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The Ubiquitin Proteome of *Toxoplasma gondii* Reveals Roles for Protein Ubiquitination in Cell Cycle Transitions

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Abstract

Protein ubiquitination plays key roles in protein turnover, cellular signalling, and intracellular transport. The genome of *Toxoplasma gondii* encodes ubiquitination machinery but the roles of this posttranslational modification (PTM) are unknown. To examine the prevalence and function of ubiquitination in *T. gondii*, we mapped the ubiquitin proteome of tachyzoites. Over 500 ubiquitin-modified proteins, with almost 1000 sites, were identified on proteins with diverse localisations and functions. Enrichment analysis demonstrated that 35% of ubiquitinated proteins are cell cycle-regulated. Unexpectedly, most classic cell cycle regulators conserved in *T. gondii* were not detected in the ubiquitinome. Furthermore, many ubiquitinated proteins localise to the cytoskeleton and inner membrane complex, a structure beneath the plasma membrane facilitating division and host invasion. Comparing the ubiquitinome with other PTM proteomes reveals waves of PTM enrichment during the cell cycle. Thus, *T. gondii* PTMs are implicated as critical regulators of cell division and cell cycle transitions.

Graphical abstract

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AUTHOR CONTRIBUTIONS

Experiments: NS guided by KK and LMW. Analysis of MS data: NS assisted by RY and EN. Bioinformatic and statistical analyses: NS. Validation of IMC protein ubiquitination: ALC and PJB. Figures: NS and ALC. Manuscript: NS and KK with input from all authors.

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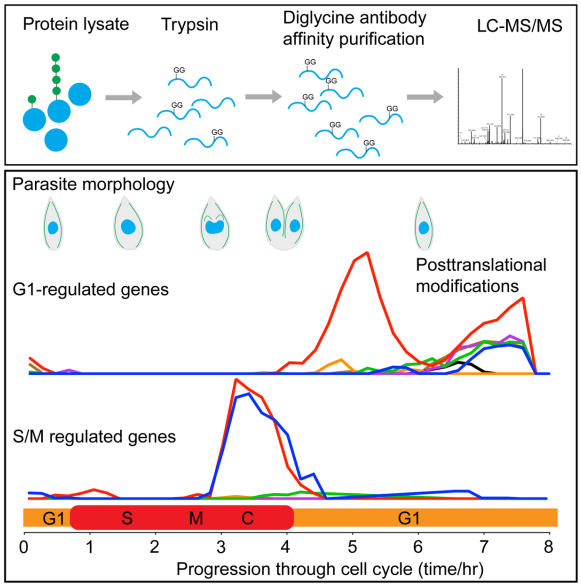

Keywords

Toxoplasma gondii; Ubiquitin; Apicomplexa; Parasite; Cell cycle; Posttranslational modification

INTRODUCTION

Ubiquitin, a 76 amino acid protein, is conjugated to lysine residues of proteins to regulate a vast number of cellular processes. Ubiquitin is either attached to a protein as a chain made up of two or more ubiquitins (polyubiquitination) or a single molecule (monoubiquitination) on one or more lysines of a substrate. Poly- and monoubiquitin have roles in protein trafficking, protein-protein interactions, subcellular localisation and transcriptional regulation; the best understood function of polyubiquitination is targeting of proteins to the proteasome for degradation (Ciechanover et al., 1980; Hershko et al., 1980). Proteins that have misfolded during transport through the endoplasmic reticulum are also polyubiquitinated and then degraded, in a process known as ER-associated degradation (ERAD) (Finger et al., 1993). The ubiquitin-proteasome pathway destroys proteins whose abundance is posttranslationally controlled, performing an essential task in cell cycle progression by degrading checkpoint complexes and cell cycle regulated proteins (Bassermann et al., 2014). In higher eukaryotes, two ubiquitinating complexes, the Anaphase Promoting Complex (APC) and Skp/Cullin/F-Box Complex (SCF) regulate cell cycle protein levels by ubiquitinating them at specific times in the cell cycle (Teixeira and Reed, 2013).

The obligate intracellular parasite *Toxoplasma gondii* replicates by a unique form of cell division called endodyogeny, wherein two daughter cells are formed within a maternal cell during division. Approximately one third of the *T. gondii* transcriptome is cell cycle regulated (Behnke et al., 2010), and cell cycle dynamics are tightly coordinated. The *T. gondii* cell cycle has a short or absent G2 phase, leading directly to M phase, and the

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checkpoints are thought to be different (Radke and White, 1998). Transition between life cycle stages involves significant changes in protein profiles and is likely to involve many levels of regulation, including posttranslational modifications (PTM) (Weiss et al., 2009).

The *T. gondii* genome encodes all of the machinery required for ubiquitination. The *T. gondii* proteasome contains homologues of many components found in other eukaryotes, and additional proteins unique to *T. gondii* (Paugam et al., 2003; Paugam et al., 2001). The *T. gondii* genome possesses a conserved and functional repertoire of ERAD proteins (Agrawal et al., 2009), a large number of candidate ubiquitin ligases and deubiquitinases, as well as ubiquitin and ubiquitin-like modifiers (Ponder and Bogyo, 2007; Ponts et al., 2008). Only one enzyme has been validated as an active deubiquitinase, dual ubiquitin and NEDD8 hydrolase, UCHL3 (Frickel et al., 2007). In addition, the apicoplast, a plastid-like organelle, has its own designated ubiquitination system required for import of nuclear-encoded proteins (Agrawal et al., 2013).

To understand the function of ubiquitination in *T. gondii,* we mapped the ubiquitin proteome and identified over 500 ubiquitin-modified proteins. A large number of ubiquitinated proteins localise to the inner membrane complex (IMC), a conserved feature of Apicomplexan parasites. Over 35% of ubiquitinated proteins have a cell cycle-regulated transcriptional profile, and a subset of ubiquitin ligases and deubiquitinases are cell cycle regulated. Homologues of known substrates of APC and SCF, such as cyclins and CDKs, are notably absent from the ubiquitinome. Analysis of ubiquitination and other PTM proteomes demonstrated that PTM proteomes are also enriched in proteins that are cell cycle-regulated. Together, these data indicate that PTMs are involved in cell cycle regulation in *T. gondii*, with significant interplay between ubiquitination and phosphorylation.

RESULTS

Ubiquitination is abundant in T. gondii and its localisation changes with cell cycle progression

We detected ubiquitin-protein conjugates in intracellular tachyzoites by Western blot and immunofluorescence assay (IFA) of replicating tachyzoites with anti-ubiquitin antibodies (Figure 1). Of the fixation conditions tested, the most extensive labelling was observed with methanol/acetone, which may reveal epitopes not readily labelled by gentler fixation conditions. Ubiquitin antibodies labelled multiple discrete compartments (Figure 1B), and ubiquitin was especially abundant in the nucleus. Ubiquitin also localised to structural components of tachyzoites that resemble centrosomes [arrowheads in Figure 1B and (Brooks et al., 2011)] and the IMC (arrows in Figure 1B). As parasites progress through the cell cycle and division, the ubiquitination staining pattern changes, suggesting that ubiquitination of proteins in *T. gondii* is cell cycle-regulated (Figure 1B).

Ubiquitin-like proteins form two phylogenetic clusters in T. gondii

Ubiquitin occurs in three forms, either as a precursor fused to one of two ribosomal subunits (RPL40 and S27A), or a linear fusion protein composed of multiple copies of ubiquitin. The genome of *T. gondii* encodes polyubiquitin, RPL40 and S27A, and a number of ubiquitin-

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like genes that are potential sources of ubiquitin or ubiquitin-like modifiers (Figure S1A). As reported, there is a single SUMO gene in *T. gondii* (Braun et al., 2009). Phylogenetic analysis showed that the genes identified cluster into two major groups (Figure S1B). Based upon PFAM domain searches of proteins in cluster 1 or 2, cluster 1 contains proteins that contain ubiquitin-binding domains. Cluster 2 contains proteins that encode ubiquitin for conjugation to target proteins.

Global identification of ubiquitination sites in intracellular tachyzoites

Ubiquitin remnant immunoaffinity profiling (Xu et al., 2010) has expanded the number of known ubiquitin targets and led to a greater understanding of ubiquitin biology. Digestion of ubiquitin-protein conjugates by trypsin results in a diglycine remnant attached to the ubiquitinated lysine residue. Anti-diglycyl lysine antibodies are used to purify ubiquitinated peptides prior to sequencing by LC-MS/MS (Figure 2A). We used this method to survey the ubiquitinome of filtered intracellular *T. gondii* RH*ΔhxprtΔku80* tachyzoites, the parasite form that represents the pathogenic stage. After LC-MS/MS of eluted peptides, spectra identified in our database were filtered to 95% confidence. Ubiquitination sites were localised using the Ascore algorithm (Beausoleil et al., 2006), which determines likelihood of PTM sites. Ubiquitinated proteins are found in Table S1, and ubiquitinated peptides and sites with a >95% localisation probability are in Table S2. Table S1 was manually annotated from available literature and annotation data available on ToxoDB.org. Table S2 was annotated automatically during generation of Scaffold files.

In total, we identified 454 ubiquitinated proteins and 800 sites in intracellular tachyzoites, with 2.2% false discovery rate (FDR) for proteins and 0.75% for peptides, at a 95% peptide confidence. These yields are comparable to a ubiquitin survey of HEK293T cells (Xu et al., 2010). Seventy-six ubiquitinated proteins were identified in *Plasmodium falciparum* using a less sensitive, whole protein immunoprecipitation method (Ponts et al., 2011). We detected known substrates such as histones and ubiquitin, as well as atypical targets. Most proteins have one or two modification sites, though a small proportion are highly modified (Figure S2A).

Ubiquitinated *T. gondii* proteins have diverse functions and localisations (Figures 2B, 2C). Cytoskeletal proteins and components of the IMC make up 18% of proteins identified (Figure 2B, 2D). A statistically significant percentage of proteins are nuclear, and 8% of the ubiquitinome comprises proteins localising to both the nucleus and cytoplasm. Thirteen ubiquitinated proteins localise to the apicoplast or mitochondrion and an additional eight ubiquitinated proteins are predicted to localise to the mitochondrion based on their putative function.

While in HEK293T (human) cells the majority of proteins identified as ubiquitinated are involved in metabolism (Xu et al., 2010), ubiquitinated *T. gondii* proteins have a wide range of functions (Figure 2C). We performed pathway analysis using Gene Ontology (GO) terms and predefined gene sets (Behnke et al., 2010; Croken et al., 2014) (Figure 2E, Figure S2B, Figure S2C). This revealed enrichment of GO term processes associated with chromatin regulation (Figure 2E). As expected, ubiquitinated proteins are significantly enriched for genes upregulated in tachyzoites (Figure S2B). Ubiquitinated proteins are highly enriched

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for GO terms associated with dimerisation and structural function (Figure 2E), and ribosomal components (Figure S2C).

Supporting this, a large proportion of ubiquitinated proteins function in translation (33 ribosomal proteins with 60 sites). Ubiquitination of ribosomal proteins regulates removal of defective ribosomes, and limits ribosome availability (Qian et al., 2006). In addition, metabolic enzymes and enzymes involved in biosynthetic processes such as glycolysis constitute a significant proportion (10%) of the ubiquitinome. Furthermore, ubiquitination was detected on a large number of proteins involved in vesicular trafficking and ion transport.

Ubiquitination occurs in regions rich in hydrophobic residues

As in human cells, ubiquitination in *T. gondii* has no strict motif for ubiquitination, as shown in a heat map of amino acid enrichment surrounding each ubiquitinated Lys (−7 to +7) (Figure 3A) and WebLogo consensus diagram (Figure 3B). Though the pattern of enrichment and depletion is distinct from humans, the trend is similar in that ubiquitinated peptides are enriched for hydrophobic residues (Xu et al., 2010).

In eukaryotes including *T. gondii*, the amino acid at the protein N-terminus often determines protein stability and lifespan (Matrajt et al., 2002). Ubiquitinated proteins have a modest preference for Ala, Gly and Ser as the N-terminal residues, compared to all predicted *T. gondii* proteins (Figure S3). Proteins tightly regulated by ubiquitination, such as cell cycle regulated proteins, often harbour a ‘destruction box’ (D-box) motif (RXXLGXIGD/N) (Glotzer et al., 1991) or a KEN box (KENXXXN/D) (Pfleger and Kirschner, 2000). We did not detect any proteins with a D-box motif in the *T. gondii* proteome, but 70 proteins have a KEN box. Of those, only 4 were ubiquitinated in intracellular tachyzoites (Table S1). It is not yet clear if degron motifs target proteins for degradation in *T. gondii*.

Ubiquitination of cytoskeletal and glideosome components

IMC proteins were identified in intracellular and extracellular tachyzoite ubiquitinomes, and multiple sites of ubiquitination were detected on several proteins (Figure S4 and Table S2). To confirm that IMC proteins are modified by ubiquitin, we performed immunoprecipitation (IP) of epitope-tagged IMC proteins (Beck et al., 2010; Chen et al., 2014) from intracellular tachyzoites followed by Western blot using anti-ubiquitin antibodies (Figure 4A). To prevent degradation of polyubiquitinated proteins, intracellular parasites were treated with the proteasome inhibitor MG-132 before lysate preparation (Shaw et al., 2000). A strong signal was detected on ILP1, suggestive of a monoubiquitinated form of ILP1, and a ladder of bands commonly associated with polyubiquitination (Figure 4A). IP-Western of other IMC proteins revealed a higher molecular weight smearing pattern consistent with polyubiquitination (Figure 4A).

IFA was performed to localise IMC components and ubiquitination during cell division (Figure 4B). To preserve structural features of the IMC, gentler fixation conditions (4% paraformaldehyde) were used. IFA was performed without proteasome inhibitors as these affect tachyzoite morphology (Shaw et al., 2000). We observed partial colocalisation of ubiquitin with ILP1 during various stages of endodyogeny (Figure 4B; note overlap in

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daughter cells in lower ILP1 panel) but minimal colocalisation with the maternal IMC18, consistent with a greater fraction of ILP1 being ubiquitinated in steady state conditions.

Motility in the Apicomplexa is powered by an actomyosin motor anchored to the IMC. The ubiquitinome contained proteins involved in motor function including glideosome-associated proteins GAP40, GAP45, GAP50, myosin light chain MLC1, actin, and several myosins. Ubiquitination of myosins may be important for rearrangement of the cytoskeleton or regulation of motor function. Tubulin is essential for parasite structure (Morrissette, 2015) and was also ubiquitinated. *T. gondii* has several microtubule structures with distinct functions: conoid and intraconoid microtubules, spindle microtubules, the centriole and subpellicular microtubules. Ubiquitination of tubulin regulates microtubule dynamics and tubulin turnover, in some cases acting to inhibit microtubule formation (Bheda et al., 2010).

The apical complex in Apicomplexans is a cytoskeletal structure located at the apical end of the cell, including the conoid, a microtubule-organising centre found in some Apicomplexans. Hu *et al* partially purified the apical complex of *T. gondii* and identified over 200 proteins (Hu et al., 2006). Ubiquitinated proteins were present in the conoid-enriched fraction and in the conoid-depleted fraction, together representing almost 50% of ubiquitinated proteins.

Self-regulation of the ubiquitination pathway by ubiquitin

Polyubiquitin chains have different functions depending on the lysine residue that is linked. K48 linkages are the most abundant linkage in yeast (Xu et al., 2009) and target proteins for degradation. Other common polyubiquitin linkages are K63, K29, K27 and K11. While ubiquitination of ubiquitin was detected on all of these sites, human and *T. gondii* ubiquitin differ by a single D to E substitution, precluding definitive assignment as *T. gondii* peptides. Ubiquitination sites were detected on S27A and three ubiquitin-like proteins on *T. gondii*-specific peptides. Unlike studies in *P. falciparum* (Ponts et al., 2011), ubiquitination of SUMO was not detected.

Little is known about how the ubiquitination system is regulated, though self-regulation by ubiquitin ligases occurs by autoubiquitination. Supporting this, diglycine was detected on ubiquitination enzymes (Table S3).

Ubiquitination of transcriptional regulators and chromatin remodelling complexes

Ubiquitination of transcription factors regulates their association with chromatin (Ndoja et al., 2014) and is essential to nuclear protein turnover. Three Apetala-like transcription factors (AP2 family) were detected as being ubiquitinated.

Transcription is mediated by RNA polymerase II, and imaging of transcriptional dynamics demonstrates frequent, prolonged arrest of transcription (Darzacq et al., 2007). During arrest, RNA polymerase II must be degraded for RNA synthesis to resume. Ubiquitin was detected on multiple RNA polymerase II complex proteins, suggesting that ubiquitination has a conserved role in removal of stalled complexes during transcriptional arrest in *T. gondii*.

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Ubiquitination was detected on chromatin modifying proteins, including acetyltransferase GCN5b, a master regulator of transcription (Wang et al., 2014) as well as GCN5b interacting proteins (Wang et al., 2014).

Ubiquitination of histones and independent validation

Covalent modification of histones regulates transcription and chromatin dynamics (Strahl and Allis, 2000). In our study, 35 ubiquitination sites were detected on eight *T. gondii* histones (Figure 5A). Modification of H2BAK112 was also identified in *P. falciparum* (Trelle et al., 2009). Ubiquitination of H2AX, which has been linked to DNA damage response (Pan et al., 2011), was also detected.

Lysine residues on *T. gondii* histones can also be acetylated, methylated or succinylated (Nardelli et al., 2013). Few PTM occur on the same lysine residues as ubiquitin (Figure 5A), but H3K24 is modified by ubiquitin, acetylation, mono- and trimethylation (Nardelli et al., 2013), implicating H3K24 as an important regulatory site.

In bulk analysis of histone PTM by mass spectrometry [(Nardelli et al., 2013), Figure S5B], only ubiquitination on H3K116 was identified, implying that ubiquitination at this site is abundant, but other modifications occur at substoichoimetric levels only detectable using an enrichment strategy. Ubiquitin-specific antibody detected several bands of higher molecular weight compared to the expected sizes of histones (Figure 5B). Antibody specific for H2BK120 ubiquitin reacted with *T. gondii* histones (Figure 5B). This antibody recognizes a C-terminus peptide that is conserved in human and *T. gondii* H2B (Figure 5C). *T. gondii* H2BV antibodies detected higher molecular weight bands similar in size to those detected by anti-H2BK120 ubiquitin antibody (Figure 5B). IFA with anti-H2BK120 ubiquitin antibodies labelled parasite nuclei (Figure 5D). Collectively, these data support the presence of ubiquitin-histone conjugates in *T. gondii* chromatin that could have conserved functions (Figure 5E).

Enrichment of ubiquitinated proteins with genes that are cell cycle-regulated

Ubiquitination regulates cell cycle dynamics through checkpoint control (Teixeira and Reed, 2013). During intraerythrocytic development of *P. falciparum* ubiquitination is altered (Lasonder et al., 2015; Ponts et al., 2011), suggesting a role for ubiquitin in cell cycle regulation. Since a large proportion of the *T. gondii* transcriptome is cell cycle regulated (Behnke et al., 2010), we hypothesised that the ubiquitinome of *T. gondii* would be enriched for cell cycle-regulated proteins.

Two transcriptional waves occur during the eight hour *T. gondii* cell cycle, corresponding to G1 and S/M phase (Behnke et al., 2010) (Figure 6A). Over 35% of the ubiquitinome comprises proteins whose mRNA abundance is cell cycle-regulated, so we tested whether the corresponding genes were enriched in gene sets that are upregulated in the G1 or S/M subtranscriptomes (Croken et al., 2014) by statistical testing. The ubiquitinome is significantly enriched for proteins whose mRNA are upregulated at two points during the cell cycle: at the end of G1 (6.8 to 7.6 hr), and in the middle of S/M phase (3 to 4 hr) (Figure 6B and 6C). Amongst the 51 ubiquitinated proteins whose mRNA are upregulated in G1 are DNA licensing factors and transcription factors. Turning to S/M phase, 70 genes for

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ubiquitinated proteins are upregulated, notably those encoding IMC and cytoskeleton proteins. IMC proteins are synthesised *de novo* during endodyogeny (Behnke et al., 2010), but the mother cell IMC and plasma membrane are recycled during cytokinesis (Ouologuem and Roos, 2014). During S/M phase, mitosis is initiated, requiring major cytoskeletal rearrangements (Anderson-White et al., 2012).

Absence of classical cell cycle regulated proteins in the T. gondii ubiquitinome

There is a notable absence of conserved ubiquitinated cell cycle regulators in this dataset, strengthening the notion that the *T. gondii* cell cycle differs from other eukaryotes (Gubbels et al., 2008). In higher eukaryotes, APC and SCF E3 ligase complexes degrade cyclins and cyclin-dependent protein kinases (Teixeira and Reed, 2013). Though some components of the APC are conserved in *T. gondii* (Baker et al., 2007), few candidate F-box containing proteins, the largest group of SCF substrates, are present in *T. gondii* (Ponts et al., 2008) and these were not detected as being ubiquitinated.

During the cell cycle, ubiquitin ligases regulate glycolysis (Tudzarova et al., 2011), implying that the cell cycle has nutrient sensitive restriction points. A large proportion of the *T. gondii* ubiquitinome comprises metabolic and biosynthetic enzymes, supporting a role for ubiquitin in nutrient pathway regulation in protozoa.

Ubiquitination of the putative T. gondii ‘replisome’

The ‘replisome’ consists of machinery required for DNA replication. ORC proteins (Origin Recognition Complex) bind to replication origins and are a binding platform for DNA replication machinery. While *T. gondii* ORC proteins were not modified by ubiquitin, two Minichromosome Maintenance (MCM) proteins and Replication factor A are ubiquitinated.

Ubiquitin was also detected on a conserved site on proliferating cell nuclear antigen 1 (PCNA), a regulator of DNA replication and repair and part of the replisome. Upon induction of DNA damage, the ubiquitination state of PCNA determines whether cells continue replication in an error-prone manner or switch to an error-free bypass pathway (Andersen et al., 2008).

AAA-type NTPase CDC48 proteins regulate cell cycle and DNA replication by shuttling ubiquitinated substrates to the proteasome. *T. gondii* has two CDC48 molecules (Agrawal et al., 2009); CDC48AP is involved in import of ubiquitinylated proteins into the apicoplast, and the function of CDC48CY is unknown. TgNoAP1, a CDC48-related protein, is a nucleolar protein that functions in pre-rRNA processing (Suvorova et al., 2013). While CDC48AP and TgNoAP1 were not detected, CDC48CY was extensively ubiquitinated.

E3 ligases and deubiquitinating enzyme expression in the cell cycle

Ubiquitination is mediated by a series of ubiquitin ligases termed E1, E2 and E3. E3 ligases directly catalyse the transfer of ubiquitin to the substrate and confers ubiquitin specificity. PFAM domain models for E3 ligases were searched against the latest *T. gondii* predicted proteome to identify potential ubiquitin enzymes beyond those identified in a prior comparative genomics study (Ponts et al., 2008). Of 94 *T. gondii* candidate E3 ligases

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identified (Table S3), 68 encode a Really Interesting New Gene (RING) domain, and ten contain a HECT ubiquitin ligase domain. mRNA for 24 putative E3 ligases were upregulated within the cell cycle with 13 exclusively upregulated during G1 and 11 exclusively upregulated during S/M phase, consistent with dedicated G1 and S/M phase E3 ligases (Figures 6D and 6E).

Deubiquitinating enzymes counteract the ubiquitinating activity of E3 ligases, by selectively removing ubiquitin chains from target proteins. Using current models for deubiquitination enzymes, we expanded the list of candidate deubiquitinating enzymes in *T. gondii* (Ponder and Bogyo, 2007; Ponts et al., 2008) to 46 proteins (Table S3) of which 30 are cell cycle regulated. Like the E3 ligases, several deubiquitinating enzymes are upregulated in the middle of S/M phase (Figures 6D and 6E).

Differences in the Ubiquitinome of Extracellular Tachyzoites

Extracellular tachyzoites are released from an infected host cell and search for a new host cell to invade. Extracellular tachyzoites are arrested in G1 (Croken et al., 2014) and differ in acetylation (Jeffers and Sullivan, 2012) and metabolism (MacRae et al., 2012), suggesting that extracellular forms represent a unique tachyzoite state (Lescault et al., 2010). We examined the ubiquitinome of extracellular tachyzoites that had naturally egressed from host cells.

The ubiquitinome of extracellular tachyzoites comprises 346 proteins (1.1% FDR for proteins, 0.46% for peptides at a 95% peptide confidence level) with 51 proteins unique to extracellular tachyzoites (Figure S6A). The amino acids surrounding ubiquitination sites do not differ from those of intracellular tachyzoites (Figure S6B, S5D). There is significant overlap between the ubiquitinomes of intracellular and extracellular tachyzoites. Fewer sites were identified in extracellular parasites, with more significant enrichment of G1 proteins (Figure 6F and 6G). Several proteins modified by ubiquitin on multiple sites in intracellular tachyzoites are not as abundantly modified in extracellular tachyzoites (Table S2, Figure S6C), while some glycolytic enzymes are modified only in extracellular tachyzoites.

Crosstalk between ubiquitin and other PTMs

Ubiquitination exhibits significant crosstalk with other modifications, particularly acetylation and phosphorylation (Mertins et al., 2013; Swaney et al., 2013; Wagner et al., 2011). We examined the overlap between the ubiquitinome and the phosphoproteome, lysine acetylome, lysine succinylome, and SUMO proteome (Braun et al., 2009; Jeffers and Sullivan, 2012; Li et al., 2014; Treeck et al., 2011), as well as the arginine methylation proteome (Yakubu *et al.*, manuscript in preparation) and O-GlcNAcylome (Silmon de Monerri & Kim, manuscript in preparation) (Figure 7A). The significance of overlaps compared to a background of all predicted *T. gondii* proteins was evaluated by hypergeometric statistical testing of enrichment (Figure 7B). There was little overlap of the ubiquitinome with succinylation or arginine methylation (less than 10% of the ubiquitinome). Twenty-one percent of ubiquitinated proteins are also acetylated; interplay between ubiquitin and acetylation may regulate protein stability by inhibiting ubiquitination (Caron et al., 2005). Although a small proportion of the ubiquitinome, 25% of the SUMO

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proteome (Braun et al., 2009) is ubiquitinated (32 proteins), suggesting that there is crosstalk between ubiquitin and SUMO modification.

There was a statistically significant enrichment of phosphoproteins in the ubiquitinome. Seventy-eight percent of ubiquitinated proteins in the intracellular ubiquitinome are also phosphorylated (Figure 6A). In yeast, phosphorylation and ubiquitination co-regulate over 400 proteins, many of which function in translation (e.g. ribosomal proteins) and transmembrane function (Swaney et al., 2013). Phosphorylation is often a prerequisite for ubiquitin-mediated degradation (Badodi et al., 2015).

Interplay between ubiquitin and phosphorylation during the cell cycle

To determine whether genes of *T. gondii* proteins found to have PTM are enriched at particular points in the cell cycle, we performed enrichment analysis of published PTM datasets (Braun et al., 2009; Jeffers and Sullivan, 2012; Li et al., 2014; Treeck et al., 2011) and our unpublished PTM datasets using the same cell cycle gene sets as above (Figure 7C). We noted three waves of phosphorylation amongst cell cycle regulated genes with two waves evident in G1 (4.5-5.5 hr; 6.5-8 hr) and one in S/M (3-4 hr). Most of the PTM proteomes demonstrate enrichment of genes upregulated at the end of G1 phase (Figure 7C). Towards the end of G1 phase, cells are preparing for S phase and there is a well-documented checkpoint at this stage in mammalian cells. Of the late G1 ubiquitinated proteins, 35 are also present in the phosphoproteome; many of these proteins are transcription factors and part of the replication machinery. In addition, the phosphoproteome is enriched for proteins whose genes are upregulated in early to mid G1, perhaps corresponding to a eukaryotic mid-G1 checkpoint that governs the decision to commit to a new round of the cell cycle (Rock et al., 1992).

While most of the PTM proteomes did not show enrichment in S/M phase genes, both the ubiquitin and phosphorylation proteomes are enriched for genes that are upregulated at the boundary of S/M phase, with an overlap of 63 proteins. Many of these ubiquitinated and phosphorylated proteins are IMC and cytoskeletal proteins. Prior studies have shown that a premitotic checkpoint exists in *T. gondii* (Radke et al., 2001), and the timing of the ubiquitinome and phosphoproteome enrichment indicates that these two PTM are important for transition through mitosis.

DISCUSSION

Ubiquitination regulates cell homeostasis by modulating a wide range of cellular processes and through crosstalk with other PTM. Protein degradation via the proteasome is essential to parasite development (Shaw et al., 2000), but it is unclear why. Our survey of ubiquitination in *T. gondii* suggests that it is an abundant modification that occurs in many different cellular compartments.

Though our data implicate ubiquitination as an important regulator of cell cycle progression, there was an underrepresentation of classical cell cycle regulators regulated by ubiquitination in other organisms. We did not detect ubiquitination of cyclins, the positive regulators of cyclin-dependent protein kinases, APC and SCF1 substrates, or the cohesin

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complex, a conserved regulator of sister chromatid separation in mitosis. Together, these data imply that the cell cycle in *T. gondii* is unique, with regulatory molecules not common in other eukaryotes.

A striking finding was large number of ubiquitinated IMC and cytoskeletal proteins. To our knowledge, ubiquitination of these proteins in *T. gondii* has not been described and suggests that ubiquitination is important for IMC biogenesis. Ubiquitination of cytoskeletal proteins may be involved in spindle assembly in late S or M phase (Radke et al., 2001), allowing rearrangement and reconfiguration of microtubules. In other organisms, a large fraction of the ubiquitinome is thought to represent newly synthesised proteins or those that require continued synthesis (Kim et al., 2011). This is consistent with what is known about the IMC; during endodyogeny, the IMC is continuously rebuilt and dissembled (Ouologuem and Roos, 2014). The IMC is highly conserved in Apicomplexa (Kono et al., 2012), but ubiquitinated IMC proteins were not identified in *P. falciparum* (Ponts et al., 2011). Ubiquitination of *Plasmodium* IMC proteins could be substoichoimetric and under the detection limit of the approach used. Alternatively, ubiquitination of IMC proteins may be specific to *T. gondii* or the process of endodyogeny, which does not occur in *Plasmodium* blood stages.

We also determined the ubiquitinome of extracellular tachyzoites. Several glycolytic enzymes were only ubiquitinated in extracellular tachyzoites. Considering the metabolic shift in extracellular tachyzoites (MacRae et al., 2012), our data implicates ubiquitination in this transition. In cancer cells, ubiquitin-mediated degradation of phosphoglycerate mutase inhibits glycolysis induces senescence (Mikawa et al., 2014). Phosphoglycerate mutase II and glucosephosphate mutase I were detected solely in the ubiquitinomes of extracellular tachyzoites; the growth-arrested state could be maintained by modulation of glycolytic enzymes by ubiquitination.

Upon exit of cells from anaphase and mitosis checkpoints in mammalian cells, only 1.5% of proteins display differential ubiquitination (Merbl and Kirschner, 2009). Similarly, a large change in ubiquitination was not observed in growth-arrested parasites. In future, a thymidylate kinase cell synchrony model (Radke and White, 1998) may help investigate when ubiquitination occurs during the cell cycle and which E3 ligases are responsible for mediating cell cycle-dependent ubiquitination.

This study is limited in that ubiquitin remnant purification does not enable the identification of sites as being monoubiquitinated or polyubiquitinated. In addition, this approach does not distinguish between ubiquitin and ubiquitin-like proteins that produce a diglycine remnant following trypsin digestion. The proteins detected here may represent only a portion of the ubiquitinome of *T. gondii* especially those modified at substoichiometric levels.

Proteins with low expression may not be detected. To examine this, data from two experiments measuring transcript abundance [ToxoDB tachyzoite microarray (Roos, UPenn) and RNA-seq data (Lorenzi, J. Craig Venter Institute)] were used to analyse transcript expression of ubiquitinated proteins (Figure S2D). Though many ubiquitinated proteins are highly expressed (>80th percentile), a significant number are expressed at low levels (<10th

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percentile). Some cyclins and cyclin-related proteins in *T. gondii* (Figure S2E) have low mRNA expression profiles while others are expressed at medium or high levels. Therefore the lack of cyclins and other mitotic regulators in the ubiquitinome cannot be attributed solely to low expression.

Comparison of cell cycle enrichment of PTM illustrates three major waves of PTM: an early G1 phase marked by phosphorylation, a second phase of PTM in later G1 when metabolism is highly active, and a final S/M phase notable for ubiquitination and phosphorylation of proteins whose mRNA are upregulated during DNA replication and cell division. These phases of PTM roughly correspond to ring, trophozoite, and schizont phases of *Plasmodium* development; it will be of great interest to determine whether the patterns of PTM are conserved in all Apicomplexa. The *T. gondii* cell cycle is rapid and under tight regulation. While specific cell cycle regulators are not conserved in Apicomplexa, coupling of phosphorylation and protein degradation to regulate cell division is a universal theme from bacteria to higher eukaryotes. Interrupting this control may lead to the development of therapeutics specifically targeting cell cycle regulation of pathogenic parasites.

EXPERIMENTAL PROCEDURES (See also Supplementary Experimental Procedures)

Cell and parasite culture

T. gondii strain RHΔHXΔku80 was passaged in human foreskin fibroblasts (HFF) in Dulbecco's Modified Eagle's Medium (Gibco) as previously described (Nardelli et al., 2013). For isolation of intracellular parasites, HFF were infected and harvested at 36-40 hr by scraping, sequential syringe lysis, and filtration through a 3μm polycarbonate filter. Extracellular tachyzoites that had lysed host cells were harvested approximately 45 hr post infection and filtered as above.

Sample preparation and mass spectrometry

All samples were lysed by sonication in urea buffer. Trypsin digestion and purification of diglycine-modified peptides were performed as described previously (Anania et al., 2014; Guo et al., 2014). Two technical and biological replicates of intracellular tachyzoites, and two technical replicates of extracellular tachyzoites were analysed. Purified diglycine peptides were analysed by LC-MS/MS and the resulting spectra were searched against a combined database of *T. gondii* and human proteins and a decoy database, using the Mascot algorithm. The dataset was filtered for peptide and protein confidence >95% in Scaffold (Proteome Software). Ubiquitination sites were localised in Scaffold PTM (Proteome Software) by the Ascore algorithm (Beausoleil et al., 2006), and filtered for localisation confidence >95%.

Bioinformatic analyses

To analyse ubiquitination motifs, amino acid sequences upstream and downstream of identified ubiquitination sites were extracted and analysed using WebLogo (weblogo.berkley.edu) and IceLogo (iomics.ugent.be/icelogoserver/main.html). Enrichment analysis using newly assembled and predefined gene sets comprised of genes upregulated at

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12 minute time points during G1 or S/M phase of the cell cycle (Croken et al., 2014), localisation or mRNA expression level (derived from microarray and RNA-seq data available on ToxoDB.org) was performed using a custom R script for testing the significance of overlap between ubiquitinated proteins (or other PTM protein datasets) compared to the entire *T. gondii* predicted proteome.

Immunofluorescence and immunoprecipitation

For immunofluorescence staining, HFF cells were grown to confluency on coverslips and infected with RH*ΔhxgprtΔku80*, IMC18-HA3 or ILP1-HA3 (Beck et al., 2010; Chen et al., 2014) and fixed after 16 hr. Coverslips fixed with paraformaldehyde or 1:1 methanol/acetone and stained with primary antibodies. For immunoprecipitations, epitope-tagged parasite lines (Beck et al., 2010; Chen et al., 2014) were used to infect two T150 plates with confluent monolayers of HFF. 36 hr post infection, 15 μM MG-132 was added to the media and parasites incubated for a further 4 hr. Parasites were lysed in radioimmunoprecipitation (RIPA) buffer and the lysates incubated with anti-HA sepharose beads (BioLegend). Eluates were analysed by Western blot using anti-HA antibody (BioLegend).

Histone extraction

Histones were acid-extracted from intracellular tachyzoites (Nardelli et al., 2013). Briefly, frozen parasite pellets were lysed and chromatin was extracted by incubation in 0.4 N HCl at 4°C for 4 hr. Solubilised histones were precipitated in acetone by incubation at −20°C overnight. The acetone mixture was centrifuged, washed in 100% acetone before drying and resuspension in water. Histones were analysed by Western blot using anti-histone and ubiquitin antibodies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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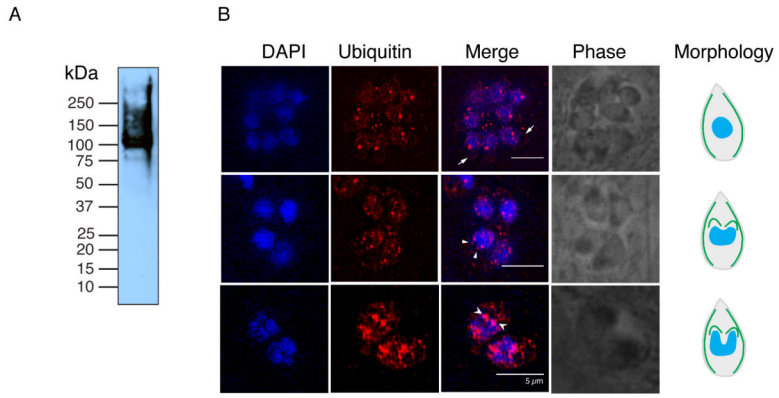

Figure 1. ***Ubiquitin is ubiquitous in*** **T. gondii** ***and changes during the cell cycle***

A. Western blot of 50 μg of intracellular tachyzoite lysate, probed with anti-ubiquitin antibody. B. Immunofluorescence staining of methanol/acetone-fixed intracellular *T. gondii* tachyzoites with anti-ubiquitin antibody (red). Nuclei are stained with DAPI (blue). Ubiquitin staining foci resembling IMC (arrows), centrosomes (see single nuclear apical dot in bottom parasite with arrow in top panel and double dots marked with triangle arrowheads in parasites beginning to divide in middle panel), and daughter buds (V-shaped arrowheads, bottom panel) are indicated. An illustration of parasite morphology shown in each microscopy image is displayed on the right; IMC (green), nucleus (blue), plasma membrane (grey). See also Figure S1.

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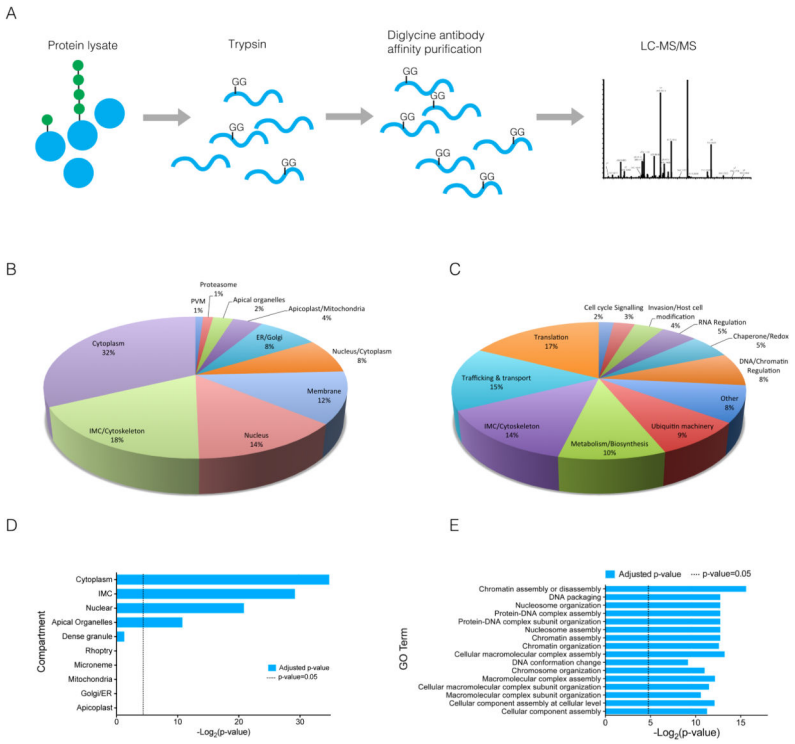

Figure 2. ***Identification of ubiquitinated proteins in*** **T. gondii**

A. Diglycyllysine remnant affinity purification. Protein lysates containing ubiquitinated proteins (green dots) and non-ubiquitinated proteins were digested with trypsin, creating a remnant diglycine moiety (GG) on the ubiquitinated lysine residue. A monoclonal antibody specific to diglycine was used to purify peptides, preceding LC-MS/MS. B. Pie charts showing ubiquitinated proteins classified by localisation, excluding proteins whose localisation is unknown. C. Pie charts showing ubiquitinated proteins classified by function, excluding proteins whose function is unknown. D. Significance of enrichment of ubiquitinated proteins in different cellular compartments, as determined by hypergeometric testing against a background of all *T. gondii* predicted proteins; $-\text{Log}_2$(p-value) is displayed. E. Gene ontology (GO) terms that are significantly enriched in ubiquitinated proteins; $-\text{Log}_2$(p-value) is displayed. See also Figure S2 and supplemental Tables S1 and S2.

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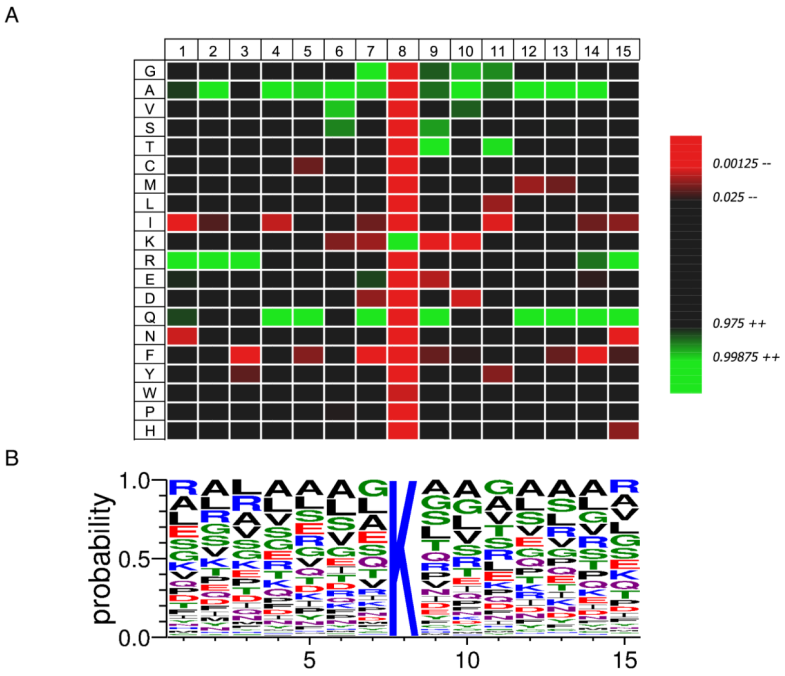

Figure 3. Amino acid environment surrounding ubiquitinated lysine residues

A. Heat map showing abundance of residues surrounding ubiquitinated lysine residues (7 residues upstream and downstream) relative to the entire *T. gondii* predicted proteome (green = enriched, red = depleted, scale as indicated). B. Consensus graphic of ubiquitination sites. Relative sizes of residue letters indicate their relative abundance across all peptides. The X-axis denotes residues surrounding the ubiquitinated Lysine residue. Residues are coloured according to side chain chemistry (green = polar uncharged, purple = polar amide, blue = basic, red = acidic, black = hydrophobic).

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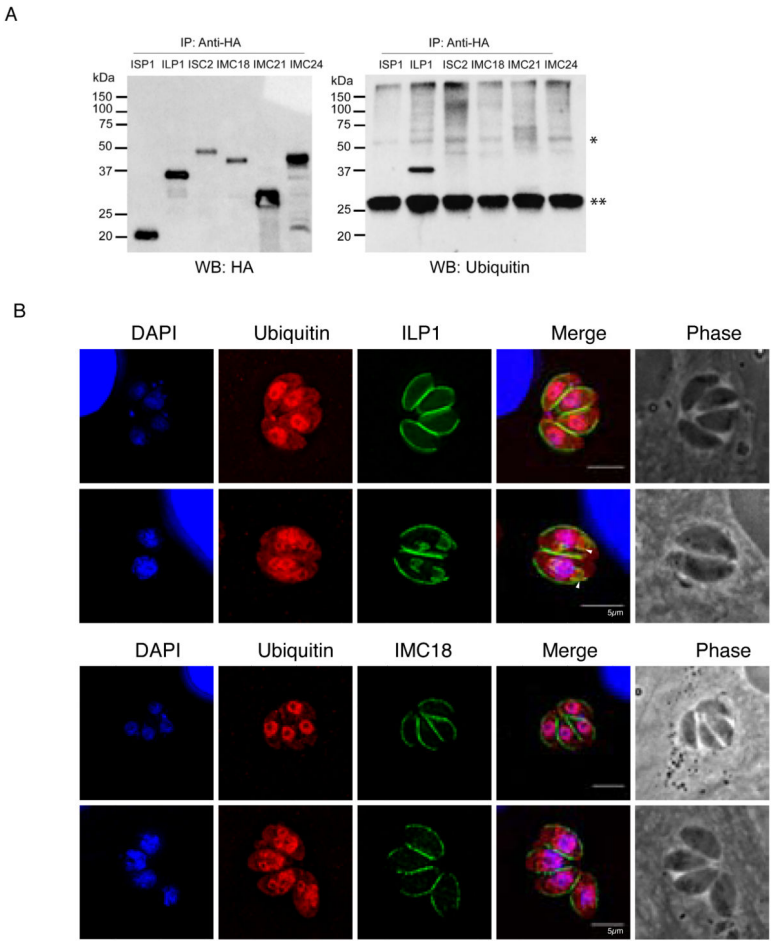

Figure 4. Detection of ubiquitinated IMC components

A. Western blots of immunoprecipitated ISP1, ILP1, ISC2, IMC18, IMC21, or IMC24, from lysates of HA-tagged intracellular parasites, were probed with anti-HA (left panel) or anti-ubiquitin antibodies (right). Similar amounts of each HA-tagged protein were enriched, and ubiquitin immunoblots showed varying degrees of higher molecular weight species consistent with polyubiquitination. For ILP1, a major band consistent with monoubiquitination was visible. Bands corresponding to antibody heavy and light chains are denoted by one or two asterisks, respectively. B. Immunofluorescence staining of paraformaldehyde-fixed ILP1-HA-tagged parasites (upper panel) and IMC18-HA-tagged parasites (lower panel) with ubiquitin antibodies (red) and anti-HA (green). Nuclei were stained with DAPI (blue). Partial colocalisation of ILP1-HA signal (yellow) with ubiquitin at daughter buds is labelled (arrowheads). See also Figure S4.

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A

Histone	Ubiquitin (K)	Succinyl (K)	Acetyl (K)	Other overlapping sites (K)**
H2Bv	113, 117, 14, 15, 19, 40, 43	None	8,13,14,18	None
H4	32*, 60, 68	31	12, 16, 31	None
H3/H3.3	116, 24, 28, 57	56, 122	9,14,18,23,79/58	56, 122
H2A1	149, 172, 173, 74	None	None	None
H2AX	124, 126, 128, 23	None	128	128ac
H2AZ	10, 143, 151, 152, 18, 24, 27	None	9,13,17,23,26,28	None
H2Ba/H2Bb	100, 108*, 112, 116, 77, 78	37, 76, 99, 107	33/34,98/99,110/111	None

* Peptide also matches human histone
**me= monomethyl, me2=dimethyl, me3=trimethyl, ac=acetyl, su=succinyl

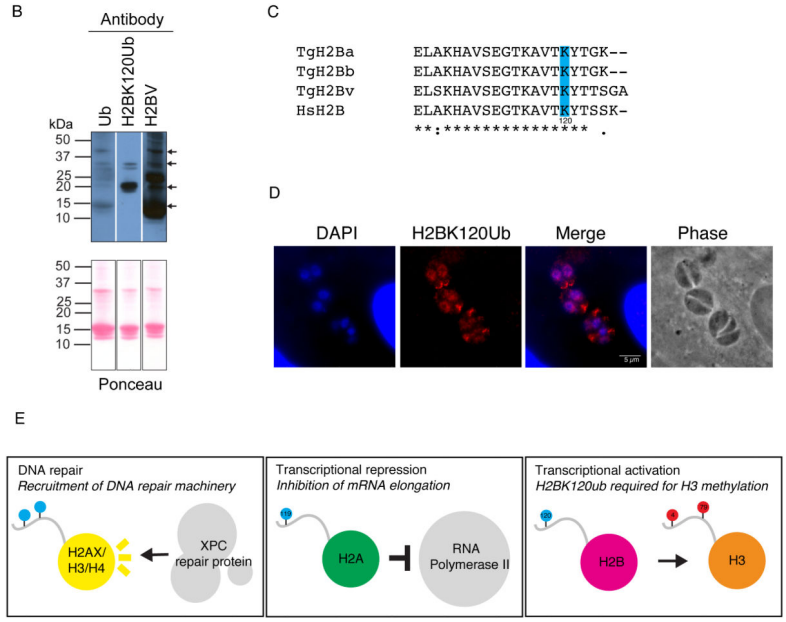

Figure 5. Ubiquitination sites related to transcriptional activation, repression and DNA repair are present in T. gondii

A. Table showing ubiquitination sites identified on *T. gondii* histones, with previously mapped histone modifications (Nardelli et al., 2013). B. Western blot on acid-extracted histones using anti-ubiquitin, H2BK120 ubiquitination (H2BK120Ub) and *T. gondii* H2BV. A Ponceau-stained image of the blot is shown in the bottom panel. Specific bands recognised by ubiquitin, H2BK120 ubiquitination antibodies correspond to bands specific to *T. gondii* H2BV (arrows). C. The antibody against H2BK120Ub was raised against a C-terminal peptide to human H2B. An alignment of H2B C-terminal peptides from human (Hs) and *T. gondii* (Tg) are shown. Consensus is indicated (* = identical, : = highly similar properties, . = weakly similar properties). D. Immunofluorescence staining of intracellular tachyzoites with antibodies against H2BK120Ub. Nuclei are labelled with DAPI (blue). E. Functions of ubiquitination sites on histones. At sites of DNA damage, ubiquitination of histones H2AX, H3 and H4 recruits DNA repair complexes e.g. XPC to damaged sites to mediate repair (left panel). Ubiquitination of histone H2AK119 inhibits RNA polymerase II elongation phase, causing transcriptional repression (middle panel). Ubiquitination of histone H2BK120 activates transcription via methylation of H3K4 and H3K79 (right panel). Blue dots, ubiquitination; red dots, methylated lysines. See also Figure S5.

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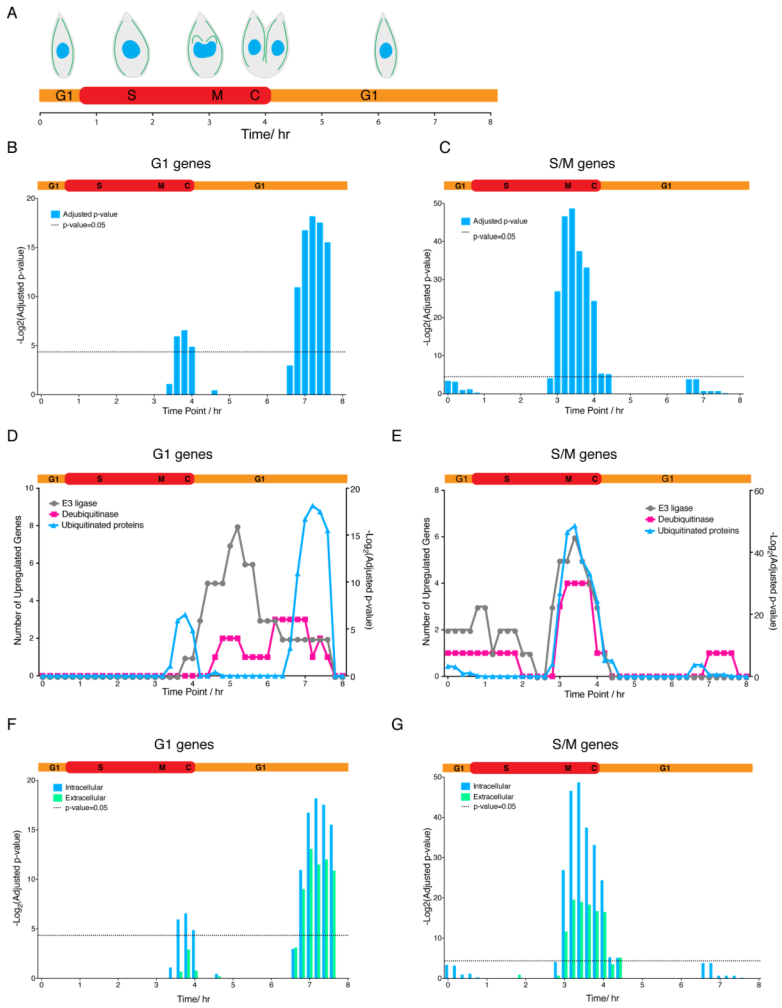

Figure 6. Ubiquitinated proteins are enriched for cell cycle regulated proteins

A. Parasite morphology across the cell cycle in *T. gondii*. Parasites are outlined in grey, nucleus in blue and IMC in green. The schema below indicates parasite cell cycle, progressing from G1 to S, M and C phases and beginning G1 again. B and C. Enrichment analysis of ubiquitinated proteins for genes in stage-specific G1 phase (B) and S/M phase (C). Adjusted p-values ($-Log_2$-transformed) are plotted. Predefined gene sets corresponding to 12 min time points during the 8 hr cell cycle were used and represent G1 and S/M subtranscriptomes during endodyogeny (Behnke et al., 2010; Croken et al., 2014). D and E. Expression trends of ubiquitination enzymes and ubiquitinated proteins in intracellular parasites. Graph showing number of E3 ligases and deubiquitinating enzymes across the *T. gondii* cell cycle in G1 (D) and S/M (E) (see Table S3); with transcriptome data from (Behnke et al., 2010), overlaid with $-Log_2$ transformed p-values from enrichment analysis of ubiquitinated proteins detected in intracellular parasites. F and G. The ubiquitinome of extracellular tachyzoites shows decreased enrichment of cell cycle regulated proteins. Enrichment analysis of ubiquitinated proteins identified in the ubiquitinomes of intracellular and extracellular tachyzoites in G1 gene sets (F) or S/M phase gene sets (G). See also Figure S6, Table S3.

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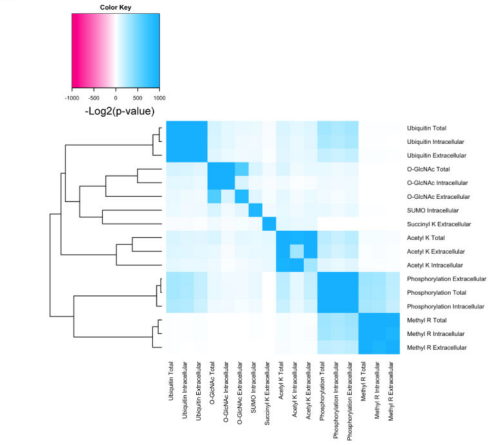
A

PTM Proteome	Reference For Proteome (DOI)	Genes In Dataset	Overlap With Total Ubiquitin Proteome	Percent of:		
				Ubiquitin Proteome	Compared Proteome	Total Predicted *T. gondii* Proteins
Acetylome (Total)	10.1128/EC.00088-12	484	108	21	22	1
Acetylome (Extracellular)	10.1128/EC.00088-12	382	91	18	24	1
Acetylome (Intracellular)	10.1128/EC.00088-12	209	60	12	29	1
Succinylome (Extracellular)	10.1021/Pr500992r	159	27	5	17	0
O-GlcNAcome (Total)	Manuscript In Preparation	260	84	17	32	1
O-GlcNAcome (Extracellular)	Manuscript In Preparation	116	41	8	35	0
O-GlcNAcome (Intracellular)	Manuscript In Preparation	184	57	11	31	1
Phosphoproteome (Total)	10.1016/J.Chom.2011.09.004	3135	395	78	13	5
Phosphoproteome (Extracellular)	10.1016/J.Chom.2011.09.004	2921	382	76	13	5
Phosphoproteome (Intracellular)	10.1016/J.Chom.2011.09.004	2390	335	66	14	4
R Methylome (Total)	Manuscript In Preparation	323	34	7	10	0
R Methylome (Extracellular)	Manuscript In Preparation	200	21	4	11	0
R Methylome (Intracellular)	Manuscript In Preparation	310	34	7	11	0
SUMO Proteome (Intracellular)	10.1016/J.Ijpara.2008.07.009	120	39	8	33	0

B



C

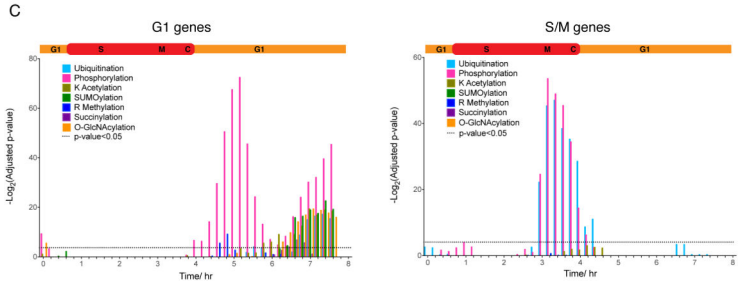


Figure 7. Cell Cycle Enrichment Analysis of PTM Proteomes

A. Table showing overlaps between ubiquitination (total ubiquitinated proteins identified from intracellular and extracellular tachyzoites) and other *T. gondii* PTM proteomes. B. Heatmap displaying the significance [$-\text{Log}_2$(p-value)] of the overlap of PTM datasets with one another, compared to the entire *T. gondii* proteome. C. Two waves of PTM modified proteins are evident in G1 (hr 4.5-5.5; hr 6.5-8) and one in S/M (hr 3-4). Proteins identified in PTM proteomic studies were tested for enrichment in predefined gene sets corresponding to the G1 or S/M subtranscriptomes. Adjusted p-values [$-(\text{Log}_2)$-transformed] are plotted.