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**Caractérisation d'un effecteur chez *Toxoplasma gondii* :
découverte d'une voie alternative d'inflammation régulée
par β -caténine**

Huan He

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THÈSE

Pour obtenir le grade de

DOCTEUR DE LA COMMUNAUTE UNIVERSITE GRENOBLE ALPES

Spécialité : **Virologie, Microbiologie, Immunologie**

Arrêté ministériel : 30 Sep 2017

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dans l'**École Doctorale Chimie et Science du Vivant**

Caractérisation d'un effecteur chez *Toxoplasma gondii* : découverte d'une voie alternative d'inflammation régulée par β - caténine.

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Abbreviations

Ahr: Aryl Hydrocarbon Receptor	Ig: Immunoglobulin
AIDS: Acquired Immune Deficiency Syndrome	IFN- γ : Interferon-gamma
AMA1: Apical Membrane Antigen 1	IFN- γ R: Interferon-gamma Receptor
APC: Antigen Presenting Cell	IL: InterLeukin
ASP: Aspartyl Protease	IMC: Inner Membrane Complex
ATG: Autophagy-related Protein	iNOS: Inducible Nitric Oxide Synthase
Bp: base pair	IRF8: Interferon Regulatory Factor 8
CAMLG: CAlcium signal-Modulating cyclophilin LiGand	IRG: Immunity Related Guanosine Triphosphatase
CCR: C-C Chemokine Receptor	IST: Inhibitor of STAT1-dependent Transcription
CNS: Central Nervous System	ISGs: Interferon-Stimulated Genes
Cy: Cyclophilin	KO: KnockOut
Da: Dalton	LDH: Lactate DesHydrogenase
DC: Dendritic Cell	LD: Lethal Dose
DG: Dense Granule	5 LO: 5 Lipoxygenase
DHFR: DiHydroFolate Reductase	MHCII: Major Histocompatibility Complex Class I
EC: Endosome Compartment	MIC: Microneme protein
ER: Endoplasmic Reticulum	MyD88: Myeloid Differentiation primary response gene 88
GAS: Gamma Activated Sequence	MYR1: Myc-Regulation-1
GBP: Guanylate Binding Protein	NFAT: Nuclear Factor of Activated T-cells
GLC: Gas Liquid Chromatography	NF- κ B: Nuclear Factor Kappa B
GPI: GlycosylPhosphate Inositol	NK: Natural Killer cell
GRA: Dense Granule Protein	NLS: Nuclear Localization Signal
GTPase: Guanosine Triphosphatehydrolase	NO: Nitric Oxide
HAUSP: Herpes virus-Associated Ubiquitin Specific Protease deubiquitinase	NTPase: nucleoside-triphosphatase
HDAC: Histone DeAcetylase	NuRD: Nucleosome Remodeling Deacetylase
HIV: Human Immunodeficiency Virus	PA: Phosphatidic Acid
HPLC: High Pressure Liquid Chromatography	PAMP: Pathogen-Associated Molecular Pattern
HSP: Heat Shock Protein	

PCR-RFLP: Polymerase Chain Reaction-Restriction Fragment Length Polymorphism
PEXEL: Plasmodium Export Element
PI: Post-Infection
PLV: Plant-Like Vacuole
PPM: Parasite Plasma Membrane
PV: Parasitophorous Vacuole
PVM: Parasitophorous Vacuole Membrane
PM: Plasma Membrane
QTL: Quantitative Trait Locus
ROM: Rhomboid protease
ROP: Rhoptry protein
ROS: Reactive Oxygen Species

SAG1: Surface Antigen 1
SEC: Size-Exclusion Chromatography
SNPs: Single Nucleotide Polymorphisms
SRS: SAG1-Related Sequences
STAT1: Signal Transducer and Activator of Transcription 1
SP: Signal Peptide
TEM: Transmission Electron Microscopy
Th1: T Helper 1 cell
TJ: Tight Junction or Moving Junction
TLC: Thin Layer Chromatography
TLR: Toll-Like Receptor
TNF- α : Tumor Necrosis Factor α
VLP: Virus-Like Particle

INTRODUCTION

Chapter I- *Toxoplasma gondii*, a model organism of Apicomplexans.

Parasites are by definition organisms that live in or on, and at the expense of, another organism, the host. The parasite benefits from this arrangement, but the host often suffers as a result. Parasitism is found throughout the tree of life and its evolutionary origin remains a central issue in evolutionary biology and has attracted intense theoretical and experimental laboratory-based attention. Parasitic diseases are among the most important causes of death worldwide. In 2013, over one million registered deaths were caused by parasites with the majority of the infectious agents being protozoan parasites (Abubakar *et al.*, 2015). Members of the *Apicomplexa* phylum of protozoa are among the most successful, life-threatening pathogens in humans and domestic animals.

Apicomplexa are single celled eukaryotes and as obligate intracellular parasites they infect a wide variety of hosts ranging from invertebrates to mammals. It is estimated that out of a probable 1.2 - 10 million apicomplexan species, only ~0.1% have been described to date (Adl *et al.*, 2007; Pawlowski *et al.*, 2012). Among the clinically and economically important apicomplexan pathogens are found *Babesia*, *Cryptosporidium*, *Eimeria*, *Neospora*, *Theileria*, *Toxoplasma* (Tenter *et al.*, 2000), and the malaria-causing parasite *Plasmodium spp.*

Among apicomplexan, *Toxoplasma gondii* (*T. gondii*) is unique as it is probably the most successful parasite with its widespread distribution worldwide causing one of the most common parasitic infection, called toxoplasmosis. Remarkably, *T. gondii*'s host range is exceptionally broad since it can infect virtually all nucleated cells of all warm-blooded animals including birds, marsupials, and mammals (Dubey, J.P, 2009). Although usually causing only mild symptoms in the adult, it can cause severe and life-threatening disease in developing fetuses and in immunocompromised individuals, especially AIDS and transplanted patients. *T. gondii* has been studied over one century, still very little is known about the mechanisms by which this parasite interact with its hosts at the molecular level. *T. gondii* offers a very exciting model to study host and parasite interactions at both the molecular and cellular levels, as well as the biological processes controlling the outcome of the infection in animals and in human.

Recognizing that pathogens can overrun crucial host-cell pathways by using a myriad of mechanisms is not sufficient. This knowledge now needs to be advanced to the point at which it can be translated into a true understanding of the disease. Only then will it be possible to target these effector mechanisms rationally as a preventive or therapeutic strategy.

1- Discovery and history of *T. gondii*.

T. gondii was fortuitously discovered in 1908 by Charles Nicolle and Louis Manceaux at the Pasteur Institute of Tunis, while searching a reservoir for *Leishmania* in a North African rodent, *Ctenodactylus gundi* (Splendore, 1908). Independently, Alfonso Splendore discovered the same parasite in a rabbit in Brazil (Splendore, 1908). The name *Toxoplasma gondii* was attributed to the isolated protozoan by Nicolle and Manceaux according to the crescent-shaped morphology of the parasite (*toxon* = arc or bow, *plasma* = form, in Greek) and *gundii* for the infected hamster-like rodent in which it was discovered. Since then, an increasing number of *T. gondii* isolates testified to its geographical widespread distribution making *T. gondii* one of the most successful parasitic organisms worldwide. Intriguingly, despite the wide host range, there was only one species consistently recognized in the genus: *Toxoplasma gondii*.

Initially, our understanding about the pathogenic potential of *T. gondii* was limited to a congenitally infectious disease in human and animals presenting the characteristic triad of symptoms, namely hydrocephalus, retinochoroiditis and encephalitis (Innes, 2010). Our knowledge about human toxoplasmosis particularly increased during the 1970/80s with the identification of *T. gondii* as a major cause of death in patients with acquired immunodeficiency syndrome, demonstrating the importance of the immune system in controlling *T. gondii* infection. A major breakthrough was achieved in 1978 with the establishment of the 'dye test' (Sabin and Feldman, 1948), that allowed the diagnosis of human infection from serum samples. Importantly, this test revealed the importance of the prevalence of the infection in human as well as in animals. To date, it is estimated that one third of the world population is seropositive for toxoplasmosis.

2- The *Toxoplasma gondii* life cycle and transmission.

Later, during the 1970s, the complete parasite life cycle and the different routes for parasite transmission were discovered by Hutchison (Hutchison, 1965) and Frenkel and Dubey (Frenkel *et al.*, 1969) (Figure 1). As a fully-fledged member of the tissue cyst-forming branch of the coccidians, *T. gondii* is transmitted through an alternating two-host life cycle, termed a heteroxenous cycle, relying on a definitive host for sexual development while undergoing asexual replication in its alternative or intermediate hosts.

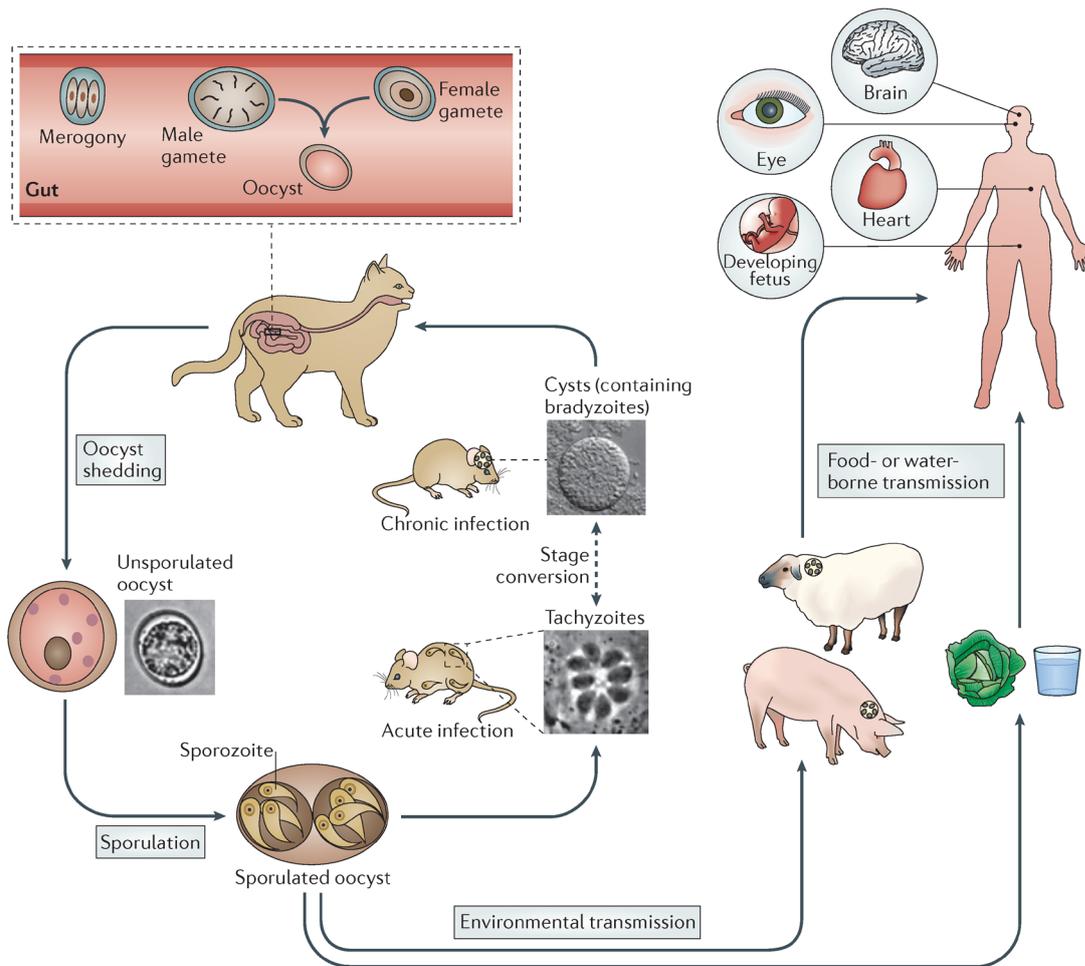


Figure 1. *Toxoplasma gondii* life cycle and modes of transmission.

Reprinted from (Hunter and Sibley, 2012). In intermediate hosts, the parasite can be encountered under the differentiated forms tachyzoites and bradyzoites. In the digestive tract of definitive Felid hosts, the parasites undergo the sexual stages leading to the excretion of the diploid oocysts that are shed in cat faeces and undergo meiosis in the environment to yield the infectious haploid progenies, the sporozoites.

2.1 Asexual replication in the intermediate hosts.

The *T. gondii* asexual replication is characterized by two forms, named tachyzoite and bradyzoite. During the acute phase of infection in the intermediate host, the parasites replicate as tachyzoites, the fast replicative form (Tachos = speed, from Greek), which expand dramatically in numbers and disseminate to many tissues in the body (Dubey *et al.*, 1997). Following a potent host immune response, the parasite differentiates to a slow-growing form, the bradyzoite (Brady = slow, from Greek), which remains quiescent within tissue cysts that reside in long-lived cells, such as neurons and skeletal muscle cells (Dubey, 1997); the latter typifies the chronic phase of toxoplasmosis and tissue cysts can persist indefinitely for the life of the host. Eventually, reactivation of the cysts may occur if the immune response is suppressed in the intermediate host, leaving the bradyzoites to differentiate back to tachyzoites, causing pathogenesis and tissue damage in the host particularly encephalitis or chorioretinitis (Frenkel and Escajadillo, 1987; Montoya and Liesenfeld, 2004). Indeed, the tachyzoite-to-bradyzoite conversion is reversible, allowing the reemergence of chronic infections in immunocompromised hosts (Odaert *et al.*, 1996) and contrasts with the restrictive cycle of the close relative, *Hammondia hammondi* that is depending on an obligatory heteroxenous cycle (Frenkel and Dubey, 2000).

The tachyzoite corresponds to the *T. gondii* form that is able to invade and multiply in any type of nucleated cell of the intermediate hosts. As mentioned earlier, *T. gondii* is remarkable

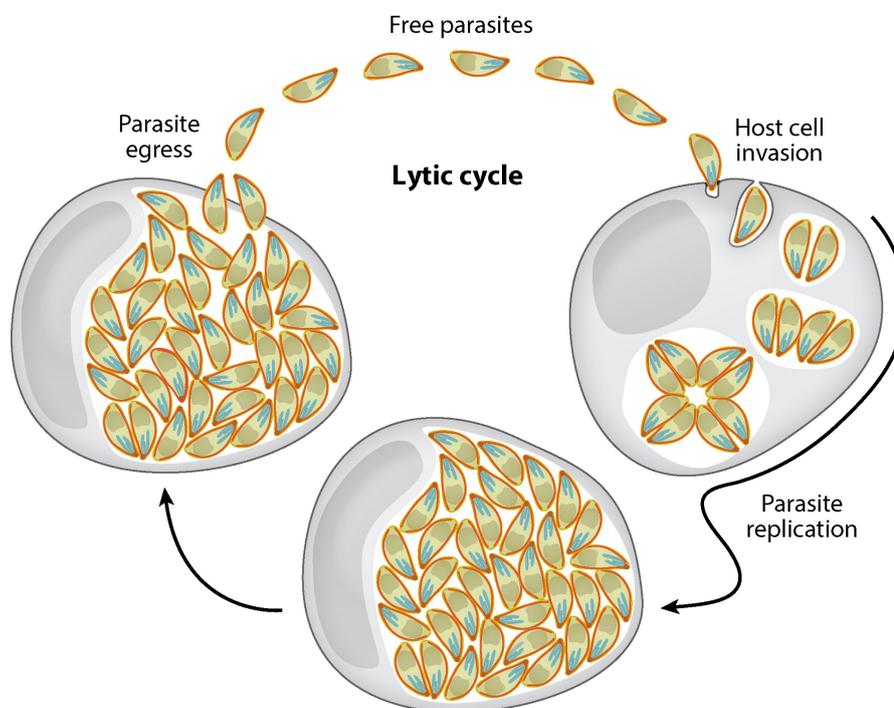


Figure 2. *Toxoplasma gondii* lytic cycle.

Reprinted from (Blader *et al.*, 2015).

in its ability to invade a wide variety of host cells. The tachyzoite enters the host cell by an active-driven process that is completed within few seconds (Dobrowolski and Sibley, 1996). The invasion starts with the formation of a tight ring-like junction bridging the apical pole of the tachyzoites with the host cell plasma membrane (PM) (Figure 2). Then, the parasite tracts itself into a PM invagination that arises below the tight-junction (TJ), and then evolves as a non-fusogenic PV that will support growth and multiplication of the tachyzoite (Mordue *et al.*, 1999). This mode of entry as well as the formation of the PV is characteristic of many other apicomplexans, including *Plasmodium spp* and *Neospora*. Notably, an actin-myosin-based motor complex has been shown of a primary importance for efficient cell entry by *T. gondii* tachyzoites as well as in related apicomplexans (Meissner, 2002; Sharma and Chitnis, 2013). Once inside the PV, the zoite divides by a particular type of asexual binary division called the endodyogeny (Blader *et al.*, 2015). The intracellular phase of the life cycle is followed by active egress from the host cell and then rapid reentry to initiate another lytic cycle (Sibley, 2010) (Figure 2).

2.2 The sexual development in Felids, the definitive hosts.

The sexual cycle of *T. gondii* takes place exclusively in enterocytes of the small intestine of Felids, the definitive hosts. Why is the sexual cycle limited to the feline intestine as well as the molecular mechanisms driving the differentiation process leading to gametocytes and oocysts are probably the most intriguing unanswered questions to date about *T. gondii*'s biology.

Once cysts or oocysts are ingested (Figure 1), the proteolytic enzymes in the stomach and intestine of the cat dissolve the cyst wall, hence releasing the bradyzoites or sporozoites, respectively, that then penetrate the enterocytes and produce morphologically distinct structural stages of *T. gondii* (Dubey and Frenkel, 1972). During these stages, parasites undergo several cycles of asexual multiplication by endopolygeny, characterized by the development of merozoites within schizonts. The formation of schizonts characterize the end of asexual development and initiate the sexual phase, which usually begins two days after tissue cyst were ingested by the cat. Then, the merozoites released from schizont initiate the gametogony, with notably the micro- and macrogametes formation (Ferguson, 2002). Microgametes swim to mature macrogamete by using the flagella yielding to the diploid zygote. Oocysts are then liberated from enterocytes by the disruption of the cell and excreted as unsporulated forms in cat feces (Figure 1). The unsporulated oocysts undergo meiosis during sporulation which occurs after few days in the environment. The sporogony phase generates two sporozoites, each containing four sporozoites. A 12 month-old cat could shed more than 20 million oocysts within 15 days following the oral infection. Oocysts are extremely resistant to chemical and physical stress conditions; even high concentrations of sodium chloride or

ozone are ineffective in fully inactivating the oocyst rendering oocysts highly infective to intermediate hosts, including humans.

2.3 Human Toxoplasmosis.

Humans are accidental hosts for *T. gondii* and play little role in its natural life cycle if any. In human, transmission occurs predominantly via three major routes: The zoonotic transmission from animal to human by the accidental ingestion of oocysts, the vertical or congenital transmission when primary infection is acquired during pregnancy, and the foodborne transmission through the ingestion of the tissue cysts in contaminated raw or undercooked meat (Hill and Dubey, 2002). Epidemiologically, it is not yet clear which of these mode of transmission is the most important although this may vary depending on culture and eating habits as well as water sanitation that is very uneven from one country to another. There have been a number of documented outbreaks of toxoplasmosis due to contamination of water supplies in British Columbia and in different regions of Brazil (Bahia-Oliveira *et al.*, 2003; Bowie *et al.*, 1997; de Moura *et al.*, 2006). In healthy adults, toxoplasmosis produces a relatively mild infection, with flu-like symptoms including elevated fever, enlarged lymph nodes, and muscle pain. While acute infection resolves rapidly, leaving the individual with a chronic, subclinical infection, more severe outcomes can occur with congenital infections, where the developing fetus can experience severe symptoms like hydrocephaly and even loss of life. Depending on the timing of infection, primo-infections can lead to few symptoms at birth but trigger ocular toxoplasmosis later in life. In immunocompromised adults, a lack of sustained immunity trigger the reactivation of chronic infection which in turn promotes severe outcome as seen in AIDS, organ transplant, or chemotherapy patients. In the 80's, toxoplasmosis was considered a *bona fide* opportunistic infection of the HIV epidemic and the introduction of Highly Active Antiretroviral Therapy (HAART) coincides with a drastic drop in the incidence of cases of reactivated toxoplasmosis. Chronic infections in humans have also been associated with an elevated risk of psychiatric illnesses, including schizophrenia however the causality of this association has not been yet well established. Globally, the serological prevalence of toxoplasmosis is highly variable, ranging from 10 to 15% in the United States and to 60% in France or in South and Central America. Overall, ~30% of the world's human population was estimated to be chronically infected with *T. gondii*. The high incidence, in general associated with subclinical chronic infections, likely reflects the lack of available therapies - pyrimethamine combined with sulfadiazine – that do not eradicate the bradyzoite encysted form in patients.

3- Population structure

T. gondii is one of the most widespread protozoan parasite of domestic and wild animals with a third of the human population infected worldwide. Paradoxically, the population structure of *T. gondii* shows relatively little genetic diversity as the vast majority of the strains

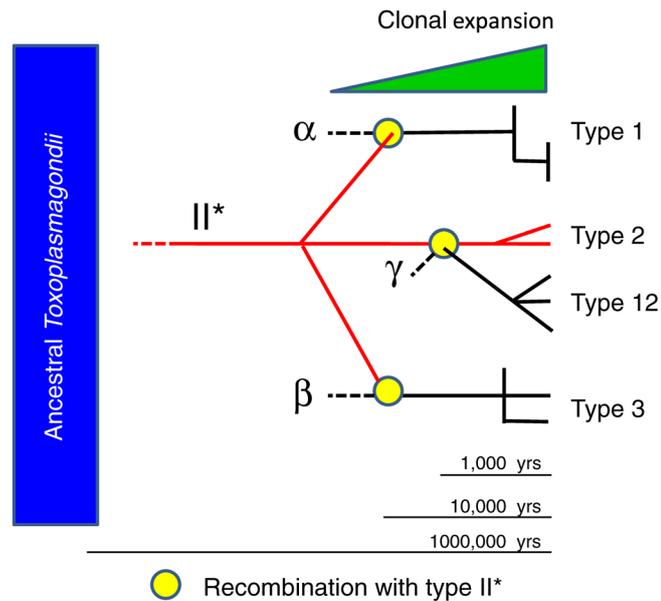


Figure 3. Model for clonal expansion of populations of *Toxoplasma gondii* in North America and Europe.

Reprinted from (Khan *et al.*, 2011). Types 1, 2, 3, and 12 represents the major clonal groups that have expanded throughout North America. Three independent crosses between an ancestral strain (II*) and separate parental types designated as α , β and γ , gave rise to types 1, 3 and 12, respectively.

isolated in North America and Europe fall into three recently derived, clonal lineages known as typical (or canonical) strains of types I, II and III (Figure 3) (Sibley, 2009). Emergence of those lineages was estimated at $\sim 10,000$ years ago and is most likely concomitant to the domestication of animals by humans (Su *et al.*, 2003). The peculiar population structure of *T. gondii* is shaped by clonal expansion (Figure 3) (Khan *et al.*, 2011), which is likely favored by the ability of *T. gondii* to be transmitted between intermediate hosts via oral tissue cysts ingestion (Su *et al.*, 2003), hence circumventing sexual reproduction in definitive host. Therefore, the geographic structure of *T. gondii* is unique with quite an uniform population across all continents except in South America where a large variety of other atypical strains were recently shown to circulate (Figure 4) (Lehmann *et al.*, 2006). Importantly, a strong association has been found in French Guiana between several atypical strains (Carne *et al.*, 2002) and the severity of the disease in particular in immunocompetent people otherwise quasi asymptomatic when infected by the typical strains. It has been proposed that the interpenetration of anthropized and wild rainforest environments favor the hybridization between strains and is at the origin of the emergence of such virulent offsprings (Mercier *et al.*, 2011). Those observations suggest that certain parasite genes segregate differentially

between populations, including those that influence pathogenesis as considered below (Figure 4). In Asia, several reports revealed a more limited genetic diversity than in South America (Robert-Gangneux and Darde, 2012). In China, a clonal lineage seems to be overrepresented across the country in favor of a clonal expansion model (Zhou *et al.*, 2010). The *T. gondii* population structure and haplogroups described in the Figure 4, were determined based on genotyping using isoenzyme analysis (69, 70), PCR-restriction Fragment Length Polymorphism (RFLP) of markers, and microsatellite analysis. A recent study based on whole-genome sequencing of multiple *T. gondii* strains suggested that coinheritance of large chromosomal haploblocks within members of a common clade shaped the actual phylogenetic branching of *T. gondii* strains (Lorenzi *et al.*, 2016). Interestingly, those haploblocks are enriched in secreted pathogenicity determinants, as discussed below in section 3.1 of Chapter II.

The pathogenicity of *T. gondii* strains in laboratory mice varies substantially and depends on both the mice and the parasite genotypes. Indeed, type 1 strains are uniformly lethal in mice and referred to as virulent strains. Infection by a single tachyzoite of type I strain can kill a mouse. Fortunately, type I strains are quite rare; as they seem to be more frequently associated with ocular toxoplasmosis and acute outbreaks (Grigg *et al.*, 2001). In contrast, type II and type III isolates are significantly less virulent in mice. Type II strains have intermediate virulence phenotype that varies with mouse strain and are most commonly associated to human infections from cases of congenital infection or in immunocompromised patients, a pattern seen in Europe and North America. Type III are significantly less virulent in mice and are considered avirulent (Sibley and Boothroyd, 1992). Although they are rarely found in human infection, they are common to domestic and wild animals. Virulence is usually determined by intraperitoneal injection of tachyzoite into outbred laboratory mice which are more resistant to infection than inbred mice. The latter mice are somewhat more susceptible to *T. gondii* infection and are therefore used to assess virulence of the type 2 and type 3 strains (Suzuki *et al.*, 2000).

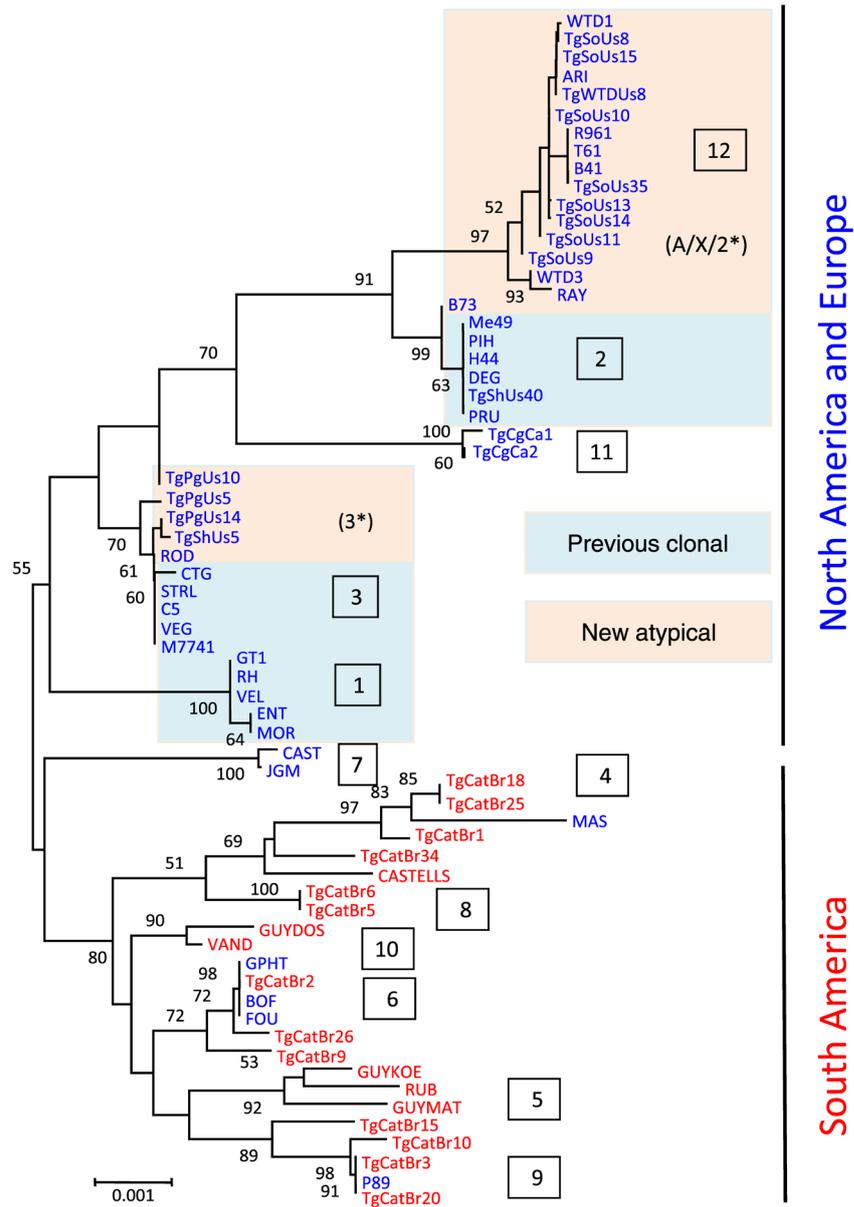


Figure 4. Population genetic structure of *T. gondii*. Reprinted from (Khan *et al.*, 2011). The phylogenetic analysis of *T. gondii* strains highlights the genetic diversity between strains isolated from North American (blue lettering) and South America (red lettering). The major branches were classified into 12 haplogroups (boxed numbers). The highly clonal strains from Europe and North America (i.e. types 1, 2, 3) are referred to as “previous clonal”, while a fourth clonal lineage, type 12, has been described in North America from isolates of wild animals and is referred to as “new atypical”.

4- Specific subcellular organization of the *Toxoplasma* zoite.

The tachyzoite is the invasive form that disseminates during the acute phase of toxoplasmosis in both intermediate and definitive hosts. The tachyzoite has a typical polarized cell structure that is often a crescent-shaped cell of approximately 2 by 7 μm with a slightly pointed anterior end named the conoid and a rounded posterior end (Figure 4A). As a protozoan, the parasite shares most of the characteristics found in other eukaryotic cells such as a nucleus, endoplasmic reticulum (ER) and Golgi apparatus, a single mitochondrion, and ribosomes.

4.1 Subcellular structures and organelles

As most *Apicomplexa*, *T. gondii* harbors unusual subcellular structures and organelles such as a plant-like vacuole (Miranda *et al.*, 2010) and a non-photosynthetic chloroplast-like organelle, the apicoplast (Figure 5A). The apicoplast is very likely derived from a red algal secondary endosymbiosis (McFadden and Roos, 1999). As an essential organelle for parasite survival, the apicoplast has been the subject of intensive investigation both because of its biological uniqueness and as a potential drug target.

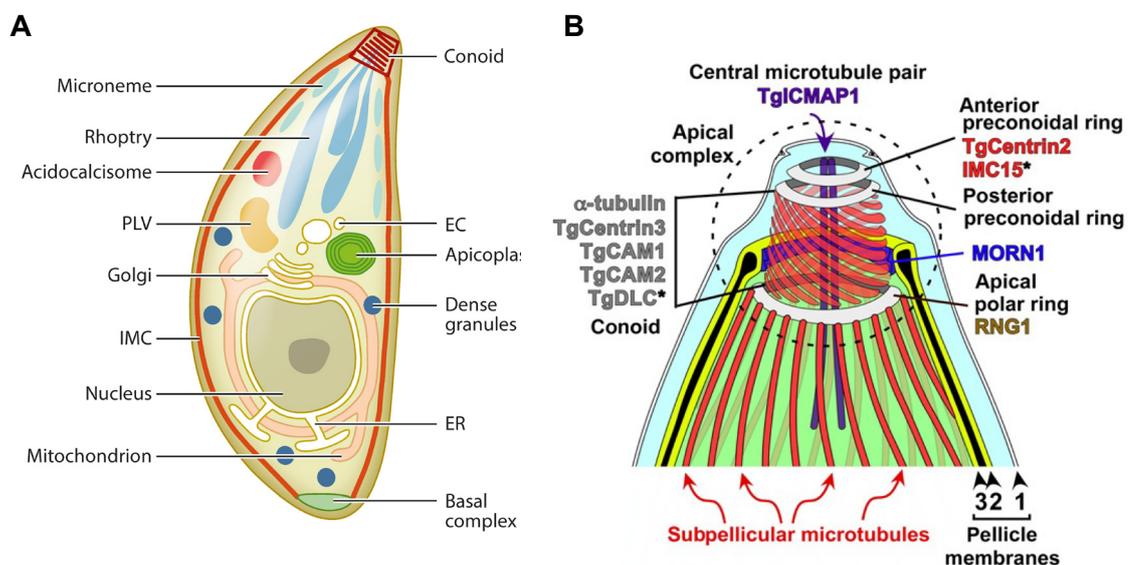


Figure 5. *T. gondii* cellular and sub-cellular organization.

Schematic representations of a *T. gondii* tachyzoite (A) and of its most apical end (B). Reprinted from (Blader *et al.*, 2015) and (Anderson-White *et al.*, 2012), respectively. A series of three cytoskeletal rings are located at the apex (gray) and the components known to localize to these structures are indicated. Abbreviations: PLV, Plant-Like Vacuole and EC, Endosome Compartment.

The shape and structural integrity of the zoite are maintained by several unique structural elements. Indeed, the outer plasma membrane and the flattened alveolar sacs of the Inner Membrane Complex (IMC) form the pellicle, a triple bilayer characteristic of apicomplexan parasites (Figure 5B). Underlying the pellicle is a filamentous cytoskeletal structure, called the sub-pellicular network, that associates on its outer face with the IMC and on its inner face with 22 subpellicular microtubules emanating from the apical end of the parasite (Anderson-White *et al.*, 2012). This structure constitutes the parasite's skeleton. The IMC covers the whole peripheral surface of the parasite except at the apical and posterior ends. Beneath the plasma membrane is located a divergent actomyosin motor system which translocates adhesive proteins from the apical to the posterior end of the parasite thus providing forces necessary for the gliding motility of the parasite (Keeley and Soldati, 2004; Tardieux and Baum, 2016).

4.2 Specialized Secretory organelles.

The apical complex, after which the phylum is named, is composed of two polar rings interconnected by an atypical microtubule structure known as the conoid (Figure 5B). It also contains a specialized set of secretory organelles, namely micronemes, rhoptries, and dense granules (GRAs) in which reside a high diversity of secreted protein families (Bradley *et al.*, 2005; Carruthers and Tomley, 2008; Leriche and Dubremetz, 1990). The apical complex plays a major role in invasion, presumably by mediating the release of secretory proteins from the apical end at the site of the entry. The content of the apical secretory organelles are sequentially discharged throughout the invasion process. Micronemes exocytosis occur at the initial step of invasion, upon host cell binding, whereas rhoptry secretion coincides with invasion, and dense granule secretion is most prominent after parasite entry into the host cell, when the PV is formed (Carruthers and Sibley, 1997).

4.2.1 The Rhoptries.

Studies based on observation by electron microscopy have shown that there are about 8 to 12 club-shaped rhoptry organelles in each zoite (Dubremetz, 2007), consisting of an extended narrow duct, the rhoptry neck, and of a bulbous base, the rhoptry bulb. Rhoptries are a secretory organelles and mass spectrometry analysis identified more than 30 different rhoptry proteins (Bradley *et al.*, 2005), which were named according to their location, RON (for rhoptry neck) and ROP (for rhoptry protein). As expected for an unusual organelle, several rhoptry proteins are unique to *T. gondii* (e.g. Toxofilin) and most of them are not conserved outside of the phylum. The rhoptry-resident proteome includes 44 member family of coccidian-specific secreted kinases (Peixoto *et al.*, 2010) and few PP2A-related phosphatases. Recently,

several rhoptry proteins were identified as major secreted pathogenicity determinants as they dictate whether a *T. gondii* strain is virulent or not in mice and is discussed below in section 3.1.

4.2.2 The Micronemes.

Micronemes are the smallest secretory organelles that are mostly located at the apical end of the parasite (Figure 5A). Secretion of micronemal proteins (MICs) is triggered in a Ca²⁺-dependent process when parasites contact the cell they infect, an event that is associated with *T. gondii* attachment and invasion of the host cells (Carruthers *et al.*, 1999a, 1999b). Among MICs are found motility-associated motors and adhesins that support host cell surface recognition and entry of the parasite, respectively. The most described MICs are MIC8 and the Apical Membrane Antigen 1 (AMA1), both being involved in parasite invasion (Bargieri *et al.*, 2014; Kessler *et al.*, 2008).

4.2.3 The Dense Granules.

Dense granules (DGs) are singular membrane rounded organelles with a diameter of 200 nm (Mercier *et al.*, 2005). Initially discovered in *Sarcocystis tenella* merozoite, they were given their name due to their high density to electrons when observed by Transmission Electronic Microscopy (TEM) (Dubremetz and Dissous, 1980). In sharp contrast to the Rhoptries and Micronemes, DGs are dispersed through the parasite cytoplasm (Figure 5A). DGs are only observed in a subset of apicomplexan parasites and seem to be restricted to those forming tissue cysts such as *Neospora*, *Sarcocystis*, *Hammondia*, *Besnoitia*, and *Frankelia* (Mercier and Cesbron-Delauw, 2015). DGs were shown to contain secreted proteins, namely GRAs, though the mechanism by which those proteins are released outside of the parasite remains largely unexplored and very few events of secretion have been captured (Souza, 2006), yet their secretion begun immediately after invasion at the onset of the vacuole formation (Carruthers and Sibley, 1997). GRAs are believed to be continuously secreted throughout the intracellular lifetime of the parasites and represent the default pathway for secretion. Most of the GRAs proteins harbor a characteristic hydrophobic N-terminal Signal Peptide (SP) for targeting the nascent GRA proteins to the ER and the secretory pathway (Mercier and Cesbron-Delauw, 2015). Then, the newly synthesized GRAs traffic from the single-stacked Golgi apparatus to the parasite's periphery through a mechanism that is dependent on the TgMyoF motor, a vesicular cargo transporter moving along the actin filaments (Heaslip *et al.*, 2016). How DGs or their content traffic through the Parasite Plasma Membrane (PPM) is still under investigation. However, it has been proposed that the DGs do not fuse with the IMC membranes, but rather navigate through small gaps of the IMC plates (Dubremetz *et al.*, 1993).

Originally, GRAs were believed to have functions restricted to the vacuolar formation or remodeling and nutrient uptake as many of which occupy the lumen of the vacuole or decorate membranous tubules called the intra-vacuolar network, or accumulate at the PVM (Cesbron-Delauw *et al.*, 2008). However, most GRAs are non-essential with the exception of GRA1 and GRA10 that were shown to be refractory to deletion and presumably essential for parasite survival within the host cell (Rommereim *et al.*, 2016). Interestingly, several GRAs form part of the cyst wall that surrounds the bradyzoites and many GRA proteins seem to be important at this stage of development (Fox *et al.*, 2011; Torpier *et al.*, 1993), which could explain why most of the GRAs are restricted to the cyst-forming coccidians. Recent studies have shown that certain GRAs have extra-PV functions and participate to the host cell remodeling as effector proteins (Hakimi *et al.*, 2017) as discussed below in section 3.2. It is now clear that several GRAs participate to the regulation of host and parasite interactions, however whether they are partitioned in a different subpopulation of DGs than the GRAs destined to PV functions remains to be further investigated.

Chapter II- Host and pathogen Interactions.

The mechanisms underlying host and parasite interactions have studied for more than 60 years and *T.gondii* has been considered not only as a model for fundamental cellular and molecular biology research, but also as a research tool to study the mechanisms of the host immune response to the intracellular pathogens, especially the innate immunity.

1- Innate Immunity in Mice.

The innate immune response to *T. gondii* infection has been well studied in the laboratory mice in part because rodents are natural intermediate hosts and thereby a well-adapted model for monitoring survival and pathogenesis. It is now well established that *T.*

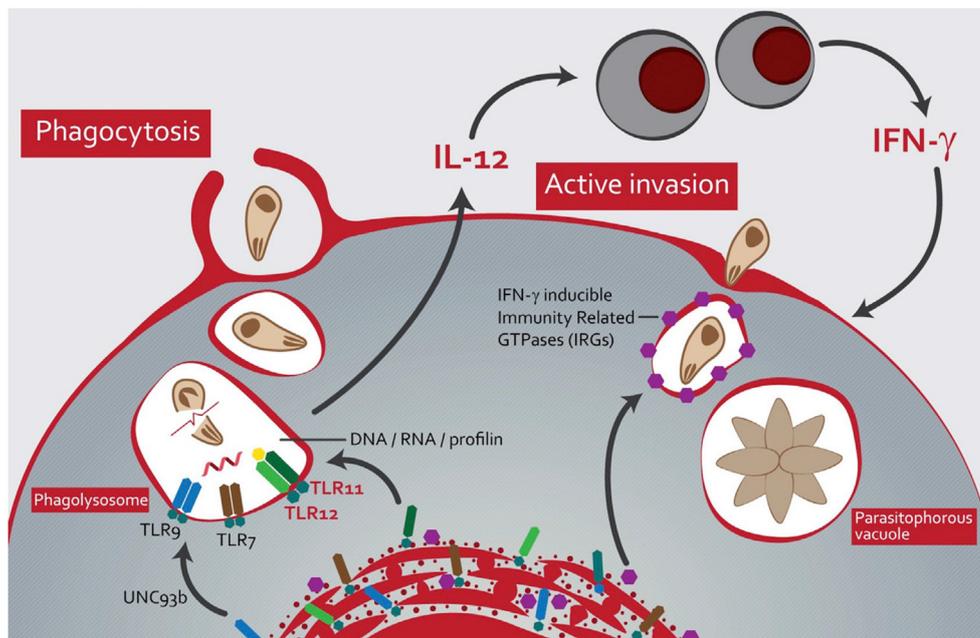


Figure 6. *T. gondii* sensing by TLR11/12 and Th1 immune response. Schematic representations of a *T. gondii*'s Pathogen-Associated Molecular Patterns (PAMPs) recognition by host TLRs. Uptake of parasite debris or, alternatively, destruction of intracellular tachyzoites leads to activation of TLR7, TLR9, and TLR11/TLR12 by *T. gondii* RNA, DNA, and profilin released in phagolysosomes. Activation of the TLRs leads to IL-12 production and consequent induction of IFN- γ by NK cells and T lymphocytes. IFN- γ is responsible for the expression of innate immune effector proteins such as the Immunity Related GTPases (IRGs) that are in charge of recognizing and destroying the PV with enclosed tachyzoites. Reprinted from (Gazzinelli *et al.*, 2014).

gondii elicits a strong and sustained Th1 immune response, typified by the IL-12-Interferon- γ (IFN- γ) axis, which is crucial to the control of the infection in mice.

1.1 Innate Immune Sensing of *T. gondii*.

1.1.1 The Profilin-TLR11/12 sensing pathway.

During the acute stage of infection in mice, the parasites rapidly disseminate from the site of infection to the different tissues and organs. Initial detection of *T. gondii*'s profiling-like protein by the Toll-like receptor 11 (TLR11) and TLR12 in mice triggers the production of the defensive host cytokine IL-12 by dendritic cells (DCs), macrophages, and neutrophils (Figure 6) (for recent review see (Yarovinsky, 2014)). Profilin is a well conserved actin binding protein that is essential for host cell invasion (Plattner *et al.*, 2008), therefore creating a close but paradoxical connection between *T. gondii* survival and recognition by the host immune system. It has been suggested that TLR11 associates with TLR12 to form a heterodimeric central sensory unit for the downstream signaling pathway (Andrade *et al.*, 2013). The high susceptibility of mice mutated for either TLR11 and TLR12 or the MyD88 TLR adaptor protein testify to the importance of this pathway in the control of the infection (Andrade *et al.*, 2013; Koblansky *et al.*, 2013; Scanga *et al.*, 2002). Then, IL-12 triggers the production of IFN- γ by Natural Killer (NK) cells and later by the T lymphocytes, characteristic of a Th1 immune response that guarantees the long-term control of *T. gondii* by the infected host (Figure 6) (Gazzinelli *et al.*, 1993; Hunter *et al.*, 1994).

1.2 IFN- γ mediated innate effector mechanisms.

IFN- γ is critical for controlling infection, as shown by the enhanced susceptibility of mice lacking IFN- γ (Scharton-Kersten *et al.*, 1996) or the IFN- γ receptors (Yap and Sher, 1999). The released IFN- γ has a pleiotropic effect on both hematopoietic and non-hematopoietic cells. IFN- γ signaling proceeds through Signal Transducer and Activator of Transcription 1 (STAT1) phosphorylation and nuclear internalization to stimulate the expression of a specific set of Interferon-Stimulated Genes (ISGs) (Darnell *et al.*, 1994; Schindler and Darnell, 1995). In line with its commitment, STAT1 was shown to be essential for the control of infection in mice (Gavrilescu *et al.*, 2004; Lieberman *et al.*, 2004).

Among the ISGs, the main effectors responsible for limiting tachyzoite growth and dissemination in mice are the IFN-inducible GTPase families, named the Immunity-Related GTPases (IRGs) and the Guanylate Binding Protein (GBP) (Figure 7) (Krishnamurthy *et al.*, 2017). As further discussed below, unlike humans, rodents express a remarkably expanded family of IRGs that are essential for the survival of mice against *T. gondii* infection, but also against other intracellular pathogens (Gazzinelli *et al.*, 2014; Howard *et al.*, 2011). Under steady state conditions, the GDP-bound inactive IRGs are sequestered to the IrgM negative

regulator that prevents GDP dissociation and therefore prevents its activation (Hunn *et al.*, 2008). When a pathogen-containing vacuole is recognized, the IRGs oligomerize at the vacuolar membrane resulting in its disruption and death of the resident tachyzoites (Martens *et al.*, 2005; Zhao *et al.*, 2009). As a major defense mechanism, mice deficient for IRGs become highly susceptible to *T. gondii* infection (Lilue *et al.*, 2013).

2- Innate Immunity in Humans.

Humans are accidental hosts for *T. gondii* and play little role in its natural life cycle. As such, humans are relatively resistant to *T. gondii* infection. However, unlike mice, humans employ distinct innate immune pathways to control the infection. While the recognition and the major defense mechanism in mice depends on TLR11/12 and the IRGs respectively, those proteins are absent in humans, pointing to a different but yet poorly understood mechanism of resistance (Gazzinelli *et al.*, 2014). Nevertheless, the IFN- γ /STAT1 signaling pathway is required for *T. gondii* growth restriction in human cells *in vitro* (Hunter and Sibley, 2012). While humans express a wide repertoire of GBPs, GBP1 is so far the only effector restricting infection in a cell type dependent manner (Johnston *et al.*, 2016). Additional mechanisms include the production of toxic Nitrogen Oxide (NO) and L-arginine depletion due to the Inducible Nitric Oxide Synthase (iNOS), and the inhibitory proteins Indoleamine 2,3-DiOxygenase (IDO) that create tryptophan depletion, an essential amino-acid for *T. gondii* (Pfefferkorn and Guyre, 1984). An alternative mechanism acting through the induction of the NALP1 inflammasome has been suggested as well (Witola *et al.*, 2011). Since the aforementioned mechanisms are not universally effective in all cell types, it is therefore possible that parasite restriction depends on their additive contributions or that still unidentified mechanisms are involved.

Recent findings pointed to the autophagy pathway as implicated in the resistance of the human cells to *T. gondii*. As such, ATG5, ATG12, and ATG16 were shown to be required for *T. gondii* restriction in IFN- γ pretreated cells, while other upstream regulatory elements such as the adaptor proteins NDP52, p62, and LC3 are likely recruited to the ubiquitylated PVM and associated with endosomes/lysosomes fusion leading to parasite death (Clough *et al.*, 2016; Selleck *et al.*, 2015). Nevertheless, further investigations will be needed to determine whether this mechanism operates in different human cell types, especially immune cells such as monocytes and macrophages that are in the front line of the defense against *T. gondii* infection.

3- Modulation of host immunity: The *T. gondii* effector proteins.

Pathogen–host interactions involve processes of host defense that are usually counteracted by microbial molecules directly delivered into the host cells. Indeed, the ability to inject effectors into mammalian or plant cells is repeatedly encountered when studying viruses or pathogenic bacteria or fungus, hence providing an efficient and elegant strategy to subvert

important host signaling pathways (Bhavsar *et al.*, 2007; Chisholm *et al.*, 2006; Roy and Mocarski, 2007).

Such effectors were originally identified in *T. gondii* by forward genetic approaches. Several research laboratory have exploited the genetic and phenotypic differences between the *T. gondii* clonal lineages in order to map virulence loci and factors responsible for changes in host cell gene expression (Behnke *et al.*, 2011; Rosowski *et al.*, 2011; Saeij *et al.*, 2006, 2007; Taylor *et al.*, 2006).

3.1 Rhopty bulb proteins as major pathogenic determinants in mice.

A major breakthrough in our understanding of the mechanisms governing virulence in mice was achieved with the identification of the rhopty proteins ROP5 and ROP18. Genetic crosses between the highly virulent type I strain (GT-1) or intermediately virulent type II (ME49) strain and the avirulent type III (CTG) strain allowed the mapping of a Quantitative Trait Locus (QTL) on chromosome VIIa containing a polymorphic pathogenicity determinant, ROP18 (Saeij *et al.*, 2006; Taylor *et al.*, 2006). *ROP18* encodes a serine/threonine kinase that is secreted from the rhoptries early during invasion and associate at cytosolic face of the nascent PVM where it prevents the IRGs-mediated clearance (Figure 7). ROP18 phosphorylates IRGs at the level of conserved threonine residues of the GTPase domain, that very likely prevents GTP hydrolysis and their oligomerization at the membrane (Fentress *et al.*, 2010; Steinfeldt *et al.*, 2010).

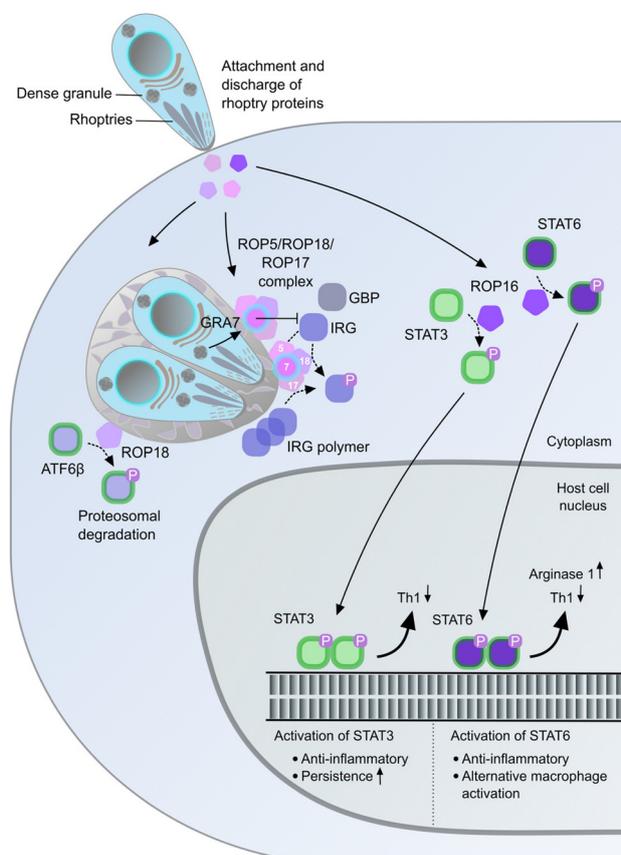


Figure 7. The Rhopty effector proteins. Schematic representations of a *T. gondii* rhopty secretion upon host cell infection. Reprinted from (Hakimi *et al.*, 2017).

The ROP5/ROP18/ROP17 complex in combination with GRA7 is assembled on the PV membrane to phosphorylate the IRG monomers/dimers (ROP18) and polymers (ROP17), thus preventing accumulation of IRGs on the PV membrane.

Compared to the type I strains, the ROP18 type III allele (*ROP18^{III}*) harbors a large insertion (~2 kb) into the promoter region preventing its expression and somehow explaining avirulence of the type III strains. As evidence, virulence can be restored by overexpressing ROP18 alleles from either type I or type II strains (*ROP18^I* and *ROP18^{II}*) in the type III strain.

However, ROP18 does not explain the difference in pathogenesis between type I and type II strains. A similar approach analyzing progenies obtained from a cross between type I and type II strains identified another QTL mapping to the chromosome XII (Behnke *et al.*, 2011; Reese *et al.*, 2011). The region contains the *ROP5* locus, a cluster of tandemly repeated genes encoding for polymorphic pseudokinases. Deletion of the *ROP5* cluster in the highly virulent type I strain caused a complete loss of virulence (Behnke *et al.*, 2011; Reese *et al.*, 2011). Subsequent functional studies revealed that ROP5 functions both as a scaffold protein for the ROP18 kinase to target the IRG family of proteins and as an inhibitor of IRG assembly at the surface of the PVM (Figure 7) (Behnke *et al.*, 2012; Reese *et al.*, 2014). In cells that express significant levels of the virulent alleles of ROP5 or ROP18, IRGs fail to accumulate on the vacuole membrane and the parasites resist to the IFN- γ -induced immune response, whereas in the absence of ROP5 or ROP18, the membrane-bound IRGs induce clearance of the parasite (Figure 7). Hence, ROP5 and ROP18 solve a long-standing question in our community about the difference in virulence observed between the *T. gondii* clonal lineages. Biochemical approach also identified ROP17 as part of ROP18 complex that acts synergistically to avoid parasite clearance by IRGs (Etheridge *et al.*, 2014). ROP17 is a non-polymorphic kinase that phosphorylates IRGs, with a preference for IRG oligomers.

Interestingly, most of the atypical strains from South America carry virulent *ROP5* and *ROP18* alleles, which likely explain the basis for acute virulence of those strain laboratory in mice (Behnke *et al.*, 2015; Niedelman *et al.*, 2012). However, it is unlikely that ROP5 and ROP18 account for severe and persistent acute toxoplasmosis observed in immunocompetent individuals infected by those atypical strains (Carme *et al.*, 2002), since their cognate targets are absent in humans as discussed above. Nevertheless, ROP18 was shown to promote the degradation of ATF6- β in both murine and human cells (Yamamoto *et al.*, 2011). ATF6- β is a transcription factor associated with CD8⁺ T cell activation, suggesting that ROP18 may have alternative functions relevant to human toxoplasmosis.

3.2 Hijacking the host cell nucleus: from ROPs to GRAs.

Forward genetic approaches have also led to the identification of several pathogenicity determinants, referred here to as effector proteins that directly counteract the activity of IFN- γ -induced host effector proteins (the IRGs). Concomitant to the identification of ROP18, another polymorphic rhoptry protein, ROP16, was identified as secreted and targeted to host

cell nucleus (Figure 7) (Saeij *et al.*, 2006, 2007). ROP16 type I allele (ROP16^I) phosphorylates STAT3 and STAT6, hence inducing their nuclear translocation and transcriptional activity associated with anti-inflammatory gene expression and M2 activation in macrophages (Jensen *et al.*, 2011; Yamamoto *et al.*, 2009). Expression of ROP16^I in the intermediately virulent type II strain lowers the virulence of the parasites, thus highlighting the importance of the anti-inflammatory response during acute toxoplasmosis.

It is now clear that *T. gondii* remodels the host cell transcriptome by subverting key components of the cell signaling transduction pathways or by targeting directly the transcription machinery. Part of the *T. gondii* molecular weapon in charge of hijacking the host cell gene expression, the GRAs have emerged as interesting regulatory elements that govern host and parasite interactions, though their effect in overall virulence are generally more subtle than those observed with ROP5 and ROP18. Historically, GRA15 was the first GRA protein shown to have an extravacuolar function, although direct evidence for secretion in the host cell is still missing (Rosowski *et al.*, 2011). GRA15 was identified by forward genetic analysis of progenies from a cross type II x type III. In macrophages, GRA15^{II} activates the NF- κ B pathway, thus promoting the M1 macrophage activation and mounting the Th1 immune response characterized by IL-12 production. Accordingly, mice infected by parasites lacking *GRA15* had a significantly higher parasite burden than mice infected with wild-type strain. The mechanism by which GRA15 activates NF- κ B remains obscure, although it was shown to be independent of the MyD88 adapter, but dependent on TRAF6 and the IKK complex.

GRA6 is another vacuole-restricted GRA Effectors. It was identified in a search for *T. gondii* regulators that were specifically modulating the expression of different host transcriptional reporters (Ma *et al.*, 2014b). GRA6 is a polymorphic secreted protein that activates the host transcription factor NFAT4 (Nuclear Factor of Activated T cells 4). In mice, GRA6^I induces the expression of the chemokines *Cxcl2* and *Ccl2* that attract the inflammatory monocytes and neutrophils to the site of infection, where they control parasite spreading. How GRA6 promote NFAT activation is still uncertain, however it is very-likely acting indirectly through the Calcium Modulating LiGand (CAMLG), a known regulator of NFAT4.

The PV membrane has been regarded as a sieve limiting the delivery of proteins secreted by the parasite beyond the vacuolar space. However, the discovery of GRA16 and its remarkable ability to cross the PV membrane and to accumulate in the host cell nucleus has changed this paradigm. GRA16 migrates to host cell nucleus embedded in a high-molecular weight complex gathering the host deubiquitinase HAUSP (Herpes-virus-Associate Ubiquitine-Specific Protease) and the holoenzyme PP2A-B55 (Bougdour *et al.*, 2013). GRA16, through its interactions with HAUSP, provokes alterations in steady-state protein levels of the tumor

suppressor p53, while it induces the nuclear translocation of the PP2A holoenzyme. It remains unclear what exactly the goal of the GRA16 induced changes in the PP2A-B56 phosphatase sub-cellular localization, however it might participate to the GRA16 *modus operandi* that leads the upregulation of host genes involved in metabolism, cell cycle progression, and the p53 tumor suppressor pathway (Bougdour *et al.*, 2013). GRA24 shares with GRA16 the ability to reach the host nucleus and to regulate gene expression. Once released in the host cell, GRA24 triggers an unusual and sustained p38 α autophosphorylation resulting in the up-regulation of pro-inflammatory cytokines, including CCL2/MCP-1 and IL-12 that promote parasite clearance by macrophages at the site of infection and accordingly control parasite burden (Braun *et al.*, 2013). The *T. gondii* Inhibitor of STAT1 Transcriptional activity (TgIST), another exported GRA effector, is a critical molecular switch that accumulates to the host cell nucleus where it inhibits STAT1-dependent pro-inflammatory gene expression normally induced by the IFN- γ cytokine. TgIST was shown not only to sequester STAT1 on dedicated loci but also to promote shaping of a non-permissive chromatin by its capacity to recruit the Nucleosome Remodeling Deacetylase (NuRD) transcriptional repressor (Gay *et al.*, 2016). Therefore, TgIST subverts STAT1 activity by using its DNA-binding ability to recruit a transcriptional repressor complex instead of an activating complex when activated by IFN- γ . At the cellular level, TgIST was shown to avoid early immune-mediated elimination by blocking the IRGs-mediated clearance of the type II persistent parasites in macrophages. Thus, during acute infection in mice, TgIST-deficient parasites are rapidly eliminated by the homing Gr1+ inflammatory monocytes, hence demonstrating the protective role of TgIST against the host innate immune response (Gay *et al.*, 2016). How *T. gondii* inhibits the IFN- γ responsive genes has been of a long-standing interest to investigators studying immune response evasion. TgIST provides a molecular explanation for the unresponsiveness to IFN- γ of the *T. gondii* infected cells and complete the function of ROP5 and ROP18 in thwarting host defenses.

The recent discovery of the exported GRA family of proteins offers new insights into trafficking through the PV membrane in *T. gondii*. The role of ASP5, the *T. gondii* homolog of *Plasmodium* Plasmeprin V that recognizes and cleaves the canonical HT/PEXEL motif in proteins destined for export in red blood cells, has recently been assessed (Coffey *et al.*, 2015; Curt-Varesano *et al.*, 2016; Hammoudi *et al.*, 2015). The PEXEL-like motif of GRA16 was shown to be directly processed by ASP5; a process that is somehow required for export through the PVM since in the absence of ASP5 or functional PEXEL sequence the amounts of GRA16 in the host nuclei were dramatically reduced and GRA16 was retained in the vacuolar space (Coffey *et al.*, 2015; Curt-Varesano *et al.*, 2016; Hammoudi *et al.*, 2015). The export of TgIST is dependent on ASP5 (Gay *et al.*, 2016); however, whether its predicted PEXEL motif corresponds to a cleavage site for ASP5 remains to be determined. Unexpectedly, GRA24,

which does not have a conserved PEXEL motif, is processed in an ASP5-independent fashion, although ASP5 is required for its export, indicating that protein maturation is a general characteristic of proteins destined for exported (Curt-Varesano *et al.*, 2016). Overall, the *Plasmodium* model of protein translocation through the PV membrane is evidently challenged by insights from *T. gondii*, whether it concerns the requirement of a PEXEL addressing signal, the ATP-powered unfolding, or an EXP2-forming pore for the *T. gondii*'s GRAs that traffic through the PVM (see review (Hakimi *et al.*, 2017)). In this regard, MYR1 was recently identified as an alternative translocation pathway (Franco *et al.*, 2016). The export of GRA16, GRA24, and TgIST is compromised in cells infected by *myr1*-deficient parasites, while functions of MAF1 and GRA15 remain unaffected, suggesting that MYR1-dependent export is devoted only to the GRAs that physically translocate across the PV membrane and accumulate in host cell compartments (Franco *et al.*, 2016) and unpublished data from Hakimi's laboratory).

4- β -Catenin and Wnt signaling pathway: a target for microbes.

β -Catenin is an evolutionarily conserved protein from *Drosophila* to human that exerts a crucial role in developmental and homeostatic processes (Clevers, 2006). More specifically, β -catenin serves as an integral structural component of cadherin-based adherent junction, and as a co-transcription factor for the Wnt signaling pathway in the nucleus, where it initiates the expression of a variety of genes involved in cell proliferation and differentiation. Additionally, several studies suggest that β -catenin is not only determining the cell fate through manipulation of gene expression, but also largely participating in the regulation of the immune system (Staal *et al.*, 2008). The capacity of β -catenin to regulate different cellular processes makes the Wnt signaling pathway a target of choice for pathogens. In this chapter, I will mainly focus on β -catenin-based Wnt signaling pathway and how the intracellular pathogens interfere with this signaling pathway for their own purpose.

4.1 The canonical Wnt signaling pathway

There are at least three different Wnt pathways: the planar cell polarity pathway, the Wnt- Ca^{2+} pathway, and the canonical Wnt pathway, in which β -catenin is the central player (Brembeck *et al.*, 2006; Staal *et al.*, 2008). In the absence of a Wnt ligand binding to the receptor complex, β -catenin will be targeted for degradation by the proteasome through the action of a complex named the β -catenin destruction complex. This complex is composed of two scaffolding proteins, including Adenomatous Polyposis Coli (APC) and Axis inhibition protein 1 (Axin1), and the serine/threonine kinases Casein Kinase 1 (CK1) and Glycogen Synthase Kinase 3 β (GSK3 β) (Behrens *et al.*, 1998). β -Catenin degradation strictly depends on a phosphorylation cascade of conserved serine/threonine residues at the amino-terminal region of the protein. Phosphorylation of β -catenin at serine residue 45 (S45) by CK1 primes the subsequent and sequential event of phosphorylation at S33, S37, and threonine 41 (T41)

by GSK3 β (Liu *et al.*, 2002). Hence, phosphorylated β -catenin is recognized by the β -transducin-repeat-containing protein (β -TRCP), resulting in the ubiquitylation and proteasomal breakdown (Aberle *et al.*, 1997). The continual elimination of β -catenin prevents its nuclear accumulation, where a transcriptional repressive complex is assembled to silence the Wnt-responsive genes (Liu *et al.*, 2008).

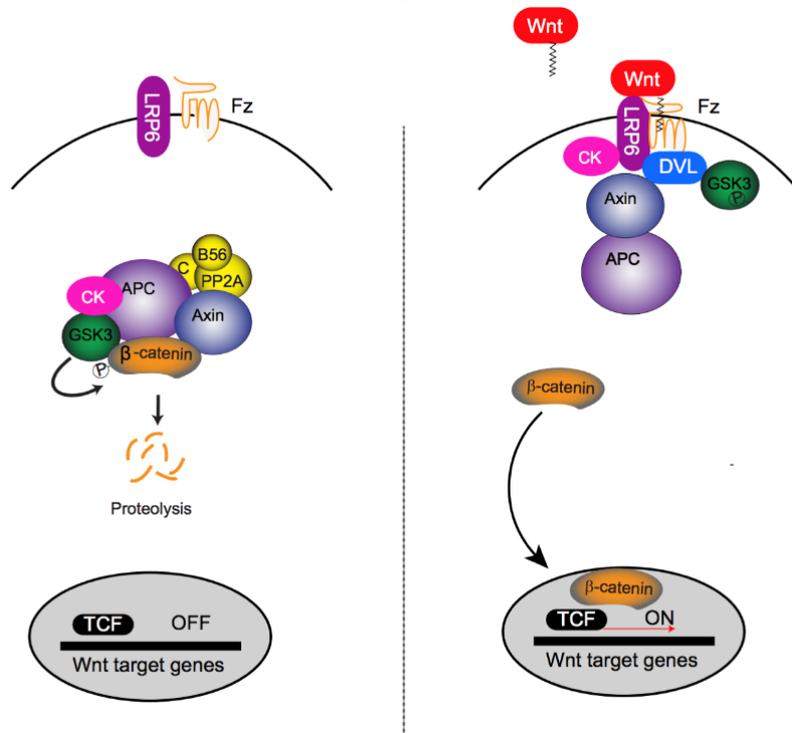


Figure 8. Canonical Wnt/ β -catenin signaling. *Left.* In the absence of Wnt signaling, β -catenin is maintained at low levels in the cytoplasm and nucleus as a result of continual phosphorylation by the CK1 and GSK3 β . Phosphorylated forms of β -catenin are recognized by β TRCP, a component of the ubiquitin-protein ligase complex, and degraded by the ubiquitin-proteasome pathway. In the absence of the Wnt ligands. The TCF/LEF proteins associate with co-repressors that shut off the expression of Wnt target genes. *Right.* Upon binding of Wnt protein to the receptor complex, a signaling cascade is initiated. LRP6 is phosphorylated by CK1 and GSK3 β , and Axin1 is recruited to the plasma membrane. The kinases associated to the β -catenin destruction complex are inactivated and β -catenin translocates to the nucleus to form an active TF complex with the TCF/LEF family of proteins, leading to transcription of a large set of target genes.

At the cell membrane, the binding of Wnt ligands to the Frizzled transmembrane receptors and LRP5/6 co-receptors (Low density lipoprotein Receptor-related Protein) leads to the inhibition of the β -catenin destruction complex (Figure 8). Wnt ligands are lipid-modified small molecules, which are secreted by various cell types. Following Wnt binding to its receptor, the cytoplasmic protein Dishevelled (Dvl) is recruited to the receptor complex, which promotes the

phosphorylation of LRP5/6 by CK1 and GSK3 β (Malbon and Wang, 2005). Hence, phosphorylated LRP5/6 “pulls out” rate-limiting component Axin1 from APC and GSK3 β and therefore destabilizes the β -catenin destruction complex (Liu *et al.*, 2005). Controversially, Li and colleagues suggested that an intact Axin1-containing β -catenin destruction complex remains bound to the LRP5/6 receptor rather than dissociation of Axin1 from the rest of the complex (Clevers, 2006). To date, it is still incompletely understood how Axin1 is contributing to the disruption of the β -catenin destruction complex. However, this disruption results in the release of an unphosphorylated form of β -catenin that escapes from degradation and translocates to the nucleus. Nuclear β -catenin associates with T Cell Factor/Lymphoid Enhancer Factor (TCF/LEF) transcription factors and promotes the transcription of the Wnt target genes (Figure 8). TCF/LEFs are multifunctional proteins that use their sequence-specific DNA-binding and context-dependent interactions to specify which genes will be regulated. Although recruitment of nuclear β -catenin to target genes serves as the hallmark of canonical Wnt signaling, mechanisms controlling stage- or tissue-specific transcriptional responses remain elusive. In contrast to invertebrates, which typically contain one TCF/LEF protein that can both activate and repress Wnt target genes, isoform complexity of TCF/LEF family observed in vertebrates have led to complex regulatory networks, in which individual TCF/LEF isoforms have distinct activities (Cadigan and Waterman, 2012).

4.2 The role of β -catenin in anti-inflammation

The expression of Wnt/ β -catenin signaling target genes regulates not only stem cell development, cell proliferation and differentiation, but also immune response, e.g., *CTLA4*, a ligand providing an anti-stimulatory signal to T cells (McCoy and Le Gros, 1999; Shah *et al.*, 2008). These findings revealed a broad control of the anti-inflammatory response by the Wnt/ β -catenin pathway. As a co-transcription factor, β -catenin is constitutively active in intestinal Dendritic Cells (DCs) and macrophages, which is critical for the induction of regulatory T cell (Treg) responses suppression of Th1 and Th17 cell responses. The depletion of β -catenin in intestinal DCs results in increased Th1 and Th17 cells over regulatory cells in the intestine, and eventually shifts the balance from anti-inflammatory cytokine production to increase the inflammatory response in the intestine (Manicassamy *et al.*, 2010). These findings demonstrate a potential tolerogenic role of β -catenin signaling. Interestingly, the role of β -catenin in Treg survival is still under debate. Through the expression of a stable form of β -catenin, Ding and colleagues obtained a remarkable enhancement of the number and survival of the Treg cells *in vitro*, which provides a substantial protection in case of inflammatory bowel disease (Ding *et al.*, 2008). Controversially, van Loosdregt *et al.* reported that the activation of Wnt signaling reduced Treg-mediated immune suppression, which could be rescued by the disruption of Wnt signaling in Tregs (van Loosdregt *et al.*, 2013). Despite conflicting evidence,

these findings argue for a role of β -catenin in the regulation of immune tolerance and provide a new view on Wnt/ β -catenin signaling pathway in the anti-inflammatory response.

4.3 Hijacking β -catenin signaling pathway by intracellular pathogens

Intracellular pathogens have evolved strategies to promote their survival by dramatically modifying the transcriptional profile of the host cells they infect, most notably through interference with host signaling pathways. Given the critical role of Wnt/ β -catenin signaling pathway in multiple biological processes, it is not surprising to find that β -catenin represents an attractive target for intracellular pathogens. Indeed, the enterobacteria *Salmonella spp.* secretes the effector protein AvrA that positively modulates the Wnt/ β -catenin signaling pathway by stabilizing β -catenin, hence activating Wnt/ β -catenin transcriptional activity in the infected intestinal epithelial cells (Liu *et al.*, 2010). Possibly, the up-regulation of the Wnt/ β -catenin signaling pathway could contribute to maintenance of the intestinal epithelium by increasing epithelial cells proliferation.

Upon infection by the type III latency Epstein-Barr Virus (EBV), which is responsible for mononucleosis, stabilizes β -catenin and increases β -catenin/TCF transcriptional activity in B lymphocytes (Shackelford *et al.*, 2003). Though the exact molecular mechanism is still unclear, an association of free cytoplasmic β -catenin with deubiquitinating enzymes may play a role in β -catenin stabilization. Hence, the activation of β -catenin/TCF by EBV infection may contribute to the lymphoproliferation characteristic of infection by type III latency EBV.

As another successful intracellular pathogen, *T. gondii* has developed the ability to rewrite the host transcriptomic profile by interfering various signaling pathways, in order to renovate the infected host cells (Hakimi *et al.*, 2017). In last decade, multiple molecular components critical to host signaling pathways have been found to be positively or negatively regulated by *T. gondii* effector proteins, including STAT1, STAT3/6, p53 and MARP kinase (Bougdour *et al.*, 2013; Braun *et al.*, 2013; Gay *et al.*, 2016; Saeij *et al.*, 2007). However, subversion of the Wnt/ β -catenin pathway by *T. gondii* has never been reported so far. In our study, we identified a novel dense granule protein GRA18, which is able to positively regulate Wnt/ β -catenin signaling pathway to modulate host cell gene expression. Our discovery did not only show the ability of *T. gondii* to regulating Wnt/ β -catenin signaling, but also enlightened a newly emerging function of β -catenin in inflammatory response.

AIM OF MY PhD PROJECT

Although the last decade has seen significant progress in identifying parasite effectors, those discovered so far do not fully explain the overall effect of the parasite on the host cell, nor the drastic remodeling of the host transcriptome. My PhD project was therefore directed to identify novel effector proteins that interfere with host functions.

We report the isolation and characterization of a new effector protein encoded by the previously uncharacterized gene *TGGT1_288840*, hereafter referred to as GRA18. We found that GRA18, once delivered in the host cell, hijacks the β -catenin destruction complex to upregulate a specific set of genes, including the chemokines *Ccl17*, *Ccl22*, and *Ccl24*. β -catenin is the main mediator of the Wnt signaling pathway and is critical for numerous cellular functions, including hematopoietic cell fate determination and proliferation (Staal *et al.*, 2008). Under normal conditions, the cytosolic β -catenin is maintained at low levels through continual phosphorylation by the Ser/Thr kinases Glycogen Synthase Kinase 3 (GSK3) and the Casein Kinase I- α (CKI- α), which promote its ubiquitination and subsequent proteosomal degradation by the 26S proteasome (Liu *et al.*, 2002). Activating Wnt ligands cause functional inactivation of the destruction complex, leaving β -catenin unphosphorylated. This fully active form then translocates in the nucleus to drive transcriptional activity in association with the T cell factor/lymphoid enhancer factor (Tcf/Lef) transcription factors (Clevers, 2006). While usually associated with embryonic development and tumorigenesis (Klaus and Birchmeier, 2008), β -catenin is now well-recognized for its role in immunity (Staal *et al.*, 2008).

Surprisingly, while *T. gondii* induces changes in the host transcriptome, analysis of the differentially regulated genes did not reveal any transcription pattern related to GSK3 or β -catenin (Melo *et al.*, 2013), whereas cluster of genes regulated by a variety of transcription factors such as NF- κ B, STATs, and p53 were repeatedly identified as commonly hijacked upon infection in both human and murine cells. Beside from the Myc (c-Myc) oncogene, none of the genes known to be regulated by β -catenin and its cofactors Tcf/Lef were found altered by *T. gondii* infection. Therefore, GRA18 unveils a novel aspect of gene expression regulation in macrophages by *T. gondii* through β -catenin hijacking.

RESULTS

Characterization of a *Toxoplasma* effector uncovers an alternative β -catenin-regulatory pathway of inflammation.

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Introduction of the manuscript

Toxoplasma gondii is the causative agent of toxoplasmosis, a widespread parasitic infection in humans which has been recognized as leading cause of deaths attributed to foodborne illness in the United States (Scallan *et al.*, 2015). Toxoplasmosis is a potentially life-threatening chronic disease in people with weakened immune systems, such as those suffering from acquired immunodeficiency syndrome or undergoing chemotherapy and graft rejection therapy (Montoya and Liesenfeld, 2004). In addition, outcomes of congenital toxoplasmosis significantly vary with the timing of infection from recurrent eye diseases to adverse motor or neurologic impairments that can cause stillbirth (Halonen and Weiss, 2013). *T. gondii* belongs to the phylum *Apicomplexa* and as most *Apicomplexa* species, it develops and proliferates inside a surrogate host cell. Remarkably, *T. gondii*'s host range is exceptionally broad since it can infect virtually all nucleated cells of mammals, marsupials and birds (Dubey, J.P, 2009).

To get access to a host cell, the *T. gondii* replicative tachyzoite stage triggers the formation of a peculiar membrane-bound compartment called the Parasitophorous Vacuole (PV). The PV is shaped as a safe niche that supports parasite growth and multiplication while being kept hidden from the harmful endocytic pathway (Jones and Hirsch, 1972; Mordue *et al.*, 1999). Aside from this direct strategy, the parasite makes use of an ever increasing list of products to specifically modulate host cell gene expression. These products are injected at the very onset of or just post-invasion and act mainly as effector proteins to counteract host defense mechanisms (Hakimi *et al.*, 2017), hence ensuring proper balance between parasite virulence and host resistance and therefore the circulation of parasite populations between hosts for life cycle completion. Known effectors are released from specialized sets of secretory organelles including the rhoptry vesicles for ROP16 and ROP38 proteins and the dense granules for GRA15, GRA16, GRA24, TgIST, GRA6, and GRA25 proteins (for review see (Hakimi *et al.*, 2017)). These effectors can be partitioned according to whether they remain exposed at PV membrane (PVM), or they travel beyond the PVM into the cytoplasm. Interestingly, the latter are all described to travel to the nucleus, where they target specific sets of genes.

The view that *T. gondii* tachyzoite effectors can cooperatively rewire host cell gene expression started to emerge recently, underscoring the need for identifying and characterizing the parasite effector repertoire to provide a comprehensive analysis of how these molecules interplay during infection at cellular and host levels. With this concern, we searched for new effectors and characterized GRA18, as the first dense granule protein that upon release is only detected in the host cell cytoplasm throughout *Toxoplasma* intracellular life. We provide evidence that secreted GRA18 forms versatile complexes with components of the β -catenin

destruction complex, which includes β -catenin, GSK3 α/β , and the PR56/B'-containing PP2A holoenzyme and thereby prevents the continual elimination of β -catenin. Instead, we observed that β -catenin accumulates and travels to the host cell nucleus, where it can activate otherwise repressed target genes. Nuclear β -catenin is known as the main effector of the canonical Wnt signaling pathway, acting as a coactivator of the Lymphoid Enhancer-binding Factor (LEF), or T Cell Factor (TCF) proteins to drive Wnt-specific transcriptional programs depending on cell lineages. We found in murine macrophages that GRA18 induces in a β -catenin-dependent fashion the expression of chemokines in particular *Ccl17*, *Ccl22*, and *Ccl24*. Intriguingly, all these chemokines, notoriously known for their role in anti-inflammatory responses (Biswas and Mantovani, 2010; Mantovani *et al.*, 2004), have not previously been identified as β -catenin targets. Therefore, besides discovering *Toxoplasma* GRA18, its partners and the down signaling pathway in the course of infection, this work also highlights a novel and unexpected β -catenin property to shape the inflammatory response.

Results

1- GRA18 is secreted and exported to the cytoplasm of infected host cells.

The gene *TGGT1_288840*, renamed hereafter *GRA18*, was originally found along with the previously characterized genes *GRA16*, *GRA24*, and *TgIST* in an *in silico* search for candidate genes encoding proteins delivered by tachyzoites into the host cell (Bougdour *et al.*, 2013; Braun *et al.*, 2013; Gay *et al.*, 2016). *GRA18* protein accommodates both a transit peptide for targeting to the secretory pathway and a canonical TEXEL motif found on vacuole residing proteins (GRAs proteins), some of which traffic through the parasitophorous vacuole membrane (PVM) to reach the host cell (Coffey *et al.*, 2015; Hakimi *et al.*, 2017; Hammoudi *et al.*, 2015; Hsiao *et al.*, 2013)

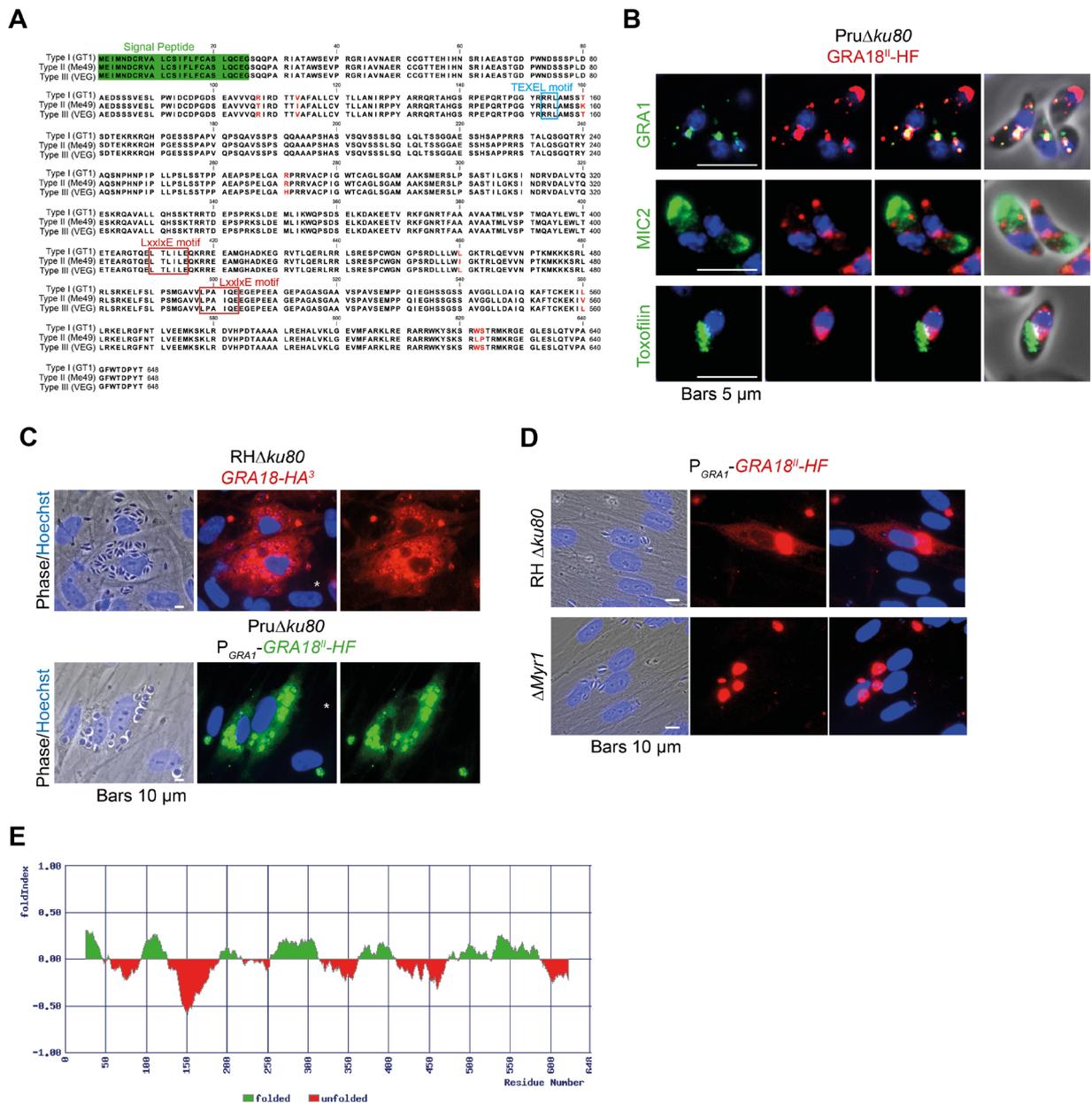


Figure 1. GRA18 is secreted and exported to the host cell cytoplasm.

(A) Alignment of GRA18 alleles from *T. gondii* strains of types I (TGGT1_288840), II (TGME49_288840), and III (TGVEG_288840). The signal peptide sequence (highlighted in green), the B56 SLiM motifs (LxxLx; boxed in red), and the *Toxoplasma* Export Element (TEXEL; RRL motif, boxed in blue) are shown. Single amino acid polymorphisms are indicated by red letters. The alignment was done using ClustalW method. (B) GRA18^{II}-HAFlag in Pru *ku80* extracellular parasites is contained in cytoplasmic organelles distinct from the apical micronemes (MIC2) and rhoptries (Toxofilin), and partially co-localizing with the dense granule protein GRA1. (C) GRA18 secretion and export to the host cytoplasm. HFFs were infected with type I RH parasites expressing endogenously tagged *GRA18* with hemagglutinin (HA) (upper panel, RH $\Delta ku80$ *GRA18*-HA³, in red) or type II Pru strain ectopically expressing a HAFlag (HF)-tagged copy of GRA18^{II} under the control of the strong promoter of *GRA1* (Pru $\Delta ku80$ P_{GRA1}-GRA18^{II}-HF). Cells were fixed 18 h post-infection (hpi) and stained with anti-HA antibodies and Hoechst DNA-specific dye (bleu). The white asterisks indicate uninfected HFF cells. (D) MYR1 is required for GRA18 export in the host cell. HFFs were infected with RH WT or RH $\Delta myr1$ parasites transiently transfected with a vector expressing an HF-tagged GRA18 (P_{GRA1}-GRA18^{II}-HF), and at 18 hpi, the cultures were fixed and stained with antibodies to the HA tag. (E) Schematic representation of GRA18 probability of disorder. Segments with values <0 are predicted to be disordered (in red), and segments with values >0 correspond to folded regions (in green).

(Figure 1A). To determine the localization of GRA18, a HA³ or HAFlag (HF)-epitope tags were inserted either at the carboxyl-terminus of the endogenous *GRA18* locus in the type I strain (RH *ku80*), or as an extra copy in type II strain (Pru *ku80*), respectively. Within extracellular tachyzoites, GRA18-HF co-localized to some extent with the known dense granule resident protein GRA1, but not with the micronemal protein MIC2, nor the Toxofilin rhoptry protein (Figure 1B). When expressed in intracellularly growing parasites, the GRA18-HA³ protein accumulated within the cytoplasm of the infected host cells (Figure 1B, upper panel), which differs from the other effectors identified so far that are preferentially targeted to the host cell nuclei. Very similar results were obtained with type II parasites (Pru *ku80*) expressing GRA18-HF under the control of the promoter of *GRA1* (Figure 1C, lower panel). Moreover, in the absence of the parasite MYR1 protein, the export through the PVM of GRA18 no longer occurs (Figure 1D), indicating that GRA18 is using a secretion route to the host cell that is shared with the other exported GRA effectors (Franco *et al.*, 2016). GRA18 is a 70 kDa protein predicted to be highly disordered (Figure 1E) with no apparent homolog counterpart outside of the coccidia. While GRA18 shows little polymorphism among the three major strain types of *T. gondii* (99% homology, Figure 1A), the protein is highly divergent in *Neospora caninum* (39% identity). Altogether, these data indicate that GRA18 is a member of the new class of exported

dense granule proteins targeted to the host cell, and adds to the growing list of exported GRAs (Hakimi *et al.*, 2017; Nadipuram *et al.*, 2016).

2- GRA18 forms versatile complexes with host elements of the β -catenin destruction complex.

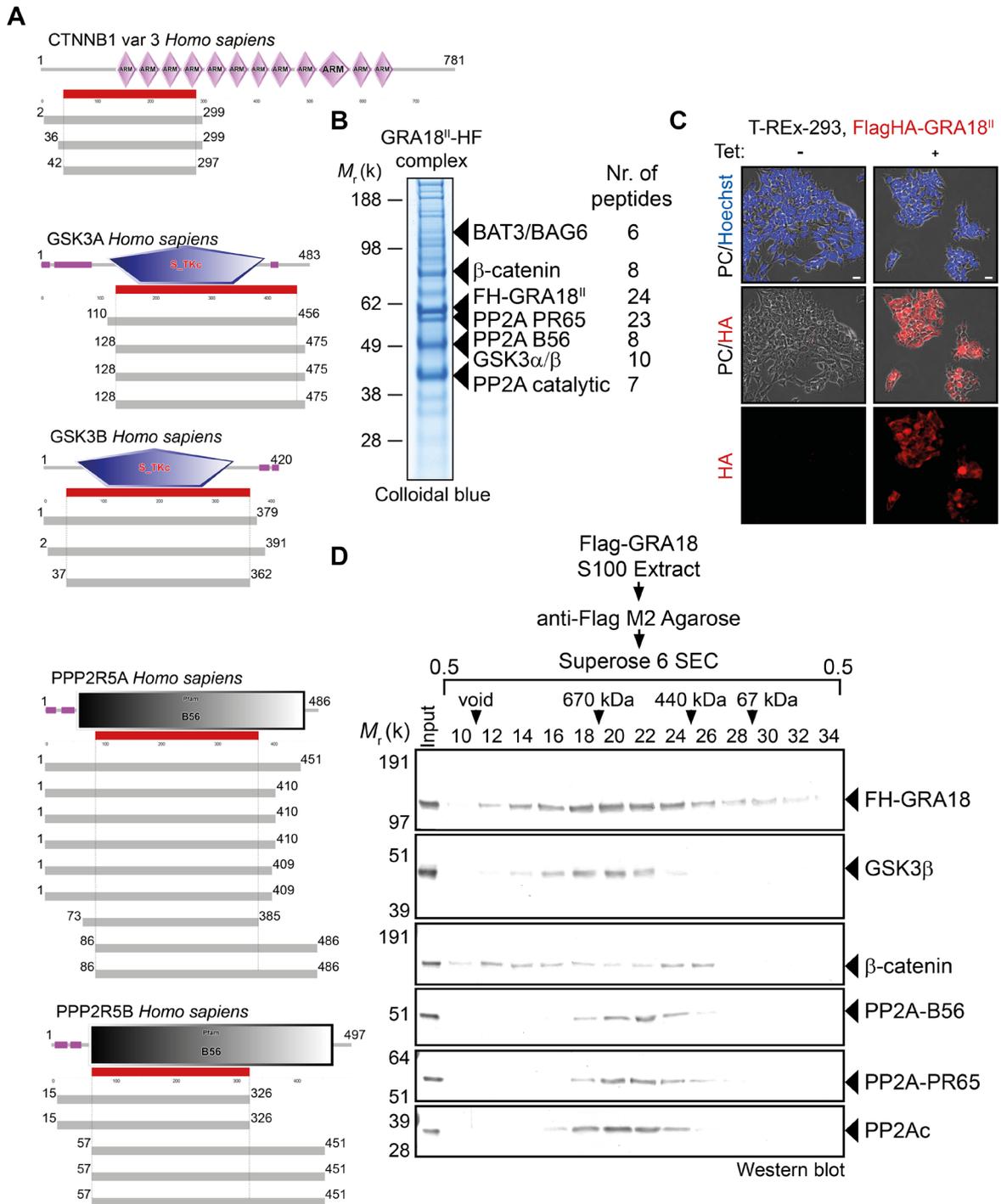


Figure 2. GRA18 binds directly to β -catenin, GSK3 α/β , and PP2A-B56 of the host cells.

(A) Yeast-two hybrid screening of a human placental cDNA library to identify potential host partners for GRA18. Schematic representation of the identified partners having the highest Global PBS® scores are shown. Summary of the prey clones that interacted with the GRA18 bait are represented as grey bars. Multiple independent interacting prey clones allowed Selected Interaction Domain (SID, in red) analysis that delineates the shortest fragment that is shared with all the interacting clones, and thus represents a potential region mediating the interaction with GRA18. (B) GRA18 associates with β -catenin, GSK3 α/β , and PP2A-B56 in infected murine macrophages. GRA18-associated proteins were purified by Flag chromatography from protein extracts of J774 cells infected with parasites expressing HF-tagged GRA18 (Pru *k80*, P_{GRA1}-GRA18^{II}-HF). Immunopurified proteins were resolved by SDS-PAGE, followed by stained by colloidal blue staining for mass spectrometry analysis. The identity of the proteins and their respective number of peptides are indicated on the right of the figure. (C) Immunofluorescence assay (IFA) of FH-GRA18 ectopically and stably expressed in T-Rex-293 cell line. Cells were either left untreated (-) or treated with 1 μ g/mL tetracycline for 12 h before fixation and staining with anti-HA antibodies (in red) and Hoechst DNA-specific dye (in blue). Scale bar, 10 μ m. (D) Size-Exclusion Chromatography (SEC) analysis of the GRA18-associated proteins. FH-GRA18 was immunopurified from tetracycline-induced T-Rex cells (T-Rex-GRA18^{FL}). SEC fractions were analyzed by immunoblot using the indicated antibodies.

Since GRA18 does not carry any recognizable structural or functional protein domains that can be used to infer its function, we thought of seeking host partners, if any, to decipher the pathway the protein could interfere with following its delivery. Initially, we used a genome-wide yeast-two hybrid (Y2H) screen to identify potential host partners interacting with GRA18. We therefore screened a human placenta complementary DNA (cDNA) library (prey) using N-terminally fused GRA18 (aa 27 to 648) from type II strain as a bait (LexA-GRA18^{II}). Of a total of 65.8 million cDNA fragments screened there were 65 positive hits for 11 different proteins. The binding proteins were given Global Predicted Biological Score (Global PBS®), with scores between A and D ('A' having the highest confidence of binding) were found (Table S1), if their coding sequences are in-frame and have no in-frame stop codons. The various prey clones that interacted with the GRA18 bait with the highest PBS® scores are depicted as grey bars in the Figure 2A. Interestingly, multiple components of the β -catenin destruction complex were found among the interactants: β -catenin itself, the glycogen synthase kinase-3 (GSK3 α/β), and the protein phosphatase 2A (PP2A) regulatory subunit B56 $\alpha/\beta/\gamma/\delta$. These interactions were subsequently confirmed by applying chromatography and mass spectrometry-based proteomics analysis of cell extracts that were prepared from primary HFF cells infected by a *T. gondii* strain expressing Flag-tagged GRA18 under the control of the *GRA1* promoter (Pru *k80*, P_{GRA1}-GRA18-HF). Following Flag affinity chromatography, GRA18 was recovered in the

eluates associated with β -catenin, GSK3 α/β , and the PP2A-B56 δ/ϵ and the interactions mediating this complex partnership were quite robust as they resisted to stringent washing conditions (0.5 M KCl and 0.1% NP-40) (Figure 2B). Interestingly, the presence of the scaffolding subunit PP2A65 RA and the catalytic subunit PP2Ac along with the PP2A-B56, testifies to the presence of a functional PP2A holoenzyme. The B56 regulatory subunit is presumably mediating the recruitment of the PP2A holoenzyme as it is the only PP2A subunit found by Y2H to interact directly with GRA18 (Figure 2A and Table S1). Thus, these data confirm the interactions between GRA18 and β -catenin, GSK3, and the PP2A-B56, and validate the relevance of these interactions in the context of infection.

To further detail the interactions between GRA18 and the host partners β -catenin, GSK3, and PP2A-B56, we generated an inducible Flag-tagged GRA18-expressing HEK293-derived human cell line (T-Rex-293, Figure 2C). After induction, the protein localized in the cytoplasm, similarly to the naturally delivered protein in infected cells. GRA18 was purified and fractionation of the Flag affinity eluate by size-exclusion chromatography (SEC) followed by immunoblot analysis revealed that GRA18 forms distinct complex(es) ranging from 400 to over 700 kDa globular sizes with β -catenin, GSK3 β , and the PP2A-B56 holoenzyme (Figure 2D). While GSK3 β and the PP2A-B56 holoenzyme eluted as discrete and overlapping complexes, in contrast, GRA18 and β -catenin were both spreading, possibly reflecting the presence of multiple sub-complexes that likely assemble with GRA18. Altogether, these data confirmed a complex partnership of GRA18 with host proteins as revealed by biochemical and Y2H approaches, the latter from which we inferred direct interactions between GRA18 and the host proteins β -catenin, GSK3 α/β , or the PP2A-B56.

3- β -Catenin, GSK3 α/β , and PP2A-B56 bind different protein domains of GRA18.

To further detail the mode of interaction between GRA18 and the aforementioned partners, we performed a domain mapping analysis by Y2H system and challenged the binding of GRA18 fragments to β -catenin, GSK3 α/β , or the PP2A-B56 regulatory subunit in yeast (Figure 3B). Interestingly, the N-terminal region of GRA18 shelters the binding site for β -catenin, whereas the C-terminal fragments accommodate the interaction to the PP2A-B56 subunit (Figures 3B and D). None of the GRA18 fragments tested other than the full-length protein could support the interactions with GSK3 in this assay, suggesting that binding to GSK3 necessitates a larger contact surface on GRA18.

In order to examine this further, we generated inducible T-Rex cell lines expressing each different flag tagged sub-domains of GRA18 and analyzed by western blot the affinity eluates from each chimeric protein. Figure 3C shows that while the full-length GRA18 (FH-GRA18^{FL}) pulled-down β -catenin, GSK3 β , and PP2A-B56, the GRA18^{Ct} truncated version was

still able to bind to PP2A-B56 in agreement with the Y2H experiments, but also retained its ability to interact with GSK3 β . Conversely, GRA18^{Nt} induced in T-Rex cells has lost its ability to interact with PP2A-B56 and the binding to GSK3 β was significantly impaired when compared to the full-length or the C-terminal region of GRA18, thus confirming that the GRA18

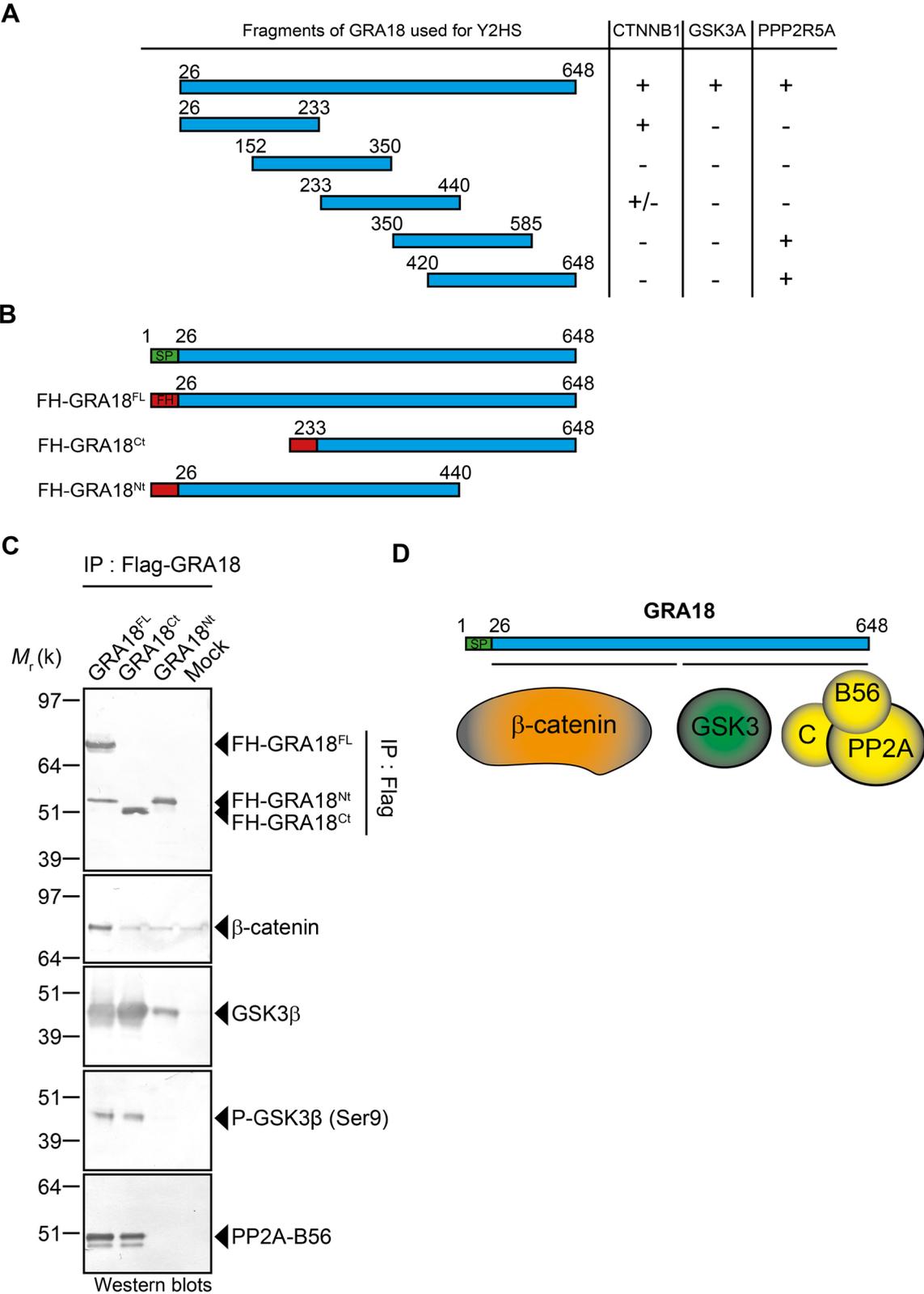


Figure 3. β -Catenin, GSK3, and PP2A-B56 recognize different domains of GRA18. (A) Interaction domain mapping by Y2H assay using the indicated fragments of GRA18 to delineate the interacting domains of GRA18 with CTNNB1 (β -catenin), GSK3A (GSK3 α) and PPP2R5A (PP2A-B56 α). CTNNB1 interacts with the N-terminus of GRA18, whereas PPP2R5A interacts with the C-terminal fragments. Although the interaction of full length GRA18 (amino acids 26-648) with GSK3A was confirmed, none of the assayed GRA18 fragments interacted with GSK3A. (B) Schematic representation of full-length (amino acids 26-648) and truncated versions of GRA18 (FH-GRA18^{Nt(aa 26-440)} and FH-GRA18^{Ct(aa 233-648)}) proteins stably expressed in T-Rex cells. (C) Cytoplasmic fractions from T-Rex cells presented in (B) were immunoprecipitated with anti-Flag antibodies and analyzed by western blotting. Untransfected T-Rex cells were used as a mock. (D) Schematic diagram summarizing the interaction domain mapping of GRA18 obtained from Y2H and biochemical approaches.

C-terminal region mediates the interaction with both the phosphatase and the kinase. It is noteworthy that both the N- and C-ter truncated versions used here have lost their ability to pull down β -catenin, which is somewhat puzzling given the data obtained in the Y2H assay. We cannot exclude here the possibility that the harsh washing conditions (0.5 M KCl and 0.1% NP-40) shattered the weak interactions between β -catenin and the GRA18 N-terminal fragment (Figure 3B). Therefore, we propose a simple mechanistic model in which β -catenin is weakly binding to the N-terminal region of GRA18, whereas GSK3 β and the PP2A-B56 holoenzyme interact with the C-terminal region (Figure 3D).

4- GRA18 functions as a positive regulator of β -catenin.

As demonstrated above, GRA18 interacts with well-known components of the β -catenin destruction complex, a central multiprotein complex of the Wnt signaling. Indeed, β -catenin is a main effector of the Wnt signaling that regulates transcription of Wnt target genes. The core of the Wnt pathway is the regulation of β -catenin by a cytoplasmic destruction complex composed, in addition to β -catenin itself, of a central scaffold protein, axis inhibition protein (Axin) that interacts with other factors, such as the adenomatous polyposis coli protein (APC), the Ser/Thr kinases GSK3 and casein kinase 1 (CK1), and the PP2A-B56 phosphatase (Reviewed by (Stamos and Weis, 2013)). In the absence of Wnt signaling, the destruction complex efficiently captures cytoplasmic β -catenin, leading to its phosphorylation by GSK3 and recognition by the β -TrCP ubiquitin ligase for degradation by the 26S proteasome. Wnt ligands cause functional inactivation of the destruction complex, with ensuing escape of β -catenin from degradation, resulting in β -catenin accumulation and nuclear entry (Clevers, 2006; Li *et al.*, 2012; Taelman *et al.*, 2010). Given the interactions between GRA18 and β -catenin, GSK3 β , and PP2A-B56, we hypothesized that GRA18 could interfere with β -catenin regulation during infection. To test this hypothesis, we generated parasites knockout for

GRA18 (Figures 4A and B). It is noteworthy that the $\Delta gra18$ mutant strains (Pru *ku80* $\Delta gra18$ and 76K $\Delta gra18$) displayed no obvious growth phenotype in cell culture (Figure 4C and data not shown), and a somewhat reduced virulence phenotype in mice when challenged by intraperitoneal injection (data not shown). While assessing β -catenin amounts and subcellular localization we showed that in both uninfected human (HFF) and murine (L929) cells, β -catenin was predominantly cytoplasmic. Intriguingly, β -catenin signals remained unaffected upon *T.*

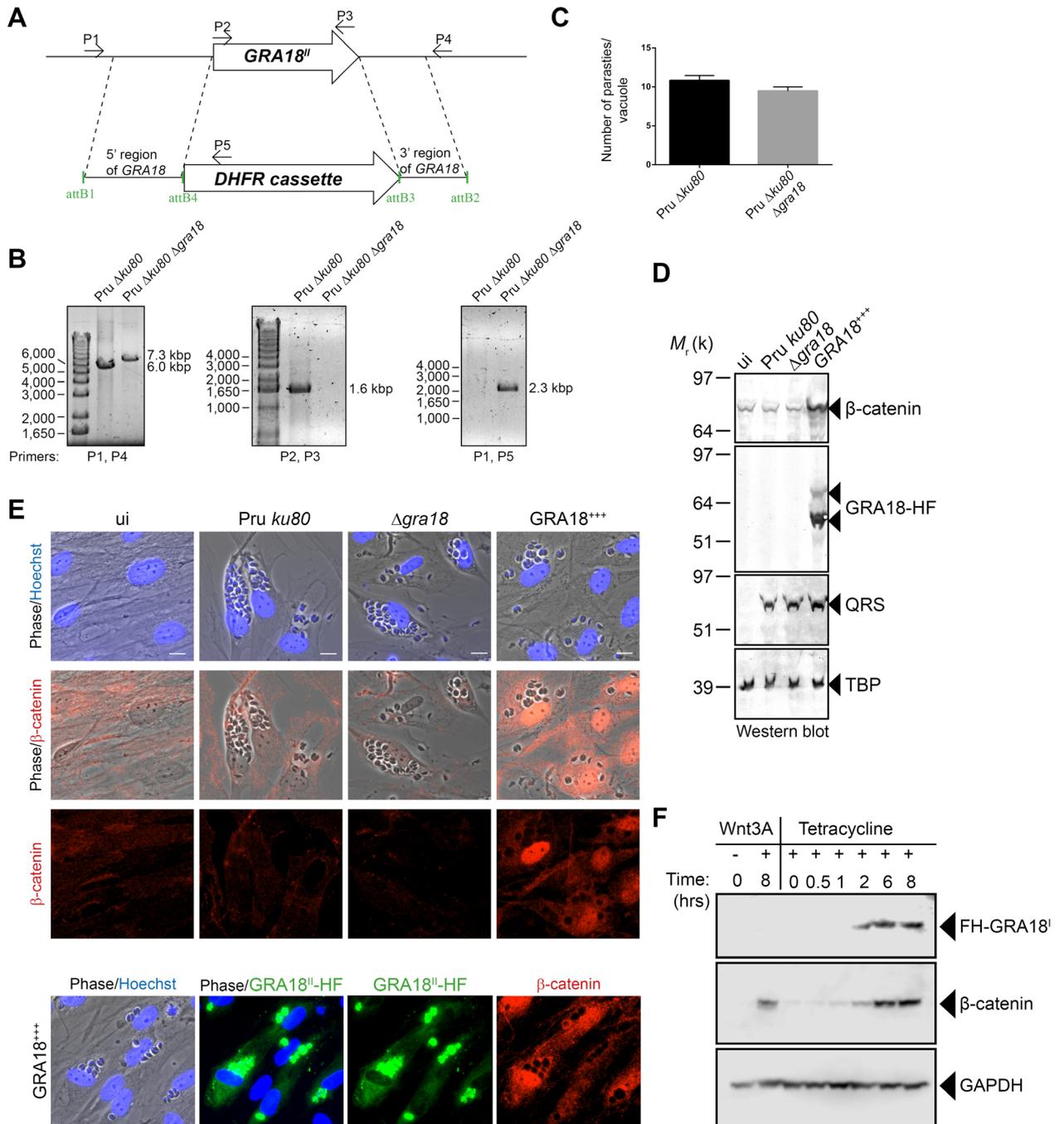


Figure 4. GRA18 is a positive regulator of β -catenin. (A) Generation and confirmation of insertion/deletion of *GRA18* in *T. gondii* type II Pru strain. Schematic representation of the *GRA16* locus with the double homologous recombination event between the knockout construct (pDEST14 KO *GRA18*) and genomic DNA replacing the *GRA18* coding sequence with the *DHFR* cassette used for positive selection. (B) PCR reactions with the indicated primers confirming the deletion/insertion of *GRA18* in the mutant parasites. (C) Parasites lacking *GRA18* exhibit no growth defect *in vitro* as determined by fluorescence imaging assays in HFFs. Data are averages for three independent replicates. (D) Effect of *GRA18* on β -catenin levels. Murine L929 cells were left uninfected (ui) or infected with wild-type (Pru *ku80*), Δ *gra18*, or the Δ *gra18* *GRA18*⁺⁺⁺ complemented (*GRA18*⁺⁺⁺) strains. At 18 h post-infection, cells were harvested and analyzed by immunoblot using the indicated antibodies. QRS was used to control parasite loading. (E) IFA of β -catenin in confluent HFFs left uninfected (ui) or infected with the indicated strains for 18 h. In the lower panel, IFA was performed using an anti-HA antibody to monitor the HF-tagged version of *GRA18* in the *GRA18*⁺⁺⁺ complemented strain. Data are representative of at least three independent experiments. (F) Immunoblot analysis of nuclear fraction of the T-Rex FH-*GRA18* cell line left untreated or induced with tetracycline for the indicated periods of time. The Wnt3A ligand was used as a positive control.

gondii infection regardless of the *GRA18* status (Figures 4E and D, compare Pru *ku80* with Δ *gra18*). Very different results were obtained with parasites expressing high levels of *GRA18*, referred to as *GRA18*⁺⁺⁺. β -Catenin was strongly induced in cells infected by the *GRA18*⁺⁺⁺ strain compared to uninfected cells and the strong accumulation of β -catenin in the host nuclei suggested that a transcriptionally active bulk of β -catenin was produced (Figures 4E and D). To determine whether additional parasite factors may contribute to the *GRA18*-mediated β -catenin induction, we used ectopic expression of *GRA18* in HEK293-derived human cell line. As a positive control, cells were treated with exogenous Wnt3A ligand, a natural inducer of β -catenin, thus confirming that the T-Rex cells carry a functional and regulatable β -catenin-destruction complex (Figure 4F and (Azzolin *et al.*, 2014)). Induction of *GRA18* expression with tetracycline promoted the accumulation of β -catenin, indicating that *GRA18* alone was sufficient to trigger β -catenin upregulation to levels comparable to those obtained with Wnt3A (Figure 4F). Therefore, these data indicate that *GRA18*, very likely through the interactions with GSK3 and PP2A-B56, functions as a positive regulator of β -catenin.

5- *GRA18* alters the expression of a specific set of genes in infected cells.

The ability of *GRA18* to promote nuclear accumulation of the transcriptional regulator β -catenin prompted us to investigate whether *GRA18*-GSK3/ β -catenin partnerships contribute to the typical changes in gene expression observed in cells infected with tachyzoites. To test this hypothesis, we performed a comparative transcriptomic analysis by RNA-sequencing of

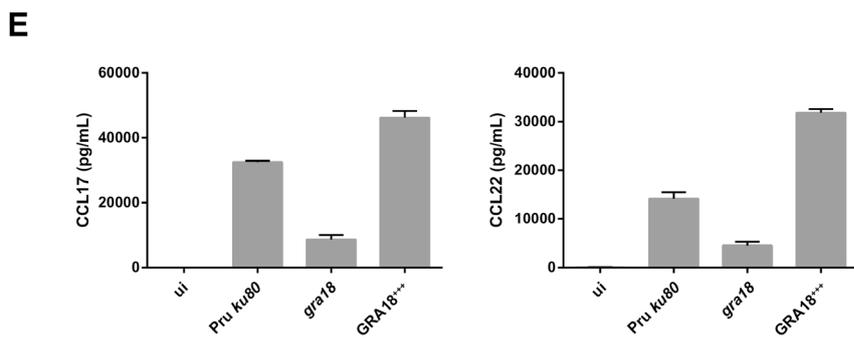
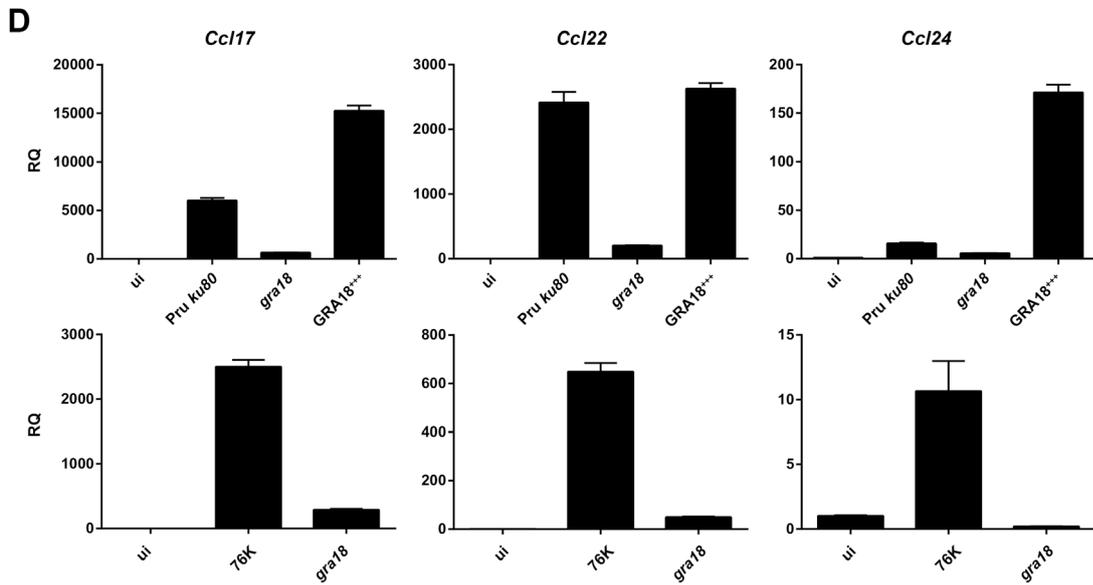
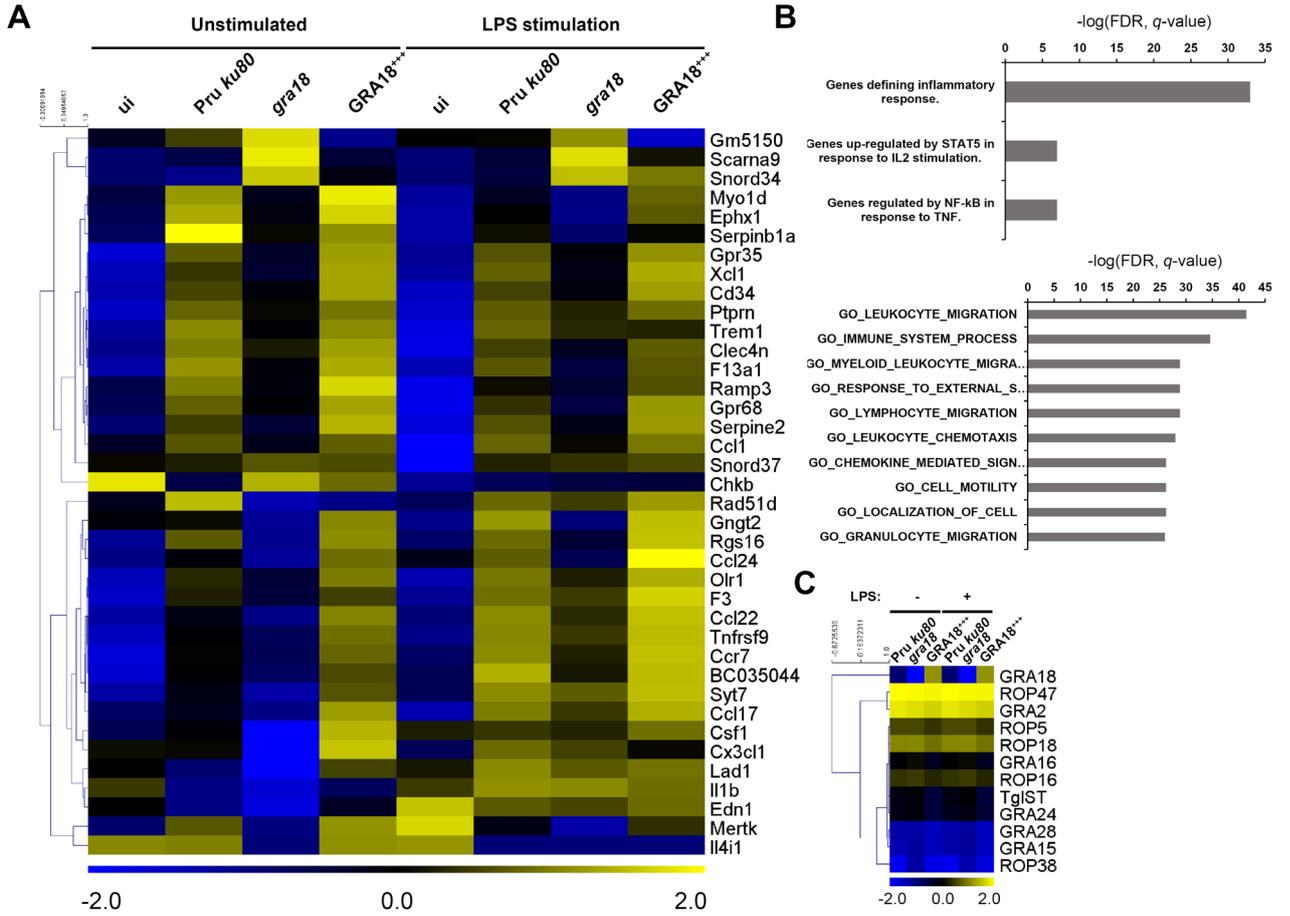


Figure 5. GRA18 alters the host cell transcriptome. (A) RNA-Seq analysis of BALB/c BMDMs that were left uninfected (ui) or infected with the indicated strains at an MOI of 1:5. At 18 hrs post-infection, cells were left unstimulated or stimulated with LPS (100 ng/mL) for 6 h. Heat map of the differentially expressed mouse genes (≥ 3 fold, RPKM ≥ 5 in at least 1 sample) between parental and $\Delta gra18$ infected cells in the absence of LPS. RPKM values were \log_2 transformed, Gene/Row normalized, and mean centered using MeV. (B) Enrichment analysis in functional annotation of the differentially expressed genes using GSEA. Top-scoring pathways regulated in a GRA18-dependent manner are shown. (C) Heat map of expression values obtained by RNA-Seq analysis of *T. gondii* genes from the samples in (A). A selected set of *T. gondii* ROPs and GRAs are shown. (D) Quantitative chemokine expression was determined by qRT-PCR on BMDMs infected with the wild-type and $\Delta gra18$ mutant strains in the type II strains Pru and 76K. Values were normalized to the amount of *Tbp* in each sample. Data are mean value \pm s.d. of three independent experiments. (E) CCL17 and CCL22 levels were measured by ELISA from supernatant collected 24 h after BMDM infection with the indicated strains. Supernatant from uninfected cells was used as a control (ui). Means of three independent experiment are shown.

mouse bone marrow derived macrophages (BMDMs) infected with parental or $\Delta gra18$ parasites. Macrophages were chosen because they are the cell type preferentially infected *in vivo* in mice and they play an essential role in the early immune response against *T. gondii* (Dunay *et al.*, 2008; Jensen *et al.*, 2011). In light of recent publications pointing to a role of GSK3 and β -catenin in the regulation of the inflammatory gene expression in response to bacterial lipopolysaccharides (LPS) (Chattopadhyay *et al.*, 2015; Jang *et al.*, 2017; Martin *et al.*, 2005; Yang *et al.*, 2010), transcriptomic analysis was also performed in the presence of LPS treatment of the infected macrophages. We focused our analysis on genes that were modulated with more than three-fold change and having a signal threshold above 5 RPKM (Reads Per Kilobase of transcript per Million mapped reads) in at least one sample when comparing the wild-type and $\Delta gra18$ mutant strains. Filtered data are presented in Table S2 (data are accessible through NCBI GEO, accession number GSExxxx). Thirty-eight genes were significantly and differentially regulated and hierarchical clustering revealed that most of them correspond to genes up-regulated upon macrophage infection by wild-type parasites, but not with the $\Delta gra18$ strain (Figure 5A). Complementation of $\Delta gra18$ mutation with GRA18-HF under the control of the GRA1 promoter ($\Delta gra18$, GRA18^{HF}) restored the expression pattern to levels observed with the wild-type parasites or even at higher levels, suggesting that the induction of those genes was GRA18-dependent. Parasite transcriptome analysis indicated that neither $\Delta gra18$ mutation nor LPS treatment had any significant impact on *T. gondii* gene expression and that GRA18 expression in the Pru *ku80* $\Delta gra18$, GRA18^{HF} complemented strain was restored with a ~ 35 fold more transcript reads than in the wild-type strain (Figure 5C, Table S2, and Figure S1), thus confirming GRA18 overexpression when under the control

of the *GRA1* promoter. It is noteworthy that the expression pattern of the aforementioned host genes regulated by *GRA18* remained similarly regulated in the infected macrophages subjected to LPS stimulation (Figure 5A and Table S2), indicating that *GRA18* function was not affected by LPS stimulation. Gene set enrichment analysis (GSEA) highlighted pathways significantly affected in a *GRA18*-dependent fashion (p values < 0.05) with functions related to the inflammatory response (e.g., *Csf1*, *Olr1*, and *Tnfrsf9*) and chemotaxis (e.g., *Ccr7*, *Ccl1*, *Ccl17*, *Ccl22*, and *Ccl24*) (Figure 5B). Surprisingly, none of the well-known Wnt/ β -catenin target genes were found differentially regulated by *GRA18* (Figure S2 and Table S2), suggesting that either β -catenin harbors various transcriptional target genes depending on the cell type, or that the *GRA18* complex fosters a singular β -catenin transcriptional activity, or that its action is independent of β -catenin.

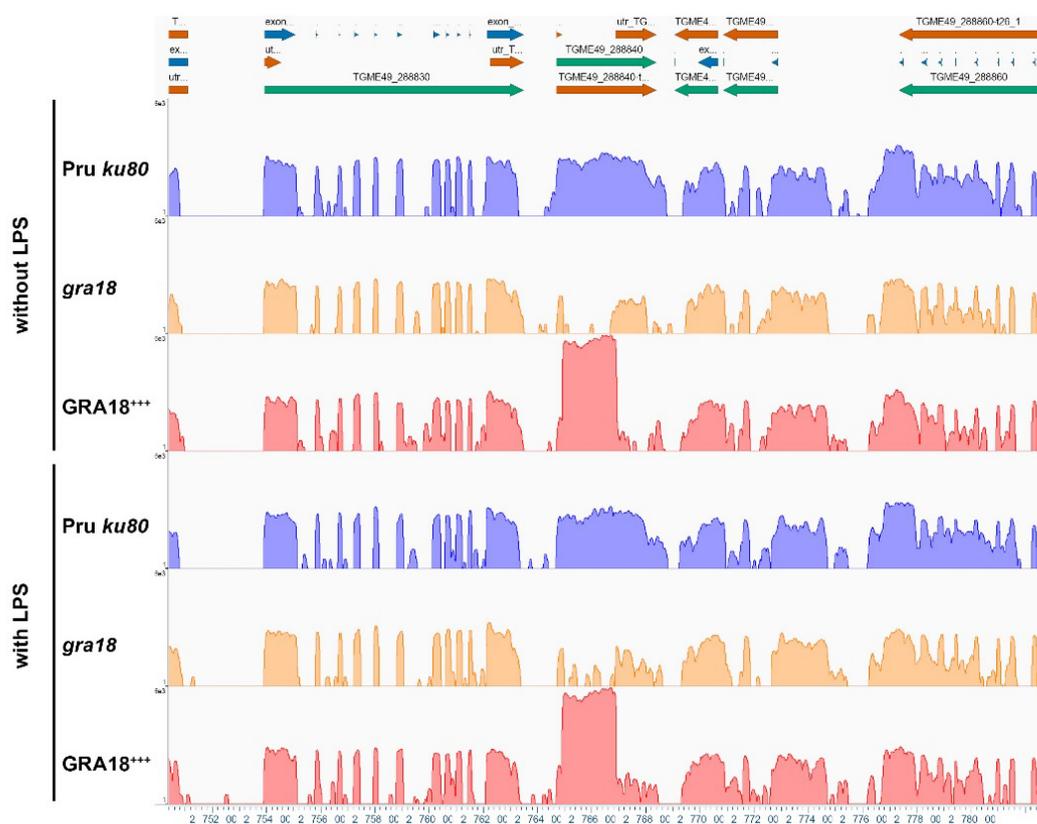


Figure S1. *T. gondii* RNA-Seq analysis at the *GRA18* genomic locus. Coverage track of the *T. gondii* RNA-Seq reads for the indicated samples obtained from GenVision Pro, Lasergene version 14 (DNASTAR, Madison, WI, USA). Assembled RNA-Seq reads obtained for each sample were plotted along the *T. gondii* genomic sequence. The graphs show the depth of sequence coverage for each nucleotide position along the *T. gondii* *GRA18* genomic sequence.

To confirm the transcriptomic profile, we focused on the chemokines *Ccl17*, *Ccl22*, and *Ccl24* previously identified as singularly up-regulated genes upon *T. gondii* infection

(Hammoudi *et al.*, 2015; Melo *et al.*, 2013). Quantitative RT-PCR (qRT-PCR) analysis reproducibly demonstrated a pronounced decrease in *Ccl17*, *Ccl22*, and *Ccl24* expression from cells infected with $\Delta gra18$ mutant parasites when compared to wild-type Pru or Pru $\Delta gra18$, GRA18⁺⁺⁺ parasites (Figure 5D). Very similar data were obtained with the 76K strain, another type II *T. gondii* cystogenic strain, indicating that *T. gondii* reproducibly regulated those chemokines in a *GRA18*-dependent manner. *GRA18* is clearly playing a major role in regulating CCL17 and CCL22 synthesis and secretion as its loss significantly compromised protein levels in the supernatant of mouse macrophages (Figure 5E). Overall, these results indicate that the *GRA18*, through its interactions with β -catenin/GSK3/PP2A-B56, aim to regulate amongst others the expression of specific chemokines.

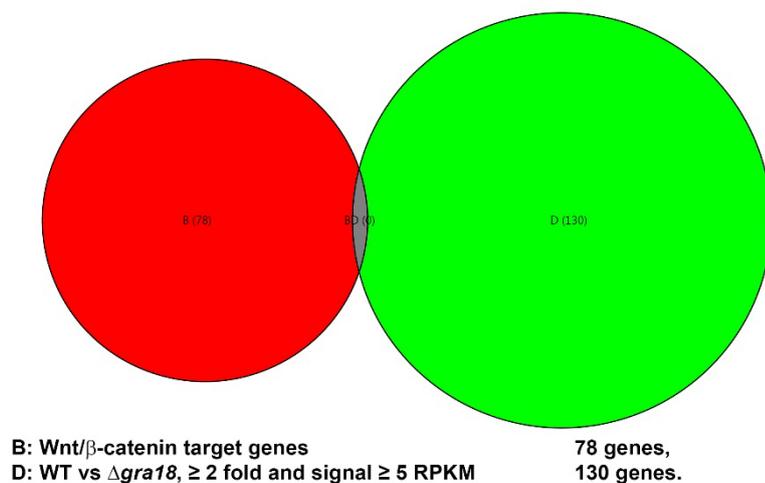


Figure S2. Venn diagram depicting the overlap between the Wnt/ β -catenin target genes and those affected in a *GRA18*-dependent fashion in infected BMDMs. A list of 78 Wnt/ β -catenin target genes were obtained from GSEA. The 130 host genes regulated by *GRA18* correspond to the genes that were found differentially regulated in our transcriptomic analysis comparing BMDMs infected by *T. gondii* wild-type and *gra18* mutant strains. Genes having at least a 2 fold-change and a signal threshold of at least 5 RPKM were selected.

6- *GRA18* alters host gene expression in a β -catenin-dependent fashion.

To evaluate whether β -catenin is required for the *GRA18*-triggered chemokine expression, we generated macrophage RAW264.7-derived cell line mutated for *Ctnnb1* using CRISPR/Cas9-mediated gene editing. Cells were transfected with a vector expressing the CAS9 endonuclease and a guide RNA targeting the third coding exon of the murine *Ctnnb1* gene (Figure 6A). Cells harboring insertion/deletion (indel) mutations at the *Ctnnb1* locus lack β -catenin expression when compared to the wild-type parental cell line (Figures 6A and B). As expected, treatment of the *Ctnnb1*^{-/-} mutant cells with the GSK3 inhibitors did not led

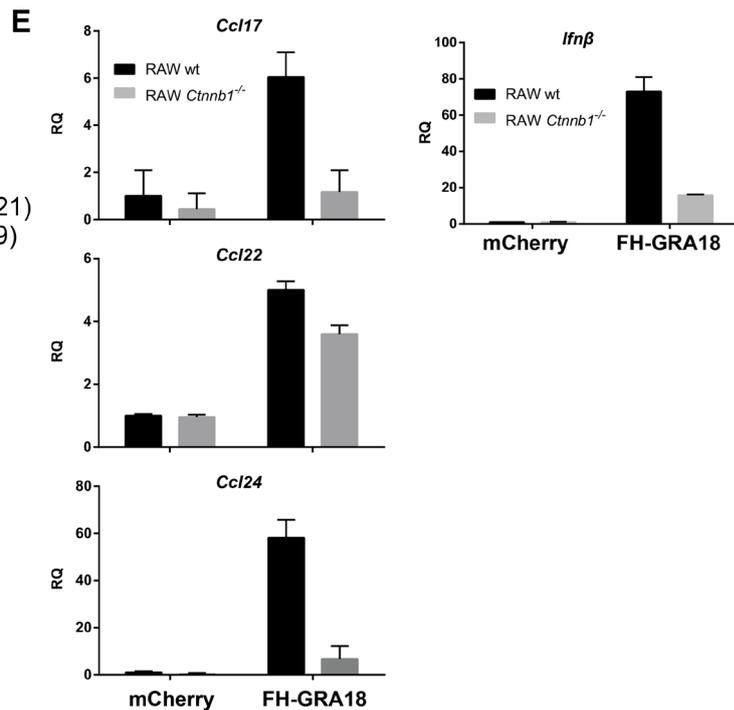
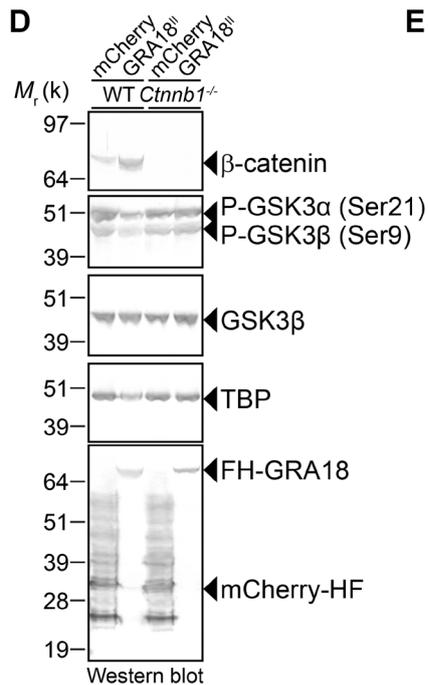
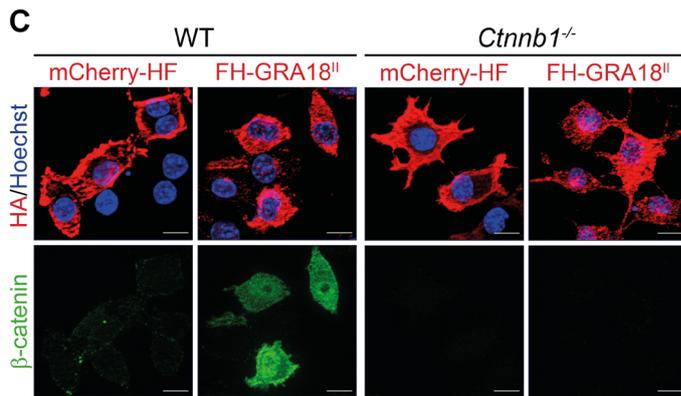
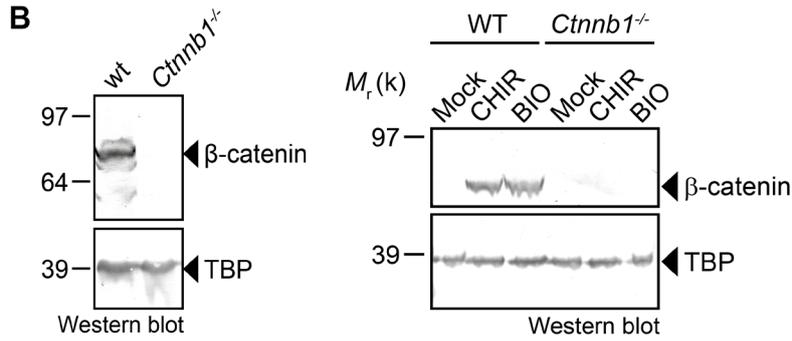
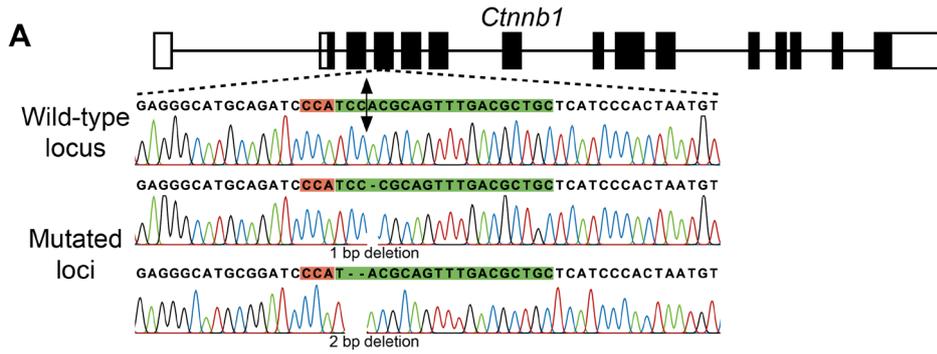


Figure 6. GRA18 promotes *Ccl17*, *Ccl22* and *Ccl24* chemokines expression in a β -catenin-dependent fashion. (A) Schematic diagram of gRNAs targeting the *Ctnnb1* locus. The Protospacer Adjacent Motif (PAM) sequence is lined and highlighted in red; the targeting sequences is shown in green. Bi-directional arrow indicates the Cas9 cleavage site. DNA sequences from the wild-type and mutated RAW264.7-derived cell lines were analyzed by DNA sequencing; the identified deletions are indicated with (-). No mutations in control samples were observed. (B) Immunoblot analysis of β -catenin in parental RAW264.7 and the *Ctnnb1*^{-/-} mutant confirmed the absence of β -catenin expression. As positive controls, cells were treated with 3 mM of BIO or 2 mM of CHIR GSK3 inhibitors for 12 h. TBP was used as loading control. (C-E) RAW264.7 (WT) and β -catenin-deficient (*Ctnnb1*^{-/-}) RAW264.7-derived cell lines were transfected with mCherry vector control (pcDNA-mCherry-HF) or the FH-GRA18^{II} expression vector (pcDNA-FH-GRA18). At 18 h after transfection, cells were either (C) fixed for IFA using anti-HA (red) and anti- β -catenin (green) antibodies or (D) cells were harvested and analyzed by immunoblot using the indicated antibodies. Anti-HA was used to detect FH-GRA18 and mCherry-HF. In (E) transcripts for *Ccl17*, *Ccl22*, *Ccl24*, and *Ifn β* were quantified by qPCR and normalized using *Tbp*. Data are mean value \pm s.d. of three independent experiments.

β -catenin expression, otherwise induced in the wild-type parental cell line. These results indicate that the RAW264.7 murine cells carry a regulatable β -catenin destruction complex and that the two alleles coding for β -catenin were successfully disrupted in the *Ctnnb1*^{-/-} mutant cells.

The ectopic expression of GRA18 in the murine RAW264.7 macrophage cell line caused a significant increase in endogenous β -catenin accumulation (Figures 6C and D, compare mCherry with GRA18), which is consistent with previous data in which GRA18 was delivered by the parasites (Figure 4E and D). The absence of β -catenin in the *Ctnnb1*^{-/-} mutant cells expressing GRA18 further confirmed that the *Ctnnb1* alleles were successfully disrupted (Figures 6C and D). Significant amounts of β -catenin were found in the nuclei of wild-type RAW264.7 macrophages ectopically expressing GRA18 and, in agreement with GRA18 promoting chemokines expression, *Ccl17*, *Ccl22*, and *Ccl24* were induced when compared to mock transfected cells (mCherry) (Figures 6D and E). In contrast, the GRA18-mediated induction of *Ccl17* and *Ccl24* expression was drastically impaired in cells lacking β -catenin, whereas *Ccl22* expression was only slightly reduced in the mutant cells (Figure 6E). Consistent with β -catenin upregulation, similar data were observed with *Ifn β* (Figure 6E), a known β -catenin target gene in macrophages (Ma *et al.*, 2014a; Rathinam *et al.*, 2010; Yang *et al.*, 2010). These results indicate that the GRA18 function toward chemokines expression is dependent on β -catenin.

7- GRA18 acts as an inhibitor of GSK3 to trigger β -catenin transcriptional activity.

In order to better understand the GRA18 *modus operandi*, we assayed the different sub-domains of GRA18 (GRA18^{Nt} and GRA18^{Ct}) for ability to upregulate β -catenin and to induce expression of the downstream target genes in macrophages. Expression of GRA18^{FL} in RAW264.7 macrophages caused a significant increase in endogenous β -catenin protein levels which in turn led to the expression of the downstream chemokines (Figures 7A and B). In cells expressing a GRA18^{Nt}, which no longer interact with GSK3 and PP2A-B56 (Figure 3), GRA18 failed to induce β -catenin and accordingly the chemokine mRNA levels remained low. Given that the N-terminal region of GRA18 has retained the ability to interact with β -catenin (Figure 3A), these data suggest that the interactions with β -catenin alone are not sufficient to promote its accumulation and transcriptional activity. On the other hand, transfection of GRA18^{Ct}, shown previously to interact with GSK3 and PP2A-B56 (Figure 3), has retained full activity on β -catenin and promoted transcription of the downstream chemokines. Thus, these data further confirm that β -catenin is mandatory for the expression of the aforementioned chemokines. While the effect of GRA18 on β -catenin protein levels is dependent on its interactions with GSK3 and/or PP2A-B56, its direct interaction with β -catenin seems to be dispensable. Altogether these results are compatible with a scenario in which GRA18, through its interactions with GSK3 and PP2A-B56, acts as a positive regulator of the intracellular amounts of β -catenin to favor gene expression. Lending further support to this model, treatment with GSK3 inhibitors induced *Ccl22* and *Ccl24* expression in a manner that resembles the action of GRA18 (Figures 7C and D). Overall, these data suggest that GRA18 binds to GSK3 and PP2A-B56 to inhibit the β -catenin-destruction complex, hence stabilizing β -catenin.

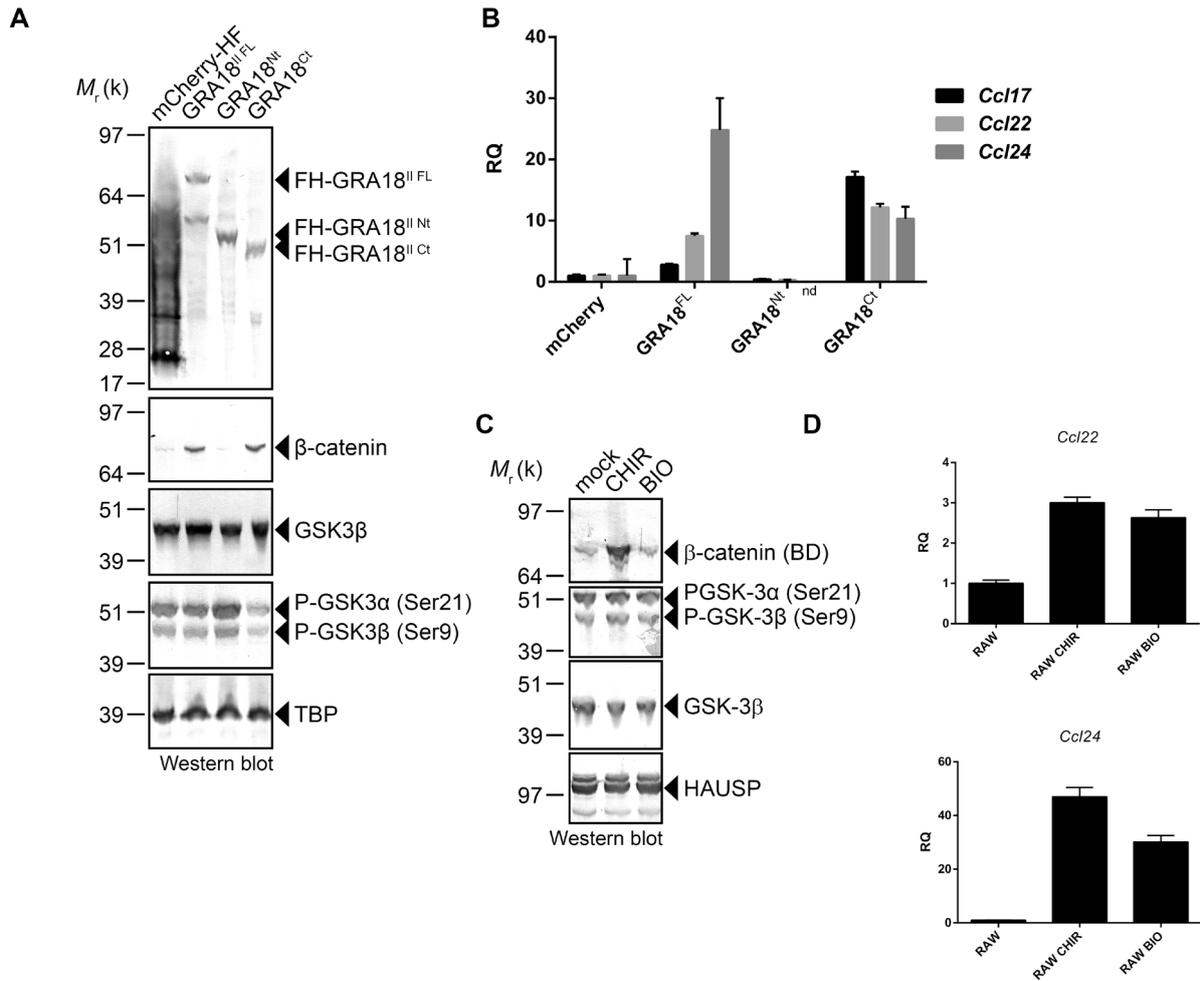


Figure 7. GRA18 activity is dependent on its interactions with GSK3 and PP2A-B56. (A-B) RAW264.7 cells were transfected with mCherry vector control or the FH-GRA18 expression vectors pcDNA-FH-GRA18^{FL} (GRA18^{FL}), pcDNA-FH-GRA18^{Nt} (GRA18^{Nt}), and pcDNA-FH-GRA18^{Ct} (GRA18^{Ct}). At 18 h after transfection, cells were harvested and (A) whole cell extract were analyzed by immunoblot using the indicated antibodies, or (B) quantitative chemokine expression was determined by qRT-PCR as in figure 6. (C) Treatment of RAW264.7 cells with the GSK3 inhibitors CHIR and BIO led to β-catenin protein accumulation and *Ccl22* and *Ccl24* upregulation as determined by qRT-PCR in (D).

Materials and Methods

Parasite and Cell Culture

T. gondii tachyzoites were maintained by serial passage on human foreskin fibroblast (HFF) monolayers. The strains used in this study were RH *ku80*, Pru *ku80*, and 76K-GFP-LUC (gift of M. Grigg, National Institutes of Health, Bethesda, MD). HFF primary cells, T-Rex-293, L929, J774, and RAW264.7 cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% heat-inactivated FBS (Invitrogen), 10 mM Hepes buffer, pH 7.2, 2 mM L-glutamine, and 50 µg/ml penicillin and streptomycin (Thermo Fisher Scientific). Cells were incubated at 37°C in 5% CO₂. Stable transgenic parasites or recombinants were selected with 25 µg/ml mycophenolic acid and 50 µg/ml xanthine or 1 µM pyrimethamine.

Reagents

Antibodies raised against Hemagglutinin (3F10; Roche), GRA1 (provided by J.-F. Dubremetz, UMR 5235 Centre National de la Recherche Scientifique, Montpellier, France), Toxofilin (provided by I. Tardieux, INSERM 1209, Grenoble, France), MIC2 (provided by D. Sibley, Washington University School of Medicine, St. Louis, MO), β-catenin (#610153, BD Transduction Laboratories), GSK3β (#D5C5Z, Cell Signaling Technology), PP2A-B56 (#MABS270, Millipore), PP2A65 RA (#2039, Cell signaling), PP2Ac (#2038, Cell signaling), Phospho-GSK3β (Ser9, D85E12, Cell Signaling Technology), Phospho-GSK3α/β (Ser9/21, #D17D2, Cell Signaling Technology), TBP (Abcam) were used in the immunofluorescence assay and/or Western blotting. Immunofluorescence secondary antibodies were conjugated to Alexa Fluor 488 or Alexa Fluor 594 (Invitrogen). Western blotting secondary antibodies conjugated to alkaline phosphatase was purchased from Promega. The inhibitors CHIR 99021(#252917-06-9) and BIO (#66746362-9) were purchased from R&D systems™. Recombinant bacterial lipopolysaccharide (LPS) from *Escherichia coli* O26:B6 (Sigma-Aldrich) was used to stimulate the BMDMs.

Plasmid Constructs

The plasmids and primers used in this work are listed in Table S1. To construct the vector pLIC-GRA18-HA³-DHFR, the coding sequence of *GRA18* was amplified using primers LICF-288840_F and LICF-288840_R using RH *ku80* genomic DNA as the template. The PCR product was cloned to pLIC-HA³-DHFR vector using the LIC cloning method as described in (Huynh and Carruthers, 2009).

The vector pDEST14 KO *GRA18*^{II} was generated to construct the deletion/insertion mutation of *GRA18* in Pru (type II) *T. gondii* strain. The Multisite Gateway Pro3-fragment Recombination system was used to clone the *DHFR* cassette flanked by the 5' and 3'

surrounding regions of *GRA18* coding sequence of type II genomic DNA. The 5' flanking region of *GRA18* of Pru strain was amplified using primer attB1-288840_F and attB4-288840_R, and was cloned into the plasmid pDONR221 P1-P4 (Invitrogen). The 3' flanking region of *GRA18* was amplified using primers attB3-288840_F and attB2-288840_R and was cloned into the plasmid pDONR221 P3-P2. The resulting vectors, pDONR221/5'*GRA18* and pDONR221/3'*GRA18*, respectively, were then recombined with pDONR221/*DHFR* (Bougdour *et al.*, 2013) into the destination vector pDEST14 KO, yielding the pDEST14 KO *GRA18*^{fl}.

The plasmid pTOXO_Cas9-CRISPR::sg*GRA18* vector was generated as previously described (Curt-Varesano *et al.*, 2016). Briefly, the sense and anti-sense oligos *GRA18*-CRISPR-FWD and *GRA18*-CRISPR-REV containing the sgRNA targeting the *GRA18* genomic sequence were phosphorylated, annealed and ligated in the pTOXO_Cas9-CRISPR plasmid linearized with *Bsa*I, yielding pTOXO_Cas9-CRISPR::sg*GRA18*.

To construct the pP_{GRA1}-*GRA18*^{fl}-HF vector, the promoter sequence of *GRA1* was amplified by PCR using the primers LICF-P_{GRA1}_F2 and P_{GRA1}_R. The *GRA18* coding sequence of type II *T. gondii* strain was amplified by PCR using the primers *GRA1*-*GRA18*^{1-x}_F and LICR-288840_R. The resulting PCR products were assembled using the Gibson assembly kit (NEB) and the resulting P_{GRA1}-*GRA18* DNA fragment was cloned into the plasmid pLIC-HF-*DHFR*, yielding the pP_{GRA1}-*GRA18*^{fl}-HF vector.

The pX330_hSpCas9::sgCTNNB1 vector was generated using the sense and anti-sense oligos, CTNNB1-CRISPR-FWD and CTNNB1-CRISPR-REV, respectively. Annealed oligos were ligated into the pX330_hSpCas9 plasmid linearized with *Bbs*I (Cong *et al.*, 2013). The expressed guide RNA targets the second armadillo repeat of genomic *Cttnb1* sequence.

Immunofluorescence microscopy

Immunofluorescence assays were performed previously (Bougdour *et al.*, 2013). Briefly, cells were fixed in PBS-3% (vol/vol) formaldehyde and permeabilized with PBS-0.1% Triton X-100 (vol/vol) or ethanol (-20°C) for 3 minutes. After blocking in PBS-3% BSA, samples were incubated in PBS-3% BSA containing the primary antibodies indicated in the figures, followed by the secondary antibodies coupled with Alexa Fluor 488 or Alexa Fluor 568 (Invitrogen) at a 1:1,000 dilution. Nuclei of both host-cells and parasites were stained for 10 min at RT with Hoechst 33258 at 2 µg/ml in PBS. After four washes in PBS, coverslips were mounted on a glass slide with Mowiol mounting medium. Images were acquired with a fluorescence microscope AxioImager M2 equipped with Apotome module (Carl Zeiss, Inc.).

Yeast two-hybrid screen

Full-length GRA18^{II} (aa 27 to 648) cloned in pB27 (N-LexA-bait-C fusion) was used in a ULTImate Y2H™ screen against a human Human Placenta_RP5 complementary DNA Gal4-activating domain-fusion library (Hybrigenics, Paris, France). The construct was checked by sequencing. Prey fragments of positive clones were amplified by PCR and sequenced at their 5' and 3' junctions and the resulting sequences were used to identify corresponding interacting proteins in the GenBank database via a fully automated procedure. A confidence score (Global Predicted Biological Score, Global PBS®) was attributed to each interaction as previously described (Formstecher *et al.*, 2005).

The interaction domain mapping was performed using Full-length GRA18^{II} (aa 27 to 648) and GRA18 fragments (aa 26 to 233, aa 233 to 440, aa 420 to 648, aa 152 to 350, and aa 350 to 585) that were PCR-amplified and cloned in frame with the LexA DNA binding domain (DBD) by Gap-repair into plasmid pB27, derived from the original pBTM116 (Vojtek and Hollenberg, 1995). The Hybrigenics reference for this construct is hgX3694v1_pB27. This construct was used previously to screen the placenta library and was used as a control in this assay. The prey fragments for the human *CTNBB1* var3, *GSK3A* and *PPP2R5A* were extracted from the above ULTImate Y2H™ screening of GRA16 against the placenta cDNA library. The prey fragments were cloned in frame with the Gal4 Activation Domain (AD) into plasmid pP6, derived from the original pGADGH (Bartel *et al.*, 1993). The AD constructs were checked by sequencing. The pP7 prey plasmid used as control in this assay is derived from the pP6 plasmid. Bait and prey constructs were transformed in the yeast diploid cells, obtained using a mating protocol with both yeast strains L40Δ*Gal4* (*mata*) and YHGX13 (Y187 *ade2-101::loxP-kanMX-loxP*, *mata*) (Fromont-Racine *et al.*, 1997). In order to identify GRA18 fragments interacting with the 3 tested preys, three diploids for each fragment were picked and the interaction tested by growth assay (robot calibrated drops) on selective medium without tryptophan, leucine and histidine (DO-3). The interaction assays are based on the reporter gene *HIS3* (growth assay without histidine). Five different concentrations of 3-AminoTriazol (3-AT), an inhibitor of the *HIS3* gene product, were added to the DO-3 plates to increase stringency. The following 3-AT concentrations were tested: 1, 5, 10, 20 and 50 mM 3-AT.

Toxoplasma growth assay by fluorescence imaging assays

T. gondii proliferation was determined by high-content fluorescence imaging as described previously (Palencia *et al.*, 2017). Briefly, 10,000 HFFs were seeded in each well of well in 96-well plate and then infected with 4×10^4 parasites. Invasion was synchronized by briefly centrifuging the plate at 400 rpm, and plates were incubated at 37°C for 2 h. Infected cells were then washed three times with PBS and further incubated for a total of 24 h of growth.

Nuclei were stained with Hoechst 33342 at 5 µg/mL for 20 min and after fixation and permeabilization, parasite vacuoles were labelled using an anti-GRA1 antibody (specific to *Toxoplasma*). Images were automatically acquired using the ScanR microscope system (Olympus) to determine the number of tachyzoites per vacuole. The experiments were done in triplicate, and data were processed using the GraphPad Prism software.

BMDMs

Bone marrow derived macrophages were generated as described previously (Bougdour *et al.*, 2013) with the following modifications. Briefly, bone marrow was flushed from femurs and tibiae of BALB/c mice and cultured for 1 week in complete macrophage medium (Dulbecco modified Eagle's minimal essential medium (DMEM) (Invitrogen, Breda, the Netherlands) supplemented with 10% fetal calf serum (FCS) (Invitrogen), 20% conditioned medium from macrophage-colony stimulating factor-secreting L929 fibroblasts (Aziz *et al.*, 2009), 50 µM 2-mercaptoethanol, 1X non-essential amino acids (Thermo Fisher Scientific), and 2% penicillin/streptomycin-glutamine. After 7-10 days in culture adherent cells were approximately 95% pure macrophages (F4/80⁺) and cells were used for experiments.

RNA-seq and sequence alignment

For each biological assay, 3 x 10⁶ BMDMs were seeded per well in 3 mL DMEM in six-well tissue culture plates. Cells were left uninfected or infected with the Pru $\Delta ku80$, Pru $\Delta ku80 \Delta gra18$, and Pru $\Delta ku80 \Delta gra18$, GRA18⁺⁺⁺ strains (MOI = 6) for 18 hours and subsequently subjected or not to LPS stimulation (100 ng/mL of bacterial lipopolysaccharide from *Escherichia coli* O26:B6; Sigma-Aldrich) for 4 hours. Total RNAs were extracted and purified using TRIzol (Invitrogen, Carlsbad, CA, USA) and RNeasy Plus Mini Kit (Qiagen). RNA quantity and quality were measured by NanoDrop 2000 (Thermo Scientific). RNA integrity was assessed by standard non-denaturing 1.2% TBE agarose gel electrophoresis. The ribosomal large subunits from *Toxoplasma* and the host cells was used to verify that the ratio between *Toxoplasma* RNA versus host RNA was equivalent between the different biological samples, thus indicating that the samples had equivalent infection rates.

RNA-sequencing was performed by GENEWIZ (South Plainfield, NJ, USA). Briefly, the RNA quality was checked with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, California, USA) and Illumina TruSEQ RNA library prep and sequencing reagents were used following the manufacturer's recommendations (Illumina, San Diego, CA, USA). The samples were paired-end multiplex sequenced (2 × 100 bp) on the Illumina HiSeq 2500 platform and generated at least 70 million reads for each sample (Table S2).

The RNA-Seq reads (FASTQ) were processed and analyzed using the Lasergene Genomics Suite version 14 (DNASTAR, Madison, WI, USA) using default parameters. The paired-end reads were uploaded onto the SeqMan NGen (version 14, DNASTAR, Madison, WI, USA) platform for reference-based assembly using either the *Mus musculus* genome package (GRCm38.p3) or the *Toxoplasma* Type II ME49 strain (ToxoDB-24, ME49 genome) as reference template. The ArrayStar module (version 14, DNASTAR, Madison, WI, USA) was used for normalization, differential gene expression and statistical analysis of uniquely mapped paired-end reads using the default parameters. The expression data quantification and normalization were calculated using the RPKM (Reads Per Kilobase of transcript per Million mapped reads) normalization method.

Transfection of RAW264.7 cells

Cells were transfected by electroporation using the Neon Transfection System (Thermo Fisher Scientific). Twenty-four hours before transfection, the RAW264.7 cells were plated at 50% confluency in T-175 tissue culture flask. Next day about 3×10^6 cells were washed once in PBS and resuspended in 100 μ L of R buffer (Provided by manufacturer). This suspension was mixed with 15 μ g of DNA followed by electroporation at 1680 V and 20 ms for 1 pulse. After electroporation, cells were cultured in penicillin and streptomycin-free DMEM medium.

Generation of β -catenin-deficient RAW264.7 cell line

RAW264.7 cells was transfected with 15 μ g of pX330-hSpCas9::sgCTNNB1 vector. At 48 h after electroporation, cells were cloned by limited dilution and individual clones were expanded and tested for β -catenin expression by IFA and immunoblotting using β -catenin antibodies. The *Ctnnb1* genomic sequences were amplified by PCR using primers Chk ctnnb1_F and Chk ctnnb1_R. The resulting fragments were then ligated into pCR2.1-TOPO for sequencing.

Chromatographic purification of GRA18-containing complex

Cytoplasmic fraction from T-Rex cells stably expressing Flag-tagged protein or J774 cells infected with Pru *ku80* expressing Flag-tagged GRA18 (GRA18⁺⁺⁺), were incubated with anti-FLAG M2 affinity gel (Sigma-Aldrich) for 1 h at 4°C. Beads were washed with 20-column volumes of the BC500 buffer (20 mM Tris-HCl, pH8.0, 0.5 M KCl, 20% Glycerol, 0.5 mM DTT, 0.5% NP40, and protease inhibitors). Bound polypeptides were eluted stepwise with 250 μ g/mL FLAG peptide (Sigma Aldrich) diluted in BC100 buffer. For size-exclusion chromatography, protein eluates were loaded onto a Superose 6 HR 10/30 column equilibrated with BC500. Flow rate was fixed at 0.35 mL/min, and 0.5-mL fractions were collected.

Co-Immunoprecipitation assay

For affinity of purification of Flag-tagged GRA18 in T-Rex, cells were harvested and washed in ice-cold PBS. Cells were resuspended in buffer A (10 mM Tris-HCl, pH7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5mM DTT, 0.2 mM PMSF, 1X Roche protease inhibitor cocktail). Swelling cells were lysed for 8 min with 0.1% NP40 followed by 10 times dounced with loose plunger to disrupt the plasma membranes. Cell lysates were centrifuged at 1,300 g for 10 min and the supernatant containing the cytosolic fraction was collected.

The cytoplasmic extracts were supplemented with 1:10th volume of 10X buffer B (0.3 M Tris-HCl, pH7.9, 1.4M KCl, 0.03 M MgCl₂) before incubation with 50 µL FLAG M2 affinity gel (Sigma-Aldrich) for 1 h at 4°C. Beads were washed three times in BC500. Bound polypeptides were eluted with 50 µL 250 µg/ml FLAG peptide (Sigma Aldrich) diluted in BC100 buffer. Samples were mixed with protein sample buffer (Invitrogen) for immunoblot analysis.

Mass spectrometry–based proteomics

Protein bands were excised from colloidal blue–stained gels (Thermo Fisher Scientific), treated with DTT and iodoacetamide to alkylate the cysteines before in-gel digestion using modified trypsin (sequencing grade; Promega). Resulting peptides from individual bands were analyzed by nanoLC-MS/MS (UltiMate 3000 coupled to LTQ-Orbitrap Velos Pro; Thermo Fisher Scientific) using a 25-min gradient. Peptides and proteins were identified and quantified using MaxQuant (version 1.5.3.17) through concomitant searches against ToxoDB (20151112 version), SwissProt (Homo sapiens taxonomy, 20151112 version), and the frequently observed contaminant database embedded in MaxQuant. Minimum peptide length was set to 7 amino acids. Minimum number of peptides, razor + unique peptides, and unique peptides were all set to 1. Maximum false discovery rates were set to 0.01 at peptide and protein levels.

Immunoblot analysis

Immunoblot analysis of protein was performed as described in (Bougdoor *et al.*, 2013). In Brief, ~10⁷ cells or infected cells were lysed in 50 µL lysis buffer (10 mM Tris-HCl, pH6.8, 0.5 % SDS [v/v], 10% glycerol [v/v], 1 mM EDTA and protease inhibitors cocktail) and sonicated. The protein extracts were separated by SDS-PAGE, and transferred to a PolyVinylidene Fluoride membrane (PVDF; immobilon-P, Millipore) by liquid transfer. Western blots were then blocked with PBS buffer containing 0.01% Tween 20 (w/v) and 5% nonfat dry milk. Appropriate primary antibodies diluted in PBS containing 0.01% Tween 20 (w/v) were used to probe the membrane and primary antibodies were detected using phosphatase-conjugated goat secondary antibodies (Promega) or with the Supersignal West Pico Chemiluminescent Substrate kit (Thermo Fisher Scientific).

Quantitative real-time PCR

Total RNA was isolated using TRIzol reagent (Thermo Fisher Scientific). cDNA was synthesized using the High Capacity RNA-to-cDNA kit (Applied Biosystem). Samples were analyzed by real time quantitative PCR for *Ccl17*, *Ccl22*, *Ccl24* and *Ifnb1* using TagMan Gene Expression Master Mix (Applied Biosystems) according to the manufacturer's instructions. *Tbp* was used as an internal control gene for normalization.

In vitro cytokine ELISA

About 10^6 BALB/c BMDMs were seeded per well of a 24-well-plate. Cells were left uninfected or infected with freshly egressed *T. gondii* tachyzoites at an MOI of 1:5. At 24 h post-infection, supernatant were collected and stored at -80°C for storage. CCL17 and CCL22 levels were determined using Mouse CCL17/TARC and Mouse CCL22/MDC Quantikine ELISA Kits from R&D Systems according to the manufacturer's instructions.

Discussion and supplementary data

GRA18 export mechanism

GRA18, as the other members of the exported GRAs family of proteins, is found secreted into the PV and then traffics through the PVM to ultimately reside in the host cell cytoplasm. The cellular distribution of GRA18 is singular and contrasts with the other recently described *T. gondii* effectors, such as GRA16, GRA24, GRA28 or TgIST, whose final destination is the host cell nuclei. As expected, the secretion mechanism in the host cell is dependent on the MYR1 effector (Figure 1D), a protein somehow involved in the trafficking of GRAs protein through the PVM (Coffey *et al.*, 2016). Furthermore, the GRA18 N-terminal region contains a putative TEXEL motif, a feature that suggests it might be processed by the aspartic protease TgASP5, which has recently been shown to be important for export of a number of exported GRA effectors (Coffey *et al.*, 2015; Curt-Varesano *et al.*, 2016; Hammoudi *et al.*, 2015). In line with an ASP5-dependent export, GRA18 was no longer detected in the

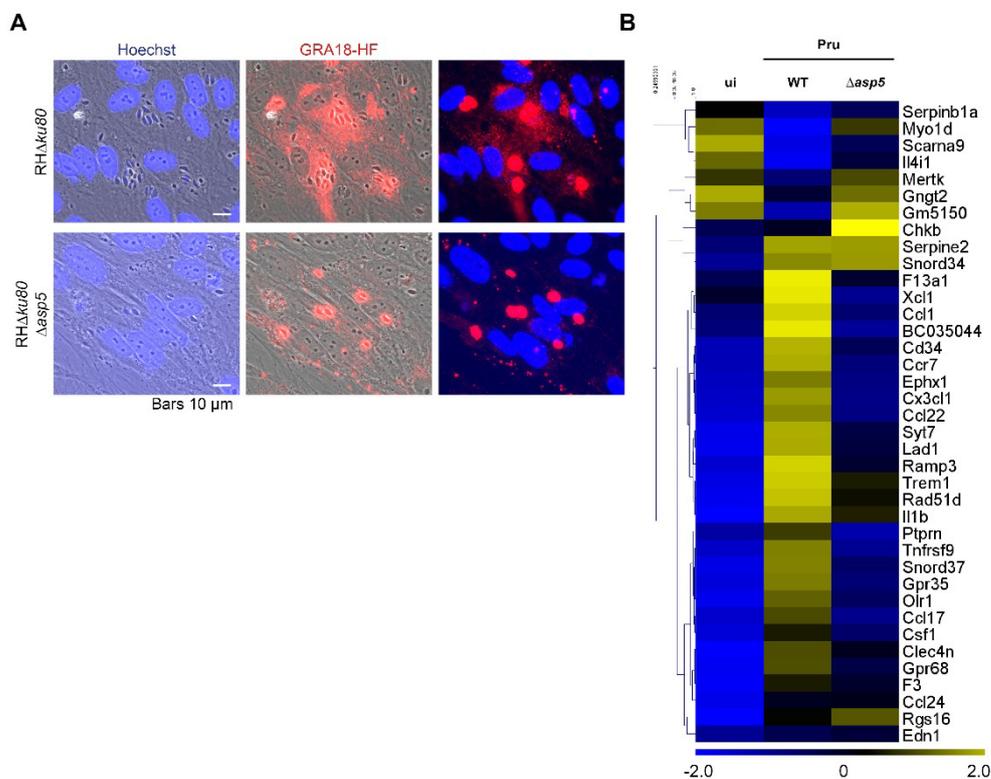


Figure S3. Deletion of the aspartyl protease ASP5 compromises GRA18 export and phenocopies the deletion of GRA18 in promoting host cell gene expression.

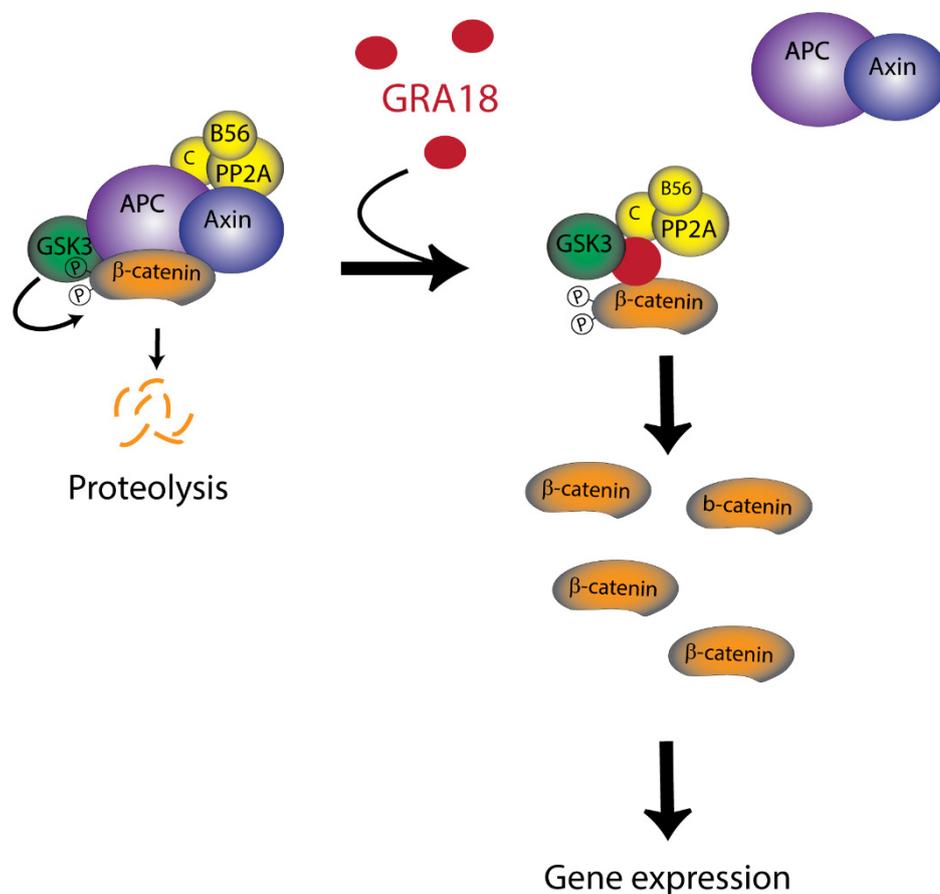
(A) In the absence of ASP5, GRA18-HF transiently transfected in type I parasites was no longer exported to the host cytoplasm and instead remained within the PV. (B) In the absence of ASP5, *T. gondii* parasites failed to induce most of the GRA18-dependent genes of BMDMs. The GRA18 specific gene-set identified in Figure 5A was analyzed in the transcriptomic data obtained from BMDMs infected by $\Delta asp5$ mutant parasites (Pru strain) from (Hammoudi *et al.*, 2015). RAW RNA-Seq data were obtained from the European Nucleotide Archive and analyzed as described in *Materials and Methods*.

host cell cytoplasm when delivered by *ASP5* mutant parasites (Figure S3A) and accordingly, the absence of *ASP5* phenocopies the defect in GRA18 in inducing its target genes in macrophages ((Hammoudi *et al.*, 2015), Figure S3B). Moreover, the secretion mechanism in the host cell is dependent on the MYR1 effector (Figure 1D), a protein somehow involved in the trafficking of GRAs protein through the PVM (Coffey *et al.*, 2016). GRA18 thus shares common characteristics with the other exported GRA effectors, including the N-terminal signal peptide and TEXEL motif both embedded in highly intrinsically disordered regions (Figure 1E), a feature that seems to be a prerequisite for effector secretion across the PVM (Hakimi *et al.*, 2017).

GRA18, an inhibitor of the β -catenin destruction complex

By using genetic and biochemical approaches, we have identified GRA18 as a strong interactor of host β -catenin, GSK3, and the PP2A-B56 holoenzyme. The resulting complex(es) leads to β -catenin accumulation. Although β -catenin levels were not seen affected upon infection with wild-type parasites (Figures 4E and D), the effects observed at the level of host cell gene expression testified to a regulatory activity of GRA18 in infected cells. The use of β -catenin mutant in RAW264.7 macrophages, provided definitive evidence for β -catenin acting epistatically to GRA18's activity on host cell transcription, though its activity was restricted to certain target genes (Figure 6). Therefore, our data indicate that the function of GRA18 aims at regulating β -catenin, very likely through its interactions with the identified host partners. β -Catenin, GSK3, and PP2A-B56 are characteristic components of the canonical Wnt signaling pathway. In the absence of Wnt ligands, cytosolic β -catenin is maintained at low levels by a multiprotein complex composed of APC, Axin, GSK3 and PP2A-B56 that constantly phosphorylates β -catenin for ubiquitylation and subsequent degradation by the 26S proteasome (Liu *et al.*, 2002). Mutation in any of these components (APC, AXIN, or β -catenin) leads to inappropriate stabilization of β -catenin, which results in cancer, most notably of the colon (Stamos and Weis, 2013). Axin, a central scaffold protein of the complex, is typified by intrinsically disordered and flexible regions that mediate direct interactions with all other core components of the destruction complex (β -catenin, APC, and GSK3) (Stamos and Weis, 2013). In light of our data, it is tempting to speculate that GRA18, by virtue of its overall disordered protein sequence (Figure 1E), could compete with Axin/APC for binding to β -catenin, GSK3, and PP2A-B56, thus disrupting the β -catenin destruction complex leading to the stabilization and nuclear translocation of β -catenin (see model). It is noteworthy that β -catenin transcripts remained unaffected by GRA18 in the RNA-Seq experiment we performed on BMDMs (Table S2), which is consistent with a post-transcriptional mechanism of regulation of β -catenin. While the exact *modus operandi* of GRA18 remains unclear, our data indicate that GRA18 is acting as a direct regulator of β -catenin and some possible scenarios are as

follows. Interestingly, the Y2H interaction assay showed that the SID analysis for interaction between GRA18 and β -catenin is mediated by a region encompassing amino acids 42–297 of the latter (Figure 2A), which harbors conserved serine and threonine residues initially phosphorylated by CK1 (Ser45), and subsequently by GSK3 (Thr41, Ser33 and Ser37). Once phosphorylated, Ser33 and Ser37 mediate the interaction with the β -TrCP adaptor protein for β -catenin degradation. Therefore, the way GRA18 binds to the N-terminal domain of β -catenin is quite peculiar and differs from the binding sites for Axin and APC mapped at central ARM domain. As such, the binding interface of GRA18 to the β -catenin N-terminal region would provide an efficient strategy to interfere with both the phosphorylation cascade catalyzed by CK1 and GSK3, but also at a later step with the binding to β -TrCP.



Model for hijacking of the β -catenin destruction complex by GRA18.

In the absence of the Wnt ligands or GRA18, β -catenin is continuously recognized and targeted for degradation by a destruction complex composed mainly of Axin, Axin, and GSK3. In *T. gondii* infected cells, GRA18 is delivered in the host cytoplasm and compete with APC/Axin for binding to β -catenin, GSK3, and PP2A-B56 leading to β -catenin upregulation and target gene expression.

In our current model, GRA18 binds to GSK3 and inhibits its activity towards β -catenin leading to its upregulation. Phosphorylation of GSK3 β Ser9 or GSK3 α Ser21 normally decreases GSK3 α/β enzymatic activity (Cross *et al.*, 1995; Peyrollier *et al.*, 2000). However, GRA18 does not alter the phospho-Ser21/Ser9 levels (Figures 6D and 7A), indicating that the parasite is acting in an independent fashion of the phosphorylation status of Ser21/Ser9.

While PP2A-B56 associates with the β -catenin destruction complex, its function in the Wnt signaling remains uncertain (Hsu *et al.*, 1999; Seeling, 1999; Yamamoto *et al.*, 2001). A recent study reported that the PP2A regulatory B56 subunit binds to a LxxIxE Short Linear Motif (SLiM) on interacting proteins as highlighted by the interaction between B56 and Axin (Hertz *et al.*, 2016). Interestingly, GRA18 carries two LxxIxE motifs within an intrinsically disordered region of the C-terminal domain, which is in agreement with the B56 binding assays (Figures 3A and C). As reported previously by (Hakimi *et al.*, 2017), SLiMs appear as general

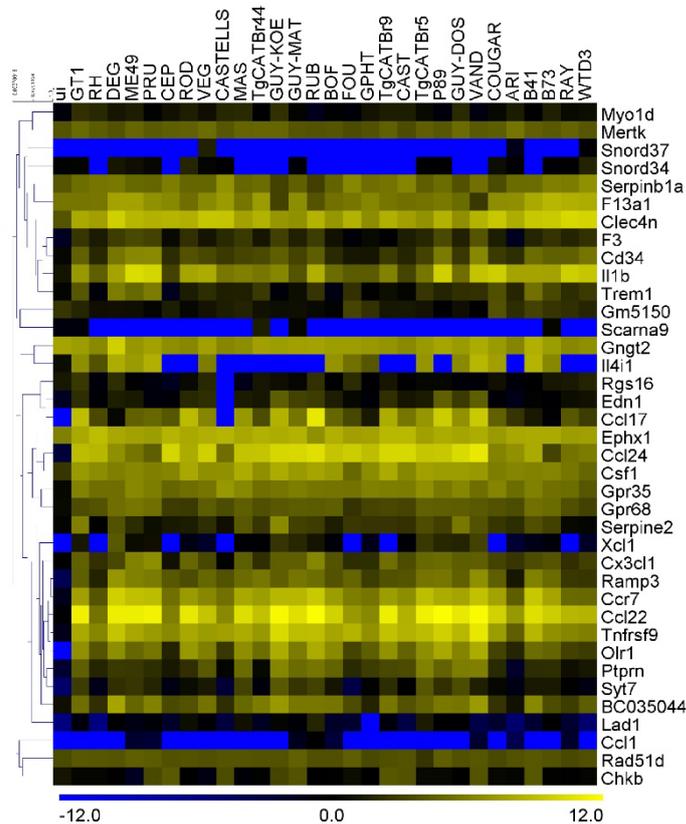


Figure S4. Analysis of the GRA18 target genes in BMDMs infected by different *T. gondii* strains.

The GRA18 specific gene-set identified in Figure 5A was analyzed in the transcriptomic data obtained from BMDMs infected by different *T. gondii* strains from (Melo *et al.*, 2013). RAW RNA-Seq data were obtained from the GEO DataSets Database and analyzed as described in *Materials and Methods*.

feature of the GRA effector family of proteins, probably reflecting a favorable evolutionary strategy to expand and diversify the binding interfaces between host and parasite proteins. The biochemical analysis suggested a simultaneous binding of GRA18 to GSK-3 and PP2A-B56 (Figure 2D). Whether the binding of PP2A-B56 is essential for GRA18 activity remains to be determined. GSK3 activity can be regulated by phosphorylation of tyrosine residues (Tyr279/Tyr216 in α/β isoforms, respectively) exposed in the activation loop, which enhance its kinase activity (Dajani *et al.*, 2003). An attractive hypothesis is that PP2A-B56 could be recruited to the GRA18 complex in order to dephosphorylate GSK3, thus participating to the inhibitory activity towards the β -catenin destruction complex. Alternatively, PP2A-B56 could be recruited to GRA18-GSK3 complex to dephosphorylate β -catenin, leading to the inhibition of β -catenin degradation (Su *et al.*, 2008).

The GRA18-GSK3- β -catenin axis induces the expression of anti-inflammatory chemokines.

Our transcriptomic data have revealed the magnitude by which *T. gondii*'s effector GRA18 is altering the expression of genes of the cell it infects, particularly the chemokines *Ccl17*, *Ccl22*, and *Ccl24*. Those genes have been found repeatedly induced by *T. gondii* regardless of the strain type in macrophages (Figure S4) (Hammoudi *et al.*, 2015; Melo *et al.*, 2013; Morgado *et al.*, 2011), which is consistent with the overall conservation of GRA18 within the *Toxoplasma* lineages. Furthermore, the GRA18-mediated induction of those chemokines was corroborated by Jon Boyle's lab in infected human placental trophoblasts (needs confirmation) (Ander *et al.*, in preparation), thereby strongly supporting the physiological importance of the GRA18-GSK3- β -catenin regulatory pathway during congenital toxoplasmosis.

We provide here genetic evidence for GRA18 acting in a β -catenin-dependent manner on chemokines expression in macrophages. How β -catenin regulates *Ccl17*, *Ccl22*, and *Ccl24* transcription remains to be determined. Analysis of the promoter regions of those genes revealed the presence of putative consensus-binding sites for the β -catenin target transcription factors Tcf/Lef (Tcf/Lef1-binding elements, CTTTGA and TCAAAG, respectively) (Scheller *et al.*, 2013; Shtutman *et al.*, 1999) in a region 1.5 kb upstream of the transcriptional start site (Figure S5), which is consistent with a direct mode of regulation by β -catenin/Tcf/Lef.

Although Wnt β -catenin signaling has originally been studied in the context of thymocyte development and stem cell biology (Reya *et al.*, 2003; Staal *et al.*, 2008; Verbeek *et al.*, 1995), it is becoming increasingly clear that it is involved in the regulation of certain aspect of innate immunity as well. Indeed, activation of β -catenin promotes differentiation and

activation of DC in a way they stimulate regulatory T cells (Tregs) and suppress the inflammatory response (Manicassamy *et al.*, 2010; Zhou *et al.*, 2009). Moreover, β -catenin accumulation triggered by GSK3 inhibitors promotes the survival of Treg cells (Ding *et al.*, 2008). Interestingly, CCL17, CCL22 and CCL24 are expressed by alternatively activated M2-polarized macrophages or tolerant macrophages and the release of these chemokines results in the recruitment of Treg cells and amplification of Th2 response (Biswas and Mantovani, 2010). Thereby, an attractive hypothesis could be that upon infection, GRA18 regulates the GSK3- β -catenin axis and thus participates to the M2 macrophage polarization characteristic of *T. gondii* infection (Jensen *et al.*, 2011). Whether GRA18 acts in concert with ROP16 in *T. gondii* type I strain to dampen the host inflammatory response appears as an interesting issue that would deserve further investigation.

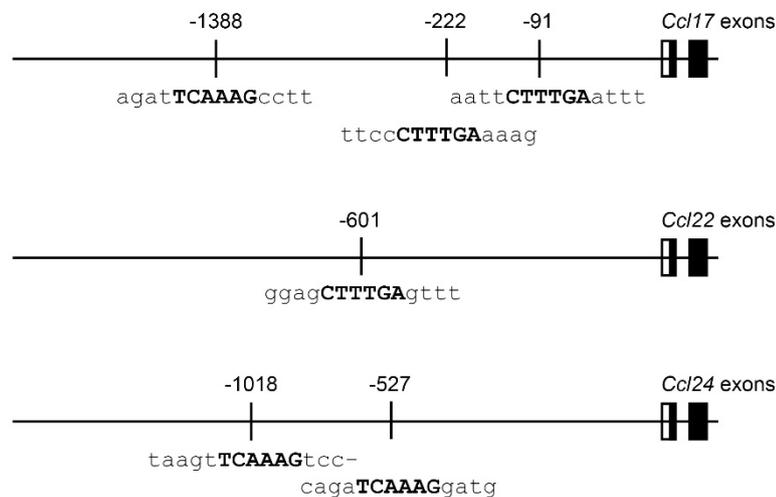


Figure S5. The promoter regions of *Ccl17*, *Ccl22*, and *Ccl24* contain putative Tcf/Lef1 binding sequences.

Positions of the Tcf/Lef1-binding elements (CTTTGA and TCAAAG, respectively (Scheller *et al.*, 2013; Shtutman *et al.*, 1999)) are indicated in the promoter regions of murine *Ccl17*, *Ccl22*, and *Ccl24* genes.

Cytokine and chemokine secretion act at the frontline of the defenses against *T. gondii* by promoting immune cells recruitment and a rapid Th1 cell-mediated pro-inflammatory response to clear the bulk tachyzoite population. Importantly, counterbalancing the Th1-induced inflammatory effects is essential to prevent immunopathology and preserve host and parasite survivals. In this context, *T. gondii* could induce Th2 chemokines that will dampen the inflammatory response in order to increase parasite chances to disseminate and access deep organs such as the brain where they form cysts. Consequently, the Δ *gra18* strains, which stimulate lower chemokines (*Ccl17*, *Ccl22*, and *Ccl24*) levels are efficiently eliminated early

during infection and are therefore less able to guarantee long-term persistence in mice when establish chronic infection in mice than wild-type parasites.

Overall, the work described here extends our view of how *T. gondii* interferes with the host cell and unveil a novel regulatory path for GSK3- β -catenin-controlled transcriptional changes in macrophages and likely in other cell types. The parasites, by hijacking the evolutionarily conserved Wnt/ β -catenin signaling pathway may provide part of an explanation for why *T. gondii* is so successful in parasitizing a wide range of hosts.

GRA18, a potential regulator of IFN- β

In the present study, we identified GRA18 as a positive regulator of β -catenin in the infected host cell. As an important co-transcription factor, β -catenin contributes to the expression of a large number of genes. Indeed, we established that the ectopic expression of GRA18 is sufficient to activate some of the β -catenin target genes, like *AXIN2* and *CTNND1* in HEK 293T cells (data not shown) (Chen *et al.*, 2015). Interestingly, in addition to the conventional Wnt target genes, we also unveiled that GRA18 promotes the expression of chemokines like CCL17, CCL22 and CCL24 in macrophages through β -catenin, which have never been defined as Wnt/ β -catenin target genes. Surprisingly, the ability of GRA18 in regulating those chemokines is only observed in macrophages, but not in fibroblast cells (data not shown), suggesting that β -catenin cooperates with other transcription factors which are specifically present in the former. In the recent years, studies on β -catenin have revealed an increasing number transcription factors working along with β -catenin, especially those involved in immune responses including Interferon Regulatory Factor 3 (IRF3) (Chattopadhyay *et al.*, 2015; Yang *et al.*, 2010).

IRF3 is a transcription factor that belongs to IRF family that is a key regulator of the type I Interferon (IFN) gene expression elicited by the pathogens. IRF3 resides in the cytosol in a latent form and undergoes a nuclear translocation following the detection of microbial nucleic acids or bacterial LipopolySaccharide (LPS) (Kawai *et al.*, 2001). In the nucleus, IRF3 forms a holocomplex with co-transcription factors and co-activators to ensure efficient transcription of its target genes, most notably, interferon- β (IFN- β). Recent studies have demonstrated that β -catenin is involved in the regulation of IFN- β expression (Servant *et al.*, 2002). Indeed, detection of microbial nucleic acids or LPS promotes the up-regulation of β -catenin expression, which associates with the C-terminal domain of IRF3 to recruit the acetyltransferase p300 to the IFN- β enhancer, hence leading to IFN- β expression. Accordingly, β -catenin deficiency substantially impairs induction of IFN- β in response to viral or bacterial infection. Moreover, β -catenin-knockout in mice revealed an important role for the β -catenin pathway in the control of

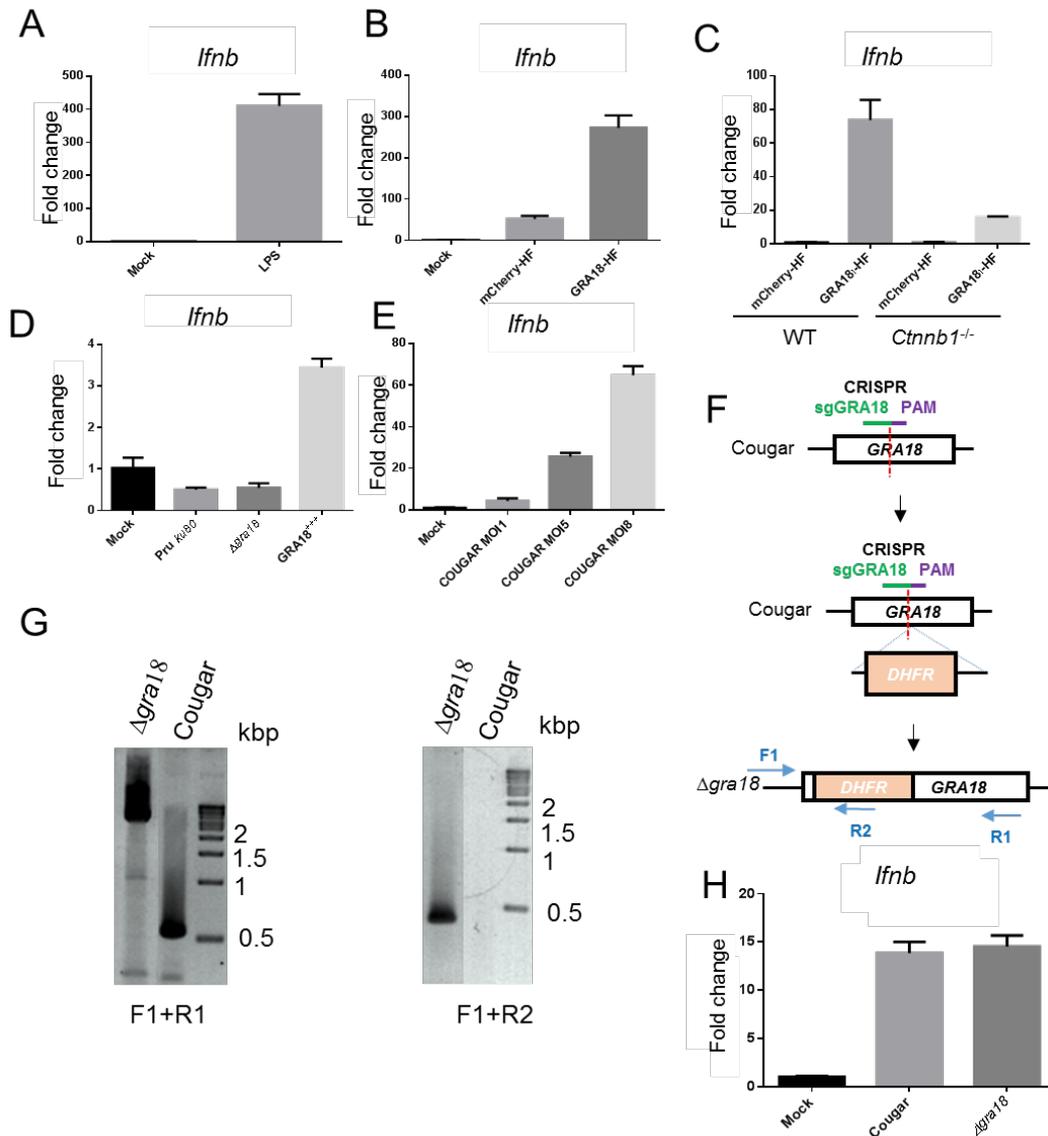


Figure S6. GRA18 triggers the expression of IFN- β . (A-H) *lfnb* mRNA levels were determined by RT-qPCR. *Tbp* was used for normalization. Data are displayed as fold difference relative to the unstimulated cells or cells left uninfected. Each assay was performed at least twice with similar results. (A) RAW264.7 cells were left unstimulated or stimulated 6 h with *E. coli* LPS. (B) RAW264.7 cells were transfected with pcDNA4_mCherry-HA and pcDNA4_HA-GRA18. (C) RAW264.7 (WT) or *Ctnnb1*^{-/-} mutant cells transfected with pcDNA_mCherry-HA and pcDNA-HA_GRA18 vectors. (D) RAW264.7 cells (left) were infected for 18 h with Pru *ku80*, Pru *ku80* Δ *gra18* (Δ *gra18*) and Pru *ku80* Δ *gra18*, P_{GRA1}-GRA18-HF (GRA18⁺⁺⁺). (E) RAW264.7 cells were infected for 18 h with COUGAR strain at the indicated multiplicity of infection (MOI). Mock: left uninfected cells. (F) Diagram showing the strategy to obtain the COUGAR *GRA18*::*DHFR* insertional mutant. The Wild-type COUGAR parasites were mutated using pTOXO_Cas9CRISPR::sgGRA18 and a PCR product containing the *DHFR* cassette. The selected clones were screened by PCR by using the indicated primers. (G) PCR analysis of the wild-type Cougar and Δ *gra18* mutant parasites using primers F1 and R1 showing the *GRA18* genomic sequence, or insertion containing the *DHFR* cassette. Parasites were analyzed with primers F1 and R2 to verify the insertion of *DHFR* cassette within the *GRA18* genomic sequence. (H) RAW264.7 cells were infected 18 h with the indicated strains or left uninfected.

viral infection *in vivo* (Yang *et al.*, 2010). Therefore, β -catenin constitute a critical component of a coactivator pathway through IRF3 that regulates transcription of the gene encoding IFN- β in response to infectious microorganisms.

IFN- β provides a protective response mainly against viral infections, but also against other intracellular microbes, including intracellular bacteria and protozoan (Silva-Barrios and Stäger, 2017; Trinchieri, 2010). Consistently, IFN- β is found as an attractive target to counteract when studying intracellular pathogens. Indeed, certain viruses and bacteria have developed the ability to regulate IFN- β by controlling its expression, or its downstream signaling pathway (Perkins *et al.*, 2015; Yang *et al.*, 2010). Several lines of evidence indicate that, similarly to other microbes, *T. gondii* evolved mechanisms to control IFN- β as most strains do not induce IFN- β production (Melo *et al.*, 2013). Intriguingly, only few atypical strains isolated from South America (RUB and COUGAR) are able to trigger IFN- β expression and downstream components of the type I interferon signaling pathway. Paradoxically, these strains are remarkably virulent in mice (Khan *et al.*, 2009) and the mechanism by which IFN- β is triggered remains enigmatic, though it has been shown to involve IRF3 and the cytoplasmic receptor retinoic acid-inducible gene 1 (RIG-I) (Melo *et al.*, 2013). Therefore, an attractive hypothesis is that GRA18, through the induction of β -catenin, promotes the expression of IFN- β .

To address this hypothesis, we assessed IFN- β mRNA levels in RAW264.7 cells ectopically expressing GRA18. As a positive control, cells were stimulated with *E. coli* LPS, a known inducer of IFN- β , which confirmed that the RAW264.7 cell line has a functional TLR4-IRF3 signaling pathway leading to the IFN- β expression (Figure S6A). In RAW264.7 cells transiently transfected with a mammalian expression vector encoding an N-terminal epitope-tagged GRA18 (FlagHA-GRA18), IFN- β was significantly induced compared to the mock transfected cells (Mock) or cells transfected with a vector encoding for a mCherry-HAFlag protein as a control (mCherry-HF) (Figure S6B). Notably, GRA18 induced IFN- β to levels comparable to those observed upon LPS treatment. The weak induction observed with the mCherry-HF vector is very-likely due to activation of the cGAS-STING pathway by the plasmid DNA introduced in cells by electroporation. The successful expression of the proteins from the expression vectors (mCherry-HF and FH-GRA18) and the ability of FH-GRA18 to trigger β -catenin nuclear accumulation were confirmed previously (Results section, Figure 6D). Thus, these data indicate that GRA18 has the ability to trigger IFN- β in murine macrophages. To confirm that GRA18 promotes IFN- β production through β -catenin, we expressed GRA18 in the RAW264.7 lacking β -catenin (RAW *Ctnnb1*^{-/-}). The ability of GRA18 to induce IFN- β was dramatically reduced in the absence of β -catenin, though remained a residual induction possibly reflecting the complex regulation of IFN- β expression by multiple transcription factors (Figure S6C). Overall, these data suggest that GRA18 is sufficient to trigger IFN- β in

macrophages in a manner that is similar to LPS with regard to β -catenin (Yang *et al.*, 2010). However, in cells infected by typical type II *T. gondii* strains expressing wild-type GRA18 (Pruku80), IFN- β remained uninduced (Figure S6D), suggesting that either the amount of GRA18 secreted in the host cell is not sufficient to trigger IFN- β expression, or that other effector(s) might prevent *T. gondii* from inducing IFN- β . Noteworthy, infection with parasites overexpressing GRA18 led to somewhat higher levels of IFN- β transcripts, suggesting that GRA18 has the potential to trigger the IFN- β pathway upon infection by *T. gondii* parasites (Figure S6D). Therefore, we assessed the ability of GRA18 to induce IFN- β in the atypical COUGAR strain, which naturally induces IFN- β expression. As expected, following infection by wild-type COUGAR parasites, IFN- β was distinguishably induced when compared to cells left uninfected (Figure S6E). Surprisingly, COUGAR parasites lacking GRA18 (COUGAR Δ *gra18*) have retained the ability to trigger IFN- β expression (Figure S6H). We concluded from

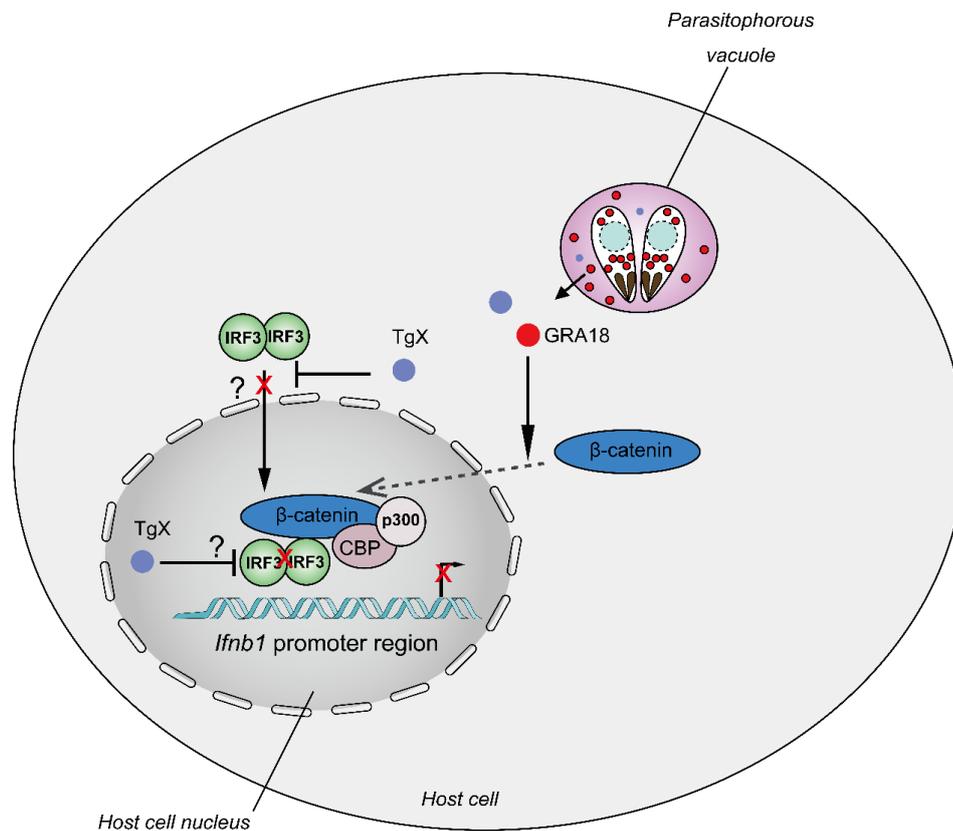


Figure S7. Hypothetical *Toxoplasma* effector (TgX) represses the expression of IFN- β in macrophages through inhibition of IRF3. In the presence of GRA18, β -catenin translocates to the nucleus, where it binds to transcriptional factors, DNA remodelers to initiate the transcription of *IFN- β* . In typical *T. gondii* strains, the hypothetical effector TgX inhibits IRF3 activity, preventing the transcriptional activation of *Ifnb1*.

this experiment that GRA18 is not responsible for the induction of IFN- β by the COUGAR strain.

The variability of the different *T. gondii* strains in regulating IFN- β expression remains very puzzling. IFNs are important mediators of the innate immune response, therefore this question deserves to be addressed in a future work. While regulation of IFN- β by the GRA18/ β -catenin was an attractive hypothesis, our preliminary experiments indicate that GRA18 is clearly not the main inducer of IFN- β expression in cells infected by *T. gondii*. Possibly, still unidentified *T. gondii* effectors are involved in the regulation of the IFN- β pathway. As mentioned above, IRF3 is a major regulator of IFN- β and therefore represent an interesting target for *T. gondii* to subvert innate immunity. Interestingly, *T. gondii* triggers IRF3 phosphorylation in the infected cells (Majumdar *et al.*, 2015). Possibly, *T. gondii* subverts the IRF3/IFN- β pathway in a similar manner to IRF1/STAT1/IFN- γ pathway by the *T. gondii* effector TgIST (Gay *et al.*, 2016; Olias *et al.*, 2016). Indeed, TgIST negatively regulates the expression of the IFN- γ responsive genes, by subverting the STAT1 transcription factor. TgIST hijacks STAT1 to recruit at the promoters

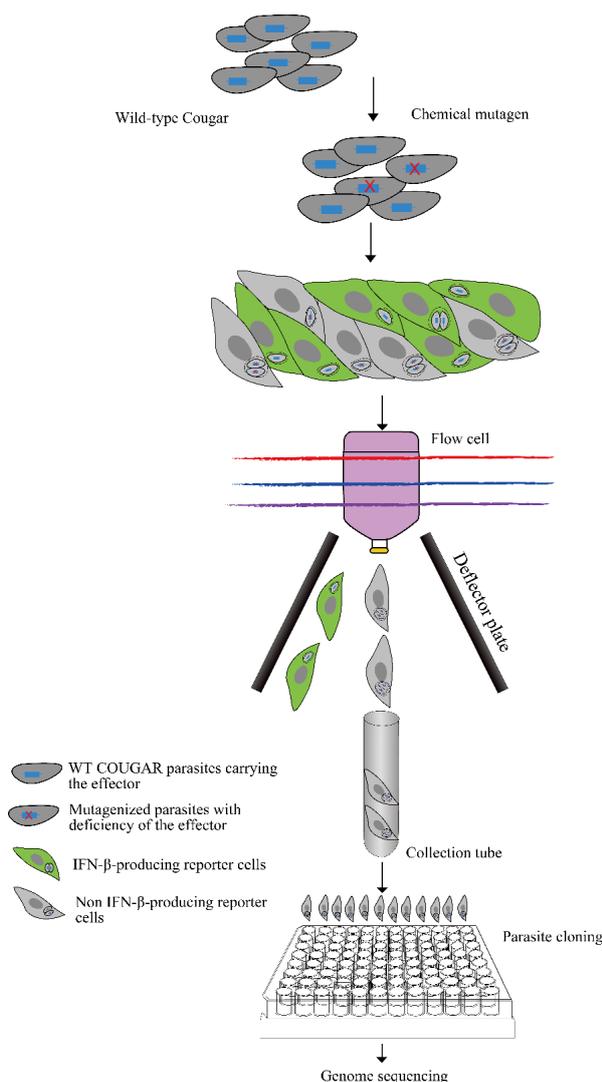


Figure S8. Schematic representation of genome wide screening for hypothetical *T. gondii* gene(s) responsible for IFN- β induction in the COUGAR strain.

COUGAR parasites will be first treated with chemical mutagen. Mutagenized parasites will be used to infect HEK cell line carrying a reporter system for IFN- β production. Infected cells containing mutant parasites that no longer trigger IFN- β production will be isolated by Flow cytometry. The isolated mutants will be expanded for genome wide sequencing in order to identify the gene(s) involved in the regulation of IFN- β in the host cell.

of the STAT1 target genes a chromatin repressor complex resulting in suppression of the STAT1-targeted genes otherwise induced by IFN- γ . Therefore, TgIST promotes *T. gondii* evasion of a major host innate immunity defense mechanism, resulting in enhanced *T. gondii* survival and virulence in mice. The identification of TgIST speaks for the possibility of the existence of a *T. gondii* effector protein - named here TgX in Figure S7 - able to inhibit the activity of IRF3, to down-regulate the expression of IFN- β (Figure S7). In that case, despite the GRA18-induced β -catenin by typical *T. gondii* strains, the IFN- β remains downregulated. In the atypical COUGAR strain, the hypothetical TgX repressor is either missing or not functional, thus leading to the upregulation of IFN- β . As a first approach to investigate the peculiar mechanism by which the COUGAR strain regulates IFN- β expression, it would be interesting to determine whether the IFN- β induction is dependent on ASP5 or MYR1, both involved in the trafficking and export of effector proteins (Coffey *et al.*, 2015; Curt-Varesano *et al.*, 2016; Franco *et al.*, 2016; Hammoudi *et al.*, 2015). This assay could provide evidence for the existence of such regulators secreted from the parasites to the host cell to regulate IFN- β .

Then, to identify the effector responsible for IFN- β induction, we could perform a genome wide screen using an IFN- β reporter system such as the HEK IFN- β reporter cell line (Figure S8) (Guo *et al.*, 2014). Mutagenized COUGAR parasites that have lost the ability to induce the IFN- β reporter would be analyzed by genome-wide sequencing to identify single nucleotide variants responsible for the lack of IFN- β induction.

Alternatively, the induction of IFN- β by COUGAR is a result of host immune sensing of parasite PAMPs. Upon PAMP recognition, some of Pattern Recognition Receptors (PRRs) present at the cell surface or intracellularly signal to the nucleus and therefore stimulate the expression of IFN- β , including TLR4 and RIG-I etc (Kawai *et al.*, 2001). In support of this hypothesis, RIG-I was found to have an essential role in the induction of IFN- β by the COUGAR strain. Initially, RIG-I was considered to serve as a sensor for short 5' triphosphate uncapped single/double stranded RNA, which are mainly found in viruses (Loo and Gale, 2011). Thus, a model has been proposed by Melo *et al.* (Melo *et al.*, 2013) in which nucleic acids from parasite origin are released into the host cell cytoplasm following infection resulting in production of IFN- β . Although the regulation of IFN- β expression during toxoplasmosis remains obscure, few studies have shown that the mouse intraperitoneal macrophages and murine embryonic fibroblast (MEF) primed with IFN- β inhibits *T. gondii* growth (Nagineeni *et al.*, 1996). This hypothesis could partially explain the abnormal growth of COUGAR as compared to other strains *in vitro* culture. This recognition of passively released nucleic acid could trigger the production of IFN- β , which eventually further promotes parasite elimination. Additionally, COUGAR strain was isolated in Vancouver Island cougars (*Felis concolor*), a feline that possibly serves as a definitive host (Aramini *et al.*, 1998). Deer, domestic animal and livestock constitute the main preys of the American cougar. Consistently, the *T. gondii* COUGAR strain may have co-evolved with these specific natural hosts resulting in a non-adapted parasite for rodents, which might explained the its high virulence in laboratory mice.

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Tables

Table S1.

Strains and Plasmids.

<i>Toxoplasma</i> Strains	Genotype	Reference
RH <i>ku80</i>	RH $\Delta ku80$	Bougdour <i>et al.</i> , 2013
Pru <i>ku80</i>	Prugniaud $\Delta ku80$ type II strain	Bougdour <i>et al.</i> , 2013
76K GFP LUC	Type II 76K strain - GFP- and Luciferase-expressing transgenes	kind gift from Michael Grigg (NIH)
Pru $\Delta gra18$	Pru <i>ku80</i> $\Delta gra18::DHFR$	This study
Pru $\Delta gra18GRA18^{+++}$	Pru <i>ku80</i> $\Delta gra18::DHFR$, P _{GRA1} - <i>GRA18</i> -HA-Flag, pMiniHX	This study
76K $\Delta gra18$	76K $\Delta gra18::DHFR$	This study
RH $\Delta myr1$	RH <i>ku80</i> $\Delta myr1::DHFR$	This study
RH $\Delta asp5$	RH <i>ku80</i> <i>ASP5</i> -Ty:: <i>DHFR</i>	Curt-Varesano <i>et al.</i> , 2016
Mammalian cell lines	Genotype	
RAW 264.7	<i>Mus musculus</i> monocytes/macrophages	ATCC, TIB-71
L929	<i>Mus musculus</i> fibroblasts	Aziz <i>et al.</i> , 2009
J774	<i>Mus musculus</i> monocytes/macrophages	ATCC, TIB-67
T-Rex	<i>Homo sapiens</i> , T-Rex™-293/pcDNA6/TR© plasmid providing the Tet repressor, Blastidicin ^R	Invitrogen
T-Rex-mCherry	T-Rex™-293/pcDNA-mCherry-HF	This study
T-Rex-GRA18 ^{FL}	T-Rex™-293/pcDNA-FH-GRA18 ²⁶⁻⁶⁴⁸	This study
T-Rex-GRA18 ^{Nt}	T-Rex™-293/pcDNA-FH-GRA18 ²⁶⁻⁴⁴⁰	This study
T-Rex-GRA18 ^{Ct}	T-Rex™-293/pcDNA-FH-GRA18 ²³³⁻⁶⁴⁸	This study
RAW 264.7 <i>Ctnnb1</i> ^{-/-}	RAW 264.7 <i>Ctnnb1</i> ^{-/-}	This study
Plasmids	Informations	Reference
pcDNA4	pcDNA4/TO/myc-His A, Zeocin ^R , Amp ^R	Invitrogen
pPuro	Puromycin ^R	Bougdour <i>et al.</i> , 2013
pcDNA-FH-LIC	pcDNA4/Flag-HA-LIC, Amp ^R , Zeocin ^S	Bougdour <i>et al.</i> , 2013
pcDNA-mCherry-HF	pcDNA4-LIC-HA-Flag/mCherry-HF	Bougdour <i>et al.</i> , 2013
pcDNA-FH-GRA18 ^{FL}	pcDNA-FH-LIC/Flag-HA-GRA18 ²⁶⁻⁶⁴⁸	This study
pcDNA-FH-GRA18 ^{Nt}	pcDNA-FH-LIC/pcDNA-FH-GRA18 ²⁶⁻⁴⁴⁰	This study
pcDNA-FH-GRA18 ^{Ct}	pcDNA-FH-LIC/pcDNA-FH-GRA18 ²³³⁻⁶⁴⁸	This study
pMiniHX	HXGPRT (HX), confers resistance to Mycophenolic acid and Xanthine	Bougdour <i>et al.</i> , 2013
pDHFR-TSc3	Confers resistance to Pyrimethamine	Bougdour <i>et al.</i> , 2013
pLIC-HF-DHFR	Amp ^R , Pyrimethamine ^R , HA-Flag tag	Bougdour <i>et al.</i> , 2013
pLIC-GRA18-HF	pLIC-HF-DHFR/GRA18-HA-Flag	This study
pTOXO_Cas9-CRISPR	pUC57/pTgTUB8-TgU6-Cas9-HA-YFP	Curt-Varesano <i>et al.</i> , 2016
pTOXO_Cas9-CRISPR::GRA18	pTOXO_Cas9-CRISPR/sgGRA18	This study
pDEST14	Kn ^R	Invitrogen
pDONR221 P3-P2	Amp ^R	Invitrogen
pDONR221 P1-P4	Amp ^R	Invitrogen
pDONR221/DHFR	pDONR221 P4r-P3r/DHFR cassette, Pyrimethamine ^R	Bougdour <i>et al.</i> , 2013
pDONR221/5'GRA18	pDONR221 P1-P4/TGME49_288840 5' region	This study
pDONR221/3'GRA18	pDONR221 P3-P2/TGME49_288840 3' region	This study
pDEST14 KO GRA18	pDEST14/TGME49_288840 5' region-DHFR-TGME49_288840 3' region	This study
pX330-hSpCas9	pX330-U6-Chimeric_BB-CBh-hSpCas9, AmpR	Cong <i>et al.</i> , 2013
pX330-hSpCas9::sgCTNNB1	pX330_hSpCas9/sgCTNNB1	This study

R: resistant
S: sensitive

Primers and Oligonucleotides.

Primer/oligo name	Primer/oligo sequence 5' to 3'
LICF-288840_F	
LICR-288840_R	
pcDNA4-FlagHA-LIC-288840_F	GGTGCTGGTGCTGGTGCCCATctttctgttgatattctccaacg
pcDNA4-FlagHA-LIC-288840_R	CTCCTTGTGCCTTGCCCATCAattacccttagtgggtgggttaacg
pcDNA4-FlagHA-LIC-288840Ct_F	GGT GCT GGT GCT GGT GCC CAT TTA CAA AGT GGA CAA ACTA GAT ATG CT C
pcDNA4-FlagHA-LIC-288840Nt_R	CTC CTT GTC GCC TTG CCC A TCA CCTACGAAGACGCTCCTGTAACG
attB1-288840_F	GGGGACAAGTTTGTACAAAAAAGCAGGCTCTTGAGAATCTTTGATTTACCTTTGATG
attB4-288840_R	GGGGACAACCTTTGTATAGAAAAGTTGGGTGATGAGCGAGAGGGGTCAACCATAAC
attB3-288840_F	GGGGACAACCTTTGTATAATAAAAGTTGATGCTTGTGAGACTCCTCACGCG
attB2-288840_R	GGGGACCACCTTTGTACAAGAAAGCTGGGTACCCTTCTGTGGAACAGTCTGAAGAG
dhfr-KO-ol1_R	GACAGAGTTGTCTCGACAACGAATG
dhfr-KO-ol1_F	CTTCATGTGGCATTTCACACAGTCTC
3'region-288840_R1	GCTCAAACTAAGTACCTTGCTGC
5'region-288840_F2	TGACTACTTCTTCTGCTGTTCGTC
GRA18-CRISP-FWD	AAGTTggggcgaatcgcggtgaatgG
GRA18-CRISP-REV	AAAACcattcaccgcatcgccccA
GRA18-DHFR - F	aacaaccggcacgaattgCaccgcctggtcagaggtagccccggggcgaatcgcggtgaaagctcgccaggctgaaatc
GRA18-DHFR - R	ctggcttcggcgatcctgaattatgaatgtgctctgttgtcccacaaccgctccgcatggaattcatcctgcaagtcgatag
Chk ctnnb1_F	TCACGCAAGAGCAAGTAGCTGG
Chk ctnnb1_R	TGAAGAGGCACCATTAGTTTCC

Table S2.

RNA-Seq Reads Mouse

Data	NumSeqs	fileCnt	Template Mer	QueryMer	Hit	Feature_Cnt	AssembledReadCnt	UnassembledReadCnt
GRA18 ⁺⁺⁺ .fq (pair)	70896190	2	2648267371	1559270601	2594572129	233771	55166802	15708810
GRA18 ⁺⁺⁺ -LPS.fq (pair)	71267108	2	2648267371	1567429814	2550831296	233771	55698466	15548051
Pru ku80.fq (pair)	77722138	2	2648267371	1709396118	2956410466	233771	61022895	16676647
Pru ku80-LPS.fq (pair)	70734904	2	2648267371	1555719561	2599670255	233771	55630489	15083764
<i>Δgra18</i> .fq (pair)	70822332	2	2648267371	1557646247	2835743714	233771	57253164	13548725
<i>Δgra18</i> -LPS.fq (pair)	77019568	2	2648267371	1693942259	2843119302	233771	60562597	16434529
ui.fq (pair)	72973464	2	2648267371	1604955607	3022834270	233771	72236336	715899
ui-LPS.fq (pair)	75125286	2	2648267371	1652280489	2768276444	233771	74412040	691295

RNA-Seq Reads *Toxoplasma*

Data	NumSeqs	fileCnt	Template Mer	QueryMer	Hit	AssembledReadCnt	UnassembledReadCnt
GRA18 ⁺⁺⁺ .fq (pair)	70896190	2	65413638	1559270601	604779810	14860387	56015216
GRA18 ⁺⁺⁺ -LPS.f.fq (pair)	71267108	2	65413638	1567429814	836986278	14918766	56327747
Pru ku80.fq (pair)	77722138	2	65413638	1709396118	558203483	16040877	61658655
Pru ku80-LPS.fq (pair)	70734904	2	65413638	1555719561	495938972	14496215	56218028
<i>Δgra18</i> .fq (pair)	70822332	2	65413638	1557646247	477867600	12847901	57953986
<i>Δgra18</i> -LPS.fq (pair)	77019568	2	65413638	1693942259	660004091	15795099	61202023

Pru vs *gra18*

(≥3 fold change, RPKM≥5 in at least one sample)

Name	ui - linear total RPKM	Pru - linear total RPKM	<i>gra18</i> - linear total RPKM	GRA18 ⁺⁺⁺ - linear total RPKM	ui_LPS - linear total RPKM	Pru_LPS - linear total RPKM	<i>gra18</i> _LPS - linear total RPKM	GRA18 ⁺⁺⁺ _LPS - linear total RPKM	Fold change Pru/ <i>gra18</i>	ui - log ₂ total RPKM	Pru - log ₂ total RPKM	<i>gra18</i> - log ₂ total RPKM	GRA18 ⁺⁺⁺ - log ₂ total RPKM	ui_LPS - log ₂ total RPKM	Pru_LPS - log ₂ total RPKM	<i>gra18</i> _LPS - log ₂ total RPKM	GRA18 ⁺⁺⁺ _LPS - log ₂ total RPKM
Ccl22	127.868	813.609	180.242	4903.932	230.126	5309.411	1781.769	9651.511	4.513 down	6.99851	9.66819	7.49379	12.25972	7.84226	12.74434	10.79909	13.23654
Clec4n	5.804	390.084	92.233	642.447	1.296	166.831	35.109	245.473	4.229 down	2.53693	8.60764	6.52722	9.32743	0.37429	7.38224	5.13377	7.93942
Ephx1	22.086	207.623	43.216	291.211	9.867	50.034	13.073	105.98	4.804 down	4.46509	7.69782	5.4335	8.18592	3.30261	5.64484	3.70849	6.72765
Il1b	2514.689	164.087	50.686	308.222	2570.242	7723.425	6996.278	4989.36	3.237 down	11.29616	7.35832	5.66353	8.26783	11.32769	12.91503	12.77237	12.28464
Csf1	65.964	100.411	24.715	238.071	118.254	133.074	121.672	172.834	4.062 down	6.04362	6.64977	4.62729	7.89525	6.88575	7.05609	6.92686	7.43325
Ccl17	27.787	67.864	17.918	544.994	10.771	386.673	184.892	667.936	3.787 down	4.79636	6.08457	4.16332	9.0901	3.42903	8.59497	7.53054	9.38357
Serpinc1a	2.64	43.26	6.426	17.762	1.367	6.835	2.332	6.288	6.731 down	1.40047	5.43498	2.68402	4.1507	0.45084	2.77295	1.2215	2.65263
Cd34	0.749	41.686	12.537	163.173	0.502	40.651	11.844	152.573	3.325 down	-0.41749	5.38148	3.64807	7.35026	-0.99524	5.34523	3.56607	7.25335
Tnfrsf9	1.573	40.263	8.991	230.318	3.827	334.514	102.145	723.267	4.477 down	0.65386	5.33139	3.16856	7.84748	1.93616	8.38592	6.67448	9.49838
Olr1	0.949	36.182	8.158	117.508	1.117	108.499	31.005	239.495	4.435 down	-0.07543	5.17722	3.02826	6.87662	0.16023	6.76153	4.95445	7.90385
Gngt2	28.092	32.274	9.238	72.885	10.102	81.833	11.738	106.754	3.493 down	4.8121	5.01228	3.20761	6.18755	3.33655	6.35462	3.55314	6.73814
Ccr7	1.28	26.591	7.948	84.953	6.324	132.579	37.876	248.27	3.345 down	3.3557	4.73288	2.99052	6.40859	2.6608	7.05071	5.24323	7.95576
F13a1	1.196	22.755	5.92	27.418	1.059	13.579	3.418	13.43	3.843 down	0.25865	4.5081	2.56565	4.77708	0.08218	3.76331	1.7731	3.74741
Ccl24	4.263	19.027	3.333	62.889	14.706	52.702	7.854	302.416	5.709 down	2.09182	4.24994	1.73667	5.97472	3.87832	5.7198	2.97347	8.24039
Myo1d	2.294	18.814	3.425	40.547	0.87	3.178	1.125	11.824	5.493 down	1.19817	4.2337	1.77699	5.34153	-0.20104	1.66792	0.17015	3.5636
Trem1	0.839	17.313	4.446	17.478	0.38	12.238	6.827	6.543	3.893 down	-0.25361	4.11375	2.15263	4.12749	-1.39727	3.61331	2.77115	2.71
Mertk	5.041	16.715	4.473	23.439	34.138	8.996	3.457	13.402	3.737 down	2.33371	4.06305	2.16108	4.55084	5.09331	3.1693	1.78954	3.74434
Mir7050	12.338	16.022	2.431	24.042	6.258	1.271	11.508	7.671	6.590 down	3.62501	4.00202	1.28161	4.58748	2.64566	0.34563	3.52457	2.9394
Rad51d	8.087	15.826	4.843	5.663	6.56	12.353	10.873	14.344	3.268 down	3.01554	3.98422	2.27581	2.50152	2.71371	3.62674	3.44271	3.8424
Il4i1	18.525	13.953	0	25.73	32.256	0	0	0	43870.292 down	4.21138	3.8025	-11.61846	4.6854	5.01149	-11.61846	-11.61846	-11.61846
Mir5132	1.898	13.733	2.431	10.123	2.682	2.541	3.452	5.114	5.649 down	0.92457	3.77962	1.28161	3.33955	1.42326	1.34563	1.78761	2.35444
Gm5150	5.123	13.212	54.892	1.689	7.388	7.809	28.394	0.88	4.154 up	2.35686	3.72378	5.77851	0.75617	2.88517	2.96506	4.82754	-0.18455
Gpr35	0.215	12.678	2.416	28.314	0.525	11.778	3.795	24.771	5.247 down	-2.21857	3.66426	1.27261	4.82343	-0.92937	3.57977	1.92429	4.63056
Serpine2	1.753	11.051	3.421	33.605	0.55	13.365	4.736	26.443	3.230 down	0.80955	3.46605	1.77427	5.07061	-0.86169	3.74036	2.24374	4.72484
Mir6910	0	10.592	3.33	7.037	1.67	9.334	3.2	5.837	3.181 down	-11.61846	3.40488	1.73533	2.81496	0.73948	3.22256	1.67806	2.54512
Ptpnrr	0.159	10.406	3.206	12.664	0.143	9.484	4.52	11.638	3.245 down	-2.65245	3.37928	1.68077	3.66265	-2.80931	3.24545	2.17633	3.54076
Cx3d1	10.334	10.034	2.278	24.671	5.942	16.15	13.263	10.034	4.405 down	3.36936	3.32686	1.18753	4.62477	2.57089	4.01347	3.72937	3.32685
Rgs16	1.513	9.915	1.601	14.032	2.2	11.053	3.31	20.513	6.194 down	0.59228	3.30959	0.67869	3.81062	1.13719	3.46632	1.72683	4.35845
Ccl1	0.357	9.901	0.549	12.601	0	14.876	1.473	23.327	18.035 down	-1.48531	3.30755	-0.86523	3.65542	-11.61846	3.89494	0.5583	4.54394
Gpr68	0.744	9.675	2.464	23.16	0.068	5.177	0.946	19.977	3.925 down	-0.42622	3.27422	1.30119	4.53358	-3.87004	2.37199	-0.07944	4.32029
F3	0.54	9.206	3.029	12.971	0.997	21.951	12.479	62.343	3.039 down	-0.88768	3.20259	1.59864	3.69722	-0.00433	4.45621	3.64148	5.96214
Xcl1	0.173	8.777	1.776	42.67	0.272	16.32	2.662	47.393	4.942 down	-2.5286	3.13381	0.82844	5.41516	-1.87791	4.02859	1.41244	5.5666
BC035044	0.621	8.588	2.632	19.945	2.926	60.217	11.417	67.217	3.262 down	-0.68658	3.10224	1.39629	4.31798	1.54908	5.91209	3.51311	6.07076
Mir1898	1.812	7.647	0	2.416	0.853	4.852	2.197	2.441	24043.562 down	0.85746	2.9349	-11.61846	1.27244	-0.22881	2.27851	1.13553	1.28732
Mir5107	1.812	7.647	0	4.946	5.242	2.426	2.249	0	24043.562 down	0.85746	2.9349	-11.61846	2.30638	2.3901	1.27851	1.16948	-11.61846
Edn1	43.559	7.34	2.054	26.529	445.321	129.699	103.136	160.649	3.573 down	5.44488	2.87584	1.03834	4.7295	8.7987	7.01903	6.6884	7.32776
Ramp3	0.718	7.145	1.552	18.194	0.096	2.104	0.98	4.354	4.602 down	-0.47753	2.83696	0.6344	4.18538	-3.38431	1.07281	-0.02953	2.12226
Lad1	19.905	6.936	1.657	34.643	24.132	61.558	41.451	50.837	4.186 down	4.31507	2.7941	0.72835	5.1145	4.59289	5.94388	5.37333	5.66782
Mir330	0	6.412	0	2.311	1.67	1.16	2.149	5.996	20160.750 down	-11.61846	2.6808	-11.61846	1.20831	0.73948	0.21438	1.10382	2.58934
Syt7	1.559	5.597	1.518	13.003	3.167	20.22	13.927	29.758	3.687 down	0.64103	2.48454	0.60192	3.70079	1.66322	4.33772	3.79985	4.8952
Mir7679	2.99	5.207	1.064	2.214	5.006	4.447	8.056	3.356	4.895 down	1.57992	2.38053	0.08897	1.14691	2.32373	2.15298	3.01	1.74676
Mir5121	3.796	2.289	8.509	2.531	0.894	7.624	2.302	7.671	3.717 up	1.92457	1.19466	3.08897	1.33955	-0.1617	2.93059	1.20264	2.9394
Mir3062	0	2.185	8.122	3.624	0	0	6.591	3.661	3.717 up	-11.61846	1.12755	3.02185	1.8574	-11.61846	-11.61846	2.72049	1.87229
Mir7059	2.718	2.185	8.122	1.208	11.947	2.426	0	3.661	3.717 up	1.44242	1.12755	3.02185	0.27244	3.57854	1.27851	-11.61846	1.87229
Snord37	1.128	1.814	6.261	5.014	0	2.014	2.736	4.559	3.451 up	0.17393	0.85906	2.64645	2.32588	-11.61846	0.10003	1.452	2.18876
Scarna9	0.949	1.144	5.47	1.265	0.894	1.271	5.179	1.918	4.779 up	-0.07543	0.19466	2.45154	0.33955	-0.1617	0.34563	2.37257	0.9394
Mir6385	12.338	1.144	7.293	2.531	5.364	7.624	10.357	2.557	6.372 up	3.62501	0.19466	2.86658	1.33955	2.42326	2.93059	3.37257	1.35444
Mir7051	0.909	1.092	18.565	3.624	4.267	16.981	13.182	9.763	16.993 up	3.17938	0.127						

Known β -catenin target genes

Name	ui - linear total RPKM	Pru - linear total RPKM	gra18 - linear total RPKM	GRA18*** - linear total RPKM	ui_LPS - linear total RPKM	Pru_LPS - linear total RPKM	gra18_LPS - linear total RPKM	GRA18***_LPS - linear total RPKM
Ncoa2	9.947	10.591	9.597	8.42	3.499	7.062	5.897	7.495
Myog	0	0	0	0	0	0	0	0
Sp5	0	0.045	0	0	0	0.025	0.081	0.05
Ywhab	268.319	260.445	276.384	275.014	206.634	236.67	244.474	263.048
Mmp9	8.252	11.233	14.469	6.252	2.517	7.909	10.392	4.615
Sall4	0	0	0	0	0	0	0	0
Tbl1xr1	19.734	36.078	33.375	22.268	11.421	26.02	22.171	18.058
Tnik	0.089	0.262	0.342	0.138	0.047	0.198	0.084	0.392
Bcl9	4.767	4.409	3.652	3.989	21.766	3.49	3.309	4.855
Pitx2	0	0	0.105	0	0	0	0	0.06
Lef1	0	0.386	0.18	0.164	0	0.215	0	0.766
Cyr61	0	0.97	1.881	6.317	0.016	1.873	0.975	8.926
Klf4	10.926	16.684	20.288	19.71	15.826	7.149	9.637	6.564
Tle1	4.637	14.211	16.774	9.254	0.885	4.387	5.038	5.712
Cdkn2a	6.082	76.724	78.002	122.649	1.443	133.564	88.691	253.479
Jun	66.851	35.393	43.129	51.4	34.334	23.392	22.123	27.169
Hdac1	5.584	6.367	6.855	5.923	12.067	8.305	9.073	7.337
Sfn	0.827	0.837	0.342	1.921	0.478	0.393	0.485	0.755
Cttnbip1	24.872	34.389	47.94	44.396	7.784	28.095	29.767	43.219
Ywhah	303.58	309.744	335.98	336.622	193.181	343.157	373.821	323.483
Ctbp1	124.196	157.682	167.511	160.45	58.773	114.949	133.46	127.583
Kcnp4	0	0.069	0	0.115	0	0.114	0.07	0.267
Ywhag	172.809	189.006	198.072	193.509	132.575	207.13	219.419	210.78
Trrap	9.751	13.792	12.781	11.485	6.035	10.165	13.562	11.117
Mdfic	117.76	132.798	122.247	157.007	289.777	150.496	126.064	158.203
Cul1	62.681	105.218	108.097	111.899	60.065	101.622	112.592	103.684
Sspo	0	0	0	0.004	0	0	0	0
Tcf7l1	0.025	0.057	0.029	0.158	0	0.016	0.043	0.11
Mitf	37.919	26.153	17.15	22.218	51.304	23.518	22.507	18.154
Ccnd2	13.791	19.254	13.027	49.052	125.911	141.184	89.279	212.903
Ruvbl2	53.348	164.16	176.688	139.793	18.113	120.772	125.244	143.441
Fgf4	0	0	0	0	0	0	0	0
Ccnd1	87.743	83.362	92.715	58.048	5.884	4.028	4.484	5.375
Dkk4	0	0	0	0	0	0	0	0
Adcy7	62.299	78.986	63.808	43.936	19.678	17.221	19.267	12.001
Mmp2	0.029	1.325	1.98	3.448	0	1.471	1.792	3.261
Cdh1	0.075	0.507	0.5	1.021	0	0.03	0.018	0.121
Smarca4	61.23	53.638	53.808	46.806	66.053	40.637	46.121	40.805
Cttnb1	323.047	374.303	369.94	268.83	179.796	323.857	282.37	289.517
Hdac2	63.486	109.453	110.441	110.257	41.066	118.581	107.277	121.888
Aes	316.792	268.546	286.145	220.102	115.185	145.419	161.397	110.961
Tle2	1.161	1.055	0.985	0.955	0.281	0.614	0.782	0.977
Myf5	0	0	0	0	0	0	0	0
Xpo1	87.798	180.54	198.927	151.081	71.907	160.838	164.44	164.765
Tcf7	0.085	0.242	0.427	2.582	0.075	0.088	0.141	1.379
Ywhae	740.948	963.282	1049.583	915.544	766.312	1080.769	1111.742	971.291
Cacna1g	0.012	0	0	0.008	0.011	0	0	0.063
Igf2bp1	0.069	0.125	0.147	1.134	0.022	0.8	0.683	1.641
Axin2	0	0.238	0.324	0.432	0	0.116	0.077	0.192
Ywhaq	33.136	41.945	42.821	42.055	25.031	36.592	39.099	41.837
Id2	277.941	465.064	418.196	669.892	569.414	756.135	827.225	1015.593
Hbp1	57.372	26.501	26.994	13.644	60.424	27.411	28.987	14.659
Neurog1	0	0	0	0	0	0	0	0
Tert	0.014	0.471	0.342	0.183	0.033	0.381	0.313	0.493
Vcan	12.27	217.319	229.611	67.807	145.529	244.419	366.455	80.823
Chd8	21.25	25.383	23.41	21.422	19.511	21.794	23.37	20.398
Ywhaz	73.673	93.506	99.088	100.76	127.2	109.87	122.182	108.518
Myc	19.361	73.456	89.483	52.269	13.272	90.689	88.825	74.506
Cby1	27.256	41.403	45.761	37.452	7.104	29.189	39.393	24.316
Ep300	20.745	21.688	21.742	15.016	21.456	15.275	20.463	13.068
Krt1	0	0	0	0	0	0	0	0
Snai2	0.148	0.178	0.41	0.525	0	0.296	0.597	0.298
Dvl3	11.935	13.6	13.399	12.885	9.142	11.334	13.035	13.76
T	0	0	0	0	0	0	0	0
Camk4	0.059	0.274	0.283	0.176	0.006	0.249	0.358	0.104
Apc	6.952	9.132	9.19	8.182	4.526	6.538	7.693	7.405
Cdx1	0	0	0	0	0	0	0	0
Tcf4	34.953	44.532	47.245	41.628	83.818	53.293	64.167	49.415
Incenp	92.367	92.652	103.224	49.375	24.37	55.83	67.204	25.943
Tle4	23.933	18.131	16.47	18.476	31.051	13.121	16.114	15.524
Dkk1	0.049	0.147	0.218	0.876	0.023	0.065	0.354	0
Tcf7l2	3.301	2.147	2.251	1.004	5.14	1.366	0.969	1.032
Zcchc12	0	0	0	0.044	0	0.044	0	0
Tbl1x	36.413	46.216	46.364	42.241	52.575	39.117	43.116	37.476
Ar	0	0.027	0.057	0	0	0.04	0.09	0
Med12	15.858	15.167	16.404	13.474	17.968	10.957	12.063	10.318
Cdx4	0.047	0	0	0	0	0	0	0
COX2	984.974	1002.136	940.977	796.479	883.229	1006.609	1087.618	950.679