New conditional expression model to study role of TgHDAC3 in *Toxoplasma gondii* growth

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Abstract

Toxoplasma gondii is an obligate intracellular protozoan parasite with worldwide distribution. The NIAID has classified *T. gondii* as a Category B Biodefense pathogen. More than 40 million men, women, and children in the U.S. carry the *Toxoplasma* parasite, but very few have symptoms due to a normally functioning immune system. However, *T. gondii* is known to cause significant opportunistic infection in AIDS and other immunocompromised persons. *T. gondii* has a complex life cycle involving asexual and sexual stages, with the development of each stage characterized by precise genetic reprogramming. Chromatin post translational modifiers (PTMs) are known to be especially active in *Toxoplasma* gene regulation when compared to other eukaryotes such as yeast or humans. In this study, a new conditional expression system under control of the auxin-inducible degron (AID) was used to examine the role of chromatin modifier *T. gondii* histone deacetylase 3 (TgHDAC3) in the asexual stage of type 1 RH *Toxoplasma gondii* growth. Immunofluorescence assays (IFAs) showed near-complete clearance of the TgHDAC3$^{AID\text{-}3xHA}$ protein after 15 minutes of indole-3-acetic acid (IAA) treatment, and complete clearance after 20-hour IAA treatment. IAA treatment caused severe growth defects as measured by plaque assays indicating the essential role of TgHDAC3 in type 1 RH *Toxoplasma* growth. IFA centrosome staining of drug-treated parasites showed most parasites with a single centrosome indicating that degradation of TgHDAC3$^{AID\text{-}3xHA}$ causes the cell cycle to arrest in the G1 phase. These results demonstrate the first time TgHDAC3 has been directly targeted in a conditional expression system. Moreover, these results provide further evidence that PTMs play a vital role in normal *T. gondii* tachyzoite cell cycle regulation.

Introduction

Epigenetics in Toxoplasma gondii

Toxoplasma gondii is an obligate intracellular protozoan parasite with worldwide distribution.[1] *T. gondii* is a member of phylum Apicomplexa, along with other dangerous parasites including *Plasmodium* (malaria), *Cryptosporidium* (cryptosporidiosis), and *Babesia* (babesiosis).[2] The NIAID has classified *T. gondii* as a Category B Biodefense pathogen.[3] More than 40 million men, women, and children in the U.S. carry the *Toxoplasma* parasite, but very few have symptoms due to a normally functioning immune system.[1] However, *T. gondii* is known to cause significant opportunistic infection in AIDS and other immunocompromised persons.[1] Humans may become infected with *T. gondii* through a number of routes including: eating undercooked meat of animals containing tissue cysts, consuming food or water contaminated with cat feces containing infective oocysts, blood transfusion or organ transplantation, and finally transplacentally from mother to fetus.[1]

The unique life cycle of this parasite, involving fast-replicating tachyzoites and encysted bradyzoites, is crucial for transmission and pathogenesis.[4] *T. gondii* differentially expresses specific sets of genes that direct phenotype transition from the fast replicating tachyzoite to the slow replicating bradyzoite in response to environmental stress such as the host immune response.[4,5] Bradyzoites form characteristic cysts that resist degradation and enhance the parasites ability to infect other organisms. Bradyzoite cysts are the form of *T. gondii* that persist in human tissue and reactivate to cause disease.[4,6] Current treatment for acute toxoplasmosis infection consists of anti-parasitic pyrimethamine (Daraprim) combined with the antibiotic sulfadiazine.[1] However, this treatment is only effective at inhibiting the growth of actively dividing parasites. This treatment does not eradicate latent tissue cysts, leading to a reactivation of infection once

treatment has stopped in immunocompromised individuals.[6] Thus, new treatments are needed that can target the slow growing bradyzoite stage of the *Toxoplasma* life cycle.

Research in Dr. Kami Kim's laboratory has focused on mechanisms of gene expression in *Toxoplasma* development, such as understanding how histone post translational modifications (PTMs) affect epigenetic regulation of gene expression.[7,8,9,10,11] Epigenetic gene regulation refers to heritable changes in gene expression that are not genetically encoded in the DNA sequence of an organism.[5] Mechanisms of epigenetic regulation describe factors that affect accessibility of chromatin, histone modification, and nucleosome location. *Toxoplasma* epigenetics has been linked with the regulation of genes expressed during oxidative stress response, cell cycle, DNA repair mechanisms, and the developmental transition from tachyzoite to bradyzoite.[5] Covalent PTMs of histones include acetylation, methylation, phosphorylation, ubiquitination, and sumoylation. These PTMs can remodel the chromatin in a manner that is either transcriptionally repressive or permissive, and have shown to play a large role in *Toxoplasma gondii* gene expression.[9]

TgHDAC3 in tachyzoites

T. gondii histone deacetylase 3 (TgHDAC3) is a member of the class I HDAC family, which are expressed ubiquitously though localized predominately in the nucleus.[12] TgHDAC3 has been shown to localize at the promoter regions of bradyzoite-specific genes that are silent during tachyzoite replication in the type 2 ME49 strain of *T. gondii*.[13] Chromatin-Immunoprecipitation (ChIP) combined with DNA microarray (ChIP-on-chip) experiments have been designed to target modified histones that were described as activation markers in other eukaryotes.[14] The modified histones are characterized by the acetylation of histone H4 (H4ac), acetylation of lysine 9 (H3K9ac), and trimethylation of lysine 4 of histone H3 (H3K4me3). These experiments were able

to show that active promoters have a characteristic pattern of histone modifications that correlate strongly with gene expression in tachyzoites.[14]

These ChIP-chip experiments have also shown that nucleosomes present in inactive bradyzoite-specific genes are hypoacetylated during the tachyzoite stage but become acetylated under bradyzoite differentiation conditions.[14] These experiments suggest that TgHDAC3 has deacetylase activity at the promoter regions of bradyzoite-specific genes which ultimately silences these bradyzoite genes. The ChIP-chip experiments provide further evidence for the role of histone modifiers in *T. gondii* developmental transition.

TgHDAC3 belongs to class I HDACs and share homology with a major HDAC in yeast, Rpd3.[15] Experiments done in yeast have indicated that one of the ways HDACs might repress transcription is by destabilizing the binding of multiprotein chromatin-remodeling complexes such as SWI/SNF, SAGA (Spt-Ada-Gcn5 acetyltransferase).[15] TgSRCAP (*T. gondii* Snf2-related CBP (CREB-binding protein) activator protein) is a SWI2/SNF2 family ATP-dependent chromatin remodeler whose expression increases during bradyzoite cyst formation, which is consistent with this hypothesis.[15] Chemical inhibition of TgHDAC3 has established this enzyme as a central regulator of gene expression and stage conversion in *Toxoplasma*.[16] The fungal metabolite FR235222 has been shown induce differentiation of the tachyzoite to the bradyzoite stage. FR235222 targets a conserved HDAC specific residue, TgHDAC3 T99, and is active against a variety of parasite species including *Plasmodium* and *Giardia.*[16,17] ChIP-chip experiments identified 369 *Toxoplasma* gene upstream regions with hyperacetylated nucleosomes upon FR235222 treatment; one third of these genes were mainly expressed in the bradyzoite stage.[16] Histone-modifying enzymes in *Toxoplasma* lack DNA-binding domains, consistent with other eukaryotes. Apicomplexa genomes do not code for traditional transcription factors seen in other eukaryotes. Instead, histone-modifying enzyme complexes are recruited by plant-like transcription

factors with AP2 (Apetala2)-integrase DNA-binding domains.[18] TgAP2s have been identified as proteins that interact with chromatin remodelers HDAC3 and GCN5 (general control nonderepressible-5), a histone acetyltransferase (HAT), as well as effectors upregulating bradyzoite-specific genes.[5,10]

Conditional Expression Models in Toxoplasma gondii

The DiCre conditional expression system is based on site-specific recombination using dimerizable Cre recombinase.[19,20] Cre recombinase is a site-specific recombinase that catalyzes the recombination between two 34 bp sequences called LoxP to excise or invert an intervening sequence. A loxP site is a 34 bp sequence made of two palindromic recognition sites, separated by an 8 bp spacer that gives directionality to the sequence.[20] Cre has become a valuable tool for geneticists due to its simplicity and efficiency in excising or inverting genes of interest (GOI).

One of the early and major problems when working with Cre was that scientists lacked temporal control over its activity.[20] If scientists crossed a Cre-expressing mouse with a mouse that contained loxP sites around a GOI in the same orientation, they would produce a mouse that had excised that particular GOI flanked by loxP sites. However, the gene being targeted may be essential for embryonic development, and thus endogenously active Cre recombinase may cause early embryonic death of the crossed mouse.[21]

Initial methods developed to better regulate the activity of this enzyme included regulation of the transcription of Cre through an inducible promoter, and posttranslational regulation of a fusion Cre protein by synthetic steroids; yet both methods proved to be “leaky”.[21,22] In 2003, scientists devised a new approach for much tighter regulation of Cre by controlled complementation of inactive Cre fragments, appropriately titled DiCre for dimerizable Cre.[20]

The DiCre system was established in *T. gondii* in 2012.[19] In this approach, Cre is split into two inactive fragments that are fused to rapamycin binding proteins FRB and FKBP respectively. Upon addition of the ligand rapamycin FRB and FKBP begin to interact with one another, bringing the two inactive Cre fragments together.[20] Thus addition of rapamycin leads to the association of the two Cre enzyme fragments, which leads to the appearance of enzymatic activity. While the DiCre system is very effective at generating conditional mutants, there are some disadvantages to its use. The main disadvantages are that gene excision is an irreversible process and full induction of DiCre takes hours to days.[23]

An inducible system based on the *Escherichia coli* tetracycline repressor-operator complex was introduced into *T. gondii* in 2001.[24] In the originally described tetracycline-controlled inducible expression system a tetracycline repressor (tetR) protein was fused to the activation domain of VP16, an essential transcription factor, from herpes simplex virus (HSV). Upon addition of tetracycline, the tetR-VP16 fusion protein binds a tetO sequence which had been added to the promoter region of a GOI allowing for RNA polymerase II to bind and transcribe the GOI. This system was originally used to show that the *MyoA* gene was essential for host cell invasion and parasite spreading in cultured cells.[24] This example describes a tet-ON scenario, because the addition of tetracycline activates the transcription of a GOI. This system may also be designed to function as tet-OFF, where the addition of tetracycline silences GOI transcription.

In the tet-OFF system, the GOI native promoter is replaced by a tetracycline-regulatable promoter with tetO sequences. The tetR protein is fused to a eukaryotic regulatory domain so that when tetracycline is added the fused protein binds to the modified promoter and represses transcription of the GOI.[25] The activity of the tetR-tetO complex thus silences GOI transcription. Conditional tet-OFF mutants have been used to further characterize the multiple Cdk-related kinases (Crks) that apicomplexan parasites use to coordinate cell division.[26]

The auxin-inducible degron (AID) system was implemented in *T. gondii* in 2017.[27] This system provides a superior method of analyzing the 8,000+ protein coding genes in *T. gondii* due to its speed of regulation at the protein level. The AID system degrades a POI within minutes, allowing for fast visualization of phenotypes associated with protein loss.[27,28] This contrasts with the hours to days it takes for other conditional expression models such as Tet-off and Di-Cre, which require natural turnover of existing mRNA and/or protein. Furthermore, because of the rapid and efficient degradation of target proteins, the AID system shows immediate consequences of protein loss before the phenotype is complicated by the accumulation of secondary effects.[28]

Auxins represent a family of plant hormones (phytohormones) that play a major role in controlling gene expression during plant growth and development.[28] Only two components of plant auxin signaling pathway are necessary for use of the AID system in *T. gondii*: a plant F-box protein called transport inhibitor response 1 (TIR1) and a POI tagged with auxin-induced degradation domain (AID).[28] Auxin (indole-3-acetic acid/IAA) catalyzes the interaction between the TIR1 protein and an AID-tagged factor. The resulting complex is recognized by SCF^{TIR1} ubiquitin ligase which exclusively targets AID-tagged proteins for ubiquitin-dependent proteasomal degradation.[28]

My research goal was to further characterize the role of post translational modifier histone deacetylase 3 (TgHDAC3 - TGME49_227290) in the type 1 RH strain of *T. gondii*. I went about this by generating a TgHDAC3 epitope-tagged transgenic type 1 RH strain of *T. gondii*, through homologous recombination, under control of an auxin inducible degron (AID) conditional expression system.

Results

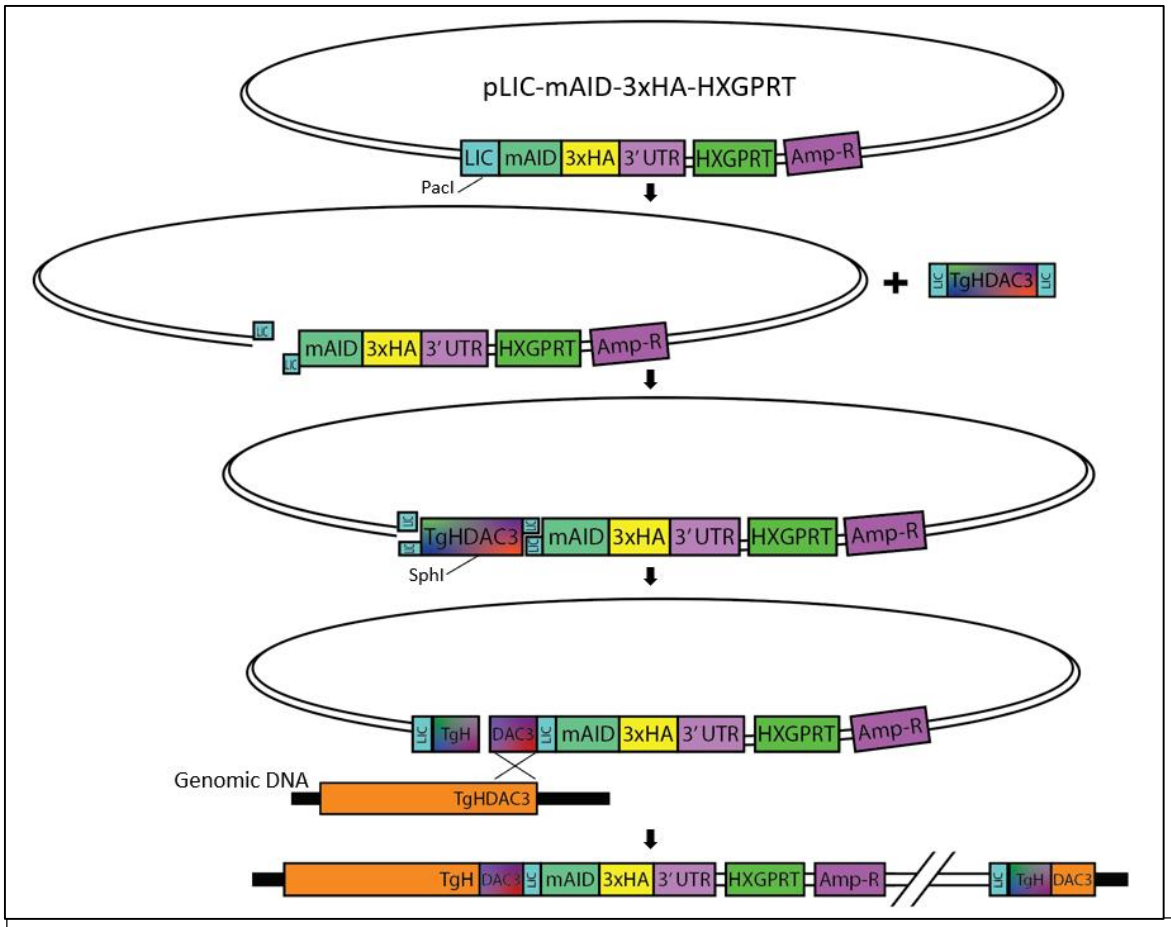

Figure 1.1: LIC of TgHDAC3 into the pLIC-mAID-3xHA-HXGPRT vector.

Ligation independent cloning (LIC) is a technique that allows one to produce expression constructs like recombinant plasmids without the use of restriction enzymes, T4 DNA ligase, or alkaline phosphatase. LIC was used in the assembly of the recombinant plasmid used for transfection and homologous recombination in these experiments.[29] LIC works by the annealing of 12 to 20 nucleotide complementary sequences between a PCR fragment and a DNA plasmid vector. The complementary sequences provide enough hydrogen bonding attraction between the vector and insert for stable transformation into chemically competent bacteria. Bacterial repair enzymes will then seal the four remaining nicks, thus removing the need for timely DNA ligase

experiments. For LIC to successfully recombine a gene fragment into a plasmid expression vector, the vector must be linearized at a unique restriction site. The type II endonuclease, PacI, is used to produce a double stranded DNA cut within the LIC cassette of an LIC expression vector. LIC exonuclease activity only occurs in linear DNA. This unique double stranded cut in the DNA allows for exonuclease activity to occur and allows for 5' overhangs to be formed in the plasmid vector. Primers must then be designed to have complementary sequences to the 5' overhangs of the plasmid vector. Complementary regions will be added to the 5' end of the forward primer and the reverse primer in order to facilitate LIC. The diagram of the TgHDAC3 LIC cloning into LIC-mAID-3xHA-HXGPRT expression vector is shown in the figure 1.1.

Section 1 – Primers design

The first step in this process was to amplify a 3'end of the *TgHDAC3* gene via polymerase chain reaction (PCR). The 3'end to be amplified by PCR was identified from Toxodb. Toxodb.org is the preeminent *Toxoplasma* genomics resource.[30] Toxodb has 19 different sections available for each gene in the database, some of which include: gene models, annotations, taxonomy, phenotype, transcriptomics, sequences, and proteomics. We examined the TgHDAC3 (TGME49_227290) gene page. Primers were designed to flank a 1000 bp region on the 3' end of the TgHDAC3 genomic sequence. Homologous recombination in Δ*KU80 Toxoplasma gondii* strain requires ~400bp homology at 3' and 5' ends. Thus, we were searching for a suitable fragment over 800 bp long.

Additionally, while the primary focus of this study was on TgHDAC3, the Kim lab has interests in generating conditional expression systems for other chromatin modifiers including TgJMJC1, TgSRCAP, TgAP2VIII-4, and TgPKR1. LIC-mAID-3xHA-HXGPRT constructs were

generated for these genes, but transgenic strains of *Toxoplasma* were only generated for TgHDAC3.

HDAC3 – (TGME49_227290); Amplicon = 1000 bp

Non-cutting enzyme: SphI (467/1000)

Forward Primer	5′ – GCAGACATTTGCGTGAACTGGTC – 3′
Forward Tm and GC%	60.00°C and 52%
Reverse Primer	5′ - GATCGGAACCTTTTGGTCTCTGTCTGC - 3′
Reverse Tm and GC%	63.00°C and 52%
Extension LIC Forward	5′-TACTTCCAATCCAATTTAATGCN – 3′
Extension LIC Reverse	5′-TCCTCCACTTCCAATTTTAGC – 3′

HDAC3_mAID_FWD	5′ TACTTCCAATCCAATTTAATGCAGCAGACATTTGCGTGAACTGGTC 3′
HDAC3_mAID_REV	5′ TCCTCCACTTCCAATTTTAGCGATCGGAACCTTTTGGTCTCTGTCTGC 3′

JMJC1 – (TGME49_307010); Amplicon = 1201 bp

Non-cutting enzyme: BstEII (578/1201)

Forward Primer	5′ – ACAAAGCACAGGGAAGAAATC – 3′
Forward Tm and GC%	55.00°C and 43%
Reverse Primer	5′ - CGGATCTTCTAGACAGAAGTCG - 3′
Reverse Tm and GC%	55.00°C and 50%
Extension LIC Forward	5′-TACTTCCAATCCAATTTAATGCN – 3′
Extension LIC Reverse	5′-TCCTCCACTTCCAATTTTAGC – 3′

JMJC1_mAID_FWD	5′ TACTTCCAATCCAATTTAATGCAACAAAGCACAGGGAAGAAATC 3′
JMJC1_mAID_REV	5′ TCCTCCACTTCCAATTTTAGCCGGATCTTCTAGACAGAAGTCG 3′

SRCAP – (TGME49_280800); Amplicon = 2055 bp

- Non-cutting enzyme: BstAPI (1021/2055)

Forward Primer	5′ – ATGCCATCGCATCGGCCA – 3′
Forward Tm and GC%	61.00°C and 61%
Reverse Primer	5′ - TTCATCGTCCTCTTCTGTGGACTC - 3′
Reverse Tm and GC%	60.00°C and 50%
Extension LIC Forward	5′-TACTTCCAATCCAATTTAATGCN – 3′
Extension LIC Reverse	5′-TCCTCCACTTCCAATTTTAGC – 3′

SRCAP_mAID_FWD	5′ TACTTCCAATCCAATTTAATGCAATGCCATCGCATCGGCCA 3′
SRCAP_mAID_REV	5′ TCCTCCACTTCCAATTTTAGCTTCATCGTCCTCTTCTGTGGACTC 3′

AP2VIII-4 – (TGME49_272710); Amplicon = 1502 bp

- Non-cutting enzyme: AarI (437/1502)

Forward Primer	5′ – GCTCCAGAGGAAATCCGTG – 3′
Forward Tm and GC%	59.00°C and 60%
Reverse Primer	5′ - GTTTTGCCGAGAGGCCTCTTCC - 3′
Reverse Tm and GC%	62.00°C and 59%
Extension LIC Forward	5′-TACTTCCAATCCAATTTAATGCN – 3′
Extension LIC Reverse	5′-TCCTCCACTTCCAATTTTAGC – 3′

AP2VIII-4_mAID_FWD	5′ TACTTCCAATCCAATTTAATGCAGGTATTCGGTGAAGAGGCCGATGC 3′
AP2VIII-4_mAID_REV	5′ TCCTCCACTTCCAATTTTAGCGTTTTGCCGAGAGGCCTCTTCC 3′

PKR1 – (TGME49_242070); Amplicon = 2165 bp

Non-cutting enzyme: PstI (1330/2165)

Forward Primer	5′ – cattctccatgtgacgtgttccttac – 3′
Forward Tm and GC%	59.00°C and 46%
Reverse Primer	5′ - ACTCGACACCAAATACCTCGACAAA- 3′
Reverse Tm and GC%	60.00°C and 44%
Extension LIC Forward	5′-TACTTCCAATCCAATTTAATGCN – 3′
Extension LIC Reverse	5′-TCCTCCACTTCCAATTTTAGC – 3′

PKR1_mAID_FWD	5′ TACTTCCAATCCAATTTAATGCAcattctccatgtgacgtgttccttac 3′
PKR1_mAID_REV	5′ TCCTCCACTTCCAATTTTAGCACTCGACACCAAATACCTCGACAAA 3′

Figure 1.2: Primer design information for TgHDAC3, TgJMJC1, TgSRCAP, TgAP2VIII-4, and TgPKR1. The A plasmid Editor (ApE) software program was used to identify the %GC and melting temperatures of the chosen primer sequences. Sequences from each gene were acquired from Toxodb. The unique non-cutting enzyme for restriction digestion of cloned plasmid constructs is shown for each of the primer pairs.

The A plasmid Editor (ApE) software program was used to identify the %GC and melting temperatures of the chosen primer sequences. ApE is an integrated program for plasmid construction and visualization.[31] The goal of the initial primer design was to find 18-22 base pair forward and reverse sequences with a 50% guanosine : cytosine ratio and similar melting temperatures. It is especially important to avoid having more than three guanosines or cytosines in

a row in your sequence as the three hydrogen bonds formed by each guanosine : cytosine nucleotide make PCR amplification less energetically favorable. The forward primer designed for TgHDAC3 cloning PCR was 23 bp and had a melting temperature of 60°C. The reverse primer designed for TgHDAC3 cloning PCR was 27 bp and had a melting temperature of 63°C. Primer design information for other LIC constructs (TgJMJC1, TgSRCAP, TgAP2VIII-4, and TgPKR1) (Figure 1.2).

Section 2 – Construction of the pLIC-GOI-AID-3xHA-Hxgprt plasmids

For insertion into the LIC-mAID-3xHA-HXGPRT vector, gene fragments were amplified using polymerase chain reaction (PCR) with primers that introduced LIC sequences at the 5' and 3' ends of the gene fragments of products, homologous to the LIC sequence in the LIC-mAID-3xHA construct. T4 DNA polymerase treatment of both the vector and the PCR product exposed compatible sequences that annealed and were subsequently ligated after transformation into chemically competent *E. coli* (Figure 1.1). Dr. Suvorova kindly provided the LIC-mAID-3xHA-HXGPRT plasmid construct used for this study.

PCR works first by raising the temperature of a reaction to denature the template DNA. Next the temperature is lowered so that the designed primers may anneal to the target genomic DNA sequences. Lastly a DNA polymerase included in the PCR mix will extend the strand of DNA starting from the site of the annealed primer.[32] The duration of the polymerization step is determined by the size of the generated DNA sequence, called an amplicon, and the DNA polymerase chosen for the PCR. PCR product is amplified by the repetitive cycles of denaturation, annealing and polymerization.

In T4 processing the linearized vector and genomic gene fragment of interest are separately incubated with T4 DNA polymerase and a single deoxyribonucleotide triphosphate. T4 DNA polymerase has dual polymerase/exonuclease functions which can be used to create overhangs of varying length based on a specific sequence. In LIC, the linearized vector is treated with a single nucleotide, for example dGTP, and T4 DNA polymerase to "chew back" the free 3' ends. The T4 *polymerase* reaction is energetically favored over the exonuclease reaction, so when the polymerase is able to add that single dGTP nucleotide the exonuclease activity ceases. The resulting vector then has two 5' overhangs that are generally 12 to 20 nucleotides in length. The overhangs generated in the linearized plasmid vector are complementary to overhangs generated in the genomic gene fragment. The annealing is strong enough to support transformation into bacteria where bacterial repair enzymes will ligate the remaining nicks.[33] T4 processing was used to build the LIC-TgHDAC3-mAID-3xHA-HXGPRT and LIC-TgJMJC1-mAID-3xHA-HXGPRT plasmid constructs.

Constructs LIC-TgSRCAP-mAID-3xHA-HXGPRT, LIC-TgAP2VIII-4-mAID-3xHA-HXGPRT and LIC-TgPKR1-mAID-3xHA-HXGPRT were generated using Gibson Assembly. Gibson Assembly describes an isothermal, single-reaction method for assembling multiple overlapping DNA molecules.[34,35] Instead of using T4 DNA polymerase, Gibson assembly requires T5 exonuclease in combination with Phusion polymerase and DNA ligase. This reaction takes place in one step rather than the two steps required for LIC. First, 5' to 3' exonuclease activity creates single stranded 3' overhangs. These complementary sequences then anneal creating a double stranded DNA of interest. DNA polymerase then extends the 3' ends, filling in the gaps, and DNA ligase seals the remaining nicks. The result is a fully sealed double stranded DNA molecule which can then be transformed into competent bacteria. Gibson Assembly master mix from New England Biolabs (NEB) can reliably assemble up to 6 fragments of DNA when

incubated in a thermocycler at 50°C for 15 minutes.[34] This method is faster and more efficient than traditional cloning methods including T4 processing.

A 50 ul analytical digest containing 5 ug of LIC-TgHDAC3-mAID-3xHA-HXGPRT plasmid DNA was done to confirm construct design. A completed digest would have a single band on gel electrophoresis due to there being only one linear product from a single cut in the construct. While an incomplete digest will show multiple bands on a gel electrophoresis due to there being both linearized product and supercoiled, undigested product. Additionally, if the restriction enzyme chosen for the digest cuts at multiple sites instead of a single unique restriction site then multiple bands would appear on the gel electrophoresis, indicating multiple DNA fragments.

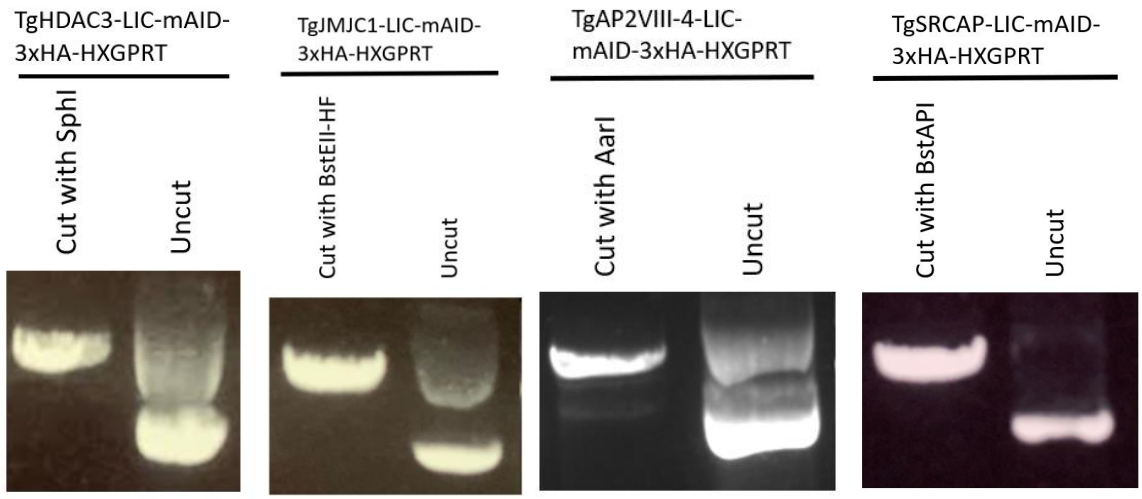

Figure 2.1: Analytical digest gel electrophoresis. Four out of the five plasmid constructs were digested with restriction enzymes unique to each gene fragment. The TgPKR1-LIC-mAID-3xHA-HXGPRT construct was unable to be digested by the available 'PstI' restriction enzyme.

Section 3 – Building *T. gondii* transgenic strains

Toxoplasma gondii is known to rely heavily on nonhomologous end-joining (NHEJ) pathways of DNA repair rather than homologous recombination pathways. NHEJ does not depend on DNA sequence homology like homologous recombination. Instead, NHEJ involves direct ligation of the ends of broken DNA strands.[37] This preference for NHEJ leads to random insertion of scientist engineered DNA, and a failure to accurately target genes of interest. This phenomenon of random insertion rather than insertion based on a homologous sequence hindered *Toxoplasma gondii* gene targeting approaches for many years. The discovery that *T. gondii ΔKU80* parasites exhibit essentially 100% frequency of double-crossover homologous recombination for DNA repair has been of enormous benefit to the community of researchers wanting to efficiently target genes for replacements, knockouts, and knock-ins.[37] Research has shown that TgKU80 protein is an essential component of the NHEJ pathway in *Toxoplasma gondii*.[38] However, type 1 and type 2 Δ*KU80* strains have been shown to exhibit normal growth rates, size, and behavior in vitro and in vivo during tachyzoite and bradyzoite stages.[40]

To further characterize the role of post translational modifier TgHDAC3, LIC-TgHDAC3-mAID-3xHA-HXGPRT construct was linearized with SphI prior to *electroporation* into *T. gondii* RH Δ*KU80* Δ*HXGPRT* parasites. Electroporation is the method of choice to introduce DNA into tachyzoites.[5] DNA enters the cell through pores generated by reversible electrical breakdown of the cell membrane.[39] Studies have shown that immediately following electroporation, cells are sensitive to the osmolarity and ionic composition of their local environment.[39] Thus, a potassium phosphate-based electroporation buffer that resembles the cytosol's ionic composition, cytomix, is used to greatly increase the chance of cell survival.

Parasites that take up the linearized pLIC-TgHDAC3-mAID-3xHA-HXPRT by homologous recombination are then positively selected for with a specific drug media containing mycophenolic acid (MPA) and xanthine (X). Individual clones are later isolated by serial dilution. The positive selection strategy used in this study was based on the HXGPRT gene. The hypoxanthine-xanthine-guanine phosphoribosyl transferase (HXGPRT) gene is of critical importance to the purine nucleotide salvage pathway of *T. gondii*. *T. gondii* is a purine auxotroph which means that it relies on acquiring purines from a host cell and is unable to synthesize purines independently. Researchers discovered that by knocking out the HXGPRT gene, they could positively select for parasites that had been tagged with a genetic construct containing this HXGPRT gene through a homologous recombination process.[41] Positive selection occurs with the addition of mycophenolic acid (MPA) and xanthine (X). The addition of MPA makes catalysis by the HXGPRT enzyme required for purine biosynthesis. Therefore, only parasites that have been tagged with the gene for the HXGPRT enzyme will be able to generate life-necessary purines while under drug selection.

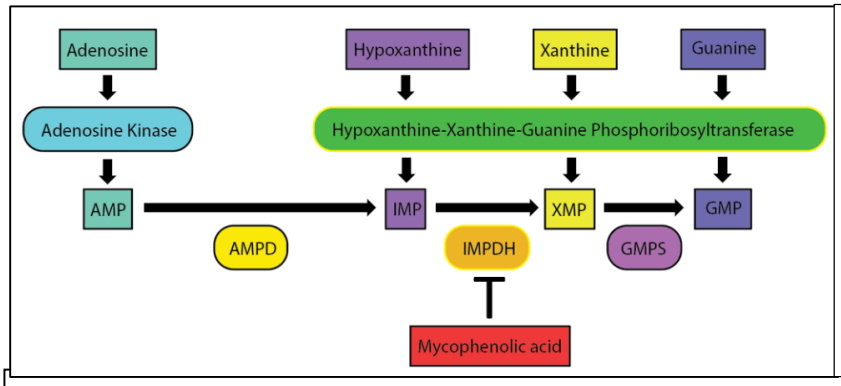

Figure 3.1: Clonal selection by addition of xanthine and mycophenolic acid.

NOTE: The hypoxanthine-xanthine-guanine phosphoribosyl transferase (HXGPRT) gene of *Toxoplasma gondii* encodes a safe, practical genetic marker suitable for both positive and negative selection.[41] In positive selection of parasites that take up HXGPRT gene, MPA inhibits the inosine 5'-monophosphate dehydrogenase (IMPDH) enzyme which is responsible for catalyzing the conversion of inosine monophosphate (IMP) to xanthine monophosphate (XMP). The addition of xanthine rescues parasites with a functional HXGPRT enzyme as HXGPRT can catalyze the conversion of xanthine to XMP, which is downstream of the IMPDH enzyme inhibited by MPA (Figure 3.1).[5]

After drug selection it is necessary to isolate individual clones for further study. The process of clonal selection involves a limiting dilution in a 96 well plate with an intact HFF monolayer. The drug selected parasites are first diluted, using a haemocytometer, to a starting concentration of 250 parasites/ml. This sample is then diluted 1:2 across the first 6 columns of a 96 well plate. This process is repeated using the same starting concentration of 250 parasites/ml across the remaining 6 columns of the 96 well plate to ensure that a clone is isolated. The starting concentration of parasites will depend on the viability of the parasite strain one is working with. These studies were done on the Type 1 RH strain which have been well characterized previously.[5]

Section 4 – Analysis of *T. gondii* RH TgHDAC3$^{AID\text{-}3xHA}$ clone

a – Confirmation of TgHDAC3$^{AID\text{-}3xHA}$ expression in tachyzoites.

Three ***T. gondii* RH TgHDAC3$^{AID\text{-}3xHA}$** candidate clones were isolated from a serial dilution 96 well plate: D11, E5, and G2. These candidates were isolated because there appeared to be only a single plaque in the corresponding wells, indicating that a single parasite had been diluted into those wells. A TgHDAC3 forward primer was designed that was upstream of the forward primer used for initial cloning described previously. A reverse primer was designed for the HA tag downstream of the genomic gene fragment located in the LIC-TgHDAC3-mAID-3xHA-HXGPRT construct. A PCR using the new TgHDAC3 forward primer paired with new HA reverse primer was performed to confirm appropriate homologous recombination in the genomic DNA of these three clones. Gel electrophoresis of this PCR showed that only clone D11 had the appropriate homologous recombination.

Indirect immunofluorescence assays (IFA) were then used to analyze expression and localization of the TgHDAC3$^{AID\text{-}3xHA}$ protein. The influenza hemagglutinin (HA) protein is one of the most widely used epitope tags, with monoclonal antibodies available commercially.[43,44,45]

Indirect IFAs work by using two sets of antibodies. The first, or primary, antibody binds a specific target molecule. The second, or secondary, antibody carries a fluorophore which can be viewed under a microscope and binds to a specific primary antibody. In these experiments, anti-HA (rat) monoclonal antibodies were used to bind the 3X hemagglutinin (HA) epitope tag on the modified HDAC3 protein and anti-IMC1 (rabbit) polyclonal antibodies were used to bind to the inner membrane complex of each parasite. In these experiments, anti-rat secondary antibodies were used to bind to the anti-HA (rat) primary antibodies; anti-rabbit secondary antibodies were used to bind to the anti-IMC1 (rabbit) primary antibodies. Nucleic acid staining was done with DAPI.

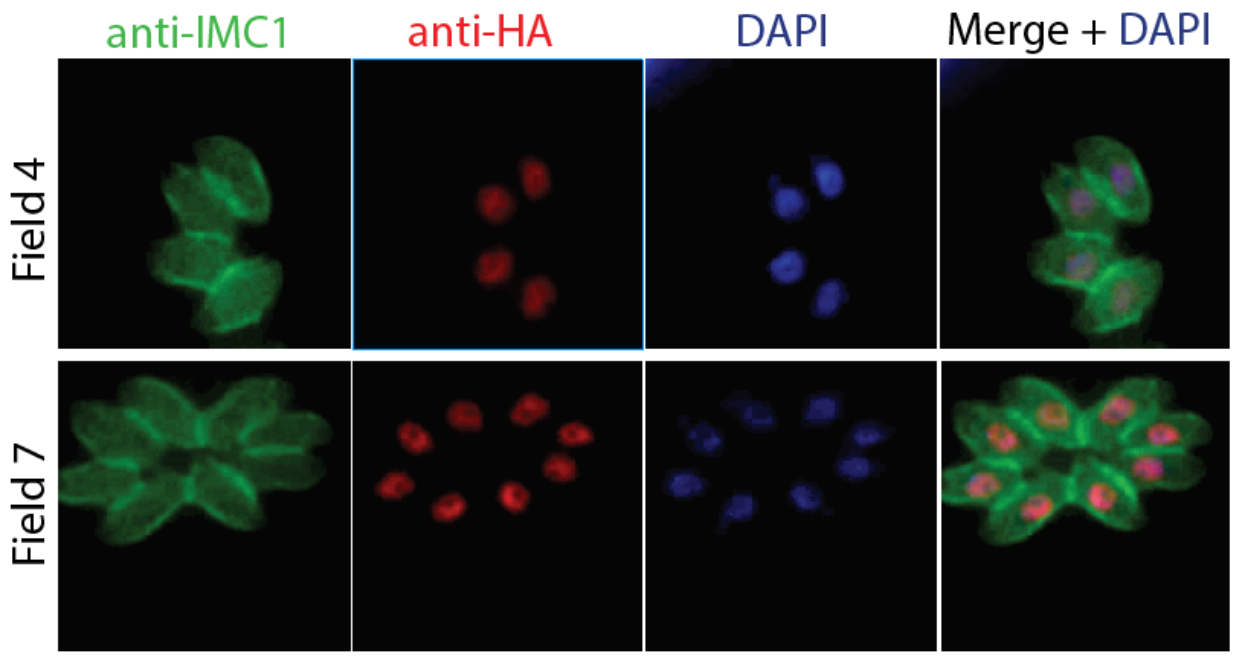

Figure 4.1: IFA confirming TgHDAC3 expression by anti-HA staining. This IFA also shows TgHDAC3 localization to the nucleus of the tachyzoite.

To find out if TgHDAC3 is expressed in a specific cell cycle phase, we co-stained **TgHDAC3$^{AID\text{-}3xHA}$** with cell cycle markers. As parasites progress into S phase from G1 they duplicate their centrosomes, therefore we identified G1 phase with anti-centrin1 monoclonal

antibodies that were used to bind to the centrosomes in each parasite; anti-mouse secondary antibodies were used to bind to the anti-centrin1 (mouse) primary antibodies.

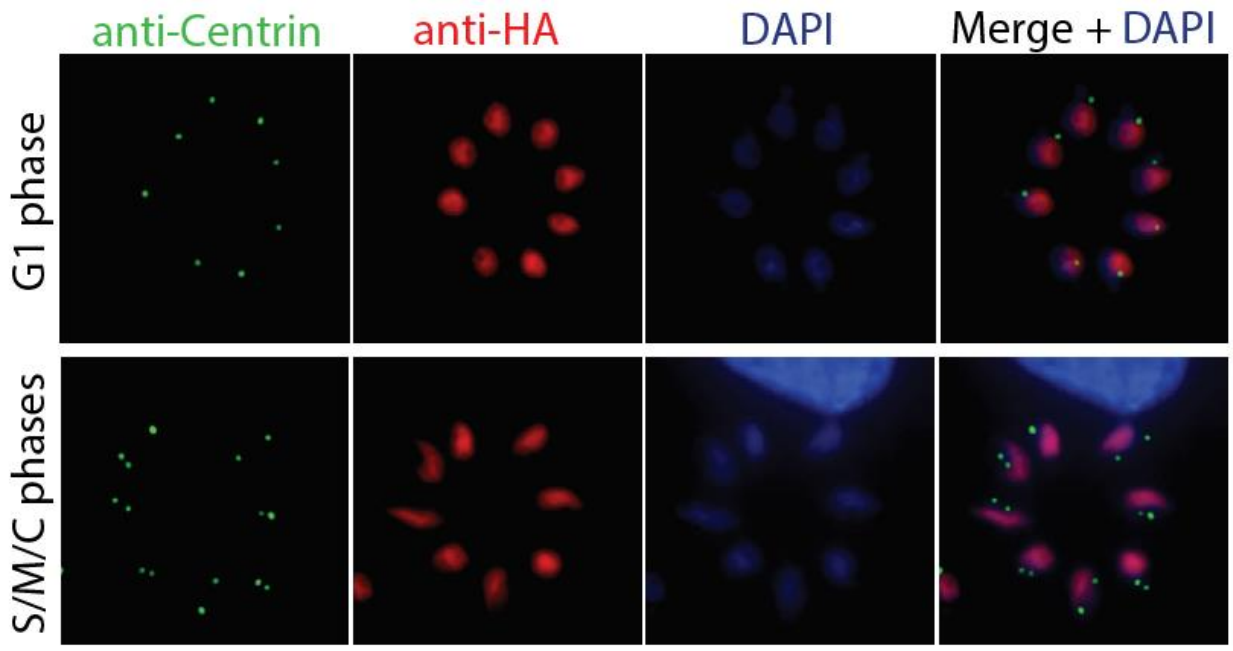

Figure 4.2: IFA showing stage dependent expression of centrosomes. The top row of images shows 1 vacuole containing 8 tachyzoites which each have 1 centrosome indicating it is in the G1 phase of the cell cycle. The bottom row of images shows 1 vacuole containing 8 tachyzoites which each have 2 centrosomes indicating this vacuole is in the S/M/C phases of the cell cycle.

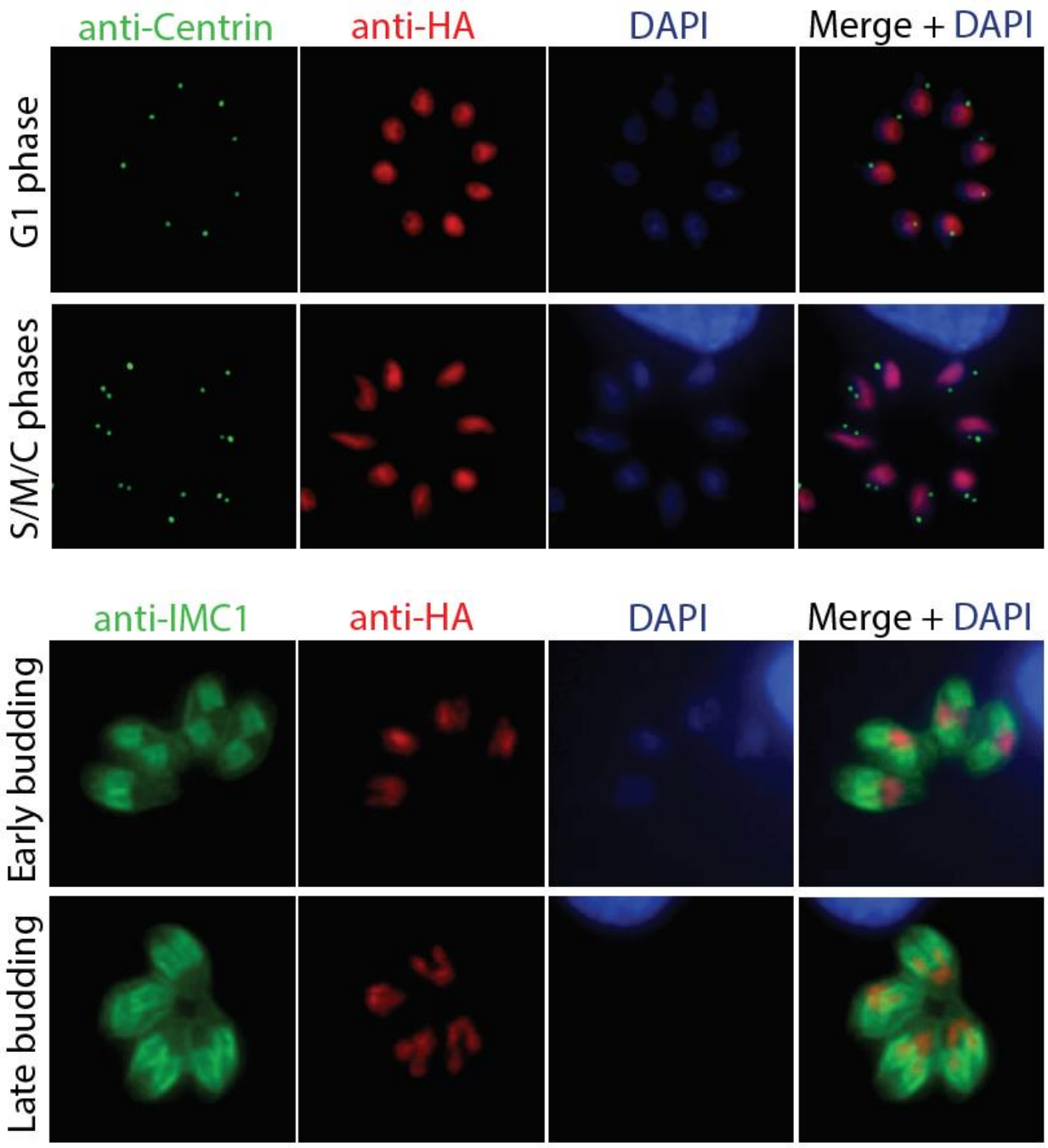

Figure 4.3: Top two rows show visualization of HA epitope tag with both single and double centrosome expression indicating the transition from G1 to S phase. Bottom two rows show parasites in early and late budding stages of lifecycle.

To identify budding parasites, we used anti-IMC1 antibodies. The inner membrane complex (IMC) of *T. gondii* is composed of flattened alveolar sacs that underlie the plasma membrane. This peripheral membrane system is joined with a supporting cytoskeletal network, and thus has important functions in parasite motility, host cell invasion, and intracellular replication.[46] There are 19 different IMC proteins in *Toxoplasma gondii*.[47] Antibodies for IMC1 were used for these IFAs because IMC1 stains throughout the IMC as opposed to specific IMC sub compartments. IMC1 staining thus provided a clear image of the parasite membrane for these studies. Additionally, IMC also serves as a structural scaffold for the formation of daughter cells within a mother cell during asexual reproduction.[47] This allowed for visualization of parasite budding in the IFAs described (Figure 4.3).

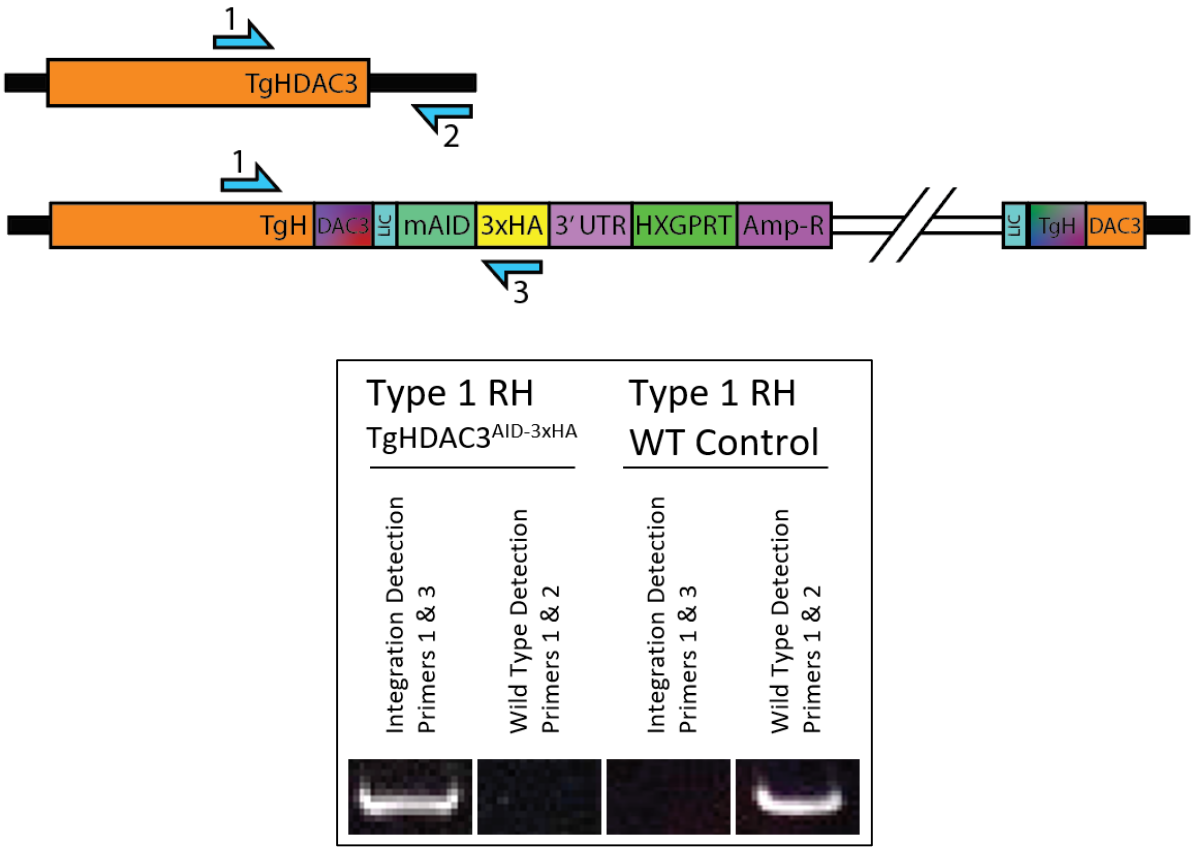

Figure 4.4: Genotypic confirmation of TgHDAC3$^{AID\text{-}3XHA}$ strain by PCR with control, schematic included for easier visualization.

Section 4b – Role of HDAC3 in tachyzoite growth

Treatment of the tagged parasites with indole-3-acetic acid (IAA) showed near-complete clearance of the TgHDAC3$^{AID\text{-}3xHA}$ after 15 minutes by IFA (Figure 4.5) and complete clearance after 20 h (Figure 4.6). Full TgHDAC3$^{AID\text{-}3xHA}$ degradation would be expected after 1 full cell cycle of 6-8 hours.

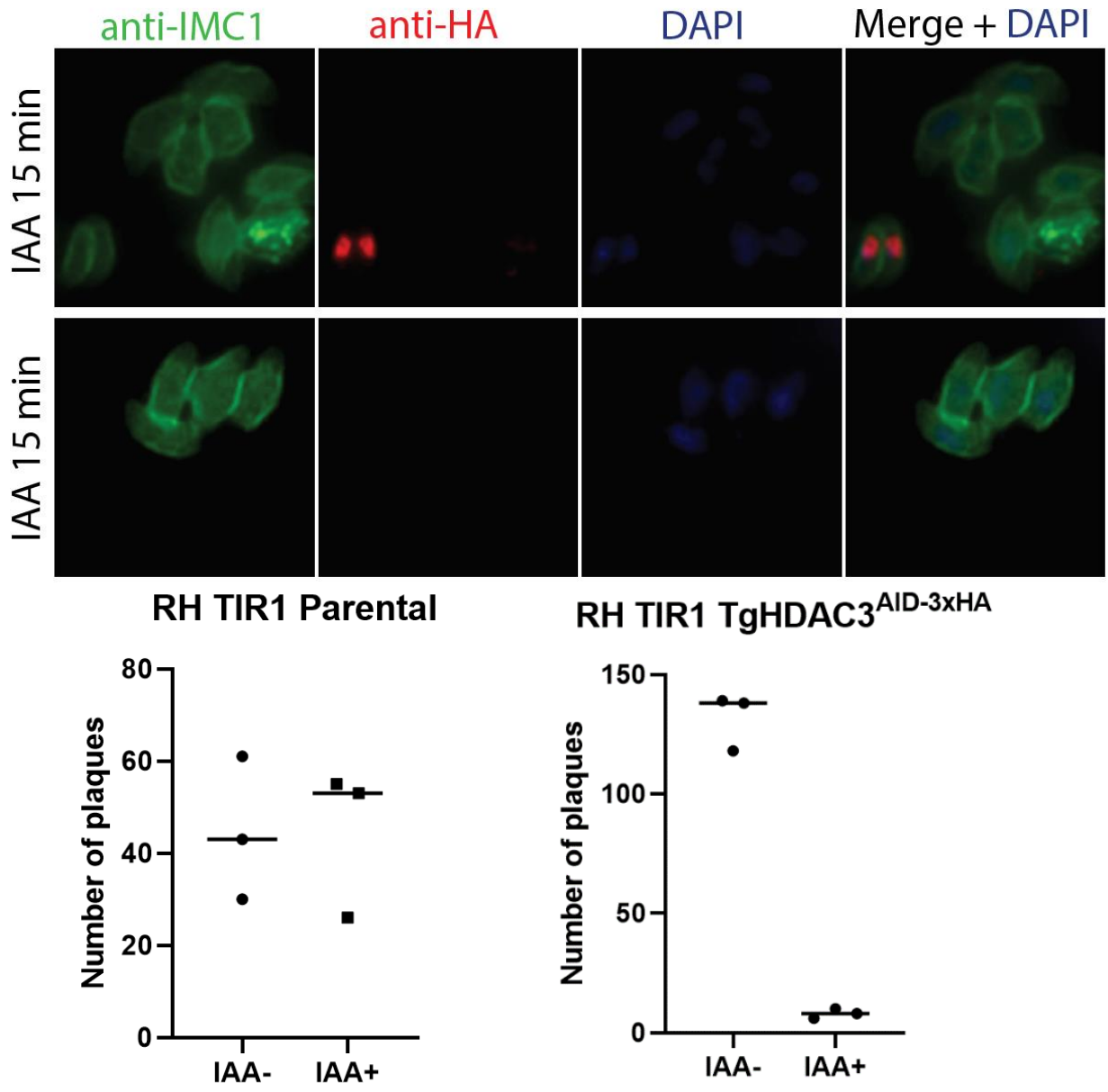

Figure 4.5: IFA showing 15-minute IAA treatment and graphs showing quantitation of 5-day plaque assays. TgHDAC3$^{AID\text{-}3xHA}$ was almost completely degraded after only a 15-minute IAA treatment. IAA treatment showed clear inhibition of transgenic parasite growth.

Plaque assays were done to determine the effect of TgHDAC3$^{AID\text{-}3xHA}$ degradation on tachyzoite growth (Figure 4.5). In plaque assays, a standard number of parasites is added to a 6-well plate which has a confluent human-foreskin-fibroblast (HFF) monolayer. The obligate intracellular protozoan parasite, *Toxoplasma gondii*, is traditionally grown in HFFs.[5] Following inoculation of parasites into the monolayer, the 6-well plate is left undisturbed in an incubator for a pre-determined amount of time. These plaque assays were left to grow for 5 days. At the end of the time period, the 6-well plates are fixed and stained with antibodies for viewing under a microscope. This study was done to determine if the TgHDAC3$^{AID\text{-}3xHA}$ strain had any differences in growth from a control strain. These studies clearly show that degradation of TgHDAC3$^{AID\text{-}3xHA}$ causes severe growth defects as indicated by the greatly reduced number of plaques when compared to a control.

TgHDAC3$^{AID\text{-}3xHA}$ parasites that were treated with IAA showed severe morphological defects including enlarged cells, a trend towards having only a single centrosome, and many vacuoles containing only a single parasite (Figure 4.8). The IAA treated samples trend towards having only a single centrosome and have only one parasite per vacuole (Figure 4.7). Taken together, this data indicates that TgHDAC3$^{AID\text{-}3xHA}$ degradation effectively halts the *Toxoplasma* in G1 phase, and thus prevents the normal cell cycle from progressing into S/M/C phases.

Toxoplasma gondii replicate by endodyogeny inside of a tight-fitting vacuole originally formed by invagination of host plasma membrane during invasion into the host cell.[5] Type 1 RH *Toxoplasma gondii* and have an average life cycle of 6-8 hours. Parasite will replicate by endodyogeny inside of a host cell until reaching a bursting point of approximately 64 – 128 parasites.[48] Parasites then break open the cell and repeat this process by invading nearby cells.

Thus, after a 20-hour period one would expect to see vacuoles containing parasites that had passed through two or three life cycles, corresponding to four or eight parasites per vacuole, respectively. This data shows that after 20 hours without IAA treatment, most vacuoles contain four parasites, many contain eight parasites, and some contain either one or two parasites (Figure 4.8). However, after IAA treatment parasite vacuoles overwhelmingly contain only a single parasite. This again indicates that TgHDAC3$^{AID\text{-}3xHA}$ degradation caused early cell cycle arrest.

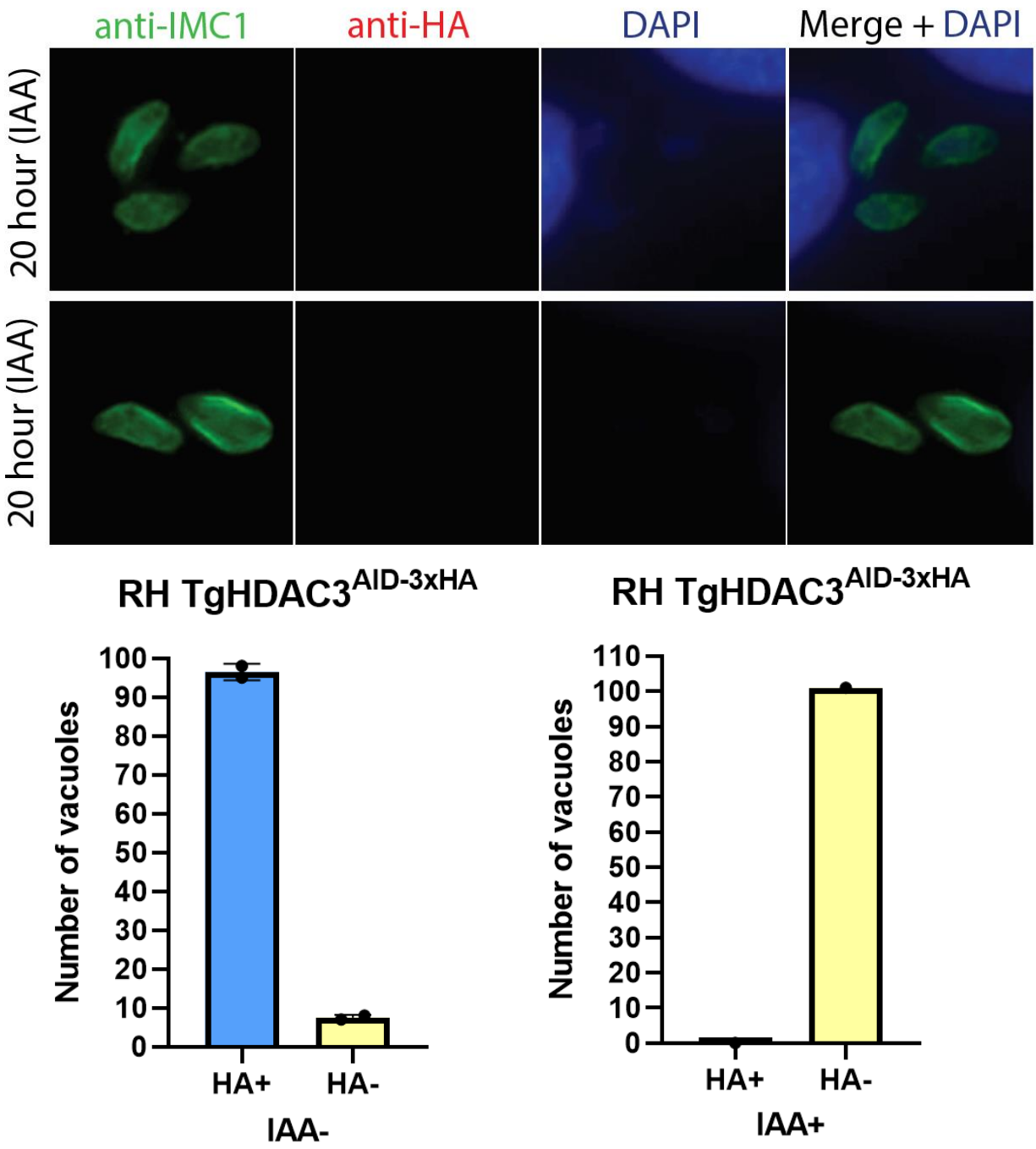

Figure 4.6: IFA showing 20-hour IAA treatment with quantitation of HA+ vacuoles in 10 microscope fields with IAA and 10 fields without IAA. TgHDAC3$^{AID\text{-}3xHA}$ is completely degraded after 20-hour IAA treatment. Parasites vacuoles contain only single parasites. There is a clear disrupted phenotype due to loss of TgHDAC3$^{AID\text{-}3xHA}$.

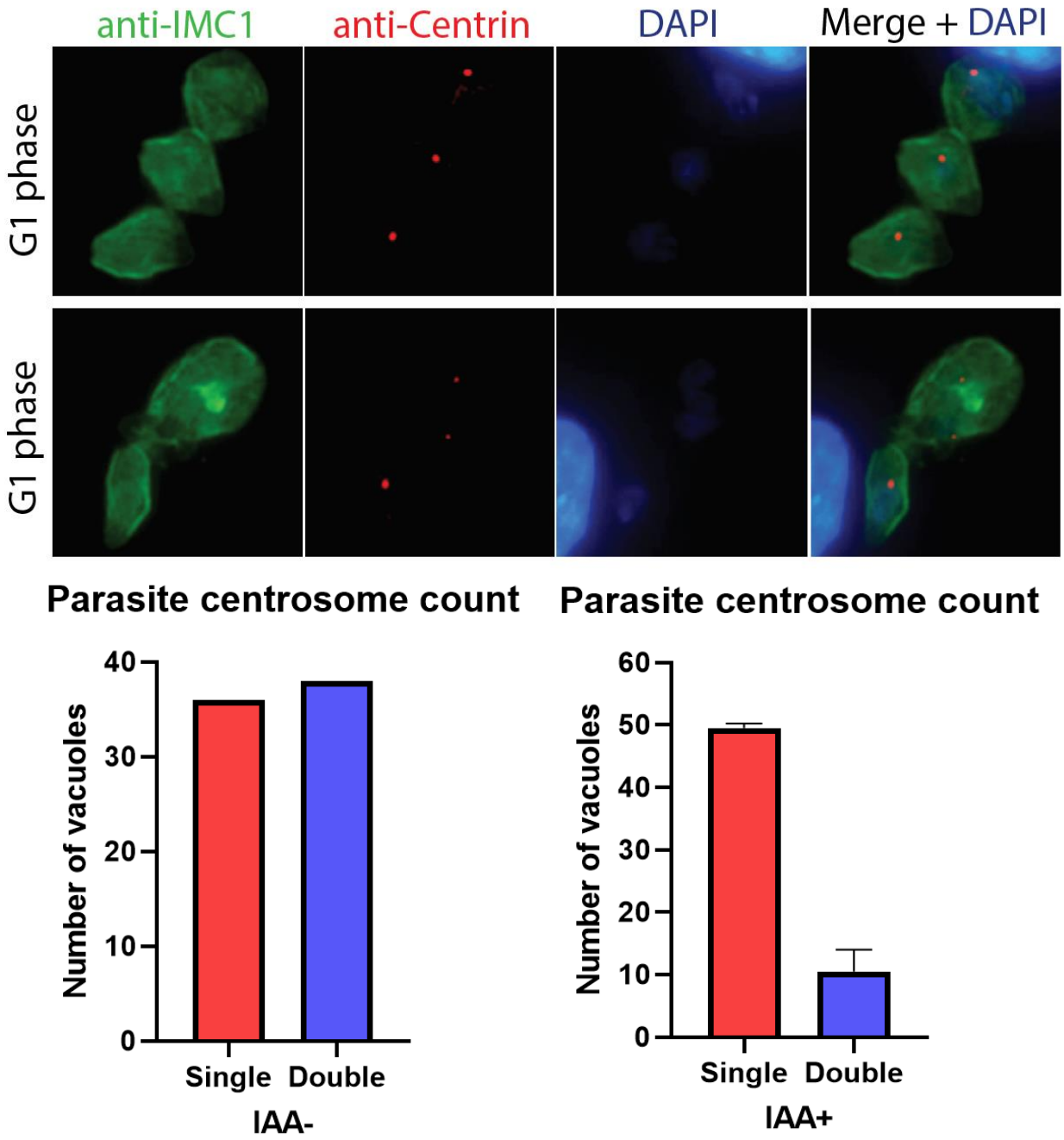

Figure 4.7: IFA showing 20-hour IAA treatment and graphs showing number of centrosomes per parasite vacuole with IAA and without IAA. IAA treated parasites show disrupted morphology and trend towards having only a single centrosome indicating cell cycle arrest in G1 phase.

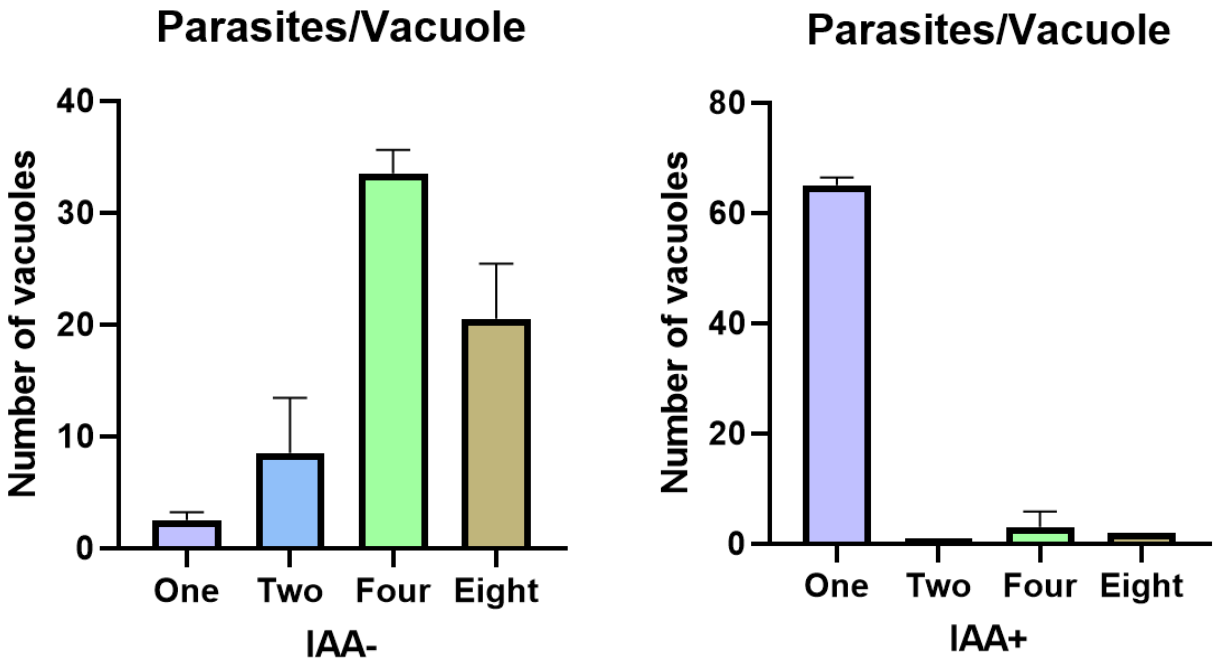

Figure 4.8: Graph showing number of parasites per vacuole in IAA- and IAA+ treated samples. IAA treated parasites overwhelmingly have one parasite per vacuole, indicating early cell cycle arrest.

Discussion

This research project demonstrates the first time that the chromatin modifier, TgHDAC3, has been directly targeted in a conditional expression system. Moreover, TgHDAC3 was studied using the novel AID system which has the advantage of acting very fast (Figure 4.5) at the protein level in a reversable manner. This also allows for visualization of phenotype defects before the accumulation of secondary defects due to protein loss.

This study clearly shows that upon degradation of TgHDAC3$^{AID\text{-}3xHA}$ the parasite becomes unable to pass from the G1 phase of the cell cycle to the S/M/C phases. This is evident by IFAs that show only single parasites per vacuole after 20 hours of growth, by 70% of parasite vacuoles

containing only single centrosomes, and by plaque assays that showed dramatically decreased growth by number of plaques.

TgHDAC3 is seen throughout the literature as a key binding partner to various *Toxoplasma* transcription factors.[15,16,49,50] TgHDAC3 was recently co-purified with a microrchidia (MORC) homologue in *T. gondii*.[51] The TgMORC was shown to act in concert with TgHDAC3 and other AP2-related transcription factors at many stage-specific genes. These findings are characteristic of Apicomplexan life cycles which are known to have precise genetic reprogramming during each developmental stage. The TgMORC-TgHDAC3-AP2 transcription factor complex was shown to silence approximately 85% of the 312 transcripts which are known to become upregulated during the merozoite sexual stage of *T. gondii* life cycle.

Toxoplasma has interesting and dynamic biology that is different than the hosts it infects, perhaps as an adaption to the extremely wide range of host cells it can inhabit (any warm-blooded nucleated cell). It relies on post translational chromatin modifiers to do a lot of gene regulation when compared to higher order metazoans. It completely lacks traditional eukaryotic regulatory elements including canonical cyclins, major cell cycle CDKs and their immediate downstream effectors.[52] It has both a sexual cell cycle (endopolygeny) which only occurs in feline enterocytes, and an asexual cell cycle (endodyogeny) which can occur in any warm-blooded nucleated cell.[5]

T. gondii tachyzoites use precise genetic "programs" to move through the cell cycle that can be seen via transcriptomic and proteomic studies. For example, the TgMORC-TgHDAC3-AP2 transcription factor complex prevents the expression of sexual stage genes when *Toxoplasma* is not in the intestines of a cat. The *Toxoplasma* sexual stage of development only occurs in enterocytes of the feline species.[5] Additionally, there have been many published studies describing

large numbers of mRNAs and proteins that are exclusively expressed in either the bradyzoite stage or tachyzoite stage.[53,54]

This study provides further evidence that TgHDAC3 is cell cycle regulated. Moreover, it indicates that TgHDAC3 plays a role in passing from the G1 phase to the other phases of the cell cycle. It is plausible that TgHDAC3 degradation destabilizes multi-protein chromatin-remodeling complexes necessary for *Toxoplasma* to pass from the G1 phase to the S/M/C phases of the cell cycle.

Future experiments would include a western blot to confirm protein expression at the expected size of 51 kDa. Due to the coronavirus pandemic, this study was not able to be completed. Cell pellets were prepared and are in storage in the -80 °C freezer in Dr. Kami Kim's lab. Future experiments could also include chromatin immunoprecipitation (ChIP) followed by DNA sequencing (ChIP-seq) and co-immunoprecipitation (co-IP) followed by mass spectrometry.

There seem to have been about 10% *T. gondii* without HA-tag expression. While these epitope tag-less parasites could be due to a contamination from a parental strain of *T. gondii*, there have been instances where the epitope tag is lost due to continuous passage (unpublished data, Kim). Moving forward, a new clone should be isolated from this sample, and these experiments should be repeated. There may be an even more obvious phenotype defect following TgHDAC3$^{AID\text{-}3xHA}$ degradation with more single centrosome parasites and more single parasites per vacuole.

Materials and Methods

Parasites and human cell culture

Human foreskin fibroblasts (HFFs) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS), 50 ug/ml penicillin and streptomycin, and amphotericin B at 37°C with 5% CO_2. Upon inoculation of *Toxoplasma* to the HFF cultures, the media was changed to DMEM supplemented with 3% heat inactivated FBS with 50 ug/ml penicillin and streptomycin, and amphotericin B. The *Toxoplasma* strains that were used in this study were maintained in vitro by serial passage on monolayers of HFFs. The cultures were free of mycoplasma as determined by plate reader in the Adams' lab.

Transfection and selection

Type 1 RH Δ*ku80* Δ*HXGPRT* parasites were cultured by passage in human foreskin fibroblasts in Dulbecco's modified Eagle's medium (DMEM) supplemented with 3% heat inactivated fetal bovine serum (FBS), penicillin, streptomycin, and amphotericin B at 37°C with 5% CO_2. Ten million parasites were filter purified, pelleted, and resuspended in cytomix buffer (2 mM EDTA, 120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4/KH_2PO_4, 25 mM HEPES, 5 mM $MgCl_2 – 6H_2O$; pH 7.6).[46,47] Eight ug of the linearized, ethanol precipitated, *Tg*HDAC3_LIC_mAID_3XHA construct was resuspended in 100 ul Cytomix with the 10 million extracellular type 1 RH Δ*ku80* Δ*HXGPRT* parasites. Program T016 on the Nucleofector Amaxa Biosystems electroporator was used for the electroporation of TgHDAC3_LIC_mAID_3XHA into the type 1 RH Δ*ku80* Δ*HXGPRT* parasites. After overnight growth, transformants were placed under selection by changing selection media to contain 25 ug/ml mycophenolic acid and 50 ug/ml xanthine. Transformants were later isolated by limiting dilution in a 96 well plate.

Auxin-induced degradation

Degradation of TgHDAC3-LIC-mAID-3XHA-HXGPRT parasites was achieved using IAA (Sigma-Aldrich, 45533). A stock of 500 mM IAA dissolved in 100% ethanol at 1:1,000 was used to deplete mAID-tagged proteins at a final concentration of 500 uM. Mock treatment consisted of an equivalent volume of 100% ethanol at a final concentration of 0.0789%, w/v.

Immunofluorescence microscopy

T. gondii infecting HFF cells were grown in 6-well plates. Coverslips were fixed with 4% BSA that is 4°C for 5 minutes, then washed one time with room temperature phosphate buffered saline (PBS) containing 2 mM sodium azide (NaN_3). Coverslips were permeabilized with 0.2% Triton X100 for 10 minutes at room temperature, then washed one time with room temperature PBS containing 2 mM NaN_3. Blocking occurred with cold 1% BSA containing 2 mM NaN_3 for 30 minutes. Cells were then incubated with primary antibodies for one hour followed by incubation with secondary antibodies for one hour. Nuclei were DAPI stained for 5 minutes at room temperature. Cells were then washed 3 times with the PBS-NaN_3 washing buffer. Coverslips were then mounted onto glass slides using Aquamount. Coverslips were sealed with three separate layers of clear fingernail polish. Fluorescence imaging was done on the ZEISS microscope located in the Adams' lab, and processed using ZEN software.

T4 Processing - Vector

Vector DNA containing LIC sequence was first digested with PacI restriction enzyme. This was purified with Agilent StrataPrep PCR purification kit. A small amount of digested sample and undigested sample was run on 0.8% agarose gel electrophoresis to confirm digest.

Approximately 500 ng of the linearized vector DNA was added to a reaction mixture on ice containing: 2ul 10X buffer, 1 ul 100mM DTT, 0.8 ul 100 mM dGTP, 0.5 ul T4 DNA polymerase (Novagen LIC qualified). dH_2O was added to the reaction mixture until the total volume was 20 ul. The mixture was allowed to incubate at room temperature (~22 ℃) for 30 minutes and was then moved to a thermocycler which held reaction mixture at 75 ℃ for 20 minutes. The mixture was then moved to 4℃ until use. The remaining mixture was added to the -20℃ freezer.

T4 Processing – Insert

PCR was used to create a 1000 bp gene fragment. PCR amplicons were purified using a Qiagen PCR spin kit. PCR product concentration was adjusted to approximately 0.04 pmol/ul. Approximately 130 ng of PCR product insert is added to a reaction mixture containing: 2 ul 10X buffer, 1 ul 100 mM DTT, 0.8 ul 100 mM dCTP, 0.5 ul T4 DNA polymerase (Novagen LIC qualified). dH_2O was added to the reaction mixture until the total volume was 20 ul. The mixture was allowed to incubate at room temperature (~22 ℃) for 30 minutes and was then moved to a thermocycler which held reaction mixture at 75 ℃ for 20 minutes. The mixture was then moved to 4℃ until use. The remaining mixture was added to the -20℃ freezer.

T4 Processing – Annealing

1 ul of the treated vector with 2 ul of the treated insert were mixed on ice. The reaction mixture was then incubated at room temperature for 5 minutes. After 5 minutes the reaction mixture was moved to 4℃. 1 ul of this reaction mixture was used to transform E. Coli TOP10 Chemically Competent Cells.

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