

**Identification of *Toxoplasma gondii* effector proteins
by pooled CRISPR-Cas9 knockout screening**

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Declaration of Originality

I, Simon William Butterworth, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this is indicated.

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Abstract

Toxoplasma gondii is an intracellular eukaryotic pathogen of global clinical significance. Secretion of effector proteins by *T. gondii* into a host cell is critical for infection. At least two hundred putative effector proteins are secreted into the host cell, of which the vast majority are uncharacterised. In this work, I used CRISPR-Cas9 knockout screening in *T. gondii* to identify effector proteins involved in parasite evasion of host immune responses.

From a screen of *T. gondii* mutants in a mouse model of infection, I identified genes that confer parasite fitness *in vivo*. I found that the first-identified rhoptry protein of *T. gondii*, ROP1, is necessary for parasite virulence and is important for evasion of the cell-autonomous, interferon-gamma-stimulated immune response in both murine and human macrophages.

Then, I adapted CRISPR-Cas9 tools in *T. gondii* to enable pooled knockout screening with a single-cell, dual host-parasite transcriptome readout. This method, which I term dual perturb-seq, enables identification of parasite effectors that modulate host cell transcription in a high-throughput and unbiased manner. Using dual perturb-seq and additional cellular assays, I show that the dense granule protein GRA59 contributes to export of other dense granule effectors from the parasitophorous vacuole to the host cell. I also identify a rhoptry protein, newly named TgSOS1, that is required for sustained parasite-induced STAT6 signalling in the host cell and M2 polarisation of infected macrophages.

Together, these genetic screens and the characterisation of newly identified effector proteins advance the understanding of how *T. gondii* manipulates the host cell and evades host immune responses.

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Introduction

Toxoplasma gondii

Origin of Apicomplexa

Apicomplexa is an eukaryotic phylum of single-celled parasitic endosymbionts, several of which are major pathogens of humans. Apicomplexans evolved from a free-living photosynthetic ancestor and many species retain a remnant plastid organelle called the apicoplast, which has lost photosynthetic capability but contributes essential biosynthetic pathways in some species (McFadden et al. 1996; McFadden and Yeh 2017). The closest known relatives of Apicomplexa are photosynthetic chromerids and free-living predatory colpodellids (together, chrompodellids), while more distantly related dinoflagellates are variously photosynthetic or non-photosynthetic and free-living or endosymbiotic, and perkinsids are non-photosynthetic parasitic endosymbionts (Adl et al. 2019).

Apicomplexans, chrompodellids, dinoflagellates, and perkinsids together comprise the Myzozoa, within which apicomplexan-like parasites have arisen independently multiple times through convergent evolution (Mathur et al. 2019; Janouškovec et al. 2019; Rueckert, Pipaliya, and Dacks 2019). Myzozoa are named after a form of “cellular vampirism”-like feeding called myzocytosis, which is present in many myzozoan lineages and may represent an ancestral behaviour of this group (Cavalier-Smith and Chao 2004). Structures and organelles involved in myzocytosis are present across Myzozoa, but are variously lost or adapted to different cell-to-cell interactions (Dos Santos Pacheco et al. 2020). In apicomplexans, these structures have elaborated into the namesake apical complex (“*apex complexus*”) that is specialised to facilitate endosymbiotic parasitism through host cell invasion and secretion of effector proteins.

Apicomplexan phylogeny

Apicomplexans are near-ubiquitous pathogens of Metazoa, with more than 6,000 named species (Levine 2018). The majority of species lack DNA or RNA sequencing data, while some species are identified only from metagenomic samples. Apicomplexan phylogeny remains in flux, with the positions of deep branching lineages and number of major groups still subject to frequent revision. Taxonomic labels therefore do not always correspond to monophyletic groups. At least seven major monophyletic groups of apicomplexans are widely recognised: hematozoans, coccidians, adeleorines, corallicolids, marosporidans, cryptosporidians and gregarines (Mathur et al. 2019; Janouškovec et al. 2019; Salomaki et al. 2021; Kwong et al. 2021). Further groups have been proposed to enable the inclusion of more distantly related apicomplexan-like parasites, though this would necessitate classifying chrompodellids as a sub-group of Apicomplexa (Mathur et al. 2019; Janouškovec et al. 2019).

Haematozoans are blood-borne parasites with complex dixenous lifecycles that alternate between mammalian or avian hosts and invertebrate vectors. The Haematozoa clade includes the genus *Plasmodium* that is the causative agent of malaria, one of the leading causes of death and disease in developing countries with an estimated 247 million estimated cases and more than 600,000 estimated deaths in 2021 (World Health Organization 2022). Haematozoa also includes the genera *Babesia* and *Theileria*, tick-borne pathogens which are minor causes of disease in humans but which have a large economic impact in endemic countries through infection of livestock (Bock et al. 2004; Morrison 2015).

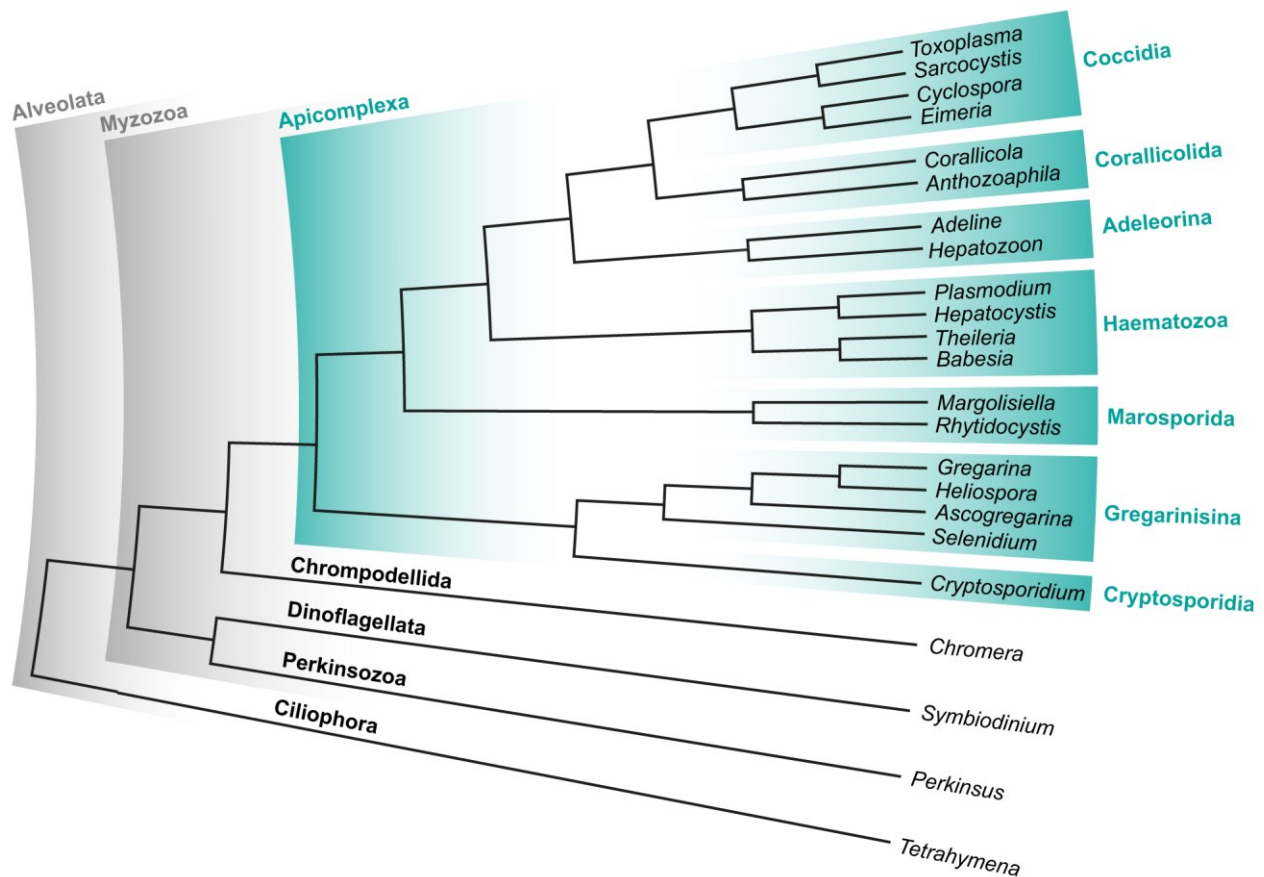


Figure 1. Phylogeny of Apicomplexa.

Cladogram of selected genera from superphylum Alveolata, with focus on phylum Apicomplexa. Branching order based on Kwong et al. 2021, noting that early branching within Apicomplexa is subject to frequent revision. Branch lengths are not to scale.

Cryptosporidians comprise a single extant genus, *Cryptosporidium*, that is sometimes grouped with gregarines to form single clade, cryptogregarines. In contrast to Haematozoa, *Cryptosporidium spp.* are monoxenous parasites that are transmitted between mammalian hosts by the faecal-oral route (Bouazid et al. 2013). *Cryptosporidium spp.* infect the small intestine epithelium and are the second leading cause of diarrhoeal disease and death in children (Kotloff et al. 2013). Gregarines, meanwhile, are parasites of invertebrates and are estimated to be the largest group of apicomplexans by number of species (Rueckert, Betts, and Tsaoasis 2019).

Marosporidans and corallicolids are recently recognised and poorly understood clades. Marosporidans infect marine invertebrates and very few species are known (Mathur et al. 2021). Corallicolids are highly unusual apicomplexans that infect corals and are abundant in coral-associated metagenomic data (Keeling, Mathur, and Kwong 2021).

Adeleorines are a diverse group containing monoxenous parasites of both invertebrates and vertebrates and dixenous parasites that alternate between vertebrate and invertebrate hosts (Votýpka et al. 2017). Different species of dixenous adeleorines may be transmitted from the definitive invertebrate host to the intermediate vertebrate host either by inoculation during blood feeding or by ingestion of an infected invertebrate by the appropriate vertebrate host. The latter mode of transmission may involve an additional paratenic host: in the case of *Hepatozoon sipedon*,

the parasite is transmitted from definitive *Culex* mosquito hosts to snakes via a tertiary frog host (Smith, Desser, and Martin 1994).

Coccidians are spore (oocyst)-forming parasites of vertebrates, primarily infecting the small intestine, although tissue cyst-forming lineages are capable of disseminating throughout the host body (Levine 2018). Coccidia can be mono- or dixenous; monoxenous species are transmitted by the faecal-oral route while dixenous species are transmitted by both the faecal-oral route and by predation. As for most apicomplexans, individual species of coccidians are typically highly restricted in host range. A remarkable exception is *Toxoplasma gondii*, which is capable of infecting any warm-blooded vertebrate as an intermediate host (Tenter, Heckeroth, and Weiss 2000). *T. gondii* is by far the most prevalent apicomplexan parasite in humans, and is the causative agent of the disease toxoplasmosis.

Discovery of *T. gondii*

T. gondii was identified independently in North Africa and in South America in 1908 and was initially thought to be related to parasitic *Leishmania* spp. (Nicolle and Manceau 1908; Splendore 1908). The name *Toxoplasma gondii* was proposed by Nicolle and Manceau based on the crescent shape of the tachyzoite life stage that they identified (“*toxon*”, arched; “*plasma*”, form) and the gundi host (*Ctenodactylus gundi*) from which it was isolated (Nicolle and Manceaux 1909). Recognition of *T. gondii* as a human pathogen did not occur for nearly another 30 years, until the identification of an infant with toxoplasmic encephalomyelitis (Wolf and Cowen 1937; Wolf, Cowen, and Paige 1939). Toxoplasmosis rose in prominence as a medical issue alongside the development of immunosuppressant drugs and the HIV pandemic, as immunocompromised individuals are much more susceptible to severe disease (Luft and Remington 1992; Nissapatorn 2017; Cohen 1970). *T. gondii* is now known to be prevalent in human populations across the world, mostly as asymptomatic dormant infections and rarely as a cause of severe disease (Furtado et al. 2011).

***T. gondii* lifecycle**

T. gondii has a dixenous lifecycle in which sexual reproduction occurs in felids, the definitive host, while asexual reproduction in intermediate hosts serves enables transmission to new definitive hosts through predation (Dubey 2014). *T. gondii* is unusual among apicomplexans in its ability to infect a diverse range of intermediate host species, including probably all mammals and birds, though not all possible intermediate hosts (for example, humans) will represent a significant vector for transmission back to the definitive host.

Felid hosts of *T. gondii* are infected by ingestion of tissue cysts during predation of an infected intermediate host. The cysts resist acid and enzymatic digestion in the stomach and release bradyzoites into the lumen of the small intestine, which invade the intestinal epithelium. Linoleic acid, which, uniquely among mammals, is present at high levels in the felid small intestine, stimulates sexual development of *T. gondii* (Martorelli Di Genova et al. 2019). Bradyzoites differentiate into pre-sexual merozoites, which can undergo further rounds of replication or differentiate into gametes. Fusion of a macrogamete and microgamete forms a diploid zygote, which develops a protective wall to become an oocyst. Oocysts are shed into the environment in faeces, following which they undergo meiosis (sporulation) to generate infective haploid sporozoites. The oocysts are highly environmentally resistant, and can remain infectious for at least a year (Hutchison 1965).

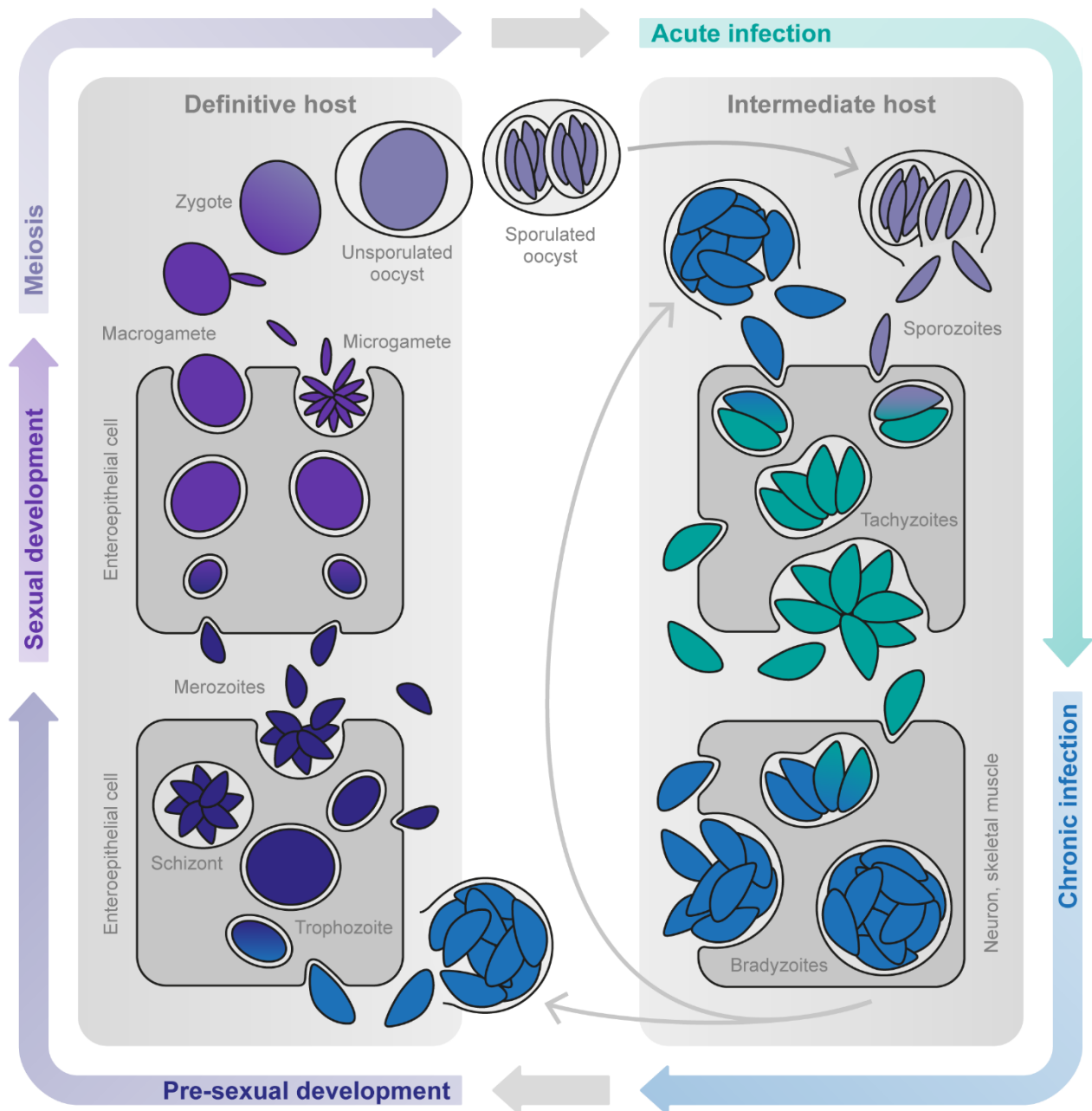


Figure 2. Lifecycle of *Toxoplasma gondii*.

Intermediate hosts are infected by ingestion of oocysts. Similarly to bradyzoite cysts, oocysts resist degradation in the stomach and release sporozoites into the small intestine to invade the epithelium. The sporozoites differentiate into fast-replicating tachyzoites that disseminate throughout the host body and are responsible for the symptoms of acute toxoplasmosis through destruction of host tissues. In neurons and skeletal muscle cells, tachyzoites differentiate into bradyzoites and develop a resistant cyst wall. Bradyzoite cysts persist following the clearance of the acute tachyzoite infection, and are thought to remain for the lifetime of the host undergoing periodic cycles of cyst rupture, bradyzoite invasion of nearby cells, and formation of new cysts. Ingestion of tissues of an intermediate host harbouring bradyzoite cysts by a definitive felid host completes the life cycle.

Human toxoplasmosis

Approximately one-third of the human population is estimated to be seropositive for antibodies indicating prior *T. gondii* infection, with prevalence varying substantially by country from 10-80% (Pappas, Roussos, and Falagas 2009). Humans can be infected by *T. gondii* through ingestion of oocysts present in contaminated water or food or through ingestion of bradyzoites in meat that is not sufficiently cooked to destroy the parasites (Montoya and Liesenfeld 2004). In both cases, the parasites invade the intestinal epithelium and differentiate into tachyzoites that disseminate throughout the body, as for any intermediate host (Matta et al. 2021). Rarely, *T. gondii* infection can be transmitted between humans by organ transplantation (Derouin, Pelloux, and ESCMID Study Group on Clinical Parasitology 2008). In naïve individuals, infection results in acute toxoplasmosis. In immunocompetent individuals infected with most strains of *T. gondii*, acute toxoplasmosis is either asymptomatic or produces mild and non-specific symptoms (fever, aches, swollen lymph nodes). Rarely, acute toxoplasmosis can result in severe retinochoroiditis. The acute infection is cleared by the immune system over the course of several weeks, following which immunocompetent individuals retain acquired immunity against future infection or recrudescence (de-differentiation of bradyzoites back to tachyzoites).

In contrast to immunocompetent individuals, toxoplasmosis can be life-threatening in immunocompromised individuals (Robert-Gangneux and Dardé 2012). In such patients, severe toxoplasmosis may result from a primary infection or recrudescence of dormant cysts. Uncontrolled replication of tachyzoites results in pathology affecting various organs including the brain, lungs, eyes, and heart. Toxoplasmic encephalomyelitis is the predominant manifestation of severe disease in immunocompromised individuals. Severe and acute toxoplasmosis can be treated with drug combinations such as pyrimethamine and sulphadiazine or trimethoprim and sulfamethoxazole, although no known drugs are able to clear bradyzoite cysts. Chronically immunocompromised individuals must therefore take continuous chemoprophylaxis to prevent recrudescence disease.

Toxoplasmosis may also be vertically transmitted between humans if an acute infection occurs during pregnancy (Robert-Gangneux and Dardé 2012). Congenital toxoplasmosis is one of the most severe forms of disease, and motivated early study of *T. gondii*. The consequences of congenital toxoplasmosis are more severe the earlier that infection occurs during gestation, although rates of transmission are correspondingly lower. Infection within the first trimester has severe consequences for foetal development, often leading to severe abnormalities or abortion; infection in the second trimester has variable pathology; infection in the third trimester is frequently asymptomatic.

***T. gondii* strains and population structure**

While most primary infections in immunocompetent individuals are mild, as described above, occasional reports have emerged of severe, even lethal, toxoplasmosis in immunocompetent individuals infected with divergent *T. gondii* strains (Robert-Gangneux and Dardé 2012).

T. gondii genetic diversity encompasses 16 haplogroups, with a notable geographic structure in which a handful of genotypes dominate in most regions whereas South America retains a highly diverse population (Su et al. 2012; Shwab et al. 2014). Expansion and dominance of certain genotypes outside South America appears to be a founder effect accompanying the domestication and spread of cats approximately 10,000 years ago (Galal et al. 2022).

Initial genotyping studies suggested a model of three clonal lineages, known as type I, II, and III (Howe and Sibley 1995). In Europe and North America, type II strains account for the majority of human infections with a lesser contribution from type III strains. Type I strains are rarely found in patients, but predominate in *T. gondii* research as the first strain isolated from a human was the type I RH strain (Sabin 1941). These clonal lineages were phenotypically characterised by virulence in mice, with type I strains being the most virulent and type III the least. Aside from type I, II and III, a handful of additional genotypes have emerged as substantial contributors to human infection in Africa and Asia (Shwab et al. 2014). Despite initial association of type I strains with ocular toxoplasmosis, it is unclear whether these clonal lineages vary substantially in severity of human toxoplasmosis.

Instead, most cases of severe toxoplasmosis in immunocompetent adults are associated with “atypical” strains (defined by exclusion from the type I/II/III model) that predominate in South America. These cases remain rare, and determinants of virulence in humans remain to be found. The population structure of *T. gondii* in South America contrasts with the rest of the world in the absence of dominant clonal lineages and the presence of a large number of diverse genotypes with frequent genetic recombination (Dardé et al. 2020). Similarly to type I strains, atypical strains tend to be highly virulent in mice.

***T. gondii* effector proteins**

The lytic cycle

The tachyzoite stage of *T. gondii* is present in during acute infection of intermediate hosts and is responsible for the symptoms of toxoplasmosis. As for all *T. gondii* life cycle stages (sporozoites arguably excepted), tachyzoites replicate within the cells of their host organism. Extracellular *T. gondii* tachyzoites exhibit an actomyosin-dependent gliding motility which enables them to traverse the extracellular matrix and attach to host cells (Meissner, Schlüter, and Soldati 2002). *T. gondii* is unusual among intracellular pathogens in that it can invade any nucleated host cell type. To invade a host cell, the tachyzoite secretes proteins from the apical complex that form a tight association between the parasite and host cell known as the moving junction (Shen and Sibley 2012). A parasite actomyosin motor draws the moving junction across the surface of the tachyzoite from the apex to the posterior, forcing invagination of the host plasma membrane and entry into the host cell. Scission of the host plasma membrane behind the parasite completes the formation of the parasitophorous vacuole, a novel compartment in which *T. gondii* resides. The parasites replicate within the vacuole until the host cell can no longer support them, following which they destructively egress from the vacuole and host cell (Black and Boothroyd 2000; Blader et al. 2015). This destruction of host cells, and accompanying inflammatory host response, are responsible for the pathology of toxoplasmosis.

Secretory organelles

T. gondii host cell invasion, survival within the host cell, and virulence depend on secretion of proteins from specialised organelles found only within Apicomplexa and closely related phyla: micronemes, elongated secretory vesicles concentrated at and secreted from the apical end of the parasite, rhoptries, long club-shaped organelles that extend to the apex of the parasite, and dense granules, relatively large electron-dense secretory vesicles distributed throughout the tachyzoite

cytoplasm. Micronemes and rhoptries are part of the specialised apical complex of Apicomplexa. Each organelle has specific protein contents and secretion dynamics during the parasite lytic cycle.

Micronemes are primarily involved in parasite egress, motility, attachment to host cells, and invasion (Dubois and Soldati-Favre 2019). Microneme contents comprise perforin-like proteins that facilitate egress from the host cell, proteases, and adhesins that enable parasite attachment and invasion of host cells in conjunction with rhoptry proteins. Microneme proteins are not extensively further considered in this thesis, which focuses on effector proteins that are secreted into the host cell from rhoptries and dense granules.

Rhoptries secrete their contents during parasite invasion of a host cell (Boothroyd and Dubremetz 2008). *T. gondii* tachyzoites have approximately 10-12 rhoptries, of which only one or two will be discharged during each invasion event. The large number of rhoptries, in comparison to *Plasmodium falciparum* merozoites and *Cryptosporidium* sporozoites, is speculated to be an adaptation that allows multiple abortive cycles of host cell invasion. The triggers and mechanism of rhoptry exocytosis are not well understood, but the process likely involves fusion of the apex of the rhoptry to an intermediating apical vesicle that in turn docks with a protein complex in the apical plasma membrane that facilitates fusion with the host cell (Sparvoli and Lebrun 2021). Each rhoptry is subdivided into two morphological zones with generally functionally distinct protein contents. The rhoptry “neck”, the most apical region, contains proteins which are mainly involved in host cell invasion, in conjunction with microneme proteins. The rhoptry “bulb”, in contrast, primarily contains effector proteins which are delivered to the host cell either in a free soluble form or in association with vesicular structures that fuse with the nascent parasitophorous vacuole. Characterised rhoptry bulb proteins encompass kinases, phosphatases, and proteases, with diverse functions in host cell remodelling and counteracting cell autonomous innate immune responses. Although dispensable in routine laboratory culture, several rhoptry proteins are major virulence factors of *T. gondii* in mice.

Dense granule proteins are secreted by intracellular tachyzoites into the parasitophorous vacuole, although there are many open questions regarding the regulation, location and mechanism of dense granule secretion (Griffith, Pearce, and Heaslip 2022). Dense granule proteins have diverse functions in biogenesis of the vacuole and membranous tubules within it, recruitment of host organelles to the vacuole, import of molecules from the host cell, and modulation of cell-autonomous host innate immunity. Others are involved in the formation of bradyzoite cysts. A subset of dense granule proteins are further exported across the parasitophorous vacuole membrane (PVM) into the host cell, where they reprogram host cell transcription to enhance nutrient acquisition and suppress host immune responses (Panas and Boothroyd 2021). This export requires four PVM-localised proteins, MYR1-4, which are presumed to form a translocon complex, the rhoptry kinase ROP17, plus at least three additional dense granule proteins that are secreted into the vacuole. At least seven dense granule effector proteins are known to be exported into the host cell in this manner.

Effector proteins that counteract host innate immune responses

A major role of rhoptry and dense granule effector proteins is to counter cell-autonomous host immune responses that target the intracellular parasites.

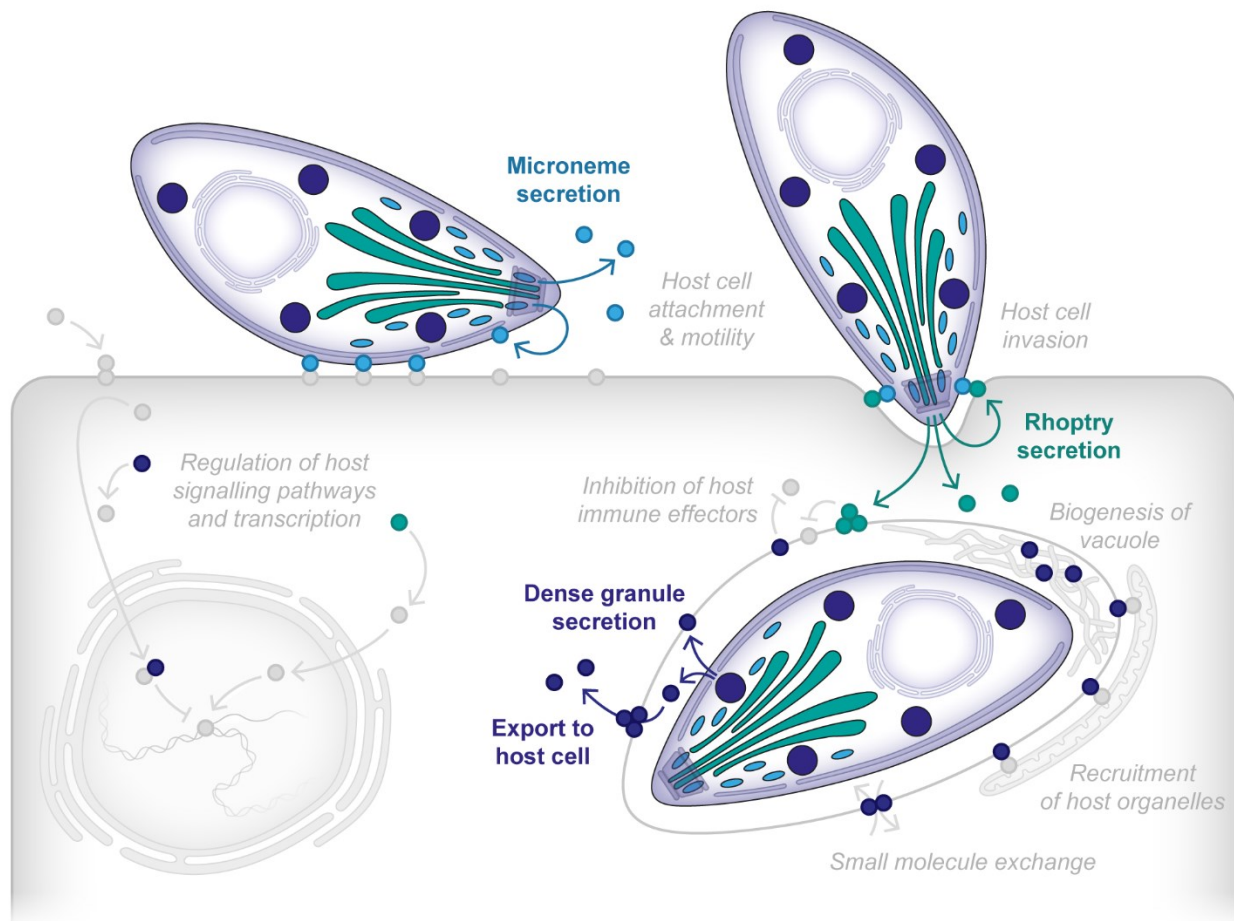


Figure 3. Secretion and function of *T. gondii* effector proteins.

The cell-autonomous immune response against *T. gondii* is best understood in mice, which are “natural” intermediate hosts and convenient for laboratory studies *in vivo* and in cultured cells. Control of acute *T. gondii* infection relies on the IL-12/interferon-gamma (IFN γ) axis (Frickel and Hunter 2021). IFN γ acts in an autocrine and paracrine manner to activate the STAT1 transcription factor via the ubiquitously expressed IFN γ receptor, inducing the expression of a large number of interferon-stimulated genes (ISGs) that encode immune effector proteins. In IFN γ -stimulated cells, the parasitophorous vacuole is recognised by an unknown mechanism and ubiquitinated, leading to the recruitment of autophagy adaptors and immune-related GTPases (IRGs). The IRGs disrupt the parasitophorous vacuole membrane, rendering the enclosed parasites non-viable (Howard, Hunn, and Steinfeldt 2011).

The ROP5 rhoptry pseudokinase family and active kinases ROP17 and ROP18 are secreted by *T. gondii* into the host cell cytoplasm and return to associate with the cytoplasmic face of the PVM. These rhoptry proteins cooperate to phosphorylate the IRGs, preventing IRG association with the PVM and inhibiting clearance of the parasite vacuole. The ROP5 family is highly polymorphic, and up to ten variants can be encoded in a single genome. Likewise, ROP18 activity and expression level varies between *T. gondii* strains. The allelic combination of ROP5 and ROP18 determines the ability to inactivate host IRGs that are also highly polymorphic, in a lock-and-key-type mechanism. ROP5

and ROP18 are thus the major determinants of virulence of different *T. gondii* strains in mice (Etheridge et al. 2014; Behnke et al. 2015). Further rhoptry and dense granule effectors have also been found which interfere with IRG loading, with the dense granule effector GRA12 in particular emerging as a critical strain-independent virulence factor (J.-L. Wang et al. 2020; Fox et al. 2019).

In human cells, mechanisms of host restriction and *T. gondii* immune evasion are comparatively less well understood and, unlike murine cells, vary substantially between different host cell types (Saeij and Frickel 2017). Humans lack the expanded repertoire of IRGs present in mice, and the remaining two members have no role in parasite restriction (Bekpen et al. 2005). The ROP5/17/18 complex therefore does not affect parasite restriction in human cell lines *in vitro*, and *T. gondii* strain-specific phenotypes in murine cells do not necessarily translate to human cells (Niedelman et al. 2012). In human macrophages, IFN γ -inducible guanylate-binding protein GTPases (GBPs) act analogously to IRGs to disrupt the parasitophorous vacuole and parasite plasma membrane; however, no parasite effectors have been identified that interfere with this restriction (Frickel and Hunter 2021). In certain other human cell lines, GBPs do not have a role in restriction of *T. gondii* and instead the vacuole is targeted by IFN γ -dependent autophagic pathways (Saeij and Frickel 2017). We recently identified a heterotrimeric complex of *T. gondii* effector proteins that inhibits parasite restriction in human fibroblasts, but not in murine fibroblasts, indicating that they specifically counteract an as-yet-unidentified mechanism of innate immune restriction present in these human cells but not in their murine equivalent (Lockyer et al. 2022).

Effector proteins that reprogram host transcription

In contrast to *T. gondii* effector proteins that directly bind and inactivate host innate immune effectors, *T. gondii* effectors that modulate host immune responses by targeting host cell transcription tend to have conserved functions in both murine and human cells.

The first *T. gondii* effector shown to modulate host transcription was the rhoptry kinase ROP16 (Saeij et al. 2007). ROP16 phosphorylates and activates the transcription factors STAT3 and STAT6, causing them to translocate to the nucleus and activate a gene expression program that is canonically induced by IL-4. Though many cell types express STAT3 and STAT6 and thus exhibit transcriptional reprogramming by ROP16, in macrophages this signalling results in M2 polarisation of the infected cell that is implicated in parasite immune evasion *in vivo* (Jensen et al. 2011; Chen et al. 2020). The expression programme induced by ROP16 is furthermore implicated in bradyzoite cyst formation and/or tolerance *in vitro* and *in vivo* (Tuladhar et al. 2019; Kochanowsky et al. 2022). Though active in type I, III and atypical strains, ROP16 has an amino acid substitution in type II strains that abolishes kinase activity. ROP16 is therefore one of the main drivers of strain-specific transcriptional responses and systemic immune responses to *T. gondii* infection.

All other effectors that have been convincingly shown to target host transcription originate from the dense granules (Panas and Boothroyd 2021). Some of these dense granule effectors (for example, GRA15) contain predicted transmembrane domains and localise to the PVM where cytosol-exposed domains can interact with host signalling pathways. However, the majority of transcription-modulating dense granule effectors are soluble and are exported into the host cell via the MYR-dependent pathway. At least seven such effectors are known: GRA16, GRA18, GRA24, GRA28, IST, NSM, and HCE1/TEEGR. In contrast to the ROP16 kinase, these exported effectors are intrinsically disordered, which may be necessary for export to the host cell, but can adopt stable

configurations upon binding to host protein targets (Pellegrini et al. 2017; Huang et al. 2022). All of these exported effectors, except for GRA18, localise to the host cell nucleus. As for ROP16, dense granule effectors that target host transcription primarily seem to modulate immune pathways and responses, although some also modify the host cell cycle and differentially regulate biosynthetic pathways to putatively enhance parasite nutrient acquisition.

IST (inhibitor of STAT transcription) is a critical virulence factor which suppresses the IFN γ response in infected cells by binding to STAT1 dimers, blocking the recruitment of transcriptional co-activators and instead recruiting repressive chromatin remodelling complexes to STAT1 target genes (Gay et al. 2016; Olias et al. 2016; Huang et al. 2022). NSM (NCoR/SMRT modulator) also inhibits IFN γ - and IFN β -driven gene expression, but has a relatively minor role compared to IST (Rosenberg and Sibley 2021).

HCE1/TEEGR (host cyclin E regulator 1/*Toxoplasma* E2F4-associated EZH2-inducing gene regulator) induces host cell cycle progression to S phase via E2F transcription factors, while activation of p53 by GRA16 causes cell cycle arrest in G2 phase (Panas et al. 2019; Bougdour et al. 2013). The benefit to *T. gondii* of this cell cycle alteration is unknown; both HCE1/TEEGR and GRA16 are dispensable *in vitro* yet are important for virulence in mice. GRA16 additionally seems to regulate host cell metabolism, in particular upregulating the expression of genes involved in cholesterol synthesis.

HCE1/TEEGR has a further role in suppressing NF- κ B signalling, in opposition to activation of NF- κ B by the PVM-resident GRA15 (Braun et al. 2019; Rosowski et al. 2011). HCE1/TEEGR selectively represses a subset of NF- κ B-regulated cytokines and chemokines, exemplifying a model in which effectors with apparently opposing phenotypes allow *T. gondii* to finely balance the host immune response. This balance varies between strains due to polymorphisms in the amino sequence and expression level of transcription-targeting effectors. For example, type I strains have a truncation in GRA15 that removes the ability to activate NF- κ B, which, together with ROP16-mediated activation of STAT3 and STAT6, produces a much less inflammatory host cell phenotype than type II strains.

Several other exported dense granule effectors influence systemic immune responses by modifying cytokine and chemokine production of infected cells. GRA24 activates p38 α MAP kinase, resulting in increased IL-12 production, which would induce an inflammatory Th1 response, but is opposed by GRA18 which enhances the production of anti-inflammatory Th2-associated cytokines (Braun et al. 2013; He et al. 2018). This regulation of immunomodulatory cytokines likely affects the recruitment of different immune cell types to the site of infection. Furthermore, differential regulation of cytokines and their receptors by GRA28 induces a dendritic cell-like migratory capacity in infected macrophages in an autocrine manner, which impacts parasite dissemination in the host (Ten Hove et al. 2022).

Overall, *T. gondii* reprogramming of the host cell is complex, involving many effectors with apparently opposing effects that combine to finely tune the host immune response. This balance may be a product of the requirement to modulate both cell-autonomous immune responses, to enable survival within the infected cell, and the systemic immune environment, to enable dissemination and persistence. In this context, it is interesting that type II strains are the most commonly isolated in North America and Europe and by this measure are more “successful” than type I or type III strains, despite inducing a strongly pro-inflammatory transcriptional response as a

result of the presence of NF- κ B-inducing GRA15 and lack of M2 polarisation-inducing ROP16. Among the canonical lineages, type II strains may represent the most “successful” balance between virulence and tolerance by the host; provided the parasites are able to disseminate and form sufficient numbers of bradyzoite cysts for transmission, it is not necessarily advantageous to suppress the host immune response to an extent that the infection overwhelms the host.

Tools to identify effector proteins

Identifying secreted proteins

For decades, a major goal of the *T. gondii* research community has been to identify secreted virulence factors and establish how they contribute to *T. gondii* infection. The first rhoptry and dense granule proteins were identified as major antigens of the immune response to *T. gondii* infection and were initially explored as vaccine candidates (Cesbron-Delauw and Capron 1993). These proteins, then termed as “excreted-secreted antigens”, are typically highly expressed and highly antigenic, which facilitated their identification as the targets of vaccine- or infection- derived monoclonal antibodies.

Genomic and transcriptomic data for *T. gondii* later allowed identification of putative secreted proteins through the presence of predicted signal peptides, by homology to known effectors, and through the tightly synchronised expression of rhoptry protein-encoding mRNAs (Talevich and Kannan 2013; Camejo et al. 2014).

Various mass spectrometry-based proteomics approaches have also been used to identify secreted proteins, including analysis of the contents of purified organelles (Bradley et al. 2005), identification of proteins which are differentially phosphorylated in intracellular versus extracellular parasites (Treeck et al. 2011), and the use of co-immunoprecipitation or proximity labelling to identify proteins in the vicinity of known secreted proteins (Nadipuram et al. 2020; Cygan et al. 2020; Cygan et al. 2021).

The most comprehensive survey of protein localisation in *T. gondii* comes from a spatial proteomics method, hyperLOPIT, that uses density-gradient fractionation of cell lysate to infer protein localisation by co-fractionation with known organelle markers (Barylyuk et al. 2020). This approach led to the assignment of nearly 2,000 tachyzoite proteins of previously unknown localisation to a subcellular niche and more than doubled the number of identified rhoptry- and dense granule-localised proteins. In total, more than 250 proteins are now localised to the rhoptries and dense granules, of which the vast majority remain uncharacterised.

Genetic crosses identify major polymorphic virulence factors

Identifying the secreted proteins that are important for infection and determining their functions has historically been challenging, and many secreted proteins have lacked functional annotation since their discovery. Most rhoptry and dense granule proteins are present only in *T. gondii* and the most closely related species comprising the sub-family Toxoplasmatinae (Barylyuk et al. 2020), and often lack conserved functional domains, meaning that it can be difficult to infer function from protein sequence alone.

Noting the dramatic variation in virulence in mice between *T. gondii* strains, initial studies sought to identify virulence factors by genetic crosses (Su et al. 2002; Saeij et al. 2006; Taylor et al. 2006; Reese et al. 2011; Behnke et al. 2011). This work led to the identification of the polymorphic (pseudo)kinases ROP5, ROP18 and ROP16. However, quantitative trait locus mapping is only able to identify virulence factors with differing phenotypes between the parental strains used, meaning they were unable to identify any of the now-known virulence factors that have conserved functions in all strains. For example, the rhoptry kinase ROP17 is active in all *T. gondii* strains, in contrast to its partners ROP5 and ROP18, and was only identified as a virulence factor following co-immunoprecipitation with these partners (Etheridge et al. 2014).

CRISPR-Cas9 knockout screening identifies conserved virulence factors

In contrast to genetic crosses, pooled CRISPR-Cas9-mediated knockout screening is able to identify conserved virulence factors by quantitatively measuring the relative fitness of *T. gondii* mutants in various conditions or selective bottlenecks. Initially, a genome-wide knockout screen identified genes that contribute to tachyzoite fitness during growth in HFFs (Sidik et al. 2016). Relatively few rhoptry and dense granule proteins were shown to be essential in this screen, with those identified likely having critical roles in the lytic cycle for host cell invasion or biogenesis of the parasitophorous vacuole.

CRISPR knockout screens were later adapted for selection of mutants *in vivo* in the mouse host, with putative virulence factors identified as those genes which contribute to parasite fitness *in vivo* but which are dispensable *in vitro* (culture in HFFs) (Young et al. 2019; Sangaré et al. 2019). The presumption is that the protein products of these genes somehow interface with the host immune system, which is absent in *in vitro* cell culture. *In vivo* CRISPR screens for *T. gondii* remain much more challenging than *in vitro* screens, as the lethal dose of parasite restricts the number target genes that can be tested in a given experiment. Furthermore, as both published *in vivo* screens were conducted before the release of the hyperLOPIT protein localisation data, many rhoptry and dense granule proteins remain to be characterised *in vivo*.

One further genome-wide screen has characterised the relative growth of CRISPR knockout mutants in unstimulated and IFN γ -stimulated murine macrophages in order to identify novel effectors that are important for parasite evasion of the IFN γ -mediated innate immune response (Y. Wang et al. 2020). However, while this screen will have covered all rhoptry and dense granule protein-encoding genes, it does not fully recapitulate the conditions *in vivo*, in which parasite dissemination, the systemic immune response, and infection of additional cell types all contribute to infection dynamics and parasite survival.

A further unresolved challenge is that pooled CRISPR knockout screens, *in vivo* and *in vitro*, have generally failed to identify effector proteins that target host cell transcription, despite the critical contributions of these effectors to parasite virulence (Young et al. 2019; Sangaré et al. 2019). Specifically, infections of mice with single knockouts have shown that the MYR-dependent effectors IST, GRA16, and GRA18, plus MYR1 itself, all contribute to parasite virulence, but are not shown to contribute to parasite fitness in the mouse peritoneum in *in vivo* screens. Although Δ MYR1 mutants were found to be defective in dissemination throughout the host (Sangaré et al. 2019), it has been shown that the MYR1-dependent effector IST is required for resistance to IFN γ -mediated clearance, therefore MYR1 would be expected to contribute to parasite fitness at the site of local

infection that is controlled by the IFN γ response. MYR1, IST, and other exported dense granule effectors also did not contribute to parasite fitness in IFN γ -stimulated BMDMs (Yifan Wang et al. 2020), although for IST this may be explained by macrophage stimulation prior to infection, in which case IST does not affect parasite susceptibility to restriction (Gay et al. 2016).

A purported explanation for the apparent lack of fitness phenotypes for these transcription-targeting effectors is that, in pooled *in vivo* screens, loss of function of a gene in a minority of the parasite population may be complemented *in trans* by the majority of the population which expresses the functional allele (Young et al. 2019). Such *trans* complementation would particularly be expected to impact effectors for which the major function is in modifying the systemic immune response, such as GRA18 and GRA24, though is less able to explain the apparent lack of phenotype for IST, which is considered to promote parasite survival primarily in a cell-autonomous manner.

Other transcription-targeting effectors contribute to parasite immune evasion in more subtle ways that are not captured by virulence assays or competitive growth in the mouse peritoneum. For example, although ectopic expression of the active ROP16 kinase from a type I or III strain renders type II strains (in which the endogenous ROP16 is inactive) less virulent following parasite injection into the peritoneum (Saeij et al. 2006), loss of ROP16 in type III strains causes stronger parasite-specific T cell responses, reduced parasite burden in the central nervous system, and reduced cyst formation (Chen et al. 2020; Tuladhar et al. 2019).

Perturb-seq

A promising new approach that may allow direct identification of *T. gondii* effectors that alter host cell transcription is single-cell CRISPR screening, or perturb-seq. Conceptually, this approach combines the throughput of pooled CRISPR screening with the data-rich output of single cell RNA-sequencing (scRNA-seq), enabling extensive characterisation of genetically modified cells far beyond the typical measures of pooled CRISPR screens (competitive growth or differential enrichment by fluorescence activated cell sorting).

Single-cell RNA-sequencing technologies are nowadays relatively mature and several platforms are commercially available. Single cells can be isolated by fluorescence activated cell sorting and deposited into well plates, or can be isolated using microwells or microfluidic devices that encapsulate the cells in water-in-oil emulsion droplets (Jovic et al. 2022). Primers for reverse transcription containing unique barcodes to identify each cell are added to or simultaneously isolated with the single cells. Reverse transcription incorporates these barcodes into every cDNA such that each sequencing read in the final data can be assigned to the cell from which the template originated. Many technologies additionally include a second random barcode in the reverse transcription primer, termed a unique molecular identifier (UMI), that allows removal of PCR duplicates and direct quantification of the number of RNA molecules obtained from the cell.

Perturb-seq refers to a collection of a relatively recent modifications of standard scRNA-seq methods to enable single-cell CRISPR screening (Przybyla and Gilbert 2022). The methodological crux of perturb-seq is the capture, reverse transcription, and sequencing of a barcode within each cell that identifies that genetic modification, alongside the mRNA transcriptome (Bock et al. 2022). Most scRNA-seq methods make use of poly(dT) primers for reverse transcription to capture polyadenylated mRNAs and limit contamination from genomic DNA and ribosomal RNAs (Haque et al. 2017). As sgRNAs are transcribed by RNA polymerase III, they are not polyadenylated and an

alternative method must be found to facilitate their capture and reverse transcription during scRNA-seq. Solutions have included linking each sgRNA to a unique polyadenylated barcode transcript (Dixit et al. 2016; Jaitin et al. 2016) or nesting the entire sgRNA expression cassette with a secondary RNA polymerase II expression cassette (Datlinger et al. 2017); however, both methods have drawbacks in terms of restricted vector design and delivery. A more flexible method termed direct-capture perturb-seq instead uses an additional primer for reverse transcription (sharing the same cell barcode as the poly(dT) primers) to capture sgRNAs that are engineered to contain a complementary sequence (a “capture sequence”) within the RNA polymerase III transcript (Replogle et al. 2020).

The few published applications of perturb-seq and related methods have been conducted exclusively in mammalian cell lines modified by lentiviral transduction. However, the relatively minimal modifications to the CRISPR screening vector required for direct-capture perturb-seq mean that this method could be straightforwardly implemented in *T. gondii*, in which knockouts are generated by electroporation with a plasmid vector. Furthermore, although genome-scale perturb-seq has been demonstrated (Replogle et al. 2022), the very high costs associated with commercial scRNA-seq reagents and sequencing of the resulting libraries to a sufficient depth per cell mean that targeted screens, as we have developed for *T. gondii* (Young et al. 2019), are a more feasible endeavour.

As for related apicomplexan *Plasmodium* spp. (Howick et al. 2019), scRNA-seq has been used to map the *T. gondii* cell cycle and differentiation (Xue et al. 2020; Waldman et al. 2020). Sequencing of intracellular parasites along with the infected cell has also been used to more finely resolve host responses to *T. gondii* infection (Rastogi et al. 2020; Sugi et al. 2022). However, newer modalities of single cell ‘omics have yet to be explored in the context of host-pathogen interaction. Adaptation of pooled CRISPR screening methods already available for *T. gondii* to enable perturb-seq would create a powerful functional genomics tool to investigate this important pathogen, and potentially allow direct identification of effector proteins that modify host cell transcription.

Objectives

The objective of this project is to identify *T. gondii* effector proteins that are involved in parasite evasion of host immune responses, particularly among newly-identified and as-yet-uncharacterised secreted proteins. Effector proteins will be identified by two complementary pooled CRISPR knockout screening methods, targeting all rhoptry- and dense granule-localised genes of *T. gondii*. Firstly, putative virulence factors will be identified through a competitive fitness screen as genes with a greater fitness contribution *in vivo* than *in vitro*, based on the assumption that these enable parasite survival of host immune responses that are absent *in vitro*. Secondly, I will develop a dual host-*T. gondii* perturb-seq method based on our existing CRISPR knockout screening platform to identify effectors that counteract host immune responses at the transcriptional level. The phenotypes and putative functions of effectors identified in these screens will then be validated by further functional assays using knockout, complemented, and tagged *T. gondii* cell lines, together aiming to reveal new mechanisms of *T. gondii* immune evasion and pathogenesis.

Chapter 1: *Toxoplasma gondii* virulence factor ROP1 inhibits murine and human innate immune restriction

Results

In vivo CRISPR-Cas9 knockout screening of the *T. gondii* secretome

To screen for *T. gondii* genes that contribute to parasite growth *in vivo*, 906 protospacer sequences targeting 235 rhoptry and dense granule protein-encoding genes were selected from an arrayed library of ssDNA oligonucleotides (Young et al. 2019). These genes were selected based on known rhoptry/dense granule localisation as determined from community annotation on ToxoDB (Harb and Roos 2020), predicted rhoptry/dense granule localisation based on spatial proteomics data (Barylyuk et al. 2020), or as positive and negative fitness-conferring controls *in vitro* and *in vivo*. The protospacer sequences were cloned by pooled Gibson assembly into a Cas9-sgRNA plasmid vector optimised for knockout screening in *T. gondii* (Young et al. 2019). The plasmid pool was linearised and transfected into the type II *T. gondii* PRUΔHXGPRT strain by electroporation. The transfected parasites were selected for plasmid integration into the genome using the HXGPRT drug-resistance marker encoded on the plasmid (**Figure 4A**). The resulting knockout parasite pool was used to infect five C57BL/6J mice with 200,000 parasites each by intraperitoneal injection. After five days, surviving parasites were recovered by peritoneal lavage and expanded for one lytic cycle *in vitro*. The sgRNA protospacer sequences were amplified from the plasmid pool and from genomic DNA extracted from the parasites following drug selection, from the leftover parasite inoculum, and from the five *ex vivo* populations, and incorporated into Illumina sequencing libraries. The libraries were sequenced on an Illumina HiSeq 4000 platform and the reads aligned to a reference of protospacer sequences.

To quantify the contribution of each target gene to parasite fitness, I calculated log₂ fold-changes (L2FCs) for each protospacer *in vitro*, using the normalised read counts in the post-drug selection population compared to the plasmid pool, and *in vivo*, using the geometric mean of the normalised read counts in the *ex vivo* populations compared to the parasite inoculum. The median L2FCs for each target gene were taken as the overall gene *in vitro* and *in vivo* phenotype scores. I obtained phenotype scores for 164 target genes after filtering out genes with fewer than three well-represented protospacers and removing the genes with the most variable L2FCs between protospacers (5th percentile of median absolute deviation *in vitro* or *in vivo*).

Both *in vitro* and *in vivo* phenotype scores correlated well with our previous study using the same system (Pearson's $r = 0.7$ and 0.88 respectively), although the dynamic range *in vitro* was notably lower (**Figure 4B and C**). *In vitro* phenotype scores also correlated moderately well with *in vitro* CRISPR phenotype scores from two genome-wide knockout screens carried out in the type I RH strain of *T. gondii* (Sidik et al. 2016; Y. Wang et al. 2020) (Pearson's $r = 0.55$ and 0.54), indicating that genes which confer fitness *in vitro* in HFFs are broadly similar in both strains of *T. gondii* (**Figure 4D and E**). Seven genes which have previously been shown to contribute to virulence of *T. gondii* PRU *in vivo* had *in vivo* phenotype scores in this screen of ≤ -1 , while nine genes which have been shown not to affect virulence had phenotype scores of > -1 , demonstrating that the *in vivo* phenotypes of this screen can accurately separate these controls (**Figure 4F**). However, the low dynamic range of the *in vitro* phenotype scores meant that we could not accurately separate essential genes (mostly identified in the RH strain) from the *in vivo* control genes (which by definition are not essential *in vitro*) with a single cut-off. Therefore, I defined a list of putative virulence factors as genes with an *in vivo* L2FC in this screen ≤ -1 , then removed genes with a Z-transformed *in vitro* phenotype in either

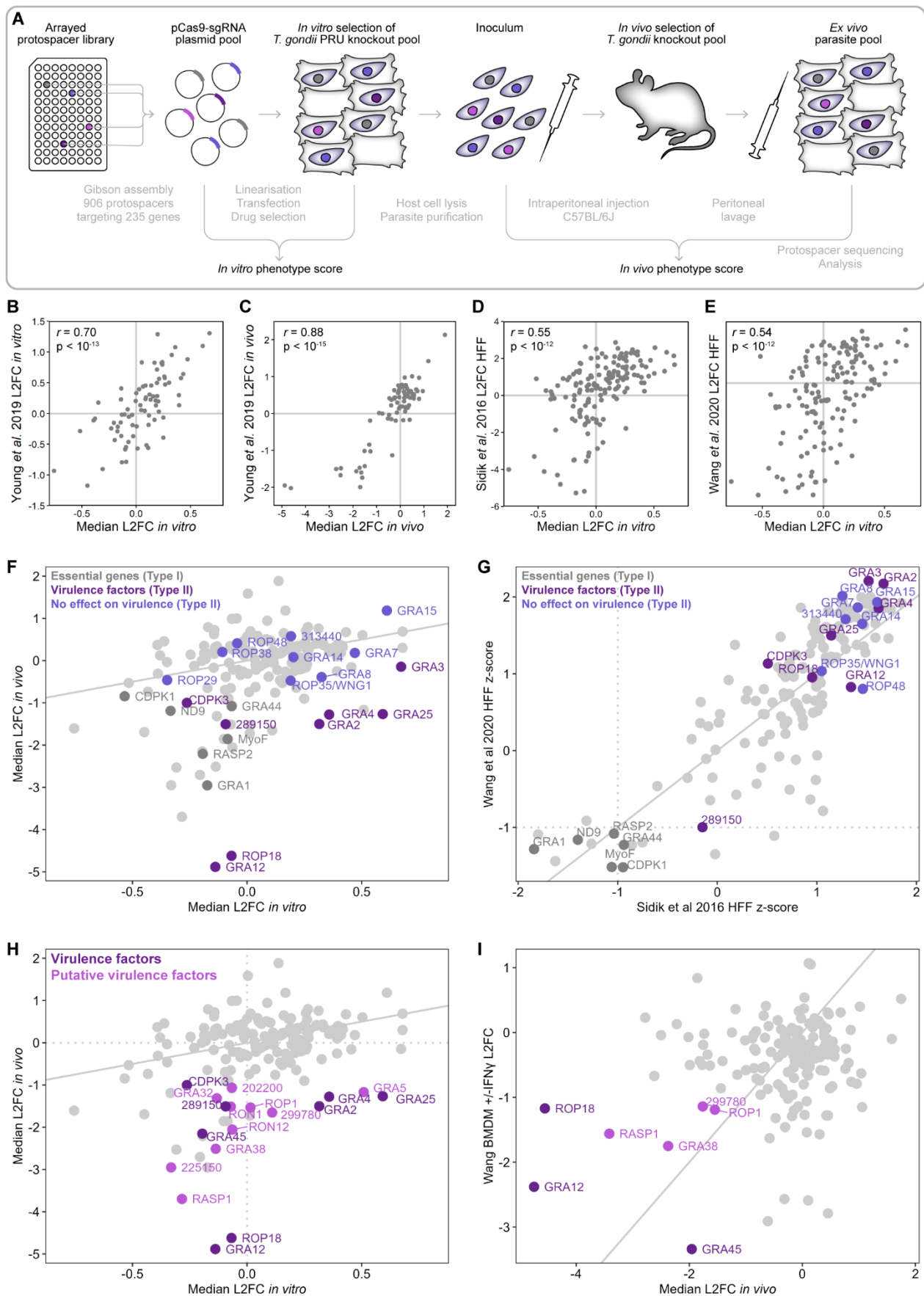


Figure 4. Pooled CRISPR-Cas9 knockout screening of *T. gondii* secreted proteins *in vivo* identifies eighteen putative virulence factors.

Continued overleaf

Figure 4 continued

Note: experimental work depicted in this figure was carried out by Jeanette Wagener and Joanna Young. Data analysis was carried out by Simon Butterworth.

A. Schematic of screening workflow.

B. Comparison of *in vitro* L2FCs to Young et al. 2019. r = Pearson's product moment coefficient.

C. Comparison of *in vivo* L2FCs to Young et al. 2019.

D. Comparison of *in vitro* L2FCs to Sidik et al. 2016.

E. Comparison of *in vitro* L2FCs to Y. Wang et al. 2020.

F. Scatter plot of median L2FCs for each gene *in vitro* and *in vivo*. Control genes for which knockouts have previously been tested for an effect on virulence in type II strains of *T. gondii* are labelled. Genes which have been found to be essential in type I strains of *T. gondii* are also labelled. The grey line indicates equal *in vitro* and *in vivo* L2FCs.

G. Z-score-transformed HFF L2FCs from Sidik et al. 2016 and Y. Wang et al. 2020 for genes screened in this study. The grey line indicates equal Z-scores in both studies and dotted lines indicate Z-scores of -1 in each study.

H. Scatter plot of median L2FCs for each gene *in vitro* and *in vivo*. Putative virulence factors are identified as genes with an *in vivo* L2FC ≤ -1 and HFF Z-score in Sidik et al. 2016 and Wang et al. 2020 ≥ -1 . The grey line indicates equal L2FCs.

I. Scatter plot of median L2FCs for each gene *in vivo* from this study and L2FCs between IFN γ -stimulated versus unstimulated BMDMs from Y. Wang et al. 2020. Genes with a L2FC ≤ -1 in both screens are labelled. The grey line indicates equal L2FCs.

of two genome-wide screens (Sidik et al. 2016; Y. Wang et al. 2020) of ≤ -1 (**Figure 4G**). All nine control genes which have been shown to be dispensable for virulence in type II strains, plus six genes which have been shown to be essential for growth *in vitro*, were excluded by these criteria. The remaining set of 18 hits contained all virulence factors that have been validated by knockouts in type II strains, except for GRA3 (Craver and Knoll 2007): GRA12 (Young et al. 2019), ROP18 (Fox et al. 2016), GRA25 (Shastri et al. 2014), GRA4 (Fox et al. 2019), 289150 (Young et al. 2019), GRA2 (Fox et al. 2019), and CDPK3 (Wu et al. 2022). Additionally, these criteria contained GRA45, which has been shown to affect virulence in the RH strain (Y. Wang et al. 2020) (**Figure 4H**).

A surprising candidate in this list of putative virulence factors was the rhoptry bulb protein ROP1, as knockout of this gene has previously been shown to have no effect on virulence of the RH strain (Soldati et al. 1995). ROP1 was not included in a recent *in vivo* knockout screen in the RH strain (Sangaré et al. 2019), but was found to have a moderate fitness phenotype in a genome-wide screen of the RH strain in IFN γ -activated versus naïve murine BMDMs (Y. Wang et al. 2020). ROP1 was one of only seven genes with a fitness score ≤ -1 in both this screen and the screen in IFN γ -activated macrophages, suggesting that it may have a function in immune evasion by both the PRU and RH strains (**Figure 4I**). As the function of ROP1 has been unknown to date, aside from an interesting rhoptry morphology phenotype (Soldati et al. 1995), I decided to focus the remaining work in this chapter on ROP1.

Knockout and complementation of ROP1

To investigate the function of ROP1, I generated knockout cell lines in both the *T. gondii* RH Δ KU80 and PRU Δ KU80 backgrounds by replacing the coding sequence of ROP1 with an mCherry-HXGPRT construct. I then complemented these lines by integrating a strain-matched ROP1-HA construct at the UPRT locus. I also generated UPRT knockout cell lines in both backgrounds to use as “wild-type-like” controls, as some downstream assays required an mCherry-expressing reference strain. Integration of these constructs into the genome was verified by diagnostic PCR (**Figure 5A**) and the expected presence/absence of ROP1/ROP1-HA confirmed by Western blot (**Figure 5B**).

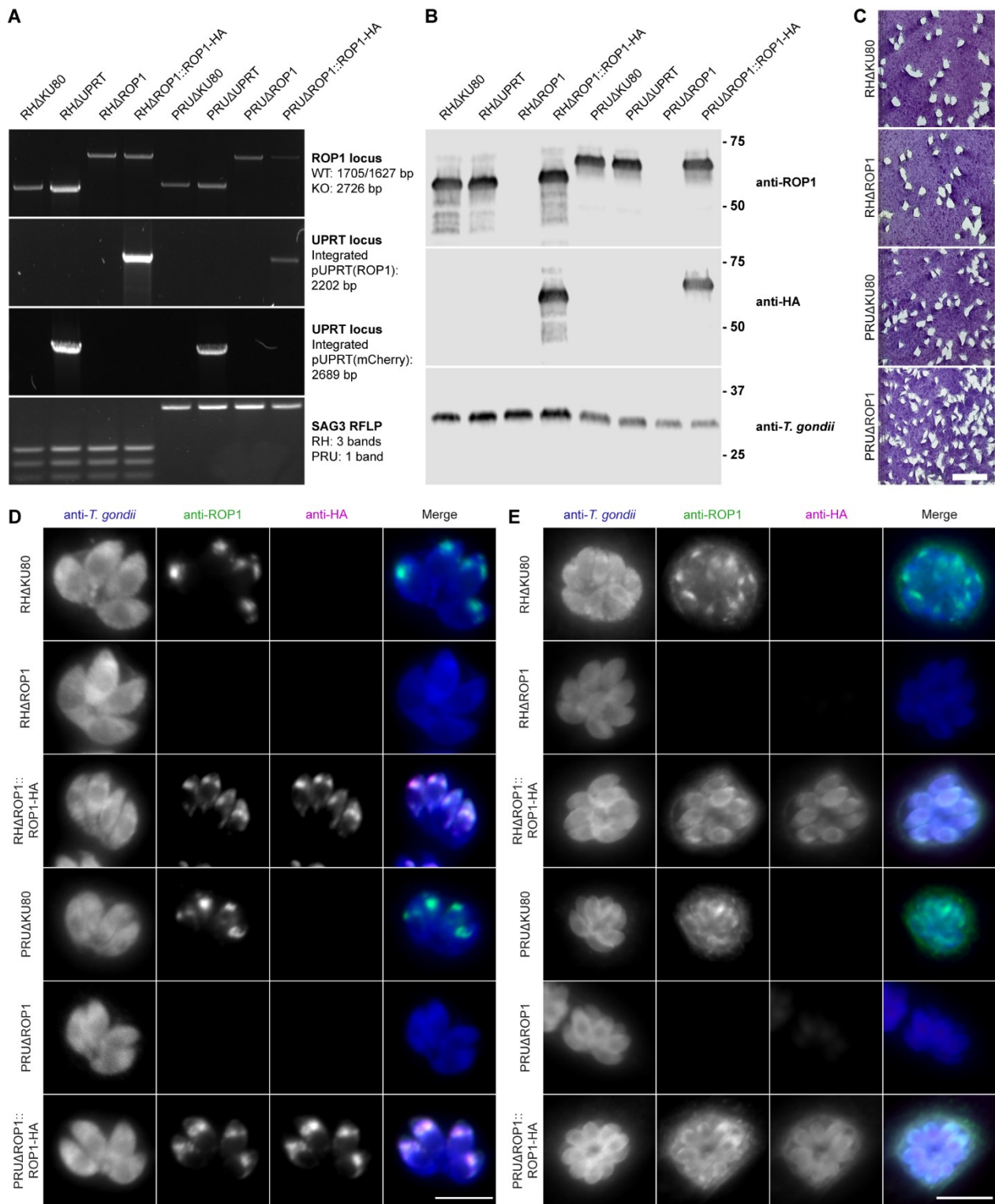


Figure 5. ROP1 is detectable at the parasitophorous vacuole until at least 24 hours post-infection.

A. Verification of ROP1 and UPRT knockout and ROP1 complementation by diagnostic PCR and verification of strain genotype by restriction fragment length polymorphism of the SAG3 gene (Su et al. 2006).

B. Verification of ROP1 and ROP1-HA expression by Western blot.

C. Plaques formed by RHΔKU80, RHΔROP1, PRUΔKU80 and PRUΔROP1 parasites after seven days' growth in a monolayer of HFFs. Scale bar = 1 cm.

D. Immunofluorescence localisation of ROP1 in *T. gondii* cell lines using 15 min permeabilisation. Scale bar = 10 μm.

E. Immunofluorescence localisation of ROP1 in *T. gondii* cell lines using 1 min permeabilisation. Scale bar = 10 μm.

Consistent with the *in vitro* screen phenotype and a previous study (Soldati et al. 1995), I did not observe a major growth defect for the RH Δ ROP1 and PRU Δ ROP1 strains by plaque assay (Figure 5C).

Interestingly, I found that ROP1 is detectable at the parasitophorous vacuole membrane (PVM) up to at least 24 hpi when the cells were gently permeabilised (1 min versus 15 min treatment with 2% Triton X-100) (Figure 5D and E). This contrasts with an early report based on immuno-electron microscopy that ROP1 was present on the PVM immediately after invasion but not at 6 h post-invasion (Saffer et al. 1992), but is supported by a recent proximity biotinylation study that demonstrated that ROP1 is accessible to host cytosolic proteins at 24 h post-invasion (Cygan et al. 2020). This localisation suggested that the function of ROP1 may require, or be facilitated by, prolonged exposure to the host cell cytosol.

PRU Δ ROP1 parasites are avirulent *in vivo*

To determine whether ROP1 is a virulence factor in the PRU strain, as suggested by the *in vivo* knockout screening results, we infected five C56BL/6J mice per strain with 20,000 PRU Δ KU80, PRU Δ ROP1 and PRU Δ ROP1::ROP1-HA parasites by intraperitoneal injection and monitored survival daily. While all PRU Δ KU80-infected mice and all but one of the PRU Δ ROP1::ROP1-HA-infected mice died within ten days, all the PRU Δ ROP1-infected mice survived until for the full duration of the experiment (Figure 6A). Furthermore, despite seroconverting against *T. gondii* tachyzoite lysate antigen, the PRU Δ ROP1-infected mice did not experience weight loss, one of the major clinical manifestations of toxoplasmosis in mice (Figure 6B and C). These results demonstrate that ROP1 is a critical virulence factor in the PRU strain of *T. gondii*. Knockout of ROP1 in the *T. gondii* RH strain was previously shown not to affect parasite virulence in Swiss Webster mice, raising the possibility that ROP1 is a strain-specific virulence factor (Soldati et al. 1995). However, the extremely high virulence of the RH strain in most laboratory mouse strains can mask virulence phenotypes, as for example in the case of cyclase-associated protein (Hunt et al. 2019).

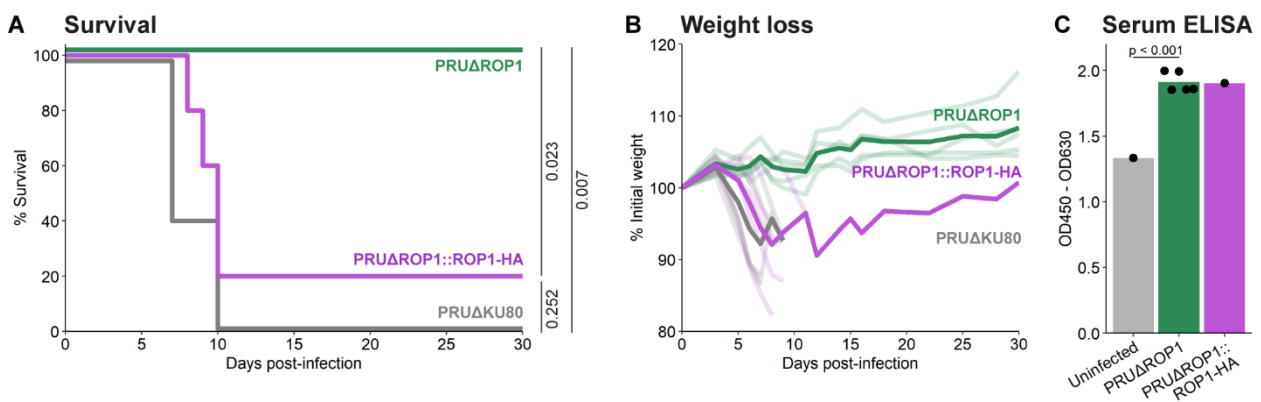


Figure 6. PRU Δ ROP1 parasites are avirulent *in vivo*.

Note: experimental work in this figure was carried out by Francesca Torelli and Aline Cristina Moreira-Souza.

A. Survival of mice infected with 20,000 parasites of each strain by intraperitoneal injection. *p*-values were calculated by two-sided log-rank test with Benjamini-Hochberg adjustment. *n* = 5 mice per group.

B. Weight change of infected mice as a percentage of initial weight. Pale lines represent individual mice while dark lines represent the mean of surviving mice.

C. Serum ELISA of mice surviving at 30 days post-infection using *T. gondii* tachyzoite lysate antigen. *p*-value calculated by one-sample two-sided *t*-test with *mu* equal to the value for the uninfected mouse.

ROP1 inhibits IFN γ -mediated parasite restriction in murine and human macrophages

I hypothesised that ROP1 might contribute to virulence of the PRU strain by preventing IFN γ -mediated restriction in macrophages. IFN γ is the principal cytokine required for control of *T. gondii* infection *in vivo* (Suzuki et al. 1988), while macrophages are the most commonly infected cell type during acute infection in the peritoneum (Jensen et al. 2011). The moderate fitness phenotype observed for ROP1 in a genome-wide knockout screen in IFN γ -stimulated BMDMs suggested that ROP1 might also have this function in the RH strain (Yifan Wang et al. 2020), even though knockout of ROP1 may not be sufficient to overcome the hypervirulence of this strain (Soldati et al. 1995).

To determine whether knockout of ROP1 makes *T. gondii* more susceptible to IFN γ -mediated restriction, I quantified parasite growth in IFN γ -stimulated murine BMDMs by high-content fluorescence imaging and automated image analysis. The number of *T. gondii* parasites, number of vacuoles, mean number of parasites per vacuole, and number of host cells were calculated in IFN γ -stimulated BMDMs as a percentage relative to unstimulated BMDMs following infection for 24 h at an MOI of 0.3.

Using total parasite number as an indicator of overall IFN γ -mediated restriction, I found that the percentage survival of both RH Δ ROP1 and PRU Δ ROP1 parasites upon IFN γ stimulation was significantly lower than Δ UPRT control cell lines, and that this phenotype was rescued by complementation (**Figure 7A**). In accordance with the *in vivo* phenotype scores, PRU Δ ROP1 had an intermediate phenotype compared to PRU Δ GRA12, a virulence factor which has been shown to be highly susceptible to IFN γ -mediated restriction (J.-L. Wang et al. 2020). The number of vacuoles was significantly reduced for both RH Δ ROP1 and PRU Δ ROP1 compared to control cell lines (**Figure 7B**), while the relative size of the vacuoles was unchanged (**Figure 7C**), indicating that the parasites are cleared through increased rates of vacuole rupture, host cell death, or IFN γ -induced early parasite egress. We did not find any significant differences in the number of remaining host cells infected with different parasite strains; however, host cell loss was highly variable between replicates with different batches of BMDMs and IFN γ , so at a relatively low MOI of 0.3 such a difference may not be detectable (**Figure 7D**).

I was curious whether ROP1 knockout parasites would also be more strongly restricted in human macrophages, which lack the IRG system primarily responsible for parasite clearance in mice (Frickel and Hunter 2021). Therefore, I repeated this IFN γ restriction assay using human THP-1-derived macrophages. Surprisingly, I found that RH Δ ROP1 and PRU Δ ROP1 were also more strongly restricted by IFN γ in THP-1-derived macrophages compared to Δ UPRT or complemented lines (**Figure 7E**). For PRU Δ ROP1 there was a similar reduction in vacuole number but not in vacuole size as in BMDMs, although for RH Δ ROP1 there was a significant decrease in the number of vacuoles compared to the complemented line but not compared to RH Δ UPRT, and instead I observed a modest but significant decrease in vacuole size compared to RH Δ UPRT, which was not rescued by complementation (**Figure 7F and G**). There were no significant differences in host cell loss (**Figure 7H**).

These results confirm that ROP1 reduces parasite clearance by IFN γ , which likely explains the reduced parasite growth *in vivo* in the knockout screen and the loss of virulence of PRU Δ ROP1 *in vivo*. Interestingly, ROP1 appears to be protective in both murine and human macrophages, which is unusual as only one other secreted protein of *T. gondii* has been shown to inhibit parasite restriction

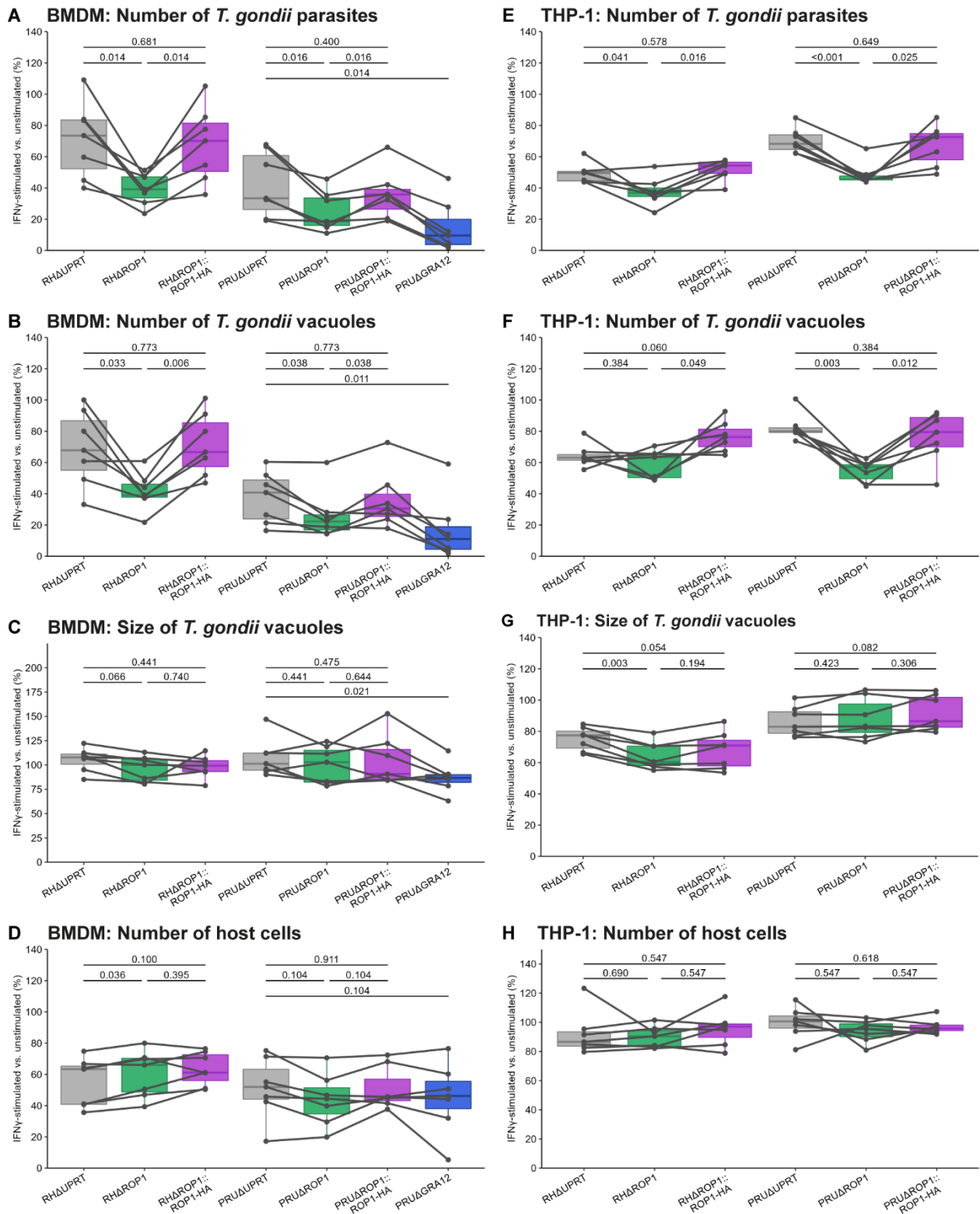


Figure 7. ROP1 contributes to *T. gondii* resistance to IFN γ in murine and human macrophages.

IFN γ -dependent growth restriction of *T. gondii* parasites in BMDMs and THP-1-derived macrophages. IFN γ -stimulated or unstimulated BMDMs and THP-1-derived macrophages were infected with indicated *T. gondii* strains at an MOI of 0.3 for 24 h. Parasite growth was quantified by high-content fluorescence imaging and automated image analysis. Data points indicate each measured parameter in IFN γ -stimulated BMDMs as a percentage of that in unstimulated BMDMs. Connecting lines indicate data points derived from the same biological replicate. *p*-values were calculated by paired two-sided *t*-test with Benjamini-Hochberg adjustment.

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Figure 7 continued

- A. *T. gondii* parasite number in IFN γ -stimulated versus unstimulated BMDMs.
- B. *T. gondii* vacuole number in IFN γ -stimulated versus unstimulated BMDMs.
- C. Size of *T. gondii* vacuoles in IFN γ -stimulated versus unstimulated BMDMs.
- D. Number of IFN γ -stimulated versus unstimulated BMDMs.
- E. *T. gondii* parasite number in IFN γ -stimulated versus unstimulated THP-1-derived macrophages.
- F. *T. gondii* vacuole number in IFN γ -stimulated versus unstimulated THP-1-derived macrophages.
- G. Size of *T. gondii* vacuoles in IFN γ -stimulated versus unstimulated THP-1-derived macrophages.
- H. Number of IFN γ -stimulated versus unstimulated THP-1-derived macrophages.

in both human and murine macrophages activated with IFN γ prior to infection, the dense granule chaperone protein GRA45 (Y. Wang et al. 2020). The parameters measured in the high-content imaging assay do not clearly indicate the mechanism of parasite clearance, but show that restriction manifests primarily through reduced vacuole number. Possible mechanisms behind this include targeted destruction of parasite vacuoles by host restriction factors, IFN γ -induced early parasite egress, or activation of host programmed cell death pathways.

Differential effects of ROP1 knockout on host cell death at low and high MOI

Host cell death has been shown to play an important role in IFN γ -mediated parasite restriction in both murine and human macrophages, although there is typically little detectable cell death following infection at MOI < 1 (Zhao et al. 2009; Gorfu et al. 2014; Fisch et al. 2019). To determine whether knockout of ROP1 affects host cell death, I carried out real-time propidium iodide uptake assays in IFN γ -stimulated BMDMs and THP-1-derived macrophages to measure cell death-associated permeabilisation of the plasma membrane.

In BMDMs infected at an MOI of 0.3, as used in the above IFN γ restriction assays, there were very low levels of parasite-induced host cell death and no differences between parasite strains (**Figure 8A and B**). However, at an MOI of 3, cumulative propidium iodide uptake at 12 hpi was significantly higher for PRU Δ ROP1 compared to PRU Δ UPRT and the complemented line (**Figure 8C and D**). Smaller increases in propidium iodide uptake were also apparent for RH Δ ROP1 in BMDMs and both RH Δ ROP1 and PRU Δ ROP1 in THP-1-derived macrophages, but were not consistently significant compared to control strains (**Figure 8C-F**).

These data indicate that increased host cell death is not the major mechanism of parasite clearance at low MOI, and plays at most a minor role at high MOI. The moderate increase in cell death at high MOI may be a secondary effect of vacuole disruption that exposes parasite molecules to the host cytosol to trigger cell death pathways, as has been documented in both BMDMs and THP-1-derived macrophages (Zhao et al. 2009; Fisch et al. 2019).

ROP1 affects rhoptry morphology but not rhoptry secretion

Knockout of ROP1 in *T. gondii* RH was previously shown to affect the ultrastructure of the rhoptries, resulting in a homogenous electron-dense texture of the rhoptry bulb by transmission electron microscopy instead of the normally observed heterogeneous texture (Soldati et al. 1995). We were able to reproduce this phenotype show that it is also apparent in the PRU strain (**Figure 9A**). This morphological change indicated that ROP1 might promote parasite survival by facilitating the secretion of other rhoptry proteins with host- or parasite strain-specific roles in counteracting IFN γ -

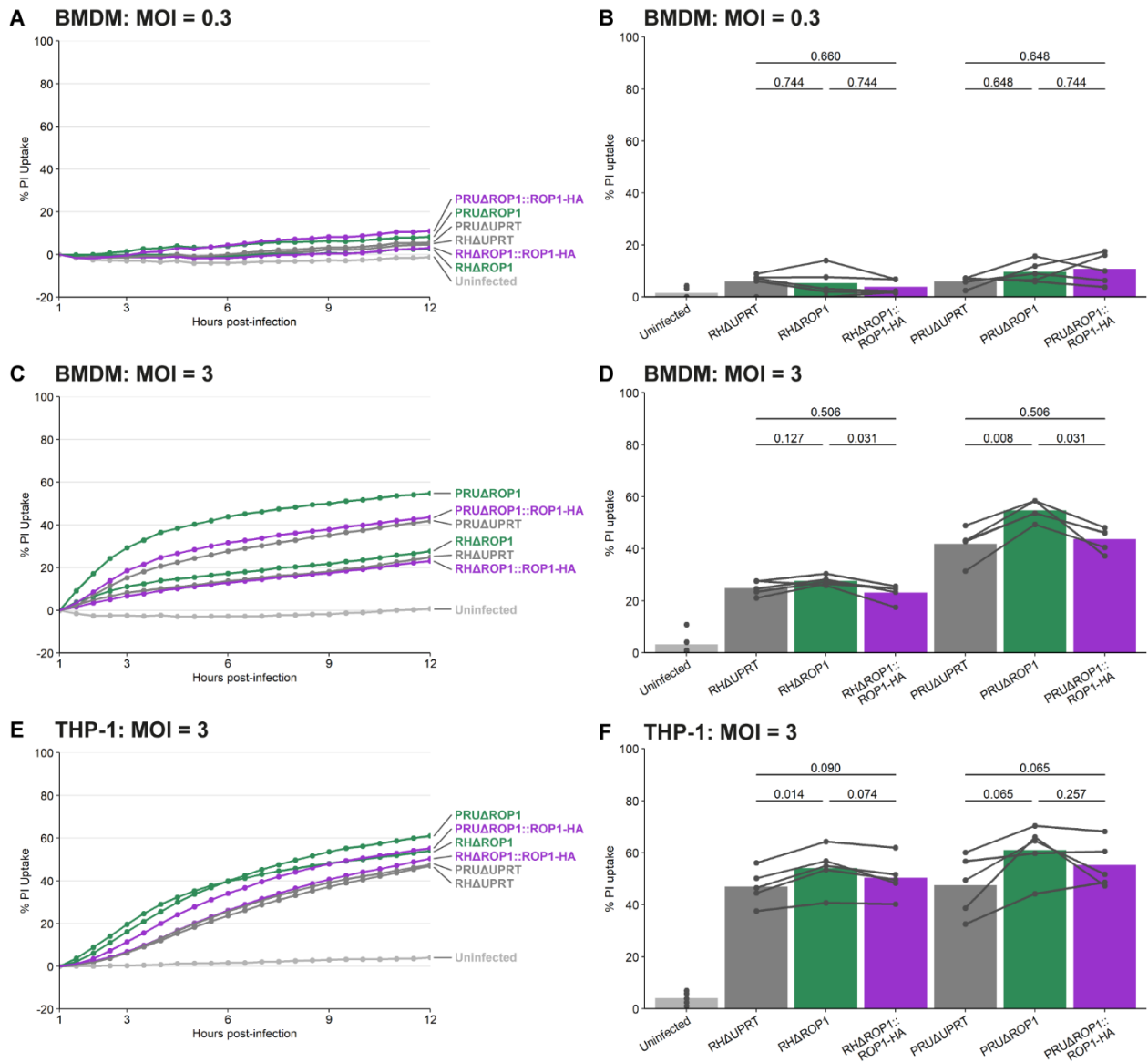


Figure 8. Knockout of ROP1 enhances parasite-induced host cell death at high MOI.

IFN γ -stimulated BMDMs or THP-1-derived macrophages were infected at the indicated MOI and propidium iodide fluorescence was measured over time. *p*-values calculated by paired two-sided t-test with Benjamini-Hochberg adjustment.

A. Propidium iodide fluorescence over time in BMDMs infected at an MOI of 0.3. Mean of all replicates shown.

B. Propidium iodide fluorescence at 12 hpi in BMDMs infected at an MOI of 0.3. Connecting lines indicate data points derived from the same biological replicate.

C. Propidium iodide fluorescence over time in BMDMs infected at an MOI of 3. Mean of all replicates shown.

D. Propidium iodide fluorescence at 12 hpi in BMDMs infected at an MOI of 3. Connecting lines indicate data points derived from the same biological replicate.

E. Propidium iodide fluorescence over time in THP-1-derived macrophages infected at an MOI of 3. Mean of all replicates shown.

F. Propidium iodide fluorescence at 12 hpi in THP-1-derived macrophages infected at an MOI of 3. Connecting lines indicate data points derived from the same biological replicate.

mediated restriction. Knockout of ROP1 was shown to not affect parasite invasion, indicating normal secretion of rhoptry neck (RON) proteins with critical roles in host cell invasion (Soldati et al. 1995); however, given the rhoptry bulb localisation of ROP1 and morphological change in the

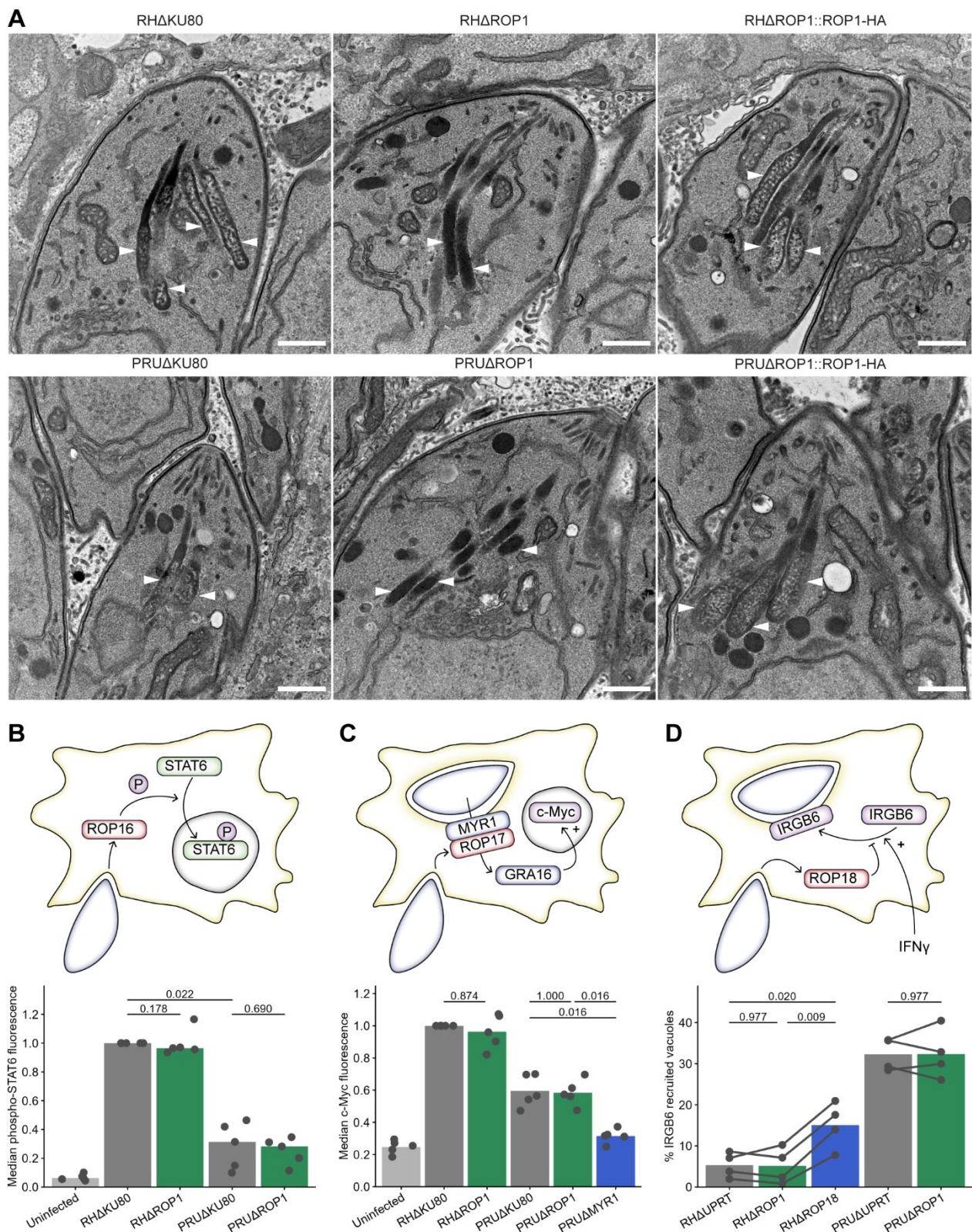


Figure 9. ROP1 knockout alters rhoptry morphology but not ROP secretion.

A. Transmission electron micrographs of the rhoptries of intracellular parasites. White arrowheads indicate rhoptry bulbs. Scale bar = 500 μ m.

B. Normalised median phospho-STAT6 immunofluorescence intensity in HFFs infected for 2 h and analysed by flow cytometry. *p*-values were calculated by unpaired *t*-test with Benjamini-Hochberg adjustment.

C. Normalised median nuclear c-Myc immunofluorescence intensity in HFFs infected for 24 h and analysed by microscopy. *p*-values were calculated by unpaired *t*-test with Benjamini-Hochberg adjustment

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Figure 9 continued

D. Mean percentage of *T. gondii* vacuoles with recruitment of IRGB6 in IFN γ -stimulated BMDMs infected for 1 h and analysed by high-content immunofluorescence microscopy. Connecting lines indicate data points derived from the same biological replicate. *p* values were calculated by paired *t*-test with Benjamini-Hochberg adjustment.

rophtry bulb upon deletion of ROP1, I hypothesised that secretion of other rophtry bulb (ROP) proteins may be affected independently of RON secretion.

As a proxy for secretion, I measured host cell phenotypes induced by three ROP proteins: phosphorylation of STAT6 by ROP16 (in the RH strain but not PRU) (Saeij et al. 2007), ROP17-dependent induction of c-Myc in the host cell nucleus (Panas, Ferrel et al. 2019), and recruitment of IRGB6 to the parasitophorous vacuole membrane, which is inhibited by ROP18 (in the RH strain but not PRU) (Fentress et al. 2010). Phosphorylation of STAT6 in was measured by flow cytometry using immunofluorescently labelled infected HFFs, c-Myc induction in HFFs was quantified from immunofluorescence microscopy images, and IRGB6 recruitment in IFN γ -stimulated BMDMs was quantified by high-content imaging and automated image analysis. In all three assays, there were no significant differences between Δ ROP1 and reference strains, although I observed the expected reduction in STAT6 phosphorylation for PRU Δ KU80 compared to RH Δ KU80 (Figure 9B) (Figure 9B), decrease in c-Myc expression of PRU Δ MYR1 compared to PRU Δ KU80 (Figure 9C), and increase in IRGB6 recruitment to RH Δ ROP18 vacuoles compared to RH Δ KU80 (Figure 9D).

These data thus indicate that ROP1 does not affect the secretion of ROP proteins, and the functional consequences, if any, of the altered rophtry morphology remain unknown. Having ruled out a function in secretion of other effector proteins, these data suggest that resistance to IFN γ -mediated restriction is an intrinsic function of ROP1.

ROP1 does not affect ubiquitination of the vacuole

In both human and murine cells, ubiquitination of the parasitophorous vacuole by host cell ligases appears to be important for subsequent recruitment IRGs, GBPs, and autophagy markers, culminating in destruction of the PVM (Haldar et al. 2015; Mukhopadhyay et al. 2020; Clough et al. 2016; Hernandez et al. 2022). Although the relative importance of different host ubiquitin ligases and types of ubiquitin linkage seems to depend on the combination of host cell species, host cell type, and parasite strain, I was therefore interested in ubiquitination of the vacuole as a conserved mechanism of restriction that may be affected by ROP1.

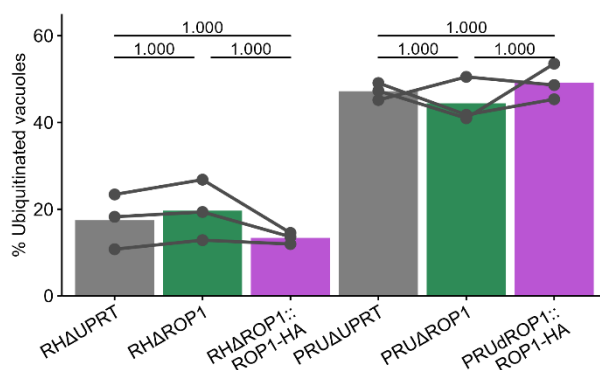


Figure 10. ROP1 does not affect vacuole ubiquitination.

IFN γ -stimulated BMDMs were infected for 3 h at an MOI of 0.3, fixed, and stained with a pan-ubiquitin antibody. The percentage of ubiquitinated vacuoles was quantified manually from blinded immunofluorescence microscopy images. Connecting lines indicate data points derived from the same biological replicate. *p*-values were calculated by paired *t*-test with Benjamini-Hochberg adjustment

To determine whether knockout of ROP1 affects vacuole ubiquitination, I measured the percentage of vacuoles in IFN γ -stimulated BMDMs at 3 hpi that were positive for pan-ubiquitin immunofluorescence staining. There were no differences between Δ ROP1 and control strains, demonstrating that total ubiquitination of the vacuole is not affected by ROP1 at this timepoint (**Figure 10**).

ROP1 co-immunoprecipitates with host cell C1QBP

To identify protein interaction partners of ROP1 that might inform on its function, I generated cell lines in which the endogenous copy of ROP1 in the RH Δ KU80 and PRU Δ KU80 strains was tagged at the C-terminus with a single haemagglutinin (HA) epitope. Integration of the tagging construct into the genome was verified by diagnostic PCR, expression of ROP1-HA was verified by Western blot, and the correct localisation of ROP1-HA confirmed by immunofluorescence microscopy (**Figure 11A-C**).

I carried out an anti-HA immunoprecipitation using lysates of IFN γ -stimulated MEFs infected with the PRU Δ KU80 or PRU ROP1-HA strains for 24 h. I used MEFs due to the difficulty of obtaining sufficient quantities of BMDMs, and because MEFs are thought to restrict *T. gondii* through the same mechanisms as BMDMs (Saeij and Frickel 2017). Immunoprecipitated proteins were identified and quantified by liquid chromatography-tandem mass spectrometry (**Figure 11D**). Aside from ROP1, all significantly enriched proteins were host cell rather than *T. gondii* proteins. Among the most highly enriched proteins were a ubiquitin-conjugating enzyme, RNF2, and a deubiquitinating enzyme, USP17LA. However, as I had previously determined that ubiquitination of the vacuole was not affected by ROP1, I instead focused on the most highly enriched host protein: C1QBP (Complement component 1Q Binding Protein, also known as GC1QR, HABP1, and p32). C1QBP is a small acidic protein that forms a homotrimer with a highly asymmetric charge distribution (J. Jiang et al. 1999). I hypothesise that a single molecule of ROP1 may interact with a C1QBP trimer, thus allowing the apparently greater enrichment of C1QBP compared to ROP1 in the mass spectrometry data (**Figure 11D**).

To validate the co-immunoprecipitation of C1QBP with ROP1, I repeated the anti-HA precipitation using both MEFs and HFFs infected with both RH and PRU strains, in addition to a PRU strain in which an unrelated rhoptry protein (TGME49_202200) was tagged with HA. I analysed the immunoprecipitate by Western blot and confirmed that C1QBP was pulled down by both RH ROP1-HA and PRU ROP1-HA in both cell types, but not by 202200-HA. Although three times the relative amount of immunoprecipitate fractions were loaded relative the post-immunoprecipitation supernatant fractions, the migration of C1QBP in the SDS-PAGE gel was slightly retarded in the supernatant fractions due to the very high protein concentration in these samples.

C1QBP is a multifunctional protein with reported roles in diverse pathways and cellular compartments (Jiao et al. 2015; Petersen-Mahrt et al. 1999; Yagi et al. 2012). Intriguingly, C1QBP has been implicated as a negative regulator of antiviral innate immune signalling pathways (Xu et al. 2009; Waggoner et al. 2005). The subcellular localisation of C1QBP has been contentious: as a complement receptor it is expected to be present on the plasma membrane, yet some studies have found that it is localised exclusively to the mitochondrial matrix (Muta et al. 1997). Another study has identified a cytoplasmic pool of C1QBP which can be recruited to the outer membrane of the mitochondrion upon infection with an RNA virus (Xu et al. 2009). I observed that C1QBP is primarily

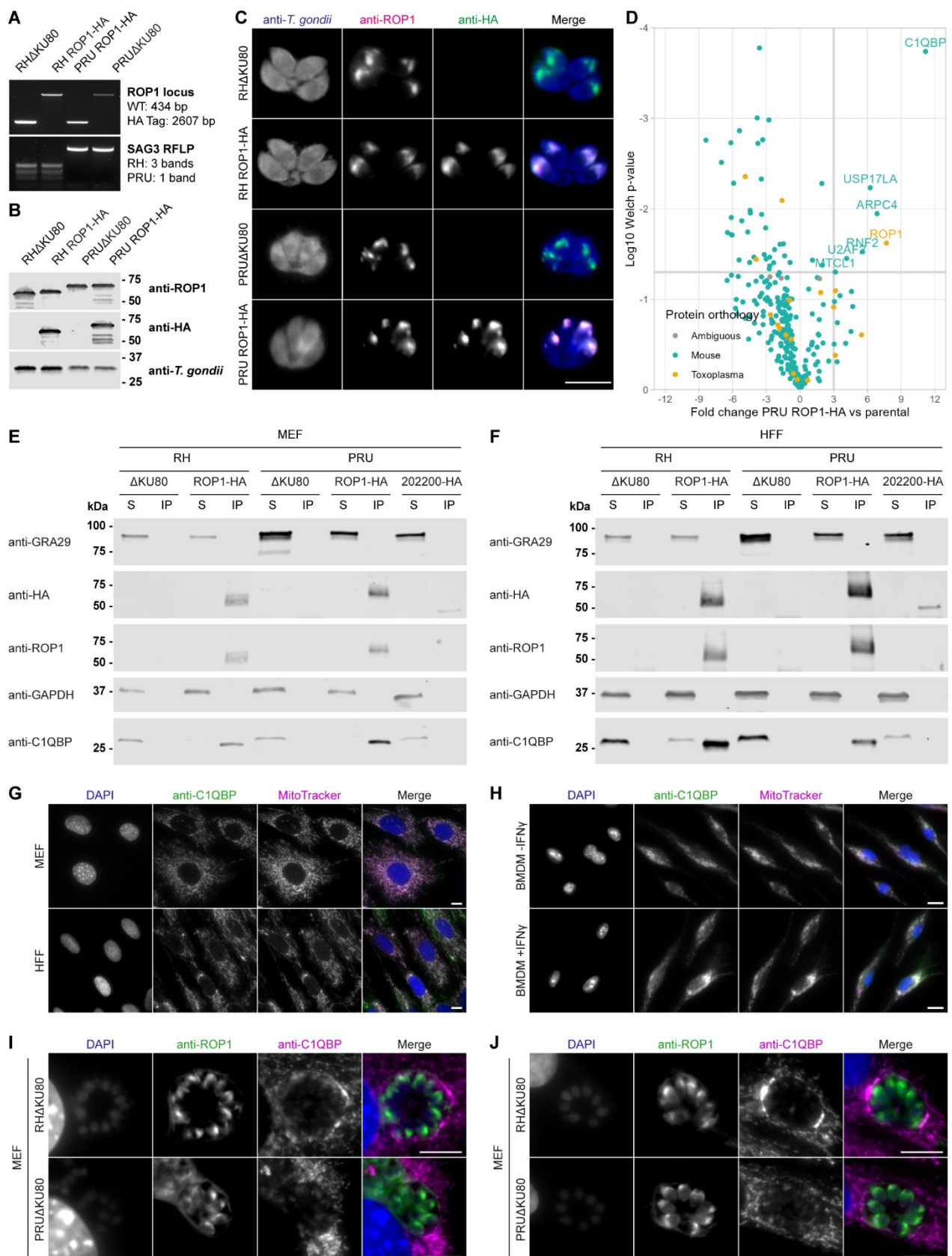


Figure 11. ROP1 co-immunoprecipitates with host cell C1QBP.

A. Verification of HA tagging construct integration by diagnostic PCR and strain validation by restriction fragment length polymorphism of the SAG3 gene.

B. Western blot verification of ROP1-HA expression.

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Figure 11 continued

C. Immunofluorescence microscopy verification of ROP1-HA expression. Scale bar = 10 μ m.

D. Enrichment of proteins that co-immunoprecipitate with ROP1-HA. IFN γ -stimulated MEFs were infected with PRU Δ KU80 or PRU ROP1-HA for 24 h, following which ROP1 was immunoprecipitated with anti-HA agarose matrix. Co-immunoprecipitated proteins were identified and quantified by liquid chromatography-tandem mass spectrometry. L2FCs were calculated from the geometric mean of the iBAQ intensities across replicates, and *p*-values calculated by two-sided Welch's *t*-test. Proteins with *p*-value < 0.05 and L2FC > 3 are annotated.

E. Western blot for co-immunoprecipitation of C1QBP with ROP1-HA in MEFs infected for 24 h. S = post-immunoprecipitation supernatant, IP = immunoprecipitate. Note that the IP fraction represents 3x the relative amount of total lysate as the S fraction.

F. Western blot for co-immunoprecipitation of C1QBP with ROP1-HA in HFFs infected for 24 h.

G. Immunofluorescence localisation of C1QBP in MEFs and HFFs.

H. Immunofluorescence localisation of C1QBP in IFN γ -stimulated and naïve BMDMs.

I. Immunofluorescence co-localisation of ROP1 and C1QBP in MEFs infected with RH Δ KU80 and PRU Δ KU80 for 24 h.

J. Immunofluorescence co-localisation of ROP1 and C1QBP in HFFs infected with RH Δ KU80 and PRU Δ KU80 for 24 h.

localised to the mitochondria in MEFs, HFFs, and BMDMs by co-staining with the fluorescent mitochondrial dye MitoTracker (**Figure 11G and H**). Still, it is possible that a smaller amount of C1QBP is present at the plasma membrane or in the cytosol that is harder to detect by immunofluorescence microscopy. There was no noticeable change in the localisation of C1QBP in BMDMs upon stimulation with IFN γ (**Figure 11H**).

In infected MEFs and HFFs, there was some co-localisation of ROP1 and C1QBP at the parasitophorous vacuole membrane for RH Δ KU80, but little co-localisation for PRU Δ KU80 (**Figure 11I and J**). Type I strains of *T. gondii* (such as RH), but not type II, recruit host cell mitochondria to the vacuole membrane via the interaction of the parasite secreted protein MAF1B with components of the mitochondrial protein import machinery and mitochondrial intermembrane space bridging complex (Pernas et al. 2014; Adomako-Ankomah et al. 2016; Kelly et al. 2017; Blank et al. 2021). As it is unclear whether C1QBP is localised exclusively to mitochondrial matrix, or whether it is also present on the mitochondrial outer membrane and in the host cytosol, it is unclear whether an interaction with ROP1 *in cellulo* is topologically possible. ROP1 has been observed in evacuoles (rhoptry derived secretory vesicles) in the host cell cytosol immediately after secretion (Håkansson, Charron, and Sibley 2001), therefore an interaction in the cytosol or at the mitochondrial outer membrane may be possible even in the absence of mitochondrial recruitment to the vacuole.

Knockout of host C1QBP may enhance IFN γ -mediated parasite restriction

Given that ROP1 co-immunoprecipitates with C1QBP, and that the absence of ROP1 results in greater parasite restriction, I hypothesised that C1QBP may have a restriction factor-like function that is inhibited by ROP1. Therefore, knockout of C1QBP in the host cell would rescue the increased susceptibility of Δ ROP1 parasites to IFN γ -mediated restriction.

To test this hypothesis, I carried out IFN γ restriction assays using immortalised MEFs derived from C1QBP^{flox/flox} mice in which C1QBP had been excised by transient transfection with Cre recombinase (Yagi et al. 2012). I validated knockout of C1QBP in these cells by immunofluorescence assay and Western blot, simultaneously confirming the specificity of anti-C1QBP immunofluorescence staining and thus mitochondrial localisation of C1QBP (**Figure 12A and B**). Neither the C1QBP^{flox/flox} nor C1QBP^{-/-} MEFs were able to strongly restrict *T. gondii* parasite

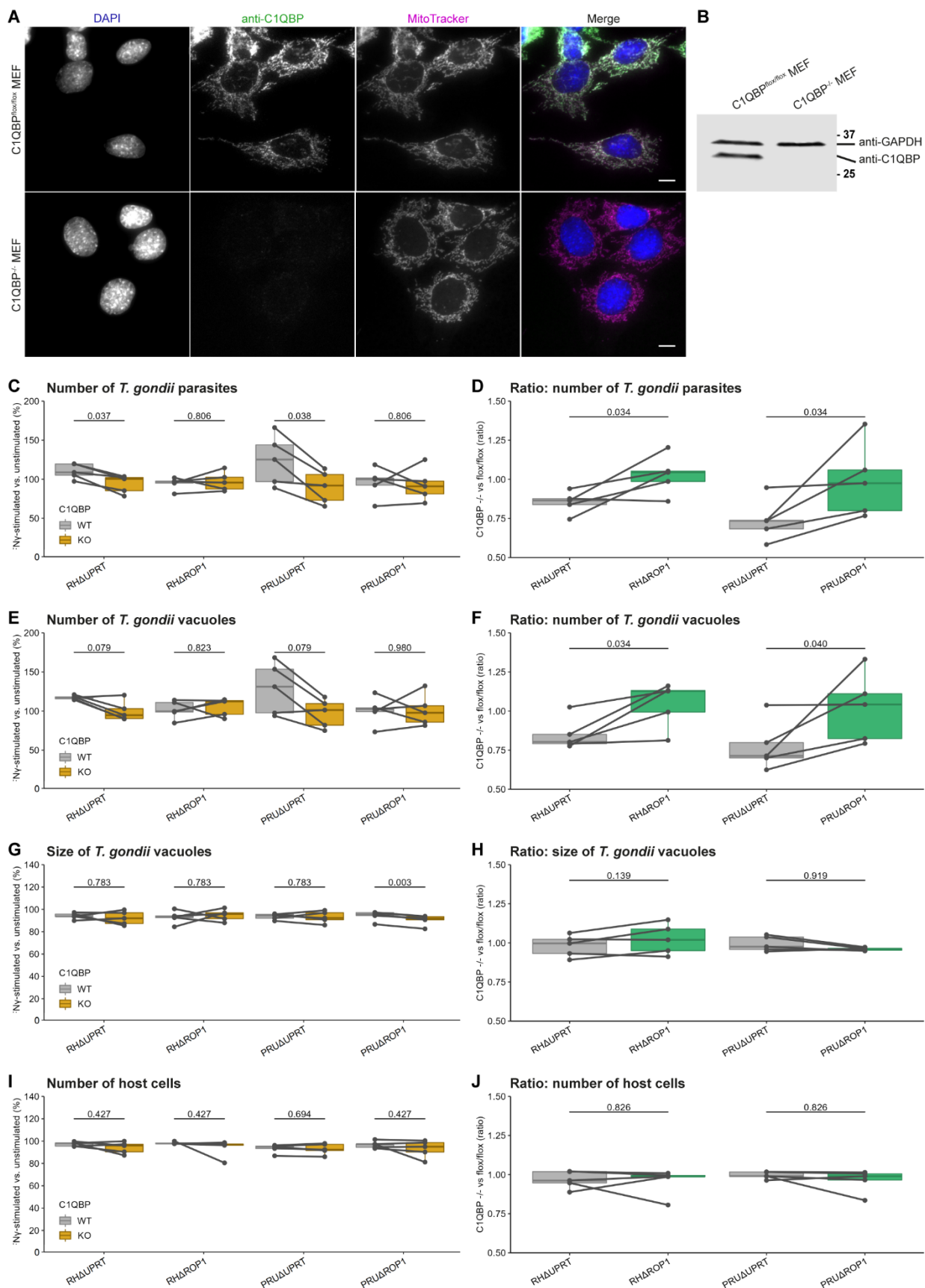


Figure 12. Knockout of C1QBP may enhance restriction of Δ UPRT parasites in IFN γ -stimulated MEFs.

C1QBP^{flx/flx} and C1QBP^{-/-} MEFs were stimulated with IFN γ and infected with indicated *T. gondii* strains at an MOI of 0.3 for 24 h. Parasite growth was quantified by high-content fluorescence imaging and automated image analysis. Connecting lines indicate data points derived from the same biological replicate. In panels C, E, G, and I, data points indicate each

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Figure 12 continued

measured parameter in IFN γ -stimulated BMDMs as a percentage of that in unstimulated BMDMs. In panels D, F, H, and J, data points indicate the ratio of these percentages in C1QBP^{-/-} MEFs compared to C1QBP^{flox/flox} MEFs. *p*-values were calculated by paired two-sided *t*-test with Benjamini-Hochberg adjustment.

- A. Verification of C1QBP knockout by immunofluorescence assay.
- B. Verification of C1QBP knockout by Western blot.
- C. *T. gondii* parasite number in IFN γ -stimulated versus naïve MEFs.
- D. Ratio of *T. gondii* parasite number in IFN γ -stimulated versus naïve C1QBP^{-/-} MEFs compared to C1QBP^{flox/flox} MEFs.
- E. *T. gondii* vacuole number in IFN γ -stimulated versus naïve MEFs.
- F. Ratio of *T. gondii* vacuole number in IFN γ -stimulated versus naïve C1QBP^{-/-} MEFs compared to C1QBP^{flox/flox} MEFs.
- G. *T. gondii* vacuole size in IFN γ -stimulated versus naïve MEFs.
- H. Ratio of *T. gondii* vacuole size in IFN γ -stimulated versus naïve C1QBP^{-/-} MEFs compared to C1QBP^{flox/flox} MEFs.
- I. Number of IFN γ -stimulated versus naïve MEFs.
- J. Ratio of number of IFN γ -stimulated versus naïve C1QBP^{-/-} MEFs compared to C1QBP^{flox/flox} MEFs

growth upon the addition of IFN γ , with parasite survival close to 100% (**Figure 12C**). Possibly, the immortalisation process or passage history of these cells has caused them to lose the ability to restrict *T. gondii*, as wild-type MEFs have previously been shown to be able to restrict both RH and PRU strains (Niedelman et al. 2012; Bando et al. 2018). However, I noticed that while both RH Δ UPRT and PRU Δ UPRT were significantly more restricted in C1QBP^{-/-} compared to C1QBP^{flox/flox} MEFs, there was no significant difference for RH Δ ROP1 and PRU Δ ROP1 (**Figure 12C**). This difference between strains could be tested by comparing the ratio of parasite restriction in C1QBP^{-/-} versus C1QBP^{flox/flox} MEFs (**Figure 12D**). While this ratio was close to one for both RH Δ ROP1 and PRU Δ ROP1, indicating that knockout of C1QBP makes no difference to parasite restriction, it was significantly reduced for RH Δ UPRT and PRU Δ UPRT, indicating that knockout of C1QBP causes significantly greater restriction of these strains than it does for RH Δ ROP1 and PRU Δ ROP1. For RH Δ UPRT and PRU Δ UPRT, the number of vacuoles in C1QBP^{-/-} MEFs was reduced compared to C1QBP^{flox/flox} MEFs, though not significant, while the vacuole size and host cell number were unchanged, mirroring the restriction of Δ ROP1 strains in BMDMs (**Figure 12E-J**).

Although it is difficult to draw firm conclusions from these data given the very low levels of parasite restriction, these data hint that C1QBP may operate as an “anti-restriction” factor, contrary to my earlier hypothesis. Knockout of C1QBP in the host cell partially phenocopies knockout of ROP1 in the parasites, in that parasite survival is reduced. This effect is absent when ROP1 is knocked out, indicating that both ROP1 and C1QBP are required to inhibit restriction, and that a putative interaction between these proteins is beneficial for the parasites, perhaps by potentiating an inhibitory role of C1QBP in innate immune signalling.

Discussion

In this knockout screen, I identified 18 putative virulence factors which contribute to *T. gondii* growth *in vivo* but not *in vitro*, indicating that they may be involved in evasion of host immune responses. While in this work I focused on ROP1, other less-well characterised effector proteins identified here also merit further study and may reveal new mechanisms of parasite immune evasion.

Out of 235 target genes in this screen, I was only able to determine phenotype scores for 164. One reason for this drop-out is that not every selected protospacer sequence was detectable in the starting plasmid pool, despite sequencing coverage of approximately 5,000x. Potentially, these protospacers were not successfully sampled from the arrayed library by the liquid handling robot, due to plate misalignment or incomplete thawing of the arrayed library. Troubleshooting and improvement of this guide selection procedure will be important to ensure that every targeted protospacer sequence is represented in the plasmid pool. Furthermore, the distribution of protospacer sequences in the plasmid pool is highly skewed, meaning that some are very highly represented while others are poorly represented. I speculate that this is due to secondary structure formation in the ssDNA oligonucleotides affecting Gibson cloning efficiency. Many of the genes for which I was not able to determine phenotype scores had several protospacers which were initially poorly represented in the plasmid pool and then dropped below the limit of detection throughout the experiment, meaning that the gene was dropped from the analysis as fold-changes could not be calculated for at least three protospacers. This loss of protospacer sequences over the course of the experiment indicates the presence of coverage bottlenecks, which should be considered carefully for future screening experiments.

Nevertheless, in combination with other emerging datasets in the field, these phenotype scores will be useful in prioritising *T. gondii* genes for further study. In particular, I found that ROP1 was one of only a small number of genes with negative fitness phenotypes both in this screen and in a genome wide-screen of *T. gondii* RH in IFN γ -stimulated versus naïve fibroblasts (Y. Wang et al. 2020), despite ROP1 being previously shown not to affect virulence of the RH strain (Soldati et al. 1995). This led me to select ROP1 for further study and show that knockout in the PRU strain leads to a complete loss of virulence. Furthermore, I showed that ROP1 is important for protecting both RH and PRU *T. gondii* against IFN γ -mediated restriction in murine macrophages. It is likely that the hypervirulent phenotype of the RH strain masks the function of ROP1 *in vivo* when time-to-death is the only measured outcome. More sensitive methods, for example CRISPR knockout screening *in vivo* using the RH strain, would likely demonstrate a role for ROP1. Such a screen was recently performed in CD-1 mice (Sangaré et al. 2019), which are less susceptible to the RH strain than C57BL/6 (Klesius and Hinds 1979; Tuttle et al. 2018); however, as with the screen here, this was a targeted screen that captured phenotypes for only 217 *T. gondii* genes, not including ROP1. Future genome-wide or effectome-wide screens in both parasite strains, ideally using the same library and methodology, would be an important resource for the community.

ROP1 is an unusual virulence factor for *T. gondii* as it inhibits parasite restriction in both murine and human macrophages. Only two other secreted effector proteins have been shown to inhibit restriction in both species: the dense granule chaperone protein GRA45 (Y. Wang et al. 2020) and the dense granule effector IST, which recruits chromatin repressors to STAT1 target genes and thereby blocks induction of interferon-stimulated genes (Gay et al. 2016; Olias et al. 2016). However, in contrast to ROP1, IST was only found to be protective against restriction when IFN γ was added to the cells after infection, not when cells were stimulated prior to infection (Gay et al. 2016). Downstream of conserved IFN γ /STAT1 signalling-mediated induction of interferon-stimulated genes, murine and human cells are thought to restrict *T. gondii* through largely different mechanisms (Saeij and Frickel 2017; Frickel and Hunter 2021). Thus, the majority of identified effector proteins that counteract these mechanisms have species-specific functions. For example, the ROP5 rhoptyr pseudokinase family together with the active kinases ROP17 and ROP18 form a

complex at the vacuole membrane that phosphorylates murine IRGs and GBPs, preventing them from loading onto the vacuole membrane (Fentress et al. 2010; Behnke et al. 2012; Etheridge et al. 2014); however, this complex does not prevent restriction in human cells (Niedelman et al. 2012; Lockyer et al. 2022). In contrast, a recently identified trimeric complex of GRA57, GRA70, and GRA71 inhibits IFN γ -mediated restriction in HFFs, but not in MEFs (Lockyer et al. 2022). Interestingly, although RH is more resistant than PRU to IFN γ -mediated restriction in BMDMs due to the activity of the ROP5/ROP17/ROP18 complex, I found that the opposite was true in human THP-1-derived macrophages.

ROP1 seems to inhibit parasite restriction in both murine and human macrophages, which suggests that it targets a mechanism of restriction that is conserved in both species, although a pleiotropic effect is also possible. Importantly, despite the change in rhoptry morphology, I ruled out ROP1 affecting the secretion of other rhoptry proteins through which such a pleiotropic phenotype could conceivably arise, and instead confirmed that inhibition of restriction in both host species is likely an intrinsic function of ROP1. Whether the change in rhoptry morphology is related to the virulence and IFN γ -mediated restriction phenotypes of ROP1 demonstrated here is unclear, as is whether these morphological changes have any functional consequences at all.

The high-content imaging-based restriction assays and propidium iodide uptake assays for host cell death indicate that there may be subtle differences in the restriction phenotype of Δ ROP1 parasites depending on the background parasite strain, though this may be due to a lack of sufficient power in the assays due to the high inter-replicate variability. For example, a small increase in host cell death was apparent for both RH Δ ROP1 and PRU Δ ROP1 in both BMDMs and THP-1-derived macrophages infected at a high MOI, but was only significant for PRU Δ ROP1 in BMDMs. Such an increase in cell death may be a downstream consequence of vacuole disruption (Zhao et al. 2009; Fisch et al. 2019) at some rate less than 100% that depends on MOI, parasite strain, and host species. There are some strain-specific differences in the protein sequence of ROP1 between RH and PRU, notably an expanded proline-rich octopeptide repeat in central region of PRU ROP1, which is noticeable in the higher apparent molecular weight of PRU ROP1 compared to RH ROP1 by Western blot. Such differences may also result in strain-specific phenotypes for ROP1 knockout, though any differences would be subtle compared to classic strain-specific effectors of *T. gondii*, such as ROP5 (Behnke et al. 2012), ROP18 (Niedelman et al. 2012), ROP16 (J. P. J. Saeij et al. 2007), and GRA15 (Rosowski et al. 2011). Confirmation or refutation of such strain- and host-specific differences in restriction phenotypes will require substantially more work with orthogonal assays to measure specific modes of parasite restriction.

The co-immunoprecipitation data and further restriction assays suggest a functional link of ROP1 to the host protein C1QBP, although this cannot be confirmed with currently available tools. Co-immunoprecipitation of ROP1 with C1QBP is robust, occurring in both human and murine fibroblasts for both the RH and PRU strains, but I was not able to validate this interaction through an orthogonal method. Although ROP1 and C1QBP can potentially interact in the host cell cytosol or at the PVM, the localisation of C1QBP is contentious and so whether such an interaction is topologically possible remains unclear. I found that C1QBP is primarily localised to the mitochondria, but I did not confirm whether C1QBP resided exclusively in the mitochondrial matrix, as some have suggested (Muta et al. 1997), or is present on the outer membrane, as others have shown (Xu et al. 2009). I attempted to validate the putative interaction *in cellulo* by proximity

biotinylation; however, C-terminal fusion of the TurboID biotin ligase to ROP1 resulted in protein mis-localisation and prevented secretion of ROP1 to the PVM (not shown). Both ROP1 and C1QBP are noted for highly asymmetric charge distributions which could provide the basis for any (real or artefactual) interaction. A crystal structure has shown that the majority of acidic charge is concentrated on one face of the C1QBP trimer (J. Jiang et al. 1999). Although there is no structure available for ROP1 and the AlphaFold prediction is poor (Varadi et al. 2022), the N-terminal region is highly acidic while the C-terminal region is highly basic. The asymmetric charge distribution of ROP1 was speculated to facilitate interaction with host cell components (Ossorio, Schwartzman, and Boothroyd 1992).

The results of restriction assays in C1QBP^{-/-} MEFs indicate a functional interaction between ROP1 and C1QBP that is beneficial for parasite growth; however, these results should also be cautiously interpreted given the overall lack of restriction in the immortalised MEF cell lines used. As knockout of C1QBP is lethal in mice, it is not possible to obtain primary C1QBP^{-/-} BMDMs. I attempted siRNA-mediated knockout of C1QBP in wild type BMDMs using a pool of three commercial siRNAs, but was only able to achieve <10% knockdown of C1QBP, as measured by immunofluorescence (not shown).

Nonetheless, C1QBP is an interesting candidate as a host partner of a pathogen virulence factor. Among the diverse apparent functions of this protein, C1QBP has been shown to act as a positive regulator of autophagy and mitophagy through stabilisation of ULK1 (Jiao et al. 2015). In both human and murine cells, autophagy proteins play key roles in IFN γ -dependent parasite restriction (Saeij and Frickel 2017); modulation of this role of C1QBP could therefore conceivably affect parasite restriction. Alternatively, activation of host autophagy through ROP1 binding to C1QBP could enhance parasite growth by providing nutrients to the parasite (Wang, Weiss, and Orlofsky 2009). Such a mechanism may counteract nutrient starvation that has been proposed as IFN γ -dependent restriction mechanism in certain cell types (Pfefferkorn, Eckel, and Rebhun 1986). Additionally, a growing body of evidence implicates C1QBP as a negative regulator of antiviral innate immune signalling pathways (Xu et al. 2009; Song et al. 2021), and a number of viral and proteins have been found to interact with C1QBP, similarly to ROP1 (Kittlesen et al. 2000; Braun, Ghebrehiwet, and Cossart 2000; Nguyen, Ghebrehiwet, and Peerschke 2000; Beatch et al. 2005; Lainé et al. 2003; Matthews and Russell 1998). Suppression of IL-12 production by ligation of C1QBP (Waggoner et al. 2005) in *T. gondii* infected-cells could potentially facilitate parasite growth by reducing IFN γ production, but would not explain the enhanced restriction of Δ ROP1 parasites in the *in vitro* restriction assays in cells that are already activated with IFN γ . In an interesting parallel, *Plasmodium berghei* was found to induce a type I interferon response in mouse liver cells by recognition of parasite RNA by the cytosolic sensor MDA5, leading to restriction of parasite growth (Liehl et al. 2014) by a canonically antiviral mechanism.

In summary, these data show that ROP1 is an important and previously-overlooked effector protein that suppresses IFN γ -mediated restriction in macrophages and thereby contributes to parasite virulence. Although the mechanism through which ROP1 suppresses restriction is unclear, the identification of an effector protein that appears to be universally important in both the RH and PRU strains of *T. gondii* and in both murine and human macrophages will be useful as tool to investigate conserved mechanisms of cell-autonomous innate immunity.

Chapter 2: Dual perturb-seq screening of the *Toxoplasma gondii* secretome

Results

Optimisation of a direct-capture perturb-seq vector for *Toxoplasma gondii*

Many intracellular pathogens reprogram host cell transcription to establish a favourable environment. This reprogramming typically involves downregulation of host immune responses and recalibration of biosynthetic pathways. Among such pathogens, *T. gondii* is remarkable in both the number of identified effector proteins that target host cell transcription and the breadth of reprogramming (Hakimi, Olias, and Sibley 2017). Although many of these effectors have been shown to have critical roles in *T. gondii* virulence, dissemination, and persistence *in vivo*, they are not typically identified in *in vivo* knockout screens (Young et al. 2019; Sangaré et al. 2019). We have hypothesised that the primary role of these effectors in modulating host immune responses is rescued *in trans* in a pooled *in vivo* infection (Young et al. 2019). Identifying secreted effector proteins which may target host cell transcription *a priori* is highly challenging, as these effectors typically represent novel orthologue groups that are found only in Apicomplexa or subgroups thereof and frequently lack conserved domains that would inform on their function (Barylyuk et al. 2020). A new method is therefore needed to specifically identify effector proteins that target host cell transcription out of the >200 rhoptry- and dense granule-localised proteins in *T. gondii* (Barylyuk et al. 2020).

In this work, I have developed single-cell perturb-sequencing as a method to screen for effector proteins that modulate host cell transcription in *T. gondii* and other endosymbionts. In perturb-seq and related methods, a pool of genetic perturbations is introduced to a target cell population using CRISPR-Cas systems and the impacts of each perturbation on the transcriptome of the cells carrying it are deconvolved by single-cell RNA-sequencing (scRNA-seq) (Adamson et al. 2016; Dixit et al. 2016; Replogle et al. 2020). This multiplexing enables the impacts of tens to thousands of genetic perturbations to be assessed in a single experiment. In *T. gondii*, I term the method developed here “dual perturb-seq” for combining elements of established perturb-seq methods (Replogle et al. 2020) with elements of dual host-pathogen RNA-sequencing (Westermann, Barquist, and Vogel 2017).

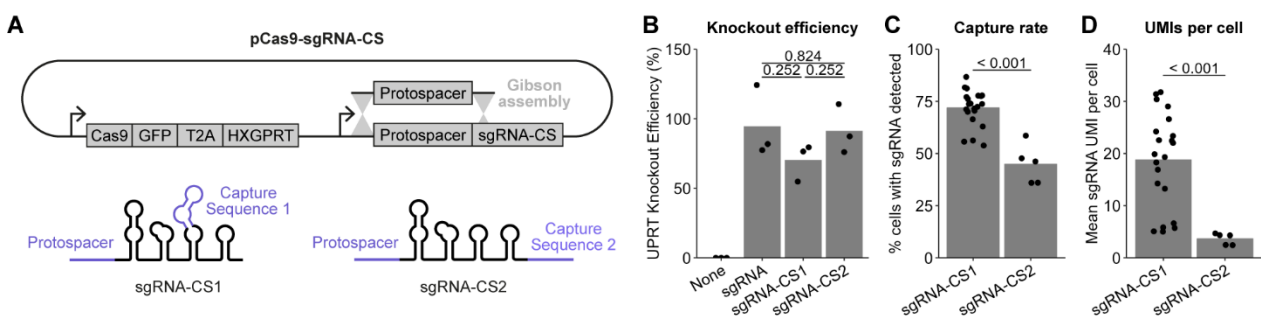


Figure 13. sgRNA-CS1 is more efficiently captured than sgRNA-CS2.

A. Diagram of *T. gondii* perturb-seq plasmid vector and sgRNA-capture sequence configurations.

B. Knockout efficiency of perturb-seq vectors. Loss of function of the *T. gondii* UPRT gene was measured by plaque assay in the presence/absence of 5-fluorodeoxyuridine. *p*-values were calculated by two-sided *t*-test with Benjamini-Hochberg adjustment.

C. Percentage of infected cells with a detectable sgRNA in all dual perturb-seq samples. *p*-value calculated by two-sided *t*-test.

D. Mean number of sgRNA UMIs in cells with a detectable sgRNA in all dual perturb-seq samples. *p*-value calculated by two-sided *t*-test.

Perturb-seq requires identification of the genetic perturbation in each cell. In this work, I used a commercially available platform from 10x Genomics (www.10xgenomics.com) that uses “direct-capture” of the sgRNA during droplet-based scRNA-seq (Replogle et al. 2020). Polyadenylated mRNAs are captured for reverse transcription by barcoded poly(dT) primers, while a “capture sequence” integrated into the sgRNA transcript binds to an additional, complementary primer, facilitating barcoded reverse transcription and sequencing of the sgRNA in each cell. I modified our existing plasmid vector for pooled knockout screening in *T. gondii* (Young et al. 2019) to introduce two previously validated sgRNA-capture sequence configurations (Replogle et al. 2020) (**Figure 13A**). There was no significant change in knockout efficiency of the non-essential *T. gondii* UPRT gene for either sgRNA compared to the unmodified sgRNA, as measured by knockout-induced resistance to the toxic nucleoside analogue 5-fluorodeoxyuridine (Donald and Roos 1995) (**Figure 13B**). As sgRNA-CS2 had a slightly higher mean knockout efficiency than sgRNA-CS1, I used this for the first pilot perturb-seq experiment. However, across all experiments I completed as part of this project, I found that the capture rate of sgRNA-CS1 was significantly higher than sgRNA-CS2 in terms of the percentage of cells in which any sgRNA transcript is detected (**Figure 3C**) and the number of sgRNA transcripts (unique molecular identifiers, UMIs) detected in those cells (**Figure 13D**). Therefore, in all later experiments I used the perturb-seq vector with sgRNA-CS1 to maximise the number of recovered single cell transcriptomes with an identifiable perturbation.

Dual perturb-seq recapitulates MYR1-dependent host cell reprogramming

As perturb-seq has not before been attempted in *T. gondii*, nor any other non-mammalian system, I carried out a pilot experiment to determine whether the transcriptional profiles of infected cells would be comparable to published bulk transcriptome data. I transfected *T. gondii* RHΔHXGPRT with perturb-seq plasmids targeting either UPRT, as a negative control, or MYR1, as a positive control (**Figure 14A**). MYR1 is essential for export of dense granule effector proteins into the host cell (Franco et al. 2016) and the majority of host cell transcriptional changes induced by *T. gondii* infection depend on MYR1 (Naor et al. 2018). Therefore, I expected the greatest change in the host cell transcriptome for MYR1-knockout-infected cells compared to UPRT-knockout-infected cells. The transfected parasites were selected for integration of the perturb-seq vectors into the genome for seven days, and the resulting knockout parasites were used to infect serum-starved HFFs for 24 h at an MOI of 0.1. Infected cells were enriched by fluorescence-activated cell sorting (FACS) based on Cas9-GFP expression and pooled for scRNA-seq using the 10x Genomics Chromium platform.

Sequencing reads were aligned to a dual *H. sapiens* and *T. gondii* reference and cell barcodes were filtered to retain only high-quality infected cells expressing a single sgRNA species (**Figure 14B**). Inspecting the cells by uniform manifold approximation projection (UMAP) using the host cell gene expression, I observed that the cells expressing the MYR1-targeting sgRNA largely partitioned into a separate cluster from cells expressing the UPRT-targeting sgRNA, indicating that knockout of these genes in the infecting parasite induces different transcriptional profiles in the host cell (**Figure 14C**). The most significant up- or down-regulated genes in these clusters were previously shown to be dependent on MYR1 in a bulk sequencing study (Naor et al. 2018) (**Figure 14D**). In contrast, when using the *T. gondii* gene expression data for UMAP, the single cell transcriptomes resolved in a circular structure that recapitulated the *T. gondii* cell cycle (**Figure 14E and F**).

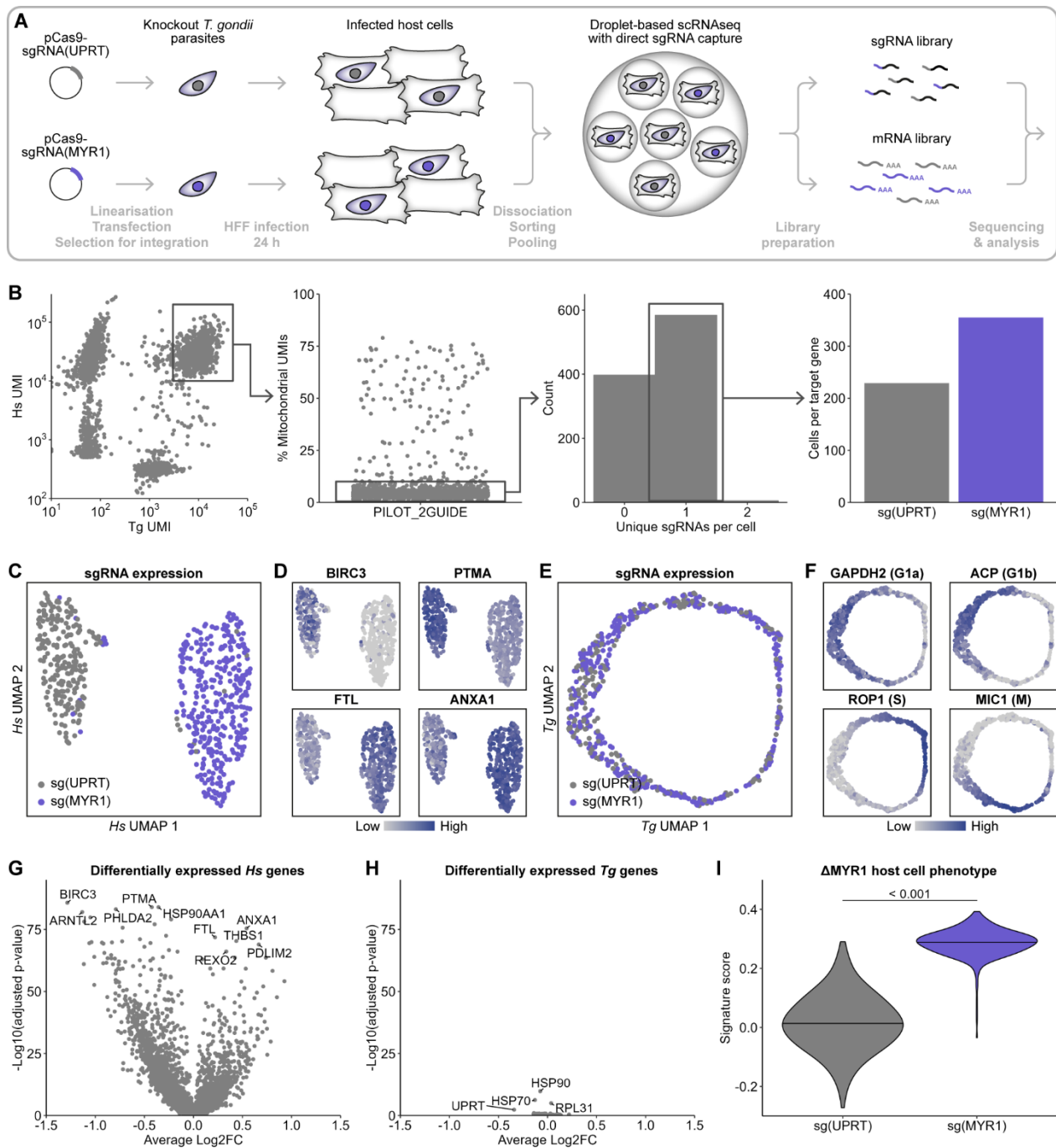


Figure 14. Dual perturb-seq recapitulates MYR1-dependent host cell reprogramming.

A. Experimental strategy.

B. Cell barcode filtering strategy.

C. UMAP of single cell transcriptomes based on host cell gene expression, coloured by sgRNA expression.

D. Top marker genes for host cell UMAP clusters (Louvain clustering, resolution = 0.1).

E. UMAP of single cell transcriptomes based on *T. gondii* gene expression, coloured by sgRNA expression.

F. Expression of *T. gondii* cell cycle marker genes.

G. Differentially expressed host cell genes between sg(MYR1)-expressing and sg(UPRT)-expressing cells, two-sided Wilcoxon rank-sum test with Benjamini-Hochberg adjustment. The top five most significant up- and down-regulated genes are labelled.

H. Differentially expressed *T. gondii* genes between sg(MYR1)-expressing and sg(UPRT)-expressing cells, two-sided Wilcoxon rank-sum test with Benjamini-Hochberg adjustment. All significant genes are labelled.

I. Scoring of single cell transcriptomes based on marker genes of established effector proteins using VISION. *p*-value calculated by two-sided Wilcoxon rank-sum test.

For further analyses, I categorised the cells by sgRNA expression as the best-available “ground truth” for cell identify. More than 5,000 host cell genes were differentially expressed in sg(MYR1)-expressing compared to sg(UPRT)-expressing cells (**Figure 14G**). However, only four *T. gondii* genes were significantly differentially expressed: ribosomal protein RPL21, two heat shock proteins, and UPRT itself (**Figure 14H**). These data show that while knockout of effector proteins can induce dramatic changes in the host cell transcriptome, there is minimal impact on the *T. gondii* transcriptome.

To determine broadly whether the single cell transcriptomes correlate with published bulk RNA-seq data, I used the VISION package (DeTomaso et al. 2019) to score the cells for a Δ MYR1-infected signature based on markers previously found to be differentially expressed in *T. gondii* RH Δ MYR1-infected HFFs compared to *T. gondii* RH wild-type-infected HFFs (Naor et al. 2018). sg(MYR1)-expressing cells scored significantly higher for this signature compared to sg(UPRT)-expressing cells, demonstrating that the phenotype of these cells concurs with bulk sequencing data (**Figure 14I**).

Deconvolution of host cell transcriptome profiles of 24 *T. gondii* mutants using dual perturb-seq

To further validate dual perturb-seq as an approach to identify *T. gondii* effector proteins with a more moderate impact than MYR1, I generated a panel of 22 additional perturb-seq vectors targeting 12 effector proteins that have been shown to impact host cell transcription and 10 rhoptry and dense granule localised proteins for which the host cell transcriptome has not been characterised. I transfected *T. gondii* RH Δ HXGPRT with these 22 vectors plus the MYR1- and UPRT-targeting vectors and pooled the resulting transfectants into a single mixed population (**Figure 15A**). I infected HFFs with this parasite pool for 24 h at an MOI of 0.1 then sorted and sequenced the infected cells as before. From a single 10x Genomics Chromium channel, I recovered 10-72 single cell transcriptomes for each target gene (**Figure 15B**).

Compared to sg(UPRT)-expressing cells, I identified differentially expressed host genes for seven out of ten effector proteins that have been shown to have a phenotype in the type I *T. gondii* RH strain used in this experiment: MYR1 (Naor et al. 2018), HCE1/TEEGR (Panas, Naor, et al. 2019; Laurence Braun et al. 2019), IST (Gay et al. 2016; Olias et al. 2016), GRA16 (Bougdoor et al. 2013), GRA24 (Braun et al. 2013), GRA28 (Rudzki et al. 2021; Ten Hoeve et al. 2022), and ROP16 (Saeij et al. 2007) (**Figure 15C**). There were no significantly differentially expressed genes for the effectors GRA6 (Ma et al. 2014), GRA7 (Yang et al. 2016), or ROP18 (J.-X. Li et al. 2020), although for these effectors there is less evidence that changes in the host cell transcriptome are apparent upon physiological infection. For GRA7, activation of TRAF6 and was shown following stimulation of macrophages with recombinant GRA7 (Yang et al. 2016). For ROP18, changes in the host cell transcriptome were identified following ectopic expression of ROP18 in HEK923T cells (J.-X. Li et al. 2020). The lack of any differentially expressed genes here suggests that caution is required in inferring the function of effector proteins from non-physiological delivery and dosage conditions. GRA7 has been convincingly shown to activate the transcription factor NFAT4, both when expressed ectopically and during *T. gondii* infection, yet no transcriptomic changes have been shown (Ma et al. 2014). However, in this dataset GRA6, GRA7 and ROP18 are represented by relatively few cells, which may not provide enough statistical power to identify differentially expressed genes. There were no differentially expressed host genes for GRA15 (Rosowski et al. 2011), GRA18 (He et al. 2018) and GRA25 (Shastri et al. 2014), effector proteins for which phenotypes have been

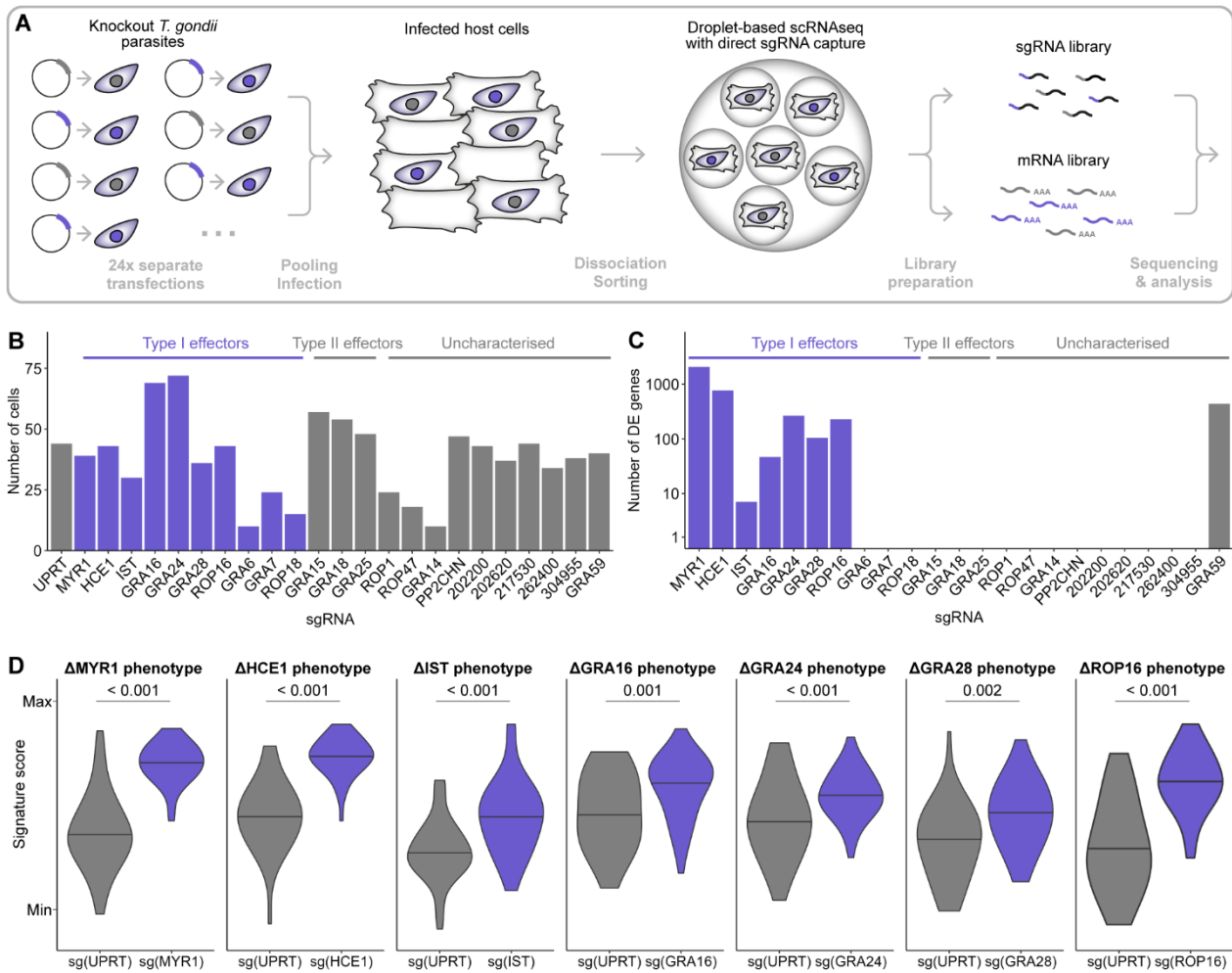


Figure 15. Transcriptional profiles resolved by dual perturb-seq reproduce published phenotypes.

A. Experimental strategy.

B. Number of single cell transcriptomes recovered for each target gene.

C. Number of significantly differentially expressed host cell genes for each target gene compared to sg(UPRT)-expressing cells, two-sided Wilcoxon rank-sum test with Benjamini-Hochberg adjustment p -value ≤ 0.05 .

D. Scoring of single cell transcriptomes based on marker genes of established effector proteins using VISION. p -values were calculated by two-sided Wilcoxon rank-sum test.

demonstrated only in type II *T. gondii* strains. Of these effectors, knockout of GRA15 has been shown to have no phenotype in type I *T. gondii*, while GRA18 and GRA25 have not been studied in a type I strain. The data here suggests that these genes may also be strain-specific effectors. Of the uncharacterised genes included in this experiment, differentially expressed genes were identified only for GRA59, which I investigate further in a later chapter.

Bulk transcriptomic datasets of *T. gondii* RH knockout strain-infected versus wild type-infected host cells are available for the seven type I effectors for which I identified differentially expressed genes, but not for the remaining three (Naor et al. 2018; Panas, Naor, et al. 2019; Matta et al. 2019; Bougdour et al. 2013; Braun et al. 2013; Rudzki et al. 2021; Saeij et al. 2007). For these seven effectors, I scored the single cell transcriptomes for expression of marker genes identified in these studies, showing that cells expressing the sgRNA targeting each of these effectors had significantly higher scores for the cognate bulk transcriptome signatures compared to sg(UPRT)-expressing cells

(Figure 15D). This data confirms that dual perturb-seq is able to deconvolve the phenotypes of host cells infected with a pool of different *T. gondii* knockouts, and that the resulting transcriptome profiles concur with all available prior datasets.

Dual perturb-seq screening of the *T. gondii* effectome

Having validated the dual perturb-seq workflow, I set up a knockout screen aiming to identify all *T. gondii* effectors that target host cell transcription. I identified 256 rhoptry and dense granule localised proteins based on spatial proteomics data (Barylyuk et al. 2020) and community annotation (Amos et al. 2022) and selected five protospacer sequences targeting each of these genes from our arrayed library (Young et al. 2019). To reduce the potential loss of protospacer sequences that I observed for the earlier *in vivo* CRISPR screen, I carried out the protospacer sampling on three different days and pooled the samples together. Additionally, I cloned the protospacer sequences by pooled Gibson assembly into three different perturb-seq vectors with different fluorescent proteins linked to Cas9 (GFP, YFP, and mCherry) and pooled these libraries together to prior to transfection to aid in filtering out parasites that take up multiple plasmids or cells that are infected with multiple parasites during sorting prior to sequencing. I verified the incorporation of 1,263 out of 1,312 selected protospacers into the plasmid pool by bulk DNA sequencing. I transfected the plasmid pool into *T. gondii* RHΔHXGPRT and used the resulting pool of knockout parasites to infect HFFs as before (Figure 16A). I carried out the screen in both unstimulated and IFN γ -stimulated HFFs to identify effector proteins that modulate the expression of interferon-stimulated genes (ISGs), similarly to the known effectors IST and NSM (Gay et al. 2016; Olias et al. 2016; Rosenberg and Sibley 2021). In order to obtain enough single-cell transcriptomes for sufficient coverage of the library, host cell infection, sorting and single-cell library preparation was carried out on three different dates with unstimulated HFFs and on two further dates with IFN γ -stimulated HFFs, using the same frozen stock of transfected parasites each time. 2-4 Chromium channels were loaded on each date, depending on the number of cells recovered after sorting, with a final total of eight channels each for unstimulated and IFN γ -stimulated HFFs. All channels were treated as technical replicates for the purpose of data aggregation and analysis.

A schematic overview of the analysis of the dual perturb-seq data can be found in Figure 17. After filtering the cell barcodes, I recovered 25,185 transcriptomes (15,920 unstimulated and 9,265 IFN γ -stimulated). Across all the transcriptomes, I identified 1,119 sgRNAs with a median of 11 single cell transcriptomes per sgRNA (Figure 16B). All 256 *T. gondii* target genes were represented by at least one sgRNA. Aggregating all sgRNAs resulted in a median coverage of 71 transcriptomes per target gene (Figure 16C). The majority of target genes were represented by five different sgRNAs, and 93% of target genes were represented by at least three different sgRNAs (Figure 16D). Both at the individual sgRNA level and when aggregated by target gene, the number of single cell transcriptomes recovered was directly proportional to sgRNA representation in the perturb-seq plasmid pool as assessed by bulk sequencing, although sgRNAs targeting genes with strongly negative fitness phenotypes (Sidik et al. 2016) tended to be less well represented (Figure 16E and F).

After cell barcode filtering, the host cell gene expression data were normalised and z-transformed, during which batch effects were removed by passing the technical replicate ID (i.e. Chromium channel) of each cell as a variable to regress out (Figure 16G and H). Inspecting the data by UMAP, one additional cluster of cells was present in the IFN γ -stimulated samples that was absent in the

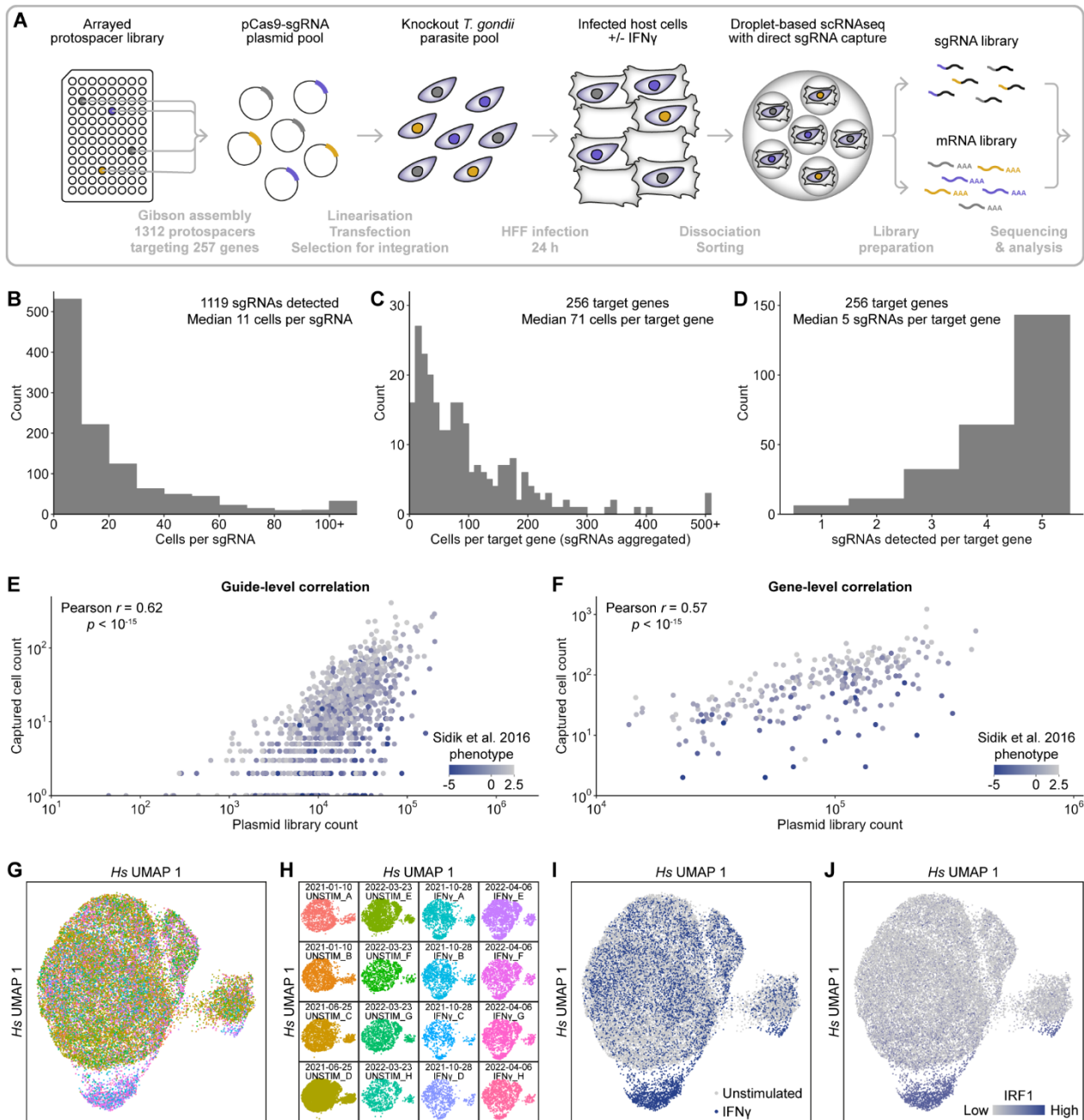


Figure 16. Dual perturb-seq screening of the *T. gondii* effectome.

- Experimental strategy of dual perturb-seq screen.
- Histogram of number of single cell transcriptomes recovered for each sgRNA.
- Histogram of number of single cell transcriptomes recovered for each *T. gondii* target gene.
- Histogram of number of detected sgRNAs for each target gene.
- Correlation between the number of read counts in bulk sequencing data of perturb-seq plasmid library and the number of single cell transcriptomes for each sgRNA.
- Correlation between the number of read counts in bulk sequencing data of perturb-seq plasmid library and the number of single cell transcriptomes summed for each target gene.
- UMAP of single cell transcriptomes using host cell gene expression, coloured by sample ID.
- UMAP of single cell transcriptomes using host cell gene expression split by sample ID.
- UMAP of single cell transcriptomes using host cell gene expression coloured by IFN γ stimulation.
- Normalised expression of IRF1.

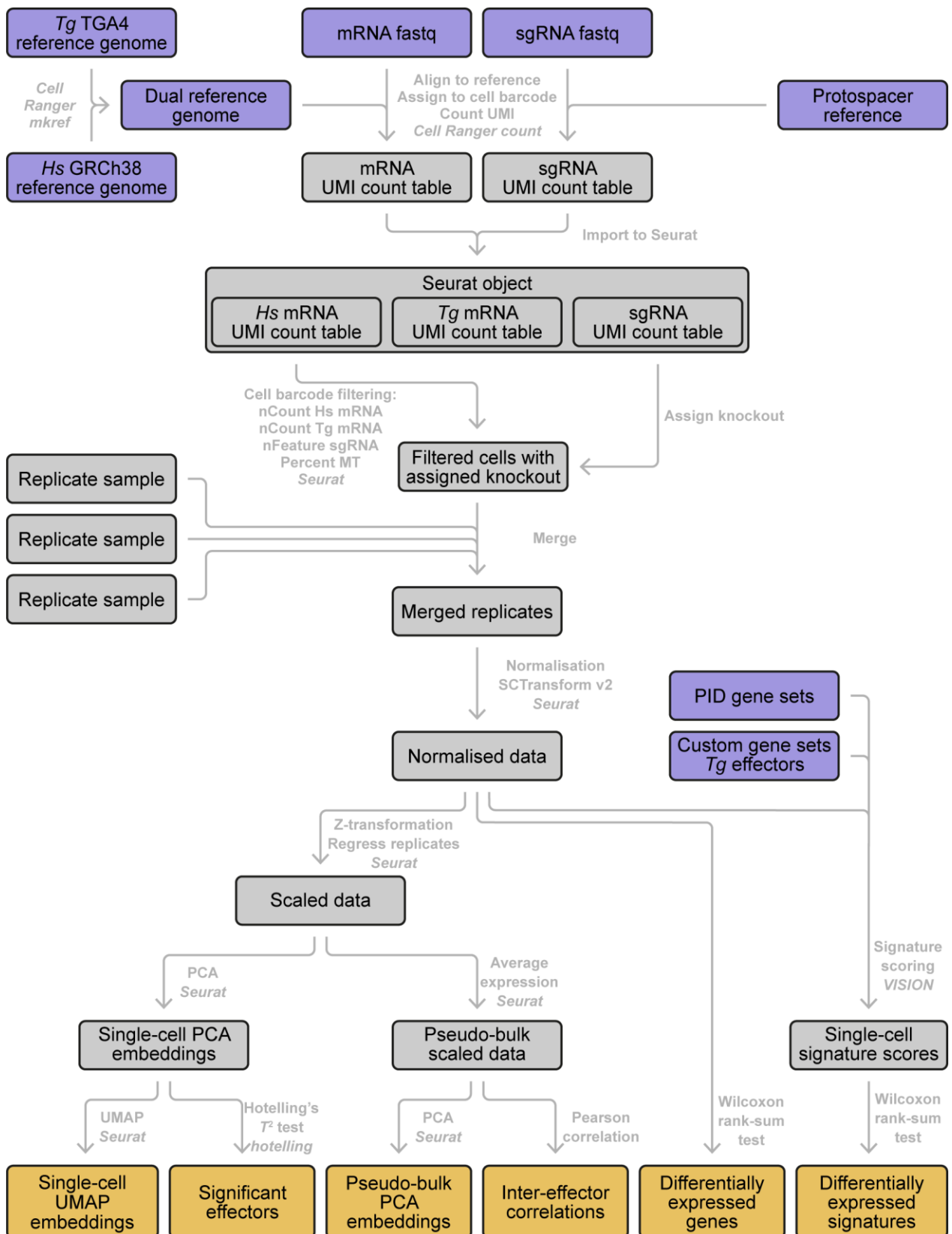


Figure 17. Overview of dual perturb-seq data analysis.

unstimulated samples, corresponding to cells in which ISGs (such as IRF1) are expressed (**Figure 16I and J**). Relatively few cells in the IFN γ -stimulated samples show expression of ISGs as the *T. gondii* effector protein IST blocks STAT1-mediated transcription following IFN γ stimulation (Gay et al. 2016; Olias et al. 2016).

Identification of *T. gondii* effector proteins from dual perturb-seq data

To identify which of the targeted *T. gondii* secreted proteins significantly perturb host cell gene expression, I adapted an approach recently used to characterise coding variants of TP53 and KRAS (Ursu et al. 2022). I analysed the single cell transcriptomes by principal component analysis (PCA) and tested for each target gene whether the distribution of single cell transcriptomes in PCA space differed from the background distribution (excluding known effector proteins) using Hotelling's t^2 -test, a multivariate generalisation of Student's t -test (Hotelling 1931) (**Figure 18A**). This test is thus able to distil the extremely high-dimension dual perturb-seq data into a single test statistic value (which I refer to as the "perturbation score") and p -value for each target gene.

Initially, I applied this test to the unstimulated and IFN γ -stimulated data separately to identify any effector proteins with a function apparent only upon IFN γ stimulation, such as IST and NSM (Gay et al. 2016; Olias et al. 2016; Rosenberg and Sibley 2021). The perturbation scores of most known effectors were highly correlated in the two analyses (**Figure 18B**). Only IST and PPM3C, which has been shown to be involved in export of dense granule effectors (Mayoral et al. 2020), were found to significantly perturb the host cell gene expression in the IFN γ -stimulated data but not the unstimulated data, indicating there may not be any further unidentified effectors that interfere with the induction of ISGs (**Figure 18C**). NSM was not included in this screen as we did not have protospacer oligonucleotides targeting NSM available.

To gain the maximum power to identify significant effectors, I applied Hotelling's t^2 -test to the combined unstimulated and IFN γ -stimulated data (**Figure 18D**). 22 targets genes significantly perturbed the host cell transcriptome, including all 14 controls included in the screen. Surprisingly, I found significant perturbations for both GRA7 and GRA25, despite not identifying any differentially expressed host genes for these knockouts in the earlier experiment targeting 24 *T. gondii* genes (**Figure 15C**). Potentially, the greater coverage of these genes in this screen in terms of number of single cell transcriptomes, and the use of multiple sgRNAs for each target, allows the detection of more subtle impacts. Most interestingly, I identified six *T. gondii* genes which have not previously been implicated in host cell reprogramming: 222100, GRA57, ROP9, GRA59, GRA3, and GRA70.

Global analysis of putative effector proteins

To further investigate these putative effector proteins, I generated pseudo-bulk transcriptomes for each target gene in the screen represented by at least 30 single-cell transcriptomes, corresponding to the 25% percentile. I analysed these pseudo-bulk transcriptomes by PCA, finding that most (non-significant) genes grouped in a central cluster, while known effector proteins tended to occupy the extremes of the principal components (**Figure 18E and F**). For example, the first principal component separated a cluster of genes known to be involved in export of dense granules effector proteins into the host cell: MYR1-4 (Franco et al. 2016; Marino et al. 2018; Cygan et al. 2020), ROP17 (Panas, Ferrel, et al. 2019), GRA45 (Cygan et al. 2020; Y. Wang et al. 2020), and PPM3C (Mayoral et al. 2020). GRA44 has also been shown to affect effector protein export, although knockout of GRA44

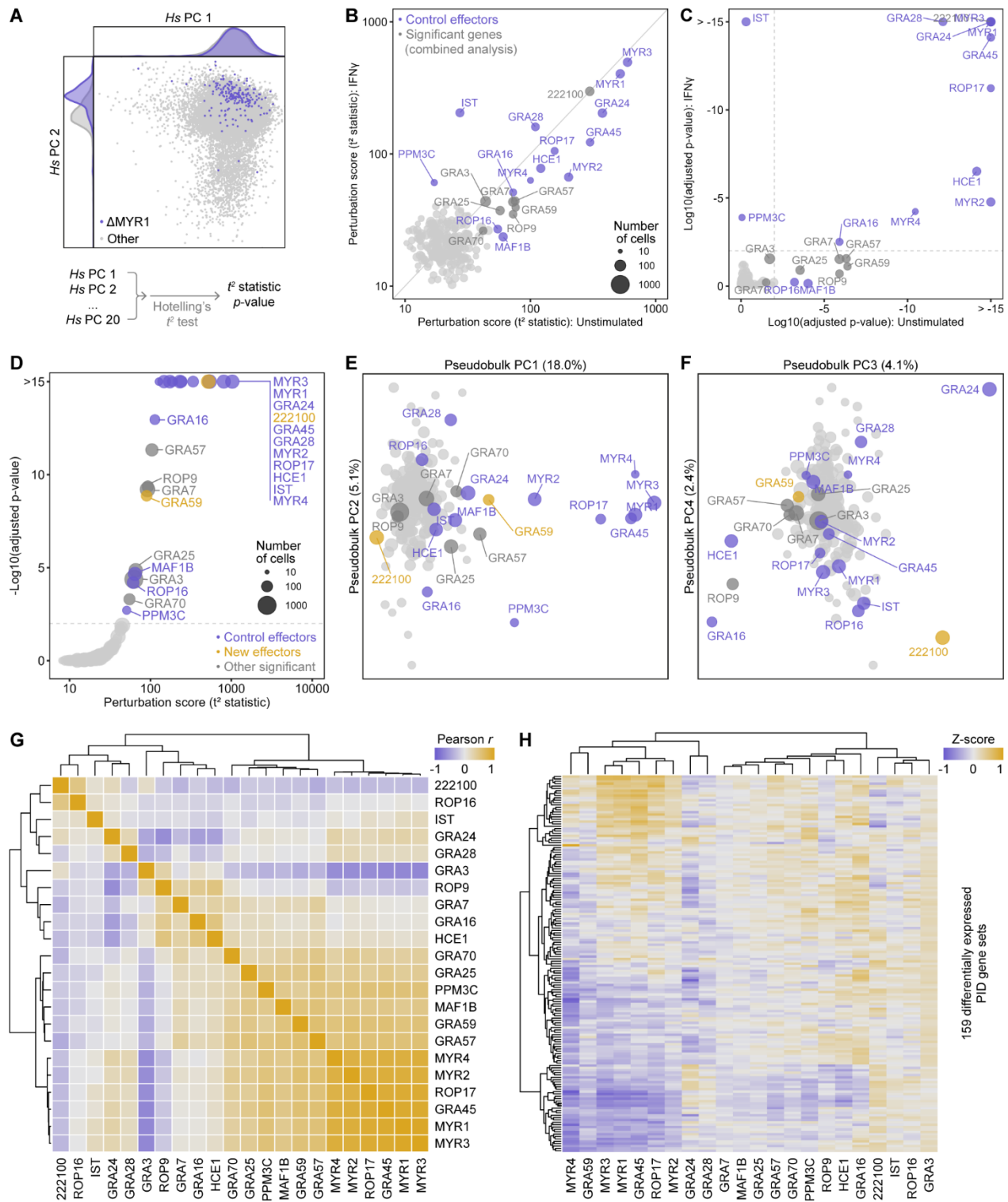


Figure 18. Analysis of dual perturb-seq data identifies 22 putative effector proteins.

- Illustration of Hotelling's t^2 -test on single cell PCA embeddings.
- Correlation between perturbation scores (Hotelling's t^2 -test statistic) in unstimulated and IFN γ -stimulated samples.
- Correlation between Hotelling's t^2 -test p -values in unstimulated and IFN γ -stimulated samples.
- Hotelling's t^2 -test results for combined dual perturb-seq data.
- PCA embeddings of pseudo-bulk transcriptomes for target genes represented by at least 30 single cell transcriptomes.
- PCA embeddings of pseudo-bulk transcriptomes for target genes represented by at least 30 single cell transcriptomes.
- Pearson correlation coefficients between pseudo-bulk transcriptomes of significant effectors.
- Average VISION signature scores of Pathway Interaction Database gene sets that are significantly differentially regulated by at least one significant effector ($p < 0.01$, two-sided Wilcoxon rank-sum test with Benjamini-Hochberg adjustment). See also **Figure 19**.

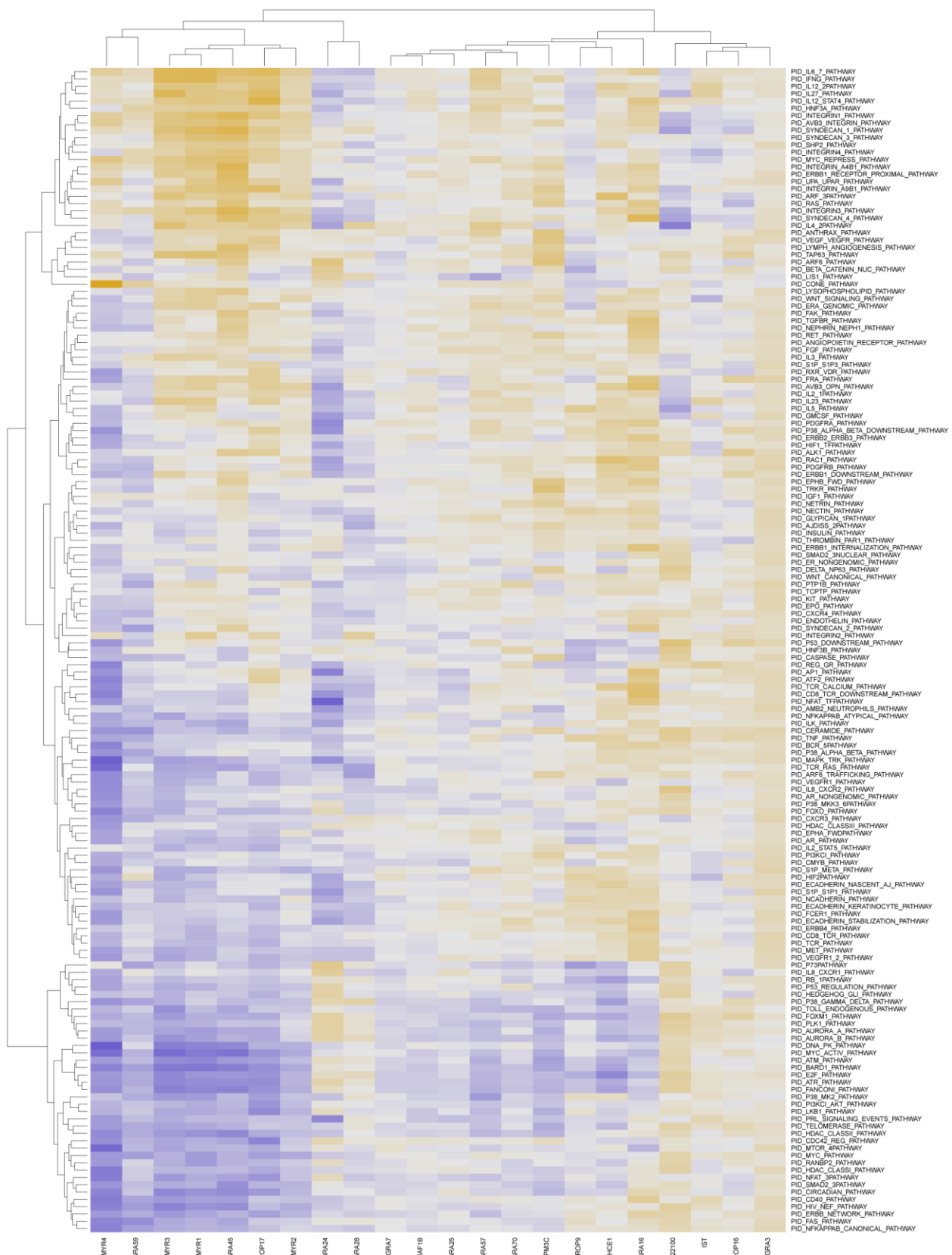


Figure 19. PID pathways differentially regulated by effector proteins.

Average VISION signature scores of Pathway Interaction Database gene sets that are significantly differentially regulated by at least one significant effector ($p < 0.01$, two-sided Wilcoxon rank-sum test with Benjamini-Hochberg adjustment).

has a strong negative impact on *T. gondii* growth, meaning we did not recover enough cells in this screen to analyse (Cygan et al. 2020; Blakely, Holmes, and Arrizabalaga 2020). As no other “exporter” protein shows such a strong contribution to parasite fitness *in vitro*, it seems likely that the apparent role of GRA44 in export is a secondary effect of loss of another function.

To more formally analyse the phenotypic relationships between the known effector proteins and putative effector proteins identified in this screen, I calculated pairwise Pearson correlation coefficients between the pseudo-bulk transcriptomes of all the target genes that significantly perturbed host cell transcription (**Figure 18G**). Hierarchical clustering based on these correlation coefficients identified four broad clusters of effector proteins. Firstly, I identified a tightly correlated cluster of six genes which have been shown to be essential for dense granule effector export: MYR1-4, ROP17, and GRA45 (Franco et al. 2016; Marino et al. 2018; Panas, Ferrel, et al. 2019; Cygan et al. 2020; Y. Wang et al. 2020). This set of genes could thus be proposed to be the “core” export machinery. Secondly, I identified a cluster of dense granule proteins which were more moderately correlated with this core export machinery: GRA59, GRA57, MAF1B, GRA25, PPM3C, GRA70. Thirdly, I identified five genes including two characterised effector proteins: HCE1, GRA16, ROP9, GRA7 and GRA3. HCE1/TEEGR has been shown to regulate the expression of E2F transcription factor targets, dysregulating the host cell cycle via Cyclin E and repressing NF- κ B target genes via EZH2 (Panas, Naor, et al. 2019; Braun et al. 2019). GRA16, meanwhile, modulates the expression of host cell metabolism- and cell cycle-related genes via p53 and PP2A (Bougdour et al. 2013). This third cluster is thus perhaps linked by regulation of the host cell cycle and/or metabolic pathways. Finally, I identified a cluster of five genes that I interpret as “immune regulators”: GRA28, which regulates host cell motility and cytokine expression through interaction with chromatin remodellers (Rudzki et al. 2021; Ten Hoeve et al. 2022), GRA24, which activates p38 α MAPK (Braun et al. 2013), IST, which inhibits the induction of ISGs by recruiting repressive nucleosome remodellers to STAT1 target genes (Olias et al. 2016; Gay et al. 2016), ROP16, which induces M2 polarisation in host cells by directly phosphorylating and activating STAT3 and STAT6 (Saeij et al. 2007; Yamamoto et al. 2009; Ong, Reese, and Boothroyd 2010), and an uncharacterised gene 222100, which is studied further in a later chapter.

Although this analysis informs which effectors are putatively functionally related, it does not inform what those functions are and instead relies on prior knowledge of established effector proteins. Therefore, to identify directly from the perturb-seq data which host cell pathways are regulated by each (putative) effector, I scored the single cell transcriptomes for expression of 196 gene sets from the Pathway Interaction Database (Schaefer et al. 2009; Liberzon et al. 2011) using VISION (DeTomaso et al. 2019). I tested whether each pathway was significantly up- or down-regulated for each effector compared to the background distribution of signature scores. I then clustered the effectors using the z-transformed mean signature scores of the gene sets that were significantly up- or down-regulated by at least one effector, with similar results to clustering using the Pearson correlation coefficients (**Figure 18H and Figure 19**).

These differentially regulated pathways confirmed the known phenotypes of many established effector proteins. For example, the most significantly downregulated pathway for sg(MYR1) and sg(GRA16)-expressing cells was “MYC ACTIV PATHWAY”; induction of c-Myc by *T. gondii* has been shown to require both MYR1 and GRA16 (Franco et al. 2016; Panas and Boothroyd 2020). The most significantly downregulated pathway for sg(HCE1)-expressing cells was the E2F pathway, which has

been shown to be induced by HCE1 (Panas, Naor, et al. 2019; Braun et al. 2019). p38 α MAPK is phosphorylated and activated in by GRA24 (Braun et al. 2013); correspondingly the “P38 ALPHA BETA DOWNSTREAM” pathway is significantly downregulated in cells infected with GRA24 knockouts. As a final example, the IL-12 and IFN pathways are upregulated in IST knockout-infected cells, as IST normally blocks these responses by inhibiting STAT1-mediated transcription (Gay et al. 2016; Olias et al. 2016).

Inspecting these differentially expressed pathways also informs on the putative functions of newly identified effector proteins. For example, sg(ROP9)-expressing cells appear to downregulate the E2F pathway, indicating this protein may cooperate with HCE1 to modulate the host cell cycle. Knockout of GRA59 appears to downregulate many of the same pathways as knockout of MYR1 and other exporter proteins, indicating that GRA59 may similarly be involved in export of dense granule effectors. Finally, the most strongly downregulated pathway for the as-yet-uncharacterised protein 222100 was the IL-4 pathway, indicating that this protein may cooperate with the rhoptyr kinase ROP16 that has been shown to directly phosphorylate and activate STAT3 and STAT6, which are the signal transducers for IL-4 stimulation (Saeij et al. 2007; Yamamoto et al. 2009; Ong, Reese, and Boothroyd 2010).

Knockout of effector proteins does not perturb the *T. gondii* transcriptome

I hypothesised that knockout of some effector proteins may have a broader impact on the transcriptome of intracellular *T. gondii* parasites, for example through nutrient starvation of the parasite or as a result of increased host immune pressure. I applied Hotelling’s t^2 -test to principal component embeddings of the single cell transcriptomes based on the normalised parasite gene expression data, and found a significant perturbation score only for GRA3 (**Figure 20B**). However, inspection of *T. gondii* genes differentially regulated in sg(GRA3)-expressing cells revealed that this perturbation was primarily driven by downregulation of GRA3 itself, with no other genes with log₂ fold-changes greater than 0.11 (**Figure 20C**).

I note, however, that the knockout parasite pool in this screen is selected *in vitro* for at least seven days, such that any knockouts which severely adversely affect parasite fitness are likely to be

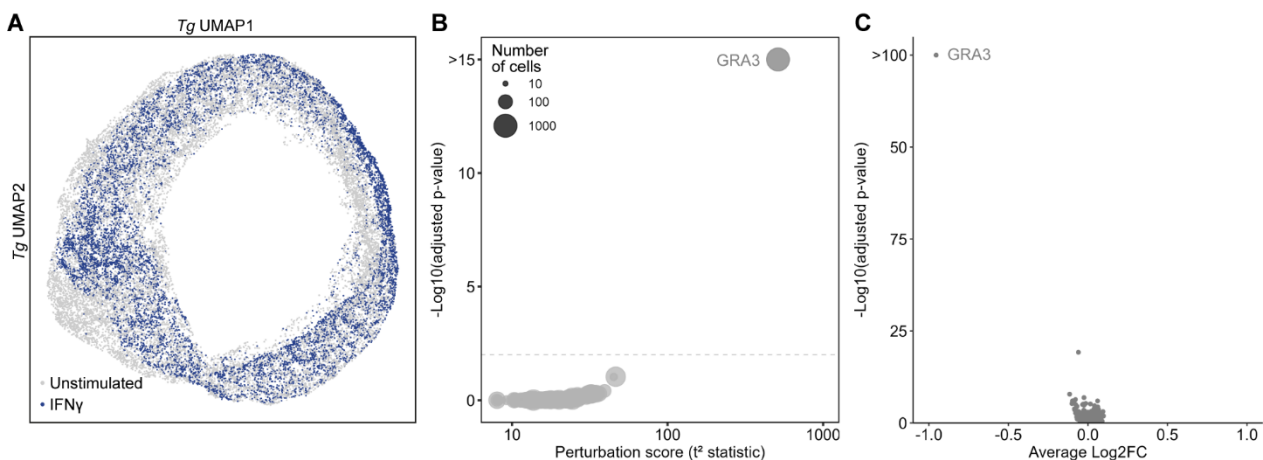


Figure 20. Knockout of effector proteins does not perturb the *T. gondii* transcriptome.

- A. UMAP of single cell transcriptomes using *T. gondii* gene expression, coloured by IFN γ stimulation.
- B. Hotelling t^2 -test results on PCA embeddings of single cell transcriptomes using *T. gondii* gene expression.
- C. Differentially expressed *T. gondii* genes for sg(GRA3)-expression cells.

depleted from the population. I observed this, for example, for GRA44 (Cygan et al. 2020; Blakely, Holmes, and Arrizabalaga 2020). A screen using rapid inducible knockout/knockdown or protein depletion would be more suited to identifying transcriptional changes caused by knockout of *T. gondii* genes (W. Li et al. 2022; Smith et al. 2022). However, from this analysis it seems that knockout of any effector protein included in this screen that is not essential *in vitro* is unlikely to cause broader changes in the *T. gondii* transcriptome.

Discussion

Intracellular bacteria, viruses, and eukaryotic pathogens all manipulate host cell gene expression using effector proteins to downregulate immune responses. Examples include the ORF6 accessory protein of SARS-CoV-2, which blocks nuclear trafficking to prevent the import of STAT transcription factors (Addetia et al. 2021; Miorin et al. 2020), and the secreted effector protein SteE of *Salmonella*, which modifies the substrate specificity of the host GSK3 kinase to drive M2 polarisation that is beneficial for bacterial replication and persistence (Panagi et al. 2020; Jaslow et al. 2018). Mutualistic endosymbionts also downregulate host immune pathways to enable colonisation, although the mediators of these interactions are less well studied. For example, colonisation of a cnidarian host by dinoflagellate *Symbiodinium spp.* involves downregulation of NF- κ B signalling, preventing expulsion of the endosymbiont (Mansfield and Gilmore 2019; Jacobovitz et al. 2021). Dual perturb-seq can therefore be envisaged as a tool to identify the molecular mediators of microbe-host transcriptional interactions not just for *T. gondii*, but also for other intracellular pathogens and endosymbionts. Although in this work, I screened a library of *T. gondii* mutants, it is also feasible to genetically manipulate the host cell and assess the transcriptional response of the pathogen/symbiont.

The principal advantage of perturb-seq methods, and by extension dual perturb-seq, is the ability to multiplex hundreds to thousands of genetic perturbations in a single experiment (Adamson et al. 2016; Dixit et al. 2016; Replogle et al. 2020). As in classic CRISPR screens, perturbations are carried out by pooled transfection/transduction and the sgRNA protospacer (or an alternative linked barcode) is retained in the cell as a record of the manipulation. However, rather than assessing the changes in the distribution of barcodes at a population level, the phenotypes of individual cells are deconvolved by scRNA-seq and the impact of specific modifications compared to a reference population. Perturb-seq thus combines the high-throughput of classical CRISPR screens with information-rich phenotypes from whole single-cell transcriptomes, making it ideally suited to systems where many target genes are poorly studied or completely uncharacterised.

While perturb-seq is typically carried out by lentiviral transduction, I instead delivered Cas9-sgRNA plasmid vectors by electroporation, highlighting that perturb-seq is possible in diverse non-mammalian cells using an extant commercial platform. The only requirement is that CRISPR-Cas9 tools are sufficiently developed to allow modification of the target species, and that expression of the sgRNA or another linked barcode (Adamson et al. 2016; Dixit et al. 2016) is maintained to identify the modification in the final dataset. I tested two previously validated sgRNA-capture sequence configurations (Replogle et al. 2020) and found that sgRNA-CS1 was much more efficiently recovered than sgRNA-CS2. The expression level of the sgRNA here appeared to be far lower than in published data (tens of UMIs per cell compared to hundreds) (Replogle et al. 2020).

Therefore, even with sgRNA-CS1, we were unable to use ~25% of captured cells as the perturbation could not be determined. Further optimisation of sgRNA capture or expression would be beneficial to increase the number of useable single cell transcriptomes in the final dataset.

Compared to bulk transcriptomics, perturb-seq reagents and sequencing costs are relatively high when only a small number of targets are included. However, at the scale of tens to hundreds to thousands of perturbations, perturb-seq benefits from economy of scale and enables screening experiments that would not be feasible by bulk RNA sequencing due to cost and labour requirements. The dual perturb-seq experiments I carried out here can theoretically be completed in under three weeks, from selection of the protospacer sequences to receipt of the sequencing data.

The experiments presented here scale two orders of magnitude in the number of target genes. The primary factor limiting further expansion of the target pool is the cost of reagents and sequencing required for sufficient coverage of the library. Importantly, I find that significant perturbations can be identified with as few as 30-50 single cell transcriptomes per target gene, in comparison to coverage ranging from hundreds to thousands of cells per target/variant in the majority of perturb-seq studies (Adamson et al. 2016; Dixit et al. 2016; Replogle et al. 2022; Ursu et al. 2022), significantly reducing the cost of such experiments. While the ability to detect differentially expressed genes is compromised by this reduced coverage due to the inherent high noise and low dynamic range of scRNA-seq, I instead used a gene set-based approach which was effective at identifying differentially regulated pathways.

Inspired by a recent study investigating coding variants of KRAS and TP53 (Ursu et al. 2022), I used Hotelling's t^2 -test to identify significant perturbations based on the distribution of single cell transcriptomes in PCA space. Despite the enormous complexity of the dual perturb-seq data, this single test efficiently identified all known effectors. The test is computationally trivial and, as it operates in a latent space embedding of the data, is likely to be generalisable to many different types of perturb-seq experiments.

With Hotelling's t^2 -test, I identified six *T. gondii* gene knockouts that significantly perturb the host cell but have not previously been studied in this regard: 222100, GRA57, ROP9, GRA59, GRA3, and GRA70. Of these candidate effectors, 222100 has the strongest and most significant impact. sg(222100)-expressing cells strongly downregulate the IL4 pathway, indicating that this gene may cooperate with the rhoptry kinase ROP16 to activate this pathway via STAT3 and STAT6 phosphorylation (Saeij et al. 2007; Yamamoto et al. 2009; Ong, Reese, and Boothroyd 2010). Concordantly, the pseudo-bulk transcriptome for sg(222100)-expressing cells correlated strongly with that of sg(ROP16)-expression cells, indicating that knockout of either these two genes has similar effects on the host cell.

GRA59 was identified as a significant effector in the perturb-seq screen and was the only uncharacterised gene included in the 24-guide pilot experiment for which a large number of differentially regulated host genes were identified. GRA59 is a dense granule protein that was previously shown to localise to the vacuole in tachyzoites and the cyst wall of bradyzoites. The pseudo-bulk transcriptome of sg(GRA59)-expressing cells correlated modestly with those of the core exporter proteins MYR1-4, ROP17, and GRA45. In addition, PCA of the pseudo-bulk transcriptomes indicated that GRA59 has an intermediate phenotype between the core exporter proteins and all other knockouts, based on its position along the first principal component. Finally,

many of the host cell pathways differentially regulated by the core exporter proteins were similarly regulated by GRA59, causing these genes to cluster together. These data strongly suggest that GRA59 is involved in export of dense granule effector proteins, although perhaps contributes more modestly than the established core exporters for which knockout completely ablates export.

GRA57 and GRA70, which are also moderately correlated with the core exporters, were recently shown to be part of a heterotrimeric complex (along with GRA71) that is important for resistance to IFN γ -mediated restriction in HFFs (Lockyer et al. 2022). Knockout of GRA57 did not affect the bulk transcriptome of unstimulated or IFN γ -stimulated cells, so it is surprising that GRA57 is a significant hit in this screen. Possibly, GRA57 may affect the transcriptome only in a subset of infected cells which may not be detectable in bulk RNA-seq data, or it may be a false positive hit here. However, the presence of two different members of this complex suggests further investigation may be warranted.

ROP9 clustered with the established dense granule effector proteins HCE1/TEEGR and GRA16. Both HCE1 and GRA16 have been shown to regulate the expression of host cell cycle genes via E2F transcription factors and p53 respectively (Panas, Naor, et al. 2019; Bougdour et al. 2013), indicating that ROP9 may also be involved in regulating the host cell cycle. As for HCE1, the E2F pathway is the most significantly downregulated for sg(ROP9)-expressing cells, while the most strongly downregulated pathway is for p73, a paralogue of p53. ROP9 might act independently of HCE1 or GRA16, or may cooperate with one or both these dense granule effectors. Cooperation between rhoptry and dense granule effectors has previously been identified in regulation of the MYR export pathway by the rhoptry kinase ROP17 (Panas, Ferrel, et al. 2019).

A putative function of GRA3 is more difficult to determine from the dual perturb-seq data, and the significant result for this gene should be interpreted with care. GRA3 is the most highly represented out of all target genes, with more than 600 single cell transcriptomes each in the unstimulated and IFN γ -stimulated data. Despite such high representation, GRA3 did not significantly perturb the host gene expression in either dataset when analysed separately, only when the data were combined for a total of >1,200 single cell transcriptomes. A large number of gene sets are significantly upregulated for GRA3, with the most significant being the sphingosine-1-phosphate and platelet-derived growth factor signalling pathways. GRA3 has been suggested to recruit either host endoplasmic reticulum or Golgi to the vacuole and potentially dysregulates host anterograde transport, meaning that it might have subtle but wide-ranging effects on the host cell phenotype (Kim et al. 2008; Deffieu et al. 2019).

Secreted proteins of *T. gondii* are under strong diversifying selective pressure, likely due to exposure to the host immune response (Barylyuk et al. 2020). As a result, there are several instances in which the functions of effector proteins are not conserved between different strains of *T. gondii*. For example, ROP16 is active in type I, III, and atypical strains (including the RH strain used in this work), but has a mutation that ablates kinase activity in type II strains (Saeij et al. 2007). In contrast, GRA15 activates host NF- κ B signalling in type II-infected cells, but does not for type I-infected cells. Although GRA18 in the PRU strain has been shown to activate gene expression by β -catenin (He et al. 2018), the lack of any phenotype for GRA18 in the work here indicates that it is likely also a strain-specific effector. Given the divergent transcriptomes of host cells infected with different *T. gondii* strains (Melo et al. 2013), it is likely that additional strain-specific effectors will be identified. As dual perturb-seq enables screening of the entire *T. gondii* secretome in a single experiment, it will enable

systematic characterisation of the roles of effector proteins in different strains of *T. gondii* and thus identify strain-specific effectors.

Furthermore, *T. gondii* is capable of infecting many different cell types and host species, and there is evidence that different cell types respond differently to infection (Swierzy et al. 2017). Thus, dual perturb-seq can also be used to explore how the *T. gondii* secretome interacts with differing host cell epigenetic landscapes and whether there are effector proteins with roles in specific host cell types or host species.

In summary, with dual perturb-seq it is possible to screen hundreds of genes in an intracellular pathogen or symbiont for an effect on the transcriptome of the host cell. In *T. gondii*, this screen identified all known transcriptional effector proteins and novel candidate effectors. By inspection of pseudo-bulk transcriptomes by PCA and inter-effector correlation, I was able to identify clusters of functionally related effector proteins and for the first time gain a global view of host cell reprogramming by the *T. gondii* effectome. Finally, from the same data I was able to identify differentially regulated host cell pathways for each of these effector proteins, as a first step to establishing their function.

Chapter 3: GRA59 contributes to export of dense granule effectors

Results

Localisation of GRA59

Data from the dual perturb-seq screen indicated that the dense granule protein GRA59 may be involved in export of other dense granule effector proteins from the vacuole into the host cytosol, along with MYR1-4, ROP17, and GRA45 (Franco et al. 2016; Marino et al. 2018; Cygan et al. 2020; Panas, Ferrel, et al. 2019; Y. Wang et al. 2020).

GRA59 was predicted to localise to the dense granules by spatial proteomics (Barylyuk et al. 2020) and, post-secretion, was shown to localise to the parasitophorous vacuole of tachyzoites and the cyst wall of bradyzoites (Nadipuram et al. 2016). To confirm and further refine the post-secretory localisation of GRA59, I tagged GRA59 with a single HA epitope at the C-terminus. Integration of the tagging construct into the genome was verified by diagnostic PCR (**Figure 21A**) and expression of GRA59-HA was confirmed by Western blot (**Figure 21B**). GRA59 migrates at an apparent molecular weight of ~40 kDa, moderately higher than the predicted molecular weight of 35 kDa. GRA59 appears to be proteolytically cleaved, leaving a C-terminal fragment with an apparent molecular weight of ~25 kDa. GRA59 has a putative transmembrane domain that is predicted with moderate confidence (~0.6 posterior probability) by Phobius (Madeira et al. 2022).

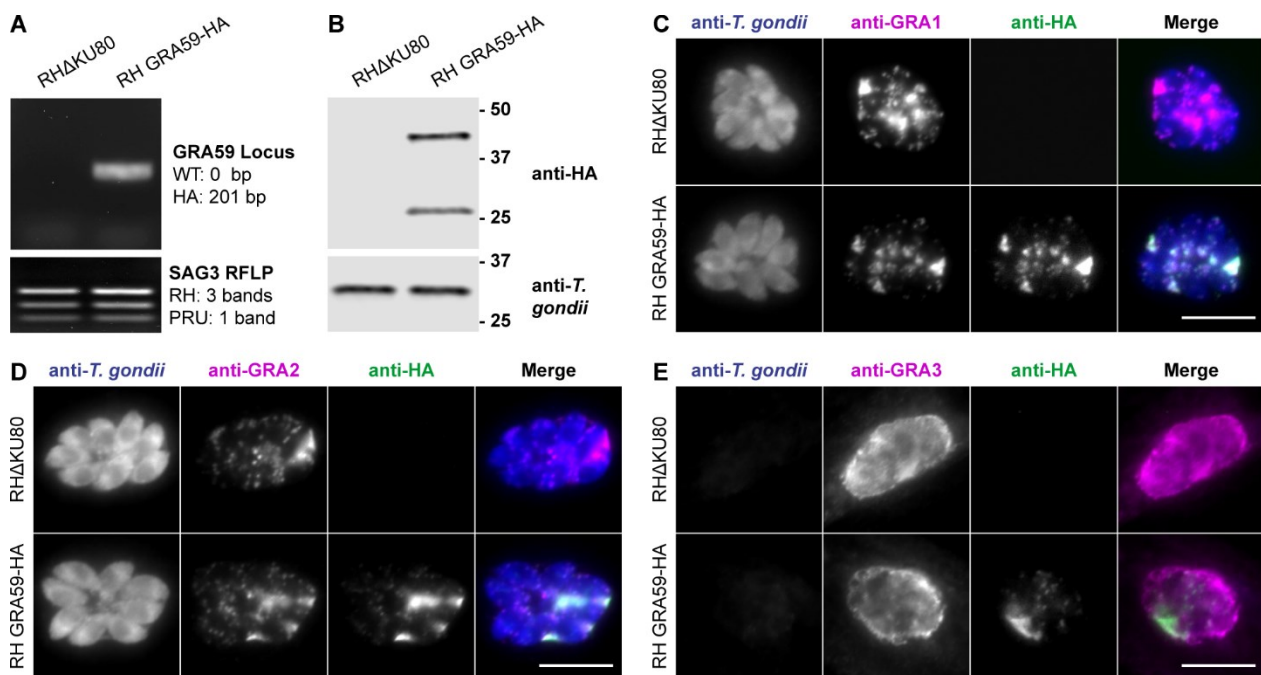


Figure 21. GRA59 localises to the parasitophorous vacuole.

A. Diagnostic PCR verification of integration of HA-tagging construct and RFLP verification of *T. gondii* strain.

B. Western blot confirmation of GRA59-HA expression.

C. Immunofluorescence co-localisation of GRA59-HA and GRA1 at 24 hpi in cells permeabilised with Triton X-100. Scale bar = 10 μm.

D. Immunofluorescence co-localisation of GRA59-HA and GRA2 at 24 hpi in cells permeabilised with Triton X-100. Scale bar = 10 μm.

E. Immunofluorescence co-localisation of GRA59-HA and GRA3 at 24 hpi in cells permeabilised with saponin. Scale bar = 10 μm.

Through immunofluorescence microscopy, I found that GRA59 co-localised with both the vacuole lumen marker GRA1 (Cesbron-Delauw et al. 1989) and with the intravacuolar network marker GRA2 (Mercier, Cesbron-Delauw, and Sibley 1998) following full permeabilisation of the vacuole (**Figure 21C and D**). Differential permeabilisation of the host cell but not the vacuole (using saponin rather than Triton X-100) showed that GRA59 partially co-localises with the PVM marker GRA3 (Dubremetz et al. 1993) and indicated that the C-terminus of GRA59 is exposed on the cytosolic face of the PVM (**Figure 21E**). Accordingly, GRA59 was recently shown to be exposed to the host cytosol by proximity biotinylation (Cygan et al. 2021).

GRA59 knockout affects host cell phenotypes induced by dense granule effectors

To further investigate the function of GRA59, I generated a knockout cell line in the *T. gondii* RH Δ KU80 background and complemented this line with an HA-tagged copy of GRA59. I verified integration of the knockout and complementation constructs into the genome by diagnostic PCR (**Figure 22A**), and confirmed the expression of GRA59-HA in the complemented strain by Western blot and immunofluorescence microscopy (**Figure 22B and C**). A plaque assay did not show any difference in the growth rate of Δ GRA59 parasites (**Figure 22D**), confirming the neutral fitness score from a prior genome-wide screen in HFFs (Sidik et al. 2016).

Based on the hypothesis that GRA59 contributes to dense granule effector export, I measured nuclear c-Myc immunofluorescence intensity in HFFs infected with these strains for 24 h. Upregulation of c-Myc by *T. gondii* is entirely dependent on the exporter protein MYR1 (Franco et al. 2016) and partially dependent on the exported effector GRA16 (Panas and Boothroyd 2020). While, as expected, the parental RH Δ KU80 strain strongly induced c-Myc compared to uninfected cells and knockout of MYR1 completely ablated this upregulation, knockout of GRA59 resulted in an intermediate phenotype that was rescued by complementation (**Figure 22E**). This phenotype is consistent either with a role for GRA59 either in effector export or in directly upregulating c-Myc, in addition to or in partnership with GRA16.

To differentiate between these hypotheses, I measured host cell phenotypes induced by three other MYR-dependent dense granule effectors: phosphorylation and nuclear translocation of p38 α MAP kinase by GRA24 (Braun et al. 2013), upregulation of EZH2 by HCE1/TEEGR (Panas, Naor, et al. 2019; Braun et al. 2019), and inhibition of IFN γ -induced IRF1 expression by IST (Gay et al. 2016; Olias et al. 2016). As for c-Myc, knockout of GRA59 resulted in an intermediate reduction in p38 phosphorylation and EZH2 upregulation compared to RH Δ KU80 and RH Δ MYR1 (**Figure 22F and G**). These results indicate that GRA59 contributes to the export of multiple effector proteins, but that knockout of GRA59 may not completely abolish export, as is the case for MYR1 (Franco et al. 2016). In contrast, suppression of IRF1 upregulation was not affected by GRA59, suggesting normal export of the effector protein IST (**Figure 22H**).

GRA59 contributes to export of dense granule effectors

To directly test whether knockout of GRA59 affects export of dense granule effectors, I introduced HA-tagged copies of GRA16 and IST to the RH Δ KU80, RH Δ GRA59, and RH Δ MYR1 strains. I verified integration of the GRA16-HA and IST-HA constructs into the UPRT locus by diagnostic PCR (**Figure 23A and C**), and confirmed expression of GRA16-HA and IST-HA protein by Western blot (**Figure 23B and D**).

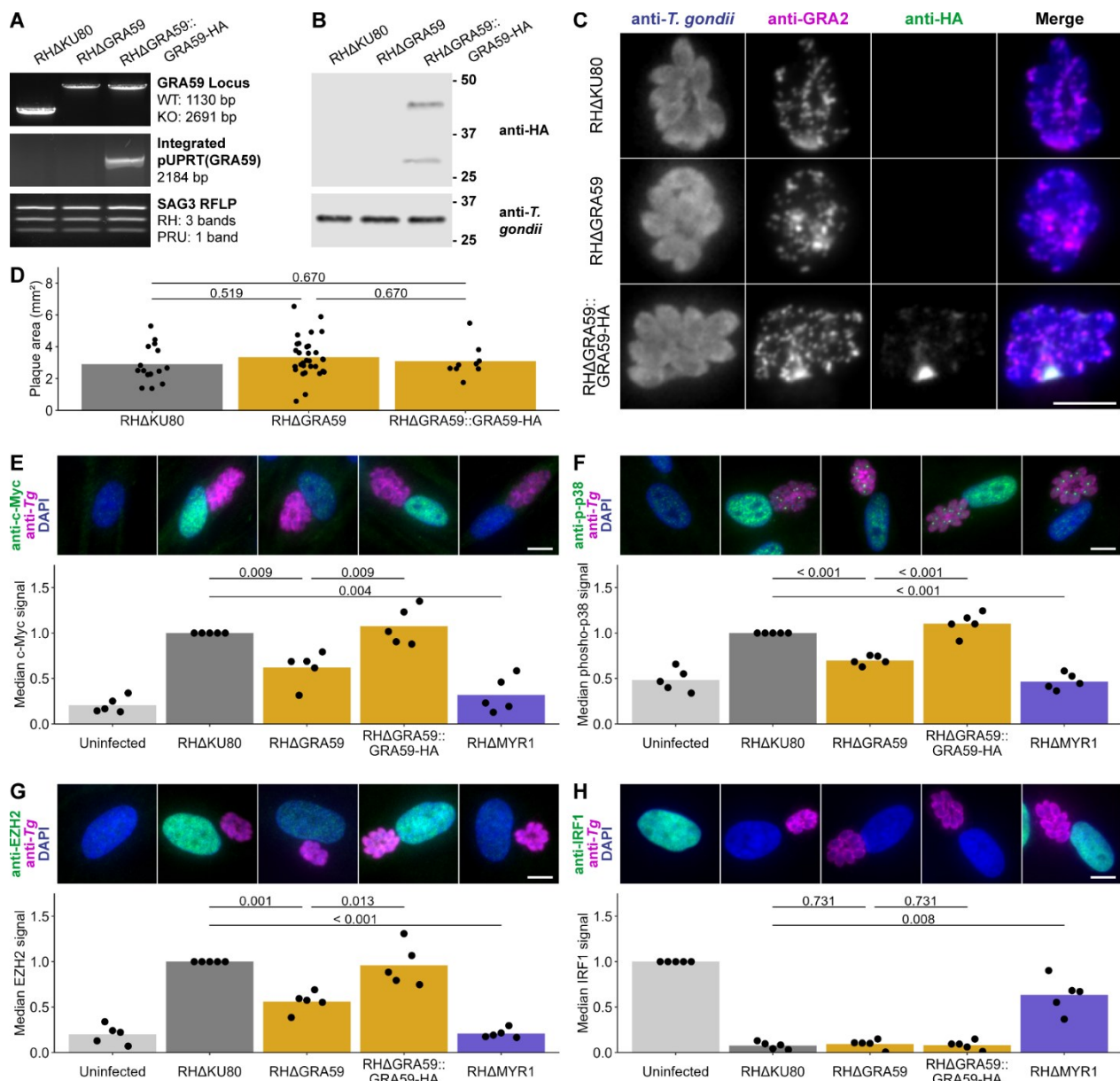


Figure 22. GRA59 knockout affects host cell phenotypes induced by dense granule effectors.

- A.** Diagnostic PCR verification of integration of knockout and complementation constructs and RFLP verification of *T. gondii* strain
- B.** Western blot confirmation of GRA59-HA expression.
- C.** Immunofluorescence verification of GRA59-HA expression. Scale bar = 10 μm.
- D.** Measurement of plaque sizes for RHΔKU80, RHΔGRA59, and RHΔGRA59::GRA59-HA. One biological replicate; points represent individually measured plaques. *p*-values calculated by two-sided *t*-test with Benjamini-Hochberg adjustment.
- E.** Immunofluorescence quantification of nuclear c-Myc in HFFs infected for 24 h. Scale bar = 10 μm. *p*-values calculated by two-sided Wilcoxon rank sum test with Benjamini-Hochberg adjustment.
- F.** Immunofluorescence quantification of nuclear phospho-p38α MAP kinase in HFFs infected for 24 h. Scale bar = 10 μm. *p*-values calculated by two-sided Wilcoxon rank sum test with Benjamini-Hochberg adjustment.
- G.** Immunofluorescence quantification of nuclear EZH2 in HFFs infected for 24 h. Scale bar = 10 μm. *p*-values calculated by two-sided Wilcoxon rank sum test with Benjamini-Hochberg adjustment.
- F.** Immunofluorescence quantification of nuclear IRF1 in HFFs infected for 24 h and stimulated with IFNγ at 1 hpi. Scale bar = 10 μm. *p*-values calculated by two-sided Wilcoxon rank sum test with Benjamini-Hochberg adjustment.

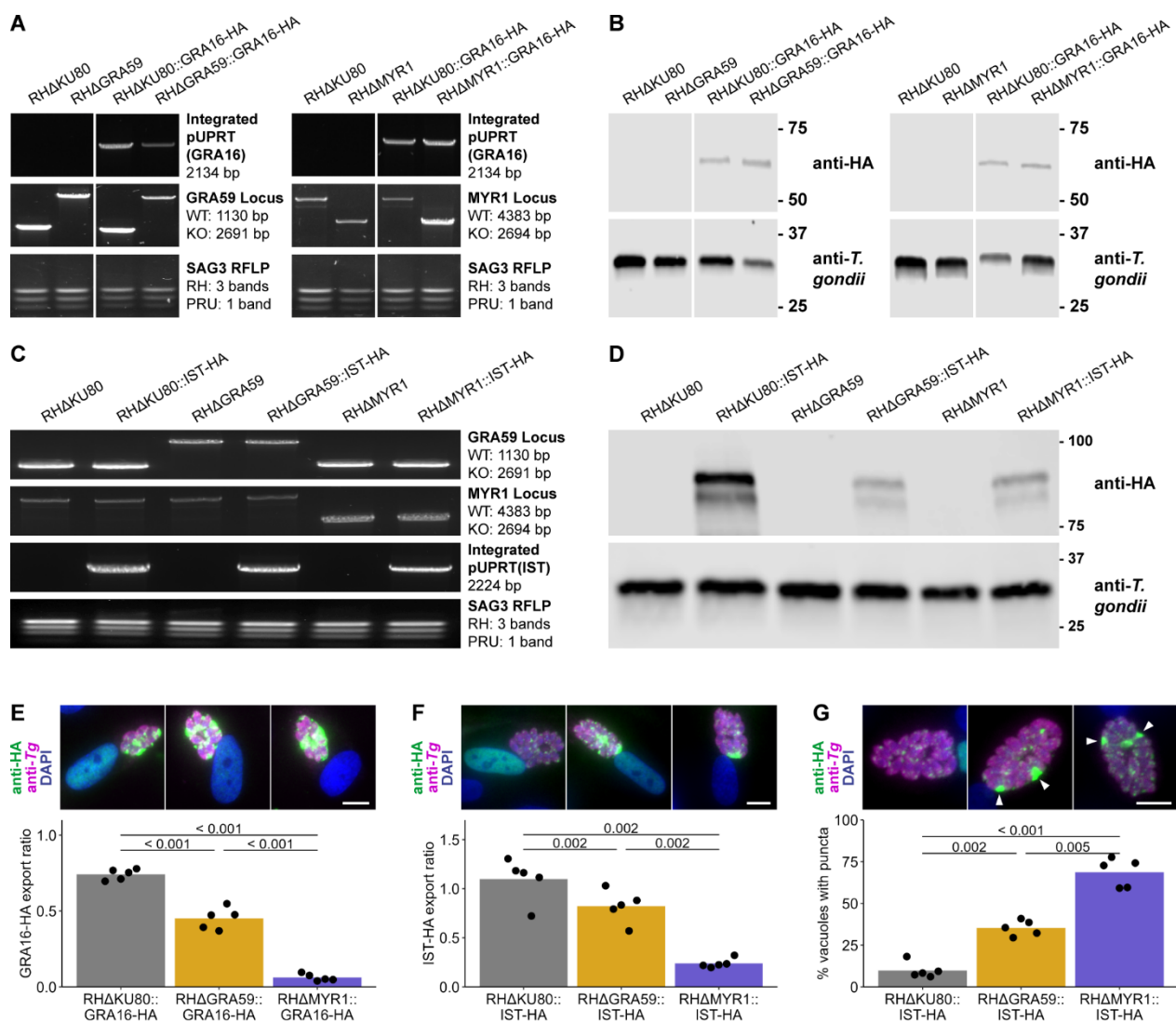


Figure 23. GRA59 affects export of dense granule effectors.

A. PCR verification of pUPRT(GRA16-HA) integration and RFLP verification of *T. gondii* strain.

B. Western blot confirmation of GRA16-HA protein expression.

C. PCR verification of pUPRT(IST-HA) integration and RFLP verification of *T. gondii* strain.

D. Western blot confirmation of IST-HA protein expression.

E. Measurement of the amount of GRA16-HA immunofluorescence signal in the host nuclei compared to the vacuole (export ratio). Scale bar = 10 μm. *p*-values calculated by paired two-sided *t*-test with Benjamini-Hochberg adjustment.

F. Measurement of the amount of IST-HA immunofluorescence signal in the host nuclei compared to the vacuole (export ratio). Scale bar = 10 μm. *p*-values calculated by paired two-sided *t*-test with Benjamini-Hochberg adjustment.

G. Percentage of vacuoles containing accumulations of IST-HA, indicated by white arrowheads. Scale bar = 10 μm. *p*-values calculated by paired two-sided *t*-test with Benjamini-Hochberg adjustment.

Both GRA16 and IST localise to the host cell nucleus after secretion via the MYR complex (Bougdoor et al. 2013; Gay et al. 2016; Olias et al. 2016). To quantify effector export, I measured the ratio of anti-HA immunofluorescence signal in the nuclei of HFFs infected for 24 h compared to the signal remaining in the vacuole and intracellular parasites. Export of GRA16-HA to the host nucleus was reduced in the RHΔGRA59 strain compared to RHΔKU80, but remained significantly higher than in RHΔMYR1, matching the phenotype expected from c-Myc induction (**Figure 23E**). Surprisingly,

export of IST-HA was also reduced in the RHΔGRA59 strain **Figure 23F**), even though suppression of IRF1 was not affected (**Figure 22H**). Potentially, this is because IST acts in an inhibitory manner, in contrast to the other effectors which activate signalling pathways. IST binds to STAT1 and recruits chromatin repressor complexes to STAT1 target genes; it may therefore be that a small reduction in the amount of IST-STAT1 complex does not affect suppression of STAT1-driven gene expression.

For IST-HA, I additionally noticed accumulations of IST-HA signal within the vacuole of RHΔGRA59 and RHΔMYR1 parasites that were largely absent in RHΔKU80. After quantifying the percentage of vacuoles containing these accumulations, I again determined an intermediate phenotype for RHΔGRA59 (**Figure 23G**). These data suggest that reduced export of IST leads to an accumulation of IST within the vacuole. Nearly all vacuoles, including those of RHΔKU80, contained accumulations of GRA16-HA, therefore it was not possible to quantify retention of GRA16-HA in the same manner.

Together, these data confirm the apparent phenotype of GRA59 in the dual perturb-seq screen: GRA59 has a MYR1-like phenotype and contributes to the export of dense granule proteins from the vacuole into the host cell. Unlike for the core export machinery, knockout of GRA59 does not completely ablate export. Instead, there is an intermediate reduction in export of GRA16 and IST, and indications that export of GRA24 and HCE1 are similarly affected.

GRA59 does not affect effector export in *T. gondii* PRU

GRA59 is highly conserved between *T. gondii* strains, with ≥99% sequence identity across all strains. In type II strains, there is only a single amino acid deletion compared to type I strains (**Figure 24A**). However, I noticed that expression level of GRA59 in publicly available RNA-seq data varies substantially between *T. gondii* strains (Minot et al. 2012). Specifically, GRA59 is highly expressed in type I and atypical strains but is expressed at a much lower level in type II and type III strains (**Figure 24B**). This contrasts with the export proteins MYR1-4, which are highly expressed in all strains.

To further explore the polymorphic expression of GRA59, I generated a knockout cell line in the PRUΔKU80 strain, in which GRA59 is expected to be expressed at only ~1% of the amount relative to RHΔKU80 at the transcript level. I then complemented this line using the same vector as for the RH strain, with the promoter and coding sequence of GRA59 cloned from RHΔKU80 genomic DNA, to determine whether increased expression or the single amino acid difference in the coding sequence would alter the phenotype of the PRU strain. I verified integration of the knockout and complementation constructs by PCR (**Figure 25A**), and verified the expression of GRA59-HA protein by Western blot and immunofluorescence microscopy (**Figure 25B and C**). As for RH, there

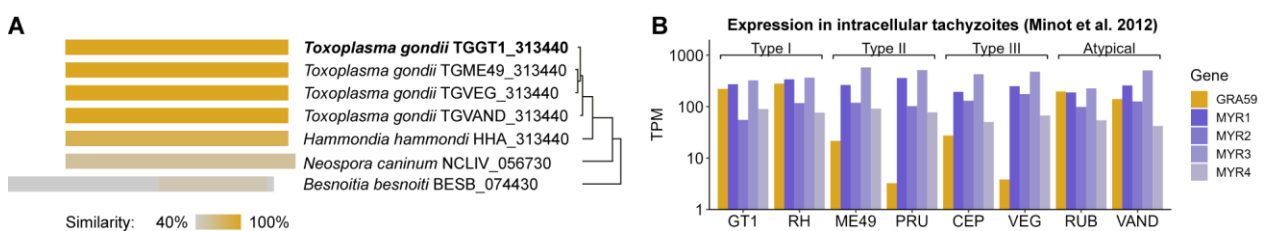


Figure 24. GRA59 mRNA expression varies between different *T. gondii* strains.

A. Protein sequence alignment of GRA59 homologues detected by BLASTP (E-value <0.01). Guide tree generated using Clustal Omega.

B. Expression level of GRA59 and MYR1-4 mRNA in intracellular tachyzoites. RNA-seq data from Minot et al. 2012.

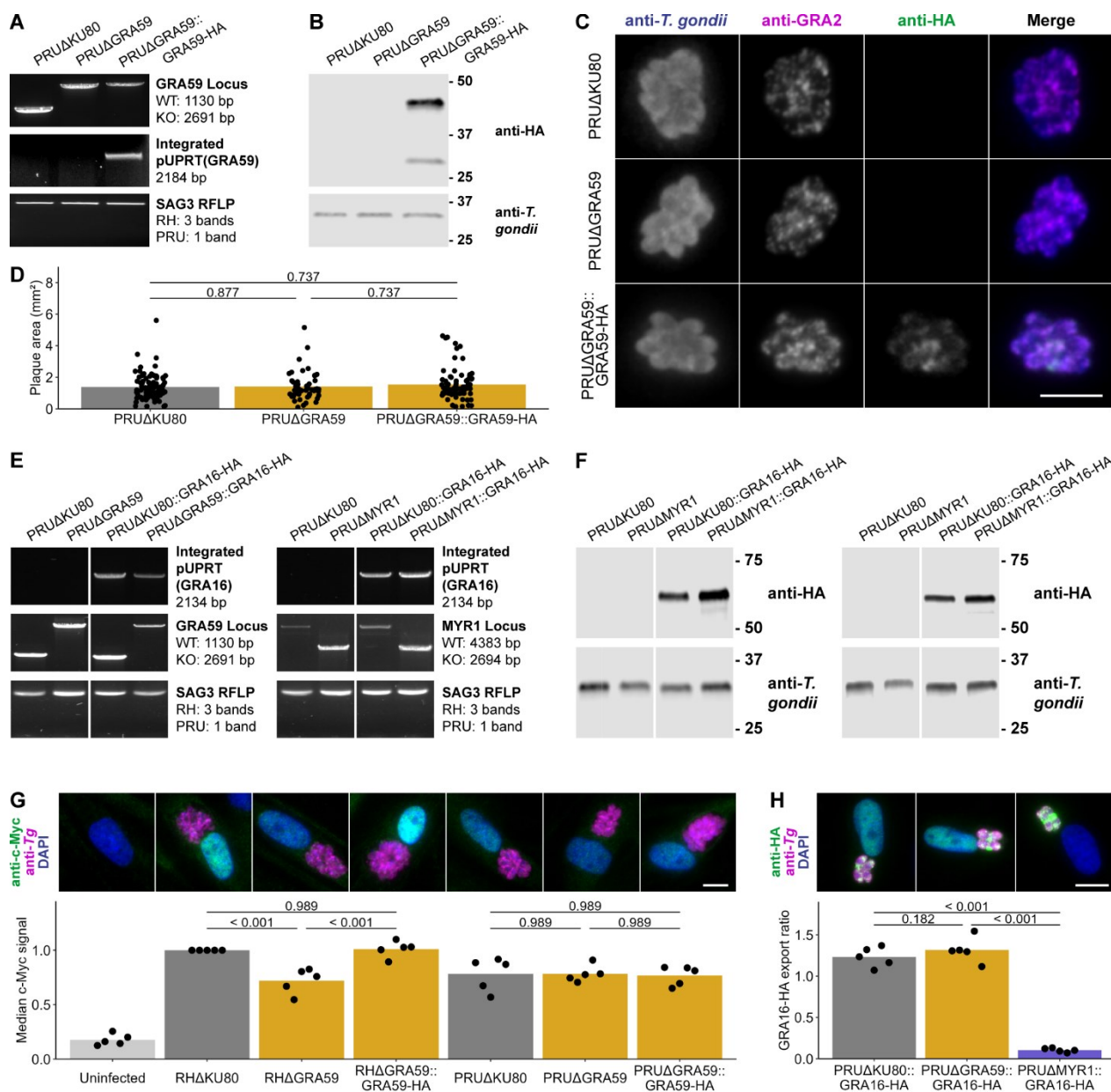


Figure 25. GRA59 does not affect effector export in the PRU strain.

- A.** Diagnostic PCR verification of integration of knockout and complementation constructs and RFLP verification of *T. gondii* strain
- B.** Western blot confirmation of GRA59-HA expression.
- C.** Immunofluorescence verification of GRA59-HA expression. Scale bar = 10 μm.
- D.** Measurement of plaque sizes for PRUΔKU80, PRUΔGRA59, and PRUΔGRA59::GRA59-HA. One biological replicate; points represent individually measured plaques. *p*-values calculated by two-sided *t*-test with Benjamini-Hochberg adjustment.
- E.** PCR verification of pUPRT(GRA16-HA) integration and RFLP verification of *T. gondii* strain.
- F.** Western blot confirmation of GRA16-HA protein expression.
- G.** Immunofluorescence quantification of nuclear c-Myc in HFFs infected for 24 h. Scale bar = 10 μm. *p*-values calculated by two-sided Wilcoxon rank sum test with Benjamini-Hochberg adjustment.
- H.** Measurement of the amount of IST-HA immunofluorescence signal in the host nuclei compared to the vacuole (export ratio). Scale bar = 10 μm. *p*-values calculated by paired two-sided *t*-test with Benjamini-Hochberg adjustment.

were no differences in plaque sizes between these strains (**Figure 25D**). I also introduced GRA16-HA to the PRU Δ KU80, PRU Δ GRA59, and PRU Δ MYR1 cell lines, using the same vector as for RH (with GRA16 cloned from RH Δ KU80). Integration of the GRA16-HA construct was verified by PCR (**Figure 25E**) and expression of GRA16-HA protein confirmed by Western blot (**Figure 25F**).

I measured nuclear c-Myc immunofluorescence in HFFs infected with these strains as before and found that, in contrast to RH Δ GRA59, there was no change in fluorescence intensity for PRU Δ GRA59 compared to PRU Δ KU80 (**Figure 25G**). Furthermore, there was no change in c-Myc intensity for the PRU Δ GRA59::GRA59-HA strain, in which expression of GRA59 is presumed to be higher than the native level in PRU Δ KU80. Measurement of GRA16-HA immunofluorescence signal confirmed that export is not affected by GRA59 knockout in the PRU strain (**Figure 25H**). Surprisingly, therefore, in contrast to the RH strain, GRA59 appears to be dispensable for effector protein export in the PRU strain.

Discussion

GRA59 is shown here to contribute to dense granule effector protein export into the host cell, in addition to nine other previously identified *T. gondii* proteins: MYR1, MYR2, MYR3, MYR4, PPM3C, GRA44, GRA45, ROP17, and ASP5. Of these proteins, it is likely that MYR1-4 comprise the presumed translocon complex, as they are all essential for effector protein export and have no other identified roles. MYR1-4 all have predicted transmembrane domains, yet do not have experimentally resolved or confidently predicted structures or conserved domains that would further inform on their function. Although export of dense granule effector proteins into the host cell has a critical role for *T. gondii* virulence *in vivo*, it is largely dispensable for the lytic growth cycle of *T. gondii* *in vitro* in HFFs (Franco et al. 2016). As such, MYR1-4 have neutral fitness phenotypes *in vitro* (Sidik et al. 2016) and show at most minor difference in growth by plaque assay (Marino et al. 2018). All other identified proteins required for export either have non-essential roles in export or appear to have wider functions that are not specific to export.

Dephosphorylation of at least some effector proteins seems to be important for their export into the host cell via the MYR translocon. This dephosphorylation is carried out by the vacuole lumen-localised phosphatase PPM3C (Mayoral et al. 2020). Knockout of PPM3C reduces the export of GRA16 and GRA28, but does not affect IST or GRA24.

Another phosphatase domain-containing protein, GRA44, is essential for export but has a strongly negative fitness score *in vitro* (Blakely, Holmes, and Arrizabalaga 2020). For this reason, I did not recover a sufficient number of sg(GRA44)-expressing cells in the dual perturb-seq screen to analyse. This phenotype suggests GRA44 has broader or additional functions in the vacuole compared to MYR1-4, which are largely dispensable *in vitro*.

The chaperone-like protein GRA45 is required for the correct localisation of transmembrane domain-containing dense granule proteins in the PVM, including GRA44 and likely also including MYR1-4 (Y. Wang et al. 2020). GRA45 is therefore essential for effector export. Interestingly, GRA45 does not have as strong an *in vitro* fitness phenotype as GRA44 does, indicating that additional functions of GRA44 other than effector export do not require PVM localisation (Y. Wang et al. 2020; Sidik et al. 2016).

The rhoptry kinase ROP17 has a regulatory role in effector export, in addition to its role in inactivation of immune-related GTPases (Etheridge et al. 2014). ROP17 is secreted into the host cell during invasion and returns to reside at the cytosolic face of the PVM. The kinase activity of ROP17 is essential for effector export (Panas, Ferrel, et al. 2019); presumably, phosphorylation of the translocon complex is necessary for its activity.

The Golgi-resident aspartyl protease ASP5, a homologue of *Plasmodium* plasmepsin V, is also necessary for export of all identified exported effector proteins (GRA16, GRA18, GRA24, GRA28, IST, and NSM) (Coffey et al. 2015; He et al. 2018; Ten Hoeve et al. 2022; Gay et al. 2016; Rosenberg and Sibley 2021; Hammoudi et al. 2015; Curt-Varesano et al. 2016). Proteolytic cleavage of GRA16 by ASP5 is necessary for export of GRA16, yet for other dense granule proteins cleavage by ASP5 is neither necessary nor sufficient for export to the host cell (Coffey et al. 2015; Hammoudi et al. 2015; Curt-Varesano et al. 2016). It would seem likely that ASP5-mediated processing of one of the proteins required for export is necessary for the function of that substrate protein. However, current data rules all proteins currently known to be involved in effector export. MYR1, GRA44, and GRA45 all appear to be processed by ASP5, yet this processing is not necessary for effector export (Naor et al. 2018; Cygan et al. 2020; Blakely, Holmes, and Arrizabalaga 2020). MYR4 does not appear to be processed by ASP5, despite containing an “RRL” ASP5 consensus motif (Cygan et al. 2020), and neither MYR2 nor MYR3 contain this motif (Marino et al. 2018). GRA59 is proteolytically cleaved, yet also does not contain an “RRL” motif. The true role of ASP5 in effector export therefore remains mysterious.

GRA59 is similar to MYR1-4 in that it is a transmembrane protein localised to the PVM and does not have any recognisable conserved domains. As for most other dense granule proteins, orthologues of GRA59 are found only in closely related genera comprising the sub-family Toxoplasmatinae (*Toxoplasma*, *Hammondia*, *Neospora*, *Heydornia*, *Besnoitia*) (Barylyuk et al. 2020). This small orthologue group combined with the lack of recognisable conserved domains means that the AlphaFold structural prediction for GRA59 (version 2022-11-01) is very low confidence (Jumper et al. 2021; Varadi et al. 2022). Therefore, as for MYR1-4, it is difficult to predict a function for GRA59 based on the protein sequence alone.

The localisation of GRA59 in the PVM and its role in effector export suggests that GRA59 may be a component of the putative translocon complex together with MYR1-4. In support of this, GRA59 was identified as a putative interaction partner of MYR1 by co-immunoprecipitation mass spectrometry (Cygan et al. 2020). GRA59 was a relatively low-ranked hit in this data, yet was more highly enriched than MYR3, which has been shown to form a stable complex with MYR1 (Marino et al. 2018). Reciprocal co-immunoprecipitation using putative interaction partners of MYR1, including GRA59, as bait would help to clarify which proteins that are required for export are part of the presumed translocon complex.

Although knockout of GRA59 appears to affect the export of all dense granule effectors tested here, it does not completely block export as is the case for knockout of MYR1-4. These data therefore suggest that GRA59 may be an accessory or regulatory component of the export complex or pathway. Of note, though, is that the contribution of most proteins to effector export has been measured as a binary (effector exported or not exported), therefore it is possible that subtle variations in the relative contributions of different proteins have been missed. For example, one study qualitatively assessed the induction of c-Myc and presence of GRA16 in the host nucleus to

determine that PPM3C does not affect export (Cygan et al. 2020). However, a quantitative measurement of c-Myc and GRA16-HA fluorescence intensity showed that PPM3C does in fact contribute to export (Mayoral et al. 2020). Although these studies used different *T. gondii* strains (RH for the former, PRU for the latter), my dual perturb-seq data confirms that PPM3C knockout in RH affects host cell transcription. This example demonstrates that to fully understand this export pathway it will be necessary for the field to transition to more precise quantitative measurements of phenotypes rather than relying on qualitative assessments.

Surprisingly, I found that GRA59 is dispensable for effector export in the PRU strain. Aside from PPM3C mentioned above, in which case the differing phenotypes are likely due to methodology, this is the first indication of strain-specific differences in effector export. PRU induces moderately lower c-Myc levels in infected cells than RH, yet it appears to export at least as much GRA16 into the host cell, despite the absence of GRA59. However, care should be taken in comparing the export ratios of RH and PRU, as RH replicates markedly faster than PRU. Therefore, although all experiments were conducted at 24 hpi, this may represent an “earlier” timepoint in the infection for PRU. Although GRA59 has a single amino acid deletion and is expressed at a much lower level in PRU than in RH, complementation with RH GRA59 did not alter c-Myc induction in the PRU strain. Potentially, this strain specific phenotype of GRA59 depends on interactions with another protein which is polymorphic between *T. gondii* strains. Alternatively, although the PRU strain does not contain any additional homologues of GRA59, there may be another protein that fulfils the function of GRA59. Furthermore, several datasets indicate that GRA59 expression in type II strains increases dramatically upon conversion to the chronic bradyzoite stage (Ramakrishnan et al. 2019; Fritz et al. 2012; Pittman, Aliota, and Knoll 2014; Waldman et al. 2020), perhaps indicating that GRA59 may have a role in export of effectors from bradyzoite cysts (Mayoral, Shamamian, and Weiss 2020; Seizova et al. 2022).

In summary, this work confirms GRA59 as a novel component of the dense granule effector export pathway. The partial reduction in export in RHΔGRA59 and the apparent strain-specific requirement for GRA59 require further study to understand, but will likely help to illuminate the mechanism of effector export.

Chapter 4: SOS1 is required for sustained host STAT6 signalling

Results

SOS1 phenocopies ROP16 in dual perturb-seq data

The *T. gondii* gene with the strongest perturbation score in the dual perturb-seq data that hasn't previously been implicated in host cell reprogramming was TGGT1_222100, which, based on the experiments described below, I propose naming *TgSOS1* (Sustainer of STAT signalling 1).

The pseudo-bulk transcriptome of sg(SOS1)-expressing cells correlated closely with that of sg(ROP16)-expressing cells, indicating these two genes have similar host cell phenotypes. ROP16 is a rhoptry-localised kinase that is secreted into the host cell and phosphorylates host STAT3 and STAT6 (Saeij et al. 2007; Ong, Reese, and Boothroyd 2010; Yamamoto et al. 2009).

The most strongly downregulated genes for sg(SOS1)-expressing cells were the cytokines CCL11 and CCL26 (Figure 26A). These genes have both been shown to be regulated by STAT6 (Matsukura et al. 1999; Hoeck and Woisetschl ger 2001) and were previously identified among the strongest differentially regulated genes in *T. gondii* RH ROP16-infected HFFs compared to RH wild type-infected HFFs (Ong, Reese, and Boothroyd 2010). In my analysis of differentially regulated pathways in the dual perturb-seq data, the "PID_IL4_2PATHWAY", indicating genes regulated by IL-4, was the most significantly downregulated in sg(SOS1)-expressing cells (Figure 26B). IL-4 is the principal activator of STAT6 (Takeda et al. 1996), further indicating that STAT6 signalling pathway is downregulated in sg(SOS1)-expressing cells. Finally, sg(SOS1)-expressing cells were significantly enriched in a signature score based on ROP16-dependent marker genes (Saeij et al. 2007), indicating that SOS1 knockout induces a similar phenotype in the host cell to knockout of ROP16 (Figure 26C).

SOS1 is a rhoptry bulb protein processed by ASP3

SOS1 is predicted to localise to the rhoptries based on spatial proteomics data (Barylyuk et al. 2020). To confirm this localisation, I tagged the endogenous copy SOS1 in the RH KU80 strain with a single HA epitope at the C-terminus. I confirmed the integration of the HA-tagging construct by diagnostic PCR (Figure 27A), and confirmed the expression of SOS1-HA protein by Western blot (Figure 27B). SOS1 migrates at an apparent molecular weight of ~130 kDa, which is similar to the predicted

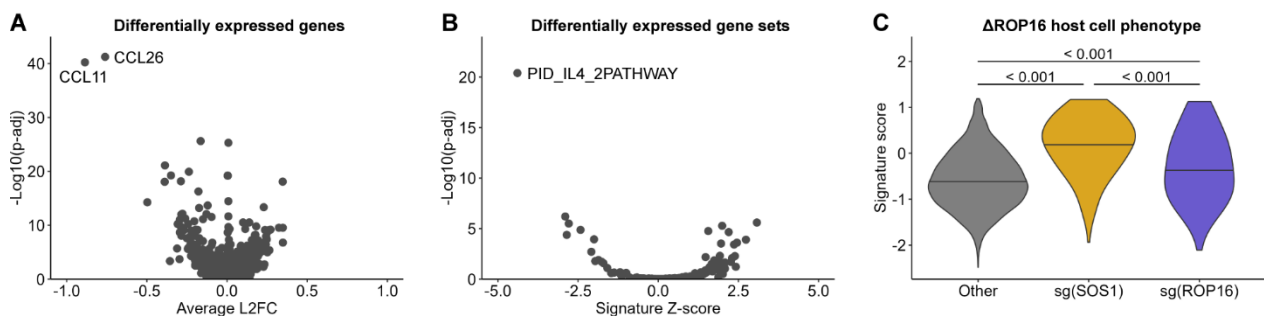


Figure 26. Knockout of SOS1 downregulates STAT6 signalling and appears to phenocopy knockout of ROP16.

A. Differentially regulated genes in dual perturb-seq data for sg(SOS1)-expressing cells; two-sided Wilcoxon rank-sum test with Benjamini-Hochberg adjustment.

B. Differentially regulated pathways in dual perturb-seq data for sg(SOS1)-expressing cells; two-sided Wilcoxon rank-sum test with Benjamini-Hochberg adjustment.

C. Scoring of cells for a  ROP16-infected-like host cell signature using VISION. *p*-values calculated by two-sided Wilcoxon rank-sum test with Benjamini-Hochberg adjustment.

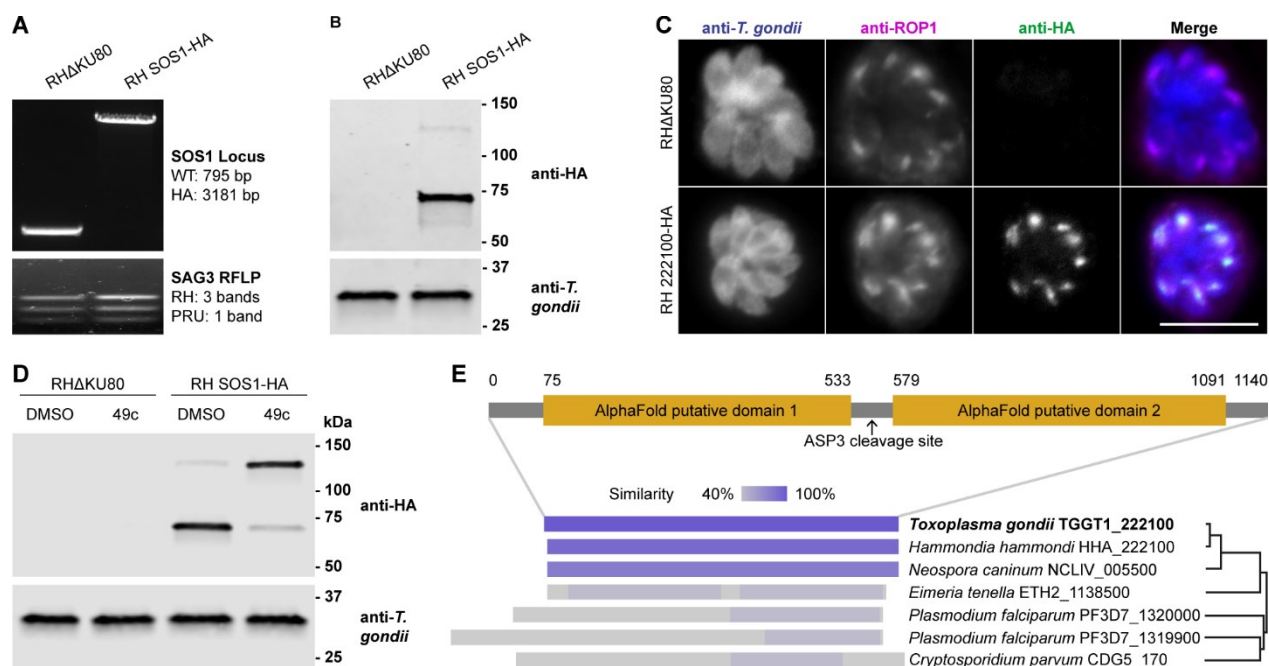


Figure 27. SOS1 is a rhoptry protein conserved across Apicomplexa.

- A.** Verification of HA-tagging construct integration by diagnostic PCR and RFLP verification of *T. gondii* strain.
- B.** Confirmation of SOS1-HA protein expression by Western blot.
- C.** Immunofluorescence co-localisation of SOS1-HA with ROP1. Scale bar = 10 μ m.
- D.** Analysis of SOS1-HA proteolytic cleavage by Western blot. The ASP3 inhibitor 49c was added to 10 μ M at 1 hpi and parasites were isolated from host cell material for analysis at 48 hpi.
- E.** Diagram of predicted protein domain structure of SOS1 and amino acid sequence alignment of homologues detected in Apicomplexa by BLASTP. Phylogenetic tree generated using Clustal Omega.

molecular weight of 128 kDa. SOS1 appears to be proteolytically processed, producing a ~70 kDa C-terminal fragment which is the major species detectable by Western blot. Immunofluorescence microscopy confirmed that this C-terminal fragment localises to the rhoptry bulb, co-localising with ROP1 (**Figure 27C**). I did not observe any anti-HA signal within the host cell or in the vacuole; however, SOS1 is not very highly expressed compared to rhoptry effector proteins, so it may not be possible to detect dispersed signal following secretion. SOS1 is not predicted to have a signal peptide by SignalP 6.0 (Teufel et al. 2022) but has a putative transmembrane domain very close to the N-terminus weakly predicted (~0.5 posterior probability) by Phobius (Madeira et al. 2022), which may instead be a cryptic signal peptide. Given that no anti-HA fluorescence is detected in the vacuole or the host cell, knowing whether SOS1 enters the secretory pathway is important in determining whether it is contained within the rhoptries, and thus is potentially secreted into the host cell, or whether it is instead associated with the cytoplasmic face of the rhoptries and so cannot access the host cytosol.

Several rhoptry proteins have been shown to be processed by the Golgi-resident aspartyl protease ASP3 (Dogga et al. 2017). One N-terminal peptide was detected for SOS1 in an N-TAILS experiment (Kleifeld et al. 2011) designed to identify substrates of ASP3 (Dogga et al. 2017), indicating that SOS1 may also be processed by ASP3. To test whether SOS1 is processed by ASP3, I treated RH SOS1-HA parasites with 49c, a small molecular inhibitor of ASP3, for one lytic cycle and compared the cleavage of SOS1-HA to a control DMSO-treated sample by Western blot. The processing of

SOS1-HA was inhibited in the 49c-treated sample, with the full length ~130 kDa band representing the major species in contrast to the DMSO-treated sample in which the majority of SOS1 is cleaved (**Figure 27D**). This result strongly indicates that SOS1 is a substrate of ASP3. Therefore, despite the lack of a clearly predicted signal peptide, SOS1 is likely within the secretory pathway and targeted to the lumen of the rhoptry bulb, rather than being associated with the cytoplasmic face of the rhoptry.

Although SOS1 does not have any recognisable conserved domains, the AlphaFold structural prediction for SOS1 (version 2022-11-01) indicates with moderate confidence the presence of two globular domains connected by a flexible linker (Jumper et al. 2021; Varadi et al. 2022) (**Figure 27E**). The putative cleavage site for ASP3 lies within this linker, based on the apparent size of the C-terminal fragment by Western blot and the position of the N-terminal peptide detected by Dogga et al. (Dogga et al. 2017).

Unusually for a *T. gondii* rhoptry bulb protein, homologues of SOS1 are present throughout Apicomplexa (**Figure 27E**). The predicted C-terminal domain is more strongly conserved, while the N-terminal domain is recognisable only in Coccidia. Interestingly, in some *Plasmodium* species, such as *P. falciparum*, there are two homologues of TgSOS1 located next to each other in genome, indicating a tandem duplication. The C-terminal regions of these proteins retain homology to TgSOS1, while the N-terminal regions are divergent both from TgSOS1 and from each other.

SOS1 is required for sustained host STAT6 signalling and M2 polarisation

To further investigate the function of SOS1, I generated knockout and complemented cell lines in the RHΔKU80 background. I verified the integration of the knockout and complementation constructs by PCR (**Figure 28A**) and confirmed the expression of SOS1-HA protein in the complemented cell line by Western blot and immunofluorescence microscopy (**Figure 28B and C**). There were no differences in plaque sizes between these strains (**Figure 28D**).

Based on the dual perturb-seq phenotype of SOS1, I hypothesised that it may cooperate with ROP16 to phosphorylate STAT6. To test this hypothesis, I measured nuclear phospho-STAT6 immunofluorescence in HFFs infected with the parental RHΔKU80 strain, SOS1 knockout and complemented strain, and an RHΔROP16 strain as a control. At 1 hpi, the RHΔKU80 strain strongly induced STAT6 phosphorylation compared to uninfected cells, yet there was no change in phospho-STAT6 signal intensity in RHΔSOS1-infected cells (**Figure 28E**). STAT6 phosphorylation was abolished by knockout of ROP16, as previously shown (Saeij et al. 2007; Ong, Reese, and Boothroyd 2010). However, at 24 hpi, the same timepoint at which the dual perturb-seq data were collected, phospho-STAT6 signal was almost completely lost in RHΔSOS1-infected cells and rescued by complementation. These data indicate that while ROP16 is required for the initial phosphorylation of STAT6, SOS1 is required to sustain STAT6 signalling throughout the infection. Hence, I propose naming this gene (TGGT1_222100) **Sustainer of STAT signalling 1**.

Activation of STAT6 signalling by ROP16 induces M2 polarisation of *T. gondii*-infected macrophages, which is implicated in dampening anti-parasitic immune responses *in vivo* (Jensen et al. 2011; Chen et al. 2020). To test if SOS1-dependent sustained STAT6 phosphorylation is important for M2 polarisation, I infected murine bone marrow-derived macrophages (BMDMs) and measured arginase activity in cell lysate, a defining feature of M2 polarisation (Mills et al. 2000). After 24 h infection, arginase activity was strongly induced by infection with RHΔKU80, compared to

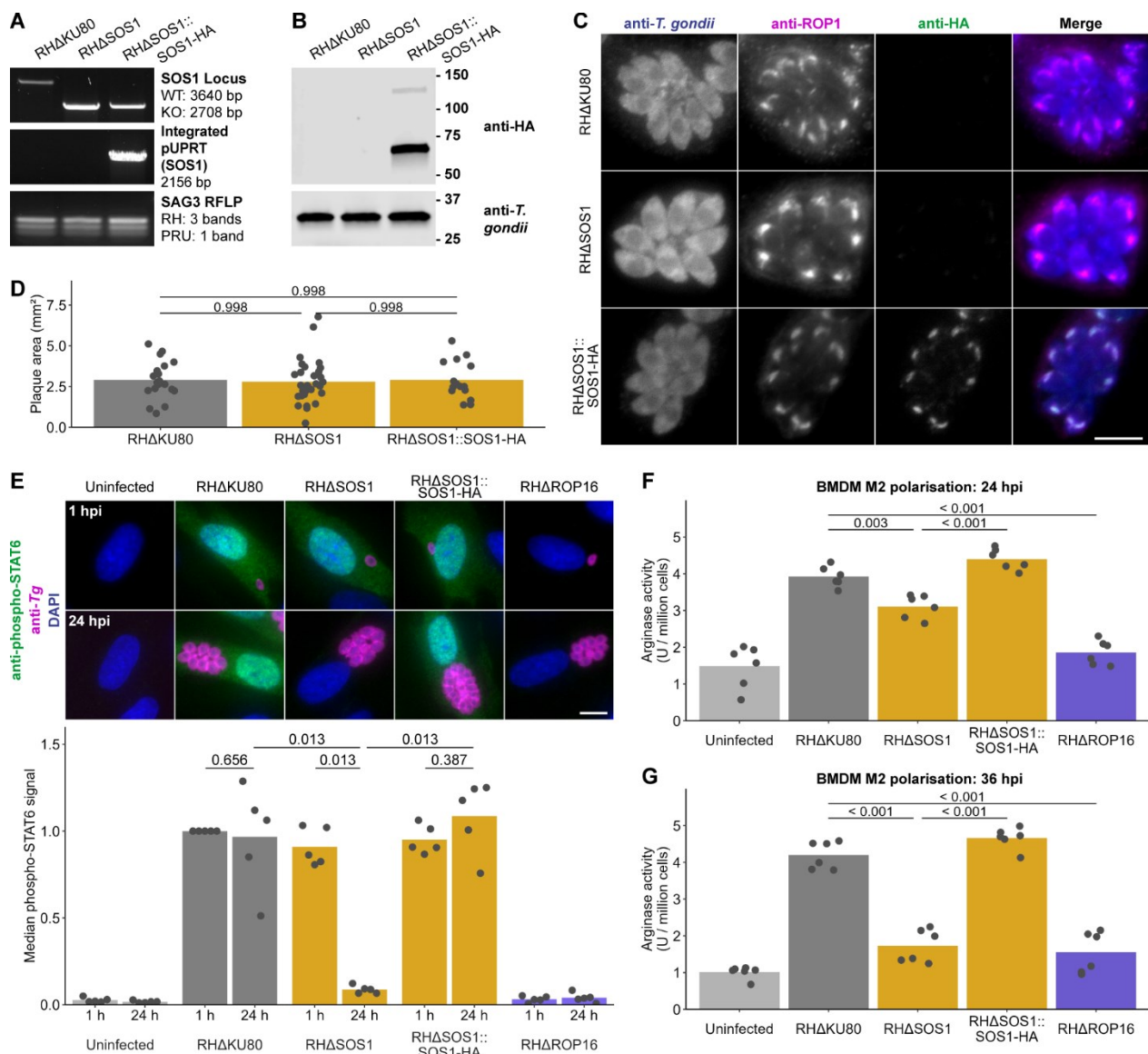


Figure 28. SOS1 is required for sustained host STAT6 signalling and M2 polarisation.

- A.** Verification of knockout and complementation construct integration by diagnostic PCR and RFLP verification of *T. gondii* strain.
- B.** Confirmation of SOS1-HA protein expression by Western blot.
- C.** Confirmation of SOS1-HA protein expression by immunofluorescence assay. Scale bar = 10 μm.
- D.** Measurement of plaque sizes for RHΔKU80, RHΔSOS1, and RHΔSOS1::SOS1-HA. One biological replicate; points represent individually measured plaques. *p*-values calculated by two-sided *t*-test with Benjamini-Hochberg adjustment.
- E.** Immunofluorescence quantification of nuclear phospho-STAT6 in HFFs infected for 1 h or 24 h. Scale bar = 10 μm. *p*-values calculated by two-sided Wilcoxon rank sum test with Benjamini-Hochberg adjustment.
- F.** Arginase activity measured by colourimetric assay in lysate of BMDMs infected with the indicated strains for 24 h. *p*-values calculated by two-sided *t*-test with Benjamini-Hochberg adjustment.
- G.** Arginase activity measured by colourimetric assay in lysate of BMDMs infected with the indicated strains for 36 h. *p*-values calculated by two-sided *t*-test with Benjamini-Hochberg adjustment.

uninfected BMDMs, and abolished by knockout of ROP16 (**Figure 28F**). There was a modest reduction in arginase activity for RHΔSOS1-infected cells compared to RHΔKU80-infected cells, which was rescued by complementation. I reasoned that transient M2 polarisation may be induced by the initial burst of STAT6 signalling in RHΔSOS1-infected BMDMs and that there would be a

delay between loss of STAT6 phosphorylation and loss of the M2 phenotype. Therefore, I also measured arginase activity at a later timepoint. At 36 hpi, arginase activity in RHΔSOS1-infected BMDMs was further reduced to near the level of uninfected and RHΔROP16-infected cells (**Figure 28G**), confirming that sustained STAT6 signalling is required to maintain M2 polarisation by *T. gondii*.

Discussion

The findings here elaborate the model of host cell reprogramming by ROP16, the first *T. gondii* effector shown to influence host transcription (Saeij et al. 2007). Although ROP16 is sufficient for the initial phosphorylation and activation of the host transcription factor STAT6, in the absence of a previously unidentified effector, SOS1, this phosphorylation is lost over time and the host cells revert to a ΔROP16-like transcriptional phenotype. Correspondingly, in the absence of SOS1, macrophage M2 polarisation by ROP16 is transient.

This identification of an additional *T. gondii* effector required for sustained STAT6 signalling is unexpected, as recombinant ROP16 has been shown to be capable of phosphorylating STAT3 and STAT6 *in vitro* in the absence of any other effector (Yamamoto et al. 2009; Ong, Reese, and Boothroyd 2010). My results here concur that in the absence of SOS1 the kinase activity of ROP16 is not completely abolished, yet reveal a novel temporal regulation of this activity during a physiological infection.

Similarly, the original study investigating the role of ROP16 in host cell reprogramming concluded that ROP16 alone is sufficient, following ectopic expression of an active type I allele in a type II strain with an inactive allele (Saeij et al. 2007). Several studies since have also made use of type II strains expressing a type I allele of ROP16 to explore its function (Jensen et al. 2011; Jensen et al. 2013; Kochanowsky, Thomas, and Koshy 2021). SOS1 is highly conserved between type I and type II strains (99.47% identity for TGME49_222100 versus TGGT1_222100); it is therefore likely that the function of SOS1 is conserved between type I and type II strains, such that when an active ROP16 kinase is expressed in a type II strain the host reprogramming is sustained by the endogenous type II SOS1. This could be tested by knockout of SOS1 in a type II::ROP16^{type I} strain.

This work has not addressed how, mechanistically, SOS1 cooperates with ROP16 to sustain STAT signalling, although certain hypotheses can be ruled out. I have also not explored activation of STAT3 by ROP16 in this work, which could reveal whether the role of SOS1 relates broadly to the function of ROP16 or is specific to regulation of STAT6. SOS1 does not affect the secretion of ROP16 into the host cell, nor the initial kinase activity, as phosphorylation of STAT6 by RHΔSOS1 parasites is at an equivalent level to wild-type parasites at 1 hpi. It is also unlikely that SOS1 affects the nuclear localisation of ROP16, as this localisation is dispensable for sustained activation of STAT6 (Saeij et al. 2007). A possible role for SOS1 is in counteracting homeostatic mechanisms that inactivate STAT signalling. A relatively small amount of ROP16 is secreted into the host cell; SOS1 may inhibit proteolytic turnover of ROP16 so that this small amount of kinase present can sustain STAT6 signalling. Alternatively, SOS1 may prevent turnover or dephosphorylation of STAT6. Both proteasome activity and the tyrosine phosphatase SHP-1 have been implicated in the decay of STAT6 phosphorylation (Hanson et al. 2003). These hypotheses could be tested by treating RHΔSOS1-infected cells with proteasome or tyrosine phosphatase inhibitors.

This work indicates a role for SOS1 in sustaining host cell M2 polarisation, which likely influences cytokine production and the systemic immune response to infection *in vivo*. Future work should investigate whether other consequences of ROP16-mediated host reprogramming also rely on sustained activation of STAT6 signally by SOS1: suppression of host reactive oxygen species production (Kochanowsky, Thomas, and Koshy 2021), suppression of T cell responses (Chen et al. 2020), and formation of bradyzoite cysts in the central nervous system (Kochanowsky et al. 2022; Tuladhar et al. 2019). The hypervirulence of the RH strain, and the fact that the parental RHΔKU80 strain used in this work has largely lost the ability for form bradyzoite cysts, mean that this further study would necessitate regenerating SOS1 knockout and complemented cell lines in a type III or atypical *T. gondii* strain that contains an active ROP16 allele and is capable of forming bradyzoite cysts.

The conservation of SOS1 across Apicomplexa implies it may have additional or broader roles, especially as ROP16 is only present in Toxoplasmatinae. Some of these homologues are annotated as rhoptry or Golgi-localised proteins, indicating that, like TgSOS1, they are within the secretory pathway. Interestingly, some of the *Plasmodium* spp. homologues of TgSOS1 are annotated with the PANTHER classification “DBF4-TYPE DOMAIN-CONTAINING PROTEIN” (Thomas et al. 2022), indicating distant homology to the protein DBF4, which acts as an activator of the cell cycle kinase CDC7 (W. Jiang et al. 1999). Notably, the putative N-terminal domain of SOS1 has a conservation profile that is more similar to ROP16, with homologues detected only in Coccidia. This may imply that the divergent N-terminal domain is responsible for maintaining STAT6 activation, while the more highly conserved C-terminal domain may have a broader function across Apicomplexa. Disentangling the roles of the N- and C-terminal domains in *T. gondii* will likely be challenging, as protein fragments would need to be correctly targeted through the secretory pathway to the rhoptries and the peptide signals or protein properties that govern this targeting are still poorly understood.

Overall, TgSOS1 exemplifies the potential of dual perturb-seq to identify effector proteins that would not be identified by any other CRISPR screening method. The requirement for SOS1 for sustained host cell reprogramming by *T. gondii* is surprising given the current model of the mechanism of action of ROP16, but is nevertheless consistent with available data. Although much work remains to determine the mechanism of action of SOS1, the identification of this effector has opened exciting new avenues of research into the temporal regulation of host cell reprogramming and parasite immune evasion.

Conclusion

Screening limitations

The screens described here go some way to further characterising the vast repertoire of secreted proteins of *T. gondii*. The motivation for these screens was to identify previously uncharacterised effector proteins that contribute to parasite immune evasion. This motivation was heightened by the recent assignment to the rhoptries and dense granules of 142 proteins of previously unknown localisation, more than doubling the number of known rhoptry- and dense granule-localised proteins (Barylyuk et al. 2020).

Although we targeted all of these predicted rhoptry and dense granule protein-encoding genes in these screens, we were unable to derive fitness phenotypes or transcriptional profiles for every target, due to the way the CRISPR plasmid library is constructed. ssDNA oligonucleotides encoding protospacer sequences and homology arms are selected from an arrayed library using a liquid handling robot and incorporated into the Cas9-sgRNA plasmid vector by pooled Gibson assembly. For incompletely determined reasons (potentially including misalignment of the liquid handling robot, incomplete thawing of guide stocks, or manual error in stock plate loading) not every selected guide is detectable in the plasmid library. This was a particular issue in the *in vivo* screen, but was greatly ameliorated in the construction the plasmid pool for the dual perturb-seq screen by selecting guides in “biological triplicate” on three different days and pooling the sampled oligonucleotides before assembly.

Furthermore, the representation of protospacer sequences in the plasmid library is highly skewed, with the number of read counts per protospacer varying over several orders of magnitude. We believe this may be due to biases in the Gibson cloning step related to sequence-dependent formation of secondary structures and dimers between the ssDNA oligonucleotides. This skew in the plasmid pools means that less-well-represented protospacers are easily lost through selective bottlenecks (e.g., infection of mice or partitioning of cells for scRNA-seq). Dropout of protospacers was particularly acute in the dual-perturb seq screen, as only a few thousand cells are captured in each replicate and it is inevitable that some target genes will not be represented well enough in the final dataset to draw any conclusions regarding their impact on the host cell. Although I was able to identify significant phenotypes for targets represented by as few as 30 single-cell transcriptomes (e.g., MYR4), other works suggest that hundreds of cells are required to detect more subtle phenotypes (Dixit et al. 2016).

That said, no high-throughput screen is able to capture every phenotype, nor is this necessary in order to find new and interesting biology and to conduct global analyses of the target gene population. The *in vivo* fitness phenotype scores and list of *T. gondii* gene knockouts that perturb host cell transcription will be useful in prioritising understudied genes for further characterisation. Follow-up assays and experiments I have conducted have assigned functions to three such effectors – ROP1, GRA59, and SOS1 – although more work remains to establish their molecular mechanisms of action. The nature of these effectors may make this work challenging. All three proteins exert functions in the parasitophorous vacuole or host cell; therefore, any manipulations of these proteins must not perturb pre-secretory trafficking or secretion. Furthermore, the lack of homology to any other proteins hinders rational protein design or mutagenesis and the inference of function, from amino acid to whole-protein level. These challenges are common to many effector proteins of *T. gondii* and other organisms.

Outlook for CRISPR-Cas9 knockout screening in *T. gondii*

Compared to *in vitro* knockout screens, the primary challenge for *in vivo* screens until now has been how to cover as many gene targets as possible without overwhelming and killing the mouse host. Different approaches have been used: we have used a less-virulent PRU strain in the highly-susceptible C57BL/6 mouse, which is the workhorse of mouse genetics; an alternative has been to use the RH strain, which is the workhorse of *T. gondii* research, in the more-resistant CD-1 mouse background (Sangaré et al. 2019). Both approaches are still limited to screening only a small subset of *T. gondii* genes in a given experiment. These screens have therefore focused on secreted rhoptry and dense granule proteins, which are expected to be disproportionately likely to have functions *in vivo* that are dispensable *in vitro*.

Close to the submission of this thesis, a genome-wide knockout screen of the *T. gondii* RH strain in CD-1 mice was described in a pre-print (Giuliano et al. 2023). This study modified prior methodology to enable a genome-wide screen by using only three sgRNAs per target and incorporating an innovative UMI system to enable tracking of individual *T. gondii* clones. Still, this screen required a total of 54 mice across nine experiments for which the results were concatenated, equivalent to approximately 1,000 *T. gondii* genes (or 3,000 sgRNAs) per experiment. This represents a modest improvement compared to the ~2,000 sgRNAs screened *in vivo* in the RH strain by Sangaré et al (Sangaré et al. 2019) and ~900 sgRNAs screened in the PRU strain here. Nevertheless, this genome-wide screen is likely to mean that the vast majority of all *T. gondii* genes, including secreted proteins, will now have an *in vivo* fitness phenotype score, such that “screening newly identified rhoptry and dense granule protein-encoding genes” will no longer be an important motivation for future work. Instead, I expect that the field will use the various CRISPR-Cas9 knockout screening platforms and methodologies now available to further explore the relationship between parasite genotype and host genotype.

A key area for further research is the comparison of different *T. gondii* strains. All current CRISPR screens have used either the RH or PRU strains, which represent only a fraction of the genetic diversity of *T. gondii*. Individual labs tend to focus on one strain, meaning there are no *in vivo* screens that directly compare the contributions of genes in the RH strain versus the PRU strain using the same sgRNA library and methodology. Although genetic crosses have identified some of the key polymorphic virulence factors in the canonical type I/II/III lineages of *T. gondii*, there are likely to be additional subtle variances in the roles of effector proteins between strains that require more precise quantitative methods to determine. Moreover, study of so-called atypical strains, which comprise the bulk of *T. gondii* genetic diversity, is likely to identify new polymorphic virulence factors. Equally valuably, comprehensive and directly comparable genetic screens will identify which effector proteins are conserved and equally important in all *T. gondii* strains.

A further aspect to explore is the influence of host genotype on the fitness contributions of *T. gondii* effector proteins. A clear example of this influence is the selection of mouse strains in the currently published *in vivo* screens: all screens with the PRU strain have used the C57BL/6 mouse background, while screens in the RH strain have necessitated the use of outbred CD-1 mice, as C57BL/6 mice would succumb to infection before the experiment could be completed. CD-1 mice have IRG alleles that are resistant to clearance by the ROP5/17/18 complex of the RH strain, such that the

hypervirulence of RH is attenuated. Consequently, although ROP5, ROP17 and ROP18 are critical virulence factors in the RH strain, in these *in vivo* screens they appear to be dispensable.

Additionally, *T. gondii* genetic screens may be combined with host genetic manipulation to provide further insight into the mechanism of action of *T. gondii* effectors. For example, comparative screens in wild-type mice and IFN γ -receptor knockout mice would reveal which effectors are important for parasite fitness *in vivo* because they counteract the IFN γ response, and which operate through IFN γ -independent mechanisms. In this manner, the roles of other cytokines or immune cell types in the control of infection and in parasite immune evasion could be explored.

Current screening methods generate single gene mutants, such that effectors with redundant functions, if they exist, are not identified. Future work could explore the functional relationships between *T. gondii* effectors through epistasis screens, in which the screen is performed simultaneously in wild-type and effector knockout *T. gondii* cell lines, or through combinatorial gene knockouts with multiple sgRNAs delivered to each parasite (R. Li et al. 2022). This work could identify synthetically lethal pairs of effectors, or establish whether the function of one effector depends on another upstream in the mechanistic pathway.

Finally, the roles of *T. gondii* effectors can be explored in different species. As *T. gondii* is capable of infecting so many different host species, it has been a long-standing question whether effector proteins have evolved host-specific functions. We and others have recently completed screens to identify *T. gondii* effectors that are required for parasite resistance to IFN γ -mediated restriction in human foreskin fibroblasts (Lockyer et al. 2022; Krishnamurthy et al. 2023). The innate immune response of human cells is markedly different to that of mouse cells – most notably in the absence of IRGs. We identified a complex of effector proteins that is necessary for parasite survival in IFN γ -stimulated human fibroblasts, but not in murine fibroblasts, indicating that they counteract a mechanism of restriction that is unique to human cells, or at least absent in mice. This is somewhat surprising, as there is not expected to be significant evolutionary pressure for *T. gondii* to evade human immune responses. Most likely, this finding is an indication of adaptation to immune pressure in an alternative, non-murine host that is significant in the *T. gondii* life cycle in nature. Nevertheless, the primary motivation for the study of *T. gondii* is its pathogenicity in humans, so, given the differences in immune responses between host species, it is important to study how *T. gondii* effectors contribute to infection of humans in the best available model systems.

Outlook for dual perturb-seq

CRISPR-Cas9 knockout screens that assess parasite fitness by bulk sequencing of a protospacer population will become accessible to more labs as the surrounding technology matures. However, perturb-seq screens in the *T. gondii* field and beyond are likely to remain beyond the reach of most labs due to the high costs of commercial reagents and sequencing necessary to reach an optimal read count per cell. These costs also currently limit the expansion of dual perturb-seq in *T. gondii* to a larger number of target genes or higher coverage of the existing library to enable robust detection of more subtle phenotypes. Nevertheless, I believe the approach outlined here represents a reasonable balance for this particular research question between number of target genes, coverage,

and cost considerations, aided by the prioritisation of a signature-level rather than gene-level bioinformatic analysis.

Newer technology, such as the 10x Genomics Chromium X partitioning device, will improve the per-cell cost of scRNA-seq and perturb-seq, although perhaps not the total expenditure per experiment. Improvements could also be made to the upstream protocol to increase the number of useable single-cell transcriptomes in each technical replicate and thereby increase the value for money. Some inefficiency can be attributed to mis-sorting of uninfected cells together with the targeted infected cells and the presence of some non-viable cells in the sample, but the greatest contributor is the failure to detect and sgRNA in one-third of otherwise high-quality infected cells. This dropout rate is most likely due to relatively low expression of the sgRNA in intracellular parasites compared to published applications in mammalian cells using lentiviral transduction for delivery of the sgRNAs (tens of UMIs versus hundreds to thousands) (Replogle et al. 2020). Increasing the expression level of the sgRNA in *T. gondii* would be challenging as alternative RNA polymerase III promoters would need to be identified and validated. Although I identified sgRNA capture sequence configuration as a key driver of capture efficiency, further optimisation is limited by the need for compatibility with commercial reagents. In addition to loss of cells with no detected sgRNA, I was unable to use cells in which multiple sgRNAs were detected. Improved capture of the sgRNA would allow the use of more sophisticated methods of assigning knockout identity to each cell, to distinguish whether two sgRNAs are really present or instead result from contamination with ambient RNA.

In the longer term, throughput may be increased by pre-indexing of cells prior to droplet-based scRNA-seq (Datlinger et al. 2021). Typically, the dissociated cells are loaded at a relatively low concentration to prevent multiple cells being captured in a single droplet, meaning that the majority of droplets are actually empty and the reagents contained within them are effectively wasted. Pre-indexing allows overloading such that most droplets contain multiple single cells, while retaining cell-of-origin information for each transcript. Using 96 pre-indexing barcodes, this method was shown to enable capture of up to 150,000 single-cell transcriptomes from a single 10x Genomics Chromium channel.

Although dual perturb-seq remains technically challenging, future applications in *T. gondii* are likely to mirror those of *in vivo* knockout screens. Dual perturb-seq in different *T. gondii* strains is likely to reveal new polymorphic effectors. Notably, neither GRA59 nor SOS1 identified here contribute to host cell reprogramming in the PRU strain. For SOS1, this is due to the lack of ROP16 activity in PRU, though for GRA59 the reason is so far unknown. As a counter example, I did not find a significant phenotype for the dense granule effector GRA18 in this screen, indicating the anti-inflammatory regulatory activity of GRA18 is specific to the PRU strain in which it was identified (He et al. 2018). Further dual perturb-seq studies may also find host cell type- and host species-dependent effectors. There is evidence of cell type-specific transcriptional responses to *T. gondii* infection (Swierzy et al. 2017), which may be due to cell type-specific effector proteins or the interaction of the same effectors with different host epigenetic and proteomic landscapes.

Furthermore, dual perturb-seq may be adapted to other organisms to disentangle transcriptional interactions between other intracellular microbes and their hosts. Both mutualistic and pathogenic endosymbioses involve transcriptional reprogramming of the host cell to downregulate immune responses and maintain a favourable environment (Jacobovitz et al. 2021). In the form used here, dual perturb-seq requires relatively high transfection efficiency, faulty repair of the Cas9-sgRNA

cleavage by non-homologous end joining to introduce loss-of-function mutations, and efficient integration of the plasmid vector into the genome so that the sgRNA continues to be expressed and can be detected (here, the combined transfection and plasmid integration efficiency was approximately 0.1%). Non-homologous end joining capacity has been lost in many parasitic protists, including other members of Apicomplexa (Nenarokova et al. 2019), yet this requirement could be obviated with straightforward modifications to the plasmid vector. Cas9 could be replaced with catalytically deactivated Cas9 to suppress gene expression by CRISPR interference, or with Cas13 to suppress gene expression by cleaving mRNA transcripts. The plasmid vector could then be integrated into the genome by homologous recombination at a safe harbour locus.

In conclusion, dual perturb-seq will be a powerful tool to identify the molecular mediators of host-microbe transcriptional interactions. As demonstrated here, dual perturb-seq has the ability to discover effector proteins that are not identified by any other method and provide insights into the mechanisms of host remodelling. Future applications in *T. gondii* will explore how parasite strain and host epigenetic landscape interact in transcriptional reprogramming, while relatively minor alterations could allow dual perturb-seq experiments in diverse organisms and higher throughput on a more cost-efficient basis.

Methods

Cell culture

All cell lines were maintained at 37 °C and 5% CO₂ and were tested monthly for *Mycoplasma spp.* contamination by PCR.

HFF

Human foreskin fibroblasts (ATCC, SCRC-1041) were cultured in Dulbecco's Modified Eagle's Medium with 4.5 g/L glucose and GlutaMAX (Gibco) supplemented with 10% v/v heat-inactivated foetal bovine serum (Gibco).

BMDM

Monocytes were isolated from the femurs of 6–12-week-old male C57BL/6J mice and differentiated into BMDMs for six days in 70% v/v RPMI 1640 medium (ATCC modification) (Gibco), 20% v/v L929 cell conditioned medium (provided by the Cell Services Science Technology Platform at the Francis Crick Institute), 10% v/v heat-inactivated FBS (Gibco), 100 U/mL penicillin-streptomycin (Gibco) and 50 µM 2-mercaptoethanol (Sigma). Following differentiation, BMDMs were cultured in the same medium without 2-mercaptoethanol.

THP-1

THP-1 cells were cultured in RPMI 1640 medium (Gibco) supplemented with 10% v/v FBS (Gibco). THP-1 monocytes were differentiated into macrophages with 100 ng/mL phorbol 12-myristate 13-acetate (Sigma) for 24 h, followed by a rest period without phorbol 12-myristate 13-acetate for 24 h.

MEF

Primary C57BL/6 MEFs (ATCC) and immortalised C57BL/6 C1QBP^{flox/flox}/C1QBP^{-/-} MEFs (a gift from the lab of Dongchon Kang) were cultured in DMEM with 4.5 g/L glucose and GlutaMAX (Gibco) supplemented with 10% v/v heat-inactivated FBS (Gibco).

Toxoplasma gondii

T. gondii cell lines were maintained by serial passage in HFFs. Parasites were harvested for experiments by mechanical lysis with a 27 G needle and passage through a 5 µm sterile filter. Parental strains used in this work were RHΔHXPGRT (Donald et al. 1996), PRUΔHXGPRT (Donald et al. 1996), RHΔKU80 (Huynh and Carruthers 2009), and PRUΔKU80 (Fox et al. 2011). Parasite genotype was verified by restriction fragment length polymorphism of the SAG3 gene as previously described (Su, Zhang, and Dubey 2006).

Mice

C57BL/6J mice were bred and housed in pathogen-free conditions at the Biological Research Facility of the Francis Crick Institute in accordance with the Home Office UK Animals (Scientific Procedures) Act 1986 and European Union Directive 2010/63/EU. All work was approved by the UK Home Office (project license PDE274B7D) and the Francis Crick Institute Ethical Review Panel.

***In vivo* screen**

ssDNA oligonucleotides encoding protospacer sequences were sampled from an arrayed library (Young et al. 2019) using an Echo 550 Acoustic Liquid Handler (LabCyte). For each protospacer, 10 nL of a 0.1 mM stock was dispensed in triplicate. The triplicate pools were combined and integrated into a Cas9-GFP-T2A-HXGPRT::sgRNA plasmid vector (Young et al. 2019) by Gibson assembly. The resulting plasmid pool was linearised with KpnI-HF (NEB) and transfected into 10^8 *T. gondii* PRUΔHXGPRT tachyzoites by electroporation using the Amaxa 4D Nucleofector system (Lonza) with buffer P3 and pulse code EO-115. Transfected parasites were selected in HFFs for integration of the plasmid vector into the genome with 5 µg/mL mycophenolic acid (Sigma) and 50 µg/mL xanthine (Sigma) for six days. Following selection, five mice were infected by intraperitoneal injection with 200,000 parasites each. Mice were monitored daily and parasites were recovered after five days by peritoneal lavage and expanded for one passage in HFFs.

Genomic DNA was extracted from the transfected parasites following the six days drug selection in HFFs, from the leftover mouse inoculum, and from the five *ex vivo* populations after recovery in HFFs. Illumina sequencing libraries were prepared by nested PCR amplification of the protospacer sequences from the plasmid pool and these genomic DNA samples. The libraries were sequenced on a HiSeq 4000 platform (Illumina) with 100 bp paired-end reads to a minimum depth of 7.5 million reads per sample (**Appendix 1**).

Analysis of *in vivo* screening data

Following demultiplexing, the reads were trimmed and aligned to a reference of protospacer sequences using a custom perl script (Young et al. 2019). Subsequent analysis was carried out using R v4.0.1 (www.r-project.org). Protospacers with fewer than 50 reads in every sample were removed from the analysis and remaining counts normalised using the median of ratios method (Anders and Huber 2010). Genes with fewer than three protospacers remaining were then removed from the analysis.

For each gene, the median *in vitro* L2FC was calculated from the normalised counts of the protospacers targeting that gene in the drug-selected parasite population and the plasmid pool. The median *in vivo* L2FC was calculated using the geometric mean of the normalised counts in the *ex vivo* parasite populations and the normalised counts in the inoculum. Genes in the top 5th percentile of median absolute deviation of the *in vitro* or *in vivo* L2FCs were removed from the analysis. *p*-values were calculated for each L2FC by paired two-sided *t*-test on log₂-transformed normalised counts and adjusted to correct for local false discovery rate (FDR) using the qvalue R package.

Dual perturb-seq experiments

Capture sequences 1 and 2 were inserted into the Cas9-GFP-T2A-HXGPRT::sgRNA plasmid vector (Young et al. 2019) by inverse PCR. To generate individual perturb-seq vectors, the protospacer was modified by inverse PCR. To generate the pool of perturb-seq vectors, ssDNA oligonucleotides encoding protospacer sequences were sampled from an arrayed library as above (Young et al. 2019).

In addition to triplicate dispensing of each ssDNA oligonucleotide, protospacers were sampled on three different days to reduce dropout through misalignment of the stock and receiving plates or incomplete thawing of the stock. The pooled protospacers were incorporated into the Cas9-GFP-T2A-HXGPRT::sgRNA-CS1, Cas9-YFP-T2A-HXGPRT::sgRNA-CS1, and Cas9-mCherry-T2A-HXGPRT::sgRNA-CS1 perturb-seq vectors by Gibson assembly. An Illumina sequencing library was prepared from the resulting Cas9-GFP perturb-seq plasmid pool to verify protospacer integration by nested PCR. The resulting library was sequenced on a HiSeq 4000 platform (Illumina) with 100 bp paired-end reads to a depth of 30 million reads. The numbers of reads matching each protospacer sequence were counted using a custom perl script as above (Young et al. 2019).

T. gondii RHΔHXGPRT parasites were transfected with KpnI-HF-digested (NEB) perturb-seq vectors by electroporation using the Amaxa 4D Nucleofector system (Lonza) with buffer P3 and pulse code EO-115. For individual perturb-seq vectors, at least 10^7 parasites were transfected with 15 µg of plasmid in one well of a 4D Nucleofector X Unit 16-well strip. For the perturb-seq plasmid pool, at least 10^8 parasites were transfected with 120 µg of an equimolar mixture of the perturb-seq vectors with different fluorophores, split across eight wells of a 16-well strip. 24 h after transfection, 25 µg/mL mycophenolic acid (Sigma) and 50 µg/mL xanthine (Sigma) were added to the parasites to select for integration of the linearised perturb-seq vector into the genome for at least seven days.

24h prior to infection of HFFs for the dual perturb-seq experiments, the HFF medium was changed to Dulbecco's Modified Eagle's Medium with 4.5 g/L glucose and GlutaMAX supplemented with 2% heat-inactivated FBS. The HFFs were infected with transfected parasites at an MOI of 0.1. After 1 h the medium was changed to remove remaining extracellular parasites. Where IFN γ stimulation was used, recombinant human IFN γ (BioTechne) was added to the infected cells at 21 h post-infection to a final concentration of 5 ng/mL (~10 U/mL).

24 h post-infection, the HFFs were dissociated using TrypLE Express (Gibco) and stained with 5 µg/mL DAPI (Sigma) for 5 minutes on ice. The cells were pelleted by centrifugation at 300 x g for 5 minutes at 4 °C, resuspended in 2% FBS DMEM without phenol red, and passed through a 30 µm filter. Infected cells were enriched based on Cas9-fluorophore expression using a FACSaria III cell sorter (BD). In the screen, in which a mixture of Cas9-GFP, Cas9-YFP, and Cas9-mCherry vectors was used, only single-positive cells were sorted to limit the number of cells with multiple parasite vacuoles and cell infected by parasites which integrated two different perturb-seq vectors.

The sorted cells were concentrated by centrifugation at 300 x g for 5 minutes at 4 °C and resuspended in DPBS (Sigma) with 0.04% w/v BSA (Sigma). The cells were partitioned into gel bead-in-emulsion droplets for barcoding and reverse transcription using the Chromium Controller (10x Genomics) and Single Cell 3' GEM, Library & Gel Bead Kit v3.1 (10x Genomics) according to the manufacturer's instructions, with a targeted recovery of 10,000 cells per channel. 3' mRNA and CRISPR sgRNA sequencing libraries were constructed following the manufacturer's protocol for 3' gene expression with Feature Barcoding technology for CRISPR screening, using the additional Chromium Single Cell 3' Feature Barcode Library Kit. The libraries were sequenced on a HiSeq 4000 platform (Illumina) with a targeted depth of 50,000 reads per cell (100,000 in the pilot experiments) for the 3' mRNA library and a minimum of 5,000 reads per cell for the CRISPR sgRNA library (Appendix 2).

Dual perturb-seq RNA sequencing data (demultiplexed FASTQ files and Cell Ranger count matrices) have been deposited at the Gene Expression Omnibus with the accession number GSE229505.

Analysis of dual perturb-seq data

Pre-processing, filtering, and normalisation

A multiple species reference genome comprising *Homo sapiens* GRCh38 release 95 and *Toxoplasma gondii* ME49 TGA4 release 42 was generated using Cell Ranger v3.0.2 (10x Genomics). Using Cell Ranger v6.1.2, sequencing reads from the 3' mRNA and CRISPR sgRNA libraries were aligned to this reference and a custom reference of protospacer sequences, including intronic reads but leaving all other settings as the default.

All subsequent analyses were carried out in R v4.0.2 (www.r-project.org). UMI count matrices from Cell Ranger were imported into Seurat v4.2.0 (Hao et al. 2021) with *H. sapiens* mRNA, *T. gondii* mRNA, and CRISPR sgRNA counts split into separate assays of a single Seurat object. Cell barcodes were filtered to retain only cells with a high total count of both *H. sapiens* and *T. gondii* mRNA UMIs, corresponding to infected cells. Cutoffs were set manually following inspection of *H. sapiens* UMI vs. *T. gondii* UMI scatter plots (see **Appendix 3** for cutoffs used for each sample). Cells with >10% UMIs derived from mitochondrial genome-encoded transcripts, characteristic of low-quality cells (Illicic et al. 2016), were removed from the analysis. Finally, cells were filtered to retain only those in which a single protospacer sequence was detected, with the aim of eliminating multiplet droplets or HFFs infected by multiple parasites. The remaining cells were classified based on the *T. gondii* gene target of the detected protospacer sequence.

H. sapiens mRNA counts were normalised using SCTransform v2 (Hafemeister and Satija 2019; Choudhary and Satija 2022). Replicates (Chromium channels) of the perturb-seq screen from both unstimulated and IFN γ -stimulated samples were merged and normalised using SCTransform v2, passing the sample ID as a variable to regress out of the SCTransform residuals.

Identification of effector proteins

The SCTransform residuals ("scale.data" slot) of the top 3,000 most variable genes were analysed by principal component analysis using Seurat. For each *T. gondii* target gene, the distribution of infected cells in the top 20 principal components was compared to all other cells (excluding cells infected with knockouts of controls) using Hotelling's t^2 -test, as implemented in the Hotelling v1.0.8 package. Hits were called based on a Benjamini-Hochberg-adjusted p -value of 0.01 or less.

Pseudo-bulk analyses

For each *T. gondii* target gene represented by at least 30 single cells, pseudo-bulk transcriptomes were generated from the mean SCTransform residuals of the top 3,000 most variable genes. These pseudo-bulk profiles were analysed by principal component analysis. For effectors identified by Hotelling's t^2 -test, Pearson correlation coefficients were calculated between the pseudo-bulk transcriptomes and clustered using the complete linkage method implemented in the hclust function of the stats package v4.0.4.

Identification of differentially expressed genes

In the pilot experiments, differentially expressed host genes were identified by two-sided Wilcoxon rank-sum test on the SCTransform-normalised data using the sgRNA(UPRT)-expressing cells as a reference population. In the screen, differentially expressed host genes were identified for each effector using all other cells (excluding cells infected with knockouts of controls) as a reference population. *p*-values were adjusted using the Benjamini-Hochberg procedure.

Identification of differentially expressed pathways

Single cells were scored for the expression of gene sets in Pathway Interaction Database (Schaefer et al. 2009; Liberzon et al. 2011) using the SCTransform-normalised gene expression data with VISION v2.1.0 (DeTomaso et al. 2019). VISION signature scores were converted to Z-scores and, for each *T. gondii* target gene represented by at least 30 single cells, up- or down-regulation of each pathway in cells infected with that knockout compared to all other knockouts (excluding cells infected with knockouts of controls) was tested by two-sided Wilcoxon rank-sum test. For the effector proteins identified by Hotelling's t^2 -test, the mean pathway Z-score was plotted for each of the pathways differentially expressed by at least one effector (Benjamini-Hochberg-adjusted *p*-value ≤ 0.05). The effectors and gene sets were clustered using these mean Z-scores by the complete linkage method.

Comparison to published bulk phenotypes using VISION signature scores

Host cell genes differentially expressed upon infection with a *T. gondii* knockout strain compared to a wild-type strain were extracted from bulk RNA-seq/microarray data for seven effector proteins: MYR1 (6 hpi RHΔMYR1-infected HFFs) (Naor et al. 2018), HCE1 (6 hpi RHΔHCE1-infected HFFs) (Panas, Naor, et al. 2019), IST (6 hpi RHΔIST-infected HFFs) (Matta et al. 2019), GRA16 (18-20 hpi RHΔGRA16-infected HFFs) (Bougdoor et al. 2013), GRA24 (18-20 hpi RHΔGRA24-infected murine BMDMs) (Braun et al. 2013), GRA28 (RHΔGRA28-infected THP-1s, timepoint not stated) (Rudzki et al. 2021), and ROP16 (24 hpi type II::ROP16^{type I} versus type II wild-type-infected HFFs) (Saeij et al. 2007). Single cell transcriptomes were scored for the expression of these gene sets using the SCTransform-normalised gene expression data with VISION v2.1.0 (DeTomaso et al. 2019), resulting in a "signature score" for each marker gene set for each cell. A higher signature score indicates that the same genes are up- or down-regulated in the single cell transcriptome data as in bulk transcriptome data. *T. gondii* knockout-infected cells were tested for higher scores of the corresponding signature using UPRT knockout-infected cells as a reference population in the pilot experiments and using all other cells (excluding cells infected with knockouts of controls) as a reference population in the screen by Wilcoxon rank-sum test.

Generation of *T. gondii* cell lines

Knockout

A Cas9-GFP::sgRNA plasmid (without HXPRT) (Shen et al. 2014) with a protospacer targeting the coding sequence of the target gene was generated by inverse PCR. A Pro^{GRA1}-mCherry-T2A-HXGPRT-Ter^{GRA2} repair construct was amplified from a template plasmid using primers to introduce 40 bp homology arms to the 5' and 3' UTRs of the target gene. 15 µg each of Cas9-GFP::sgRNA

plasmid and repair construct were co-transfected into RH Δ KU80 or PRU Δ KU80 parasites by electroporation using the Amaxa 4D Nucleofector system (Lonza) with buffer P3 and pulse code EO-115. Parasites were selected for integration of the repair construct with 25 μ g/mL mycophenolic acid and 50 μ g/mL xanthine starting 24 h post-transfection for at least six days, following which clonal cell lines were obtained by limiting dilution. Integration of the repair construct into the target locus, deleting the coding sequence of the target gene, was verified by diagnostic PCR using extracted *T. gondii* genomic DNA.

Complementation

The coding sequence and 1000-1500 bp upstream of the start codon of the target gene was amplified from RH Δ KU80 or PRU Δ KU80 genomic DNA and integrated into the pUPRT plasmid (Shen et al. 2014) by Gibson assembly. 15 μ g each of linearised pUPRT plasmid and Cas9-GFP::sgRNA plasmid targeting the UPRT locus were co-transfected into *T. gondii* parasites by electroporation as described above. Parasites were selected for integration of the pUPRT vector into the UPRT locus with 5 μ M 5-fluorodeoxyuridine (Sigma) starting 24 h post-transfection for at least six days, following which clonal cell lines were obtained by limiting dilution. Integration of the pUPRT plasmid into the UPRT locus was verified by diagnostic PCR using extracted *T. gondii* genomic DNA.

Epitope tagging

A Cas9-GFP::sgRNA plasmid (without HXPRT) (Shen et al. 2014) with a protospacer targeting the 3' UTR of the target gene was generated by inverse PCR. An in-frame HA-Ter^{GRA2}::Pro^{DHFR}-HXGPRT-Ter^{DHFR} repair construct was amplified using primers to introduce 40 bp homology arms to the end of the coding sequence and 3' UTR of the target gene. 15 μ g each of Cas9-GFP::sgRNA plasmid and repair construct were co-transfected into *T. gondii* parasites by electroporation as described above. Parasites were selected for integration of the repair construct with 25 μ g/mL mycophenolic acid and 50 μ g/mL xanthine starting 24 h post-transfection for at least six days, following which clonal cell lines were obtained by limiting dilution. Integration of the repair construct into the target locus, appending a single HA tag to target gene, was verified by diagnostic PCR using extracted *T. gondii* genomic DNA.

Immunofluorescence assays

Anti-HA localisation

HFFs were infected with parasite strains for 24 h, then washed with DPBS, fixed with 4% w/v formaldehyde (Thermo) for 15 min, permeabilised with 0.2% Triton X-100 (Sigma) for 15 min (unless otherwise indicated), and blocked with 2 % w/v bovine serum albumin (Sigma) for 1 h. For non-fluorescent parasite strains, the cells were stained with rat anti-HA for 1h, followed by anti-rat Alexa 594 for 1 h. The cells were then stained with either rabbit anti-*T. gondii* or mouse anti-*T. gondii* plus one of mouse anti-ROP1, mouse anti-GRA1, mouse anti-GRA2 or mouse anti-GRA3 for 1 h. Where rabbit anti-*T. gondii* was used, the cells were then stained with anti-rabbit Alexa 647 plus anti-mouse Alexa 488 plus 5 μ g/mL DAPI for 1 h. Where mouse anti-*T. gondii* was used, cells were stained with anti-mouse Alexa 647 plus anti-rabbit Alexa 488 plus 5 μ g/mL DAPI for 1 h. When mCherry-

expressing parasite strains were used, the cells were stained with rat anti-HA for 1h, followed by anti-rat Alexa 488 for 1 h. The cells were then stained with rabbit anti-*T. gondii* plus either mouse anti-ROP1 or mouse anti-GRA2 for 1 h, followed by anti-rabbit Alexa 405 plus anti-mouse Alexa 647 for 1 h. Images were acquired on a Nikon Ti-E inverted widefield fluorescence microscope with a Nikon CFI APO TIRF 100x/1.49 objective and Hamamatsu C11440 ORCA Flash 4.0 camera running NIS Elements (Nikon).

C1QBP

Where the cells were uninfected and MitoTracker staining was used, cells were stained with 200 nM MitoTracker Red CMXRos (Invitrogen) in complete medium for 30 min prior to fixation. The cells were fixed, permeabilised, and blocked as described above. The cells were stained with rabbit anti-C1QBP overnight at 4 °C followed by anti-rabbit Alexa 488 plus 5 µg/mL DAPI for 1 h. Where the cells were infected and MitoTracker staining was not used, the cells were stained with mouse anti-ROP1 plus rabbit anti-C1QBP overnight at 4 °C, followed by anti-mouse Alexa 594 plus anti-rabbit Alexa 488 plus 5 µg/mL DAPI for 1 h. Images were acquired on a Nikon Ti-E microscope with a 100x/1.49 objective as described above.

cMyc

HFFs were serum starved for 24 h (0.1% FBS), then infected for 24 h, fixed, permeabilised and blocked as described above. The cells were stained with rabbit anti-cMyc plus mouse anti-*T. gondii* for 1 h, followed by anti-rabbit Alexa 488 plus anti-mouse Alexa 647 plus 5 µg/mL DAPI for 1 h.

For each sample, a 3x3 tiled image was acquired as above using a Nikon Plan APO 40x/0.95 objective. cMyc fluorescence intensity was quantified in ImageJ (Schneider, Rasband, and Eliceiri 2012). DAPI signal was used to generate a mask for each host nucleus and the median cMyc intensity in each infected cell nucleus was measured with the median background (non-nucleus) intensity subtracted. The median nuclear signal per sample was taken as representative of a replicate, and normalised by scaling to RHΔKU80 = 1 AU. Five biological replicates were carried out using HFFs prepared independently and infected on different days. Differences between strains were tested by two-sided Wilcoxon rank-sum test with Benjamini-Hochberg adjustment.

EZH2

HFFs were infected for 24 h, fixed, permeabilised and blocked as described above. The cells were stained with mouse anti-EZH2 plus rabbit anti-*T. gondii* overnight at 4 °C, followed by anti-mouse Alexa 488 plus anti-rabbit Alexa 647 plus 5 µg/mL DAPI for 1 h. Images were acquired and median nuclear EZH2 fluorescence quantified as described above for cMyc.

Phospho-p38

HFFs were infected for 24 h, fixed, permeabilised and blocked as described above. The cells were stained with rabbit anti-phospho-p38 plus mouse anti-*T. gondii* overnight at 4 °C, followed by anti-rabbit Alexa 488 plus anti-mouse Alexa 647 plus 5 µg/mL DAPI for 1 h. Images were acquired and median nuclear phospho-p38 fluorescence quantified as described above for cMyc.

IRF1

HFFs were infected and 50 ng/mL (~100 U/mL) recombinant human IFN γ (Bio-Techne) added 1 hpi. The cells were fixed 24 hpi, permeabilised and blocked as described above. The cells were stained with rabbit anti-IRF1 plus mouse anti-*T. gondii* overnight at 4 °C, followed by anti-rabbit Alexa 488 plus anti-mouse Alexa 647 plus 5 μ g/mL DAPI for 1 h. Images were acquired and median nuclear IRF1 fluorescence quantified as described above for cMyc, normalising to uninfected = 1 AU.

Phospho-STAT6 – microscopy

HFFs were infected for 1 h or 24 h, with remaining extracellular parasites washed off at 1 hpi. The cells fixed with 100% methanol for 15 min and blocked with 2% w/v BSA for 1 h. The cells were stained with rabbit anti-phospho-STAT6 plus mouse anti-*T. gondii* overnight at 4 °C, followed by anti-rabbit Alexa 488 plus anti-mouse Alexa 647 plus 5 μ g/mL DAPI for 1 h. Images were acquired and median nuclear phospho-STAT6 fluorescence quantified as described above for cMyc.

Phospho-STAT6 – flow cytometry

HFFs were infected for 2 h, dissociated with TrypLE Express (Thermo) and fixed with 100% methanol for 15 min. The cells were stained with rabbit anti-phospho-STAT6 plus mouse anti-*T. gondii* overnight at 4 °C, followed by anti-rabbit Alexa 488 plus anti-mouse Alexa 594 plus 5 μ g/mL DAPI for 1 h. Data were collected on an LSR II flow cytometer (BD) running FACSDiva v9 (BD) and analysed with FlowJo v10 (www.flowjo.com). The median anti-phospho-STAT6 signal in the infected cells was determined for each sample, and the median technical replicate taken to represent the biological replicate. The data were scaled to RH Δ KU80 = 1 AU and differences between strains tested by two-sided Wilcoxon rank sum test with Benjamini-Hochberg adjustment.

GRA16-HA/IST-HA export

HFFs were infected for 24 h, fixed with formaldehyde, permeabilised, and blocked as described above for cMyc. The cells were stained with rat anti-HA, followed by anti-rat Alexa 594, followed by rabbit anti-*T. gondii*, followed by anti-rabbit Alexa 647. For each sample, a 3x3 tiled image was acquired as above using a Nikon Plan APO 40x/0.95 objective. The proportion of exported GRA16-HA/IST-HA was quantified using ImageJ. DAPI signal was used to generate a mask for each host nucleus and anti-*T. gondii* signal was used to generate a mask for each vacuole. The median anti-HA signal in the background (non-nucleus and non-vacuole) was subtracted across the whole image, and the total anti-HA signal in the host nuclei and *T. gondii* vacuoles. was measured. The export ratio was calculated as the ratio of nuclear signal to vacuolar signal. To determine the percentage of vacuoles with IST-HA puncta, the images were blinded and the vacuoles manually scored for the presence/absence of puncta. Five biological replicates were carried out using HFFs prepared independently and infected on different days. Differences between strains were tested by unpaired, two-sided *t*-test with Benjamini-Hochberg adjustment.

IRGB6 recruitment

75,000 BMDMs per well were seeded in a 96-well μ -plate (Ibidi) and stimulated with 10 ng/mL (~100 U/mL) recombinant mouse IFN γ (Gibco) for 24 h prior to infection. The BMDMs were infected with parasite strains with an MOI of 0.3 for 1 h, fixed with 4% w/v formaldehyde for 15 min, permeabilised with 0.1% w/v saponin (Sigma) for 15 minutes and blocked with 2% w/v BSA for 1 h.

The cells were stained with rabbit anti-IRGB6 for 1 h, followed by anti-rabbit Alexa 488 plus 5 µg/mL DAPI plus 5 µg/mL CellMask Deep Red plasma membrane stain for 1 h. Images were acquired on an Opera Phenix High-Content Screening System (PerkinElmer) as above, and analysed in Harmony v5. Vacuoles were counted as recruited if the median anti-IRGB6 intensity in a 6 pixel-wide ring around the vacuole (defined by parasite-expressed mCherry signal) was more than 2.3–2.6x (depending on the maximum signal intensity in the replicate) higher than the median anti-IRGB6 signal in the rest of the infected cell. For each well the % IRGB6-recruited vacuoles was determined, and the median % recruitment per strain taken as representative of a biological replicate. Differences between strains were determined by paired two-sided *t*-test with Benjamini-Hochberg adjustment.

Vacuole ubiquitination

150,000 BMDMs per well were seeded in an 8-well µ-slide (Ibidi) and stimulated with 10 ng/mL (~100 U/mL) recombinant mouse IFN γ for 24 h prior to infection. The BMDMs were infected with parasite strains at an MOI of 0.3 for 3 h, then washed and fixed with 4% w/v formaldehyde for 15 min. Prior to permeabilisation, the cells were blocked with 2% w/v BSA for 1 h and extracellular parasites were stained with rabbit anti-*T. gondii* for 1 h, followed by anti-rabbit 405 for 1 h. The cells were then permeabilised with 0.2% v/v Triton X-100 for 15 minutes, blocked again with 2% w/v BSA for 1 h, stained with mouse anti-ubiquitinated proteins overnight at 4 °C, and finally stained with anti-mouse 488 for 1 h. For each sample, a 3x3 tiled image was acquired as above using a Nikon Plan APO 40x/0.95 objective. The images were blinded and the percentage of ubiquitinated vacuoles was determined manually using ImageJ, excluding *T. gondii* cells which were positive for extracellular staining. Differences between strains were determined by two-sided *t*-test with Benjamini-Hochberg adjustment.

Western blot

For purified *T. gondii* samples, parasites were isolated from host cell material by syringe-lysis, filtering, and washing in DPBS. For *T. gondii* plus host cell samples, infected cells were washed with DPBS and dissociated with a cell scraper. Samples were lysed in RIPA buffer (Thermo) supplemented with 2x cOmplete Mini EDTA-free Protease Inhibitor Cocktail (Roche) and 2 U/mL benzonase nuclease (Sigma) for 30 min on ice. The samples were centrifuged at 15,000 x g for 5 min at 4 °C and the pellet discarded. Protein concentration was quantified using the Pierce BCA protein assay kit (Thermo). 10 µg protein per sample was heated to 95 °C for 5 min in sample loading buffer and separated by SDS-PAGE using the Mini-PROTEAN electrophoresis system (Bio-Rad). Proteins were transferred to a nitrocellulose membrane using the Trans-Blot Turbo transfer system (Bio-Rad), blocked in 2% w/v skim milk powder, 0.1% v/v Tween 20 in PBS for 1 h at room temperature. Primary and secondary antibodies as indicated were incubated with the membrane for 1 h each. Secondary antibodies used were anti-rat IRDye 800CW, anti-mouse IRDye 680LT, anti-mouse IRDye 800CW, anti-rabbit IRDye 680 LT, and anti-rabbit IRDye 800CW. Images were acquired using an Odyssey CLx Imager (Li-Cor).

Transmission electron microscopy

HFFs grown on glass coverslips were infected with *T. gondii* cell lines for 24 h, fixed with 2.5% w/v glutaraldehyde and 4% v/w formaldehyde in 0.1 M phosphate buffer (PB) for 30 min and transferred to a BioWave Pro+ microwave for processing (Pelco; Agar Scientific). The cells were washed with PB twice on the bench and twice in the microwave 250 W for 40 s, stained with 1% reduced osmium for 14 min under vacuum (with/without 100 W power at 2 min intervals), and then washed twice on the bench and twice in the microwave with PB. A further stain with 1% tannic acid for 14 min (with/without 100 W power at 2 min intervals under vacuum) was followed by a quench with 1% sodium sulphate at 250 W for 2 min under vacuum and bench and microwave washes in water (as for PB). The blocks were then dehydrated in a graded ethanol series of 70%, 90%, and 100%, each performed twice at 250 W for 40 s. Exchange into Epon resin (Taab Embed 812) was performed with 50% resin in ethanol, followed by three 100% resin steps, each at 250 W for 3 min, with 30 s vacuum cycling. Finally, the samples were baked for 24 h at 60°C. 80 nm sections were stained with lead citrate and imaged in a JEM-1400 FLASH transmission electron microscope (JEOL).

Plaque assay

100 tachyzoites were inoculated onto a T25 flask of confluent HFF and left undisturbed for 10 days. The cells were stained with 0.5% w/v crystal violet (Sigma), 0.9% w/v ammonium oxalate (Sigma), 20% v/v methanol in distilled water, then washed with DPBS. Images were taken using a GelDoc Go System (Bio-Rad) and plaque area quantified using ImageJ. One biological replicate was carried out. Differences in plaque sizes between strains was measured by unpaired, two-sided *t*-test using all measured plaques as replicates, with Benjamini-Hochberg *p*-value adjustment.

In vivo virulence assay

T. gondii parasites were syringe-lysed from intact vacuoles, passed through a 5 µm filter and resuspended in PBS. Five C57BL/6J mice per *T. gondii* strain were randomly assigned to each experimental group and infected with 50,000 parasites by intraperitoneal injection. Parasite viability was determined by plaque assay using inoculum left over after the mouse injections to be 40% for all three parasite strains used, resulting in an infective dose of 20,000 parasites per mouse. Mice were monitored daily for 30 days and euthanised if the humane endpoint was reached. All surviving mice were checked by serum antibody ELISA to confirm seroconversion against *T. gondii* tachyzoite lysate antigen as previously described (Young et al. 2019).

IFN γ restriction assay

For BMDMs, 75,000 cells per well were seeded in a 96-well µ-plate (Ibidi). For THP-1-derived macrophages, 75,000 THP-1 monocytes were seeded per well and differentiated into macrophages. For MEFs, 10,000 cells were seeded per well and grown to confluence prior to infection. Cells were stimulated with 10 ng/mL (~100 U/mL) recombinant mouse IFN γ (Gibco) or 50 ng/mL (~100 U/mL)

recombinant human IFN γ (BioTechne) 24 h prior to infection or left unstimulated. The cells were infected with *T. gondii* parasite strains at an MOI of 0.3, with at least three wells for each strain with and without IFN γ per replicate. The plates were fixed at 24 hpi with 4% w/v formaldehyde for 15 min and stained with 5 μ g/mL DAPI plus 5 μ g/mL CellMask Deep Red Plasma Membrane Stain (Thermo) for 1 h. Biological replicates were carried out on different days with independently prepared host cells.

The plates were imaged on an Opera Phenix High-Content Imaging System (PerkinElmer) with a 40x 1.1 NA water immersion objective. 25 fields of view with 3-5 focal planes (depending on host cell type) were captured per well. Analysis was performed in Harmony v5 (PerkinElmer) on a maximum projection of the focal planes. For each well, the total number of host cell nuclei and *T. gondii* vacuoles in the captured fields of view was determined by thresholding on the DAPI and mCherry signal. The number of parasite nuclei in each vacuole was determined based on DAPI signal to define the total number of parasites in the captured fields of view and the mean number of parasites per vacuole in each well. A pseudo-count equivalent to one vacuole containing four parasites (the BMDM dataset-wide mean vacuole size) was added to each well in the BMDM data to prevent a division by zero error, as no parasites were detected in some IFN γ -stimulated wells infected with PRU Δ GRA12. For each *T. gondii* line, IFN γ -mediated restriction was calculated as the median tachyzoite number/vacuole number/vacuole size/host cell number in the IFN γ -stimulated wells as a percentage of the median in the unstimulated wells. Differences between strains were tested by paired two-sided t-test with Benjamini-Hochberg adjustment.

Propidium iodide uptake assay

60,000 BMDMs per well of a Falcon black-walled, clear-bottom 96-well plate were stimulated with 10 ng/mL (~100 U/mL) recombinant mouse IFN γ (Gibco) for 24 h prior to infection. The cells were infected with *T. gondii* strains at an MOI of 0.3 or 3, at the same time that propidium iodide (Thermo) was added to a final concentration of 5 μ g/mL. Images of each well were acquired every 30 min between 1 hpi and 12 hpi on a Nikon Ti-E inverted widefield fluorescence microscope maintained at 37 °C with a Nikon CFI Plan Fluor 4x/0.13 NA objective and Hamamatsu C11440 ORCA Flash 4.0 camera running NIS Elements (Nikon). After 12 h, Triton X-100 was added to a final concentration of 1% v/v to fully permeabilise the cells, and a final image captured of each well. In each image, the total propidium iodide fluorescence signal was measured in ImageJ. The initial measurement in each well was subtracted from subsequent measurements to remove background fluorescence signal, and the percentage propidium iodide uptake calculated relative to the signal following full permeabilisation. The mean propidium iodide uptake across three technical replicates for each strain was taken to represent the biological replicate. Differences between strains were tested by paired two-sided t-test with Benjamini-Hochberg *p*-value adjustment.

Arginase activity assay

10⁶ BMDMs per sample were infected with *T. gondii* parasites at an MOI of 1 for 24 h or 36 h. Arginase activity in the cell lysate was measured using a colorimetric assay (Abcam). Two biological

replicates were carried out for each timepoint with BMDMs prepared and infected on different days, with triplicate infections for each strain in each biological replicate. Differences between strains were tested by two-sided *t*-test with Benjamini-Hochberg *p*-value adjustment.

Co-immunoprecipitation mass spectrometry

Primary MEFs/HFFs were grown to confluence in T150 flasks and infected with 5 million parasites per flasks (approx. MOI of 3) for 24 h, in triplicate for each strain. The cells were washed three times with DPBS, dissociated with a cell scraper, centrifuged at 3,000 x *g* for 5 min at 4 °C, and lysed in 1 mL chilled IP buffer on ice (50 mM Tris, 150 mM NaCl, 0.2% v/v Triton X-100, 2x cOmplete Mini EDTA-free Protease Inhibitor Cocktail (Sigma), pH 7.5). The samples were incubated with 100 µL anti-HA agarose matrix (Thermo) for mass spectrometry analysis or 30 µL anti-HA magnetic beads (Thermo) for Western blot analysis overnight at 4 °C with rotation. The matrix/beads were washed three times with chilled IP buffer and the bound proteins eluted with 30 µL 3x Sample Loading Buffer (NEB) at 95 °C for 10 min.

Samples were prepared for LC-MS/MS analysis by in-gel tryptic digestion. The eluted proteins were run 1 cm into a NuPAGE 10% Bis-Tris gel (Thermo) and stained with Coomassie Brilliant Blue. The gel was cut into 1 mm cubes, destained using 50% ethanol, 50 mM ammonium bicarbonate, and dehydrated with 100% ethanol. Proteins were then simultaneously reduced and alkylated with 10 mM tris(2-carboxyethyl)phosphine and 40 mM chloroacetamide in water at 70°C for 5 min. The gel cubes were washed in 50% ethanol, 50 mM ammonium bicarbonate and dehydrated as above. Proteins were digested with 250 ng of mass spectrometry-grade trypsin (Thermo) in 50 mM HEPES, pH 8, at 37°C overnight. Peptides were extracted from the gel into acetonitrile and dried by vacuum centrifugation.

Digested samples were solubilised in 0.1% formic acid and loaded onto Evotips (Evosep), according to the manufacturer's instructions. Following a wash with aqueous acidic buffer (0.1% formic acid in water), samples were loaded onto an Evosep One system coupled to an Orbitrap Fusion Lumos (ThermoFisher Scientific). The Evosep One was fitted with a 15 cm column (PepSep) and a predefined gradient for a 44-minute method was employed. The Orbitrap Lumos was operated in data-dependent mode (1 second cycle time), acquiring IT HCD MS/MS scans in rapid mode after an OT MS1 survey scan (*R* = 60,000). The MS1 target was 4E5 ions whereas the MS2 target was 1E4 ions. The maximum ion injection time utilized for MS2 scans was 300 ms, the HCD normalized collision energy was set at 32 and the dynamic exclusion was set at 15 seconds.

Acquired raw files were processed with MaxQuant v1.5.2.8 (Cox and Mann 2008). Peptides were identified from the MS/MS spectra searched against *Toxoplasma gondii* (ToxoDB) and *Mus musculus* (UniProt) proteomes using the Andromeda search engine (Cox et al. 2011). Methionine oxidation, acetylation (N-term), and deamidation (NQ) were selected as variable modifications whereas cysteine carbamidomethylation was selected as a fixed modification. The enzyme specificity was set to trypsin with a maximum of two missed cleavages. The precursor mass tolerance was set to 20 ppm for the first search (used for mass re-calibration) and to 4.5 ppm for the main search. The datasets were filtered on posterior error probability (PEP) to achieve a 1% false discovery rate on protein,

peptide and site level. Other parameters were used as pre-set in the software. “Unique and razor peptides” mode was selected to allow identification and quantification of proteins in groups (razor peptides are uniquely assigned to protein groups and not to individual proteins). Intensity-based absolute quantification (iBAQ) in MaxQuant was performed using a built-in quantification algorithm (Cox and Mann 2008) enabling the “Match between runs” option (time window 0.7 minutes) within replicates.

MaxQuant output files were processed with Perseus, v1.5.0.9 (Tyanova et al. 2016). Data were filtered to remove contaminants, protein IDs originating from reverse decoy sequences and only identified by site. iBAQ intensities and the total intensity were $\log_2$ and $\log_{10}$ transformed, respectively. Samples were grouped according to sample type (PRU Δ KU80 or PRU ROP1-HA) and the iBAQ intensities were filtered for the presence of two valid values in at least one group. Next, missing values were imputed from the normal distribution in order to generate $\log_2$ fold-changes (L2FCs) between tested conditions and perform statistical analysis (Welch’s *t*-test, $p < 0.05$, $-3 > \text{L2FC} > 3$). The L2FC threshold was set at three times the median absolute deviation.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository (Perez-Riverol et al. 2019) with the dataset identifier PXD032319.

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Appendices

Appendix 1: *In vivo* CRISPR screen sequencing statistics

"Mapped reads" refers to reads that contain a perfect match of one of the protospacer sequences. "Library coverage" is calculated by dividing the number of mapped reads by the number of protospacer sequences (906) and is thus the mean number of mapped reads per protospacer.

Sample	Description	Total reads	Mapped reads	Mapped reads (% of total)	Library coverage
1	Plasmid pool	12,649,881	7,712,898	60.97	8,513x
2	<i>In vitro</i> selected parasites	21,256,434	13,042,729	61.36	14,395x
3	Parasite inoculum	24,190,350	14,391,496	59.49	15,884x
4	<i>Ex vivo</i> parasites: mouse 1	17,272,510	10,704,384	61.97	11,814x
5	<i>Ex vivo</i> parasites: mouse 2	7,747,796	4,725,825	61.00	5,216x
6	<i>Ex vivo</i> parasites: mouse 3	20,219,463	12,046,399	59.58	13,296x
7	<i>Ex vivo</i> parasites: mouse 4	23,238,863	14,369,094	61.83	15,859x
8	<i>Ex vivo</i> parasites: mouse 5	23,740,344	14,552,495	61.30	16,062x

Appendix 2: Dual perturb-seq sequencing statistics

“Total reads” is the number of read pairs assigned to the library in demultiplexing. “Reads per cell” is the mean number of read pairs per cell, based on the Cellranger estimate of the number of host cells. “Sequencing saturation” is the percentage of read pairs originating from an already-observed cell barcode, UMI, reference gene combination. “Confidently mapped reads” is the percentage of read pairs that contain a valid cell barcode and UMI and map to a unique gene in the dual reference transcriptome in a manner consistent with annotated splice junctions; these reads are considered for UMI counting. “GRCh38 reads” and “TGA4 reads” are the percentages of confidently mapped reads mapped to the GRCh38 or TGA4 transcriptomes respectively, together totalling 100%. “Useable guide reads” is the percentage of CRISPR sgRNA library read pairs with a valid cell barcode and UMI and recognised protospacer sequence; these reads are considered for UMI counting.

Sample (Chromium channel)	Experiment date	Total reads (mRNA)	Reads per cell (mRNA)	Sequencing saturation (mRNA)	Confidently mapped reads (mRNA)
PILOT_2_GUIDES	2019-03-06	275,321,407	100,850	44.9%	79.0%
PILOT_24_GUIDES	2020-10-08	218,114,168	107,287	40.8%	73.5%
SCREEN_UNSTIMULATED_1	2021-02-10	309,117,785	54,393	39.2%	82.4%
SCREEN_UNSTIMULATED_2	2021-02-10	272,053,524	43,837	32.0%	82.0%
SCREEN_UNSTIMULATED_3	2021-06-25	290,184,793	52,418	22.6%	78.7%
SCREEN_UNSTIMULATED_4	2021-06-25	697,191,402	32,160	35.6%	76.8%
SCREEN_UNSTIMULATED_5	2022-03-23	479,913,974	49,701	36.7%	80.0%
SCREEN_UNSTIMULATED_6	2022-03-23	198,395,232	56,123	35.1%	69.7%
SCREEN_UNSTIMULATED_7	2022-03-23	262,446,599	56,186	35.8%	71.5%
SCREEN_UNSTIMULATED_8	2022-03-23	133,572,213	53,344	36.0%	78.2%
SCREEN_IFNG_1	2021-10-28	427,099,355	77,066	31.8%	80.6%
SCREEN_IFNG_2	2021-10-28	398,564,731	78,073	31.7%	81.3%
SCREEN_IFNG_3	2021-10-28	266,597,394	68,569	32.8%	79.8%
SCREEN_IFNG_4	2021-10-28	174,671,170	72,418	30.2%	81.3%
SCREEN_IFNG_5	2022-04-06	512,184,073	59,336	37.4%	79.0%
SCREEN_IFNG_6	2022-04-06	324,184,312	53,302	36.7%	79.8%
SCREEN_IFNG_7	2022-04-06	378,421,340	51,824	36.7%	78.9%
SCREEN_IFNG_8	2022-04-06	236,049,048	51,993	33.7%	79.2%

Continued overleaf

Sample (Chromium channel)	Total reads (sgRNA)	Reads per cell (sgRNA)	Sequencing saturation (sgRNA)	Useable guide reads (sgRNA)
PILOT_2_GUIDES	18,837,863	6,900	96.4%	0.5%
PILOT_24_GUIDES	187,808,809	92,380	93.7%	0.3%
SCREEN_UNSTIMULATED_1	38,716,364	6,813	92.9%	0.8%
SCREEN_UNSTIMULATED_2	39,646,888	6,388	94.7%	1.3%
SCREEN_UNSTIMULATED_3	35,292,237	6,375	89.1%	4.3%
SCREEN_UNSTIMULATED_4	121,848,672	5,621	96.6%	3.9%
SCREEN_UNSTIMULATED_5	82,598,109	8,554	82.0%	2.2%
SCREEN_UNSTIMULATED_6	36,943,678	10,451	49.3%	0.4%
SCREEN_UNSTIMULATED_7	36,321,188	7,776	45.1%	0.5%
SCREEN_UNSTIMULATED_8	16,428,042	6,561	66.0%	1.6%
SCREEN_IFNG_1	278,764,623	50,300	90.7%	0.5%
SCREEN_IFNG_2	263,982,927	51,711	91.0%	0.5%
SCREEN_IFNG_3	184,613,205	47,483	86.0%	0.2%
SCREEN_IFNG_4	126,764,135	52,556	87.0%	0.4%
SCREEN_IFNG_5	47,619,443	5,517	63.2%	1.6%
SCREEN_IFNG_6	40,220,036	6,613	66.4%	1.8%
SCREEN_IFNG_7	38,309,698	5,246	56.1%	0.9%
SCREEN_IFNG_8	36,214,028	7,977	76.5%	3.3%

Appendix 3: Dual perturb-seq UMI filtering criteria and statistics

“Estimated cells” is the Cellranger estimate of the number of human host cells. “Filtering criteria” are the cutoffs used to select cells for further analysis. “Hs UMIs” is the total number of *Homo sapiens* host cell UMIs per cell barcode. “Tg UMIs” is the total number of *T. gondii* UMIs per cell barcode. “% MT” is the percentage of *Homo sapiens* UMIs that are derived from mitochondrial genome-encoded transcripts. “Unique sgRNAs” is the number of unique sgRNA protospacer sequences that were detected with a given cell barcode. Hs UMI and Tg UMI cutoffs were selected manually based on inspection of scatter plots (as in **Figure 14B**).

Sample (Chromium channel)	Estimated cells	Filtering criteria: Hs UMI	Filtering criteria: Tg UMI	Filtering criteria: % MT	Filtering criteria: unique sgRNAs
PILOT_2_GUIDES	2,730	≥ 10,000	≥ 3,000	≤ 10	= 1
PILOT_24_GUIDES	2,033	≥ 10,000	≥ 3,000	≤ 10	= 1
SCREEN_UNSTIMULATED_1	5,683	≥ 5,000	≥ 1,000	≤ 10	= 1
SCREEN_UNSTIMULATED_2	6,206	≥ 5,000	≥ 1,000	≤ 10	= 1
SCREEN_UNSTIMULATED_3	5,536	≥ 5,000	≥ 4,000	≤ 10	= 1
SCREEN_UNSTIMULATED_4	21,679	≥ 5,000	≥ 1,000	≤ 10	= 1
SCREEN_UNSTIMULATED_5	9,656	≥ 5,000	≥ 2,000	≤ 10	= 1
SCREEN_UNSTIMULATED_6	3,535	≥ 5,000	≥ 2,000	≤ 10	= 1
SCREEN_UNSTIMULATED_7	4,671	≥ 5,000	≥ 2,000	≤ 10	= 1
SCREEN_UNSTIMULATED_8	2,504	≥ 5,000	≥ 2,000	≤ 10	= 1
SCREEN_IFNG_1	5,542	≥ 5,000	≥ 3,000	≤ 10	= 1
SCREEN_IFNG_2	5,105	≥ 5,000	≥ 3,000	≤ 10	= 1
SCREEN_IFNG_3	3,888	≥ 5,000	≥ 3,000	≤ 10	= 1
SCREEN_IFNG_4	2,412	≥ 5,000	≥ 3,000	≤ 10	= 1
SCREEN_IFNG_5	8,632	≥ 5,000	≥ 3,000	≤ 10	= 1
SCREEN_IFNG_6	6,082	≥ 5,000	≥ 3,000	≤ 10	= 1
SCREEN_IFNG_7	7,302	≥ 5,000	≥ 3,000	≤ 10	= 1
SCREEN_IFNG_8	4,540	≥ 5,000	≥ 3,000	≤ 10	= 1

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Sample (Chromium channel)	Filtered cells	Median <i>Hs</i> UMIs per filtered cell	Median <i>Tg</i> UMIs per filtered cell	Median sgRNA UMIs per filtered cell
PILOT_2_GUIDES	584	30,240	11,590	2
PILOT_24_GUIDES	919	42,084	9,197	9
SCREEN_UNSTIMULATED_1	1,243	26,804	3,400	2
SCREEN_UNSTIMULATED_2	1,436	23,439	3,054	2
SCREEN_UNSTIMULATED_3	1,739	21,206	9,320	6
SCREEN_UNSTIMULATED_4	6,215	11,347	3,581	2
SCREEN_UNSTIMULATED_5	2,356	17,664	5,306	6
SCREEN_UNSTIMULATED_6	910	17,744	7,232	5
SCREEN_UNSTIMULATED_7	1,231	17,834	7,626	4
SCREEN_UNSTIMULATED_8	790	20,010	6,026	7
SCREEN_IFNG_1	1,122	39,490	10,690	8
SCREEN_IFNG_2	1,004	39,385	10,993	8
SCREEN_IFNG_3	507	41,790	11,769	6
SCREEN_IFNG_4	688	34,469	10,309	7
SCREEN_IFNG_5	1,947	20,332	10,113	8
SCREEN_IFNG_6	1,344	19,058	8,883	9
SCREEN_IFNG_7	1,421	19,626	9,585	7
SCREEN_IFNG_8	1,232	16,656	7,628	10

Appendix 4: List of *in vivo* CRISPR screen fitness phenotype scores

Genes are sorted by median *in vivo* L2FC. Putative virulence factors identified in this screen (median L2FC *in vivo* ≤ -1 and *in vitro* Z-score from Sidik et al. 2016 and Wang et al. 2020 > -1) are listed in bold. Of these, previously validated virulence factors are annotated with an asterisk.

Gene ID	Gene name	Median <i>in vitro</i> L2FC	Median <i>in vivo</i> L2FC
TGME49_288650	GRA12*	-0.14	-4.88
TGME49_205250	ROP18*	-0.07	-4.62
TGME49_235130	RASP1	-0.28	-3.70
TGME49_270250	GRA1	-0.17	-2.95
TGME49_225150	225150	-0.33	-2.95
TGME49_205240	CLPTM1	-0.21	-2.70
TGME49_289380	GRA39	-0.31	-2.53
TGME49_312420	GRA38	-0.14	-2.51
TGME49_315160	RASP2	-0.19	-2.21
TGME49_316250	GRA45*	-0.20	-2.15
TGME49_232020	RON12	-0.06	-2.05
TGME49_278870	MyoF	-0.08	-1.86
TGME49_301270	301270	-0.10	-1.86
TGME49_222310	222310	0.01	-1.72
TGME49_205190	205190	-0.45	-1.69
TGME49_299780	299780	0.11	-1.65
TGME49_245580	245580	-0.75	-1.60
TGME49_309590	ROP1	0.02	-1.53
TGME49_310010	RON1	-0.07	-1.51
TGME49_289150	289150*	-0.09	-1.51
TGME49_227620	GRA2*	0.32	-1.50
TGME49_212300	GRA32	-0.13	-1.31
TGME49_310780	GRA4*	0.36	-1.28
TGME49_290700	GRA25*	0.59	-1.27
TGME49_249730	ND9	-0.33	-1.19
TGME49_286450	GRA5	0.51	-1.17
TGME49_228170	GRA44	-0.07	-1.08
TGME49_202200	202200	-0.07	-1.06
TGME49_305860	CDPK3*	-0.26	-1.00
TGME49_313330	ROP27	-0.09	-0.92
TGME49_278510	PPM3F	0.25	-0.90
TGME49_276130	CPC2	-0.08	-0.89
TGME49_301440	CDPK1	-0.53	-0.84
TGME49_227010	ROP30	0.12	-0.76
TGME49_261750	RON10	0.01	-0.74
TGME49_244080	244080	-0.43	-0.74
TGME49_205360	205360	-0.10	-0.69
TGME49_269885	TLN1	-0.07	-0.65
TGME49_225320	225320	-0.17	-0.65

TGME49_249670	Toxopain-1	0.21	-0.64
TGME49_222100	222100	0.04	-0.62
TGME49_262420	262420	0.15	-0.53
TGME49_220240	GRA31	-0.52	-0.53
TGME49_304740	ROP35_WNG1	0.19	-0.47
TGME49_312950	312950	0.14	-0.47
TGME49_242230	ROP29	-0.35	-0.46
TGME49_254720	GRA8	0.33	-0.39
TGME49_200360	200360	-0.09	-0.39
TGME49_242250	ROP19B	0.09	-0.38
TGME49_201130	ROP33	0.18	-0.38
TGME49_203520	203520	-0.03	-0.34
TGME49_308970	308970	0.00	-0.31
TGME49_246178	246178	-0.39	-0.31
TGME49_316360	316360	-0.02	-0.26
TGME49_230180	GRA24	0.17	-0.26
TGME49_258230	ROP20	0.39	-0.25
TGME49_220950	MAF1B	0.21	-0.24
TGME49_202610	PPM3D	0.13	-0.22
TGME49_258660	ROP6	-0.04	-0.22
TGME49_320630	320630	0.04	-0.21
TGME49_310790	CST9	-0.06	-0.21
TGME49_210095	210095	0.23	-0.20
TGME49_226380	GRA35	0.24	-0.19
TGME49_262400	262400	-0.15	-0.17
TGME49_265120	RON2L2	0.26	-0.16
TGME49_215220	GRA22	0.46	-0.16
TGME49_227280	GRA3	0.67	-0.14
TGME49_239600	ROP23	-0.23	-0.13
TGME49_225200	225200	0.06	-0.12
TGME49_212410	GRA11A	0.04	-0.10
TGME49_208830	GRA16	0.26	-0.09
TGME49_243730	ROP9	0.23	-0.08
TGME49_285290	285290	0.08	-0.07
TGME49_211260	ROP26	0.12	-0.06
TGME49_263220	ROP21	-0.08	-0.05
TGME49_240090	ROP34_WNG2	0.20	-0.05
TGME49_304490	304490	0.08	-0.05
TGME49_246550	ASP3	-0.35	-0.03
TGME49_266100	ROP41	0.24	-0.01
TGME49_229500	229500	-0.20	0.00
TGME49_202780	ROP25	0.03	0.03
TGME49_254880	254880	-0.18	0.04
TGME49_315940	315940	0.12	0.05
TGME49_251540	GRA9	0.23	0.07
TGME49_239740	GRA14	0.20	0.08
TGME49_203790	203790	0.12	0.09
TGME49_219810	GRA40	0.39	0.10

TGME49_315210	315210	-0.01	0.12
TGME49_272540	272540	0.27	0.12
TGME49_259830	259830	-0.38	0.12
TGME49_204320	204320	-0.25	0.14
TGME49_205140	205140	0.08	0.15
TGME49_314780	314780	-0.17	0.16
TGME49_315490	ROP10	-0.14	0.16
TGME49_291960	ROP40	0.14	0.17
TGME49_297960	RON6	-0.19	0.18
TGME49_203310	GRA7	0.47	0.18
TGME49_241610	GRA21	0.41	0.18
TGME49_267740	GRA49	0.15	0.19
TGME49_237800	GRA11B	0.21	0.20
TGME49_242110	ROP38	-0.11	0.20
TGME49_309760	309760	0.36	0.21
TGME49_262730	ROP16	0.03	0.21
TGME49_269075	269075	0.00	0.22
TGME49_270240	MAG1	0.19	0.23
TGME49_275850	275850	0.16	0.23
TGME49_270320	PPM3C	0.11	0.24
TGME49_280670	280670	-0.13	0.25
TGME49_214080	Toxofilin	0.26	0.25
TGME49_242118	242118	-0.01	0.25
TGME49_253780	253780	-0.06	0.26
TGME49_209985	209985	0.27	0.27
TGME49_215775	ROP8	0.36	0.29
TGME49_205050	205050	-0.14	0.29
TGME49_242240	ROP19A	-0.03	0.29
TGME49_288840	GRA18	0.68	0.30
TGME49_308950	308950	0.19	0.31
TGME49_314500	SUB2	-0.14	0.31
TGME49_274170	ROP49	0.42	0.32
TGME49_297880	GRA23	0.38	0.32
TGME49_234950	234950	-0.13	0.34
TGME49_277720	277720	-0.07	0.34
TGME49_245432	245432	0.22	0.35
TGME49_253370	RON4L1	-0.19	0.35
TGME49_200010	GRA20	0.19	0.35
TGME49_247520	WIP	0.42	0.35
TGME49_203600	GRA47_CST2	0.33	0.36
TGME49_229680	229680	-0.04	0.37
TGME49_253330	BPK1	0.26	0.37
TGME49_258800	ROP31	-0.39	0.37
TGME49_203990	ROP12	0.40	0.40
TGME49_289540	SFP1	0.25	0.40
TGME49_218270	ROP48	-0.04	0.41
TGME49_232000	GRA30	0.30	0.42
TGME49_314250	BRP1	0.33	0.43

TGME49_204560	204560	0.06	0.45
TGME49_262920	262920	-0.02	0.45
TGME49_217400	217400	-0.36	0.46
TGME49_201180	MSF	0.10	0.48
TGME49_203290	GRA34	0.05	0.50
TGME49_230470	ROP46	0.08	0.51
TGME49_201390	201390	0.26	0.55
TGME49_225330	225330	0.00	0.57
TGME49_270920	ROP32	0.18	0.57
TGME49_313440	313440	0.19	0.58
TGME49_201520	PPM3G	0.05	0.58
TGME49_268970	268970	0.50	0.60
TGME49_211290	ROP15	-0.14	0.63
TGME49_266050	266050	0.16	0.65
TGME49_204340	GRA54	0.43	0.67
TGME49_277270	NTPase II	0.68	0.69
TGME49_208450	PI-2	0.02	0.69
TGME49_280380	280380	0.26	0.71
TGME49_252360	ROP24	-0.14	0.72
TGME49_231960	GRA28	0.31	0.76
TGME49_258580	ROP17	-0.17	0.79
TGME49_202620	202620	0.27	0.80
TGME49_254470	MYR1	0.09	0.94
TGME49_242090	242090	-0.07	0.99
TGME49_210370	ROP54	-0.05	1.00
TGME49_252500	252500	0.17	1.18
TGME49_275470	GRA15	0.61	1.18
TGME49_281450	281450	0.00	1.59
TGME49_314430	314430	0.14	1.89

Appendix 5: List of perturbation scores from dual perturb-seq screen

Results of Hotelling t^2 -test on principle component embeddings of single cell transcriptomes from combined dual perturb-seq data (unstimulated and IFN γ -stimulated), sorted by t^2 -test statistic (perturbation score). Target genes represented by at least three single cell transcriptomes are included. Effectors that induce a significant perturbation (Benjamini-Hochberg-adjusted p -value < 0.01) are listed in bold. Control effectors are indicated with an asterisk.

Gene ID	Gene name	Perturbation score	Adjusted p -value	Number of cells
TGME49_237230	MYR3*	1029.26	<1E-15	216
TGME49_254470	MYR1*	807.92	<1E-15	204
TGME49_230180	GRA24*	538.62	<1E-15	244
TGME49_222100	SOS1	519.59	<1E-15	237
TGME49_316250	GRA45*	337.02	<1E-15	96
TGME49_231960	GRA28*	240.76	<1E-15	123
TGME49_270700	MYR2*	231.68	<1E-15	180
TGME49_258580	ROP17*	187.91	<1E-15	68
TGME49_239010	HCE1*	173.97	<1E-15	175
TGME49_240060	IST*	147.46	<1E-15	162
TGME49_211460	MYR4*	127.31	<1E-15	33
TGME49_208830	GRA16*	113.47	1.10E-13	71
TGME49_217680	GRA57	104.30	4.67E-12	155
TGME49_243730	ROP9	93.00	4.38E-10	114
TGME49_203310	GRA7	91.97	6.21E-10	331
TGME49_313440	GRA59	89.82	1.38E-09	89
TGME49_290700	GRA25	65.74	1.39E-05	225
TGME49_220950	MAF1B	64.34	2.19E-05	203
TGME49_227280	GRA3	62.35	4.26E-05	1226
TGME49_262730	ROP16*	61.09	6.35E-05	128
TGME49_249990	GRA70	55.06	5.01E-04	104
TGME49_270320	PPM3C*	50.86	1.97E-03	41
TGME49_240090	ROP34_WNG2	44.90	0.01	345
TGME49_208370	GRA46	43.92	0.02	145
TGME49_298040	298040	43.62	0.02	19
TGME49_258800	ROP31	42.71	0.02	114
TGME49_309590	ROP1	39.82	0.05	294
TGME49_310740	310740	38.99	0.06	47
TGME49_252360	ROP24	36.37	0.12	167
TGME49_314500	SUB2	36.15	0.13	13
TGME49_260520	GRA53_CST6	35.41	0.15	30
TGME49_297650	297650	35.12	0.16	26
TGME49_201180	MSF	34.87	0.16	84
TGME49_239600	ROP23	34.85	0.16	51
TGME49_268970	268970	34.79	0.16	160

TGME49_315470	315470	34.69	0.16	54
TGME49_242110	ROP38	34.49	0.16	96
TGME49_234540	234540	33.66	0.19	25
TGME49_291630	291630	33.35	0.20	18
TGME49_235130	235130	33.15	0.20	37
TGME49_202610	PPM3D	33.14	0.20	75
TGME49_253330	BPK1	32.86	0.21	71
TGME49_202620	202620	32.80	0.21	389
TGME49_319340	GRA52_CST5	32.40	0.23	3
TGME49_262920	262920	32.13	0.23	39
TGME49_241610	GRA21	32.13	0.23	232
TGME49_253130	253130	30.96	0.30	8
TGME49_297280	297280	30.89	0.30	15
TGME49_306560	NUP67	30.87	0.30	10
TGME49_211290	ROP15	30.58	0.31	73
TGME49_297160	297160	30.53	0.31	52
TGME49_285290	285290	29.84	0.36	90
TGME49_247530	247530	29.67	0.36	193
TGME49_304740	ROP35_WNG1	29.55	0.36	30
TGME49_207700	ROP22	29.35	0.36	34
TGME49_264600	264600	29.25	0.36	33
TGME49_234370	SRS42	29.16	0.36	22
TGME49_297510	297510	29.15	0.36	20
TGME49_275860	275860	29.12	0.36	74
TGME49_291960	ROP40	29.02	0.36	90
TGME49_258660	ROP6	29.01	0.36	121
TGME49_237880	GRA13	28.97	0.36	27
TGME49_258230	ROP20	28.72	0.37	136
TGME49_225160	225160	28.71	0.37	116
TGME49_217530	217530	28.65	0.37	192
TGME49_270920	ROP32	28.05	0.42	131
TGME49_276130	CPC2	28.02	0.42	70
TGME49_258360	258360	27.69	0.44	88
TGME49_271270	271270	27.58	0.44	19
TGME49_248990	248990	27.54	0.44	38
TGME49_215220	GRA22	27.30	0.46	32
TGME49_309760	309760	27.19	0.46	346
TGME49_227810	ROP11	27.06	0.46	63
TGME49_275460	275460	27.04	0.46	80
TGME49_229010	RON4	26.50	0.50	96
TGME49_227010	ROP30	26.44	0.50	158
TGME49_318525	318525	26.41	0.50	90
TGME49_269460	EFPP	26.38	0.50	31
TGME49_225320	225320	26.31	0.50	67
TGME49_239740	GRA14	26.19	0.51	88
TGME49_305590	305590	25.89	0.53	22
TGME49_204340	GRA54	25.84	0.53	205
TGME49_210095	210095	25.73	0.53	59

TGME49_269950	269950	25.71	0.53	17
TGME49_282055	PP2CHN	25.66	0.53	47
TGME49_251540	GRA9	25.48	0.54	239
TGME49_203990	ROP12	25.41	0.54	190
TGME49_300220	300220	25.41	0.54	43
TGME49_270250	GRA1	25.08	0.57	20
TGME49_258870	258870	24.91	0.58	73
TGME49_315910	315910	24.88	0.58	90
TGME49_320630	320630	24.81	0.58	92
TGME49_236860	236860	24.78	0.58	36
TGME49_236870	236870	24.62	0.58	84
TGME49_211260	ROP26	24.59	0.58	46
TGME49_234950	234950	24.40	0.60	109
TGME49_280670	280670	24.37	0.60	41
TGME49_278510	PPM3F	24.10	0.62	75
TGME49_237180	237180	24.04	0.62	64
TGME49_268790	268790	24.01	0.62	20
TGME49_277270	NTPasell	23.86	0.62	620
TGME49_203290	GRA34	23.79	0.62	145
TGME49_266050	266050	23.75	0.62	77
TGME49_313330	ROP27	23.74	0.62	155
TGME49_288650	GRA12	23.70	0.62	177
TGME49_225150	225150	23.42	0.65	99
TGME49_232000	GRA30	23.11	0.66	181
TGME49_314260	314260	23.05	0.66	84
TGME49_269885	TLN1	22.99	0.66	61
TGME49_228170	GRA44	22.92	0.66	8
TGME49_277240	NTPasel	22.91	0.66	42
TGME49_215775	ROP8	22.79	0.66	533
TGME49_315490	ROP10	22.79	0.66	73
TGME49_311470	RON5	22.77	0.66	10
TGME49_215910	215910	22.75	0.66	193
TGME49_308950	308950	22.73	0.66	175
TGME49_253370	RON4L1	22.42	0.68	60
TGME49_254070	254070	22.39	0.68	22
TGME49_208450	PI2	22.34	0.68	21
TGME49_203930	203930	22.32	0.68	19
TGME49_297910	297910	22.32	0.68	97
TGME49_259830	259830	22.26	0.68	59
TGME49_321710	321710	22.24	0.68	55
TGME49_203600	GRA47_CST2	22.16	0.68	177
TGME49_225330	225330	22.12	0.68	194
TGME49_269920	269920	22.10	0.68	81
TGME49_246178	246178	21.96	0.69	29
TGME49_226690	226690	21.76	0.70	25
TGME49_275440	GRA6	21.71	0.70	65
TGME49_258462	258462	21.65	0.70	27
TGME49_277720	277720	21.53	0.71	31

TGME49_304955	PPM11C	21.46	0.71	191
TGME49_289380	GRA39	21.46	0.71	42
TGME49_294970	294970	21.32	0.72	171
TGME49_273980	273980	21.26	0.72	46
TGME49_210820	210820	21.21	0.72	12
TGME49_205360	205360	21.13	0.72	89
TGME49_297960	RON6	20.96	0.74	96
TGME49_227620	GRA2	20.93	0.74	404
TGME49_312270	ROP13	20.86	0.74	165
TGME49_316280	316280	20.66	0.74	32
TGME49_289540	SFP1	20.63	0.74	79
TGME49_252500	252500	20.62	0.74	52
TGME49_242118	242118	20.54	0.74	163
TGME49_261740	ROP47	20.51	0.74	9
TGME49_213069	213069	20.51	0.74	11
TGME49_220240	GRA31	20.51	0.74	166
TGME49_321650	321650	20.39	0.74	45
TGME49_254720	GRA8	20.37	0.74	171
TGME49_286450	GRA5	20.28	0.74	67
TGME49_244250	244250	20.27	0.74	66
TGME49_246710	246710	20.25	0.74	22
TGME49_280480	280480	20.23	0.74	8
TGME49_208730	MCP4	20.12	0.74	204
TGME49_217430	PI1	19.87	0.77	30
TGME49_297410	297410	19.74	0.77	65
TGME49_309600	309600	19.69	0.77	100
TGME49_237800	GRA11B	19.40	0.80	259
TGME49_202200	202200	19.15	0.82	39
TGME49_210370	ROP54	19.13	0.82	95
TGME49_232020	RON12	19.05	0.82	139
TGME49_222170	GRA17	18.83	0.83	48
TGME49_272460	272460	18.82	0.83	13
TGME49_268900	GRA10	18.71	0.83	74
TGME49_263220	ROP21	18.69	0.83	129
TGME49_209985	209985	18.65	0.83	38
TGME49_226380	GRA35	18.54	0.84	156
TGME49_225200	225200	18.43	0.85	98
TGME49_310780	GRA4	18.29	0.85	285
TGME49_321700	321700	18.28	0.85	24
TGME49_288840	GRA18	18.28	0.85	262
TGME49_272420	LCAT	18.16	0.85	120
TGME49_212410	GRA11A	18.11	0.85	117
TGME49_280380	280380	17.87	0.87	216
TGME49_312420	GRA38	17.84	0.87	92
TGME49_305270	305270	17.71	0.87	33
TGME49_299780	299780	17.68	0.87	25
TGME49_266435	266435	17.63	0.87	28
TGME49_297880	GRA23	17.61	0.87	149

TGME49_273860	273860	17.53	0.87	65
TGME49_243690	243690	17.52	0.87	7
TGME49_247520	WIP	17.49	0.87	81
TGME49_264660	CST1	17.40	0.87	108
TGME49_275470	GRA15	17.30	0.87	168
TGME49_221210	CYP18	17.22	0.87	5
TGME49_279420	279420	17.17	0.87	60
TGME49_229500	229500	17.16	0.87	59
TGME49_269690	GRA29	17.05	0.88	139
TGME49_219810	GRA40	16.96	0.88	129
TGME49_207610	ROP36	16.95	0.88	92
TGME49_201520	PPM3G	16.87	0.88	102
TGME49_310010	RON1	16.84	0.88	18
TGME49_204320	204320	16.74	0.88	67
TGME49_209170	209170	16.70	0.88	120
TGME49_309560	309560	16.69	0.88	31
TGME49_305250	305250	16.53	0.89	22
TGME49_208740	MCP3	16.35	0.90	43
TGME49_225860	225860	16.29	0.90	39
TGME49_254080	254080	16.21	0.90	18
TGME49_202420	202420	16.18	0.90	7
TGME49_228760	228760	16.10	0.90	17
TGME49_308970	308970	16.03	0.90	211
TGME49_248510	248510	16.02	0.90	105
TGME49_203790	203790	15.85	0.90	152
TGME49_205250	ROP18	15.84	0.90	113
TGME49_310790	CST9	15.66	0.91	43
TGME49_261750	RON10	15.64	0.91	20
TGME49_262400	262400	15.48	0.91	109
TGME49_237500	PPM3A	15.43	0.91	178
TGME49_281675	ROP45	15.33	0.92	228
TGME49_268760	268760	15.29	0.92	263
TGME49_214080	Toxofilin	15.21	0.92	44
TGME49_244530	GRA47	15.14	0.92	93
TGME49_270240	MAG1	15.08	0.92	71
TGME49_315220	ROP14	14.72	0.93	27
TGME49_247220	247220	14.66	0.93	20
TGME49_200010	GRA20	14.60	0.93	158
TGME49_277230	277230	14.46	0.93	22
TGME49_202780	ROP25	14.44	0.93	138
TGME49_212300	GRA32	14.36	0.93	46
TGME49_242230	ROP29	14.35	0.93	199
TGME49_236890	GRA37	14.29	0.93	26
TGME49_294630	294630	13.94	0.95	11
TGME49_246130	246130	13.91	0.95	28
TGME49_242240	ROP19A	13.77	0.95	167
TGME49_270200	270200	13.42	0.96	18
TGME49_241000	ROP51	13.41	0.96	17

TGME49_315210	315210	13.31	0.96	19
TGME49_267740	GRA49	13.29	0.96	39
TGME49_218270	ROP48	13.23	0.96	147
TGME49_201130	ROP33	13.19	0.96	81
TGME49_314250	BRP1	12.96	0.96	71
TGME49_300100	RON2	12.73	0.96	3
TGME49_306890	306890	12.68	0.96	32
TGME49_254000	GRA48	12.59	0.96	49
TGME49_297900	297900	12.56	0.96	38
TGME49_262050	ROP39	12.48	0.96	274
TGME49_200360	200360	12.22	0.97	76
TGME49_258370	ROP28	12.04	0.97	128
TGME49_215360	215360	11.84	0.98	18
TGME49_304720	304720	11.67	0.98	16
TGME49_217400	217400	11.24	0.98	86
TGME49_230350	RON11	11.21	0.98	5
TGME49_223920	RON3	10.84	0.99	15
TGME49_242820	242820	10.61	0.99	4
TGME49_254880	254880	10.47	0.99	16
TGME49_246550	ASP3	10.37	0.99	6
TGME49_201630	PPM3H	10.21	0.99	88
TGME49_288000	288000	10.10	0.99	55
TGME49_214410	214410	9.56	0.99	74
TGME49_315160	RASP2	9.51	0.99	35
TGME49_276210	276210	9.09	0.99	17
TGME49_279350	279350	8.81	0.99	58
TGME49_306060	RON8	8.03	0.99	23

Appendix 6: List of *T. gondii* cell lines generated in this work

Strain name	Parental strain	Gene ID	Description
RHΔUPRT	RHΔKU80	TGGT1_312480	Knockout of UPRT (mCherry+)
PRUΔUPRT	PRUΔKU80	TGME49_312480	Knockout of UPRT (mCherry+)
RHΔROP1	RHΔKU80	TGGT1_309590	Knockout of ROP1 (mCherry+)
RHΔROP1::ROP1-HA	RHΔROP1	TGGT1_309590	Complementation of ROP1 at UPRT locus
PRUΔROP1	PRUΔKU80	TGME49_309590	Knockout of ROP1 (mCherry+)
PRUΔROP1::ROP1-HA	PRUΔROP1	TGME49_309590	Complementation of ROP1 at UPRT locus
RH ROP1-HA	RHΔKU80	TGGT1_309590	C-terminal HA tagging of endogenous ROP1
PRU ROP1-HA	PRUΔKU80	TGME49_309590	C-terminal HA tagging of endogenous ROP1
RHΔMYR1	RHΔKU80	TGGT1_254470	Knockout of MYR1 (mCherry+)
PRUΔMYR1	PRUΔKU80	TGME49_254470	Knockout of MYR1 (mCherry+)
RH GRA59-HA	RHΔKU80	TGGT1_313440	C-terminal HA tagging of endogenous ROP1
RHΔGRA59	RHΔKU80	TGGT1_313440	Knockout of GRA59 (mCherry+)
RHΔGRA59::GRA59-HA	RHΔGRA59	TGGT1_313440	Complementation of GRA59 at UPRT locus
PRUΔGRA59	PRUΔKU80	TGME49_313440	Knockout of GRA59 (mCherry+)
PRUΔGRA59::GRA59-HA	PRUΔGRA59	TGME49_313440	Complementation of GRA59 at UPRT locus
RHΔKU80::GRA16-HA	RHΔKU80	TGGT1_208830	Expression of GRA16-HA at UPRT locus
RHΔGRA59::GRA16-HA	RHΔGRA59	TGGT1_208830	Expression of GRA16-HA at UPRT locus
RHΔMYR1::GRA16-HA	RHΔMYR1	TGGT1_208830	Expression of GRA16-HA at UPRT locus
PRUΔKU80::GRA16-HA	PRUΔKU80	TGGT1_208830	Expression of GRA16-HA at UPRT locus
PRUΔGRA59::GRA16-HA	PRUΔGRA59	TGGT1_208830	Expression of GRA16-HA at UPRT locus
PRUΔMYR1::GRA16-HA	PRUΔMYR1	TGGT1_208830	Expression of GRA16-HA at UPRT locus
RHΔKU80::IST-HA	RHΔKU80	TGGT1_240060	Expression of IST-HA at UPRT locus
RHΔGRA59::IST-HA	RHΔGRA59	TGGT1_240060	Expression of IST-HA at UPRT locus
RHΔMYR1::IST-HA	RHΔMYR1	TGGT1_240060	Expression of IST-HA at UPRT locus
RH SOS1-HA	RHΔKU80	TGGT1_222100	C-terminal HA tagging of endogenous SOS1
RHΔSOS1	RHΔKU80	TGGT1_222100	Knockout of SOS1 (mCherry+)
RHΔSOS1::SOS1-HA	RHΔSOS1	TGGT1_222100	Complementation of SOS1 at UPRT locus
RHΔROP16	RHΔKU80	TGGT1_262730	Knockout of ROP16 (mCherry+)
RHΔROP16-GFP	RHΔKU80	TGGT1_262730	Knockout of ROP16 (GFP+)

Appendix 7: List of antibodies used in this work

Antibody	Dilution factor	Source	Catalogue number
Mouse anti-T. gondii	1:200	Santa Cruz Biotechnology	SC-52255; RRID:AB_630350
Rabbit anti-T. gondii	1:1000	Abcam	ab138698
Rat anti-HA	1:500	Sigma-Aldrich	11867423001; RRID:AB_390918
Mouse anti-ROP1	1:500	Abnova	MAB17504
Mouse anti-GRA1	1:1000	Laboratory of Jean-Francois Dubremetz	NA
Mouse anti-GRA2	1:1000	Laboratory of Jean-Francois Dubremetz	NA
Rabbit anti-GRA3	1:1000	Laboratory of Jean-Francois Dubremetz	NA
Rabbit anti-GRA29	1:1000	Young et al. 2019	NA
Rabbit anti-C1QBP	1:100	Abcam	ab270032
Rabbit anti-cMyc	1:800	Cell Signaling Technology	5605
Mouse anti-EZH2	1:200	BD	612666
Rabbit anti-phospho-p38	1:800	Cell Signaling Technology	4511
Rabbit anti-phospho-STAT6	1:200	Cell Signaling Technology	56554
Rabbit anti-IRGB6	1:4000	Laboratory of Jonathan Howard	NA
Rabbit anti-GAPDH	1:10,000	Proteintech	10494-1-AP
Mouse anti-ubiquitinated proteins	1:200	Sigma-Aldrich	04-263
Goat anti-rat Alexa 488	1:1000	ThermoFisher Scientific	A-11006; RRID:AB_141373
Goat anti-rat Alexa 594	1:1000	ThermoFisher Scientific	A-11007; RRID:AB_10561522
Goat anti-mouse Alexa 488	1:1000	ThermoFisher Scientific	A-11001; RRID:AB_2534069
Goat anti-mouse Alexa 594	1:1000	ThermoFisher Scientific	A-11005; RRID:AB_2534073
Goat anti-mouse Alexa 647	1:1000	ThermoFisher Scientific	A-21235; RRID:AB_2535804
Goat anti-rabbit Alexa 405	1:1000	ThermoFisher Scientific	A-31556; RRID:AB_221605
Goat anti-rabbit Alexa 488	1:1000	ThermoFisher Scientific	A-11008; RRID:AB_143165
Goat anti-rabbit Alexa 647	1:1000	ThermoFisher Scientific	A-21244; RRID:AB_2535812
Goat anti-rat IRDye 800CW	1:10,000	Li-Cor	925-32219; RRID:AB_2721932
Goat anti-mouse IRDye 680LT	1:10,000	Li-Cor	925-68020; RRID:AB_2687826
Goat anti-mouse IRDye 800CW	1:10,000	Li-Cor	925-32210; RRID:AB_2687825
Goat anti-rabbit IRDye 680LT	1:10,000	Li-Cor	925-68021; RRID:AB_2713919
Goat anti-rabbit IRDye 800CW	1:10,000	Li-Cor	925-32211; RRID:AB_2651127