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Toxoplasma gondii and *Neospora caninum* induce different host cell responses at proteome-wide phosphorylation events; a step forward for uncovering the biological differences between these closely related parasites

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ABSTRACT

Toxoplasma gondii and *Neospora caninum* are closely related intracellular protozoan parasites and tissue-cyst forming Coccidia of the phylum Apicomplexa. There are remarkable similarities between the morphology, genomes and transcriptomes of both parasites. *Toxoplasma* is zoonotic, with a wide host range and is mainly transmitted horizontally between its definitive host, the cat, and its intermediate hosts. *Neospora* causes disease within a narrow host range and with reduced virulence potential to the hosts. The dog is the definitive host of *Neospora* and its epidemiology in cattle mainly depends on vertical transmission. What causes these biological differences is not well understood. Since these parasites secrete an array of secretory proteins, including kinases, during infection to manipulate host cell responses. Host-parasite interactions due to phosphorylation of host cell proteins by *T. gondii* kinases enhance virulence and maintenance of infection. In this study, proteome-wide phosphorylation events of host cell proteins were investigated in response to infection with *T. gondii* and *N. caninum* using phosphoproteomic analyses, followed by pathway analysis on host signalling pathways. A few interesting differences in host responses at both the qualitative and quantitative levels were identified between the two infections; about one-third of the phosphoproteomes, approximately 21 % of the phospho-motifs and several pathways such as glycolysis/gluconeogenesis and mTOR pathways of the host cell were found differentially enriched between infection with these parasites. Identifying the differences in host-parasite interactions represents a promising step forward for uncovering the biological dissimilarities between both parasites.

Keywords: *Toxoplasma gondii*, *Neospora caninum*, proteome-wide phosphorylation, phosphopeptides enrichment, Tandem mass spectrometry, biological differences.

Introduction

Toxoplasma gondii and *Neospora caninum* are closely related obligatory intracellular protozoan parasites and tissue-cyst forming Coccidia of the phylum Apicomplexa (Dubey et al. 1988). There is remarkable similarity in morphology and ultrastructure of the infective stages; the tachyzoites, bradyzoites and sporozoites (Lindsay et al. 1999; McAllister et al. 1998; Speer et al. 1998; Speer et al. 1999) and at the genome and transcriptome level (Ramaprasad et al. 2015; Reid et al. 2012). However, there are several marked biological differences between the two parasites including host range, zoonotic capacity, transmission routes, virulence potential and definitive host (Davison et al. 1999; Dubey 2004; Dubey et al. 2007; Lei et al. 2014; McAllister et al. 1998; McCann et al. 2008; Tenter et al. 2000; Williams et al. 2009). The definitive host for *T. gondii* is the cat while for *N. caninum,* it is the dog (Frenkel et al. 1970; McAllister et al. 1998). *Toxoplasma* causes toxoplasmosis, a zoonotic disease that infects a wide range of animal species and humans (Tenter et al. 2000). Congenital toxoplasmosis occurs as a result of a primary acute infection in pregnant women leading to abortion and congenital defects of the infected foetus (Montoya and Remington 2008). *Neospora* causes neosporosis with a narrower host range in comparison to *T. gondii* and has not been reported in humans (Dubey et al. 2007; McCann et al. 2008). Neosporosis is restricted mainly to cattle and dogs and is considered the major cause of abortion in cattle, resulting in great economic losses in cattle industry worldwide (Dubey 2003). The epidemiology of *T. gondii* is primarily based on horizontal transmission particularly between the definitive host, the cat and its main prey, the rodents (Dubey et al. 1998). But *Neospora* is mainly transmitted vertically from the pregnant dam with an endogenous infection to the foetus by crossing the placenta (Davison et al. 1999; Dubey et al. 2007; Paré et al. 1997; Trees and Williams 2005; Williams et al. 2009). In addition, *T. gondii* has strain-specific virulence to the host cells and is considered more pathogenic compared with *N. caninum* which has reduced virulence (Lei et al. 2014; Reid et al. 2012; Saeij et al. 2005).

Toxoplasma gondii and *N. caninum* have to invade host cells successfully in order to survive, grow, multiply and evade the immune system of the host (Besteiro et al. 2011; Hemphill et al. 1996; Ojo et al. 2014). The invasive stages of these parasites use the apical complex and the sequential release of proteins from the invasion-related organelles (rhoptries, micronemes and dense granules) to facilitate host cell invasion, manipulation of the host cell genome and establishment of infection (Besteiro et al. 2011; Besteiro et al. 2009; Bougdour et al. 2013; Carruthers and Sibley 1997; Coppens et al. 2006; Mercier et al. 2005; Naguleswaran et al. 2002). Therefore, the prerequisite to understand the cause and effect in response to infection with these parasites is to dissect the functional molecules of the parasites (effectors) and the target molecules of different host cells throughout the infection.

The transcriptomes and proteomes of these parasites and host in response to infection have been studied using DNA microarray, transcriptional analysis and quantitative gel-based proteomics (Blader et al. 2001; Nelson et al. 2008; Pittman et al. 2014; Reid et al. 2012; Xia et al. 2008). These studies showed that many host cell genes, transcripts and proteins associating with the immune response to pathogens (e.g. chemokine receptor 8 and interleukin 18 receptor 1) and metabolism (e.g. hexokinase, phosphofructokinase and phosphoglycerate kinase were increased by almost 3.3-4 folds) were modulated in response to infection with *T. gondii*. Nelson et al. (2008) suggested that studies of the functional form of proteins including post-translational modification (PTMs) provide a more robust interpretation of host response to infection than transcriptional studies. A study has reported hundreds of PTMs of proteins (Garavelli 2004). Protein phosphorylation is one of the most common types of PTMs which is implicated in signal transduction and is crucial for regulation of almost all cellular processes (Villen and Gygi 2008).

In apicomplexan parasites, protein phosphorylation plays an important role in the regulatory mechanisms within and outside the parasites after the invasion of the host cells (Treeck et al. 2011). Difference gel electrophoresis (DIGE) analysis of host cell protein in response to infection with *T.*

gondii demonstrated that many of protein changes were a result of PTMs and protein phosphorylation was found as one of the most important modifications (Nelson et al. 2008). Phosphorylation of host cell proteins by *T. gondii* is related to adaptation and protection from the immune system within the host environment (Butcher et al. 2011; Reid et al. 2012). In addition, *TgROP18* overexpression was found related to an increase in the multiplication rate and virulence (El Hajj et al. 2007). Furthermore, TgROP18 is protected from IFN-γ mediated clearance processes by ROP5 in activated macrophages (Behnke et al. 2012). Moreover, TgROP16 is also involved in virulence through phosphorylation of the host signalling pathway signal transducer and activator of transcription (STAT3/6). Phosphorylation of STAT3/6 modifies the early inflammatory response of the host mediated by interleukin-12 (IL-12) (Butcher et al. 2011; Ong et al. 2010; Saeij et al. 2007; Yamamoto et al. 2009). Wang et al. (2009) reported the phosphorylation and activation of mTOR signalling pathway substrates in different cell types such as human foreskin fibroblasts (HFF) cells, HeLa cells and murine peritoneal exudate macrophages in response to infection with *T. gondii*.

There is evidence of differential host cell protein phosphorylation between *T. gondii* and *N. caninum* infections. For example, Immunity-related GTPases (IRGs) were found phosphorylated by *T. gondii* rhoptry protein 18 (TgROP18) in a virulent type I strain, whereas *N. caninum* was found not to phosphorylate IRGs due to the pseudogenisation of *ROP18* (Lei et al. 2014; Reid et al. 2012). However, a comparison of genome-wide protein phosphorylation of *T. gondii* and *N. caninum* infected host cells has not been studied and could be critical for understanding the subtleties of host-parasite interactions.

To understand the critical role of protein phosphorylation in the biological differences between *T. gondii* and *N. caninum* infections, large-scale phosphoproteome analyses of the host cell during infection is very important. This study aimed to analyse the comparative protein phosphorylation of host cells in response to infection with these parasites at 20 hours post infection (p.i) using Mass spectrometry (MS)-based phosphoproteomics.

MATERIALS AND METHODS

Host cell and parasite maintenance

Both *T. gondii* VEG strain and *N. caninum* Liverpool strain were grown and maintained as described previously (Sohn et al. 2011). Briefly tachyzoites of both parasites were cultured by serial passage in confluent monolayers of human foreskin fibroblasts (HFF) cells grown in medium consisting of Iscove's Modified Dulbecco's Media (IMDM) (Lonza) supplemented with 10 % bovine foetal serum (BFS) (Biosera) and 1 % penicillin 10,000 units/streptomycin 10 mg/ml (Sigma).

Time course study of HFF cell infections with *T. gondii* and *N. caninum*

Both *T. gondii* and *N. caninum* infected cells were grown for 20 hr p.i. at a ratio of 20:1 parasites to the initial number of HFF cells seeded. The final number of the parasites per HFF cell is equivalent to 5:1 or less, respectively, due to the doubling time of HFF cells which is approximately 24-30 hours. In addition, a higher MOI resulted in a higher signal to noise ratio, which is particularly important for phosphoproteome studies. The time point of 20 hr p.i. was chosen as the majority of the *T. gondii* and *N. caninum* tachyzoites were at the 2nd and 3rd stage of endodyogeny and before egress.

Three biological replicates of each infection were analysed for phosphopeptide identification and phosphoprotein quantification. Briefly, a total of 2×10^7 parasites were used to infect the host cells that were maintained for three days following seeding with 1×10^6 cells/T75 flask. At 20 hr p.i., growth medium was discarded and cells were collected in 5 ml of growth media. Cell suspension was centrifuged at $1{,}000 \times g$ for 5 min at 4 °C, the pellet was resuspended in 10 ml cold PBS and re-centrifuged at 4 °C. The last step was repeated and the pellet was collected in 1 ml cold PBS followed by a final centrifuge at $12{,}000 \times g$ for 3 min at 4 °C.

Protein preparation from harvested pellets of HFF cells infected with *T. gondii* and *N. caninum*

Harvested pellets from 1-2 T75s were prepared for phosphoprotein analysis. Each pellet was resuspended in 250 µl of a master mix lysis buffer and incubated on ice for 30 min. Master mix lysis buffer was comprised of 22.5 mM ammonium bicarbonate, 200 µg of RapiGest™ SF surfactant (Waters), 1 × phosphatase inhibitor cocktail 2 (Sigma), protease inhibitor cocktail-EDTA free (Sigma) (containing 4.16 mM of AEBSF, 3.2 µM of aprotinin, 200 µM of bestatin, 56 µM of E-64, 8 µM of leupeptin and 6 µM of pepstatin A), 100 µM of benzimidine, 200 µM of phenylmethanesulfonyl fluoride, 400 µM of β-glycerophosphate, 400 µM of sodium fluoride and 12 µM of sodium orthovanadate. Samples were further lysed and homogenised using a probe sonicator (Sonics®) on ice for 5 times using a 30 second pulse (74 Hz) and 1 min off at 40 % amplitudes output. Cell lysates were centrifuged at 16,000 × *g* for 1 hr at 4 °C and the supernatant was collected. The protein concentration measured using Coomassie (Bradford) protein Assay Kit (Thermo scientific) and the absorbance was measured at 595 nm using Tecan-Infinite® F50 (Tecan Group Ltd.). An equivalent of 1 mg was taken for enrichment of phosphopeptides using Titanium dioxide (TiO_2) SpinTips (Protea®).

Analysis of sample lysates on an Orbitrap Velos mass spectrometer

In-solution digestion of sample protein and analysis

Each protein sample was diluted in 25 mM ammonium bicarbonate to a volume of 170 µl before being heated at 80 °C for 10 min with a brief vortex at 5 min. Samples were then reduced with 3 mM dithiothreitol and the sample incubated for 10 min at 60 °C. Samples were cooled to room temperature (RT) before alkylation with 9 mM iodoacetamide and incubated for 30 min in the dark at RT. Proteins were then digested with proteomic grade porcine trypsin (Sigma-Aldrich®) at a ratio

of 1 µg: 50 µg of trypsin to lyse proteins and were incubated overnight at 37 °C. Digestion was stopped by acidification with a final concentration of 0.5 % trifluoroacetic acid (TFA) and incubated for 45 min at 37 °C. The solution was centrifuged at 13,000 × *g* for 15 min at RT. The supernatant containing the digestion was further processed using TiO_2 SpinTips kit for enrichment of phosphopeptides.

Enrichment of phosphopeptides from the digestion

Enrichment of phosphopeptides was performed using a TiO_2 SpinTip kit as per the manufacturer's instructions (Protea®). The eluates were dried in a vacufuge Concentrator plus (Eppendorf) and resuspended in 20 µl recombination buffer containing 3 % acetonitrile and 0.5 % TFA followed by bath sonication for 10 min at 4 °C.

Tandem mass spectrometry analysis (Velos –orbitrap)

Peptide mixtures were analysed by on-line nanoflow liquid chromatography using the nanoACQUITY-nLC system (Waters MS technologies, Manchester, UK) coupled to an Linear Trap Quadropole (LTQ)-Orbitrap Velos mass spectrometer equipped with the manufacturer's nanospray ion source. The analytical column (nanoACQUITY UPLCTM BEH130 C18 15 cm × 75 µm, 1.7 µm capillary column) was maintained at 35 °C and a flow-rate of 300 nl/min. The gradient consisted of 3-40 % acetonitrile in 0.1 % formic acid for 90 min then a ramp of 40-85% acetonitrile in 0.1% formic acid for 5 min. Full scan MS spectra (m/z range 300-2000) were acquired by the Orbitrap at a resolution of 30,000. Analysis was performed in data dependant mode. The top 20 most intense ions from the MS1 scan (full MS) were selected for tandem MS by collision induced dissociation (CID) and all product spectra were acquired on the LTQ ion trap.

Protein identification using multiple search engines

Raw data from mass spectrometric analyses of three biological replicates of HFFs infected with *T. gondii* and *N. caninum* were submitted to PEAKS® Studio (version 7.0, BSI, Waterloo, ON, Canada). The following settings were used for the searches: data refinement was performed with no merged scans, with precursor charge correction and no filtering. InChorus searching was performed including search engines comprised of Peaks, Mascot (Matrix Science), OMSSA (Geer et al. 2004) and X!Tandem (Craig and Beavis 2004) with a mass tolerance of 10 ppm for the precursor and 0.8 ppm for the fragment ion. Trypsin was used as enzyme specificity; carbamidomethyl cysteine was set as the fixed modification, and oxidation of methionine (M), phosphorylation of serine, threonine and tyrosine (STY) were set as the variable modifications. Peptides with a maximum of 3 variable modifications and one missed cleavage were allowed. The peptides were searched for protein identification against locally-installed databases including the annotated protein sequences for *N. caninum* (ToxoDB-9.0_NcaninumLIV_AnnotatedProteins. fasta) and for *T. gondii* (ToxoDB-10.0_TgondiiME49_AnnotatedProteins.fasta) [downloaded from ToxoDB (Gajria et al. 2008)]. UniProt-human reference proteome databases release 2014_02 was used for identification of human proteins (http://www.uniprot.org/). A p value < 0.05 with false discovery rate (FDR) of ≤ 1 % was used as a cut-off for significant phosphopeptide identifications.

Pathway analysis

The phosphoproteins identified from HFF cells infected with *T. gondii* and *N. caninum* were submitted to the online functional annotation tool DAVID Bioinformatics Resources version 6.7 (Huang et al. 2008). The protein accession numbers from proteins of interest were submitted as gene lists. Within the DAVID tool, the KEGG Pathway (Kanehisa and Goto 2000) was chosen for pathway analysis. A P-value cut-off ≤ 0.05 was applied to pathways to be considered statistically significant enriched in the annotation categories. The phosphoproteins enriched in each pathway for HFF cells infected with *T. gondii* were also mapped manually for *N. caninum* (and vice versa) to

compare the discrepancies of phosphoprotein coverage when the same pathway was not statistically enriched.

Phosphopeptide motifs analysis

Phosphopeptide motifs were analysed using a web-based motif-x programme (Chou and Schwartz 2011) using default settings. Briefly, the phosphopeptides were queried against background data in the international protein index (IPI) human proteome. Each time one amino acid (S or T or Y) was used to identify the motif with +7 and -7 up and down of the selected amino acids (total of 15 amino acids). The occurrence of the motif was set to at least 5 × with significant threshold of 0.000001 which correspond to actual alpha-value of approximately 0.0003 by Bonferroni method.

Phosphoprotein quantification

For quantitation of enriched phosphopeptides, Progenesis LC-MS software (Nonlinear Dynamics, U.K.) was used to carry out label-free quantification. Peptide and protein quantification using Progenesis LC-MS consisted of several consequent steps. Collected spectra from Orbitrap Velos mass spectrometer where aligned and monoisotopic spectra with +2 to +7 charges were maintained for analysis. Recovered spectra were quantified from both groups (each comprised of three biological replicates) of infected cells. Quantified features were identified at both the peptide and protein level by PEAKS® software. Identified peptides and proteins were matched to the quantified spectra and standardised using the built in normalisation tool in Progenesis LC-MS. Label-free quantification was carried out based on cut-off filters including a p value < 0.05 for significant phosphopeptides quantification and a fold change of > 1.5 to determine the differentially expressed phosphoproteins. The fold change of > 1.5 is due to the fact that the stoichiometry of a

phosphoprotein is less than one percent of the total of that protein (Hunter and Cooper 1985); therefore any small changes in phosphorylation profile of a protein may indicate great differences.

Subcellular localisation and types of identified and quantified phosphoproteins

Subcellular location and types (based on molecular function) of quantified phosphoproteins were determined using ingenuity pathway analysis (IPA) software (Ingenuity® Systems, Redwood City, CA). In addition, an upstream regulator analysis for pathways or kinases involved in phosphorylation of the quantified phosphopeptides was performed using IPA.

RESULTS

Growth comparison of *T. gondii* and *N. caninum* tachyzoites in HFF cells at 20 hr p.i.

The growth comparison of tachyzoites was based on the doubling processes of *T. gondii* and *N. caninum* in infected HFF cells. As shown in Figure 1, most of the tachyzoites at 20 hr p.i from both parasites were found at 2nd (4 tachyzoites) or 3rd (8 tachyzoites) doubling rounds.

Total identified phosphopeptides using TiO_2 and data analysis using PEAKS®

Redundancy in the identified phosphopeptides obtained from the biological replicates of the infected host cells was eliminated. The total number of identified phosphopeptides from host cells infected with *T. gondii* and *N. caninum* were 1,796 and 1,630, respectively. Phosphopeptides from HFF cells infected with *T. gondii* and *N. caninum* were compared. There are 1101 host phosphopeptides shared between *T. gondii* and *N. caninum* infections (Figure 2a) which accounted for 61.30 % and 67.54 % of the identified phosphopeptides, respectively. A total of 695 and 529 phosphopeptides were found different from HFF cells infected with *T. gondii* and N. caninum,

respectively. At the phosphoprotein level, there were 359 phosphoproteins that shared between host cells infected with either parasites which accounted for 70.4 % and 78 % of the identified phosphoproteins for infection with *T. gondii* and *N. caninum*, respectively. A total of 151 phosphoproteins from HFF cells infected with *T. gondii* differed from those infected with *N. caninum*. A total of 101 phosphoproteins were found in infection with *N. caninum* but not in *T. gondii* (Figure 2b).

Subcellular location and types of identified phosphoproteins

Ingenuity pathway analysis (IPA) was used to determine the types (based on molecular function) and the distribution of the subcellular locations of identified phosphoproteins of HFF cells in response to infection with *T. gondii* and *N. caninum*. Host cells infected with *N. caninum* and *T. gondii* showed very similar distribution in both subcellular location and types of enriched phosphoproteins as shown in Figure 3 and 4, respectively.

Comparison of phosphorylation sites of phosphopeptides identified from HFF cells infected with *T. gondii* and *N. caninum*

Investigating phosphorylation sites is important to understand the differences in signalling pathways and the kinases most frequently involved. Phosphorylated proteins of HFF cells were investigated for differences in their phosphorylation sites in responses to infection with *T. gondii* and *N. caninum*. The phosphorylation sites (phosphoserine, phosphothreonine and phosphotyrosine) were found distributed similarly between infected host cell with *T. gondii* and *N. caninum* (Table 1).

Motif analysis from enriched phosphopeptides

Motif analysis was performed in order to investigate the involvement of different kinases with various phospho-motifs between the two infections. Motif analysis of phosphoserine containing peptides showed differences between host cells infected with *T. gondii* and *N. caninum*. A total of

19 motifs were enriched where four motifs were differentially enriched for each infection, as shown in Table 2, which is equivalent to 21.05 % (4/19) differences. On the other hand, phosphothreonine containing peptides showed that only four motifs were enriched where two of them were differentially enriched in *T. gondii,* as shown in Table 3.

Pathway analysis for the enriched phosphopeptides from HFF cells infected with *T. gondii* and *N. caninum*

The lists of identified phosphoproteins enriched from HFF cells infected with each parasite were submitted to DAVID functional annotation tool for pathway analysis. The results are shown in Table I and II (Online Resource 1) for *T. gondii* and *N. caninum*, respectively. Comparing the pathways from both conditions showed that three pathways (Mammalian target of rapamycin (mTOR) signalling pathway, glycolysis/gluconeogenesis and endometrial cancer) were enriched in HFF cells infected with *T. gondii* but not *N. caninum*. While five pathways (erbB signalling pathway, renal cell carcinoma, gap junction, long-term potentiation, and vascular smooth muscle contraction) were enriched in *N. caninum* infected cells but not those infected with *T. gondii* (Table 4). The detailed information about individual proteins involved in the differentially enriched pathway is shown in Table III (Online Resource 1).

Mammalian target of rapamycin (mTOR) signalling pathway

The mammalian target of rapamycin (mTOR) signalling pathway is significantly enriched from phosphoproteins of HFF cells infected with *T. gondii* but not with *N. caninum*. Among the phosphoproteins identified as shown in Table III (Online Resource 1), three of them play roles in the regulation of the core pathway (AMPK1, PDK1, and TSC2) while the other three (EIF4EBP1, RPS6 and EIF4B) are substrates for the core pathway (Figure 5). However, only the last three molecules (EIF4EBP1, RPS6 and EIF4B) were found phosphorylated in *N. caninum* infection as well.

Glycolysis/gluconeogenesis pathway

Pathway analysis showed that more phosphopeptides from HFF cells infected with *T. gondii* were enriched in the glycolysis/gluconeogenesis pathway than *N. caninum* infection at 20 hr p.i. as shown in Table III (Online Resource 1) and Figure 6.

Phosphopeptides quantification from host cell infected with *T. gondii* and *N. caninum*

In total, 2540 phosphopeptides were quantified in TiO_2 enrichment of *T. gondii* and *N. caninum* infections. When comparing between the two infections, only 397 phosphopeptides were statistically significant ($p < 0.05$). A total of 95 unique phosphopeptides showed a fold change (FC) of ≥ 1.5 between the two infections, as shown in Table IV (Online Resource 1). In total, 57 out of the 95 phosphopeptides had higher expression in *T. gondii* infection and the remaining 38 have higher expression in *N. caninum,* as shown in Table IV (Online Resource 1) and Figure 7. Phosphoproteins with high fold changes were observed in *T. gondii* infection that play critical role in cellular process involved in protein productions/translation such as eukaryotic translation initiation factor 4B (EIF4B) and 60S acidic ribosomal protein P1. The EIF4B was increased 26 times more in cells infected with *T. gondii* than *N. caninum*. In addition, phosphoproteins with a high fold change of proteins involved in glycolysis were found in infection with both parasites; triosephosphate isomerase was found in infection with *T. gondii* while glyceraldehyde-3-phosphate dehydrogenase with *N. caninum* infection.

Upstream regulator analysis of quantified phosphopeptides from host cell infected with *T. gondii* and *N. caninum*

The quantified phosphopeptides were found to be regulated by different molecules that play vital role in normal cellular physiology. The upstream regulator analyses showed that kinases were distributed similarly between both infections. In addition, transcriptional regulators and enzymes

were among the most regulated molecules enriched from infection with *T. gondii* and *N. caninum* as shown in Table V and VI (Online Resource 1) and Figure 8.

DISCUSSION

The primary aims of the present study were to analyse the comparative protein phosphorylation of host cells in response to infection with *T. gondii* and *N. caninum* at 20 hr p.i using MS-based phosphoproteome approaches. In the present study, HFF cells infected with both parasites displayed phosphorylation of peptides and proteins approximately two times higher compared to uninfected cells in the mock infections (data not shown).

Here in this study, very close similarity in the host response to infection with these parasites through phosphorylation events has been found, however many differences have also been identified. Firstly, about one-third of the enriched phosphoproteomes from host cell infected by *T. gondii* and *N. caninum* were different. Secondly, four different motifs were found differentially enriched between infection with *T. gondii* and *N. caninum,* which is equal to 21.05 % of the differences. Thirdly, pathway analysis showed that several pathways were differentially enriched between infections such as glycolysis/gluconeogenesis and mTOR signalling pathway in infection with *T. gondii* but not *N. caninum*. Finally, the upstream regulator analysis of the quantified phosphoproteins showed that transcriptional regulators and enzymes were more highly processed in *N. caninum* than *T. gondii* infection (Figure 8). Small modifications in the genomes of these parasites have been found to create great differences in host-parasite interactions; pseudogenisation of the *Neospora* gene *ROP18* was found associated with reduced ability to resist immunity of the host due to inability of the NcROP18 to phosphorylate IRGs enzyme. The latter enzyme is responsible for the destruction of the PVM in Interferon gamma (IFNγ)-induced cells (Lei et al. 2014; Reid et al. 2012).

Pathway enrichment analyses coupled with quantitative data, revealed some interesting differences in host responses following infection with both parasites. mTOR signaling pathway is a Ser/Thr kinase that plays a key role in integrating responses arising from certain vital conditions (Hay and Sonenberg 2004; Tsalikis et al. 2013; Wullschleger et al. 2006). In this study, downstream (E4-BP1 and S6 and eIF4B) and upstream (PDK1, TSC2 and AMPK) substrates of mTOR signalling pathway were found in HFF cells infected with each parasite and *T. gondii* only, respectively. However, quantitative data showed that eIF4B was enriched 26 times more in infection with *T. gondii* than *N. caninum*. The PDK1 and TSC2 were, however, found to be phosphorylated in *T. gondii* infected host cells only, but not in uninfected cells (data not shown). Together, results associated with mTOR signalling pathway suggested that *T. gondii* and *N. caninum* potentially phosphorylate host mTOR substrates in a parasite-dependent manner for selective translation of certain mRNAs.

Glycolysis/gluconeogenesis is another metabolic pathway that is highly regulated in HFF cells infected with *T. gondii*. In this study, HFF cells infected with *T. gondii* involved in more metabolic processes and highly up-regulated than *N. caninum* as shown in Table I, III and IV (Online Resource 1). The enzymes identified in this study catalyse most of the reactions involved in glycolysis/gluconeogenesis. Acetyl-coA synthase was also phosphorylated which catalyses the formation of acetyl-coA (the citric acid cycle substrate). In addition, this study also showed the activation of the glycolysis pathway and energy production under aerobic conditions, which provided complementary evidence to the results of microarray study from Blader et al. (2001), which reported that *Toxoplasma*-stimulated transcripts of host cell were up-regulated in anaerobic glycolysis.

High energy production is required to fulfil the demand of fast growing and multiplying tachyzoites; these processes are critically involved in the expenditure of cellular energy (Sanli et al. 2014). This is supported by the study of Weilhammer et al. (2012), which demonstrated that glucose

and glycolytic metabolites such as lactate play critical roles in inhibition of conversion of tachyzoites to bradyzoites in permissive cells. The enrichment of phosphoproteins involved in the mTOR, glycolysis and insulin pathways together (Table I) indicated that high energy and proteins production are required for biogenesis and rapidly dividing tachyzoites in *T. gondii* infection comparing to *N. caninum*. High energy expenditure in cells infected with *T. gondii* potentially determines virulence through modulation of host cell metabolic processes to support tachyzoite replication, early egression, cell death and re-invasion of new neighbouring cells (Hoff and Carruthers 2002).

On the other hand, ErbB signalling pathway enriched in *N. caninum* infection may be associated with the parasite's transmission route. ErbB signalling pathway plays important roles in the normal physiology of cells and in cancer (Hynes and MacDonald 2009). In this study, the downstream substrates of the ErbB2, 3, 4 and EGFR receptors were found to be phosphorylated. Upstream analysis of the quantified phosphoproteins indicates that different kinases such as Mitogen-activated protein kinases (MAPKs) are involved in the phosphorylation of the proteins important for cell survival or death, as shown in Table V and VI (Online Resource 1). Many pathogens modulate the Phosphatidylinositol 3-kinase (PI3K)/AKT survival pathway of the host cell which is primarily associated with activation of anti-apoptotic pathways that assure a safe place for the pathogens within the host cells (Carter 2009). Maintenance of the infected host cell by *N. caninum* may guarantee minimum exposure to the immune system of the host and apoptosis. It could be also related to the transmission route through converting tachyzoites to bradyzoites; this process is necessary to maintain the vertical transmission route and allows transmission from infected pregnant mother to subsequent generation.

To summarise, comparative host-parasite interactions have been studied by determining host protein phosphorylation in response to infection with *T. gondii* and *N. caninum*. The differences in host protein phosphorylation and modification of signalling pathways, e.g.

glycolysis/gluconeogenesis and mTOR signalling pathway potentially associate with the differences in the biology of *T. gondii* and *N. caninum*. High modulation of glycolysis/gluconeogenesis and mTOR signalling pathway during *T. gondii* infection is potentially more associated with the differences in virulence and transmission routes compared to infection with *N. caninum*.

The results of the present study will encourage future research through investigation of the parasite factors that mimic the host factors. Mimicry could be used as a strategy to selectively activate different host substrates necessary for maintaining its biological activities. Therefore, using functional protein microarrays would be important to screen and identify substrates of parasite kinases especially rhoptry kinases that associate with regulation of host cells transcription factors.

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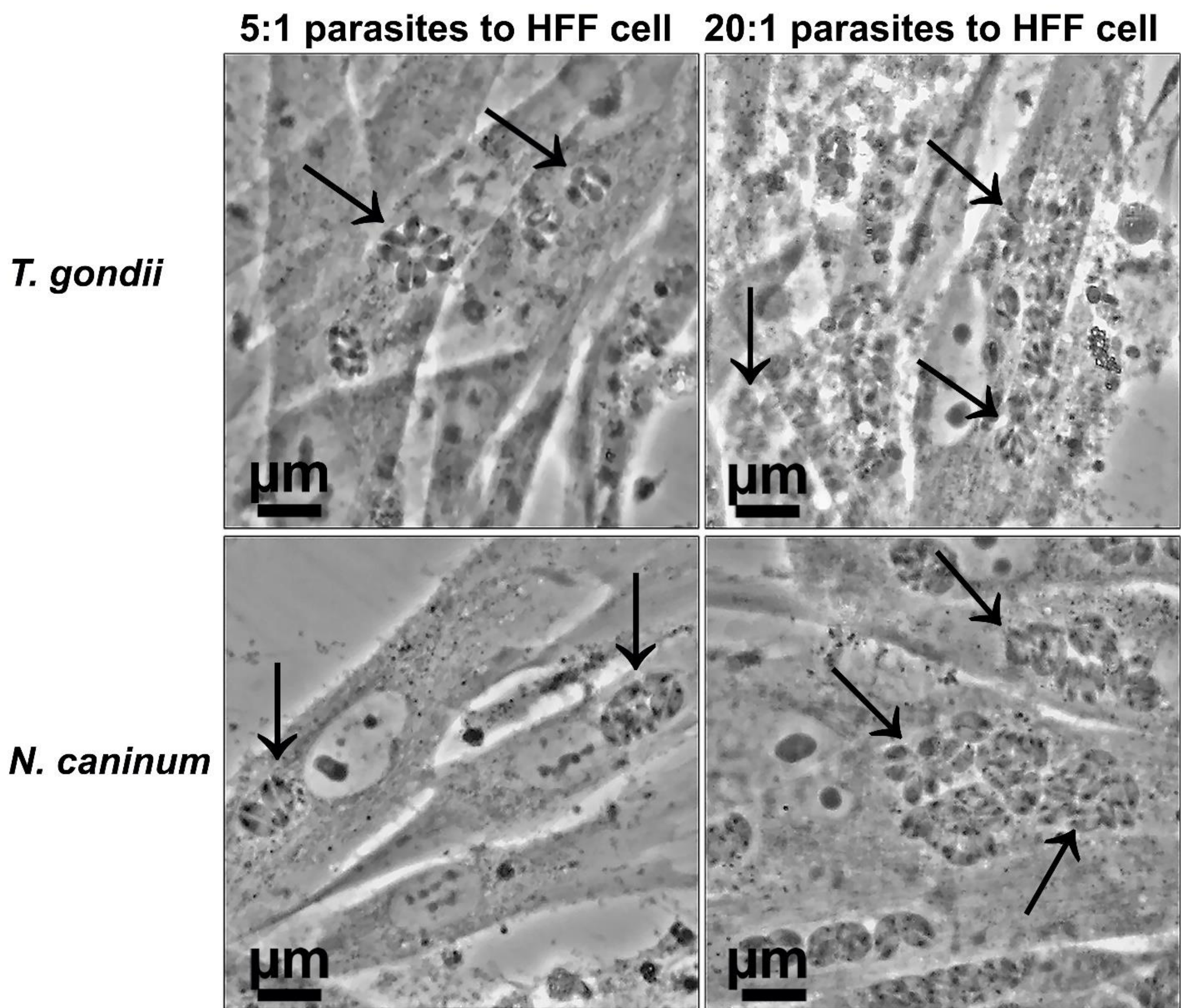

Fig. 1 The doubling processes of *T. gondii* and *N. caninum* in infected HFF cells at 20 hr p.i. (a) and (c) show HFF cells infected with *T. gondii* and *N. caninum* respectively at a ratio of 5:1 parasites to cells, arrows show parasites tachyzoites at the 2nd and 3rd doubling stages at 400 × magnification. (b) and (d) show HFF cells infected with *T. gondii* and *N. caninum* respectively at a ratio of 20:1 parasites to cells at 400 ×. Pictures were taken by AxioCam ERCs % Rev. 2.0 (2 mega pixels) connected to an inverted microscope (Carl Zeiss Microscopy GmbH, Germany) with 400 × magnification

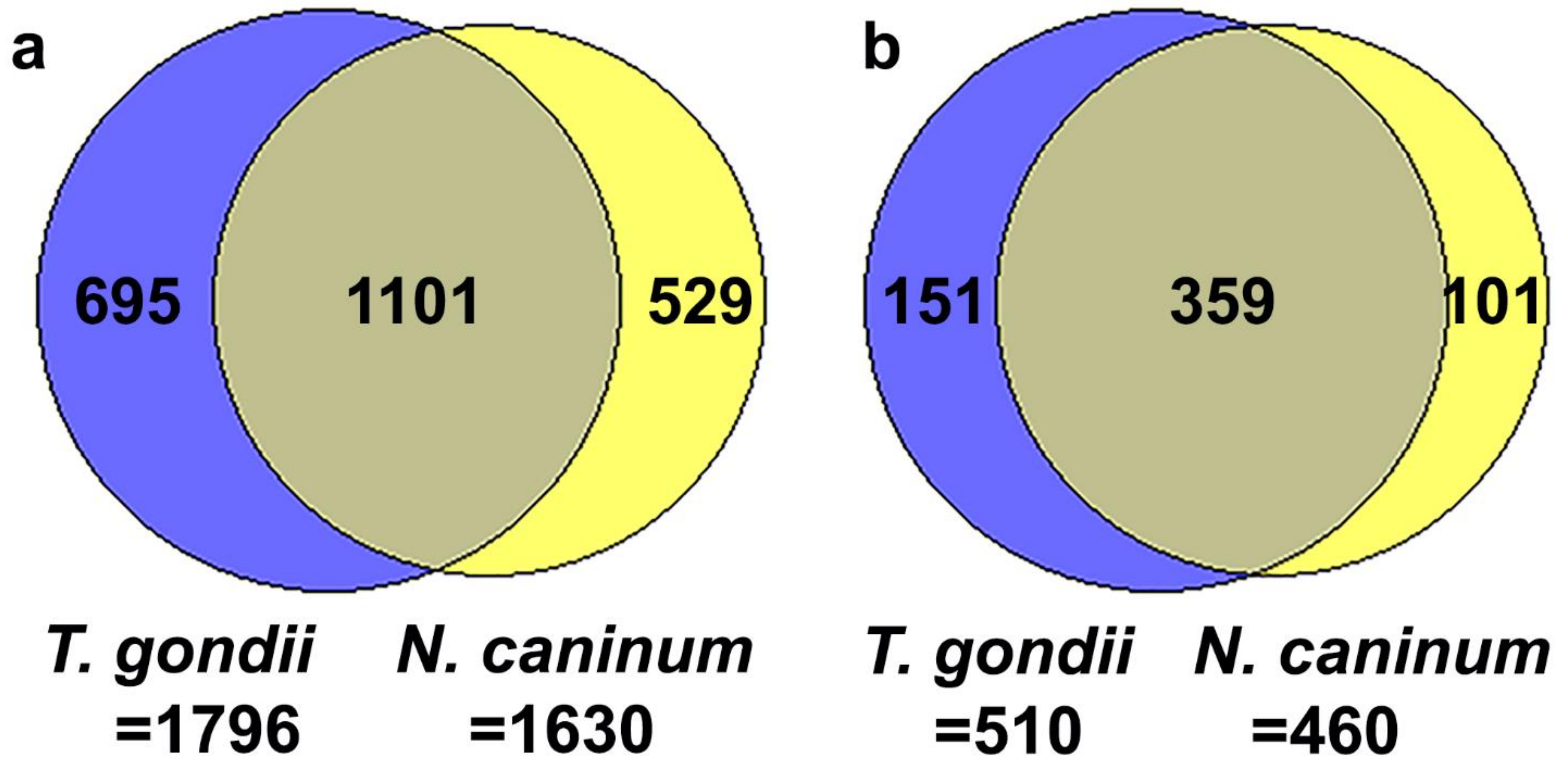

Fig. 2 Comparison of non-redundant phosphopeptides and phosphoproteins enriched using TiO_2 from infected HFF cells with *T. gondii* and *N. caninum*. The total number of phosphopeptides (a) and phosphoproteins (b) identified are shown

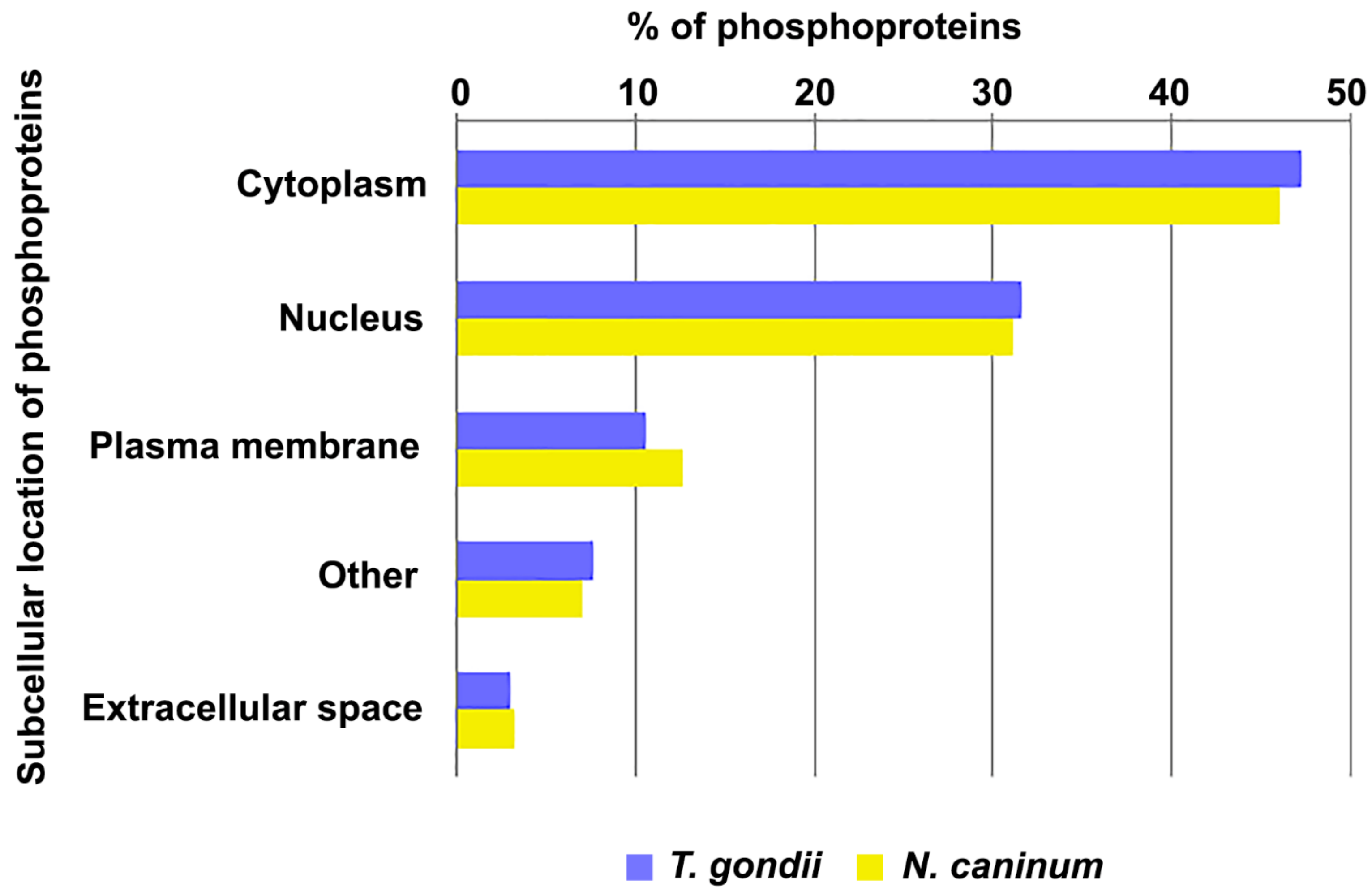

Fig. 3 Subcellular location of enriched phosphoproteins from host cells infected with *T. gondii* and *N. caninum*.

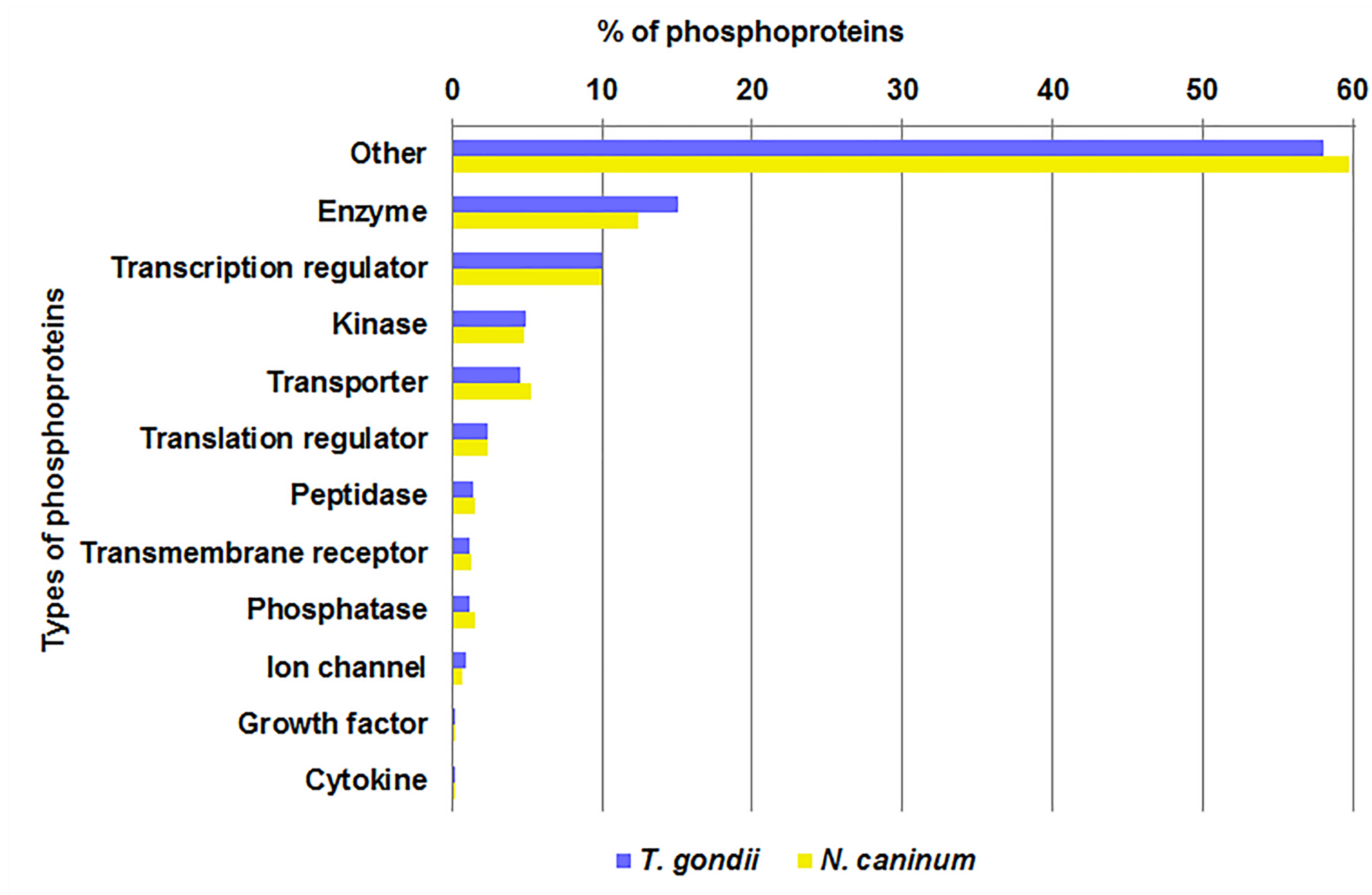

Fig. 4 Types of enriched phosphoproteins from host cell infected with *T. gondii* and *N. caninum*

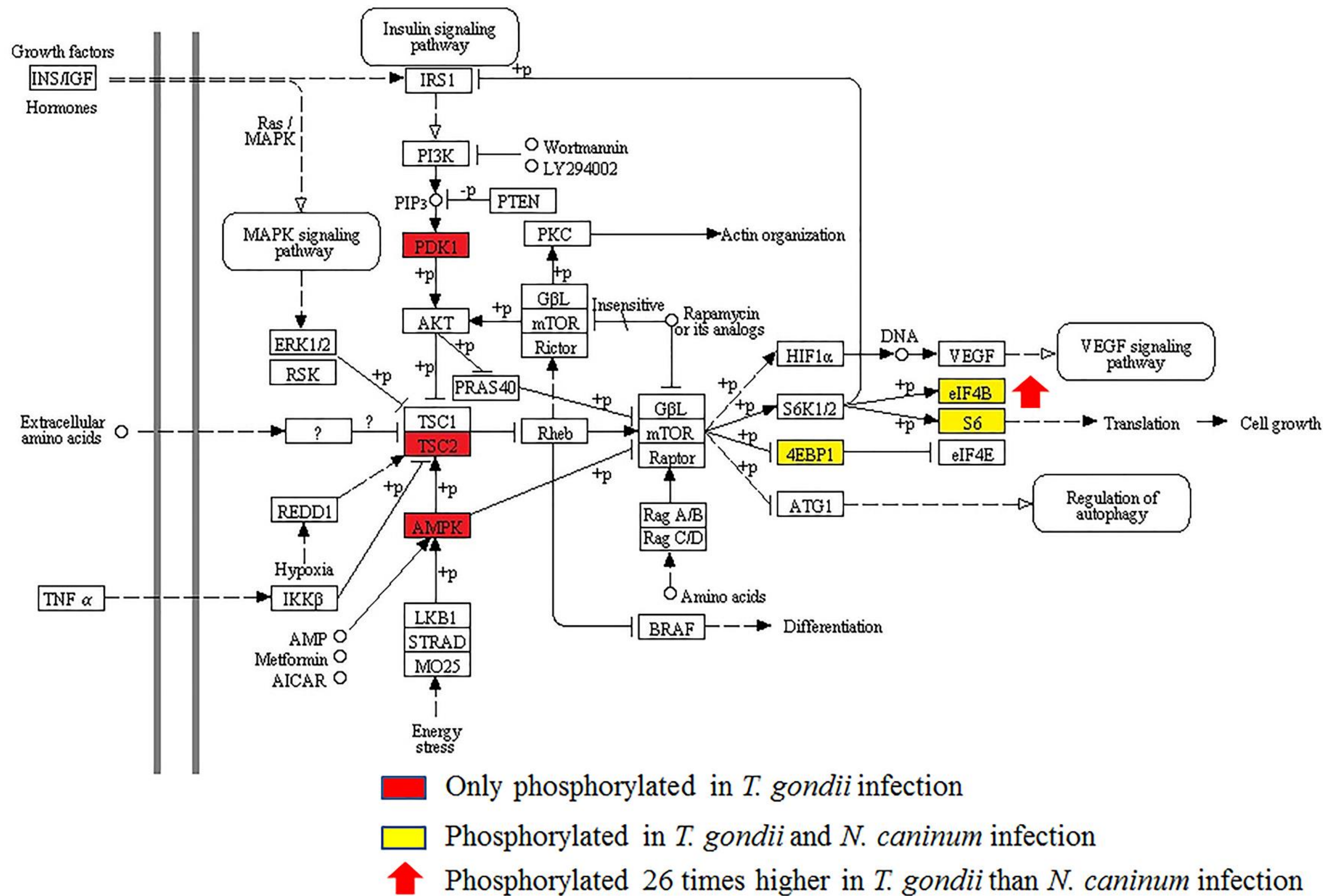

Fig. 5 mTOR signalling pathway of host cell. Enriched phosphopeptides coverage from HFF cells infected at 20 hr p.i. with *T. gondii* and *N. caninum*. Red boxes: phosphopeptides phosphorylated only in *T. gondii* infection. Yellow boxes: phosphopeptides phosphorylated in *T. gondii* and *N. caninum* infections. Pathway is available online on the KEGG pathway site http://www.genome.jp/kegg/pathway.html (Kanehisa and Goto 2000).

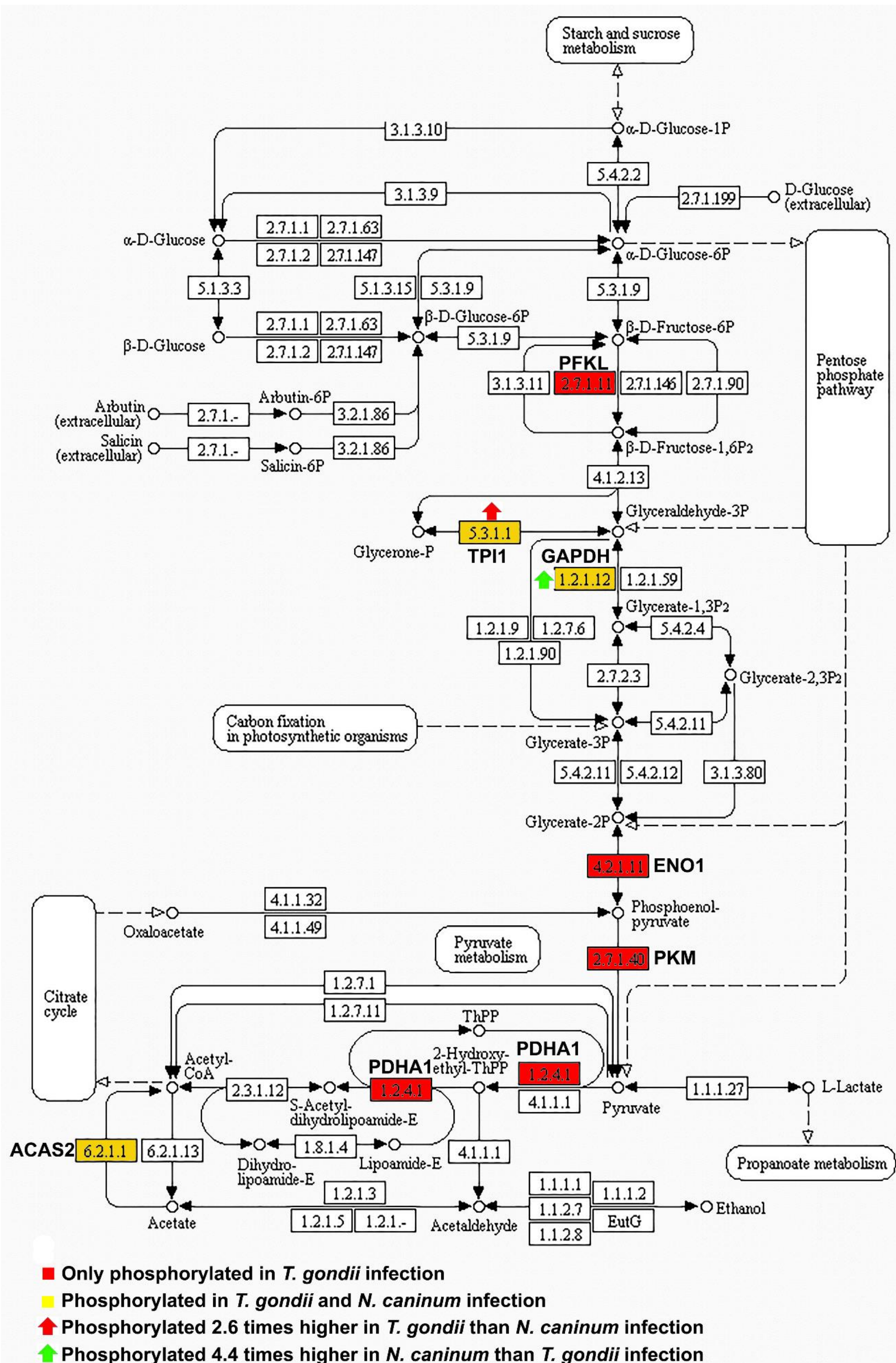
Starch and sucrose metabolism
α-D-Glucose-1P
3.1.3.10
5.4.2.2
3.1.3.9
2.7.1.199
D-Glucose (extracellular)
α-D-Glucose
2.7.1.1
2.7.1.63
2.7.1.2
2.7.1.147
α-D-Glucose-6P
5.1.3.3
5.1.3.15
5.3.1.9
β-D-Glucose-6P
β-D-Glucose
β-D-Fructose-6P
PFKL
3.1.3.11
2.7.1.11
2.7.1.146
2.7.1.90
Pentose phosphate pathway
Arbutin (extracellular)
2.7.1.-
Arbutin-6P
3.2.1.86
Salicin (extracellular)
Salicin-6P
β-D-Fructose-1,6P2
4.1.2.13
Glyceraldehyde-3P
5.3.1.1
Glycerone-P
TPI1
GAPDH
1.2.1.12
1.2.1.59
Glycerate-1,3P2
1.2.1.9
1.2.7.6
5.4.2.4
1.2.1.90
2.7.2.3
Glycerate-2,3P2
Carbon fixation in photosynthetic organisms
5.4.2.11
Glycerate-3P
5.4.2.12
3.1.3.80
Glycerate-2P
4.2.1.11
ENO1
4.1.1.32
4.1.1.49
Oxaloacetate
Phosphoenol-pyruvate
Pyruvate metabolism
2.7.1.40
PKM
Citrate cycle
1.2.7.1
1.2.7.11
ThPP
2-Hydroxy-ethyl-ThPP
PDHA1
1.2.4.1
Acetyl-CoA
2.3.1.12
S-Acetyl-dihydrolipoamide-E
4.1.1.1
Pyruvate
1.1.1.27
L-Lactate
ACAS2
6.2.1.1
6.2.1.13
1.8.1.4
Dihydro-lipoamide-E
Lipoamide-E
Propanoate metabolism
1.2.1.3
1.1.1.1
1.1.1.2
1.1.2.7
Ethanol
Acetate
1.2.1.5
1.2.1.-
Acetaldehyde
EutG
1.1.2.8
Only phosphorylated in T. gondii infection
Phosphorylated in T. gondii and N. caninum infection
Phosphorylated 2.6 times higher in T. gondii than N. caninum infection
Phosphorylated 4.4 times higher in N. caninum than T. gondii infection

Fig. 6 Metabolic pathway coverage: Glycolysis and gluconeogenesis enriched from phosphopeptides of HFF cells infected with *T. gondii* and *N. caninum* at 20 hr p.i. Red boxes: phosphopeptides phosphorylated only in *T. gondii* infection. Yellow boxes: phosphopeptides phosphorylated in *T. gondii* and *N. caninum* infection. Pathway is available online on the KEGG pathway site (http://www.genome.jp/kegg/pathway.html (Kanehisa and Goto 2000_ENREF_29)

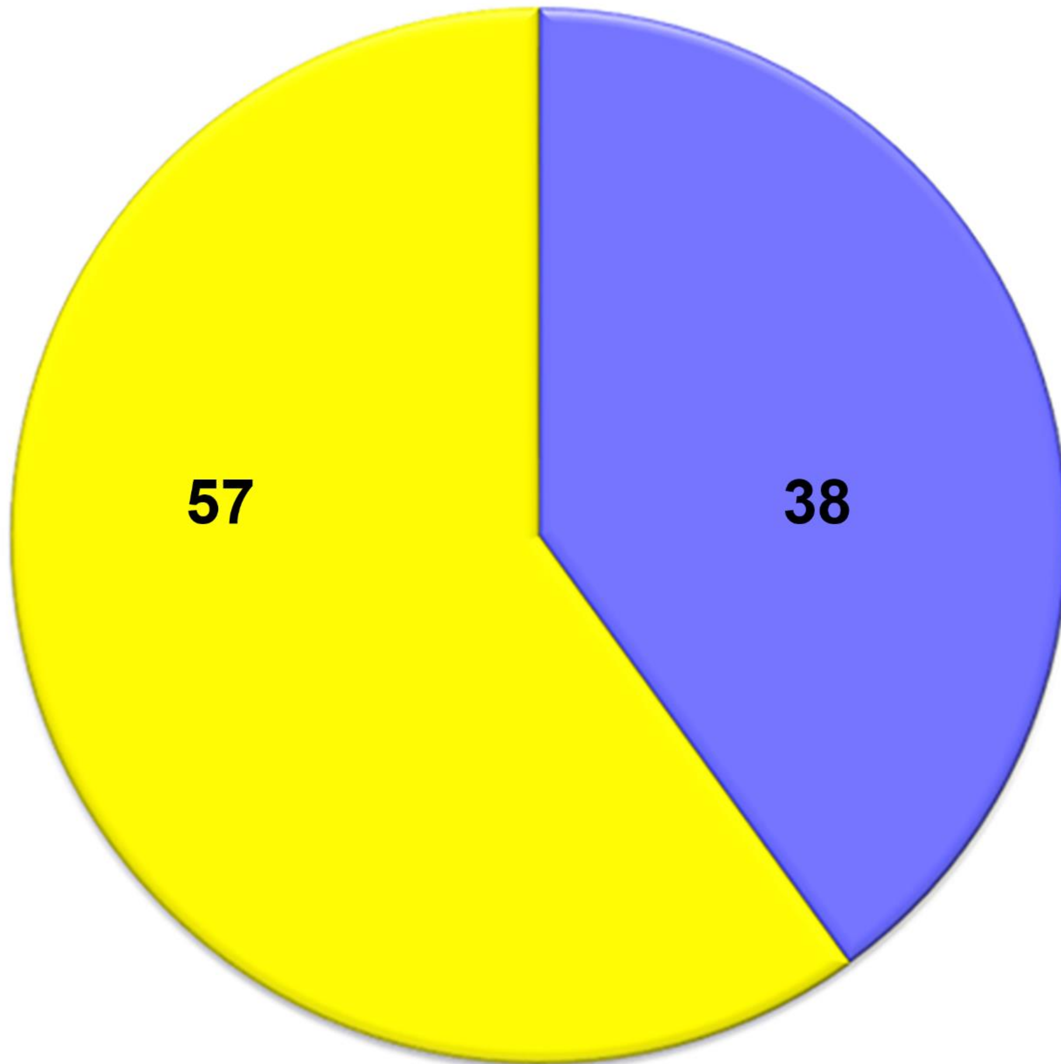

Fig. 7 Quantified phosphopeptides enriched in HFF cells infected with *T. gondii* and *N. caninum* at 20 hr p.i

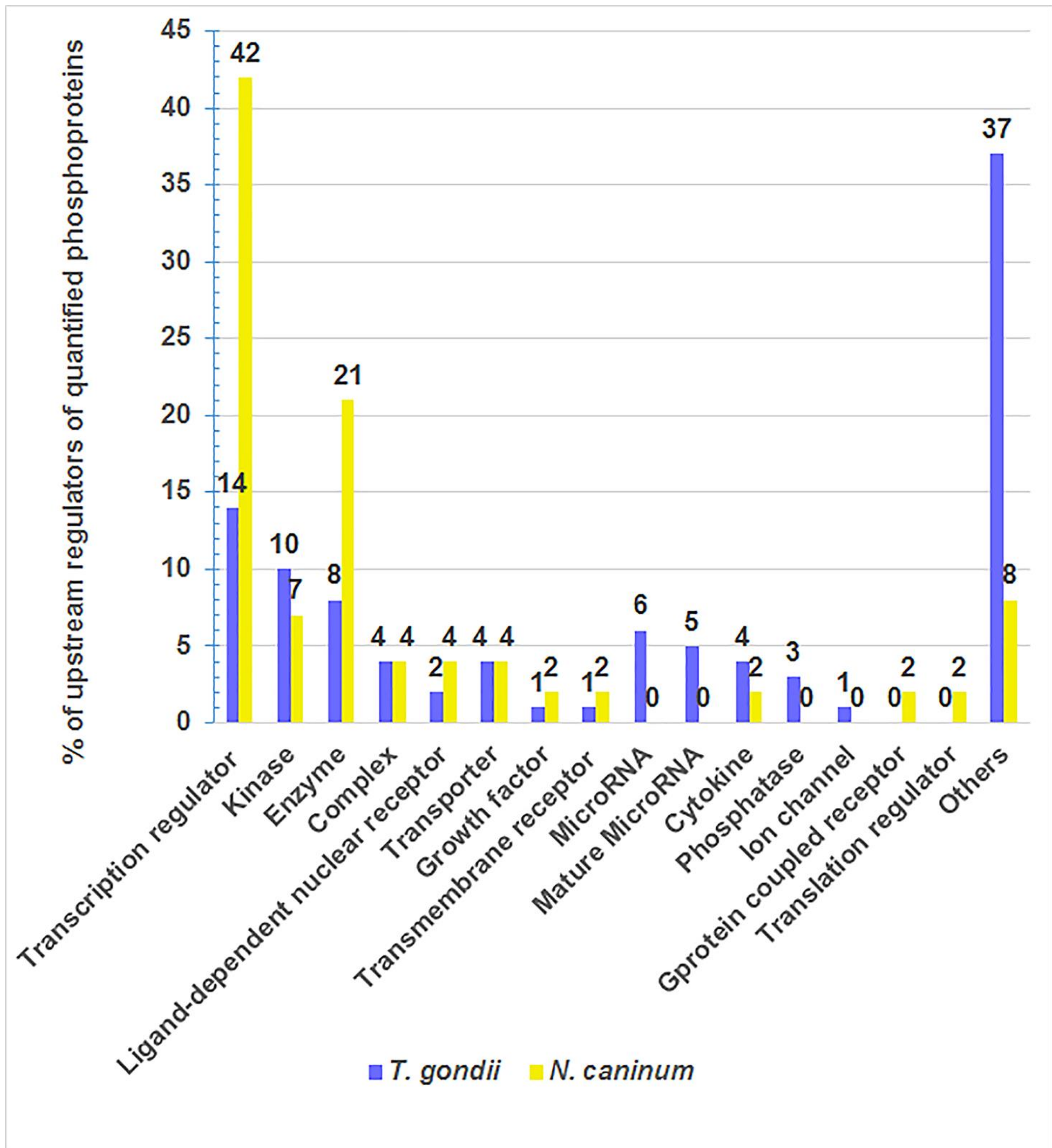

Fig. 8 Upstream analysis of quantified phosphoproteins. Types of upstream regulator of phosphoproteins quantified from host cell infected by *T. gondii* and *N. caninum*

Table 1: The phosphorylation sites identified in HFF cells infected with *T. gondii* and *N. caninum* at 20 hr p.i.**Phosphorylation type**	**No. of phosphosites in *T. gondii* infected cells**		**No. of phosphosites in *N. caninum* infected cells**	
	# sites	**% of total**	**# sites**	**% of total**
Phosphoserine	1985	85.93	1795	85.6
Phosphothreonine	310	13.42	287	13.69
Phosphotyrosine	15	0.65	15	0.72
Total	2310		2097	

Table 2: Phosphopeptides motifs enriched from host cells infected with *T. gondii* and *N. caninum* based on the phosphorylated serine amino acid.

Motif	**Motif score (*T. gondii* infected)**	**Fold increase [a]**	**Motif score (*N. caninum* infected)**	**Fold increase [a]**
....R..sP.P....	40.67	41.46	40.32	42.70
.....P.sP......	26.10	9.48	27.04	9.87
.......sP......	16.00	4.48	16.00	4.49
.......sD.E....	32.00	19.69	32.00	19.00
....RR.s.......	22.09	11.98	22.62	12.23
....R..s.......	16.00	4.35	16.00	4.40
.......sE.E....	30.26	12.94	27.56	12.33
.......sSP.....	27.88	9.43	28.55	9.80
.......s.E.....	13.39	2.79	9.84	2.55
.......s.SP....	20.29	8.42	23.52	10.54
.......s....E..	6.44	2.38	6.64	2.60
.......sP...R..	24.83	11.47	-	-
....R..s.S.....	23.65	10.32	-	-
......Rs...S...	13.19	10.97	-	-
.......s....D..	8.57	2.93	-	-
.....S.sP......	-	-	24.95	9.02
....RS.s.......	-	-	23.44	9.12
.......s.DE....	-	-	16.82	17.73
.....R.s.......	-	-	8.10	2.79

[a]: Fold changes of phosphopeptides motifs were queried against background data in international protein index (IPI) human proteome

Table 3: Phosphopeptides motifs enriched from host cells infected with *T. gondii* and *N. caninum* based on the phosphorylated threonine amino acid.

Motif	Motif score from infection with *T. gondii*	Fold increase[a]	Motif score from infection with *N. caninum*	Fold increase[a]
.......tP......	**15.95**	**3.72**	**16.00**	**4.41**
.....SPt.......	**15.56**	**14.51**	**20.93**	**18.11**
....K..tP......	**22.11**	**20.72**	**-**	**-**
.......tSP.....	**14.10**	**14.74**	**-**	**-**

[a]: Fold changes of phosphopeptides motifs were queried against background data in international protein index (IPI) human proteome

Table 4: Pathways identified from phosphatides of HFF cells infected with *T. gondii* and *N. caninum*.

Enriched pathway	HFF cells infection with *T. gondii*	HFF cells infection with *N. caninum*
Adherent junction	✓	✓
Arrhythmogenic right ventricular cardiomyopathy	✓	✓
Focal adhesion	✓	✓
Insulin signalling pathway	✓	✓
Pathogenic *Escherichia coli* infection	✓	✓
Regulation of actin cytoskeleton	✓	✓
Spliceosome	✓	✓
Glycolysis/gluconeogenesis	✓	×
MTOR signalling pathway	✓	×
Endometrial cancer	✓	×
ErbB signalling pathway	×	✓
Gap junction	×	✓
Long-term potentiation	×	✓
Renal cell carcinoma	×	✓
Vascular smooth muscle contraction	×	✓