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The Methods employed in Mass Spectrometric Analysis of Posttranslational Modifications (PTMs) and Protein–Protein Interactions (PPIs)

Rama R. Yakubu[1], **Edward Nieves**[2,3], **Louis M. Weiss**[1,4,*]

[1]Department of Pathology, Albert Einstein College of Medicine, Bronx, NY, USA.

[2]Department of Biochemistry, Albert Einstein College of Medicine, Bronx, NY, USA.

[3]Department of Developmental and Molecular Biology, Albert Einstein College of Medicine, Bronx, NY, USA.

[4]Department of Medicine – Division of Infectious Diseases, Albert Einstein College of Medicine, Bronx, NY, USA.

Abstract

Mass Spectrometry (MS) has revolutionized the way we study biomolecules, especially proteins, their interactions and posttranslational modifications (PTM). As such MS has established itself as the leading tool for the analysis of PTMs mainly because this approach is highly sensitive, amenable to high throughput and is capable of assigning PTMs to specific sites in the amino acid sequence of proteins and peptides. Along with the advances in MS methodology there have been improvements in biochemical, genetic and cell biological approaches to mapping the interactome which are discussed with consideration for both the practical and technical considerations of these techniques. The interactome of a species is generally understood to represent the sum of all potential protein-protein interactions. There are still a number of barriers to the elucidation of the human interactome or any other species as physical contact between protein pairs that occur by selective molecular docking in a particular spatiotemporal biological context are not easily captured and measured.

PTMs massively increase the complexity of organismal proteomes and play a role in almost all aspects of cell biology, allowing for fine-tuning of protein structure, function and localization. There are an estimated 300 PTMS with a predicted 5% of the eukaryotic genome coding for enzymes involved in protein modification, however we have not yet been able to reliably map PTM proteomes due to limitations in sample preparation, analytical techniques, data analysis, and the substoichiometric and transient nature of some PTMs. Improvements in proteomic and mass spectrometry methods, as well as sample preparation, have been exploited in a large number of proteome-wide surveys of PTMs in many different organisms. Here we focus on previously published global PTM proteome studies in the Apicomplexan parasites *T. gondii* and *P. falciparum* which offer numerous insights into the abundance and function of each of the studied PTM in the Apicomplexa. Integration of these datasets provide a more complete picture of the relative importance of PTM and crosstalk between them and how together PTM globally change the

*corresponding author: Louis.Weiss@einstein.yu.edu.

cellular biology of the Apicomplexan protozoa. A multitude of techniques used to investigate PTMs, mostly techniques in MS-based proteomics, are discussed for their ability to uncover relevant biological function.

Keywords

Mass Spectrometry; Posttranslational Modifications; Protein-Protein Interactions; In-silico databases; Toxoplasma gondii; Posttranslational Crosstalk; Experimental Techniques; Cell Cycle

Protein-Protein Interactions

Introduction

Proteins are generally understood to achieve their functions in concert with other molecules such as DNA, RNA and other proteins by mediating metabolic, signaling pathways and cellular processes. Protein-protein interactions have been found to be an important factor in the regulation of molecular and cellular mechanisms responsible for both healthy and diseased states of organisms. There is an increasing amount of literature that examines the role of protein-protein interactions in the etiology of disease and studies have demonstrated that some diseases are the result of mutations in protein-protein interaction sites that lead to a change in binding and function, e.g. Von Hippel-Lindau syndrome [12]

One definition of a protein-protein interaction (PPI) presented by De Las Rivas et. al is a specific physical contact between protein pairs that occur by selective molecular docking in a particular biological context [3]. Importantly this definition indicates the selective nature of PPIs should exclude random interactions and all those made when the protein is being synthesized, folded and degraded. The interaction interface of proteins involved in PPIs will most likely feature selective biomolecular events and will have evolved to fulfill a particular purpose distinguishable from generic functions such as protein production and degradation [3]. Further these interactions will be physiologic, i.e. occurring within a biological context and thus are dependent on cell type, cell cycle phase, developmental stage, protein modifications, cofactors and other binding partners.

Protein-Protein interactions function as highly precise and regulated networks, adding additional layers of complexity to cellular systems. Information concerning the structural coverage of protein-protein interfaces confers a deeper understanding of molecular mechanisms of protein function and recognition. Practical applications of this information can be seen in drug design of protein interaction inhibitors and rational protein design for therapeutic strategies [45].

Goncearenco et al. have argued that our ability to understand these complex interactomes is facilitated by the availability of structurally characterized interfaces and efforts to structurally characterize protein-protein interfaces [6]. However, this comes with the drawbacks that current interactome datasets suffer from high false positive rates and even with high-throughput methods, current growth rates of domain databases and structural PPI deposition push complete domain coverage with PPI into the distant future. Further newly proposed PPIs require verification in order to ensure high accuracy and prevent erroneous

entries based on insufficient evidence [78]. An ideal analytical scenario is one in which high-throughput data is supplemented with binding site locations, physiochemical properties or structure-derived interaction interfaces.

The human interactome is predicted to comprise 130,000– 600,000 protein-protein interactions (PPIs) and its mapping is regarded as the latest large scale scientific challenge faced by biotech and pharmaceutical companies since completion of the human genome project [9]. In this context interactomes are meant to represent all potential protein-protein interactions of a given species [6]. This large number of PPIs is a barrier to its elucidation as many PPIs have numerous specific functions and the component proteins may be involved in multiple signaling pathways concurrently. Furthermore, signaling pathways may be interconnected and make up complicated signaling pathways for basic cellular processes [101112]. Some of the technical challenges of the characterization of this interactome are linked to the tools employed to elucidate PPIs, such as yeast two-hybrid assays [1314] and mass spectrometry [1516], which while useful for high-throughput studies however are unable to consistently generate high accuracy and validated results on their own [1718].

Experimental Techniques for detecting Protein-Protein Interactions (PPI)— Obtaining data on PPIs must be met with considerable forethought regarding available background information on the PPI sought such as knowledge of binding affinity, temporality of the PPI and of course the available tools and reagents required to carry out the studies. For example, the difficulty of characterizing transient interactions that are dependent on posttranslational modifications is well known. Other factors such as the involvement of second messenger molecules as drivers of the interaction, the requirement of growth factor stimulation, subcellular location or knowledge of binding domain interfaces will also have an impact on the necessary experimental approach to characterize a PPI [19].

Throughout this chapter techniques will be discussed with a focus on both their advantages and the limitations of a particular methodology. Previously genetic and biochemical techniques made up the bulk of tools employed to investigate PPIs. However, with the advent of big data and omics research there has been a shift towards the use of mass spectrometry (MS) and bioinformatics tools [2021]. Among the various approaches for PPI identification yeast two hybrid (Y2H) and tandem affinity purification coupled to mass spectrometry (TAP-MS) have proven to be the most used. MS-based investigation of disease-related PPIs opens the door for the determination of novel protein functions and thereby the unraveling of yet undiscovered novel pathogenic mechanisms.

One of the impediments to uncovering a complete interactome map is set by experimental limitations to identifying all biologically relevant protein-protein interactions. Upwards of 80% of cellular proteins are known to interact with other molecules to construct metabolic and signaling pathways [22]. The challenge of the sheer number of these potential interactions is further complicated by the spatial and temporal variability in PPIs. Many of these interactions are transient, only briefly associating and then disassociating [23], require chemical modification in the form of posttranslational modification, are location dependent and have restrictions to their transportability, have varying expression levels in various tissues and cell types and ultimately have an intrinsically linked three-dimensional structure-

function relationship which is dependent on the chemical milieu of their environment. The 3D structure while adding complexity to the interactome also represents one way of improving reliability of PPIs. The direct protein-protein interactions are mediated through specific structural interfaces that are often made up of domains of known 3D structures and thus these structural domain-domain interactions may be associated with the specific PPIs to reduce false positives and validate computational results [24]. Similar strategies will be required to vet the vast number of PPIs that are predicted computationally and also to ensure the reliability of PPIs that are already present in current PPI databases (which are likely fraught with false positives). Identifying and removing false positive along with determining what constitutes and adequate biological context to differentiate between PPIs that are true and physiologic and those that are not, represents the greatest challenge to the future study and completion of an accurate and reliable interactome [3]. One approach to these challenges comes in the form of computational methods of protein interactions that utilize sequence, evolution, expression and structural data to inform prediction modeling to reconstruct pathway maps and fill in gaps in our current interactome.

The various techniques employed in investigating PPIs have advantages as well as disadvantages and, therefore, it is important to analyze PPIs with more than one approach for both confirmation and validation in order to avoid false-positive results, false negative results and PPI's with weak interactions [25] (summarized in Figure 1). There are several confirmatory techniques available to verify PPI's depending on the binding strength of the PPI, subcellular location, whether protein function need be retained and many other parameters. Of course, all of these techniques come with their own advantages and limitations.

Functional validation of PPIs is important to understand the role of a PPI within a given signaling pathway, and once a PPI has been validated, it is important to undertake studies aimed at functional validation by carrying out truncation mutations and specific point mutations to disrupt protein binding [26].

Yeast Two-Hybrid System

One of the earliest and still popular methods used to investigate PPIs is the yeast two hybrid assay [27]. The yeast two-hybrid system is a genetic high-throughput system capable of detecting a binary interaction between two proteins *in vivo*. Although this method is capable of determining interactions between proteins and nucleic acids and small molecules [2829], it is classically a genetic method, wherein transcriptional activation of a reporter gene occurred upon interaction of a bait-protein with a prey-protein [27]. The bait protein is fused to a DNA-binding domain and the prey-protein is fused to a transcription activation domain and when these two entities bind or come into close proximity to each other function as a transcription factor and lead to the expression of a reporter gene, indicating if and when binding occurs [27]. This technique is simple, rapid, high-throughput, cost-effective, reproducible and has numerous applications from PPI identification to interactome mapping [3031]. Other advantages include the fact that the assay is carried out *in vivo*, representing a physiologic milieu and avoids artifacts such as those that may occur with cell lysis [2829]. However, the drawbacks of yeast two hybrid are that it: (1) does not account for the dynamic nature of

PPIs such that proteins expressed in different subcellular locations and at different times may be shown to interact, leading to greater false positives; (2) interactions requiring posttranslational modifications are not picked up leading to more false negatives [3130]. (3) it has low reliability rate of at most 50%; and (4) it cannot detect interactions with full-length integral membrane proteins and interactions involving some cofactors and binding partners [323325]. Since this genetic model essentially forms a transcription factor upon binding of the bait and prey protein both of these fusion proteins must have access to the nucleus. Proteins investigated with this method are also often overexpressed in order to obtain results and can lead to high false positive rates. There are other technical issues that represent limitations to this technique such as spontaneous transcriptional activation in the absence of binding and toxicity in yeast [3428]. Further PPIs in some organisms may not be detected in a yeast model. As with all techniques there is a clear need for follow-up experiments to confirm and validate any potential binding partners.

Membrane Yeast Two Hybrid System

The membrane yeast two hybrid (MYTH) represents an adaptation of yeast-two-hybrid that enables the detection of PPIs from membrane proteins [3536]. In this technique the bait protein is fused to a C-terminal fragment of the ubiquitin (Cub), which is linked to a transcription factor and the soluble or membrane-bound prey protein is fused to an N-terminal ubiquitin fragment (Nub) that has been mutated to prevent spontaneous reconstitution [37] {Paumi, 2007 #1020}. Upon binding of bait and prey proteins a pseudo-ubiquitin is formed and can be cleaved by deubiquitinating enzymes which release the transcription factor and reporter gene. MYTH has some of the same advantages of Y2H in that it requires no specialized equipment and can be used to detect interactions between “full length” proteins. Moreover, it can detect interactions taking place within the context of a membrane environment [38393640]. Similarly, MYTH is burdened by some of the same drawbacks as the Y2H assay with regard to limitations arising from proteins being overexpressed, modified and non-native to the yeast organism. MYTH can only investigate membrane proteins that have at least one terminus in the cytosol due to the deubiquitinating enzymes residing in the cytosol and soluble proteins may not be used a bait protein as they could easily diffuse into the nucleus causing erroneous transcription activation in an interaction-independent manner, leading to false positives. Soluble proteins require anchoring to intracellular structures or very large molecular mass [25].

Additional modifications to the yeast two hybrid have emerged in order to address limitations and specific experimental requirements e.g., hSOS/Ras recruitment system, three-protein system, small ligand-dependent systems, dual bait systems, reverse two hybrid systems, bacterial cell two-hybrid and mammalian two hybrid (MaMTH) systems [4142434445].

Mammalian Two Hybrid System

(MaMTH) is an extension of the Y2H system into a mammalian system, it is based on reassembly of inactive split-ubiquitin fragments Nub and Cub fused to bait and prey proteins. When the target proteins interact the rejoined ubiquitin fragments release an artificial transcription factor causing expression of a reporter gene such as luciferase [46]. A significant advantage of this system is that full-length mammalian proteins are analyzed in

their homogenous cellular environment. This method is quite sensitive allowing for the measurement of both weak, transient and dynamic interactions. However, this technique shares some of the same limitations as MYTH, requiring bait protein to be anchored in the membrane or other intracellular structure to prevent diffusion into the nucleus as well as requiring the termini of the protein fused to Cub to be cytosolic in order to interact with cytosolic deubiquitinating enzymes [46].

Kinase Substrate Sensor & Mammalian Protein-Protein Interaction Trap

More recently the kinase substrate sensor (KISS) has been developed to address incompatibility of some mammalian proteins with the Y2H system. KISS is a mammalian two-hybrid approach, which measures PPI by fusing the bait protein to the kinase domain of the TYK2 with prey protein bound to the gp130 cytokine receptor fragment harboring TYK2 substrate motifs. Protein interaction is indicated by phosphorylation of TYK2 by gp130 which leads to the activation of STAT3. Commonly a STAT3-dependent reporter system is expressed by STAT3 translocating into the nucleus to activate transcription of said reporter such as luciferase [47]. This technique allows for PPI detection in living mammalian cells, is sensitive enough to detect physiologic and pharmacologic perturbations and can be used with both membrane and cytosolic protein. However, there are limitations as it is an indirect readout preventing direct spatiotemporal analysis of PPIs and is reliant on endogenous STAT3, rendering the technique inapt for the study of proteins or signals affecting STAT3 pathways.

A similar mammalian protein-protein interaction trap (MAPPIT) uses STAT3 in a signal transduction approach to measure protein interaction by fusing bait proteins to the C-terminus of STAT3 deficient in a cytokine receptor, causing a signal transduction defect. A prey protein is fused to receptor fragments containing functional STAT3 recruitment sites. Interaction between bait and prey proteins produce a functional receptor, which is capable of STAT3 activation upon cytokine ligand stimulation. Once this occurs STAT3 is able to enter the nucleus to induce transcription of luciferase or another reporter system [48].

Protein complex-immunoprecipitation

Protein complex-immunoprecipitation (Co-IP) is a biochemical method and represents one of the most robust means of detecting physical interactions between two or more proteins. This method utilizes specific antibodies against an epitope on one of the protein components of a protein complex and is able to detect and isolate the protein and its binding partners from a cell lysate or homogenate. Isolation generally occurs with the help of protein A or G immobilized to a bead matrix (agarose or sepharose) allowing for pull-down followed by MS identification of the proteins within the complex [4950]. Some of the advantages of this technique include proteins being assayed in their native state thus allowing for detection of physiologic PPIs and allowing for post-transcriptional modifications. Further the relatively minimal sample preparation with exception of cell lysis makes this approach highly compatible with other techniques such as mass spectrometry or affinity tagging [51].

Affinity purification (AP) is a popular method for isolating a target or bait protein from a soluble phase onto a solid substrate which is generally agarose or magnet beads. The bait

protein is conjugated to an affinity tag which typically is a small entity such as TAP-, FLAG-, c-myc-, HA-, His-, protein A-, Strep-Tag. The tagged protein can then be affinity or immunoaffinity purified in a pull-down step. In a more advanced version of this technique the captured protein along with its protein binding partners within a molecular complex are then digested by proteases such as trypsin to generate peptides which are separated by high pressure liquid chromatography (HPLC) to be ionized and detected by a mass spectrometer, in a technique known as AP-MS [525354]. The specific experimental requirements will determine which purification method, antibodies and other parameters are best suited for identification of a target protein.

An improvement from standard immunoprecipitation can be achieved by tandem affinity purification (TAP), where the conjugated tag has an IgG fragment and calmodulin binding protein attached to it, which allow for the bound protein and its binding partners to be directly purified using an IgG sepharose column, followed by cleavage of the complex by a protease and subsequent pulldown and elution from a calmodulin-sepharose column. TAP-MS allows for the identification of multimolecular complexes in their native conditions however this carries with it the drawback of under identifying transient interactions and over identifying non-physiologic indirect interactions [19]. Some of these issues have been addressed through subcellular fractionation, crosslinking and quantitative proteomics.

Some of the advantages of immunopurification are that this technique theoretically surveys the entire proteome without restrictions on which proteins can be detected in a complex with the target protein. In addition, proteins can be isolated in their native state from cells and tissues, which eliminates some artefacts associated with tagging proteins but also allows for the simultaneous capture of all isoforms of that protein simultaneously. Affinity tagging while similar to immunoprecipitation enables interrogation of native proteins for which there are no available antibodies and further allows for parallel analysis of multiple proteins with a single epitope tag [54].

Both Co-IP and affinity purification face some of the same challenges beginning with the need to lyse the cell to gain access to the proteins of interest, which inadvertently hinders the discovery of the spatial and temporal relationships of PPIs but also obstructs detection of transient and weak protein interactions [2554]. Further, as with any assay using antibodies for protein identification –the accuracy is dependent on the specificity and avidity of the chosen/available antibody. Depending on the antibody and purification technique a certain degree of non-specifically binding proteins, especially abundant proteins, are expected to co-purify as contaminants and make up a large portion of the background experienced with these methods. Proteins may also be affected by lysis regents, epitope tagging, overexpression, which may all lead to denatured, misfolded and mislocalized proteins, ultimately mischaracterizing the nature of the relationship of PPIs. Proteins may also not be detected at all if the target proteins are part of large molecular complexes, if such molecular complexes require their natural cellular environment to attain their native state of association or endogenous proteins have low expression levels [52552554].

Both affinity purification and immunoprecipitation may be improved by appropriate negative controls, by using quantitative approaches such as SILAC, isotopic labeling, label-free

quantitation and by subcellular fractionation if the cell compartment of the protein of interest is known in order to reduce background and non-specific binding. Further crosslinking, is a technique used to stabilize transient or labile PPIs by covalently binding them with the help of a linker containing amine-, sulfhydryl-, and photo-reactive groups [5657].

Luminescence-based mammalian interactome mapping

Luminescence-based mammalian interactome mapping (LUMIER) is a co-IPP based approach to PPI discovery, wherein one binding protein is fused with *Renilla luciferase* and the other binding protein is tagged with FLAG, HA or protein A. The tagged constructs are introduced into an appropriate cell line and overexpressed. These cells are then lysed and the tagged protein is purified by immunoprecipitation using the tag. The interaction between tagged binding protein and fused luciferase binding protein is assessed based on the degree of luciferase activity that is measured upon interaction of the two protein constructs [5859]. The greatest advantage of this approach is its practicality –It is relatively simple, reproducible, does not require specialized equipment and lends itself to a high-throughput format. Further this technique can be used in various cell lines allowing for the investigation of PPIs in many different organisms. It is best suited for the study of binary interactions however can also detect indirect interactions [5859]. One of the main drawbacks to this approach is the necessity for cell lysis prior to immunoprecipitation which may lead to the disruption of weak and transient interactions but more importantly has the potential for introducing artifact due to mixing of proteins in the lysate that would be separated in the cell, exposing masked non-native binding sites and destabilizing true native PPIs. These effects together may lead to an increase in both false positives and negatives. Further due to the requirement of genetic modification including transfection and expression of gene constructs this method requires strict controls to minimize background signal and ensure reproducibility. Generally, this technique is not suited for spatial and temporal determination of PPIs [5859].

Biophysical Methods for the Detection of Protein Complexes

Biophysical methods are also employed to understand the formation of macromolecular complexes with analytical ultracentrifugation, sedimentation equilibrium experiments and sedimentation velocity experiments with the latter likely being the most popular. All three techniques rely on centrifugal force to facilitate a sedimentation process wherein proteins within a protein mixture separate by sedimentation based on their shape and size/mass. In analytical ultracentrifugation proteins above 10 kDA can be analyzed and followed by absorbance [606162]. In sedimentation equilibrium the macromolecular diffusion rate is compared to the sedimentation rate with this technique being most useful for dynamic interactions [6364]. Sedimentation velocity experiments provide sedimentation coefficients and molecular weights of macromolecules and are most suited for static interactions, unstable systems, complex systems involving intermediates, cooperative monomer-multimer systems and systems involving conformational changes [636566]. Advantages of SV experiments are that stoichiometry and stepwise binding constants may be determined [67]. Drawbacks include experimental run time such as in the case of sedimentation equilibrium experiments which may take up to one week, which is concerning for sample stability and other issues associated with such a time-consuming experiment [686970].

Direct Visualization of Protein Interactions by Microscopy (FRET, FLIM, BRET, BiFAC and FRAP)

One of the most popular methods of validating PPI is visualization of co-localized proteins. It should be appreciated, however, that a direct interaction of two proteins is not proven by most of these techniques. An ever-expanding array of microscopic and fluorescent techniques are available to capture co-localization at very small scales and at increasingly low concentrations. Immunofluorescence utilizes antibodies to specific target proteins and fluorophore conjugated antibodies detect proteins of interest. The detection of PPIs by this method requires the available of antibodies with high target specificity, physiologic target protein expression levels, and high-resolution instruments to resolve the spatial co-localization signals [71].

Fluorescence resonance energy transfer (FRET) is capable of visualizing physical interactions between two proteins *in vivo* and in real-time using either fluorescence or confocal microscopy. FRET works by having a donor fluorophore that is conjugated to one protein binding partner transferring non-radiative energy to an acceptor fluorophore that is conjugated to another protein binding partner. The donor fluorophore is chosen so that its emission spectrum overlaps with the absorption spectrum of the acceptor fluorophore. Thus energy transfer with subsequent fluorescence only occurs when the protein binding partners (e.g. the conjugated fluorophores) are within a certain distance (Foerster radius) of each other. When the donor fluorophore is excited in the proximity of the acceptor instead of releasing a photon, energy is transferred to the acceptor which produces a distinct emission signal which allows resolution and monitoring of PPIs on the nanoscale [72]. Some of the key advantages of this technique are the ability to measure PPIs instantaneously in real-time, within living cells, transient interactions, determination of interaction sites, monitoring of complex interaction dynamics such present during dynamic equilibrium between complex formation and dissociation [72].

Combining FRET with laser scanning confocal microscopy enables control of the depth of the imaging field eliminating out-of-focus fluorescence, reducing background fluorescence, and improving signal-to-noise ratio. FRET allows co-localization of proteins in vivo within their cellular environment, which has added advantage of being able to localize protein interactions to their subcellular compartments with the help of organelle specific probes [71]. Requirements for this technique are highly specific monoclonal antibodies to the target protein and preferably cellular proteins at endogenous expression rates, although this poses the challenge of potentially having to work with very low concentrations of target protein [22]. Drawbacks of FRET are: (1) the technically challenging task of generating fusion proteins with fluorophores; and (2) the proximity requirement of the fluorophores which poses a challenge when investigating multi-complex or large molecules. Among fluorescence-based approaches FRET exhibits decreased sensitivity due to auto fluorescence in illuminated cells as compared to methods like BiFC or BRET. For that reason this technique requires many controls to quantify the intensity of fluorescence differences. This is true in particular for weak interactions. Photobleaching is another problem that is inherent to some of the fluorophores [7372].

Fluorescence lifetime imaging microscopy (FLIM) is a technique wherein the fluorescence of a fluorophore is measured over its fluorescent lifetime. The fluorescence lifetime is the average time an electron spends in an excited state before returning to the ground state and releasing a photon in the process. The fluorescence lifetime is a property intrinsic to the fluorophore, however is influenced by the local environment such as temperature, pH and ion concentration, changes and the concentrations of which may be measured by FLIM [74]. In this manner FLIM is capable of measuring dynamic signaling events within living cells and detecting protein-protein interactions. FLIM is frequently combined with FRET as energy transfer is a quenching process that decreases the excited state of the donor fluorophore's lifetime fluorescence. With FLIM it is possible to map the spatial distribution of a fluorophore lifetimes within living cells and measure the shortened donor lifetimes that result from FRET [74]. Some of the advantages of using this technique are the lack of signal contamination as FLIM is affected solely by be cellular microenvironment. Additionally, the coupling with FRET allows it to resolve an increase in FRET population and monitor multiple interacting proteins in real time. Drawbacks include high cost of the techniques, requirement of specialized equipment and unreliable use with fixed cells [51].

Bioluminescence resonance energy transfer (BRET) represents an improvement on FRET and was created in order to reduce some of the background fluorescence that occurs when illuminating samples. BRET uses the *Renilla* luciferase protein (RLuc) as the energy donor, fused to the protein of interest and green or yellow fluorescent protein fused to a protein binding partner as the energy acceptor. When the binding partners are brought into proximity of each other and interact the RLuc transfers its bioluminescence to YFP or GFP, which leads to a fluorescent signal that can be measured as a surrogate for protein binding [7375]. This technique has some of the same advantages as FRET for detecting PPIs with the addition that BRET has greater sensitivity and lower background signal [73]. Similarly, BRET has some of the same limitations as FRET, with the BRET signal generally being significantly weaker than the FRET signal and this technique not scaling well to high-throughput applications.

Biomolecular fluorescence complementation (BiFC) is a visual fluorescence detection method, wherein a fluorescent protein like YFP is split into two distinct non-fluorescent fragments. These two fragments are then fused to a bait and prey binding protein of interest each and upon interaction of bait and prey form a fluorescent complex which can be monitored by microscopy or flowcytometry [7677]. BiFC is capable of detecting proteins at endogenous expression levels and can pick up weak and transient PPIs. Practically BiFC is a simple method, reproducible, cost-effective and can detect multiple protein-protein interaction events within a cell by using different colored fluorescent proteins [787677]. Unlike FRET and BRET, BiFC is irreversible and has a delay in fluorescence once the conjugated binding partners interact; therefore BiFC is not suitable for investigation of interaction dynamics or real-time measurements. BiFC limitations include: (1) issues with functionality of the fusion protein due to the endogenous protein being modified through tagging, and (2) the possibility of false positive fluorescent signal due to reconstituted fluorescent protein in the absence of a true protein interaction [79].

Fluorescence Recovery After Photobleaching (FRAP) is a technique wherein fluorescent molecules are irreversibly bleached in one area of the cell under study using a high intensity focused laser beam. Surrounding non-bleached fluorescent molecules then diffuse into the bleached area and recover fluorescence at a particular velocity, which is recorded by a laser. FRAP was originally created to measure diffusion in cellular membranes [8081], however with the development of fluorescent proteins and confocal microscopy, it is now used to study protein mobility in the cell interior. The most common uses for FRAP include the study of dynamics in cell biology (e.g. cytoskeletal dynamics, vesicle transport, cell adhesion, mitosis, chromatin structure, transcription, protein recycling and signal transduction [82]. Other uses include study of subcellular compartmentalization, measuring protein or molecule exchange speed between compartments, binding characteristics between proteins and the effect of mutations and small molecules on this process, and immobilization of proteins binding to large structures such as DNA, the nuclear envelope or cytoskeleton [82]. FRAP may also be used to measure dynamics of 2D and 3D molecular mobility. In a modified technique known as inverse FRAP (iFRAP) all but one small area of a cell is bleached and subsequent diffusion of fluorescent molecules out of the unbleached area is recorded. This technique is particularly useful for the study of cellular organelles e.g. the nucleus as the time required for diffusion reflects the releasing properties of the fluorescent molecule from the organelle [82]. The advantages of FRAP include the ability to detect intracellular protein mobility within living cells. The limitations of this technique are mainly technical in nature in that is requires specialized instrumentation to conduct, requires temperature control and is not very specific for protein-protein interactions [51]. One of the drawbacks of iFRAP is that since it requires a relatively long time to photobleach the entire cell under study it is not useful for rapid translocations and is more suitable for studying dissociation kinetics of molecules immobilized to intracellular structures [82].

Proximity-dependent biotin identification coupled to mass spectrometry

Proximity-dependent biotin identification coupled to mass spectrometry (BioID-MS) is a technique that uses a bait protein of interest fused to a prokaryotic biotin ligase (BirA) to biotinylate prey protein in its proximity upon expression in the cell. The biotinylated complexes may then be purified by an avidin/streptavidin-based biotin affinity approach. The isolated biotinylated proteins can then be identified by MS to generate a list of potential interacting prey proteins [83]. The advantages of BioID-MS are that this technique is library-independent and capable of detecting protein complexes in their native state within the cellular milieu as biotinylation occurs before cell lysis. This has the added advantage that complex formation and stability is not affected by the lysis process or reagents. Since this is a proximity-based method where interactions are covalently fixed through biotinylation it is ideal for detecting weak and transient interactions and has been found to be more effective than e.g. AP-MS in terms of identifying low-abundance proteins [8384]. One of the challenges of the BioID-MS approach is that it requires fusion of the bait protein to the BirA biotin ligase, which adds considerably to the size of the construct and has the potential for hampering function, behavior, interaction and targeting capabilities of the protein. Additionally low expression levels may lead to false negatives. And as with all MS-based approaches analysis of results requires specific bioinformatic tools adding to the complexity of this technique [2583].

In situ Proximity Ligation Assay

Proximity ligation assay (PLA) similarly to BioID-MS uses a proximity based approach to discover PPIs however it varies in the method of detection. It is an *in situ* technique that uses proximity probes, which are antibodies conjugated to DNA oligonucleotides, in fixed cells and tissues. These probes recognize two target binding proteins, which when interacting bring the probes into proximity with the DNA strands functioning as templates for direct ligation of two subsequently added oligonucleotides. This process creates a circular DNA molecule which is amplified by rolling circle amplification, which is primed by one of the probe oligonucleotides. The result is a long DNA strand that is linked to the appropriate antibody and thus binding protein. Repetitive elements found on this DNA strand bind to fluorophore-labeled complimentary oligonucleotide probes, which can then be detected by fluorescence microscopy [85]. Advantages of this technique are that it is able to localize PPIs down to single molecule resolution, quantify PPIs in cells and tissues and measure both weak and transient interaction [85]. The main drawbacks to this method are technical in nature such as the dependence of this approach on enzymes for ligation and polymerization as well as its dependence on antibodies for protein binding partner detection, which can be expensive and unavailable. In a study by Chen et al an in situ proximity ligation assay (In situ PLA) was employed to quantify and visualize PPIs in cells and tissues [86]. This study provided a proof of concept for an integrated approach for mapping large-scale PPIs onto signaling pathways. Chen et al identified 557 endogenous PPIs using primary antibodies in HeLa cells to create a PPI and pathway map [86]. This tool holds significant promise for investigating PPIs and was able to map identified PPIs to annotated ones in public PPI databases such as the Human Protein Reference Database, but also identified 8 new PPIs (CDC42-MPK8, CDC42-MAPK9, FGF5-EGF, MAPK3- BRAF, PIAS2-CDKN2B, PLCG2-PLCG1, RPS6KA5-EGF, IL6-HRH1) that had not been cataloged in the PubMed database [86].

Methods for the detection of receptor-ligand interactions

Other PPI approaches have focused on identifying receptor-ligand interactions. Ligand-receptor-capture- trifunctional chemoproteomics regents (LRC-TriCEPS) uses three components to bind ligands of interest, covalently link them to glycosylated receptors and finally biotin tag bound proteins for purification [87]. In an Avidity-based extracellular interaction screen (AVEXIS) extracellular receptor-ligand pairs are systematically screened by expressing secreted recombinant bait and prey proteins in a mammalian system, allowing for PTMs to occur. In this approach the bait protein is biotinylated and captured via a streptavidin solid phase, while the prey protein is tagged with beta-lactamase and a sequence to promote pentamerization to increase its concentration. The isolated bait and prey proteins are then exposed to each other in an ELISA-type system in order to detect directly binary interactions [88].

Other biophysical methods that are employed for the study of PPIs include but are not limited to biosensor analysis, size exclusion chromatography, gel electrophoresis (SDS-PAGE, BN-PAGE, CN-PAGE), scattering techniques, nuclear magnetic resonance, X-ray crystallography, single particle electron microscopy, peptide array technologies and spectroscopy [2151].

In-silico Databases—Small scale experiments at the bench are highly informative and have traditionally represented the backbone of biochemical research. However, there has been an increasing recognition over the past few decades for the need of systems biology approaches to understand biological networks and generate knowledge on a larger scale. Examples of this approach include multidisciplinary projects such as the RAS initiative, which was established in 2013 to explore ways of identifying the mechanisms of proteins encoded by RAS genes and develop novel and effective therapies for RAS-related cancers [8990], and the human genome project [91]. These projects require collaboration between various scientific disciplines including mathematical modeling, cell biology and bioinformatics [91]. For PPIs there is a critical need is to develop a comprehensive map of dynamic protein interaction networks. To this end a number of both *in silico* and *in vitro* tools have been developed by researchers. With the number of publications on PPIs steadily increasing over the last two decades there has also been a shift from molecular biology and biochemical methods such as yeast two hybrid, co-immunoprecipitation and affinity-precipitation to computational methods used to predict PPIs in a high throughput fashion [21]. However, with the advantages of these innovations also come challenges such as a greater need for confirmation and validation of PPIs in order to avoid both false positive and negative results. In this regard, there is still a vital necessity for the genetic, molecular and biochemical methods used in the past to validate PPIs [21]. This need arises from the fact that although many PPIs are reported in various databases, far fewer have been experimentally validated, which should always be the next step for PPI that are based on *in silico* prediction or otherwise computationally derived.

There are a number of practical challenges that arise from the vast amount of protein interaction data that has been created to date including the problem of how to organize this data into structured databases. While individual studies generally report their findings in the form of journal articles, this free-form text creates a lot of heterogeneity. These data must be gathered and converted into accessible interaction information with the appropriate metadata (e.g. experimental description in a standardized format). The key challenges in accomplishing this have been identified as: Describing experiments in a controlled vocabulary; Vast amounts of curated data must be made easily accessible; and Proteins must be unambiguously identified. The first task was addressed by the Human Proteome Organization Proteomics Standard Initiative (HUPO-PSI), who created a common controlled vocabulary for experimental techniques, molecular features, interaction types [92], and XML and tab-delimited formats [93]. The majority of databases, including the ones discussed here, have adopted this vocabulary and data format. The second task of making PPI data readily accessible was first addressed by the DIP [94] and BIND [95] databases, which initially focused on the yeast and human entries, but have since expanded to include all of the major model organisms. Today there are more than 100 PPI containing databases available online [96], all featuring various differences with regard to experimental detail, data acquisition (e.g. by manual curation or automatic text-mining approaches), information source, species and other distinguishing factors. One way to bridge all of these resources, to obtain the most comprehensive PPI data on a given query, is through a platform called PSICQUIC that enables multiple PPI databases to be searched with the same query and provides merged results by using a clustering algorithm based on primary identifiers [97]. The third task of

unambiguously identifying proteins still presents a challenge to PPI databases, which for the most part use UniProtKB protein identifiers, to represent anything from peptides to proteins and their isoforms. However, some databases employ other protein identifiers, e. g. Ensemble, Entrez, RefSeq as well as species-specific identifiers. The heterogeneity and inconsistency in such nomenclature complicates efforts of mapping PPIs between the different protein identifiers [25].

In a systems biology approach to PPI discovery, the focus is shifted from investigating individual PPIs to looking at dynamic protein interaction networks within organisms [91]. To this end a number of online databases have been created for the *in silico* detection of PPIs using computational methods, relying on available structural information of proteins [98], genomic data, known PPI networks or sequence information [99]. Some of the in silico databases which provide high quality data on experimentally determined interactions between proteins, protein complexes and pathways include: database of interacting proteins (DIP), Biomolecular Interaction Network Database (BIND), Biological General Repository for Interaction Datasets (BioGRID), Munich Information center for Protein Sequences (MIPS), IntAct and Molecular Interaction Database (MINT). The advantages of these databases are the availability of relationship information between protein interactions and domain architecture along with various tools and resources for PPI analysis [51]. Some of the limitations of these databases include the inability to distinguish between various protein isoforms, interacting proteins may be components of a larger complex and it is not known whether the interactions between proteins are direct [51].

The aforementioned databases: DIP, BIND, BioGRID, IntAct, MINT and MIPS comprise *in silico* experimental databases, containing high quality data on experimentally derived protein-protein interactions, complexes and pathways. Like the other databases MINT is still incomplete, however at present holds 117,001 interactions from 24,421 interactors in 607 organisms. Entries include PPIs and interactions between any other molecular entities including protein, nucleic acid, lipid and artificial. Although the majority of entries are binary PPIs, MINT was designed to store other types of functional interactions like enzymatic modifications, direct and indirect relationships and information on kinetic and binding constants. The entries are expert curated and also gathered with help of text mining software “MINT Assistant”, which is still limited by heterogeneity in language used in the scientific literature with regard to protein names [100]. This database also provides confidence scores of experimentally detected PPIs which aide in ensuring reliability of the interactions [99].

IntAct is an open source database and toolkit that was designed for the storage, presentation and analysis of PPIs with the aim of creating a platform to reduce duplication of work and counter incompatibility between different protein datasets by providing the same infrastructure and annotation system. Currently the IntAct database contains 862,676 binary interactions and 572,063 interactions from 107,900 interactors including information on DNA, RNA and small molecule interactions [99], all extracted from the scientific literature and curated with the Swiss-Prot team. This ensures consistency as data attributes like experimental methods must be annotated, intensive use of controlled vocabulary and the use of existing reference systems such as NCBI taxonomy database or Gene Ontology (GO) [101].

The MIPS database contains systematically organized genome-related information from both automatically generated and manually annotated genome-specific databases. It also contains systematic classification schemes for the functional annotation of protein sequences and tools for their analysis. MIPS draws upon data from the yeast genome (CYGD), *Neurospora crassa* genome (MNCDB), PENDANT genomes, annotated human EST clusters (HIB), cDNAs from German Human Genome Project (DHGP), Genome Analysis in Plants (GABI), Helmohotlz-Netzwerk Bioinformatik (HNB), *Arabidopsis thaliana* database (MATDB), database of mitochondrial proteins and International Protein Sequence Database (PIR) [102].

DIP seeks to integrate diverse experimental data from PPI studies into a single database, where quality assessment is performed to identify the most reliable interactions. Initially the platform was designed to store binary PPI data however now is capable of storing multi-protein complexes. DIP documents experimentally derived PPIs and provides integrated tools for browsing, extracting and visualizing information about protein interaction networks. The information provided includes proteins participating in interactions, protein localization and cellular function, information on interacting domains, amino acids required for the interaction, experimental techniques and the published source of the experimental data. Experiments are annotated using a list of controlled vocabulary terms. Further information on the topology and protein location are collected in order to infer the type of interaction [94]. Historically the vast majority of entries in the database are from high throughput experiments in microorganisms [100]. The data is obtained mainly through automated searches using a strict set of keywords in a data-mining strategy or through the transfer of a known interaction between a pair of proteins to the homologs of the interacting proteins [103]. DIP also draws on data from other databases including the Yeast Protein Database (YPD), EcoCyc, FlyNet and Kyoto Encyclopedia of Genes and Genomes (KEGG) [99]. Data from high throughput technologies such as large-scale yeast two hybrid screens, protein microarrays, and mass spectrometry analysis have shown little overlap with small scale experiments, calling into question the reliability of high throughput methods [104]. DIP addresses this discrepancy by attempting to assess the quality of the data based on reliability of individual experimental methods and analysis of the patterns of interactions between analogous proteins among others [94]. Currently there are 28,826 protein entries comprising 81,762 PPIs in the database with proteins mostly from *Saccharomyces cerevisiae, Drosophila melanogaster, Escherichia coli, Caenorhabditis elegans, Mus musculus and Homo sapiens*.

BioGRID is one of the most comprehensive open access databases for genetic and protein interactions of all major model organisms and human and is capable of storing complex multi-gene/protein interactions. Protein data on the effect of posttranslational modifications such as phosphorylation and ubiquitination are also captured. The data is manually curated from primary biomedical literature and coordinated through an interaction management system (IMS), which serves to compile interaction records through structured evidence codes, phenotype ontologies and gene annotation. Semi-automated text-mining approaches aide in this process as well. BioGRID aims to ensure usage of unambiguous and appropriate gene identifiers while providing proper data representation of high throughput datasets particularly in the case of quantitative datasets. Some of the quality control measures

employed include automated random recuration of datasets to ensure consistency and supplying information about the experimental methods for interaction detection [10599]. Additionally, BioGRID performs themed curation for topics areas of particular interest to the field of biomedical science such as interactions associated with the ubiquitin-proteasome system [105]. Currently BioGRID holds 1,623,645 protein and genetic interactions and 726,378 posttranslational modifications from all major model organism species.

Pathguide is an *in silico* meta-database which provides information and resources for the study of molecular interactions, biological pathways, signaling pathways, transcription factor targets, gene regulatory networks, genetic interactions, protein compound interactions and protein-protein interactions [5195]. Pathguide contains a large variety of PPI related data however the listed databases mainly store pairwise interactions and like many other databases the information is biased towards proteins, pathways and interactions that are deemed of interests to the biological community rather than covering a representative spectrum of the available interaction and pathway data [95].

String is an *in silico* prediction database that is used for the retrieval of gene and protein interactions. The database contains data on known and predicted PPIs with the stated aim of both integrating critically assessing physical and functional protein associations [5198]. Among databases that integrate both known and predicted interactions STRING focuses on interaction confidence scoring and comprehensive coverage protein numbers, prediction methods and organisms. Currently it contains information on more than 2000 organisms with algorithms capable of transferring interaction information between these organisms. This is accomplished by instating hierarchical and self-consistent orthology annotations for interacting proteins and establishing protein groupings at various levels of the phylogenetic tree. STRING derives the information to predict the basic interaction unit between two proteins which is a specific and productive functional relationship from five main sources: known experimental interactions, from manually curated databases, automated text-mining for statistical and semantic associations between proteins, algorithms predicting de novo interactions based on genomic information and observed interactions that are systematically transferred to other organisms. Some of the limitations of STRING include the fact that alternative splice isoforms and posttranslationally modified proteins are not resolved and the database like many others does not perform well in predicting transient interactions [5198].

PrePPI is a structure driven database of protein-protein interactions that uses three-dimensional structural information to predict PPIs. The database combines structural information with nonstructural information such as essentiality of the proteins in the interaction, co-expression level, gene ontology functional similarity, MIPS functional similarity and phylogenetic profile similarity. The combination of both structural and non-structural approaches has proven to perform better than either alone based on the yield of identified interactions in a high confidence data set. Interestingly, many of the interactions identified by PrePPI differed from those determined by high-throughput methods, indicating that the use of three-dimensional structural information effectively expands the coverage of PPIs. It is important to note that this method is not constructing three dimensional models but rather bases an interaction model off of structure-based sequence alignment of the two query proteins. Advantages of this technique are the integration of both predicted and

experimentally obtained PPIs, however the PPI coverage in this database as in many others is sparse and may pose a challenge in investigating a protein of interest [106].

The discussion of databases here is by no means comprehensive and for the most part merely scratches the surface of primary databases (e.g. Human Protein Reference Database (HPRD)) containing PPI experimental data that is manually curated and/or captured by various text-mining approaches from both large high throughput and small-scale experiments. Several meta-databases contain PPI experimental datasets that are integrated and unified from various public repositories e.g. Agile Protein Interaction Data Analyzer (APID), Microbial Protein Interaction Database (MPIDB), Protein Interaction Network Analysis platform (PINA). Prediction databases house PPI experimental and predicted data on protein functional interactions and interactions derived from various types of data, examples of such databases are Michigan Molecular Interactions (MiMI), Human PPI Prediction database (PIPs), Online Predicted Human Interaction Database (OPHID), Unified Human Interactome (UniHI), and STRING.

Post-translational Modifications (PTMs)

Introduction

Posttranslational modifications (PTM) are enzyme-catalyzed modifications in the form of functional groups that are mostly reversibly added to the protein backbone at any time after protein biosynthesis. The innumerable structure changes possible in a modified protein compared to the nascent protein reflect the multitude in changes possible to the functionality of that protein [107]. PTMs have a wide array of functions; they can alter protein localization, cause protein-protein interactions to form or be prevented or activate and inactivate a protein. PTMs massively increase the complexity of organismal proteomes and play a role in almost all aspects of cell biology, allowing for fine-tuning of protein structure, function and localization. There are an estimated 300 PTMS with a predicted 5% of the eukaryotic genome coding for enzymes involved in protein modification [108], largely conserved across species with phosphorylation, glycosylation, acetylation, methylation, and ubiquitination being the most studied [109]. It is possible that there remain a large number biologically relevant PTMs, which play a role in disease pathogenesis however have not been detected due to limitations in sample preparation, analytical techniques, data analysis, and the substoichiometric and transient nature of some PTMs. The enzymes responsible for catalyzing PTMs (kinases, acetyl-transferases, ubiquitin ligases and methyl-transferases etc.) are under tight but dynamic transcriptional regulation and are often times themselves regulated by other PTMs, leading to a complex network of PTMs interacting with each other. PTMs may also be created non-enzymatically by covalent cleavage of the peptide backbone (i.e. proteolysis) in the case of glycation and oxidation.

The transient and often substoichiometric nature of PTM’s, meaning that they occur on some but not all molecules of a protein, make them difficult to detect and study. This makes detection of PTMs by immunoblot or unenriched proteomics approaches difficult and often enrichment is required to detect these modifications (e.g. [110111]). Improvements in proteomic and mass spectrometry methods, as well as sample preparation, have been exploited in a large number of proteome-wide surveys of PTMs in many different organisms

(reviewed in: [112113114]). The literature reveals that there a multitude of techniques used to investigate PTMs, however the most employed and advanced techniques are MS-based proteomics, which when paired with in vitro or in vivo biological assays uncover relevant biological function [115116].

The Apicomplexa are a phylum of obligate intracellular pathogenic protozoa that cause significant morbidity and mortality in animals and humans. This phylum includes among many others the etiologic agents of malaria (Plasmodium spp.) and toxoplasmosis (Toxoplasma gondii). Apicomplexan parasites undergo complex life cycles involving several stages, morphologies and host cells. One of the mechanisms by which parasites likely regulate responses to extracellular stimuli and life cycle transitions is through post-translational modifications (PTMs). Common PTMs found in the Apicomplexa and elaborated upon in more detail are phosphorylation, methylation, acetylation, palmitoylation and ubiquitination.

Alongside studies of the impact of PTM on individual proteins, several proteome-wide studies of PTMs have surveyed specific modifications in Apicomplexan parasites, mostly in *T. gondii* and *P. falciparum* (summarized in Table 1).

Phosphorylation

One of the most common PTMs is phosphorylatio, which occurs with the transfer of a phosphoryl group from adenosine triphosphate (ATP) or guanosine triphosphate (GTP) to primarily serine (86.4 %), threonine (11.8 %), and tyrosine (1.8 %) residues through formation of a phosphoester bond [115117]. However, phosphorylation of histidine, aspartate, and arginine has also been observed [118119]. Genomic sequencing indicates that 2–3% of eukaryotic genes code for protein kinases [120121]. Kinases are the enzymes that mediate phosphorylation, of which there are 500 kinase-coding genes in human as compared to a repertoire of more than 100 identified in the Apicomplexa [122123124125126], which releases orthophosphate and is catalyzed by enzymes known as phosphatases [127106], of which more than 100 are encoded in the human genome [128]. Phosphorylation plays a role in a multitude of cellular processes including protein synthesis, protein degradation, inter/intracellular signaling, transcriptional and translational regulation, cell survival, apoptosis, metabolism, homeostasis and differentiation among many others [129130120]. Phosphorylation is an important reversible modification as it commonly acts as an on/off switch for biological processes and is estimated to at some point modify up to 30% of all expressed proteins in the eukaryotic cell [131]. The aberrant phosphorylation status of proteins has been associated with defects in protein function.

Analysis of phosphorylated proteins is challenging due to a number of reasons including the target proteins generally being low abundance and phosphorylation being substoichiometric, challenges in selectively isolating phosphopeptides and the unique chemistry of each phosphorylated residue [132]. These properties are leveraged in chemical methods of phosphopeptide detection such as beta-elimination of the phosphate group on phosphoserine and phosphothreonine, leading to double bond formation, capable of reacting with nucleophilic groups used to isolate the phosphopeptide [133134135]. Other chemical methods involve linking phosphoserine, phosphotyrosine and phosphothreonine to a solid phase

support by phosphoramidate chemistry (PAC) [136], amino-derivatized dendrimer [132] or controlled pore glass derivatized with maleimide [137]

The investigation of this PTM usually calls for highly selective and efficient techniques for enriching phosphorylated proteins and subsequent identification of phosphorylation sites. There are a number of approaches that have been traditionally employed to study phosphoproteins including immunoprecipitation using serine/threonine- or tyrosine-specific antibodies [138139] separation of proteins using two-dimensional gel electrophoresis according to their isoelectric point and molecular mass and detection by autoradiography in proteins that have been labeled with ^{32}P [140]. Other methods for phosphopeptide recognition include peptide mapping, post-source decay, precursor ion scan, neutral loss scan, stepped skimmer potential, 31P detection and for phosphorylated residue identification include collision-induced dissociation, post-source decay, in-source decay and electron-capture dissociation [141]. These MS strategies share the same general work flow of enzymatically digesting proteins into peptides, which are then either first enriched or directly analyzed by MS.

Phosphorylation is identified based on molecular mass of phosphopeptides which show an increase of an 80 Da mass on MS, corresponding to the addition of a phosphate (HPO_3) group as compared to the mass of the predicted amino acid sequence. Currently, widely available MS/MS is capable of direct sequencing of the peptide for verification of phosphorylation. In addition, this technique is able to provide the exact phosphorylation site when the phosphopeptide is fragmented to produce fragment ions. This is known as collision-induced dissociation (CID) and may produce inadequate fragmentation of the peptide backbone, only causing loss of a phosphoric acid and limited sequence information when low-energy collisional activation of phosphorylated peptides takes place. However, the fragment ion created in this process may be subjected to multistage MS/MS to further fragment it in a second round of CID [142]. In an alternative MS approach called electron capture/transfer dissociation (ECD/ETD) the peptide backbone is fragmented to yield fragment ions without loss of the phosphate group. Other methods include mass spectrometric parent ion scanning in negative mode to identify diagnostic fragment ions which have an m/z of 79 for PO_3 and neutral loss scanning used to detect phosphorylated peptides in LC-MS by continuously collecting mass spectra with or without CID which produced mas spectra of both phosphorylated and dephosphorylated peptides [143144].

Even with these techniques it is extremely difficult to analyze phosphorylated peptides in a complex biological sample containing un-phosphorylated peptides. In order to facilitate the detection of phosphopeptides there are selectively separated before MS/MS using various techniques such as strong anion/cation exchange [145] e.g. LC-MS/MS. In strong cation exchange chromatography (SCX), phosphopeptides contain an additional negative charge at acidic pH of 2.7 and have a reduced retention on the negatively-charged stationary phase and therefore can be easily separated from non-phosphorylated peptides [145]. Other strategies focus on phosphopeptide enrichment following fractionation as in the case of immobilized metal ion affinity chromatography (IMAC). This method significantly improves identification of phosphopeptides by purifying them based on their affinity for Fe^{3+} and Ga^{3+} [146142]. One drawback of this method is that unphosphorylated peptides with multiple acidic residues have affinity for Fe^{3+} and Ga^{3+} and co-purify with the phosphopeptides.

Even though the affinity of the acidic residues for metal ions can be reduced by blocking them with O-methyl-esterification [147], this reaction is not 100% efficient and leads to an increase in the complexity of the sample by increasing partial deamidation and subsequent methylation of this moiety. These changes complicate peptide separation and affect MS analysis and interpretation [148]. Other studies have observed a tendency of this method to isolate more double phosphorylated peptides [147146].

An alternative to IMAC is phosphopeptide enrichment by titanium dioxide chromatography (TiO_2) before MS analysis [149]. Some methods of TiO_2 also require O-methylation, however other methods have found ways of modifying the TiO_2 micro-column such as with 2,5-dihydroxybenzoic acid (DHB) or phthalic acid in acetonitrile to increase the affinity for phosphopeptides [150].

SCX can be combined with IMAC or TiO_2 for improved enrichment of phosphopeptides [151] and this approach has proven to be most comprehensive for mapping the phosphoproteome [117149152]. In a study by Villen et al IMAC and TiO_2 were combined and shown to identify around 2,000 and 3,000 phosphorylation sites from *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, respectively [153154]. The same group has also previously identified more than 5,500 phosphorylation sites in mouse liver and over 13,000 in fly embryos by combination of an SCX and IMAC protocol [155156]. IMAC and TiO_2 produce incomplete and partially overlapping phosphoproteomes such that neither method by itself provides a complete global phosphoproteome capture [132]. LC-MS/MS is not a reliable means of global phosphoproteome mapping because only a fraction of the peptides sampled and analyzed in each run [157]. Detected differences may represent true differences in phosphorylation status or merely differences in sampling by the mass spectrometer [132].

There are also antibody-based strategies using antibodies raised against phosphorylation motifs such as by Pease et al who used a phosphor-tyrosine monoclonal antibody (P-Tyr-100) to enrich for phosphopeptides in *Plasmodium falciparum* [158]. SCX has also been combined with pTyr antibodies, IMAC or TiO2 to reduce sample complexity and increase coverage.

To date no complete phosphoproteome from a single cell line has been mapped. Even with rapid and deep whole proteome level characterization on the horizon, comprehensive PTM-level characterization is likely years if not a decade away [151]. Thus there is no gold standard for benchmarking and therefore it is difficult to know how well any one method performs in mapping phosphoproteins as the true distribution over the proteome is not currently known [132]. Phosphorylation is the most prevalent PTM in both *T. gondii* and *P. falciparum*, covering over 30% of the predicted proteomes [159160] (see Table 1).

Ubiquitination

Ubiquitination involves the attachment of a 76-amino acid protein to a lysine residue of a protein, playing key roles in protein turnover, cellular signaling, intracellular transport, protein-protein interactions, transcriptional regulation and most notably in targeting proteins to the proteasome for degradation. It is one of the main mechanisms by which protein

homeostasis, cell cycle progression, gene transcription, receptor transport, and immune responses

are controlled [161]. Ubiquitination is a complex process that requires the action of three different enzymes: ubiquitin-activating enzyme E1, ubiquitin-conjugating enzyme E2, and ubiquitin-ligating enzyme E3. E1 transfers ubiquitin to its active cysteine site through a thioester bond formation in an ATP-dependent process, followed by ubiquitin conjugation to the active cysteine site of E2 and thus allowing E3 to attach it to a lysine residue on a particular protein through thioesterification [162]. De-ubiquitinating enzymes (DUBs) can remove the covalently attached ubiquitin, making this PTM reversible [163164]. Ubiquitination was detected as being similarly abundant, on up to 5% of the *T. gondii* or P. falciparum predicted proteomes [165166]. Its role in *T. gondii* was investigated only recently on a proteome-wide level by [165]. Through this survey of the *T. gondii* ubiquitinome we found that Ub-proteins are highly enriched for gene ontology (GO) terms related to structural function, ribosomal components and dimerization. Further proteins involved in vesicular trafficking, ion transport and translation were found to be ubiquitinated, which in the case of translation points to ubiquitin's role of removing defective ribosomes and limiting ribosome availability [167].

Some of the techniques used to identify ubiquitination include site-directed mutagenesis and affinity purification-mass spectrometry (AP-MS). One diagnostic approach to ubiquitinated endogenous proteins is protein-level immunoprecipitation with subsequent Western blot analysis using an anti-ubiquitin antibody. However one of the challenges of this approach is the short-lived nature of ubiquitinated proteins, which can be overcome by creating recombinant target protein that is overexpressed in cells and treating cells with proteasomal or lysosomal inhibitors to prevent endoplasmic reticulum-associated destruction (ERAD) [168]. In site-directed mutagenesis lysine residues are substituted with arginines, incapable of being ubiquitinated, followed by the mutated protein undergoing immunoprecipitation and subsequent Western blot analysis. This approach faces challenges associated with the size of the target protein and the number of lysines contained within it. Functional redundancy may also lead to alternative lysine being modified if the preferred site is mutated [168].

During the ubiquitination process a 76-amino acid ubiquitin residue is covalently attached by its C-terminal di-glycine (K-GG) to a lysine on the modified protein. This fact has been leveraged to perform peptide-level immunoaffinity enrichment on K-GG modified peptides to identify Ub-modified proteins. In AP-MS a typical gel-based method for endogenous proteins includes protein-level immunoprecipitation, protein separation by SDS-PAGE, excision of the bands of interest, in-gel tryptic digestion, which generates di-glycine remnants attached to the ubiquitinated lysine residue. The residue has a characteristic mass of +114.0429 Da and can accurately detected by MS/MS. Anania et al observed that K-GG peptide immunoaffinity enrichment consistently outperformed protein level AP-MS by producing a greater protein yield. The same group went on to show by SILAC that more than fourfold higher levels of modified peptides were obtained by K-GG peptide immunoaffinity than by AP-MS [168]. Other advantages of this approach include greater depth of coverage and information regarding the ubiquitination status of proteins other than the protein of interest [168]. However, with any gel-based method that involves multiple steps

there is a potential for sample losses and recovery of tryptic peptides from polyacrylamide gel is variable which can lower the yield. Further cell lysis often involves lysis buffers that are only partially denaturing, subjecting the lysate to deubiquitinases and proteases, capable of modifying proteins [168].

Palmitoylation

Another PTM that may act as an on/off switch for protein function (including localization) is palmitoylation. The addition of a 16-carbon palmitic acid to a cysteine residue via a labile acyl-thioester linkage [169], i.e. palmitoylation, has been shown to play an important role in regulating how proteins associate with membranes and lipid rafts by increasing hydrophobicity. Many G-proteins have been shown to have palmitoylation-dependent membrane localization such as H- and N- Ras, Rho and the alpha subunits of most heterotrimeric G proteins. Additionally, many ion channels, receptors, cytoskeletal proteins, kinases and signaling proteins, some believed to play roles in cancer and synaptic signaling have been found to be palmitoylated [170171]. Several *T. gondii* proteins display palmitoylation-dependent localization e.g. IMC-sub compartment proteins (ISPs) [172] and TgHSP20 [173] home to the IMC. Proteins localizing to the rhoptries and other organelles of the invasion machinery are also palmitoylated, implicating this PTM in invasion in *T. gondii* [174]. In a comprehensive analysis of palmitoylated proteins in *T. gondii* by a metabolic labeling approach, Foe et al. confirmed palmitoylation of motor and glideosome-associated protein (MLC1, MyoA, GAP45, GAP40, GAP50, GAP70, GAP80), providing evidence that the glideosome (a structure required for motility in *T. gondii*) is heavily palmitoylated [175]. In Apicomplexa the few proteome-wide surveys of palmitoylation that have been conducted use one of two (and sometimes both) fundamentally different approaches to global purification of palmitoyl proteins– acyl-biotin exchange (ABE) and metabolic labeling with palmitic acid analog followed by click chemistry (MLCC).

ABE was initially described in a global analysis of palmitoylation in *Saccharomyces cerevisiae* [176]. This method exchanges a thioester-linked palmitoyl group for biotin which can then be used to specifically affinity-purify the previously palmitoylated protein. In performing ABE the whole proteome is extracted and treated with N-ethylmaleamide (NEM), which permanently blocks free thiol groups on unmodified cysteines in order to prevent erroneous labeling with biotin. Hydroxylamine is then used to cleave thioester bonds releasing thioester-linked palmitoyl groups restoring modified cysteines to thiols. The remaining thiol group is then covalently bound to sulfhydryl-reactive biotinylation reagent Biotin-HPDP-N-[6-(Biotinamido)hexyl]-3’-(2’-pyridyldithio)propionamide (HPDP-biotin), which forms a reversible disulfide linkage. The advantages of using HPDP-biotin is that disulfide linkages that are formed between avidin and substrate can later by cleaved by a reducing agent, like dithiothreitol or beta-mercaptoethanol-mediated cleavage of the biotin-cysteine linkage [171]. Protein preparations equal in quantity that are not hydroxylamine treated serve as a control, as their palmitoyl groups are not exchanged for biotin and should not purify.

In MLCC, an orthogonal method of palmitoylome purification, cells are metabolically labeled with 17-octadecynoic acid (17-ODYA), which is a palmitic acid analog that contains

an alkyne group. Once 17-ODYA labeling has occurred, the proteome is extracted from the cells and the labeled protein are permanently biotinylated with biotin-azide and copper (I)-catalyzed azide-alkyne cycloaddition (click chemistry). The 17-ODYA and biotin labeled proteins are then purified using streptavidin-agarose affinity purification [170].

Although robust and validated both of these techniques suffer from their own specific limitations. Since ABE requires complete blockage of thiols by NEM, high abundance proteins in which only partial blockage of thiols occurs may erroneously produce enrichment. Further enzymes which use thioester-linked acyl intermediates in their reaction mechanism (as in the case of ubiquitin ligase) may be purified [177]. Martin & Cravatt were the first to use biorthogonal labeling to survey global palmitoylation in human cells using 17-ODYA, identifying many previously verified palmitoylated proteins but also many putative novel palmitoylation sites. Th2is for one validated the method in human but also brings up the issue of false positive detection, which is a given in any large-scale mass spectrometry-based assay. MLCC, which is reliant on metabolic labeling with 17-ODYA, may enrich non-palmitoyl proteins that have enriched palmitic acid analog erroneously or other non-palmitoyl proteins which contain palmitoyl linked structures such as GPI anchors which contain palmitoyl-linked inositol ring and thus incorporates 17-ODYA [178].

Martin & Cravatt were the first to use biorthogonal labeling to survey global palmitoylation in human cells using 17-ODYA, identifying many previously verified palmitoylated proteins but also many putative novel palmitoylation sites. This for one validated the method in human but also raised the issue of false positive detection, which is a given in any large-scale mass spectrometry-based assays especially when dealing with low-abundance signals. Some of the ways in which they addressed the drawbacks of the method were by using the sum of the number of tandem mass spectrometry spectra assigned to a specific protein, known as spectral counts, to estimate protein amounts in each sample [170]. Spectral counts are highly correlated with protein abundance [179]. Further they used a simple gel-based method to rapidly validate palmitoylated proteins by click chemistry conjugation of 17-ODYA labeled proteins to rhodamine-azide. The rhodamine-labeled proteins can then be visualized by fluorescence scanning in a high throughput fashion that is simpler and faster than LC-MS [170].

In a study investigating the palmitoylome in *P. falciparum* Jones et al for the first time used MLCC in conjunction with ABE and found that both methods on their own reliably purify palmitoyl proteins and produce a wide range of enrichment values across the proteome when paired with SILAC [180]. However, these techniques do not isolate congruently overlapping protein datasets, with ABE capable of theoretically capturing the entire palmitome, and MLCC due to its reliance on metabolic labeling only capturing proteins palmitoylated at the time of labeling, producing a far more dynamic snapshot of the palmitome [180]. The difference in these two purification methods is likely reflected in two palmitome surveys conducted in *T. gondii*, one purified using ABE approach identified 401 *T. gondii* palmitoylated proteins, representing 4.80% coverage of the entire proteome [174], while the other study used MLCC and only identified 282 proteins, representing 3.40% of the proteome [175].

Glycosylation

Glycosylation is the most prevalent protein modification making up approximately 50 % of modified proteins in the human proteome [181]. Enzymes called glycosyltransferases recognize specific protein motifs and then transfer the first monosaccharide (or preformed oligosaccharide for N-glycosylation) onto the recognition site. Other glycosyltransferases (and glycosidases for N-glycosylation) then sequentially elongate the glycan sequence. Glycosylation of proteins play a major role in the proper folding and stabilization of proteins as well as in cell–cell adhesion and communication (Spiro 2002). In some cases of cancer and infectious diseases, deficient or absent glycosylation has been proposed as a possible leading cause of these diseases [182]. Moreover, numerous drugs on the market are glycoproteins that have well- characterized glycan entities necessary for their function, efficacy, and safety [183].

Of the two forms of glycosylation (N- and O-glycosylation), N-glycosylation is the most common. O-linked glycosylation occurs at the hydroxyl oxygen of serine, threonine, tyrosine, hydroxylysine, or hydroxyproline side chains of extracellular, nuclear, and cytoplasmic proteins [184185186187188]. O-GlcNAcylation is a dynamic and abundant PTM, known to occur in the cytosolic, nuclear and more recently in the mitochondrial compartments of eukaryotes [189]. O-GlcNAc is catalyzed and removed by O-GlcNAc transferase (OGT) and O-GlcNAcase, respectively. OGT genes have been found in protists e.g. *Giardia*, *Cryptosporidium*, *Toxoplasma*, and *Dictyostelium*, confirming the presence of active OGT enzymes these eukaryotes [190]. O-GlcNacylation was shown in *T. gondii* for the first time by Perez-Cervera et al. using antibodies against the PTM and numerous high molecular weight (above 130 kDa) O-GlcNAc modified proteins were found [191]. A proteomic analysis of the *T. gondii* glycoproteome confirmed the abundance of both O-linked and N-linked glycoproteins with numerous modified proteins found in surface proteins, microneme proteins, rhoptry proteins, heat shock proteins and hypothetical proteins [192]. Although not much is known about the functional consequences of glycosylation on *T. gondii* proteins, tunicamycin-treated parasites were found to have defects in invasion and motility with TgGAP50 and TgMyoA implicated in these defects, suggesting a role for glycosylation in glideosome function [193194]. Studies have using lectin affinity chromatography and identified over a hundred glycosylated proteins in *T. gondii* [192195]. Overall, these proteomic surveys indicate that PTMs are abundant in the Apicomplexa and are found on proteins involved in many critical biological pathways.

Glycation

Glycation refers to the nonenzymatic addition of sugar aldehyde or ketone to the Ɛ-amino group of lysines or the N-terminal amino group of proteins. The glycan reaction, also known as the Maillard reaction, leads to the irreversible formation of advanced glycation end products (AGE), which can cross-link proteins thus rendering them detergent insoluble and protease resistant [196197].

Arginine methylation

Arginine methylation is a common posttranslational modification found mostly on lysine and arginine residues and to a lesser extent on histidine, cysteine, aspartic acid, glutamic

acid, serine, and threonine [198199] amino acids of nuclear and cytoplasmic proteins. Arginine methylation has roles in epigenetic and transcriptional regulation, RNA processing, metabolism, signal transduction and DNA repair [200199201]. Arginine methylation is catalyzed by a family of Protein Arginine Methyltransferases (PRMT), capable of transferring one or more methyl groups from S -adenosyl-methionine (SAM) to arginine to form mono-di- and tri-methyl arginine groups [202]. Individual PRMTs differ significantly in terms of their biochemical properties and substrate specificities [203], suggesting that they have non-redundant functions.

In the past, studies have focused mainly on arginine methylation of histones [204], however this posttranslational modification (PTM) also occurs on a large number of non-histone proteins that have diverse functions. In *T. gondii*, arginine methylation has been implicated in transcriptional regulation and splicing biology [205]. Other studies found arginine methylation of transcription factors can inhibit their degradation by preventing phosphorylation events required for ubiquitin-mediated destruction [206], pointing toward a high degree PTM crosstalk in the Apicomplexa. In a recently published study we obtained an arginine monomethylome from *T. gondii* by immunoprecipitation and MS/MS using two distinct commercially-available methyl arginine antibodies– one against a single methyl-modified arginine (Me-R4–100) and the other against a motif-specific methyl-modified arginine in a glycine-arginine rich environment (R*GG) in order to reflect previous work indicating that arginine methylation preferentially took place in the setting of these GAR motifs [205]. Further this study employed a sequential PTM enrichment workflow, wherein MMA peptides from one data set were purified from the flow-through samples that had been depleted of ubiquitinated peptides in another experiment [165]. A similar yield of MMA peptides was obtained with intracellular parasite flow-through as from other samples used in the monomethyl arginine (MMA) study prepared from whole cell lysates, indicating that in the case of *T. gondii*, there is little concordant proteome wide modification of these two PTMs. Thus we were able to leverage this finding to more efficiently utilize samples to detect two proteome wide sets of PTMS. In addition, in error tolerant searches of the ubiquitin data sets (Mascot from Matrix Science, version 2.5.1) we did not detect any MMA sites, indicating that few, if any, MMA peptides were lost in the preceding ubiquitin enrichment step [205]. The MMA proteome was found to cover almost 5% of the *T. gondii* proteome (Yakubu et al., 2017).

Lysine [207] and arginine methylome studies have been reported in P. falciparum, using anti-monomethylarginine and anti-dimethylarginine cross-linked antibodies to detect mono-/di-/tri-methyl arginine in the context of RGG, RGx, RxG, GxR and WxxxR motifs [208].

Acetylation

Acetylation has been found to be involved in numerous cellular functions such as mRNA translation, metabolism, DNA packaging, the cytoskeletal system and protein folding. Acetylation is catalyzed by histone acetyltransferases (HATs), which transfer the acetyl group from acetyl co-enzyme A to the amino group of lysine at the N-terminus of histones to form 3- N -acetyl lysine. This reaction can be reversed by the action of histone deacetylases (HDACs) (chapter;96). Both, HATs and HDACs are potential drug targets for

many disorders such as obesity, cancer, and neurodegenerative disorders [209210183211]. Similar to methylation, acetylation is a dynamic and reversible PTM affecting protein stability, localization, activity and protein-protein interactions [212]. Acetylation has been predominantly studied as a histone modification. It was previously thought to take place only on histones but has now been reported on transcription factors and other nuclear regulatory molecules, implicating it mainly in transcription and metabolic regulation [213214]. It has also been found on non-histone substrates in proteome-wide acetylation studies in *T. gondii* and predicted to play a role in mRNA translation, metabolism, DNA packaging, the cytoskeletal system and protein folding. It is a dynamic and reversible PTM affecting protein stability, localization, activity and protein-protein interactions [212]. A study in P. falciparum [215] demonstrated that acetyllysine heavily features on ApiAP2 transcriptional regulators. Acetylation influences AP2 DNA-binding capacity and is altered in response to perturbations of the acetyl-CoA/acetate pool. This suggests that acetylation is subject to metabolic regulation, thereby making it a candidate regulator of parasite sensing. Supporting this, Xue et al found that extracellular *T. gondii* are enriched in acetylated proteins involved in metabolism, translation and chromatin biology [212]. The most common means of isolating acetylated proteins or mapping the acetylome on a cellular or subcellular level in *T. gondii* and other organisms is anti-acetyl-lysine immunoprecipitation followed by ultra-/high performance liquid chromatography (UPLC/HPLC) and mass spectrometry analysis (MS/MS) for protein and amino acid sequence identification (see Table 1) [213212216]. This work flow may be preceded by stable isotope labeling with amino acids in cell culture (SILAC) in order to measure the relative ratios of acetylated amino acid of interest bearing heavy atoms (^{13}C and ^{15}N) as compared to acetylation from cultures grown in unenriched media with light amino acids [215].

SUMOylation

SUMOylation, the covalent attachment of small ubiquitin-related modifier to a lysine residue, is ubiquitously expressed in eukaryotes and is involved in a large number of cellular functions including transcription, DNA replication and repair, chromosome segregation, mitochondrial fission, ion transport and signal transduction [217]. About 1% of the *T. gondii* and P. falciparum predicted proteomes have been shown to be SUMOylated [218]. In the Apicomplexa there are only few studies investigating SUMOylation on a proteome wide level. In *Plasmodium falciparum* the SUMO orthologue was identified in a two-step process both by detection with polyclonal antibodies against synthetic peptides of the 100-amino amino acid PfSUMO and also using parasites expressing FLAG-tagged PfSUMO with similar performance of the two techniques [219], followed by LC-MS/MS for protein fractionation and sequence identification. A similar technique using only polyclonal antibody detection was used to map the SUMOylome in *T. gondii* [218] (Table 1).

Integrated Analysis of *T. gondii* PTM proteomes

Apicomplexa, such as *Plasmodium spp.* and *Toxoplasma gondii*, undergo complex life cycles involving multiple stages with distinct biology and morphologies. Posttranslational modifications (PTM), such as phosphorylation, acetylation, methylation, ubiquitination and glycosylation, regulate numerous cellular processes, playing a role in virtually every aspect of cell biology. PTMs have been implicated in functioning as key regulators of

developmental transitions, biology and pathogenesis of apicomplexan parasites. Here disucuss studies that investigate roles of PTM and the potential crosstalk between PTMs, that together have been proposed to regulate the intricate biological processes of these protozoan parasites. Numerous insights into the abundance and targets of modifications in the Apicomplexa are provided by recent proteome-wide studies of PTM. Table 1 summarizes data from a number of PTM proteomic studies conducted on members of the Apicomplexa.

Interactions Between PTM Proteomes in *T. gondii*

The studies mentioned provide abundant data supporting crosstalk between PTMs in these parasites. The combination of different PTMs on a protein is read by PTM-binding proteins and is important for protein-protein interactions. To obtain insights into the interactions between different PTM in *T. gondii*, published and unpublished proteomic data on PTMs were used to perform pairwise comparisons between PTM datasets using a statistical test of enrichment [165]. The analysis of these comparisons is presented as a heat map in Figure 2. Significant overlap between arginine monomethylated and phosphorylated proteins were identified. Phosphorylation targets are significantly enriched in several other PTM proteomes. Ubiquitination, O-GlcNAcylation and Palmitoylation proteomes are also significantly enriched in the proteins in each dataset. However, proteins that are succinylated and phosphorylated do not significantly overlap. While this analysis suggests that there is significant crosstalk between PTM proteomes in *T. gondii*, it has yet to be discovered whether PTMs harbor a large

degree of redundancy or function in a tightly organized network of PTM crosstalks. An additional caveat with these proteome-wide studies of varying degrees of proteome coverage, is the lack of information on how representative these studies are with regard to modifications on the proteins that are captured.

PTMs in *T. gondii* Metabolic Pathways

In some organisms, PTM accumulate on specific types of protein or proteins in a particular pathway. To gain an understanding of the biological targets of PTM at the pathway level, we performed enrichment analysis using gene ontology (GO) gene lists, which are gene annotations curated by function or pathway. Figure 3 depicts a heatmap of enrichment scores (p values derived from a statistical test of enrichment) of GO pathways for each set of proteins in a PTM proteome.

The heat map shows differential enrichment of PTM gene sets in GO pathways, suggesting that PTM tend to target proteins in distinct biological pathways, e.g. proteins in the ubiquitome, O-GlcNAc-ome, palmitoylome and acetylome are significantly enriched in GO pathways related to protein biosynthesis (e.g. translation) and in contrast, the succinylome, phosphoproteome, arginine monomethylome and SUMO proteome are not. Additionally, the phosphoproteome is enriched in kinase and phosphotransferase functions, as well as nuclear proteins. The role of succinylation in metabolism and mitochondrial processes has been noted previously [220221222] (reviewed in [223]). Here the succinylome is found to be enriched in GO pathways related to the tricarboxylic acid cycle, mitochondrial pathways and

metabolism. Together, these data suggest that PTMs have unique functions in *T. gondii* and target specific pathways and compartments in this organism.

PTMs in the Cell Cycle of *T. gondii*

Like other eukaryotes, *T. gondii* undergoes cell cycle, during which parasites grow and divide. *T. gondii* has an 8-hour cell cycle, proceeding with G1 phase followed by S, M and C phases. The G2 phase is either lacking or very short [224]. Mitosis in *T. gondii* is closely linked to cytokinesis, due to the organism's specialized form of division known as endodyogeny. In this process, daughter cell budding takes place within the mother cell in late S phase immediately before entering mitosis. There are distinct G1 and S/M regulated subtranscriptomes in this organism [225]. Similar to classic eukaryotic cell cycle, checkpoints assess progress of the cell through various phases of the cell cycle in *T. gondii*. There are also at least START and M-phase checkpoints, as well as a mid-G1 checkpoint [224226].

PTM typically occur in a spatiotemporal manner, and in other organisms have been identified as key cell cycle regulators. Ubiquitination, for example, is an essential regulator of checkpoint control during cell cycle; ubiquitination of checkpoint complexes enables progression of cells through cell cycle checkpoints [227]. To determine whether proteins of specific PTM proteomes are enriched for cell cycle regulated genes, Silmon de Monerri et al examined the enrichment of PTM-modified proteins in gene sets consisting of genes upregulated at different time points in G1 and S/M phase. This analysis was expanded to include proteins identified to be palmitoylated in *T. gondii* [175]. Silmon de Monerri et al demonstrated that a large number of ubiquitination targets in *T. gondii* are regulated in a cell cycle-dependent manner at the level of transcription [165]. Of the ubiquitinated proteins upregulated in G1, 35 are also present in the phosphoproteome; many of which are transcription factors and part of the replication machinery [165], which supports the cell being in an anabolic state. Figure 4A shows PTM proteomes enriched in genes upregulated at 4.5–5.5 hr and 6.5–8 hr during G1 phase. Only the phosphoproteome and arginine monomethylome are enriched in genes upregulated in mid-G1 (4.5– 5.5 hr) which probably corresponds to a mid-G1 checkpoint in *T. gondii* [224226]. The enrichment of upregulated mid-G1 proteins in the phosphoproteome and monomethylome suggests a need for increased signaling, gene regulation and protein synthesis associated with cell growth. The wave of enrichment seen at 6.5 – 8 hr, at the end of G1 phase in which all analyzed PTMs show enrichment for the upregulated genes, likely precedes or coincides with a START (G1/S) checkpoint, responsible for allowing parasites to reenter cell cycle (White et al., 2005). At this G1/S checkpoint the cell is preparing for replication and duplicates the Golgi and centrioles [228229230] indicating a commitment to DNA replication.

Proteins that are targets of ubiquitin, phosphorylation and palmitoylation are significantly enriched in genes upregulated between hours 3 and 4 of the S/M subtranscriptome. During the S/M phase, chromosome replication, mitosis and budding occur. The inner membrane complex (IMC), a parasite organelle important for cytokinesis and invasion, is central to these processes, and many IMC genes are upregulated in mid-S/M phase. Accordingly, IMC proteins are highly decorated with numerous PTM. Silmon de Monerri et al reported that several E3 ligases (ubiquitinating complexes) are upregulated at the same mid-S/M time

point [165]. This stage of *T. gondii* cell cycle is crucial for ensuring parasite integrity is maintained and mitosis occurs correctly; it is thus likely to represent an important checkpoint, associated with a proposed 1.8 N DNA content checkpoint in late S phase where chromosome replication slows or halts right before entering mitosis [224]. Although *T. gondii* encodes some eukaryotic ubiquitinating complexes, responsible for marking cell-cycle proteins and cyclins for destruction by the proteasome [231227] such as APC components [232] and SCF proteins [233]; homologues of cyclins and CDK substrates were not identified in the *T. gondii* ubiquitin proteome [165]. Other authors have suggested that *T. gondii* does not exploit classic eukaryotic cell cycle control mechanisms [224]. Over 35% of ubiquitinated proteins are cell cycle-regulated, with 63 proteins being both ubiquitinated and phosphorylated at the boundary of S/M phase, many of which are IMC and cytoskeletal proteins [165]. This suggests coordinated phosphorylation and ubiquitination in regulating transition through cell division in IMC. Together, these findings provide evidence that PTMs are coordinated throughout cell cycle in *T. gondii* and probably serve important functions in regulating cell cycle transitions.

Conclusion

The number of reported PPI has significantly increased over the past decade in part due to recognition of the role of protein-protein interactions in disease pathogenesis, but also due to the development of high throughput experimental technologies in line with the ongoing “omic” revolution. This generation of vast amounts of data poses a number of challenges including the need for improved false positive detection in PPI data and databases as well as robust methods for the distinction between PPI of biological relevance occurring in a physiologic context and those that are not relevant[3]. The importance of studying and understanding protein PTMs and PPIs in order to elucidate biological processes and develop strategies for identifying and treating pathological disorders has been demonstrated in the literature.

Despite the limitations, MS is still the best available method to identify and quantify PPIs and PTMs in complex samples. It enables identification of proteins in the femto- to picomole range and is a powerful tool for the determination of proteins involved in complex formation. Proteomic studies have identified a vast number of diverse PTMs on apicomplexan proteomes, providing a wealth of information and opportunities for future research. To date, very few PTMs have been assigned a biological function and this can be extremely challenging. Until functional studies can be performed in a high throughput manner, studies need to be performed on individual proteins. New approaches are required to fuel discovery of the numerous important biological functions PTMs are likely to have in the Apicomplexa and other organisms. An exciting avenue of future research into PTMs will be the discovery of how the complement of PTMs present in an organism interact with one another to regulate parasite biology. It is yet to be discovered whether PTMs harbor a large degree of redundancy or function in a tightly organized network of PTM crosstalk. Although there are a number of proteome-wide studies with varying degrees of proteome coverage, we still do not know exactly how well we manage to capture all of the modifications on the proteins that are surveyed. Though many protein modifications have been discovered and are well described, it is still believed that many are missed in proteomic experiments. A reason

for this could be that experimental data are always matched against a database of known proteins, where unassigned or unmatched experimental data are automatically not considered. Examinations of proteomic data have demonstrated that the vast majority of peptides in shotgun proteomics approaches are unassigned, with modified peptides making up a large fraction of these spectra [234]. This problem persists despite strides in the field of proteomics towards very high mass accuracy, improved acquisition rates for MS/MS and higher resolution mass spectrometers. Incorporation of ultra-tolerant database searches, which consider upwards of 1000-fold more peptides for matching than directed searches and match more unassigned spectra, may circumvent this issue [234].

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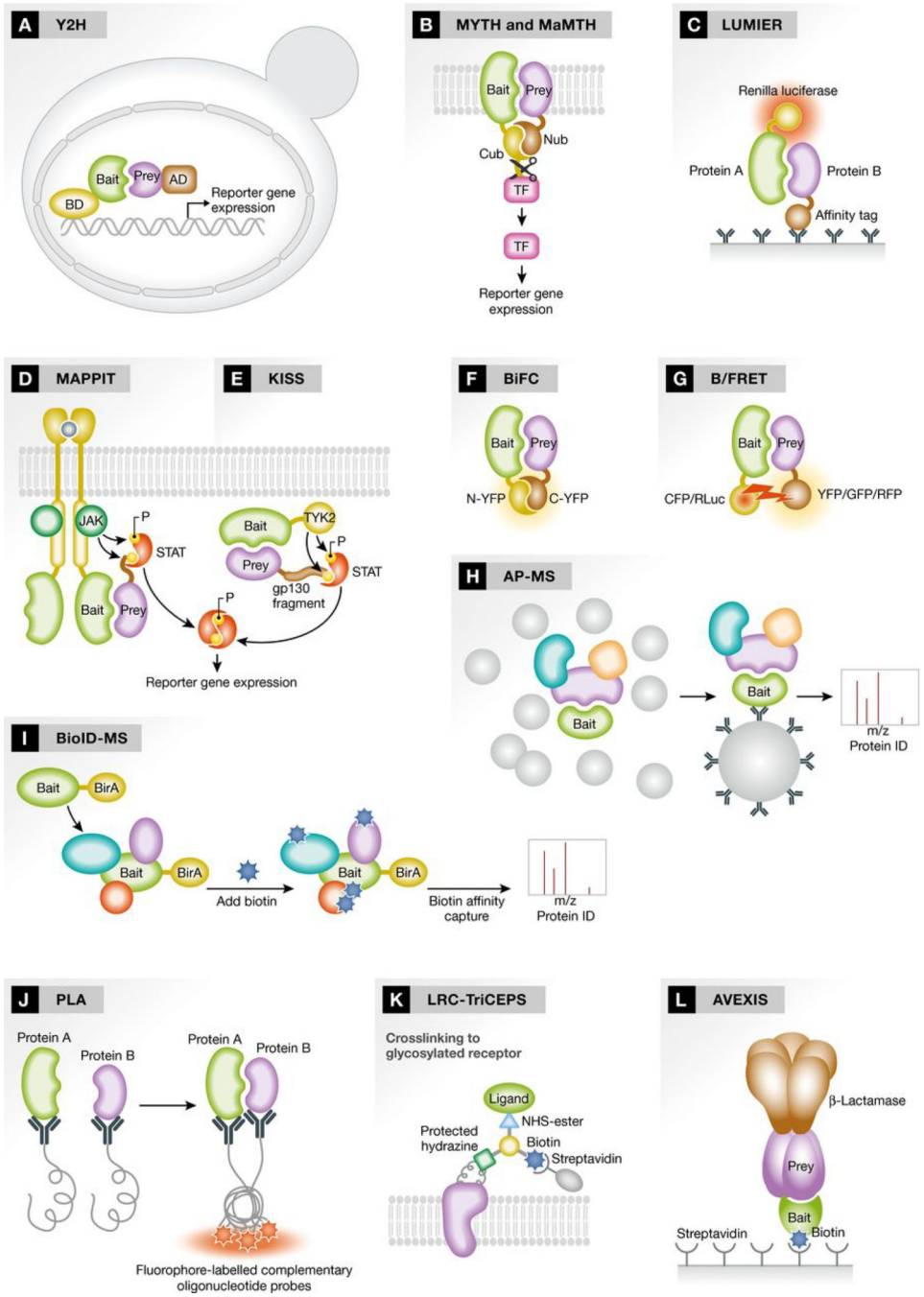

Figure 1.

Schematic representations of selected PPI assays. (A) Yeast Two Hybrid (Y2H). (B) Membrane Yeast Two Hybrid (MYTH) and Mammalian Membrane Two Hybrid (MaMTH). (C) Luminescence-based Mammalian Interactome Mapping (LUMIER). (D) Mammalian Protein-Protein Interaction Trap (MAPPIT). (E) Kinase Substrate Sensor (KISS). (F) Bimolecular Fluorescence Complementation (BiFC). (G) Bioluminescence/Fluorescence Resonance Energy Transfer (B/FRET). (H) Affinity Purification-MassSpectrometry (AP-MS). (I) Proximity-dependent Biotin Identification Coupled to Mass Spectrometry (BioID-

MS). (J) Proximity Ligation Assay (PLA). (K) Ligand-Receptor Capture-Trifunctional Chemoproteomics Reagents (LRC-TRiCEPS). (L) Avidity-based Extracellular Interaction Screen (AVEXIS). Reprinted and adapted with permission under the terms of the Creative Commons Attribution License.

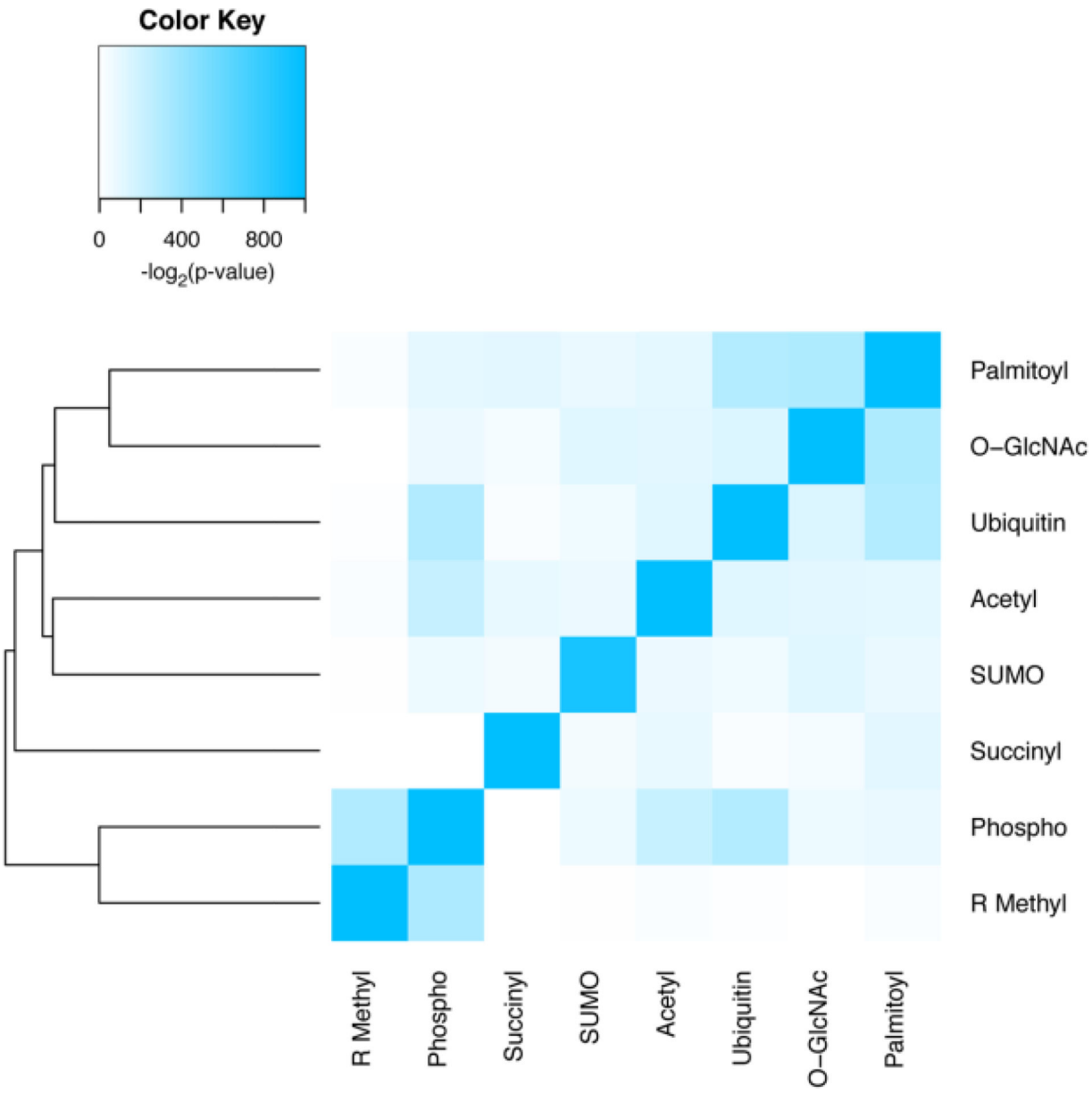

Figure 2.

Interactions between PTM proteomes in *T. gondii*. Lists of proteins detected by proteomics to be modified by different PTMs in *T. gondii* were compared to one another using a hypergeometric test of enrichment using methods described by Silmon de Monerri et al. (2015). Both published (Braun et al., 2009; Treeck et al., 2011; Jeffers and Sullivan, 2012; Li et al., 2014; Foe et al., 2015; Silmon de Monerri et al., 2015; Yakubu et al., 2017) and unpublished (O-GlcNAc dataset, Silmon de Monerri and Kim, in preparation) PTM datasets were analyzed. Color key is in -log2 (p-value). Reprinted and adapted with permission from *John Wiley and Sons*.

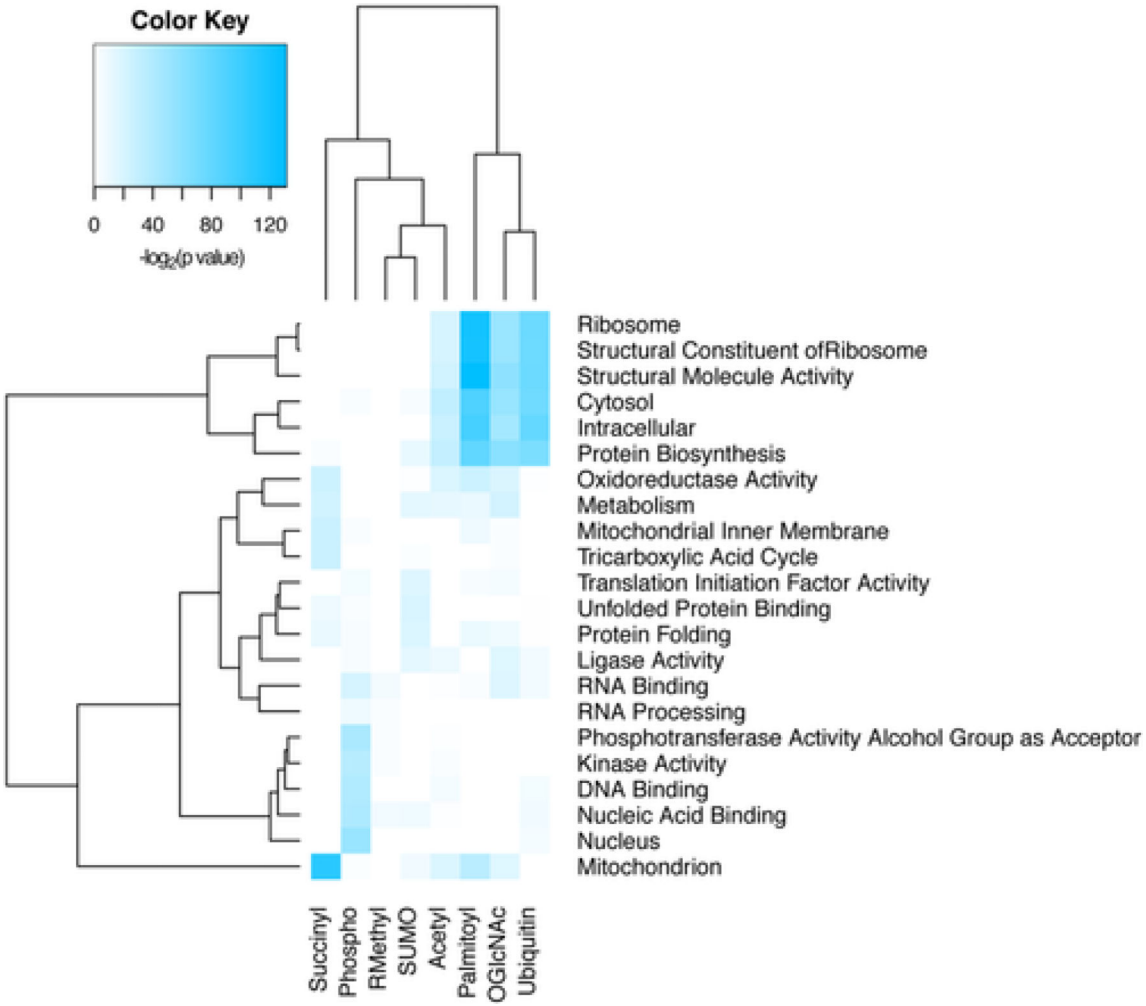

Figure 3.

PTM display preferences for different metabolic pathways in *T. gondii*. Proteins identified as PTM targets by proteomic studies in T. gondii were compared to sets of genes with different functions (classified by Gene Ontology [GO] terms) using a hypergeometric test of enrichment using methodology described by Silmon de Monerri (Silmon de Monerri et al., 2015). The five most significantly enriched GO terms are shown in a clustered heatmap. Color key is in -log2 (p-value). Reprinted and adapted with permission from *John Wiley and Sons*.

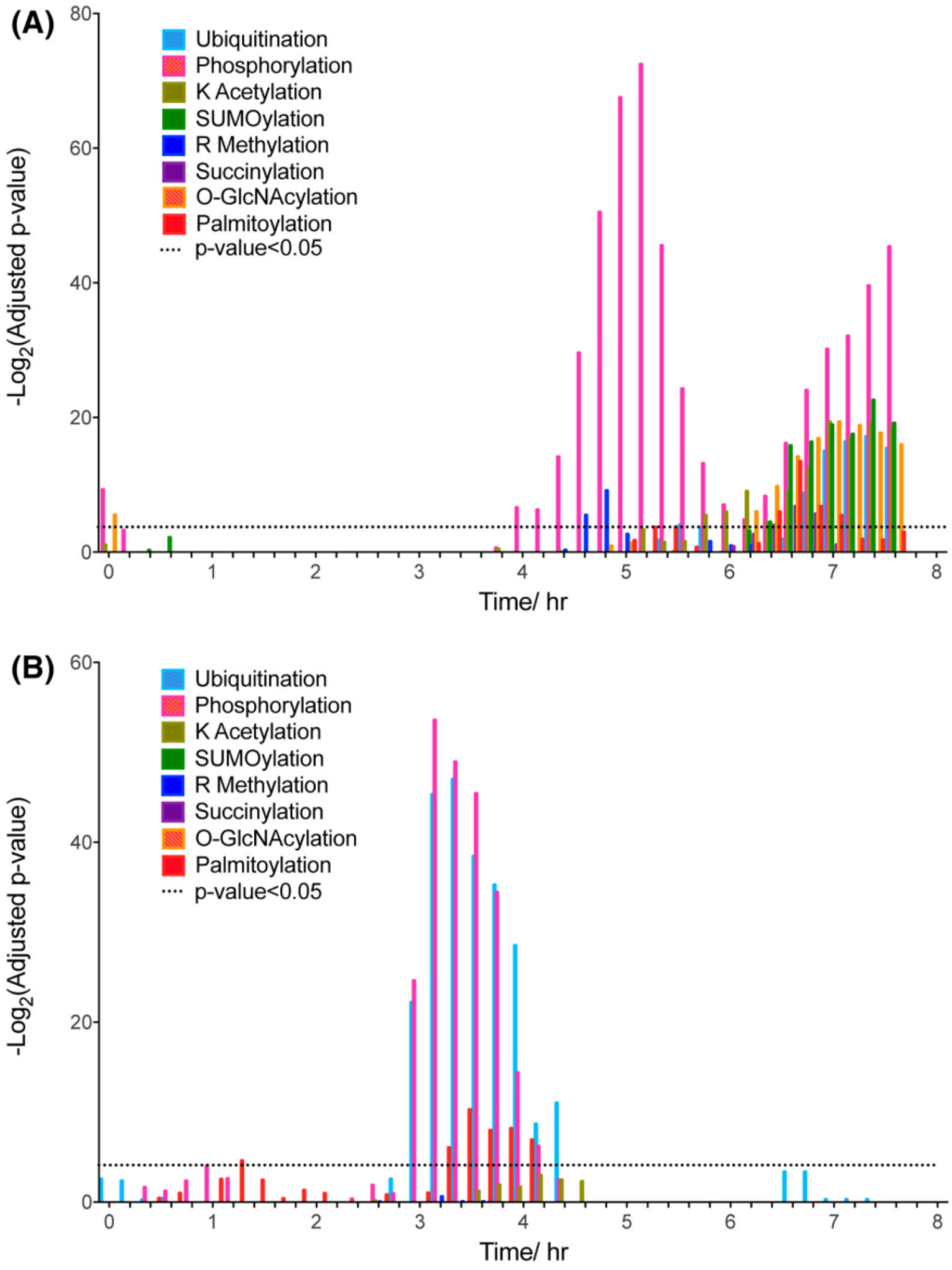

Figure 4.

Proteins modified by PTMs are enriched in cell cycle regulated genes in *T. gondii*. Cell cycle enrichment analysis of published (Braun et al., 2009; Treeck et al., 2014; Jeffers and Sullivan, 2012; Li et al., 2014; Foe et al., 2015; Silmon de Monerri et al., 2015; Yakubu et al., 2017) and unpublished (O-GlcNAc, Silmon de Monerri and Kim, in preparation) PTM datasets. Proteins identified in proteomic studies of PTM were compared against cell cycle gene sets by a hypergeometric test of enrichment. Gene sets were composed of genes that are transcriptionally upregulated in G1 or S/M subtranscriptomes at time points during the 8

h *T. gondii* cell cycle, as described by Croken (Croken et al., 2014). (A) Two peaks of enrichment are seen in G1 phase (h 4.5–5.5; h 6.5–8) and (B) one peak of enrichment in S/M phase (h 3–4). Adjusted p-values (-(log2)- transformed) are plotted. Reprinted and adapted with permission from *John Wiley and Sons*.

Table 1:

Proteomic Studies of Posttranslational modifications

Organism	Parasite Stage	PTM	PTM sites	Proteins	% Proteome coverage	Detection/ purification method	Mass Spectrometer	Reference
P. falciparum	Trophozoite	Lysine acetylation	2,876	1,146	21.0%	Anti-acetyl-lysine immunoprecipitation	VelosPro-Orbitrap Elite hybrid mass spectrometer	(Cobbold, Santos, Ochoa, Perlman, & Llinas, 2016)
T. gondii	Intracellular tachyzoite	Lysine acetylation	411	274	3.30%	Anti-acetylated lysine antibody	LTQ-Orbitrap Velos mass spectrometer	(Jeffers & Sullivan, 2012)
T. gondii	Extracellular tachyzoite	Lysine acetylation	571	386	4.60%	Anti-acetylated lysine antibody (Cell Signaling Technology)	LTQ-Orbitrap Velos mass spectrometer	(Xue, Jeffers, Sullivan, & Uversky, 2013)
P. falciparum	Trophozoite	Lysine acetylation	421	230	4.20%	Anti-acetyllysine antibodies	LTQ mass spectrometer	(Miao et al., 2013)
T. gondii	Intra- and extracellular tachyzoite	Arginine methylation	618	370	4.50%	Methylation motif specific antibody immunoprecipitation Me-R4–100 and R*GG	LTQ-Orbitrap Elite mass spectrometer	(Yakubu, Silmon de Monerri, Nieves, Kim, & Weiss, 2017)
P. falciparum	Ring, trophozoite, and schizont	Arginine methylation		843 (MMA & DMA)	15.40%	Anti-MMA and anti-DMA	Orbitrap Velos Pro mass spectrometer	(Zeeshan et al., 2017)
P. falciparum	Ring, trophozoite and schizont	Lysine methylation	>605	422	7.7%	Anti-mono/dimethyl or anti-trimethyl lysine	Orbitrap Velos Pro mass spectrometer	(Kaur et al., 2016)
T. gondii	Intracellular tachyzoites	Cysteine palmitoylation, myristoylation, prenylation		401	4.80%	Acyl-biotin exchange (ABE)	Linear quadrupole ion trap mass spectrometer	(Caballero et al., 2016)
T. gondii	Intracellular tachyzoites	Cysteine palmitoylation		282	3.40%	17-ODYA bioorthoganal tagging	Quadrupole ion mobility time of flight mass spectrometer	(Foe et al., 2015)
P. falciparum	Schizont	Cysteine palmitoylation		494	9.00%	Acyl-biotin exchange (ABE), metabolic labeling with a palmitic acid analog followed by click chemistry (MLCC)	LTQ Orbitrap Velos mass spectrometer	(Jones, Collins, Goulding, Choudhary, & Rayner, 2012)
T. gondii	Purified and intracellular tachyzoites	Phosphorylation	*T. gondii*: 12,793	*T. gondii:* 2,793	33.60%	immobilized metal affinity chromatography	LTQ-Velos Orbitrap mass spectrometer	(Treeck, Sanders, Elias, &

Organism	Parasite Stage	PTM	PTM sites	Proteins	% Proteome coverage	Detection/ purification method	Mass Spectrometer	Reference
P. falciparum	Schizont					(IMAC) phosphopeptide enrichment		Boothroyd, 2011)
			Purified *T. gondii*: 24,298	Purified T. gondii: 3,506	42.10%			
			P. falciparum: 8,463	P. falciparum: 1,673	30.60%			
P. falciparum	Schizont	phosphorylation	107 (PKG dependent)	69 (PKG dependent);	1.30%	Immobilized metal affinity chromatography (IMAC) phosphopeptide enrichment	LTQ-Orbitrap-Velos mass spectrometer	(Alam et al., 2015)
			Wildtype: 2,931	Approx. 2000	Approx. 36.6%			
P. falciparum	Merozoite	phosphorylation	1765	740	13.50%	TiO_2 bead incubation based on Filter Based Affinity Capturing and Elution (FACE)	linear ion trap cyclotron resonance Fourier transform (LTQ-Ultra FT) mass spectrometer	(Edwin Lasonder, Green, Grainger, Langsley, & Holder, 2015)
P. falciparum	Schizont	phosphorylation	2541	919	16.80%	TiO_2 bead incubation based on Filter Based Affinity Capturing and Elution (FACE)	7-T linear ion trap cyclotron resonance Fourier transform (LTQ-Ultra FT) mass spectrometer	(E. Lasonder et al., 2012)
P. falciparum	Schizont	phosphorylation	1177	650	11.90%	Immobilized metal affinity chromatography (IMAC) phosphopeptide enrichment	LTQ Velos Orbitrap mass spectrometer	(Solyakov et al., 2011)
P. falciparum	Ring, trophozoite, and schizont	phosphorylation	6293	1337	24.50%	Phosphor-tyrosine monoclonal antibody (P-Tyr-100)	LTQ-Velos-Orbitrap mass spectrometer	(Pease et al., 2013)
T. gondii	Intra- and extracellular tachyzoites	ubiquitination	800	454	5.40%	Ubiquitin di-glycine Remnant Motif (K-ε-GG) antibody	LTQ-Orbitrap Elite mass spectrometer	(Silmon de Monerri et al., 2015)
P. falciparum	Ring, trophozoite, and schizont	ubiquitination		437	8.00%	Anti-conjugated ubiquitin mouse IgG1 (clone FK2)	Orbitrap (MudPIT) mass spectrometer	(Ponts et al., 2011)
P. falciparum	Trophozoite	SUMO		23	0.42%	PfSUMO antisera	LTQ ion trap mass spectrometer	(Issar, Roux, Mattei, & Scherf, 2008)

Organism	Parasite Stage	PTM	PTM sites	Proteins	% Proteome coverage	Detection/ purification method	Mass Spectrometer	Reference
T. gondii	Extracellular tachyzoites	SUMO		120	1.40%	Whole cell extract of transgenic tachyzoites ectopically expressing HA Flag–TgSUMO, anti-FLAG M2 affinity gel	QTOF Ultima mass spectrometer	(Braun et al., 2009)
T. gondii	Extracellular tachyzoites	Lysine succinylation	425	147	1.80%	Anti-succinyl lysine antibody	Q Exactive Orbitrap mass spectrometer	(Li et al., 2014)