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Author/s:

Batty, MB;Schittenhelm, RB;Dorin-Semblat, D;Doerig, C;Garcia-Bustos, JF

Title:

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Date:

2020-06-01

Citation:

Batty, M. B., Schittenhelm, R. B., Dorin-Semblat, D., Doerig, C. & Garcia-Bustos, J. F. (2020). Interaction of Plasmodium falciparum casein kinase 1 with components of host cell protein trafficking machinery. *IUBMB Life*, 72 (6), pp.1243-1249. <https://doi.org/10.1002/iub.2294>.

Persistent Link:

<https://hdl.handle.net/11343/275697>

Interaction of *Plasmodium falciparum* Casein kinase 1 (PfCK1) with components of host cell protein trafficking machinery.

Mitchell B Batty¹, Ralf B Schittenhelm², Christian Doerig^{1,3*}, Jose Garcia-Bustos^{1*}.

¹Infection and Immunity Program and Department of Microbiology, Biomedicine Discovery Institute, Monash University, Clayton 3800, Victoria, Australia

²Monash Proteomics & Metabolomics Facility, Department of Biochemistry and Molecular Biology, Monash Biomedicine Discovery Institute, Monash University, Clayton 3800, Victoria, Australia.

³Biomedical Sciences Cluster, School of Health and Biomedical Sciences, RMIT University, Bundoora, 3083, Victoria, Australia

*Corresponding authors

Abstract

A pool of *Plasmodium falciparum* casein kinase 1 (PfCK1) has been shown to localise to the host red blood cell (RBC) membrane and be secreted to the extracellular medium during trophozoite stage of development. We attempted to identify mechanisms for secretion of PfCK1 and its appearance on the RBC membrane. We found that two host proteins with established functions in membrane trafficking in higher eukaryotes, GTPase-activating protein and Vps9 domain-containing protein (GAPVD1) and Sorting nexin 22 (SNX22), consistently co-purify with PfCK1, suggesting the parasite utilises trafficking pathways previously thought to be inactive in RBCs. Furthermore, reciprocal immunoprecipitation experiments with GAPVD1 identified parasite proteins suggestive of a protein recycling pathway hitherto only described in higher eukaryotes. Thus, we have identified components of a trafficking pathway involving parasite proteins that act in concert with host proteins, and which we hypothesise mediates trafficking of PfCK1 to the RBC during infection.

Key words: *Plasmodium falciparum*, PfCK1, GAPVD1, SNX22, protein trafficking

Abbreviations

PtdIns3, phosphatidylinositol-3-phosphate; *PX*, phox homology domain; *PfCK1*, *Plasmodium falciparum* casein kinase 1; *RBC*, red blood cell; *SNX22*, sorting nexin 22; *GAPVD1*, GTPase-activating protein and Vps9 domain-containing protein 1; *Vps51*, vacuolar protein sorting-associated protein 51; *CK1 α* , casein kinase 1, isoform alpha; *PTM*, post-translational modifications; *PEXEL*, *Plasmodium* export element; *PNEPS*, *PEXEL*-negative exported proteins; *GFP*, green fluorescent protein; *PCA*, principle component analysis; *PI3K*, phosphatidylinositol-3 kinase; *PfRab5*, *Plasmodium falciparum* Ras-related protein 5; *PfRab7*, *Plasmodium falciparum* Ras-related protein 7; *AMPK*, AMP-activated protein kinase; *IGEPAL CA-630*, Octylphenoxy poly(ethyleneoxy)ethanol, branched; *DTT*, Dithiothreitol; *ACN*, acetonitrile; *ABC*, ammonium bicarbonate; *SDS-PAGE*, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; *LFQ*, label free quantification.

1. Introduction

Post-translational modifications (PTMs) are essential biochemical switches that regulate protein interactions and activity, and thereby many cellular functions in all eukaryotes, including Apicomplexa (1). A PTM of major importance is protein phosphorylation, a dynamic process regulated by protein kinases and phosphatases, with over 500 protein kinase and more than 200 protein phosphatase coding genes identified in humans (2, 3). In the malaria parasite *Plasmodium falciparum*, the number of kinases is smaller, with 86-99 annotated protein kinases depending on the parameters used in each study (4-7). Of these, 36 have been identified as likely essential for completion of the asexual proliferation cycle in red blood cells (RBCs) (8), the

part of the parasite's life cycle that is responsible for malaria pathogenesis and therefore the target of most current antimalarials. In view of the success in targeting protein kinases in other diseases such as cancer (9), *P. falciparum* protein kinases are attractive targets for drugs with novel modes of action and, therefore, free of any pre-existing

resistance mechanisms (10).

Casein kinase 1 (CK1) is highly conserved in eukaryotic organisms and exists as seven active isoforms in mammalian cells whose activity is necessary for multiple cellular processes (reviewed in (11)). CK1 recognises and phosphorylates substrates "primed" by previous phosphorylation (12). Early studies on the determinants of substrate recognition by CK1 also showed that clusters of

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3-5 acidic residues upstream of a Ser/Thr are capable of directing CK1 phosphorylation, albeit with lower affinity than pre-phosphorylated sites (13). Mutations in CK1, or inhibition of its kinase activity, results in the dysregulation of biochemical pathways that can lead to disease, including cancer (14); human CK1 is therefore an attractive therapeutic target. *P. falciparum* CK1 (PfCK1) is present as a single 36kDa isozyme and is one of the 36 aforementioned parasite-encoded protein kinases identified as likely essential for asexual blood stages. Recent work has identified the subcellular distribution of PfCK1, and interactomic analysis pointed to cellular processes involving this enzyme (15). During the ring to early trophozoite phase, a large proportion of PfCK1 appears to be associated with the host RBC membrane, making it an ectokinase (a protein kinase with an extracellularly facing kinase domain). PfCK1 activity has been detected in the supernatant of trophozoite stage parasite cultures, indicating that this protein is also secreted during the asexual lifecycle (15). These observations are similar to those made in *Leishmania*, where ectokinase status and secretion to the culture medium have been observed for one of the CK1 homologues present in this species (16, 17). During progression to the schizont stage, PfCK1 loses its association with the host cell membrane and localisation within the parasite is more prominent, until PfCK1 becomes detectable only in daughter cells inside segmented schizonts. The association with the host cell membrane at the trophozoite stage is intriguing, because PfCK1 lacks a PEXEL (Plasmodium EXPORT ELEMENT) motif or any form of recognisable export signal (18), and it is therefore probably not trafficked by canonical pathways. Thus, PfCK1 can be added to list of "PEXEL-negative exported proteins (PNEPs)" (19) highlighting the existence of alternative export pathways.

We hypothesised that trafficking of PfCK1 to the host cell plasma membrane involves interactions with a host protein transport machinery. In this study, we used immunoprecipitation coupled with mass spectrometry to show that PfCK1 co-precipitates with host proteins with known functions in protein trafficking and secretion in higher eukaryotes. Further, reciprocal immunoprecipitation experiments identified parasite homologs to components of

protein recycling pathways, hinting to a hitherto unexplored mechanism for PfCK1 transport to the RBC membrane.

2. Materials and methods

2.1. Parasite culture

Wild type 3D7 *P. falciparum* blood stage parasites, and 3D7 parasites expressing PfCK1-GFP from the endogenous locus were cultured as previously described (20). Parasites expressing endogenous GFP-tagged PfCK1 were cultured under 5.4 μ M Blasticidin S-HCl selection. To estimate lifecycle stage and parasitaemia, 2 μ l of parasite culture was prepared as a thin blood smear on a glass slide, fixed in 100% methanol and stained in a 10% v/v aqueous Giemsa solution. Ring stage parasites were synchronised by sorbitol lysis and trophozoite stage parasites were harvested by magnetic isolation.

2.2. Affinity purification

PfCK1-GFP immunoprecipitations were performed according to the GFP Trap manufacturers protocol (Chromotek). Briefly, 1x10⁸ packed RBCs infected with trophozoite stage parasites expressing PfCK1-GFP from the endogenous locus or wild-type control parasites were resuspended in 2mL of ice-cold filtered lysis buffer supplemented with protease and phosphatase inhibitors and incubated for 30min with end-over-end rotation at 4°C followed by centrifugation at 20,000 x g for 10min at 4°C. Clarified supernatants were precleared with 50 μ l of washed unconjugated agarose beads for 1hr at 4°C and incubated with 30 μ l of equilibrated GFP-Trap agarose beads for 2hrs at 4°C. Beads were washed with 3x 1ml of wash buffer supplemented with protease inhibitors, 500mM NaCl and 1% v/v IGEPAL CA-630 and 2x 1ml of wash buffer before eluting in 60 μ l of 2X reducing sample buffer. GAPVD1 immunoprecipitation was performed by resuspending 1x10⁸ magnet-purified trophozoite stage parasites in 500 μ l of cold lysis buffer, incubated for 30 min at 4°C with rotation and clarified as above. Supernatants were incubated overnight at 4°C under rotation with 4 μ g/ml rabbit anti-Gapex-5 antibodies (GAPVD1, Abcam) bound to Protein A agarose. Beads were centrifuged, washed as above and complexes eluted firstly by incubating beads with 50 μ l of 2X

non-reducing sample for 10min at 50°C (elution 1), followed by a second elution step in 2X reducing sample buffer and incubated for 10min at 50°C (elution 2). DTT (100mM) was added to elution 1 and samples were boiled for 5min at 98°C and both elution's pooled together. An equal number of uRBCs were prepared as a control. Samples were briefly run on SDS-PAGE for 5 mins, stained with Coomassie and a single band containing all proteins was excised from the gel for subsequent analysis.

2.3. In-gel digestion

Coomassie-stained protein bands were excised and destained with 50% acetonitrile (ACN) in 100 mM ammonium bicarbonate pH 8.5 (ABC). The proteins were subsequently reduced with 10 mM DTT (Astral Scientific) and carbamidomethylated with 20 mM chloroacetamide (Sigma). The gel was completely de-hydrated using 100% ACN and re-hydrated in digestion solution, which contained 20 ng/μl trypsin (Promega), 100 mM ABC, 5% ACN. After overnight digestion at 37°C, the tryptic peptides were extracted from the gel with 50% ACN, 5% formic acid, lyophilized in a vacuum concentrator and purified using OMIX C18 Mini-Bed tips (Agilent Technologies) prior to LC-MS/MS analysis.

2.4. Mass spectrometry and data analysis

A Dionex UltiMate 3000 RSLCnano system equipped with a Dionex UltiMate 3000 RS autosampler was used. Samples were loaded via an Acclaim PepMap 100 trap column (100 μm x 2 cm, nanoViper, C18, 5 μm, 100Å; Thermo Scientific) onto an Acclaim PepMap RSLC analytical column (75 μm x 50 cm, nanoViper, C18, 2 μm, 100Å; Thermo Scientific). The peptides were separated by increasing concentrations of buffer B (80% acetonitrile/0.1% formic acid) for 128 min and analyzed with an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific) operated in data-dependent acquisition mode using in-house, LFQ-optimized parameters. Acquired raw data files were analyzed using MaxQuant (21) to obtain protein identifications and their respective label-free quantification values. Statistical analysis was performed using an in-house generated R script based on the ProteinGroup.txt file. First, contaminant proteins, reverse sequences and proteins identified "only by site" were filtered out. In addition, proteins that have been only

identified by a single peptide and proteins not identified/quantified consistently in the same condition have been removed as well. The LFQ data was converted to log₂ scale, samples were grouped by conditions and missing values were imputed using the 'Missing not At Random' (MNAR) method, which uses random draws from a left-shifted Gaussian distribution of 1.8 standard deviations apart with a width of 0.3. Protein-wise linear models combined with empirical Bayes statistics were used for the differential expression analyses. The Limma package (R Bioconductor) was used to generate a list of differentially expressed proteins for each pair-wise comparison. A cutoff of the adjusted p-value of ≤0.05 (Benjamini-Hochberg method) along with a log₂ fold-change of ≥1.5 has been applied to determine significantly regulated proteins in each pairwise comparison.

3. Results and discussion

Using mass spectrometric analysis, we interrogated the protein composition of immunoprecipitates obtained by GFP-trap purification, using lysates from cells infected with parasites expressing PfCK1-GFP from the endogenous chromosomal locus. A 3D7 wild-type parasite line was used as a negative control and the results of three independent experiments are presented in a volcano plot in Fig. 1A. Good separation between PfCK1-GFP and wild-type samples was observed in the principal component analysis (PCA) (Supp. Fig. S1) and PfCK1 was identified in precipitates obtained from PfCK1-GFP lines, but not from wild-type-infected samples, as expected (Fig. 1A).

We reproducibly identified ten proteins as significantly more abundant in PfCK1-GFP than in wild-type samples (Supp. Table S1), the three strongest hits being host proteins: (i) GTPase-activating protein-VPS9 domain-containing protein 1 (GAPVD1, also known as Gapex-5), (ii) Sorting Nexin 22 (SNX22) and (iii) CK1α. This is consistent with published observations that GAPVD1 and SNX24 (which clusters with SNX22 in phylogenetic trees) co-purify with mammalian CK1 isoforms in bait-prey pull-down experiments (22, 23). Sorting nexins anchor cargo proteins to membranes enriched in phosphatidylinositol-3-phosphate (PtdIns3P) *via* their Phox homology (PX) domain (Fig. 1C) and have been implicated in protein secretion (24).

Although no functions are currently described for SNX22, other members of this family, such as SNX3, are well-studied and known to function in directing retromer-mediated vesicle transport to the *trans*-Golgi network (TGN), a conserved trafficking pathway which guides the retrieval, sorting, recycling and retrograde transport of membrane receptors (25). Phosphorylation within the PX domain of SNX3 abolishes PtdIns binding, resulting in cytosolic localisation (26) and exemplifying the modulation of SNX function by protein kinases.

GAPVD1 contains an evolutionarily conserved Vps9 domain (Fig. 1C) that functions as a guanine nucleotide exchange factor (GEF) for Ras-related GTP-binding proteins (27) and it has well-known functions in receptor trafficking (28, 29). Trafficking of GAPVD1 to the plasma membrane also results in the synthesis and turnover of PtdIns3, through the coordination of the GTPase Rab5 and PtdIns3P-kinases (PI3K) (30). *P. falciparum* does not encode homologs of GAPVD1 or SNX22, but it does encode Rab GTPases such as PfRab5 (involved in trafficking PfCK1 to the parasite plasma membrane (31)) and PfRab7, which co-localises with components of the retrograde transport complex in punctate structures near the Golgi apparatus (32).

In light of their various roles in protein trafficking and their high abundance in our PfCK1-GFP immunoprecipitates, we tested the association of PfCK1 with GAPVD1 and SNX22. To this end, we performed Western blot analysis of immunoprecipitates obtained with GFP-Trap beads from cells infected with PfCK1-GFP parasites, probing each blot with antibodies against GAPVD1 and SNX22. Multiple bands were observed in blots probed with anti-GAPVD1 antibody (Fig. 1B), suggesting multiple phosphorylation states. Indeed, eight phosphopeptides that mapped to various regions of GAPVD1 (Table 1 and Supp. Table S2) were exclusively identified in PfCK1-GFP precipitates. None of these phosphopeptides map to the Vps9 domain or the Ras-GAP domain (Fig. 1C), suggesting they probably do not directly regulate GTP turnover (although allosteric effects cannot be excluded), but likely function in regulating other essential functions of GAPVD1, such as adaptor protein interactions. Five of the eight phosphosites (T391, S903, S904, S972 and S999) have been previously described in GAPVD1 from

human and mouse cells (33, 34), including S903 (corresponding to PfCK1 residue S902) in uninfected RBCs (35). The other three phosphosites (Y461, S915 and S1104) are hitherto undescribed in GAPVD1 proteins (Table 1). As mentioned above, CK1 enzymes are adept at phosphorylating acidic substrates. Intriguingly, GAPVD1 S1104 is located downstream of a cluster of Asp residues, suggesting that phosphorylation at this site may be due to PfCK1 activity. Although the functional consequences of phosphorylating GAPVD1 at these sites remains to be elucidated, it is known that phosphorylation of S903 is directed by AMP-activated protein kinase (AMPK) (36), indicating that GAPVD1 is indeed a substrate for cellular kinases.

We next verified the interaction between GAPVD1 and PfCK1 in infected cells by performing reciprocal immunoprecipitation using an antibody against GAPVD1. Immunoprecipitates were prepared from lysates of wild-type 3D7 parasite-infected RBCs, using antibodies against human GAPVD1 bound to Protein A Sepharose beads. Bound proteins were eluted, digested and analysed by mass spectrometry as described in Materials and Methods. Mass spectra were compared to those from human and *Plasmodium* proteomes and the results from independent triplicate experiments are displayed as a volcano plot in Fig. 2A. Good separation between wild-type infected and uninfected RBCs was again observed in the PCA analysis (Supp. Fig. S2) and PfCK1 was detected among the 48 proteins identified as potential GAPVD1 interactors (Fig. 2A). This result is consistent with PfCK1 forming a complex with GAPVD1. Within this set of 48 proteins (Supp. Table S1), we also identified *P. falciparum* Vacuolar protein sorting-associated protein 51 (PfVps51) as a high probability interactor with GAPVD1 (Fig. 2A). In eukaryotes, Vps51 is a known binding partner of SNARE proteins (37) and could thus participate in the trafficking of PfCK1, as the SNARE-associated protein SNAPIN is a known substrate of CK1 δ and localises to Golgi membranes in mammalian cells (38). Importantly, Vps51 and SNARE homologues are not only present in *Plasmodium* but also in *Leishmania* (39, 40), indicating that this protein transport pathway is probably conserved across parasitic protists. This is consistent with protein trafficking and export being one of the most

represented functional groups among parasite proteins identified in our analysis of GAPVD1 precipitates (Fig. 2B). The identification of components from multiple trafficking pathways in our dataset strongly supports the hypothesis that PfCK1 is transported by non-conventional processes involving host and parasite proteins. We cannot exclude that at least some of the parasite proteins identified in this experiment result from non-specific binding; this dataset nevertheless provides hypotheses that can be tested in future work.

In summary, we provide evidence for (i) the presence of GAPVD1 and SNX22-dependent protein trafficking pathways in human RBCs, and (ii) the physical interaction of PfCK1 with these host RBC proteins. Furthermore, our co-precipitation data suggests that GAPVD1 and SNX22 collaborate in the process, providing initial evidence of crosstalk between these transport pathways. We are currently investigating human host interactors of SNX22 and GAPVD1 to gain insights into their roles in RBCs, previously assumed not to have functional protein transport pathways. The identification of PfCK1 as an interactor with these proteins suggests that, during infection, *P. falciparum* parasites commandeer remnant but functional components of the endosomal trafficking machinery in RBCs, repurposing these for trafficking parasite proteins to membranes and to the external medium. Our data also suggests an interaction between parasite endosomal processes and host protein trafficking machinery.

Conflicts of interest

None declared

Acknowledgements

We would like to thank the Monash Proteomics & Metabolomics facility for their expertise. We acknowledge Megan Bird for general laboratory support, Simona John von Freyend, Jack Adderley and William Poole for useful discussion and we are thankful to the Australian Red Cross Blood Service for providing RBCs for this study.

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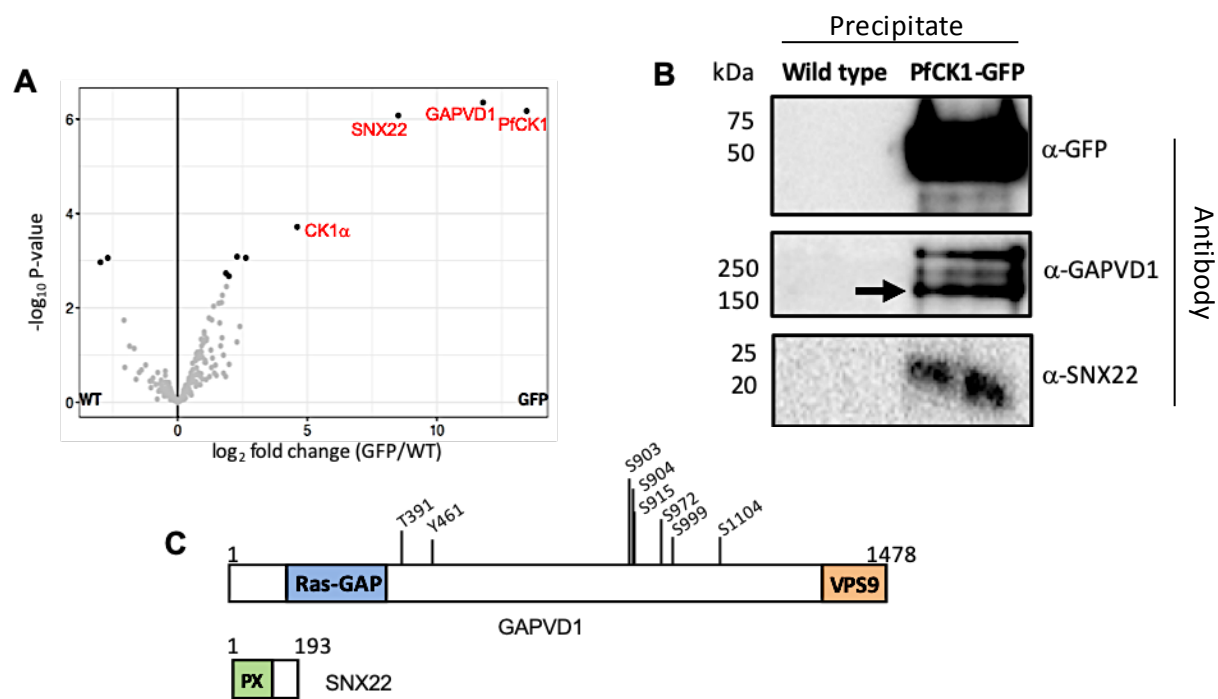


Figure 1. Label-free quantitative analysis of human interactors of PfCK1. (A) Volcano plot representing the logarithmic ratio of LFQ values between PfCK1-GFP/wild-type (WT) samples, plotted against the negative logarithm of p-values obtained from Student's t-test of triplicate experiments. High probability interactors (black) were separated from background (grey) using an adjusted p-value cut-off of ≤ 0.05 and log fold change ≥ 1.5 . (B) Analysis of immunoprecipitation experiments by Western blot. For each experimental condition, rabbit polyclonal antibodies against GFP, GAPVD1 and SNX2 were used to detect the presence of each protein in the precipitates. Arrow indicates the estimated size of GAPVD1. (C) Schematic diagram of the domain organisation of GAPVD1 and SNX2; GTPase activating protein (Ras-GAP), Guanine nucleotide exchange factor (VPS9) and Phox homology domain (PX). GAPVD1 phosphosites identified in PfCK1-GFP precipitates are annotated.

Table 1. Number of GAPVD1 phosphopeptides obtained from PfCK1-GFP immunoprecipitations.

Phosphosite	GFP 1	GFP 2	GFP 3	Peptide
T391	-	2	1	RAVETPPLSSVNLEGLSRT
Y461*	-	2	-	KSSSLEMTPYNTPLSPATTPANKKN
S903	2	2	4	RSRSDIVSSVRR
S904	-	2	3	RSDIVSSVRR
S915*	-	1	-	RRPMSDPSWNRR
S972	-	1	-	KDDPSPRLSAQAQVAEDILDKY
S999	2	4	-	KRTSPSDGAMANYESTEVMGDGEAHDSPRD
S1104*	2	4	1	RDEALQNISADDLPDSASQAHPQDSAFSYRD

Red lettering indicates identified phosphosites and possible CK1 recognition motifs are underlined. GFP 1, 2 and 3 refers to individual replicate experiments performed using the parasites expressing PfCK1-GFP from the endogenous locus. *GAPVD1 phosphorylation sites identified in this study that have not been observed previously.

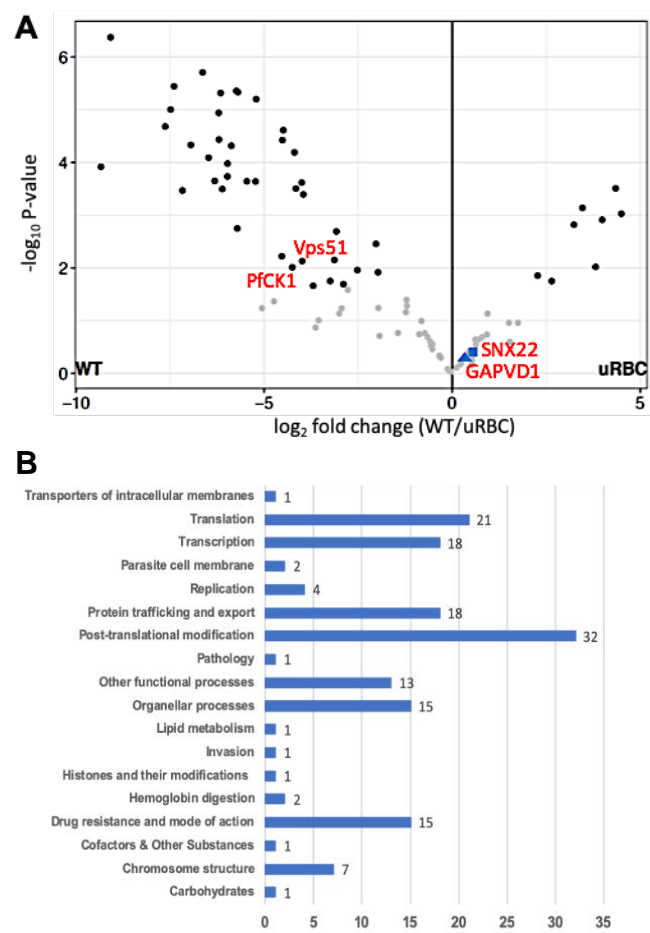


Figure 2. Label-free quantitative analysis of parasite interactors of GAPVD1. (A) Volcano plot representing the logarithmic ratio of LFQ values between wild type (WT) infected RBS/uninfected RBCs (uRBCs) incubated with GAPVD1 antibody which are plotted against the negative logarithmic p-values obtained from student's t-test of triplicate experiments. Cut-off values of ≤ 0.05 for p-value and ≥ 1.5 log fold change was used to separate high probability interactors (black) from background (grey) proteins. Key parasite proteins are annotated in red and GAPVD1 and SNX22 (solid triangle and square respectively) are also marked. (B) Parasite proteins identified as high-probability interactors of GAPVD1 distributed across the various metabolic pathways of the malaria parasite. The histogram represents the number of parasite proteins mapped to each pathway described in the Metabolic Pathways of Malaria Parasites website (<http://mpmp.huji.ac.il/>).