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In silico prediction of antimalarial drug target candidates

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ABSTRACT

The need for new antimalarials is persistent due to the emergence of drug resistant parasites. Here we aim to identify new drug targets in *Plasmodium falciparum* by phylogenomics among the *Plasmodium* spp. and comparative genomics to *Homo sapiens*. The proposed target discovery pipeline is largely independent of experimental data and based on the assumption that *P. falciparum* proteins are likely to be essential if (i) there are no similar proteins in the same proteome and (ii) they are highly conserved across the malaria parasites of mammals. This hypothesis was tested using sequenced Saccharomycetaceae species as a touchstone. Consecutive filters narrowed down the potential target space of *P. falciparum* to proteins that are likely to be essential, matchless in the human proteome, expressed in the blood stages of the parasite, and amenable to small molecule inhibition. The final set of 40 candidate drug targets was significantly enriched in essential proteins and comprised proven targets (e.g. dihydropteroate synthetase or enzymes of the non-mevalonate pathway), targets currently under investigation (e.g. calcium-dependent protein kinases), and new candidates of potential interest such as phosphomannose isomerase, phosphoenolpyruvate carboxylase, signaling components, and transporters. The targets were prioritized based on druggability indices and on the availability of in vitro assays. Potential inhibitors were inferred from similarity to known targets of other disease systems. The identified candidates from *P. falciparum* provide insight into biochemical peculiarities and vulnerable points of the malaria parasite and might serve as starting points for rational drug discovery.

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1. Introduction

Drug discovery programs launched by the Medicines for Malaria Ve... na... pounds such as the synthetic peroxide OZ439 (Charman et al., 2011) and the spiroindolone NITD 609 (Rottmann et al., 2010), which are currently undergoing clinical trials. In spite of these recent successes, it is pivotal to maintain early phase drug discovery to prevent the antimalarial drug development pipeline from draining. Due to the propensity of the parasite to become drug-resistant (Muller and Hyde, 2010; Sa et al., 2011), the need for new antimalarial chemotypes will persist until the human-pathogenic *Plasmodium* spp. are eventually eradicated.

Rational post-genomic drug discovery is based on the screening of large chemical libraries – either virtually or in high-throughput format – against a given target enzyme of the parasite. A persistent ... tial for survival of the parasite and sufficiently different from its closest counterpart in the human host to be inhibited selectively. Experimental tools to validate candidate drug targets are limited for the malaria parasites. Gene silencing by RNAi does not seem to be feasible (Baum et al., 2009). Gene replacement with selectable markers is (Triglia et al., 1998), but it is inherently problematic to call a gene essential from failing to knock it out. Inducible degradation of *Plasmodium falciparum* proteins that have been fused to a FKBP-destabilization domain (Armstrong and Goldberg, 2007) is currently the most conclusive method for antimalarial target validation. However, none of the reverse genetic methods is practicable at the genome-wide scale. Adding up to the challenges with *Plasmodium* molecular biology is the lack of a phylogenetically close model organism that could serve as a point of reference – as is the case with parasitic nematodes, where essentiality of genes may be estimated based on the RNAi phenotypes (Schindelman et al., 2011) of orthologues in *Caenorhabditis elegans*.

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Several bioinformatic approaches have previously been employed to help identify or prioritize drug targets for *Plasmodium* parasites. These include techniques based on automated identification of important steps in metabolic pathways (Yeh et al., 2004; Fatumo et al., 2009; Huthmacher et al., 2010; Plata et al., 2010), techniques that combine chemical starting points and protein-based queries (Joubert et al., 2009), as well as the use of the TDR-targets web-resource (http://www.tdrtargets.org) (Magarinos et al., 2012) to prioritize drug targets through the combination of multiple data types relevant to drug development (Crowther et al., 2010).

Here we try to predict antimalarial drug targets in silico, building on previous approaches by other labs for predicting essentiality of proteins based on phylogeny (Doyle et al., 2010; Waterhouse et al., 2010). We define a protein as a candidate antimalarial drug target if it (i) has conserved orthologues in all of the mammalian-pathogenic *Plasmodium* spp.; (ii) has no other match in *P. falciparum*; (iii) does not have a good match in the human proteome either; (iv) is expressed in the trophozoite and gametocyte stages; and (v) is predicted to function as an enzyme, receptor, or transporter. The rationale is that conserved genes often fulfill essential functions – or rather that genes fulfilling essential functions are under negative selection. A conserved single copy gene that lacks close matches in the same genome is likely to be indispensable. Starting from the complete predicted proteome of *P. falciparum* (Gardner et al., 2002), we applied consecutive filters to extract all candidate drug targets that meet the above criteria.

2. Material and methods

2.1. Datasets

The predicted *Plasmodium* spp. proteomes were downloaded from PlasmoDB (http://www.plasmodb.org/common/downloads) (Aurrecoechea et al., 2009), the *Saccharomyces cerevisiae* proteome from SGD (Saccharomyces genome database; http://www.down-loads.yeastgenome.org/) (Engel et al., 2010), the *Homo sapiens* proteome from EBI (ftp://www.ftp.ebi.ac.uk/pub/databases/integr8/fasta/proteomes) (Mulder et al., 2008), and all others from UniProt (http://www.uniprot.org/taxonomy) (Magrane and Consortium, 2011). *P. falciparum* 3D7 cell cycle expression data (Le Roch et al., 2003) were obtained from PlasmoDB, using as a threshold for expression $E > 10$ and probe signal distribution $\log P < -0.5$ as proposed by the authors (Le Roch et al., 2003). *S. cerevisiae* deletion phenotype data were from SGD (http://www.downloads.yeastge-nome.org/curation/literature/phenotype_data.tab). Proteins were termed essential if the phenotype of the knock-out (mutant type = 'null') of the corresponding gene was 'inviable'. The TDRtar-gets web resource (http://www.tdrtargets.org) (Magarinos et al., 2012), as well as the BRENDA database (http://www.brenda-enzy-mes.org) (Scheer et al., 2011) was used to identify proteins with precedence for interaction with small molecule chemical inhibitors. Essentiality of *Plasmodium berghei* orthologues was found through RMgmDB (Rodent Malaria genetically modified Parasites; http://www.pberghei.eu/) (Janse et al., 2011).

2.2. Programs

InParanoid was obtained from http://www.inparanoid.sbc.su.se and QuickParanoid from http://www.pl.postech.ac.kr/QuickPara-noid. BLAST 2.2.17 (Altschul et al., 1990) was downloaded from NCBI (ftp.ncbi.nlm.nih.gov), Needle (from the EMBOSS suite (Rice et al., 2000)) from http://www.emboss.sourceforge.net. All programs were run on the Basel Biocomputing Linux cluster (http://www.bc2.unibas.ch).

2.3. Algorithms

The pipeline for comparative genomics and target prediction was run in a semi-automated way, combining the existing programs (see Section 2.2) with self-made Perl scripts for parsing, re-formatting, and analysis of outputs. Druggability scores were extracted from the TDRtargets website (Magarinos et al., 2012). These druggability predictions are numerical values between 0 and 1, with 1 corresponding to more druggable targets. Scores reflect a combination of factors including degree of similarity to a large library of targets within the ChEMBL database (Hopkins et al., 2011; Gaulton et al., 2012), with empirically determined interactions with drug like compounds, possession of physiochemical features enriched in known drug targets, and the drug-like quality of the inhibitors known to interact with those homologues. These scores are outputs of a combination of multiple discriminators based on empirically-characterized drug targets, but are untested in *Plasmodium*, other than through concordance of high-scoring proteins with the very few known antimalarial drug targets. Proteins with higher druggability scores are likely to be more amenable to inhibition with drug-like molecules than proteins with low druggability scores, and hence are used here as one aspect of the ranking system, but there is lack of parasite-specific direct data for these algorithms with which to assign a cutoff for designating an acceptable score.

3. Results and discussion

3.1. Using yeast as a touchstone for essentiality prediction

The central hypothesis implemented in the present target discovery pipeline is, that a protein is likely to be essential if there are no other similar proteins in the same proteome but conserved orthologues in all the related species. Before applying it to malaria parasites, we tested the hypothesis on the yeast *S. cerevisiae* where essentiality of proteins has been tested by systematic knock-out of the respective genes (Winzeler et al., 1999). The current null mutant dataset in the *Saccharomyces* Genome Database comprises 5477 genes, of which 1109 were found to be essential. To test whether a given *S. cerevisiae* protein is unique, its best match (excluding itself) in the same proteome was identified with Blastp (Altschul et al., 1990), then and a Needleman-Wunsch global alignment (Needleman and Wunsch, 1970) was performed between the two proteins. The median global identity between a *S. cerevisiae* protein and its best match was 15.7% (red bar in Fig. 1). 14% of the proteins with a match above the median were essential, compared to 24% of those below the median (Fig. 1). The difference was
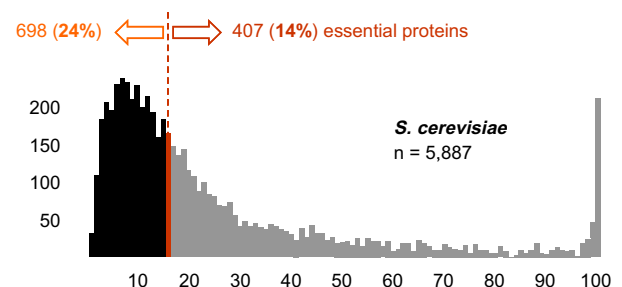
Fig. 1. *S. cerevisiae* non-redundant proteins. Frequency distribution of the similarities for all predicted *S. cerevisiae* proteins to their next best match in the same proteome, 698 were below the median identity of 15.7% (red bar) and 407 above (4 proteins had exactly 15.7% identity to their next best match). The black area represents the proteins below the threshold of ≤15% global identity. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

significant ($p < 0.0001$, two-sided Chi-square test) and supported the hypothesis that 'unmatched' proteins are more likely to be essential (Hannay et al., 2008). Setting the cut-off at ⩽15% global identity 2648 *S. cerevisiae* proteins passed (black area in Fig. 1), 26% of which were essential (Fig. 2).

To determine which of the *S. cerevisiae* proteins are conserved across the Saccharomycetaceae, a set of related (Fitzpatrick et al., 2006) and fully sequenced yeasts served as the reference: *Candida glabrata*, *Ashbya gossypii*, *Kluyveromyces lactis*, *Candida albicans*, *Debaryomyces hansenii*, and *Yarrowia lipolytica*. The predicted proteomes (Table 1) were fed into InParanoid and QuickParanoid (Ostlund et al., 2010), programs that utilize reciprocal, proteome-wide Blastp searches to build groups of orthologous proteins and rank them. A total of 3546 *S. cerevisiae* proteins possessed orthologues in all the included yeasts. The highly conserved orthology clusters, i.e. those above median similarity, were significantly enriched for essential proteins (Fig. 2): 29% of the *S. cerevisiae* proteins that belonged to a conserved cluster were essential ($p < 0.0001$, two-sided Chi-square test). Finally the intersect of the two sets, unmatched and conserved, contained 632 proteins of which 40% were essential (Fig. 2). The yeast results supporting our working hypothesis, we went on to apply it to malaria parasites.

3.2. Essentiality prediction in P. falciparum

The predicted *P. falciparum* proteome exhibits a strikingly low degree of redundancy, with the large majority of proteins exhibiting but a low level of similarity to their next best match in the same proteome (Fig. 3). As described above (Section 3.1) for *S. cerevisiae*, the closest hit to any *P. falciparum* protein was determined with Blastp. Similarity was measured by global alignment and expressed as percent identical amino acids. The median global identity between a *P. falciparum* protein and its next best match was only 13% (red bar in Fig. 3), and 3078 of the 5410 proteins passed the threshold of ⩽15% global identity to their next best match (black area in Fig. 3).

The conserved *Plasmodium* proteins were identified by comparing *P. falciparum* to all other available genome sequences of mammalian malaria parasites, namely *P. berghei*, *Plasmodium chabaudi* and *Plasmodium yoelii* (mouse), *Plasmodium knowlesi* (primate), and *Plasmodium vivax* (human tertian malaria). The six genomes encode a total of 33,704 predicted proteins (Table 1). The common intersect of proteins from orthology clusters represented in all the

Table 1
Proteomes used in this study, their sizes and sources.

| Species	Proteins	Source	Version
P. falciparum	5410	PlasmoDB	8.1
P. vivax	5396	PlasmoDB	8.1
P. berghei	4861	PlasmoDB	8.1
P. chabaudi	5119	PlasmoDB	8.1
P. knowlesi	5194	PlasmoDB	8.1
P. yoelii	7724	PlasmoDB	8.1
H. sapiens	67,172	Integr8	08/2011
S. cerevisiae	5887	SGD	R64-1-1
A. gossypii	4761	UniProt	08/2011
C. albicans	5727	UniProt	08/2011
C. glabrata	5197	UniProt	08/2011
D. hansenii	6242	UniProt	08/2011
K. lactis	5074	UniProt	08/2011
Y. lipolytica	6414	UniProt	08/2011
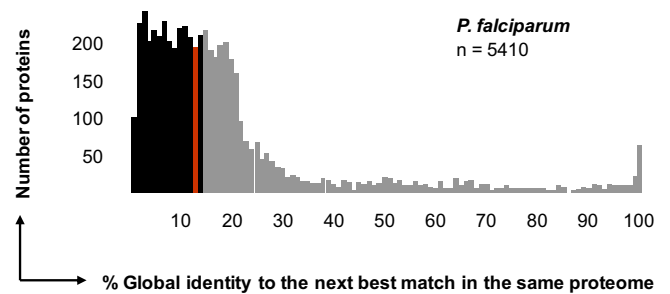
Fig. 3. *P. falciparum* non-redundant proteins. Frequency distribution of the similarities for all predicted *P. falciparum* proteins to their next best match in the same proteome. The median is shown in red, the black area represents the proteins which, passing the threshold of ⩽15% global identity, are assumed to be enriched for essential ones.

six species was 26,377, as determined with InParanoid and Quick-Paranoid (Ostlund et al., 2010). This amounts to 78% of all proteins, reflecting the close relationship of the malaria parasites. The number of proteins in the common clusters was conserved in all the
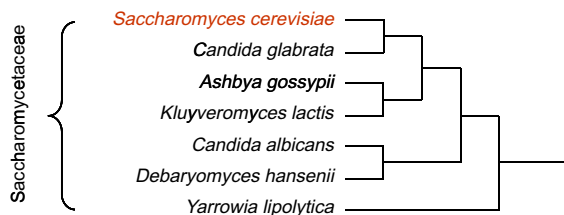
	Total	Essential	
S. cerevisiae proteome	5,477	1,109	20%
Unmatched >15% global identity	2,648	677	26%
Conserved in Saccharomycetaceae	1,773	514	29%
Unmatched & conserved	**632**	**253**	**40%**

Fig. 2. Testing for essentiality with yeasts. The cladogram depicts the phylogeny of the fully sequenced Saccharomycetaceae (Fitzpatrick et al., 2006). Eliminating the *S. cerevisiae* proteins which possess matches in the same proteome (gray area in Fig. 1), and those which are not conserved across all the analyzed yeasts, enriches for essential proteins.
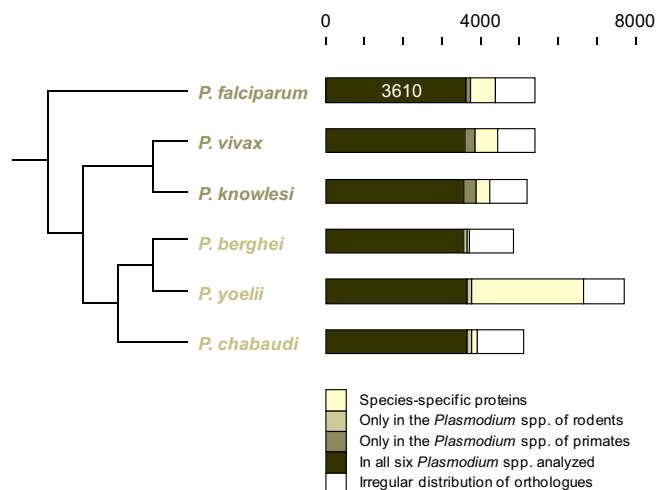
Fig. 4. The conserved malaria proteome. The cladogram depicts the phylogeny of the fully sequenced malaria parasites of mammals (Martinsen et al., 2008). The bars divide each predicted proteome into proteins present only in the species itself, proteins with orthologues only in the *Plasmodium* spp. sharing intermediate hosts, and proteins with orthologues in all the analyzed species (3610 in *P. falciparum*). The white sections represent proteins with an irregular distribution insofar as the presence of orthologues did not correlate with host specificity (nor with phylogeny).

analyzed *Plasmodium* spp., and the size of their proteomes correlated with the number of species-specific proteins (Fig. 4). Of the 5410 predicted *P. falciparum* proteins, 3610 (67%) possessed at least one orthologue in all of the other *Plasmodium* species (Fig. 4). 630 *P. falciparum* proteins did not have an orthologue in any other species and 139 had an orthologue in *P. vivax* and *P. knowlesi* but not in the malaria parasites of rodents (Fig. 4). The different protein subsets outlined in Fig. 4 for each parasite are available as a Supplementary excel file (supplement1.xls). The intersect of the two subsets, *P. falciparum* unique (n = 3078) and *P. falciparum* highly conserved (i.e. above median, n = 1805), contained 944 proteins which we posit – based on the results with *S. cerevisiae* – to be enriched for essential ones.

3.3. Comparative genomics between parasite and host

For each predicted protein of *P. falciparum* the best match in the human proteome was identified with Blastp, ranking the obtained hits by ascending expectancy (*E*-values). The similarity between a *P. falciparum* protein and this closest match from *H. sapiens* was then quantified with a Needleman-Wunsch global alignment (since the local alignment provided by Blast may be misleading if the similarity between two otherwise unrelated proteins is confined to a smaller common domain). The frequency distribution of global identity to human proteins in the *P. falciparum* proteome is shown in Fig. 5. The most conserved proteins between parasite and host were ubiquitins, histones, calmodulin, and tubulin. The median global identity between a *P. falciparum* protein and its best match in *H. sapiens* was 10% (red bar in Fig. 5). Proteins exhibiting >10% identity to the host (gray area in Fig. 5) were removed from the potential target space, which thereby was trimmed down to 426 proteins (Fig. 6). Some of these still had highly significant Blastp *E*-values to a human protein, e.g. the *P. falciparum* kinases listed in Table 2, since a short region of high similarity in two otherwise disparate proteins can return a high local alignment score (in the case of the *P. falciparum* kinases, this region is the catalytic domain). Nevertheless, selective targeting might be possible against other domains.

3.4. Blood-stage expression of putative targets

The majority of genes in *P. falciparum* are expressed in a stage-dependent manner (Llinas and DeRisi, 2004), and it is in the asexual and gametocyte stages that we attempt to find targets given their roles in pathology and transmission. We relied on a published microarray dataset (Le Roch et al., 2003) of *P. falciparum* cultures synchronized by sorbitol treatment to identify the potential targets that are present in the blood stages. Retaining only the products of genes which are expressed in the ring stages, trophozoites, or schizonts, reduced the target space to 288 proteins (Fig. 6). The
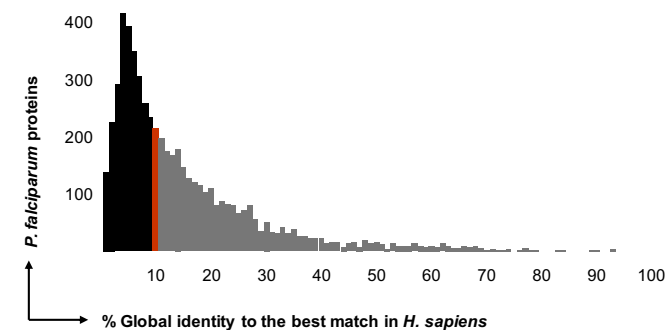

Fig. 5. Parasite vs. host. Frequency distribution of percent global identity to *H. sapiens* in the *P. falciparum* proteome. The red bar marks the median, the gray areas the *P. falciparum* proteins that were excluded from the potential target space.
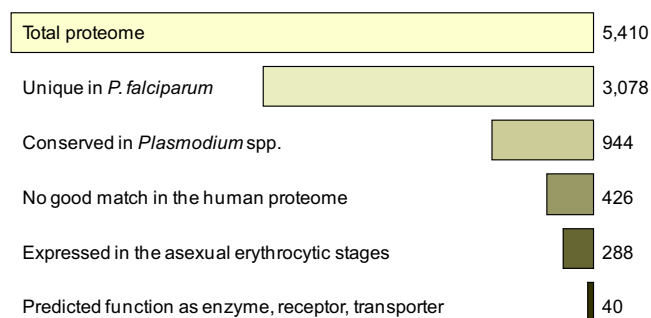

Fig. 6. From genome to target. Overview on the in silico pipeline to narrow down the potential target space of *P. falciparum*. The 40 identified candidates are listed in Table 2.

complete list is available as a Supplementary excel file (supplement2.xls). Over half of these (n = 178) could not be assigned a putative function. Although of potential interest since conserved in the malaria parasites and absent in humans, these proteins were (for the time being) removed from the candidate target set. Other proteins were removed manually because they were not likely to have a function that is susceptible to inhibition, e.g. structural proteins or surface antigens. Focusing on proteins with a clear, though sometimes putative, functional annotation in PlasmoDB as enzyme, receptor or transporter resulted in a final set of 40 *P. falciparum* candidate drug targets (Fig. 6).

3.5. Assessing essentiality of the 40 identified candidate targets

For four of the predicted essential proteins attempts have been made to disrupt the corresponding gene in *P. falciparum* (Table 3). In three cases disruption was attempted through deletion by recombination but no gene deletion could be obtained (Santiago et al., 2004; Wang et al., 2004; Abdi et al., 2010). For the fourth gene ispH (HMBPP reductase; PFA0225w), introduction of a hammerhead ribozyme targeting the transcript was lethal (Vinayak and Sharma, 2007). Thus, each of these four genes experimentally examined appeared to be essential. Similarly, a small number of these proteins' orthologues in *P. berghei* or *Toxoplasma gondii* have been the subject of directed gene knock-out or mutagenesis experiments aimed at assaying their essentiality. The orthologues of the kinases PF13_0166 and CDPK7 (PF11_0242) have been suggested to be essential in *P. berghei* (Tewari et al., 2010). In *T. gondii*, the orthologue of the apurinic/apyrimidinic endonuclease (PF13_0176) has been shown to essential by conditional knockout (Onyango et al., 2011) and GAP40 (PFE0785c) is part of the essential glideosome complex, though the protein itself has not been verified as essential (Frenal et al., 2010). Only one gene was found to be non-essential. The *P. berghei* CDPK6 (PF11_0239) orthologue has been successfully deleted by double crossover (Coppi et al., 2007). These experimental assays for essentiality thus indicate eight of the predicted proteins to be essential, and only one to be non-essential, supporting our method to enrich for essentiality.

3.6. Prediction of druggability

In order to recognize the important role of chemical accessibility in ranking of putative drug targets we looked for druggability information on the predicted essential targets. Druggability describes the properties of a protein that make them able to interact with a drug-like molecule. Some proteins have physiochemical and structural properties that make them unlikely to ever bind a compound with the necessary characteristic to be a drug, and whole genome analyses have estimated that only 10% of a genome may represent druggable proteins (Hopkins and Groom, 2002). As part

Table 2
Forty candidate drug targets from *P. falciparum* identified as outlined in Fig. 6, their putative function, Blastp *E*-value to the best hit in the human proteome, and druggability index (D.I.). The categories (*italics*) are arbitrary and mainly meant to illustrate the functional diversity of the identified targets.

Accession	Putative function	%Id *Hsa*	E *Hsa*	D.I.
Metabolism				
PF08_0095	Dihydropteroate synthetase (2.5.1.15)	0.8	8.5	0.8
MAL8P1.156	Mannose-6-P isomerase (5.3.1.8)	6.9	1E-8	0.6
PF08_0068	FAD-dependent monooxygenase	6.0	0.07	0.5
PF14_0334	Ornithine aminotransferase (2.6.1.13)	4.3	1E-14	0.3
PF13_0234	PEP carboxykinase (4.1.1.49)	4.0	3.6	0.2
PF14_0246	PEP carboxylase (4.1.1.31)	3.8	4.5	0.1
PFI1180w	Patatin-like phospholipase	6.3	7.4	0.1
PF10_0221	HMB-PP synthase (GcpE, 1.17.7.1)	6.5	2.2	
PFA0225w	HMB-PP reductase (1.17.1.2)	7.3	5.3	
MAL7P1.88	Thioredoxin-like protein	3.1	4.9	
PFI1340w	Fumarate hydratase (Fumarase, 4.2.1.2)	10.0	2.7	
PF08_0132	Glutamate dehydrogenase C (1.4.1.2)	8.1	7.2	
PFD0285c	Lysine decarboxylase (4.1.1.18)	5.6	7.4	
PFL0620c	Glycerol-3-P acyltransferase (2.3.1.15)	9.8	1.0	
PF14_0250	Lipase	3.3	8.1	
Nucleic acids				
PF14_0273	RNA methyltransferase	9.8	7E-30	0.7
PF13_0080	Telomerase reverse transcriptase (2.7.7.49)	0.3	0.04	0.5
PF13_0176	Apur./apyr. endonuclease (4.2.99.18)	2.6	2.6	0.2
MAL13P1.311	Exonuclease	5.9	7E-4	0.1
MAL13P1.328	DNA topoisomerase VI, b subunit	4.9	7.1	
PFE0675c	DNA photolyase (4.1.99.3)	3.7	0.06	
PF13_0048	NUDIX hydrolase (3.6.1.17)	8.4	8E-35	
PF08_0111	RNA helicase	7.8	8E-15	
PFC0980c	RNA triphosphatase	8.5	8.6	
Signaling				
PF11_0239	Ca^{2+}-dependent protein kinase 6	7.8	9E-34	0.5
PF11_0242	Ca^{2+}-dependent protein kinase 7	9.0	6E-41	0.5
PF13_0258	Serine/threonine protein kinase TKL3	4.8	3E-21	0.5
PFA0515w	Phosphatidylinositol-4-P-5-kinase (2.7.1.68)	7.0	1E-24	0.5
PF13_0166	Protein kinase	8.9	7E-4	0.3
PF07_0024	Inositol phosphatase	7.3	1E-31	0.3
MAL7P1.18	Serine/threonine protein kinase	4.7	5E-13	0.1
MAL7P1.64	G protein-coupled receptor	2.5	93	0.1
PF14_0143	Atypical protein kinase, ABC-1 family	3.8	4E-26	
PF14_0614	Phosphatase	6.1	4E-5	
Protease/chaperone				
PF14_0382	Stromal-processing peptidase	4.4	0.001	0.5
PF10_0032	DnaJ protein	2.4	7E-4	
Transporter				
PF11_0092	Mechanosensitive ion channel	5.0	11	0.1
PF13_0019	Na$^+$:H$^+$ antiporter	6.6	0.01	0.1
PFI0785c	Sugar transporter	2.8	0.09	0.1
PFE0785c	Metabolite transporter/glideosome component	0.7	10	

of the TDRtargets project a druggability score was assigned to enzymes from the *P. falciparum* proteome (Aguero et al., 2008; Magarinos et al., 2012). This score predicts the suitability of the protein for binding a drug like molecule, and is an aggregate of several factors including physiochemical and structural features of the protein and sequence similarity to validated druggable proteins from other organisms (Al-Lazikani et al., 2007; Gaulton et al., 2012). Of the 40 targets predicted as essential, 23 have positive druggability scores (Table 2). Those with the highest scores are predicted to have more druggable structures and might therefore be prioritized over potential targets with low or zero druggability scores. The higher druggability scores are more likely to be indicative of promising drug target-like proteins, but any positive score indicates structural or physiochemical similarity to proteins with some precedence for interaction with a drug-like molecule.

To search for proteins with specific compounds as starting points for inhibitor development we also searched for proteins that had been identified as the targets of inhibitors, or that had orthologues with identified inhibitors. Searches were conducted of the TDRTargets database, the ChEMBL database and the BRENDA database to identify such targets. Of the 40 predicted essential *P. falciparum* genes, 2 have been directly validated as the targets of

inhibitors. One of these, dihydropteroate synthetase (DHPS; PF08_0095) is one of the very few validated targets of clinically used antimalarials, consistent with the value of our approach for identifying essential targets. An additional 13 proteins have orthologues with identified small molecule inhibitors. Identification of these inhibitors not only provides useful starting points for identifying small molecules with which to interrogate the *Plasmodium* targets, but also indicates that these are likely to be potentially druggable targets. Compounds were curated to exclude facile inhibitors such as natural amino acids, ATP and NaCl, however no systematic attempt was made to rate the drug-like quality of the predicted inhibitors. For the kinases in the list a slightly modified approach was taken, as many kinase inhibitors are well known to be cross reactive against multiple kinases. An empirical cut-off of 30% local sequence identity in the kinase domains was therefore used to identify homologues for potential inhibitors of from the TDR/ChEMBL data (Table 3).

3.7. Experimental amenability of the predicted targets

Tractability of a target for developing an assay in which inhibition is a major factor in prioritizing promising drug targets in

Table 3
Identified targets, evidence for essentiality in apicomplexan parasites (*Pfa, P. falciparum*; *Pbe, P. berghei*; *Tgo, T. gondii*), known and inferred inhibitors, and published in vitro assays.

Accession	Enzyme	Essentiality	Inhibitors	Assay	References
PF08_0095	DHPS	*Pfa*	Sulfonamides	Yes	Wang et al. (2010)
PF14_0273	RNA methyltransferase		8 inferred	Yes	Riguet et al. (2005), Leber et al. (2009)
MAL8P1.156	M6P isomerase		8		Wells et al. (1995), Salvati et al. (2001) and Foret et al. (2009)
PF11_0242	CDPK7	*Pbe*	72 inferred		12 Publications
PF11_0239	CDPK6	Not in *Pbe*	2488 inferred		189 Publications
PFA0515w	PI-4-P-5-kinase		1		Kobayashi et al. (2005) and Santiago et al. (2004)
PF13_0080	Telomerase RT		Berberine		Sriwilaijareon et al. (2002)
PF13_0258	TKL3	*Pfa*		Yes	Abdi et al. (2010)
PF13_0166	Protein kinase	*Pbe*			
PF14_0334	OAT		>10	Yes	Strecker (1965), Levillain et al. (2000), Wang et al. (2007), Stranska et al. (2008) and Gafan et al. (2001)
PF13_0176	AP endonuclease	*Tgo*	>20	Yes	Onyango et al. (2011), Zawahir et al. (2009), Bapat et al. (2010), Adhikari et al. (2012) and Haltiwanger et al. (2000)
PF13_0234	PEP carboxykinase			Yes	Hayward (2000)
PF14_0246	PEP carboxylase		9	Yes	Pairoba et al. (1996), McDaniel and Siu (1972) and Jenkins (1989)
PFD0285c	Lysine decarboxylase		4		Yamamoto et al. (1991) and Takatsuka et al. (1999)
PFI1340w	Fumarase		2	Yes	Flint (1994), Beeckmans and Van Driessche (1998) and Bulusu et al. (2011)
PFL0620c	G3P acyltransferase	*Pfa*	>40	Yes	Santiago et al. (2004), Yamashita and Numa (1981), Coleman (1988) and Wydysh et al. (2009)
PF13_0048	NUDIX hydrolase		>20		Guranowski et al. (2003, 2009) and Branson et al. (2009)
PF08_0132	GDH C		>20	Yes	Li et al. (2007, 2009), Rodriguez-Acosta et al. (1999)
PFA0225w	HMB-PP reductase	*Pfa*		Yes	Vinayak and Sharma (2007) and Rohrich et al. (2005)

infectious diseases because the smaller research communities, compared to those working on human proteins, means fewer protein reagents are available. This is exacerbated in malaria research because the *P. falciparum* genome is extremely A + T rich, and many proteins have long low-complexity insertions, making recombinant protein production challenging. High throughput attempts to generate recombinant *Plasmodium* enzymes have relatively high failure rates (Abdi et al., 2010), so the presence of an existing assay is a particularly relevant criteria for target prioritization. We therefore prioritized proteins where researchers had already developed a direct in vitro assay for the *P. falciparum* enzyme. Eleven of the 40 predicted essential enzymes have had in vitro assays described in *P. falciparum*, although in some cases these may not be readily amenable to high throughput inhibitor screening.

3.8. Candidate antimalarial drug targets

The approach presented here, although starting from the complete proteome, is by no means all-embracing in the sense that it would discover every potential drug target. Many essential proteins and known drug targets were missed, such as *P. falciparum* enzymes which possess human orthologues but exhibit exploitable differences nevertheless. These could be differences in sequence, as is the case with glyceraldehyde-3-phosphate dehydrogenase (Satchell et al., 2005) or in regulation, as demonstrated for dihydrofolate reductase (Zhang and Rathod, 2002). Other proven targets were eliminated because they possess several paralogues, e.g. the falcipains (Teixeira et al., 2011). Using rigorous filters, we tried to maximize the specificity of target prediction at the cost of sensitivity, taking into account a large number of false negatives. Our aim was not to identify all potential antimalarial drug targets but rather to come up with a sizeable list of promising targets that (i) may serve as starting points for rational drug discovery and (ii) provide new insights into peculiarities and vulnerable points of the malaria parasites. The applied selection criteria were rigorous (Fig. 6), so the list of potential targets could be expanded by using less stringent filters. The present approach identified 40

candidate drug targets from *P. falciparum* which are shown in Table 2. We are confident that this set contains promising leads because it includes known targets such as dihydropteroate synthetase (PF08_0095), the target of sulfonamides (dihydrofolate synthetase is not included because it is 20% identical to human mitochondrial tetrahydrofolylpolyglutamate synthase, EC 6.3.2.17), and enzymes of the non-mevalonate pathway for isoprenoid synthesis. Another potential target in the apicoplast could be stromal processing peptidase (PF14_0382), which has already been characterized in *P. falciparum* (van Dooren et al., 2002). Investigational targets identified include glutamate dehydrogenase C (PF08_0132) (Aparicio et al., 2010; Storm et al., 2011) and Ca²⁺-dependent protein kinases (PF11_0239, PF11_0242) (Kato et al., 2008; Ojo et al., 2010; Lim et al., 2012). In addition, there are enzymes which are being targeted in other systems, e.g. apurinic/apyrimidinic endonuclease (PF13_0176) (Tell et al., 2010) or telomerase reverse transcriptase (PF13_0080) (Chen et al., 2009) by antitumor agents, or phosphomannose isomerase (MAL8P1.156) by antifungals. Phosphomannose is a key enzyme in yeast cell wall synthesis (Mastrolorenzo et al., 2000; Wills et al., 2001); to our knowledge, the *P. falciparum* orthologue has so far not been studied. A surprising find in the list of candidate targets was phosphoenolpyruvate carboxylase (PF14_0246), the enzyme responsible for carbon fixation in C4 plants. The *P. falciparum* orthologue was characterized and proposed to fix atmospheric carbon as well (McDaniel and Siu, 1972). If this process is essential, PEP carboxylase could be an attractive target since inhibitors have been identified as C4 plant herbicides (Madhusudana et al., 1980; Jenkins, 1989; Pairoba et al., 1996). Further candidate targets of interest could be the identified signaling enzymes and nutrient transporters (Table 2).

4. Conclusion

Translating a pathogen's genome sequence into new drugs is a key challenge to post-genomic biology. Several strategies for the prediction of drug targets that are essential and unique to the parasite have been proposed (Payne et al., 2001; Joyce and Palsson,

2008; Holman et al., 2009; Crowther et al., 2010; Deng et al., 2010; Doyle et al., 2010; Magarinos et al., 2012). Here we implement phylogenomic comparison within a clade of related parasites to infer on protein essentiality, rather than referring to a model organism which may be understood in detail but phylogenetically distant to the parasites. In addition, we estimate redundancy across the parasite's proteome based on global alignments to quantify protein similarity. Thus the proposed target identification pipeline does not rely on experimental data to predict essentiality, and therefore is generally applicable. The identified candidate targets from *P. falciparum* are, by definition, present in all the *Plasmodium* spp. analyzed. Conservation of targets between *P. falciparum* and *P. vivax* is important since eventual new drugs need to be active against both parasites. Conservation of the candidate targets between human- and rodent-pathogenic *Plasmodium* spp. is desirable as well, since mouse models are indispensable in the drug development process. The presented target discovery pipeline is corroborated by the presence of known antimalarial targets in the output list (Table 2). We hope that the newly identified candidate targets will support the development of novel antimalarials.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpddr.2012.07.002.

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