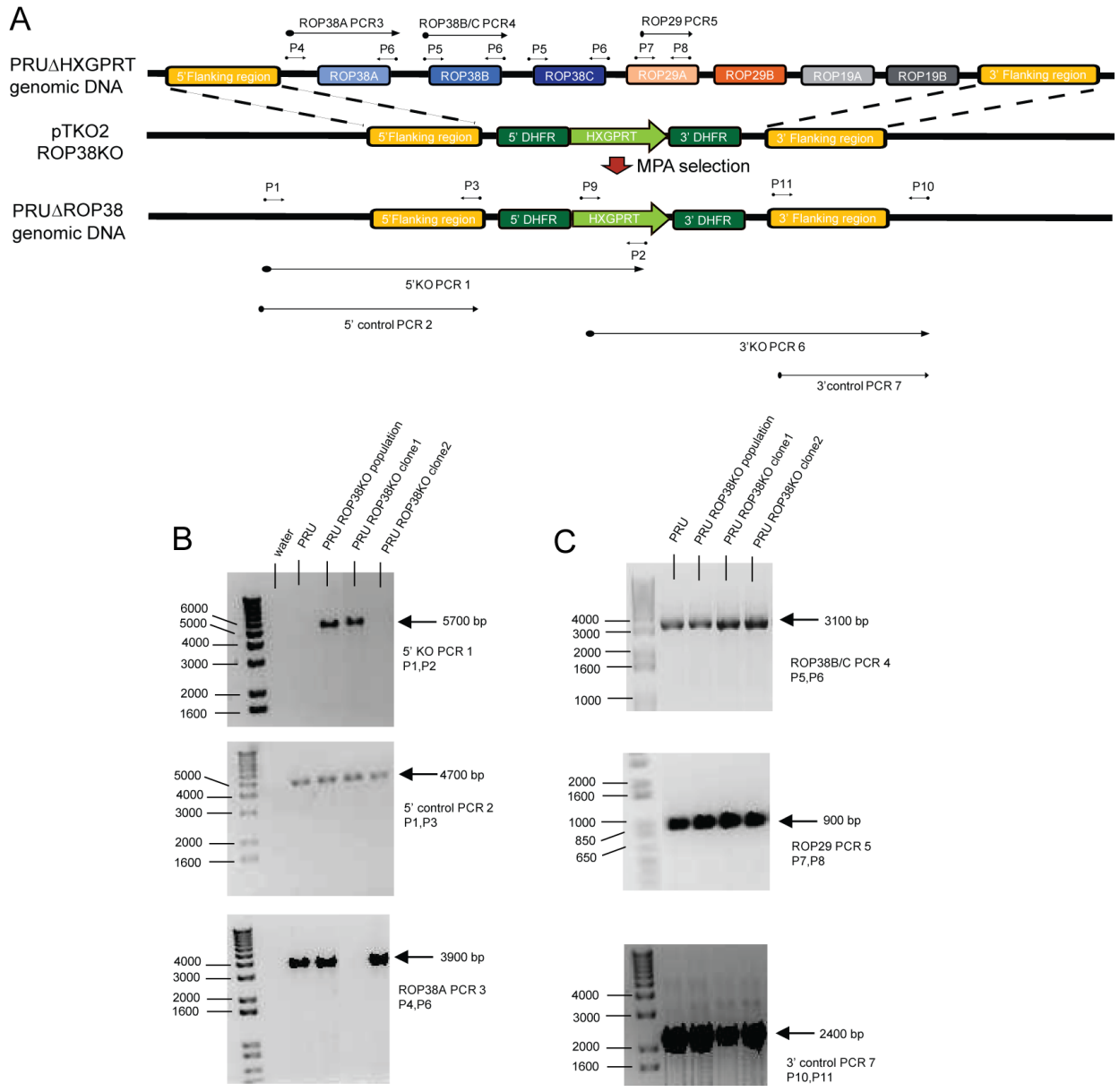


Figure 3. Generation of ROP38 KO in type II parasites. (A) Schematic of ROP38, ROP29 and ROP19 locus, not drawn to scale. Double homologous recombination between knockout construct (pTKO2 ROP38KO) and genomic DNA replaces the ROP38, ROP29, ROP19 locus with the *hxgprt* gene, which was used for positive selection. Primers used to confirm knockout and loss of loci are shown (P1-P11).

Genomic DNA was isolated from transfected PRUΔ*ku80*Δ*hxgprt* population and clones, and used for subsequent PCRs. (B) 5' knockout bands were obtained for the parental, transfected population and isolated clones (top, P1/P2 amplifying 5700bp fragment); 5' control bands were obtained to ensure genomic DNA quality control (middle, P1/P3 amplifying 4700bp fragment) and loss of ROP38A was checked by ROP38A allele specific primers (bottom, P4/P6 amplifying 3900bp fragment). (C) Loss of ROP38B/C loci was checked by ROP38 non allele specific primers (top, P5/P6 amplifying 3100bp fragment); loss of ROP29 loci was checked by amplifying ROP29 (middle, P7/P8 amplifying 900bp fragment) and 3' control bands were obtained to ensure genomic DNA quality control (P10/P11, amplifying 2400bp fragment), but 3' knockout bands (using P9/P10) were unable to be obtained.

ROP38 affects weight loss and cyst burden in vivo

To assay the effects of ROP38 *in vivo* in a type II background, we infected C57BL/6 mice with 500 tachyzoites intraperitoneally with type II parasites as follows: PRUΔ*ku80*Δ*hxgprt* (PRU), PRU overexpressing ROP38 (ROP38OE), PRUΔ*ku80*ΔROP38A clone 1 (ROP38KO) and PRUΔ*ku80*ΔROP38A clone 2 as a heterologous control (Het). We then monitored mouse weight loss and survival during the acute phase of infection (**Figures 4A and 4B**). We observed that even though there was no difference in survival between type II, type II overexpressing ROP38 and type II ROP38A knockout parasites, parasites overexpressing ROP38 (II) had less weight loss compared to the type II parental strain (**Figure 4A**). All mice were successfully infected, as seroconversion was tested day 30 post infection and all animals tested seropositive (data not shown), thus the effect of ROP38 on weight is not likely due to lack of invasion and replication *in vivo*.

As ROP38 is strongly differentially expressed during bradyzoite differentiation, we investigated whether chronically infected mice at day 30 post i.p. infection had differences in brain cyst burden. We observed that mice infected with type II ROP38A knockout parasites had increased number of brain cysts compared to mice infected with type II parental strains (**Figure 4C**), but due to the high variability between individual mice infected with the same strain, this was not significant (p-value = 0.18, Student's t test). We observed that the heterologous control had significantly decreased number of cysts compared to type II parental (p-value = 0.05, Student's t test), consistent with reports of lower cyst numbers where *hxgprt* is ectopically present in the PRUΔ*ku80*Δ*hxgprt* background (Fox et al. 2011). In addition, as IFN-γ has been established as a major mediator of resistance to *Toxoplasma*, we investigated whether ROP38 has an effect on IFN-γ levels *in vivo*. IFN-γ serum levels of mice infected with type II ROP38A deleted or ROP38 overexpressing strains were not significantly different from type II parental (**Figure 4D**), either early during acute infection (day 7) or after chronic infection has been established (day 30).

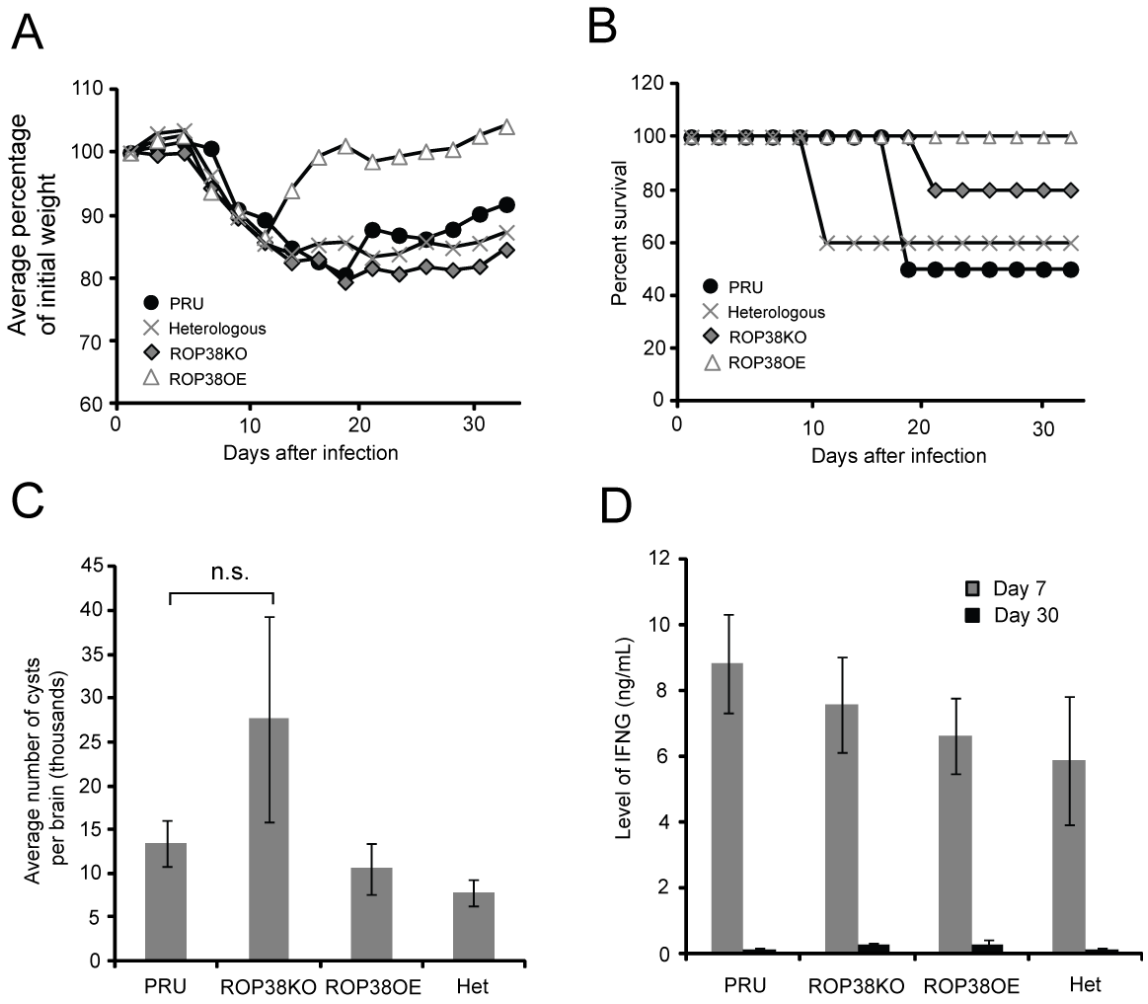


Figure 4. ROP38 affects cyst formation in vivo, but does not affect virulence. (A) Average percentage change of weight compared to initial weight, measured over 35 days after intraperitoneal injection with 500 tachyzoites. Points on graph show average weight of at least three individual animals infected per parasite strain. (B) Percent survival after i.p. injection over 35 days, of at least three mice per indicated strain. (C) Average number of cysts per brain at day 35 following i.p. injection, of at least three mice per strain. Graph shows average, error bars represent standard deviation. (D) IFN-γ cytokine levels in peripheral blood serum of infected mice were measured by ELISA day 7 and day 30 post infection. Graph shows average, error bars represent standard deviation. PRU- PRUΔ*ku80*Δ*hxgprt*; ROP38KO - PRUΔ*ku80*ΔROP38A clone1; ROP38OE – PRU overexpressing ROP38 (II); Het – heterologous control - PRUΔ*ku80*ΔROP38A clone2.

We also wanted to investigate whether ROP38 could have an effect *in vivo* upon oral infection, which is the natural route by which *Toxoplasma* is transmitted in the environment. Thus, we performed oral gavage of 1000 cysts derived from chronically infected mice, and monitored weight loss and survival across the acute phase of infection. We observed that there were no differences in survival between mice infected with type II wild type, ROP38A deleted or ROP38 overexpressing strains (data not shown). However, we observed a similar gain in weight for mice infected with the ROP38 overexpressing strains compared to the type II wild type and slightly increased weight loss in ROP38A knockout strain (**Figure 5A**). We also investigated cyst burden when these orally infected mice became chronically infected, and observed that similar to i.p. infection, the ROP38A knockout strain had increased cyst numbers, though it was not significant (p-value = 0.08, Student's t test) while the ROP38 overexpressing strain had decreased cyst numbers compared to the type II wild type strain (p-value = 0.11, Student's t test) (**Figure 5B**).

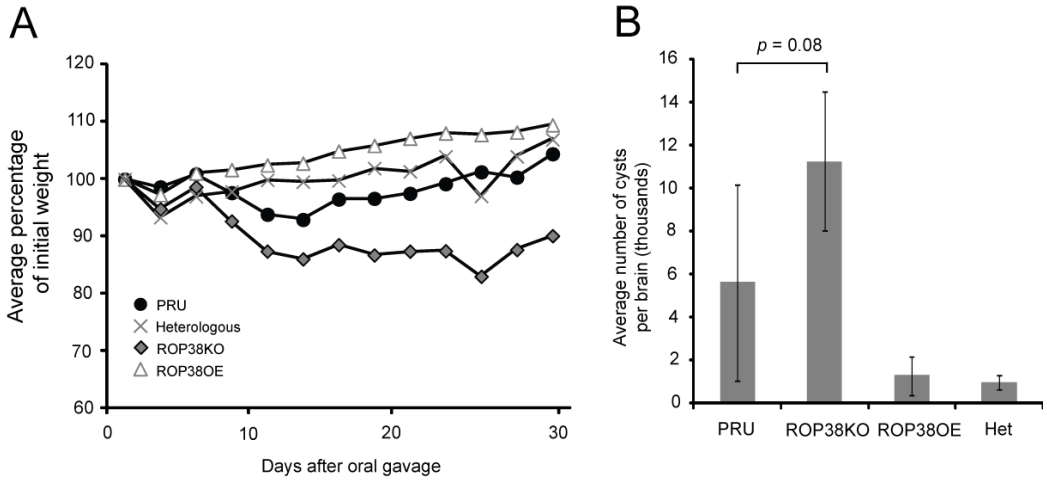


Figure 5. ROP38 affects weight loss and cyst counts upon oral infection. (A) Average percentage change of weight compared to initial weight, measured over 30 days after oral gavage with 1000 cysts. Points on graph show average weight across at least 4 individual animals. (B) Average number of cysts per brain at day 30 following oral gavage, of at least 3 mice per strain. Graph shows average, error bars represent standard deviation. PRU- PRUΔ*ku80*Δ*hxgprt*; ROP38KO - PRUΔ*ku80*ΔROP38A clone1; ROP38OE – PRU overexpressing ROP38 (II); Het – heterologous control - PRUΔ*ku80*ΔROP38A clone2.

Discussion

We observed that the ROP38 sequence is conserved across canonical and atypical *Toxoplasma* strains. This could be a sign of ROP38 under purifying selection and suggests a conserved functional role for ROP38, as nonsynonymous mutations are deleterious and eliminated under selective pressure (Nielsen 2005). *Toxoplasma* genes under diversifying selection have been increasingly characterized, such as the expanded ROP5 family (Reese, Shah & Boothroyd 2014) and ROP16 (Melo et al. 2013), but purifying selection has been reported for *Toxoplasma* surface antigen genes (Wasmuth et al. 2012), which mediate parasite attachment to host cells and are involved in chronic infection. In addition, we and others (Adomako-Ankomah et al. 2014) have observed that ROP38 is tandemly duplicated, and it has been reported that duplicated genes evolve under purifying selection (Kondrashov et al. 2002).

Overexpression of ROP38 in the type II background caused repression of NF-κB activation, but this NF-κB repression was not reflected in p65 nuclear translocation in cells infected with type II overexpressing ROP38 parasites. This difference could be because other subunits such as c-Rel or p52 are involved in ROP38 repression but the NF-κB reporter cell line has promoter binding sites for all the NF-κB subunits. There are numerous examples of pathogen effectors which inhibit NF-κB activation (Rahman, McFadden 2011), but with GRA15 (II) as the *Toxoplasma* effector responsible for NF-κB activation in type II strains, there is likely a more complex interplay of effectors present. The spatio-temporal regulation of *Toxoplasma* effectors and how they interact with one another have begun to be investigated (Etheridge et al. 2014, Alaganan et al. 2014), but the detailed mechanisms of this interplay of effectors remain to be elucidated.

In addition, ROP38 did not significantly affect downstream NF-κB dependent responses such as IL-12 cytokine secretion. Even though IL-12p40 regulation has been linked to NF-κB activation (Robben et al. 2004), mice deficient in NF-κB subunits such as p50, p52 or RelB do not have significantly decreased levels of IL-12 upon *Toxoplasma* infection (Caamano et al. 1999, Caamano et al. 2000). Moreover, production of IL-12p40 involves NF-κB independent pathways such as the interferon response (Wang et al. 2000, Mason et al. 2002). It is likely that other *Toxoplasma* effectors, in addition to GRA15 and ROP16, modulate the interferon response as *Toxoplasma* infection leads to inhibition of global IFN-γ induced gene expression (Rosowski, Saeij 2012). Thus, though ROP38 represses NF-κB activation, it may not affect other NF-κB independent pathways involved in production of IL-12p40, and this could be a likely reason as to why no significant difference in IL-12p40 levels between type II and type II overexpressing ROP38 infected macrophages was observed.

Though ROP38 overexpression delayed the kinetics of ERK1/2 phosphorylation (Peixoto et al. 2010), it is uncertain whether ROP38 acts on MAPKs directly, or interact with upstream adaptors of MAPKs. To answer this question, we identified candidate ROP38 interacting host proteins upon ectopic expression of ROP38 through immunoprecipitation and our results included upstream kinases such as calmodulin dependent protein kinase IV (CaM-KIV) and receptor type tyrosine protein phosphatase gamma. CaM-KIV is involved in the transmission of signal to the MAPKs, especially p38 and JNK (Enslen et al. 1996), and interaction between serine/threonine phosphatase 2A (PP2A) and CaM-KIV in a stable complex results in inhibition of CaM-KIV (Anderson et al. 2004). Identification of these host proteins suggests that ROP38 acts upstream of the MAPKs, rather than direct interaction with MAPKs. However, these mass spectrometry

results require validation through further immunoprecipitations using the ROP38 overexpressing cell line and detection of candidate interacting proteins in immunoprecipitates using specific antibodies against the candidate host proteins identified. In addition, it would be important to validate this interaction in the parasite setting, by infecting host cells with parasites overexpressing ROP38, immunoprecipitating ROP38 and probing for these candidate interacting proteins.

We have been unable to obtain a deletion of the entire region encompassing ROP38 and the other related kinases, ROP29 and ROP19. A possible reason for this could be synthetic lethality, where deletion of single genes leads to a viable phenotype but the combination of deletions in two or more genes lead to an inviable organism (Tong, Boone 2006). Another possibility is one or more of the targeted rhoptry kinases being essential for parasite invasion or replication. An essential *Toxoplasma* kinase, calcium-dependent protein kinase 1 (CDPK1), is required for host cell invasion and exit from the host cell and viable parasites can only be obtained under conditional knockout conditions (Lourido et al. 2010).

We observed weight gain and weight loss during acute infection when ROP38 was overexpressed and partially deleted respectively, indicating that ROP38 could function as a repressor of virulence. Moreover, there were increased brain cyst burdens in mice infected with the type II ROP38A knockout strain, though this was not significant due to high experimental variability. In *Toxoplasma*, deletion of serine protease inhibitor 1 (TgPI1) also led to a similar increased cyst burden phenotype (Pszenny et al. 2012). How ROP38 act to repress bradyzoite differentiation *in vivo* remain to be elucidated. It could be differences in parasite dissemination leading to different numbers of tachyzoites reaching the brain or differences in immune

responses leading to varying efficacy rates in tachyzoite to bradyzoite conversion *in vivo*, though we did not see differences in serum cytokine levels upon ROP38 knockout or overexpression.

The increase in cyst burden and weight loss in mice infected with the type II ROP38A knockout strain was observed to be magnified upon oral gavage with derived brain cysts, in comparison to intraperitoneal infection. Oral gavage is more reflective of how *Toxoplasma* is transmitted in the natural environment, and different kinetics of *Toxoplasma* dissemination through the host has been reported, depending on the route of infection (Zenner et al. 1998). It is possible that ROP38 affects the efficiency of oral infection by affecting bradyzoite to tachyzoite conversion in the intestine, and previous reports have shown that a *Toxoplasma* pseudokinase, bradyzoite pseudokinase 1 (BPK1), is required for efficient oral infection (Buchholz et al. 2011). Another possibility is ROP38 affecting the ability of the host to regulate cyst burden through the host immune response, but differentiation between these mechanisms leading to cyst burden differences await further investigation.

We report that ROP38 is able to repress the NF-κB response, though downstream NF-κB dependent responses were not affected. In addition, our results suggest that ROP38 likely interacts with upstream MAPK adaptors to repress host transcriptional responses. ROP38 also represses morbidity and cyst formation *in vivo*, and the mechanistic understanding of the effects of ROP38 *in vivo* warrant further examination.

Materials and Methods

Phylogenetic alignment

Toxoplasma strains were cultured on human foreskin fibroblasts, and the following strains were used in this study: RH (type I), RH-JSR (type I), GT1 (type I); ME49 (type II); CEP (type III); FOU, CAST, MAS, CASTELLS, TgCatBr5, COUGAR, GUYMAT (Melo et al, 2013). ROP38 was PCR amplified with the following primers (Forward 5'-ATCGTGCCAATGTGTGTTGT-3', Reverse 5'-ACACCATCTTGCTCGTTGC-3'). Evolutionary analysis was performed on the nucleotide alignment using MEGA5, with the tree inferred from 500 replicates using the Maximum Likelihood method. The synonymous-nonsynonymous analysis program (SNAP) was used to calculate the proportion of synonymous and non-synonymous changes in coding regions (Korber, 2000).

Reporter cell line construction

The HEK293 NF-κB reporter cell line was obtained from System Biosciences, and used as previously described (Yang et al. 2013). SRF (TR029PA-1) pGreenFire1 (pGF1) lentiviral reporter vectors containing a Neo selection cassette and a minimal CMV promoter followed by four tandem consensus SRF sites driving the expression of Firefly luciferase was purchased from System Biosciences. Each individual vector was co-transfected using FuGene 6 reagent (Roche) with vectors containing gag, pol and VSV-G proteins, according to manufacturer's instructions. Supernatant containing virus was collected three days after transfection, and added to HEK293 cells (ATCC) with polybrene (Sigma). Geneticin (Invitrogen) was used at 750 µg/ml to select HEK293 cells with the integrated constructs, and were subsequently cloned by limiting dilution.

Phorbol 12-myristate 13-acetate (PMA, Enzol Life Sciences) was used at 10 ng/ml to test the SRF reporter.

Luciferase assays

HEK293 pGF1-SRF cells were plated in 96 well plates (Corning) at 4*10^4 cells per well, and grown for 16 hours. Cells were then stimulated with either inducing chemicals for 24 hours or infected with PRUΔ*hxgprt*, or PRU overexpressing ROP38 (II) at varying MOIs for 24 hours. Cells were lysed with 40µl of cell culture lysis reagent (Promega) containing 1x protease inhibitor (Roche), and stored at -80°C. 100µl of luciferase assay substrate (Promega) was added per well, and luciferase readings read using Varioskan flash reader (Roche). Luciferase readings were normalized to unstimulated, uninfected cells.

Nuclear p65 quantification

HFFs were left uninfected, stimulated with TNF-alpha for 24 hours or infected with PRUΔ*hxgprt*, or PRU overexpressing ROP38 (II) at varying MOIs for 24 hour. These were fixed with 3% formaldehyde (vol/vol) for 20 minutes, blocked with 5% BSA (vol/vol) and incubated with p65 (anti-rabbit, sc-109, 1:500) at 4°C overnight. Secondary and Hoechst antibodies were incubated for 1 hour at room temperature to visualize p65 and DNA respectively, and quantification of mean nuclear p65 intensity for at least 10 cells per condition was measured using Nikon microscope.

In vitro cytokine ELISAs

C57BL/6 bone marrow derived macrophages were isolated (Jensen et al, 2010) and plated in DMEM, supplemented with 20% L929 supernantants, two days before infection. Parasites were syringe lysed, washed once with PBS and three different MOIs per strain were used to infect uninfected macrophages. After 24 hours infection, supernants from uninfected and infected cells were collected and stored at -80°C until ELISAs were performed. IL12-p40 and CCL2 levels in culture supernatants were determined using commercially available ELISA kits (ELISA DuoSet, R&D Biosystems), according to manufacturer's instructions. IFN-γ levels in peripheral blood serum from mice were determined using commercially available ELISA kits (eBioscience), according to manufacturer's instructions.

Inducible TRex 293 cell line construction

Primers were designed to amplify after the predicted signal peptide to the predicted stop codon. Forward primers to amplify ROP38 (5'-**TGGCTGGTGCTGGTGCCCAT**CATGGCAGCAGCACTGATGGATCAG-3') together with reverse primers (5'-**GCTCCGGCTCCTGCCCCAGC**AAATTGATGCGTTCTTATCCGA-3'), contained Ligation independent cloning (LIC) sequences (in bold), and were used to amplify ROP38 from RH (I) genomic DNA. PCR products were treated with T4 DNA polymerase (using only TTP at 100mM). The pcDNA-LIC-HF vector (gift from A. Boughdour and M.A. Hakimi) was digested with SmaI, and treated with T4 DNA polymerase (using only ATP at 100mM) to generate long overhangs. The PCR fragment and vector were then annealed for 15 minutes at room temperature, generating expression vectors with ROP38 C terminally tagged with HA-

FLAG. TRex-293 cells (gift from J. Niles) were seeded at 75% confluency, and cotransfected with expression vectors and puromycin resistance vector (ratio of 10:1), using XtremeGENE 9 DNA transfection reagent (Roche). Cells were split 2 days post transfection, and subjected to puromycin (Calbiochem) selection at 1µg/ml. Foci were picked one and a half weeks post selection and expanded, and positive foci were selected through HA expression by immunofluorescence and immunoblotting.

Co-immunoprecipitation and immunoblotting

TRex-293 overexpressing ROP38 (I) or TgME49_247520 (II) cells were grown in T25s until 100% confluency, and induced with tetracycline (1µg/mL) for 24 hours. Cells were then scraped in ice-cold PBS and centrifuged at 1500rpm for 5 minutes, and washed twice with 1x PBS with 1mM PMSF (phenylmethylsulfonylfluoride, Thermo Scientific) through centrifuging at 13,000 rpm for 1 minute at 4°C. Cells were lysed in 500µl lysis buffer (300 mM NaCl, 50 mM HEPES pH 7.5, 0.5% NP-40) for 1 hour at 4°C, and soluble fraction obtained by centrifuging at 13,000 rpm for 15 minutes at 4°C. The soluble fraction was incubated with conjugated Dynabeads G:HA beads overnight at 4°C, rotating, and washed 5 times with HBS (10 mM HEPES-KOH, pH 7.5, 150 mM NaCl). Beads were then boiled in sample buffer with DTT (dithiothreitol, 100mM) for 5 minutes and subject to either Western blotting or colloidal Coomassie (Invitrogen) staining for further mass spectrometry analysis. Cell lysates and immunoprecipitates were subject to 10% SDS-PAGE, proteins transferred to PVDF membrane and blocked with PBS/0.1% Tween-20/5% nonfat dry milk, incubated with anti-rat HA (Roche, 1:500) or anti-GAPDH (Santa Cruz, sc-32233, 1:500) overnight at 4°C and followed by secondary antibodies.

Mass spectrometry

SDS Page gels were immersed in staining solution (20% methanol, 80% colloidal Coomassie) for 3 hours on a rotating platform, and destained using milliQ water overnight on rotating platform, until visible bands were seen. Each lane encompassing the entire molecular weight was then excised and subject to trypsin digest. These were then analyzed by reversed phase HPLC and a ThermoFisher Orbitrap linear ion trap mass spectrometer. Peptides were identified from the MS data using SEQUEST algorithms 44 that searched a species specific database generated from NCBI's non redundant database.

Generation of ROP38 knockout parasites

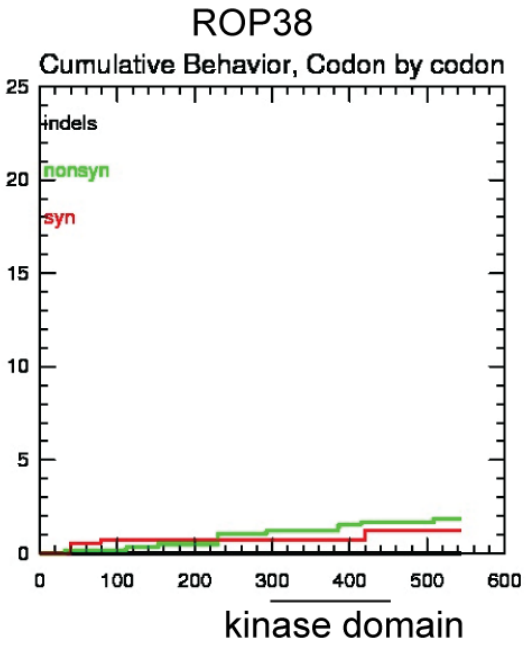
A targeting construct was engineered using pTKO2 (Rosowski et al. 2011) using the Multisite Gateway Pro 3-fragment Recombination (Invitrogen). The 5' and 3' flanking regions of ROP38 was cloned from type II (PRU) genomic DNA. Primers contained att recombination sites (denoted in bold) and amplified 1,982 base pairs, 2386 bp upstream of the annotated ROP38 start codon (5' forward primer, 5'-**GGGGACAAGTTTGTACAAAAAAGCAGGCTT** AGAAATTAGAAACTCTT-3', 5' reverse primer, 5'-**GGGGACAACTTTGTAT AGAAAAGTTGGGTG**CACGTGTTCCGTCCAG–3'), or amplified 2,103 base pairs, 968 bp downstream of the annotated ROP19B stop codon (3' forward primer, 5'-**GGGGACA ACTTTGTATAATAAAGTTGCTGCT**AACTGGGTAGCCTGCTG-3', 3' reverse primer, 5'-**GGGGACCACTTTGTACAAGAAAGCTGGGTA**TCGCAAGACCGACCTATACC-3'). These flanking regions were then cloned around the hypoxanthine-xanthine-guanine-ribosyl transferase (*hxgprt*) selectable marker flanked by 5' and 3' untranslated regions from DHFR.

Before transfection, the knockout vector was linearized with restriction enzyme Not1-HF (New England Biosystems), and the linearized vector was transfected into PRUΔ*hxgprt*Δ*ku80* through electroporation in 2mm cuvettes (Bio-Rad) with 2mM ATP and 5mM GSH in Gene Pulser Xcell (Biorad), with the following settings: 25uFD, 1250kV, ∞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. Sequences of primers used in the diagnostic PCRs are in Supplementary Table 2.

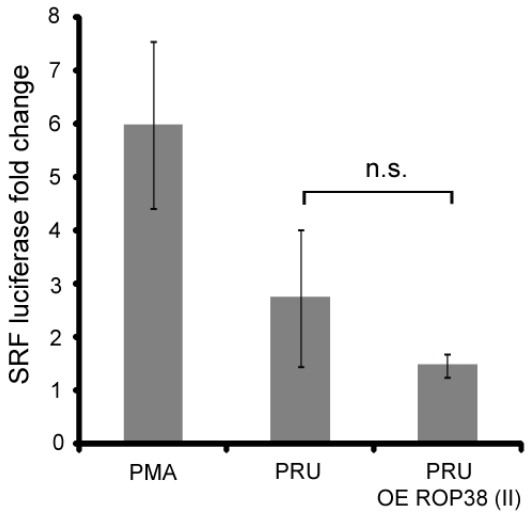
Animal infections

Six to 10 week old female C57Bl/6 mice (Jackson Laboratory) were used in all experiments. For intraperitoneal infections, tachyzoites were grown *in vitro* and syringe-lysed through 30-gauge needles and washed twice with PBS. Mice were then inoculated intraperitoneally with 500 tachyzoites using a 28-gauge needle. For oral gavage of mice, brains from chronically infected mice were harvested, emulsified in phosphate buffered saline (PBS) and stored at 4°C before use, where 1000 cysts were administered per mouse via feeding needle. Brain homogenates of chronically infected mice after day 30 were collected and one tenth was fixed with methanol, stained with dolichos biflorus-FITC overnight at 4°C and total number of cysts per brain was counted through microscopy. Peripheral blood serum was collected on days 7 and days 30 after i.p. injection, and levels of IFNγ and IL12-p70 were measured using commercially available ELISA kits (eBioscience, USA), according to the manufacturer's instructions. The Massachusetts Institute for Technology Committee on Animal Care approved all protocols. All mice were maintained under specific pathogen-free conditions, in accordance with institutional and federal regulations.

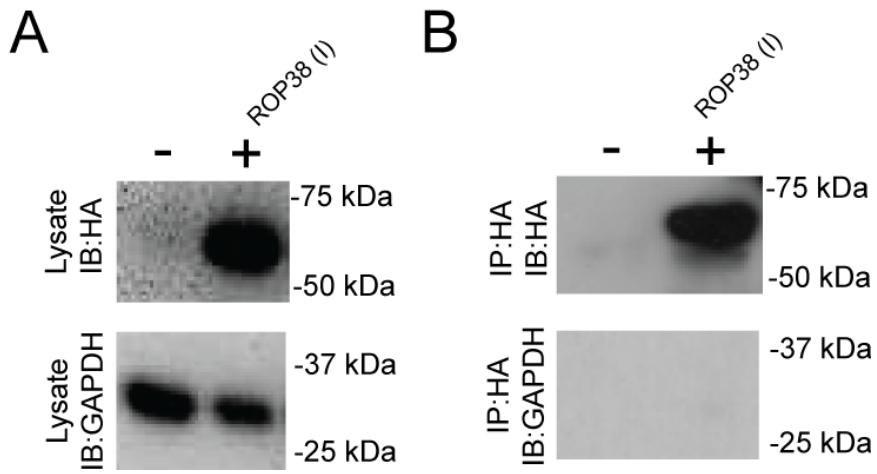
Supplementary Figures



Supplementary Figure 1. ROP38 is not under diversifying selection. Cumulative behavior, codon by codon, of synonymous (red) and nonsynonymous (green) and insertion/deletion (black) mutations in ROP38.



Supplementary Figure 2. ROP38 does not repress SRF. An SRF reporter cell line was stimulated with PMA as a positive control, or infected with PRU (II) and PRU overexpressing ROP38 (II) strains, and luciferase activity was measured 24 hours post infection. Graph is the average of three independent experiments, and error bars represent standard deviation.



Supplementary Figure 3. Identification of candidate host interacting proteins with ROP38. (A) Whole cell lysates of TRex-293 ROP38 (I) were induced with tetracycline (1µg/ml), harvested 24 hours post-induction and probed with anti-HA (top) or anti-GAPDH (bottom) as a loading control. (B) TRex-293 ROP38 (I) were immunoprecipitated with anti-HA antibodies and probed with anti-HA(top) or anti-GAPDH (bottom).

Supplementary Tables

Primer_name	Primer_sequence
Forward primer for 5' flanking region	**GGGGACAAGTTTGTACAAAAAAGCAGGCTT**AGAAATTAGAAACTCTT
Reverse primer for 5' flanking region	**GGGGACAACTTTGTATAGAAAAGTTGGGTG**CACGTGTTCCGTCCAG
Forward primer for 3' flanking region	**GGGGACAACTTTGTATAATAAAGTTGCTGCT**AACTGGGTAGCCTGCTG
Reverse primer for 3' flanking region	**GGGGACCACTTTGTACAAGAAAGCTGGGTA**TCGCAAGACCGACCTATACC

Supplementary Table 1. Primer sequences of primers used in generating the ROP38 knockout vector. Bold indicate att sites used in Gateway Recombination cloning.

Primer_name	Primer_sequence
P1	CGGACGTACGAACCGAGTAT
P2	GATCCAGACGTCTTCAATGC
P3	**GGGGACAACTTTGTATAGAAAAGTTGGGTG**CACGTGTTCCGTCCAG
P4	ATCGTGCCAATGTGTGTTGT
P5	CACCCGAGAGGGAAGCAACGTTTA
P6	ACACCATCTTGCTCGTTGC
P7	GGTGTCCTCTTCCCAGTTGA
P8	*CGCGTAGTCCGGGACGTCGTACGGGTA*AATGCATGTGTCACCGCCAGAA
P9	ACACCTTCTACAACGCTGAT
P10	GACTCAGACGACGACGTGAA
P11	**GGGGACAACTTTGTATAATAAAGTTG**CTGCTAACTGGGTAGCCTGCTG

Supplementary Table 2. Primer sequences of primers used in checking ROP38KO in type II background. Italics indicate HA tag, bold indicate att sites used in Gateway Recombination cloning.

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Chapter Five – Conclusions and Future Directions

Conclusions

This thesis presents work on using strain differences to identify and characterize *Toxoplasma* effectors leading to differences in parasite phenotypes and modulation of innate immune pathways by *Toxoplasma gondii* (**Figure 1**). Using comparative genomics between type I strains, we identified polymorphisms in *Toxoplasma* dense granule protein, GRA2, to account for differences in IFN-γ susceptibility between type I parasite strains. In addition, polymorphisms in another *Toxoplasma* effector, GRA15, accounted for differences in NF-κB activation upon infection by different type I strains. These findings show that genetic differences between distinct *Toxoplasma* strains lead to differences in modulation of host innate immune pathways. These differences in the host immune response in turn likely lead to varying outcomes upon *Toxoplasma* infection of the host.

We further characterized the mechanism of action for GRA15 leading to NF-κB activation through immunoprecipitation of GRA15 upon ectopic expression. We identified a likely interaction between GRA15 and TRAF2, an upstream adaptor protein of the NF-κB pathway. In addition, an N terminal region of GRA15 was determined to be required for NF-κB activation through GRA15 structure-function analysis. Interestingly, both alleles of GRA15 from type II and III *Toxoplasma* strains are able to cause NF-κB activation upon ectopic expression, though infection by type II strains leads to NF-κB activation and infection by type III strains do not cause NF-κB activation. This suggested other inhibitory *Toxoplasma* effectors present in type III strains, such as rhoptry kinase ROP38, which is highly expressed in type III strains.

We then showed that ROP38 represses NF-κB activation, though ROP38 did not affect downstream NF-κB dependent cytokine levels such as IL-12. We investigated the mechanism of action for ROP38 leading to host transcriptional repression through immunoprecipitation of ROP38 upon ectopic expression. Several candidate ROP38 interacting host proteins were identified to be upstream kinases of the MAPK pathways, suggesting that ROP38 likely acts upstream of the MAPKs. Deletion of ROP38 leads to increased weight loss during acute infection of mice and increased brain cyst burden upon onset of chronic infection in mice, suggesting that ROP38 functions as a repressor of virulence. Characterization of these two *Toxoplasma* effectors GRA15 and ROP38, which have opposing effects on the NF-κB pathway, will enable further insights into the interplay of *Toxoplasma* effectors.

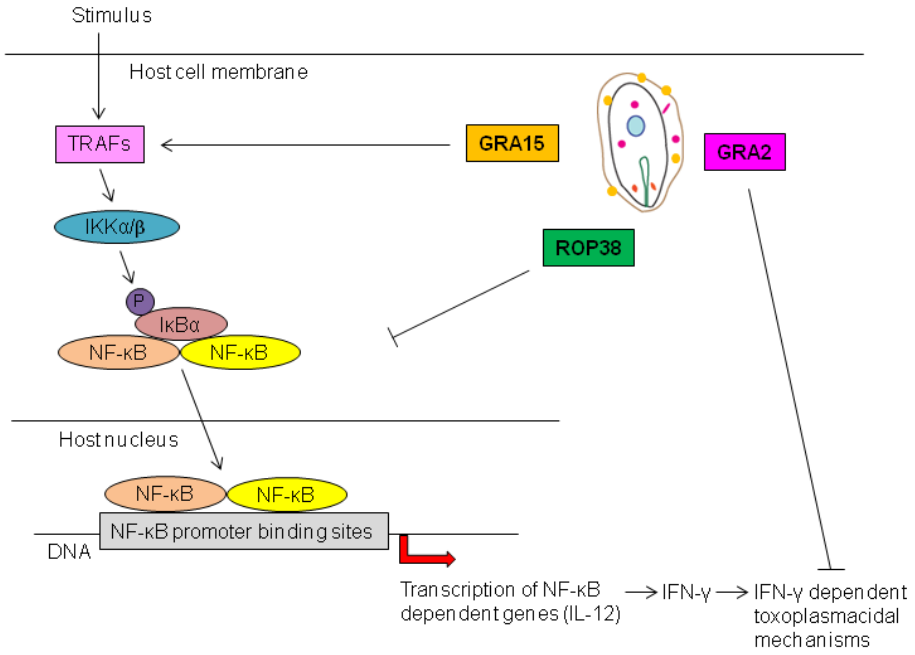

Figure 1. Proposed model of strain specific *Toxoplasma* effectors modulating innate immune pathways. GRA15 likely interacts with TRAF2 to cause NF-κB activation upon *Toxoplasma* infection while ROP38 represses NF-κB activation, though the mechanism remains unclear. Strain specific polymorphisms in GRA2 lead to differences in IFN-γ susceptibility between different *Toxoplasma* strains, which is important in determining *in vivo* virulence of *Toxoplasma*.

Discussion and Future Directions

We systematically identified genetic differences and parasite gene expression differences within *Toxoplasma* type I strains, and identified strain specific polymorphisms in *Toxoplasma* effectors leading to differential host responses. We also furthered understanding of the mechanism of action for GRA15, and the role of ROP38 during *in vivo* infection, but many more questions remain in understanding how these *Toxoplasma* effectors modulate innate immune pathways.

Why does the GRA2 polymorphism between RH and GT1 lead to differences in IFN-γ evasion?

We observed that complementing RH GRA2KO with either RH GRA2 or GT1 GRA2 led to differences in evading the IFN-γ response *in vitro*, but did not lead to differences in *Irgb6* coating (**Chapter II, Figure 1**). It is possible that other IRGs, such as *Irga6*, *Irgb10 or Irgd*, could be involved in addition to *Irgb6*. Indeed, knockout mice lacking *Irga6* or *Irgd* are highly susceptible to acute *Toxoplasma* infection (Liesenfeld et al. 2011, Collazo et al. 2002). IFN-γ also induces expression of guanylate binding proteins (GBPs), which belong to the same superfamily of IFN-inducible GTPases as the IRGs. Particular GBP members localize to the PV together with the IRGs (Virreira Winter et al. 2011) and mice deleted for a GBP cluster, including six GBP members, exhibit increased susceptibility upon acute *Toxoplasma* infection (Yamamoto et al. 2012). Another distinct possibility is that other IFN-γ dependent pathways independent of IFN-inducible GTPases are involved, such as nitric oxide production (Yarovinsky 2014), leading to differences in the IFN-γ evasion response. Even though GRA2 is involved in the tubulovesicular network formation of the PVM (Mercier et al. 2002), it is possible that GRA2 has functions independent of PVM formation.

Would GRA2 and GRA15 polymorphisms affect the RH/GT1 difference in virulence upon re-infection during chronic infection in vivo?

We identified GRA2 and GRA15 to be polymorphic between RH and GT1, and showed that GRA2 and GRA15 lead to differences in IFN-γ evasion and NF-κB activation between RH and GT1 infected cells respectively *in vitro* (**Chapter II, Figures 1 and 4**). Both of these innate immune responses are crucial in mediating resistance *in vivo* to acute and chronic *Toxoplasma* infection (Suzuki et al. 1988, Caamano et al. 1999). Though both RH and GT1 are acutely virulent with LD_{100}~1, it has been observed that mice chronically infected with type III strains do not exhibit weight loss or death upon re-infection with RH, but die rapidly upon re-infection with GT1 (Jensen et al, submitted). IFN-γ is important in controlling *Toxoplasma* cyst reactivation during chronic infection, thus polymorphisms in GRA2 leading to IFN-γ evasion differences could be involved in this RH/GT1 *in vivo* difference. The RHΔ*gra2* deleted strain and RHΔ*gra2*+GT1-GRA2 or RHΔ*gra2*+RH-GRA2 complemented strains could be used to infect chronically infected mice to determine the role of GRA2. Polymorphisms in GRA15 leading to NF-κB activation differences could also be involved in this RH/GT1 difference *in vivo*, but there were no differences in mortality or morbidity between RH and RH+GRA15(GT1) parasites upon re-infection of chronically infected mice (Kirk Jensen, personal communication). Thus it is unlikely that GRA15 is involved in the RH/GT1 mortality difference upon re-infection. Interestingly, another *Toxoplasma* surface antigen SRS29C has a RH/GT1 polymorphism, is highly expressed in type I strains and overexpression of SRS29C in RH attenuates its virulence during acute infection (Wasmuth et al. 2012).

How does GRA15 interact with candidate host proteins to cause NF-κB activation?

It is possible that GRA15 interacts with one or more proteins to achieve NF-κB activation. This has been observed in the Epstein Barr Virus effector LMP-1, which contain two domains, CTAR1 and CTAR2, interacting with upstream NF-κB adaptors such as TRAF2, TRAF6 and RIPK1 (Kung, Raab-Traub 2010, Song, Kang 2010). To further elucidate the mechanism of action for GRA15, we identified candidate host proteins that interact directly with GRA15 or are in the same complex as GRA15 through immunoprecipitation, followed by mass spectrometry. We identified the upstream adaptor TRAF2, which could be involved in GRA15 dependent NF-κB activation, and detected TRAF2 in GRA15 (II) and GRA15 (III) immunoprecipitates through immunoblotting (**Chapter III, Figure 3D**), but this requires further validation through reciprocal immunoprecipitations. In addition, immune cell types could have different expression of TRAFs or other host proteins interacting with GRA15 compared to HEK293, so it would be important to confirm the GRA15 interaction by ectopic expression in macrophages. Furthermore, ectopic expression of GRA15 in the cell alone excludes the parasite, and this interaction has to be validated in the context of infection. Thus, knockdown (using RNAi) or knockout (using the CRISPR-Cas system) of these GRA15 host interacting partners such as TRAF2 should abolish NF-κB activation upon infection with type II or RH overexpressing GRA15 (II) parasites.

Identifying interacting proteins with GRA15 still elicits questions about how GRA15 functions on a molecular level. GRA15 could modify host interacting proteins to subvert their normal cellular functions for NF-κB activation or alternatively, GRA15 could act as a scaffold protein to assemble two or more signaling adaptors (Shaw, Filbert 2009), leading to NF-κB activation. GRA15 does not have homology to conserved functional domains, but this does not exclude

functional activity for protein modification or scaffold. To address this, we could examine the difference in molecular weight between nascent and modified interacting host proteins using mass spectrometry or probe using specific antibodies that recognize these modifications to determine the possible post-translational modification (like ubiquitination or glycosylation).

Furthermore, GRA15 has no primary sequence homology to other known proteins and has no predicted secondary and tertiary structure with structural resemblance to conserved functional domains, using numerous protein prediction programs (data not shown, (Kelley, Sternberg 2009)). However, with the GRA15 truncation mutant-function analysis, we found that an N terminal region of GRA15 close to the signal peptide was necessary for NF-κB activation and this region has increased order compared to the whole GRA15, which is relatively disordered. We also noted that another middle region of GRA15 has increased order using the same algorithm (**Chapter III, Figure 5C**), and GRA15 truncation mutants deleting this region could be engineered to determine whether it is also necessary for NF-κB activation. To determine whether these GRA15 regions are required for interaction with host proteins, we would transfect cells with epitope tagged GRA15 full length and truncation mutants, immunoprecipitate GRA15 using epitope specific antibodies and immunoblot for interacting partners.

What is the role of GRA15 in vivo in species without TLR11/12, such as humans?

During i.p. *in vivo* infection, there was no significant difference in mouse survival between type II and type II GRA15 knockout, though there was increased parasite burden and significantly reduced IFN-γ early (Rosowski et al. 2011). It is likely that later release of parasite profilin binds TLR11/12 and causes downstream NF-κB activation and enables control of type II parasites

during i.p. infection (Yarovinsky 2014). However, in other species such as humans, where TLR11 is a pseudogene, GRA15 likely plays a more important role in activating NF-κB and controlling acute infection, or starting chronic infection by stimulating tachyzoite-bradyzoite conversion. GRA15 is the *Toxoplasma* effector responsible for IL-1β secretion in human immortalized monocytic cell lines (Gov et al. 2013), and an intracellular sensor (NLRP1) which leads to IL-1β processing has been implicated in susceptibility to human congenital toxoplasmosis (Witola et al. 2011). In addition, GRA15 is also responsible for CD40 expression in infected human THP-1 differentiated macrophages (Morgado et al. 2014), and CD40/CD40L interaction is required to induce IL-12 secretion and generate an effective IFN-γ response in humans (Subauste et al. 1999).

It is possible that GRA15 plays a role in chronic *Toxoplasma* infection, since bradyzoite conversion is dependent on an active immune response (Weiss, Kim 2000). For example, IFN-γ dependent nitric oxide has been implicated in bradyzoite conversion in macrophages (Skariah, McIntyre & Mordue 2010). By the natural oral route of infection, there is increased parasite burden upon GRA15 deletion, though there were no significant differences between type II parental strains and GRA15 deleted strains for *ex vivo* cytokine production (Jensen et al. 2013). Currently, it is unclear whether there are differences in brain cyst numbers or tachyzoite to bradyzoite conversion efficiency between type II and type II GRA15 KO chronically infected mice either by intraperitoneal or oral infection.

What is the cellular pathway affected by ROP38 and its mechanism of action during infection?

ROP38 could possibly affect one of the MAP kinases, the ERK1/2 pathway, as RH overexpressing ROP38 was shown to change the kinetics of ERK phosphorylation similar to that of CEP (Peixoto et al. 2010). Moreover, it is unclear whether ROP38 acts directly on the MAP kinases, or on upstream adaptors of MAPKs. To answer this question, we overexpressed ROP38 ectopically using the same tetracycline inducible system as GRA15, and sent the immunoprecipitates for mass spectrometry analysis (**Chapter IV, Table 1**). We noted that ROP38 immunoprecipitates yielded host protein adaptors upstream of MAPKs including calmodulin dependent protein kinase type IV, predicted MAPKKKK4 and G protein coupled receptor kinase 6. This indicates that ROP38 is likely to act upstream of the MAPKs, rather than interacting directly with MAPKs. However, these mass spectrometry results require validation through further immunoprecipitations using the ROP38 overexpressing cell line and detection through immunoblot of candidate interacting proteins.

In addition, though ROP38 is predicted to be an active kinase, experimental evidence is still lacking for this prediction, with *in vitro* kinase assays such as demonstrated in ROP16 (Ong, Reese & Boothroyd 2010). Substrates for kinases have been shown to be more easily identified upon mutation of the conserved catalytic motif, as kinase interactions tend to be transient, such as the interaction between ROP18 and *Irgb6* (Fentress et al. 2010). Thus, a possible approach would be to generate a type II strain overexpressing kinase-dead ROP38 to determine whether the parasite phenotypes observed are dependent on the catalytic motif, and to pull down host interacting proteins which may have transient interactions due to kinase phosphorylation.

What are the functional differences between ROP38, ROP29 and ROP19?

Though ROP38, ROP29 and ROP19 are reported to be from the same gene family, and are predicted active kinases, it remains unclear whether they play redundant roles or are functionally divergent. A way to address this would be complementation of a full ROP38 gene locus deletion with either ROP29 or ROP19, followed by *in vivo* intraperitoneal infections of mice. Should the mouse virulence phenotypes observed be rescued by complementation, this would indicate possible functional redundancy. Indeed, functional redundancy is present in other pathogen and their effectors as well, such as in *Yersinia* spp. where three different effectors, YopE, YopT and YpkA target the Rho family GTPases to prevent phagocytosis and subsequent elimination of the bacteria (Trosky, Liverman & Orth 2008). Functional redundancy is often present to ensure a robust system allowing efficient attachment, invasion and replication of pathogens (Galan 2009).

It is possible that ROP38 has evolved to be similar to that of ROP5, which is another heavily duplicated gene family. There is much stronger evidence for the different alleles of ROP5 to have differences in countering the IRG system, with crystal structures showing that numerous strain specific ROP5 nonsynonymous amino acid changes are centered at the interacting site of ROP5 and IRG (Reese et al. 2011). There is also high diversity in the IRGs across different mouse species with different susceptibilities to *Toxoplasma* (Lilue et al. 2013), and it is possible that the different species have slightly different MAPKs for which *Toxoplasma* requires different copies of ROP38 to adapt to. However, unlike ROP5, ROP38 is relatively conserved in its sequence across strains, without the strong nonsynonymous substitutions observed (**Chapter IV, Figure 1**), thus the selective pressure would be purifying rather than diversifying selection.

Are ROP38 differences in weight loss and cyst burden due to differences in immune response?

Our experiments show that ROP38 might have repressive effects *in vivo* upon type II infection of susceptible mice, with increased weight loss and cyst burden upon ROP38 deletion. Even though *Toxoplasma* requires successful parasite replication and dissemination during acute infection, it is crucial to encode effectors with attenuating effects on virulence to ensure host survival for lifelong chronic infection, enabling successful transmission. The increased weight loss and cyst burden due to ROP38 could be due to increased parasite dissemination or altered immune responses. However, there were no differences observed in IFN-γ and IL-12 secretion in serum levels of infected mice for i.p. infection, but it is not clear whether there are differences in cytokine secretion and immune cell recruitment at the site of infection, especially for the oral infection route (in the small intestine). A possible way to answer this is through *ex vivo* cytokine measurement by organ culture after infection or isolation of peritoneal cavity cells and using surface markers by flow cytometry to identify different immune cell populations recruited.

How do Toxoplasma effectors regulate one another?

There has been extensively characterization of individual pathogen effectors affecting pathogen virulence, but there are few investigations examining the interplay of effectors (Shames, Finlay 2012). The importance of effector interplay stemmed from early work in *Salmonella*, with two different T3SS encoded on two separate pathogenicity islands (SPI1 and SPI2). A SPI1 dependent effector is SopE, secreted early during infection, which activate the Rho GTPase dependent signaling pathways. This activation causes host cell membrane ruffling to enable phagocytosis of Salmonella into the host cell (Zhou, Galan 2001). However, a SPI2 dependent

effector, SptP, is injected late during infection, and inactivates the Rho GTPases, allowing recovery from invasion induced damage (Gruenheid, Finlay 2003). Regulation of these proteins with opposing effects is critical, and it involves the rate of control of degradation by the proteasome of these two proteins (Srikanth et al. 2011). An example in *Toxoplasma* would be ROP5, a pseudokinase, that binds and allosterically changes the conformation of an immunity related GTPase (IRG), Irga6, inhibiting further IRG oligomerization (Reese, Shah & Boothroyd 2014). This is turn allows ROP18, an active kinase, to phosphorylate and inactivate IRGs and allow virulent *Toxoplasma* strains to evade killing mediated by IRGs (Behnke et al. 2012).

We report that ROP38 represses NF-κB, but both GRA15 (II) and GRA15 (III) are sufficient to activate NF-κB. Infection of human and mouse cells with RH (type I) and CEP (type III) strains do not activate NF-κB, but overexpression of GRA15 (II) in the RH background is sufficient to cause NF-κB activation. However, overexpression of GRA15 (II) in the type III background does not cause NF-κB activation, even though both transgenic strains express GRA15 (II) to similar levels (Melo et al. 2013). As ROP38 is able to repress NF-κB in the type II background, it is possible that the high expression of ROP38 in type III strains ((Melo et al. 2013); www.toxodb.org) is repressing NF-κB activation due to the overexpression of GRA15 (II). To confirm that ROP38 is the effector contributing to the lack of NF-κB activation in the transgenic type III overexpressing GRA15 (II) strain, we would delete ROP38 in this transgenic strain and assay NF-κB activation. However, deletion of ROP38 is not trivial, as it is part of a tandemly duplicated family (**Chapter IV, Figure 3**) and the limitation of genetic selective markers would complicate the generation of this strain. However, with the CRISPR-Cas system (Cong et al. 2013), where high specificity for the gene of interest can be achieved without selective markers

and without long regions of homology (unlike deletion by double homologous recombination), short guide RNAs targeting ROP38 specifically could be designed.

Another direction that extends from examining the interplay of particular effectors would be systematically investigating binding host cell partners of identified *Toxoplasma* effectors (Selbach et al. 2009). ROP16 interacts directly with STAT3 and STAT6 through its kinase domain (Yamamoto et al. 2009), but ROP16 is likely to have STAT3/6 independent effects as several of the identified host partners do not have a STAT motif in their promoter regions (Ong, Reese & Boothroyd 2010). A comprehensive recent analysis of the host binding partners of another *Toxoplasma* calcium dependent kinase, TgCDPK3, also confirmed that the host targets for phosphorylation are not confined to the egress phenotype described for TgCDPK3 (Treeck et al. 2014). Systematic analysis of host binding partners for *Toxoplasma* effectors such as ROP38, GRA15 and ROP16 to examine host partners that are not involved in the expected effector phenotypes could lead to identification of novel functions and regulation of these *Toxoplasma* effectors.

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