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Plasmodium Helical Interspersed Subtelomeric (PHIST) Proteins, at the Center of Host Cell Remodeling

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SUMMARY

During the asexual cycle, *Plasmodium falciparum* extensively remodels the human erythrocyte to make it a suitable host cell. A large number of exported proteins facilitate this remodeling process, which causes erythrocytes to become more rigid, cytoadherent, and permeable for nutrients and metabolic products. Among the exported proteins, a family of 89 proteins, called the *Plasmodium* helical interspersed subtelomeric (PHIST) protein family, has been identified. While also found in other *Plasmodium* species, the PHIST family is greatly expanded in *P. falciparum*. Although a decade has passed since their first description, to date, most PHIST proteins remain uncharacterized and are of unknown function and localization within the host cell, and there are few data on their interactions with other host or parasite proteins. However, over the past few years, PHIST proteins have been mentioned in the literature at an increasing rate owing to their presence at various localizations within the infected erythrocyte. Expression of PHIST proteins has been implicated in molecular and cellular processes such as the surface display of PfEMP1, gametocytogenesis, changes in cell rigidity, and also cerebral and pregnancy-associated malaria. Thus, we conclude that PHIST proteins are central to host cell remodeling, but despite their obvious importance in pathology, PHIST proteins seem to be understudied. Here we review current knowledge, shed light on the definition of PHIST proteins, and discuss these proteins with respect to their localization and probable function. We take into consideration interaction studies, microarray analyses, or data from blood samples from naturally infected patients to combine all available information on this protein family.

INTRODUCTION

Malaria is an infectious disease caused by the protozoan parasite *Plasmodium* and is transmitted by female *Anopheles* mosquitoes to humans during a blood meal. Of the five *Plasmodium* species that cause human malaria, two are of major public health interest: *P. falciparum* causes the most severe form of malaria, while *P. vivax* is the most widespread *Plasmodium* species (1, 2). The success of *P. vivax* is due to the presence of undetectable

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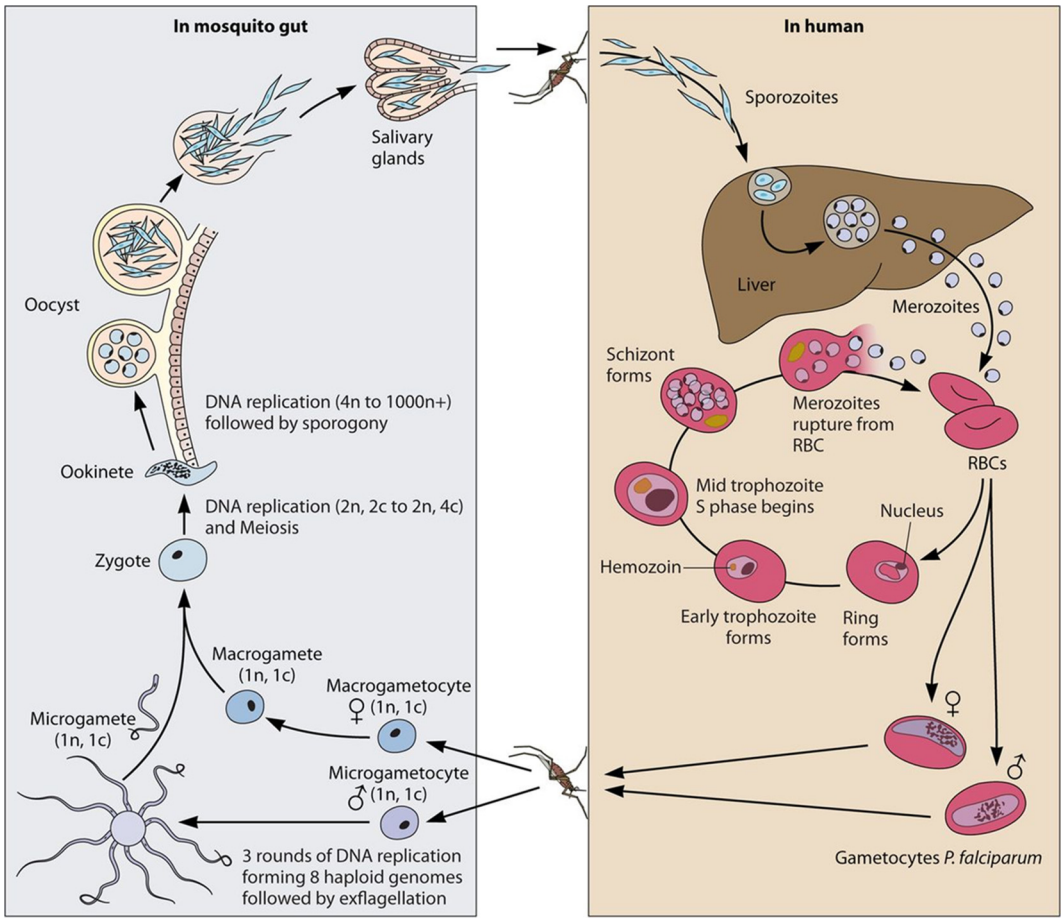


FIG 1 Life cycle of *P. falciparum*. (Right) Upon the bite of a *Plasmodium falciparum*-infected female *Anopheles* mosquito, sporozoites are injected into the dermal tissue of the human host. Sporozoites quickly enter the bloodstream and are transported to the liver, where they invade liver cells and develop into liver schizonts. Through merosomes, thousands of merozoites are released into the bloodstream, where they invade erythrocytes, starting the asexual replication cycle. Each cycle, a few parasites cease replicating, commit to the sexual cycle, and develop into gametocytes. (Left) Mature gametocytes are taken up by a mosquito during a blood meal; rapidly develop into male and female gametes, which fuse together in the gut of the mosquito; form an ookinete that penetrates the gut wall; and undergo sexual replication in the oocyst, producing thousands of sporozoites. At the end, sporozoites migrate to the salivary gland and are ready to be transmitted to a human host during the next blood meal. (Adapted from reference 148.)

hypnozoites, which represent dormant stages in the liver and pose a huge problem for malaria control. With half of the human population at risk, roughly 200 million malaria cases, and an estimated 438,000 deaths in 2015, malaria still remains a major threat to public health (3).

Plasmodium has a complex life cycle, alternating between the arthropod vector and its vertebrate host (Fig. 1) (1). During the bite of an infected female *Anopheles* mosquito, sporozoites are injected into dermal tissue and transported through the bloodstream to the liver. There, the sporozoites penetrate and invade hepatocytes, which is followed by several rounds of asexual replication. Subsequently, thousands of merozoites are released into the bloodstream through so-called merosomes, starting the blood stage cycle (4, 5).

Once in the bloodstream, merozoites invade erythrocytes and multiply during an ~48-h intraerythrocytic development cycle. At the end of the cycle, ~16 to 32 merozoites are released and reinvade new erythrocytes, starting the cycle anew. Once a merozoite has invaded the erythrocyte, it extensively refurbishes and remodels the host cell. This remodeling of the cell ensures parasite ion homeostasis within the cell, allows nutrient uptake, and is accompanied by changes in host cell membrane structure and rigidity. All this is achieved by the export of a large number of proteins from the parasite into the host cell, leading to dramatic changes of infected erythrocytes. Since such a remodeled erythrocyte would be eliminated in the spleen, the parasite also exports the major virulence factor *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), which conveys binding of infected cells to endothelial receptors such as CD36, intracellular adhesion molecule (ICAM), or endothelial protein C receptor (EPCR) (6, 7). PfEMP1 is displayed on the surface of the infected red blood cell (iRBC) and is considered to be the key factor for morbidity and mortality. Overall, these refurbishing processes are considered to be responsible for most symptoms and the pathology of malaria (1, 8). Hence, recently, research focusing on exported proteins and processes involved in this host cell remodeling has attracted increased attention.

Protein Export

Because exported proteins of *P. falciparum* play such a central role in the remodeling of infected red blood cells, the description of the

pentameric amino acid motif RxLxE/Q/D, called the *Plasmodium* export element (PEXEL) (9, 10), was a breakthrough that allowed the prediction of ~400 exported proteins. The PEXEL motif was recently expanded into a more relaxed PEXEL motif (RxLxxE) (11) or a noncanonical motif (K/HxL/IxE/Q/D) (12), resulting in a total number of >460 proteins predicted to be exported in *P. falciparum*. This "exportome" provides the basis to study exported proteins and their involvement in the pathology of remodeled infected erythrocytes much more specifically. Besides the defined PEXEL exportome, there are additional exported proteins that lack a known or discernible export element, referred to as PEXEL-negative proteins (PNEPs) (13, 14). Although the definitive number of proteins exported into the erythrocyte is unknown, it is without doubt that they play important roles in pathology through the significant changes that they induce in infected erythrocytes and that they are major contributors to disease. Figure 2 shows a schematic representation of differences in cytoskeleton organization between uninfected and infected erythrocytes and the localization of exported proteins during the asexual replicative blood stage of *P. falciparum*.

PHIST PROTEINS

Methodology for Review

We focused on *P. falciparum* and included all *Plasmodium* helical interspersed subtelomeric (PHIST) proteins and genes that were either identified by Sargeant and colleagues (15) or later identified as PHIST proteins by Frech and Chen (16). We did not attempt to identify new PHIST proteins but compiled only data already reported. Gene identifications in PlasmoDB have changed over time; therefore, some PHIST proteins have two or even three gene identifications, and various articles use different gene identifications, although they refer to the same protein or gene. We also scanned supplemental material for information on PHISTs. which in some instances was found only there and would not be available through a standard literature search. We compiled any available information on each member of the PHIST family by using all gene identifications or names used now or previously. Complete lists of PHIST proteins or genes found in the literature and reviewed here are shown in Tables 1 to 5 and represent an in-depth description of this important protein family.

The PHIST Protein Family

In the large number of exported proteins, Sargeant and colleagues (15) identified a new protein family, which they termed the PHIST protein family. This protein family is characterized by a conserved domain of ~150 amino acids predicted to form four consecutive alpha helices (determined by Fugue [17]). Some members of this family comprise little more than an export signal sequence, the PEXEL motif, and the PHIST domain, whereas other members are substantially elongated and include additional domains, such as a DnaJ domain (Fig. 3). Based on the presence and position of several conserved tryptophan residues within the PHIST domain, the PHIST protein family has been further divided into three subgroups: PHISTa, PHISTb, and PHISTc (15). With more recent data included, PlasmoDB has annotated additional PHIST domains in proteins, resulting in a total of 89 currently known PHIST proteins in *P. falciparum* (15, 16). Some of the newly added PHIST proteins were not grouped into the already existing subgroups but were classified as PHISTa-like or simply PHIST proteins. Thus, PlasmoDB annotations give the impression that there are more than three PHIST subgroups. However, a phylogenetic tree based on a multiple-sequence alignment shows that PHISTa-like and PHIST proteins cluster with the PHISTa subgroup and the PHISTb-DnaJ protein group among the PHISTb subgroup, giving rise to three distinct PHIST subgroups (Fig. 3).

When the protein family was first described, Sargeant and colleagues used the presence and position of conserved tryptophan residues in the amino acid sequence of the PHIST domain to distinguish between the three subgroups (15). Comparison of multiple-sequence alignments for each subgroup in which PHISTb-DnaJ and PHISTa-like/PHIST proteins were treated as individual subgroups reveals a unique pattern of the conserved tryptophan residues for each of the subgroups (Fig. 4 and 5A). For each subgroup, a different positional pattern of conserved tryptophan residues was found, with PHISTa and PHISTa-like/PHIST proteins possessing only two conserved tryptophan residues within the PHIST domain and the remaining subgroups having four. There is only slight variation in the positions of these residues between the PHISTb and PHISTb-DnaJ subgroups and between the PHISTa and PHISTa-like/PHIST subgroups. We therefore use the original three subgroups introduced by Sargeant and colleagues unless otherwise stated.

Tables 1 to 5 list all 89 proteins that we identified as PHIST proteins in *P. falciparum*, 64 of which contain a classical PEXEL motif and are thus predicted to be exported (11, 15). Although PlasmoDB lists 19 *phist* genes as pseudogenes in reference strain 3D7, some have been found to be present as proteins or transcripts in other studies. Thus, PHISTs represent a substantial group of exported proteins comprising ~14% of all PEXEL proteins or nearly 2% of the complete *P. falciparum* proteome. Despite their potentially important role in host cell remodeling and (in)direct involvement in pathogenicity, most PHIST proteins remain completely uncharacterized, rendering it difficult to assign specific functions or roles to the PHIST protein family and/or the three subgroups.

Comparison of PHIST and PRESAN Domains

There has been confusion in the literature on the definition of PHIST and *Plasmodium* ring-infected erythrocyte antigen (RESA) N-terminal (PRESAN) domains, which in fact are virtually identical. The confusion was generated when, independent of the sequence analysis reported by Sargeant and colleagues (15), transcriptome analysis of *P. falciparum* parasites grown at elevated temperatures mimicking febrile conditions revealed a number of upregulated genes that coded for proteins containing a DnaJ domain (Pfam family PF00226) (18). Subsequent alignments identified an extended protein family with at least 67 members, all sharing a particular N-terminal domain, with a DnaJ domain being present in only some members. Because some of the DnaJ domain-containing proteins showed similarity to the RESA, this N-terminal domain was termed the PRESAN domain (Pfam family PF09687). Examination of the sequence alignments by Sargeant and colleagues (15) and Oakley and colleagues (18) revealed that the domain boundaries of the PHIST and PRESAN domains are virtually identical. Secondary-structure predictions by Oakley and colleagues (18) suggested the presence of six α-helices in PRESAN domains; however, these α-helices overlapped the predicted four α-helices of the PHIST domain (15). Thus, the PRESAN domain can be regarded as being highly similar to the

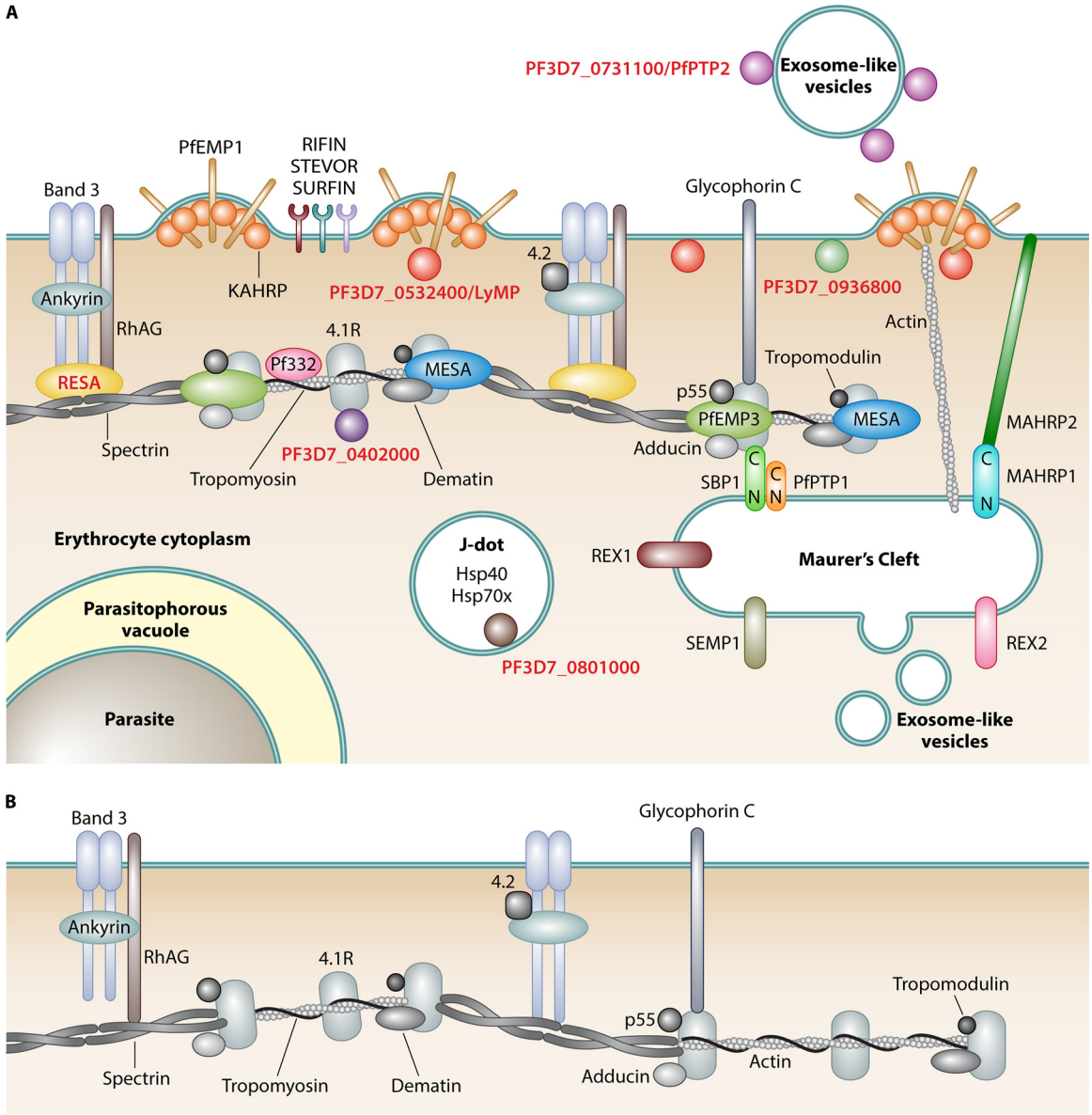


FIG 2 PHIST proteins in the remodeled iRBC. (A) Remodeled iRBC. During the asexual blood stage of *P. falciparum*, human erythrocytes are subject to extensive remodeling. All erythrocyte proteins are shown in gray (reviewed in reference 59). Parasite structures, compartments, and organelles are labeled in boldface type. PHIST proteins are labeled in red boldface type, and all parasite proteins are represented by colored shapes. References for PHIST proteins are indicated in the text. Knobs are parasite-derived protrusions in the host cell membrane, with knob-associated histidine-rich protein (KAHRP) being a prominent protein of these structures (149, 150). Maurer's clefts (reviewed in reference 151) are parasite-derived membranous structures in the iRBC cytoplasm involved in protein trafficking and are connected with knobs via actin filaments (152, 153). Maurer's cleft-associated histidine-rich protein 1 (MAHRP1) is a Maurer's cleft-resident protein (154) that potentially interacts with MAHRP2, the tether protein anchoring Maurer's clefts to the iRBC membrane (155). REX1 (156), REX2 (107), SEMP1 (157), SBP1 (158), and PfPTP1 (159) are other exported parasite proteins that localize to Maurer's clefts, with the latter two being located in a high-molecular-weight complex (159) and SBP1 interacting with the erythrocyte cytoskeleton proteins spectrin and band 4.1R (160). J dots are mobile, dot-like structures in iRBCs (161, 162). PfPTP2 is associated with exosomes, which are parasite-derived vesicles that are involved in cell-cell communication between iRBCs (43). RhAG, rhesus-associated antigen. (B) Cytoskeleton of an uninfected red blood cell (reviewed in reference 59). (Inspired by references 42 and 100.)

PHIST domain, with differences depending mainly on the prediction algorithms. An example of such close similarity is shown with the PHIST/PRESAN protein PF3D7_0532400 (Fig. 5B). The overall domain structure of all expressed PHIST proteins is shown in Fig. 3. Crystallographic and nuclear magnetic resonance (NMR) studies provided clear evidence that the PHIST/PRESAN domain forms a four-helix bundle (19). Subsequently, Tarr and colleagues (20) defined an "extended PRESAN" domain, which was N terminal to the original PHIST domain and which was thought to comprise a domain targeting membranes. This extended domain includes additional helix-forming sequences.

Confusingly, the current InterPro database uses the term "PHIST domain" (accession number IPR006526) to refer to a small protein fragment spanning approximately the N-terminal half of the PHIST/PRESAN domains, as defined by the original authors (15, 18). In contrast, the InterPro (accession number

TABLE 1 PHISTa genes

Gene identification[a]	Other gene identification(s) or name(s)[b]	Presence of PEXEL[c]	Gene status[d]	Molecular mass (kDa)[e]	Length (aa)[e]	KO[f]	Description (source or reference[s])[g]
PF3D7_0102000	PFA0100c, MAL1P1.11		PG	30.4	252		
PF3D7_0115100	PFA0735w, MAL1P4.22	x		28.6	239		Only 5-aa difference compared to PF3D7_0800600 (PlasmoDB)
PF3D7_0402000	PFD0090c, MAL4P1.18	x		41.7	357	x	Interaction between band 4.1R (58), not silenced in the 3D7 strain (28), overexpressed in samples from patients with cerebral malaria (34)
PF3D7_0424900	PFD1185w, MAL4P1.232	x		25.3	216		
PF3D7_0425300	PFD1210w, MAL4P1.237		PG	27.5	232		Exported despite the lack of any export signal (79)
PF3D7_0425400	PFD1215w, MAL4P1.238	x		19.5	164		
PF3D7_0601700	PFF0085w, MAL6P1.21	x	PG	26.3	222		
PF3D7_0800600	MAL8P1.163	x		28.3	237		Adjacent to a PfEMP1 variant on the gene locus; weak binding to the ATS domain of PfEMP1 compared to other PHIST proteins tested (19), only 5-aa difference compared to PF3D7_0115100 (PlasmoDB)
PF3D7_1001100.1	PF10_0014, PF10_0015, acyl-CoA binding protein			36.5	313	x	Grouped as PHISTa (15), deleted in parasites with PfCRT mutations, annotated as a putative lipid transporter (80), upregulated under lumefantrine pressure and deletion of the gene found under chloroquine pressure (81), unusual PHIST member with an N-terminal acyl-CoA binding protein domain (16)
PF3D7_1001100.2	PF10_0014, PF10_0015, acyl-CoA binding protein			31.0	267	x	Same as PF3D7_1001100.1
PF3D7_1001300	PF10_0017	x		22.8	190		Diffuse localization in the RBC cytosol (20), differentially expressed in parasites under lumefantrine pressure (81)
PF3D7_1100600	PF11_0012		PG	33.3	281		
PF3D7_1149700	PF11_0514			10.5	85		Once mentioned in relation to PHISTa structure (58)
PF3D7_1253100	PFL2555w, MAL12P1.506	x		26.5	221		HSP40 chaperone with a DnaJ domain (82), used as a negative control in an interaction study (58)
PF3D7_1253300	PFL2565w, MAL12P1.508	x	PG	18.3	157		Not silenced in 3D7 (28)
PF3D7_1253800	PFL2590w, MAL12P1.513	x		26.2	219		
PF3D7_1253900	PFL2595w, 2600w, MAL12P1.514	x	PG	24.3	207		Once mentioned in relation to PHISTa structure (58)
PF3D7_1301100	MAL13P1.11, MAL13P1.11a	x	PG	24.6	203		
PF3D7_1301500	MAL13P1.59			37.5	308		Downstream of a *clag* gene, and its expression is affected by H3K9me3 acetylation (83); not located in the telomeric region (27)
PF3D7_1372000	MAL13P1.470	x		41.0	349		String database mining suggested interaction with PFI1785w, PFB0115w, and PFL0050c (84)
PF3D7_1400900	PF14_0009	x	PG	24.6	209		
PF3D7_1477700	PF14_0748, Pfg14-748			34.5	291		Highest transcript concn in bone marrow comparing different organs (37); marker for early and midgametocytes, and its promoter can drive gametocyte-specific gene expression (36, 37); expressed in sexually committed schizonts with >100-fold upregulation (27); used as a marker for sexual commitment (85)
PF3D7_1478000	PF14_0752, GEXP17	x		25.5	215		Overexpressed in field samples compared to 3D7, suggested surface protein (30); differentially expressed in different 3D7 clones (33)
PF3D7_1478500	PF14_0757	x	PG	25.1	215		
PF3D7_1479200	PF14_0763	x		26.2	219		
PF3D7_1479300	PF14_0764 PF14_0765	x	PG	24.6	209		

[a] Current gene identification from PlasmoDB.
[b] Other/previous names.
[c] The presence of a PEXEL motif is indicated by "x," as identified in reference 15 or 1.
[d] Annotated as a pseudogene (PG) in PlasmoDB.
[e] For proteins with a PEXEL motif, molecular mass and length were calculated for the PEXEL-cleaved form of the protein (the molecular mass for PEXEL-cleaved proteins was calculated by using the ExPASy Web tool [http://web.expasy.org/compute_pi/]). aa, amino acids.
[f] Existing viable knockout (KO) parasites or natural gene deletions are indicated by "x" (see reference 44 or the reference[s] indicated).
[g] Information or references referring to the corresponding gene or protein.

TABLE 2 PHISTb genes

Gene identification[a]	Other gene identification(s) or name(s)[b]	Presence of PEXEL[c]	Gene status[d]	Molecular mass (kDa)[e]	Length (aa)[e]	KO[f]	Description (reference[s] or source)[g]
PF3D7_0201600	PFB0080c, PF02_0016	x		48.0	394	x	Affected by large chromosome break, which causes a loss of cytoadherence and permanent expression of var2csa, regulates *var2csa* and *var* gene expression (86); displayed on iRBC surface (86); cytosolic localization with weak accumulation at the erythrocyte periphery (20); PHISTb domain-containing RESA-like protein 1 (PlasmoDB); overexpressed in samples from patients with cerebral malaria (34)
PF3D7_0401800	PFD0080c, MAL4P1.16, PfD80	x		54.8	512		Upregulated within 90 min after artesunate treatment (87), suggested to interact with PFD0985w based on data mining (88), putative Maurer's cleft protein (89, 90), differentially expressed in Pf-FRC parasites selected for CSA or CD36 binding (91), among the most significantly upregulated genes in children with *P. falciparum* malaria (92), discrepancy observed between RNA and protein levels (93, 94)
PF3D7_0402100	PFD0095c, MAL4P1.19	x		61.7	522		*var* gene *chr4var7* showed recombination with PFD0100c (95), essential for *in vitro* growth (44), apparently linked to invasion ligand RH1 abundance in FCR3 (80, 95), contains a MEC motif (39)
PF3D7_0424600	PFD1170c, MAL4P1.229	x		26.2	221	x	Required for correct KAHRP transport and knob formation, and deletion has an effect similar to that in KAHRP KO parasites but does not affect PfEMP1 transport (44); protein shows peripheral localization (20); no interaction with the ATS of PfEMP1 (19); involved in knob formation and cytoadherence (96); present in peripheral blood of malaria patients (77)
PF3D7_0424800	PFD1180w, MAL4P1.231	x		31.1	266		
PF3D7_0532300	PFE1600w, MAL5P1.314	x		49.9	419		Exported and tyrosine phosphorylated in the RBC cytoplasm (97), shown on immunoblots with candidate vaccine antigens (98), shown in an interaction cluster with PFE1605w (99)
PF3D7_0532400	PFE1605w, MAL5P1.315, LyMP	x		50.6	439		Showed interaction with ATS of PfEMP1 variants localized to the knobs (19); localization at membrane cytoskeleton between knobs (40); recently reviewed, indicating both knob and membrane localizations (100); referred to as Hsp40 protein, with interaction with MSP1 (101); HSP40 protein with J domain (102); yeast two-hybrid interaction with SBP1 and PFE1600w (102); also computationally predicted to be a nuclear pore protein and to be part of a signaling pathway in the FIKK protein family (103)
PF3D7_0601500	PFF0075c, MAL6P1.19	x		50.0	417		Has two MEC motifs, with only a 1-aa difference compared to PF3D7_0631100 (39)
PF3D7_0631100	PFF1510w	x		49.9	416		Has two MEC motifs, with only a 1-aa difference compared to PF3D7_0601500 (39)
PF3D7_0702100	MAL7P1.7	x	PG	69.1	586		Part of an interaction network with SBP in the center (99), hexadecyl-trimethyl-ammonium bromide treatment changed its expression (104), identified as a RESA-like protein (82), identified as a RESA-like protein but not HSP40 (105)
PF3D7_0731300	MAL7P1.174, Pfg174	x		31.6	263	x	Putative Maurer's cleft protein (89, 90), suggested location of a surface protein (106), soluble protein (107)
PF3D7_0831000	MAL8P1.2, GEXP09			51.0	426		Listed as an HSP40 chaperone with a J domain (102)
PF3D7_0902700	PFI0130c	x	PG	44.0	372		Silencing of this gene resulted in inhibition of apoptosis without affecting parasite growth (108), potentially links an unknown surface protein to the iRBC cytoskeleton (109), contains a MEC motif (39)
PF3D7_0936900	PFI1785w	x	PG	32.9	274		Particularly abundant protein in samples from pregnant women but not in samples from children (49, 110, 111), affected by deletions on chromosome 9 (112, 113), String database mining suggested interaction with var2csa and MAL13P1.470-1 (84), one of the earliest-upregulated genes in the asexual cycle (28), often mentioned together with PFD1140w

(Continued on following page)

TABLE 2 (Continued)

Gene identification[a]	Other gene identification(s) or name(s)[b]	Presence of PEXEL[c]	Gene status[d]	Molecular mass (kDa)[e]	Length (aa)[e]	KO[f]	Description (reference[s] or source)[g]
PF3D7_0937000	PFI1790w			43.0	357	x	Yeast two-hybrid interaction with band 4.1R (99, 114), potential involvement in host cytoskeleton remodeling (39), located in a region prone to deletion in *P. falciparum* strains IT and FCR3 (113), contains a MEC motif (39)
PF3D7_1102500	PF11_0037, GEXP2	x		64.6	547	x	Immunoprecipitation using this protein pulled down *Plasmodium* translocon of exported proteins (PTEX) components HSP101, PTEX150, and EXP2 (45); differentially expressed in HP1-depleted parasites (53); involved in cytoadherence (109)
PF3D7_1201000	PFL0050c, MAL12P1.10	x		71.7	605	x	Putative Maurer's cleft protein (89, 90), String database mining suggests interaction with var2csa and MAL13P1.470-1 (84)
PF3D7_1252700	PFL2535w, MAL12P1.502	x		42.1	363		RESA-like protein (115)
PF3D7_1252800	PFL2540w, MAL12P1.503	x		66.6	559		Contains a MEC motif (39)
PF3D7_1372100	MAL13P1.475, GEXP04	x		67.0	558	x	Frequently deleted in field samples, affected by the same deletion as HRP2 and HRP3 (116, 117)
PF3D7_1401600	PF14_0018	x		45.7	391	x	Knockout resulted in less rigid but viable parasites (39, 44), putative TM domain or GPI anchor (118), contains a MEC motif (39)
PF3D7_1476200	PF14_0731 or PF14_0730	x		49.3	410		Upregulated *in vitro* when cultured with human serum compared to AlbuMAX (119)
PF3D7_1476300	PF14_0732	x		61.5	514	x	Frequently deleted in HB3 and other strains (120)
PF3D7_1477500	PF14_0746	x		49.9	411		

[a] Current gene identification from PlasmoDB.
[b] Other/previous names.
[c] The presence of a PEXEL motif is indicated by "x," as identified in reference 15 or 1.
[d] Annotated as a pseudogene (PG) in PlasmoDB.
[e] For proteins with a PEXEL motif, molecular mass and length were calculated for the PEXEL-cleaved form of the protein (the molecular mass for PEXEL-cleaved proteins was calculated by using the ExPASy Web tool [http://web.expasy.org/compute_pi/]).
[f] Existing viable knockout (KO) parasites or natural gene deletions are indicated by "x" (see reference 44 or the reference[s] indicated).
[g] Information or references referring to the corresponding gene or protein. TM, transmembrane; GPI, glycosylphosphatidylinositol.

IPR019111) and Pfam (family PF09687) entries for the PRESAN domain are identical to these original alignments. This discrepancy needs correction urgently. In this review, we refer to this conserved domain type as the PHIST domain.

PHIST Proteins in Other *Plasmodium* Species

PHIST proteins are found exclusively in the genus *Plasmodium*, and the protein family has been significantly expanded in *P. falciparum* and in the laveranian species *P. reichenowi*, in which there is a one-to-one representation of the PHIST genes (21). While little is known about PHIST proteins in *P. falciparum*, even less is known about PHIST proteins in other *Plasmodium* species. There are fewer PHIST proteins in other plasmodia, but their exact number is not yet clear, and different publications report various counts. Initially, Sargeant et al. reported 39 PHIST proteins for *P. vivax* (15), but the complete analysis of the *P. vivax* genome (22) revealed the presence of a gene family (Pv_fam_e), which contained 44 *rad* genes and 21 *phist* genes, with both groups showing structural similarities. However, PlasmoDB currently has only 18 genes annotated as *phist* (PlasmoDB) for *P. vivax*. Supplemental material in two publications provided expression data for *P. vivax* PHIST proteins (23, 24). For *P. knowlesi*, Sargeant and colleagues (15) initially reported 27 PHIST proteins, but this has been reannotated to 38 proteins by Pain et al. (25), and PlasmoDB currently lists 39 records. For the monkey malaria parasite *P. cynomolgi*, 21 PHIST genes were identified, while the number of PHIST genes in the rodent malaria parasite is unclear and varies in different publications or databases. For *P. berghei*, 1 to 3 *phist* genes have been found, and 1 to 2 have been found in both *P. chabaudi* and *P. yoelii* (15, 16, 26). Moreira et al. recently characterized two PHIST proteins in *P. berghei* and demonstrated potentially similar roles for these PHIST proteins, as has been attributed to *P. falciparum* PHIST proteins (26). So far, to our knowledge, no *phist* genes have been identified in the avian *Plasmodium* lineage. Whether there are other gene families with a similar structure or function in these species remains to be seen.

Except for CVC-81_{95}, a PHIST protein of *P. vivax* that has been investigated in more detail, we do not further discuss PHIST proteins in other plasmodia.

The PHIST Subfamilies

PHISTa. Proteins of the PHISTa subgroup are very short and besides the PHIST domain consist of only a signal sequence and a PEXEL motif if present. PHISTa proteins are found exclusively in *P. falciparum* (15) and currently amount to 26 different proteins (Table 1). In contrast to the other subgroups, PHISTa and PHISTa-like proteins possess only two conserved tryptophan residues (Fig. 4). These proteins were previously grouped together and described as a subtelomeric protein superfamily (27), although two of them, PF3D7_1301500 and PF3D7_1301300, are not located subtelomerically and are referred to as PHISTa-like proteins today (Table 5).

Except for PF3D7_0402000 and PF3D7_1253300, PHISTa proteins have been reported to be transcriptionally silent in reference strain 3D7 (15, 28). Initially, it was proposed that this transcriptional silencing might be caused by mutually exclusive expression (15). However, as shown in the heat map in Fig. 6, several studies recently showed that other PHISTa proteins are upregu-

TABLE 3 PHISTb genes with a DnaJ domain

Gene identification[a]	Other gene identification(s) or name(s)[b]	Presence of PEXEL[c]	Gene status[d]	Molecular mass (kDa)[e]	Length (aa)[e]	KO[f]	Description (reference[s] or source)[g]
PF3D7_0102200	PFA0110w, MAL1P1.13, Pf155, RESA			126.5	1,085	x	RESA-KO iRBCs have reduced cell rigidity (44, 65), RESA stiffens iRBCs and protects them from cell damage at febrile temperatures (71), binds to spectrin (68), expressed in ring stages of early gametocytes (51), part of an interaction network with SBP in the center (99), found in peripheral blood of malaria patients (77), RESA-positive and parasite-free RBCs observed (121), subject of research in vaccine development (search term, *Plasmodium* Pf155 vaccine)
PF3D7_0201700	PFB0085c, PF02_0017	x		99.7	846	x	Part of an upregulated gene cluster in C4S binding parasites but suggested to be nonessential for PfEMP1 expression (86); HSP40 chaperone with a DnaJ domain, RESA-like protein (82); chromosomal deletion also affecting KAHRP and resulting in the absence of knobs and reduced cytoadherence (15, 122)
PF3D7_0220100	PFB0920w, PF02_0188	x		106.8	909	x	Identified as type III HSP40 (105, 123), deletion leads to increased rigidity and increased CS2 binding (44), putative DnaJ protein (PlasmoDB)
PF3D7_1038800	PF10_0378	x		96.8	822	x	Coprecipitated full-length band 4.1R (39), identified as type III HSP40 (123), RESA-like protein with PHIST and DnaJ domains (PlasmoDB)
PF3D7_1149200	PF11_0509, RESA 3	x		117.2	1,003		Part of an interaction network with SBP in the center but also present in a different interaction network with RESA, SBP1, and other PHISTs (99); identified as type IV HSP40 (105); absent in parasites treated with T4, which causes arrest of the cell cycle (124); less abundant in proteomic data than RESA (65); described as an essential gene by Maier et al. (44); expression peak in late ring, early trophozoite stage (125); high level of sequence homology with RESA (126)
PF3D7_1149500	PF11_0512, RESA 2	x	PG	89.0	839	x	Although annotated as a pseudogene, it has been found to be poorly translated, with very low protein levels, function is not known, protein might be degraded (127); identified as RESA2 (65); mutation T1526C frequently found in cases of severe malaria, upregulated *in vivo* (128); identified as type IV HSP40 (105); not expressed in laboratory strains according to Maier et al. (42) but highly upregulated when two isogenic 3D7 strains were compared (33); peak expression in trophozoite stage (125); overexpressed in *in vivo* samples (30); transcript found at high levels in samples from patients with cerebral malaria from Malawi (129) (data not shown)
PF3D7_1201100	PFL0055c, MAL12P1.11	x		96.2	806		Identified as type III HSP40 (105, 123), RESA-like protein with PHIST and DnaJ domains (PlasmoDB), contains a MEC motif (39)

[a] Current gene identification from PlasmoDB.
[b] Other/previous names.
[c] The presence of a PEXEL motif is indicated by "x," as identified in reference 15 or 1.
[d] Annotated as a pseudogene (PG) in PlasmoDB.
[e] For proteins with a PEXEL motif, molecular mass and length were calculated for the PEXEL-cleaved form of the protein (the molecular mass for PEXEL-cleaved proteins was calculated by using the ExPASy Web tool [http://web.expasy.org/compute_pi/]).
[f] Existing viable knockout (KO) parasites or natural gene deletions are indicated by "x" (see reference 44 or the reference[s] indicated).
[g] Information or references referring to the corresponding gene or protein.

lated in pregnancy-associated malaria or in cerebral malaria (29–34). Additional studies reported that members of the PHISTa group are differentially expressed in parasites committed to becoming gametocytes (27, 35–37). Whether the transcriptional silencing of PHISTa proteins in 3D7 is an adaptation to *in vitro* growth remains to be tested, but PHISTa proteins in natural infections seem to play an important role in pathogenesis.

PHISTb. With 24 members, PHISTb proteins make the second largest subgroup (Table 2). Members of this subgroup are slightly longer than PHISTa proteins, with 300 to 600 residues. Characteristic of the PHISTb subgroup is a unique, long, C-terminal amino acid stretch that follows the PHIST domain (15) and that might indicate a unique and different function of these proteins compared to that of proteins of the other PHIST subgroups. It is conceivable that the PHIST domain provides a general binding motif, while the C terminus might provide a more specific interaction domain. Such a dual binding capacity was recently shown by Oberli and colleagues (38) for the PHIST protein

TABLE 4 PHISTc genes

Gene identification[a]	Other gene identification(s) or name(s)[b]	Presence of PEXEL[c]	Gene status[d]	Molecular mass (kDa)[e]	Length (aa)[e]	KO[f]	Description (reference[s] or source)[g]
PF3D7_0202100	PFB0105c, LSAP2	x		25.4	216		Not recognized by pooled immune sera (98); continuously upregulated, with predicted TM, granular pattern in immunofluorescence assays (IFA) of older parasites, expressed in liver stages (130); located at periphery in liver stage parasites similarly to circumsporozoite protein (CSP) (131); polymorphic gene with SNPs (80); differentially expressed in parasites under lumefantrine pressure (81)
PF3D7_0219700	PFB0900c, PF02_0184, GEXP20			35.3	295		
PF3D7_0219800	PFB0905c, PF02_0185	x		31.9	263		
PF3D7_0424000	PFD1140w, MAL4P1.223	x		35.2	296	x	Antigenic properties, antibody recognition in several reports, potential vaccine candidate (29, 49, 111, 132, 133); stronger expression in culture supplemented with albumin instead of human serum (119); String database mining suggests interaction with var2csa and PFB0115w (84); often referred to together with PFI1785w
PF3D7_0532200	PFE1595c, MAL5P1.313			27.3	226		Involved in response to chloroquine treatment (134, 135)
PF3D7_0731100	MAL7P1.172, GEXP11 PfPTP2	x		92.5	799	x	Involved in cell-cell communication and plasmid transfer, located on budding vesicles from Maurer's clefts (43); knockout with very reduced levels of surface PfEMP1, which was trapped in Maurer's clefts, no cytoadherence to CSA (44); indirect evidence for PfPTP2 being located inside the lumen of Maurer's clefts (44); coprecipitated with plasmepsin V (136); RESA-like protein found among soluble proteins from parasitophorous vacuole (PV) lumen and iRBC cytosol (82)
PF3D7_0801000	PF08_0137	x		147.1	1,127		Found among soluble proteins from PV lumen and iRBC cytosol (82), coprecipitated PTEX components such as HSP101 and PTEX150 (45), associated with J dots (J. Przyborski, personal communication), *P. yoelii* orthologue is annotated as a putative dentin phosphoryn protein possibly involved in mineral nucleation or functions as an extracellular matrix protein (137), contains a relaxed PEXEL motif (1), elicits an immune response (138)
PF3D7_0830600	MAL8P1.4	x		45.1	380		Located in Maurer's clefts, no interaction with the ATS of PfEMP1 (19); expression downregulated in HB3, 3D7, and Dd2 (28); expression upregulated with treatment with histone deacetylase inhibitors (trichostatin A, suberoylanilide hydroxamic acid) (139)
PF3D7_0936600	PFI1770w, GEXP5	x		25.2	212		Originally classified as PHISTb (15); reclassified as PHISTc (16); sole "PHISTb" that showed only cytosolic localization (20); upregulated in gametocytes (107, 140); earliest-known postinvasion gametocyte marker, expressed at 14 h postinvasion and independent of major gametocyte marker, cannot promote gametocyte maturation alone when other factors are not present (51)
PF3D7_0936800	PFI1780w	x		38.6	324		Protein of unknown function, several TM domains predicted (15); interacts with the ATS of PfEMP1 (56); located at the iRBC periphery with PfEMP1 but not cotransported with PfEMP1 (19); stuttering motif identified not found in any other PHIST protein (141); noncanonical PEXEL motif that is correctly cleaved (12); suggested presence in figure displaying exported proteins (100)
PF3D7_1001700	PF10_0021	x		27.6	226		Significantly downregulated after chloroquine treatment (80)
PF3D7_1001800	PF10_0022	x		23.8	204		Corrected gene model with adjusted exon and intron sizes (142, 143)
PF3D7_1016500	PF10_0161	x		81.3	675		Wrongly annotated as PF3D7_1016600 previously (15), evidence for sumoylation (144)
PF3D7_1016600	PF10_0161 or PF10_0161a	x		27.8	226		Had been annotated together with PF3D7_1016500 (15), evidence for sumoylation (144), IFA shows bright punctuate pattern in iRBCs (20)
PF3D7_1016700	PF10_0162			98.8	830		
PF3D7_1016800	PF10_0163	x		30.0	241		
PF3D7_1148700	PF11_0503, GEXP12	x		38.1	323		
PF3D7_1200900	PFL0045c, MAL12P1.9	x		37.6	310		Noncanonical PEXEL, localizes to small dotted structures in iRBCs (12)

[a] Current gene identification from PlasmoDB.
[b] Other/previous names.
[c] The presence of a PEXEL motif is indicated by "x," as identified in reference 15 or 1.
[d] Annotated as a pseudogene (PG) in PlasmoDB.
[e] For proteins with a PEXEL motif, molecular mass and length were calculated for the PEXEL-cleaved form of the protein (the molecular mass for PEXEL-cleaved proteins was calculated by using the ExPASy Web tool [http://web.expasy.org/compute_pi/]).
[f] Existing viable knockout (KO) parasites or natural gene deletions are indicated by "x" (see reference 44 or the reference[s] indicated).
[g] Information or references referring to the corresponding gene or protein. LSAP2, liver stage-associated protein 2; SNPs, single-nucleotide polymorphisms.

PF3D7_0532400. C-terminal interaction motifs have indeed been identified in several other PHISTb proteins (39). In this respect, it is important to note that all PHISTb proteins characterized today were localized at and might interact with the host cell cytoskeleton (19, 20, 39, 40).

The non-PHIST protein mature parasite-infected erythrocyte surface antigen (MESA) (PF3D7_0500800) interacts via an N-terminal 19-amino-acid motif with band 4.1R. This motif has been termed the MESA erythrocyte cytoskeleton binding (MEC) motif (39) and has been found in 14 other exported *P. falciparum* pro-

TABLE 5 PHISTa-like and PHIST proteins

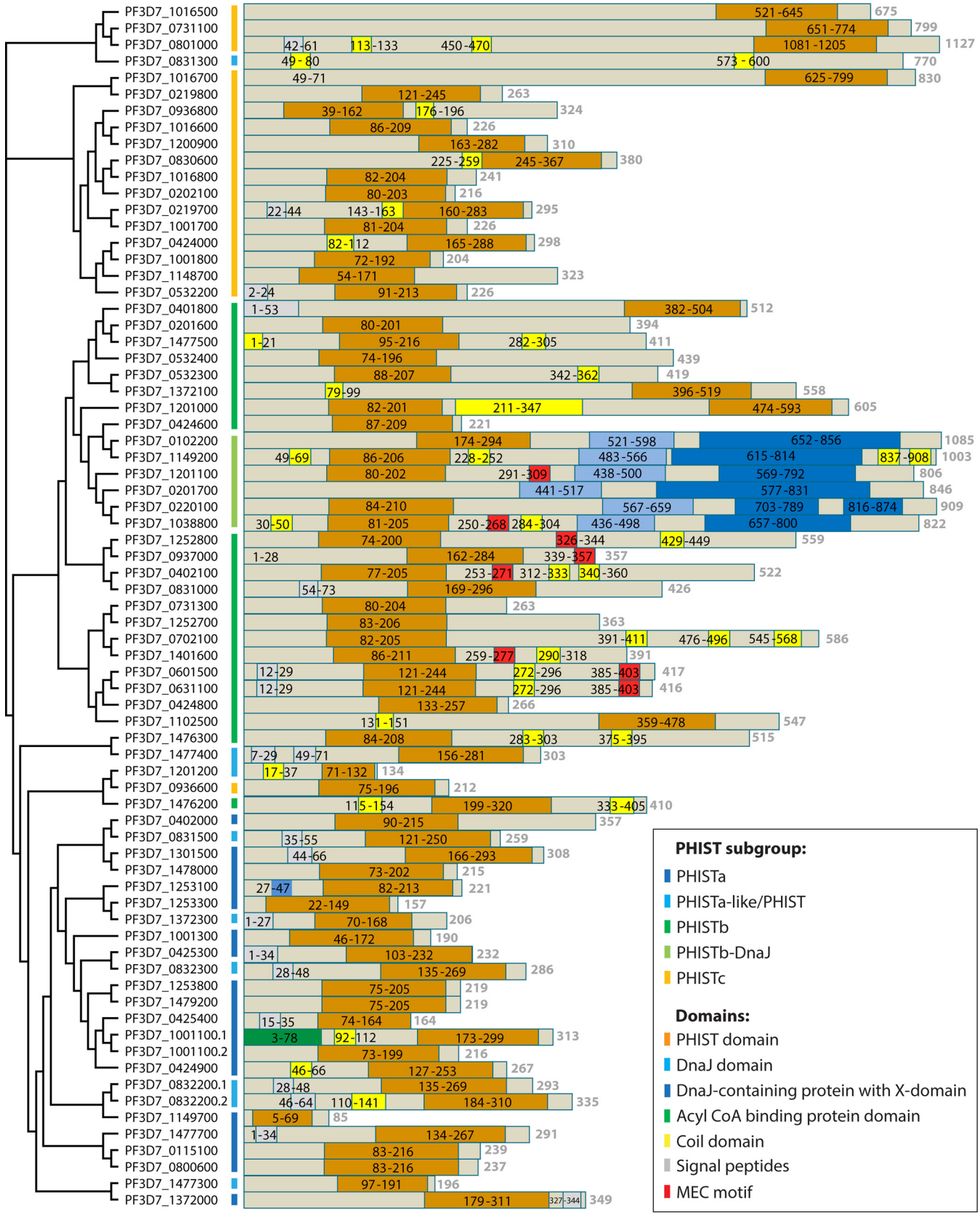
Gene identification[a]	Other gene identification(s) or name(s)[b]	Presence of PEXEL[c]	Gene status[d]	Molecular mass (kDa)[e]	Length (aa)[e]	KO[f]	Description (reference[s] or source)[g]
PF3D7_0831300	MAL8P1.205, GEXP13	(x)		93.0	770		Classified as a PHIST protein (16), although no PHIST domain identified in InterPro; detected in early gametocytes (35); has a PEXEL motif (RKLSE), although this is not yet indicated in any publication
PF3D7_0831500				39.1	335		
PF3D7_0831750			PG	34.0	288		
PF3D7_0831900	MAL7P1.230		PG	33.5	287	x	Deletion in field samples from South America (116), no deletion in samples from Central America (117)
PF3D7_0832200.1	MAL7P1.225			33.5	286		One of the earliest-upregulated genes in the asexual cycle (28), one splice form of MAL7P1.225 (GeneDB)
PF3D7_0832200.2	MAL7P1.225			34.3	293		Same as PF3D7_0832200.1
PF3D7_0832300	MAL7P1.224			30.8	259		Upregulated during the commitment phase for the sexual developmental cycle (53, 145)
PF3D7_0832700	MAL7P1.220		PG	30.8	260		
PF3D7_1000700	PF10_0007, PF10_0008	x	PG	26.1	209		Both old gene identifications have different domains (27), PHISTa protein with N-terminal PHIST domain (15)
PF3D7_1201200	PFL0060w, MAL12P1.12	x		16.0	134		Downregulated in several strains (28), PHISTa-like protein (15)
PF3D7_1301300	MAL13P1.58		PG	28.9	247		Not located in telomeric region (27)
PF3D7_1372300				24.2	206		
PF3D7_1477300	PF14_0744, Pfg14-744	x		22.8	196		Strongly upregulated at gametocytogenesis (27); expression peak in stage I gametocytes but also present at stage II (85, 145, 146); grouped as PHIST or PHISTa-like protein (1, 15, 16); tested as a gametocyte marker (129); expressed as early as in committed schizont stage, regulated by AP2-G, and HP1 is reported to have a silencing effect (54)
PF3D7_1477400	PF14_0745			36.2	303		Expressed in parasites committed to the sexual cycle (27), found in the nuclear proteomic fraction (147), classified as a PHIST protein (16)

[a] Current gene identification from PlasmoDB.
[b] Other/previous names.
[c] The presence of a PEXEL motif is indicated by "x," as identified in reference 15 or 1. A newly identified PEXEL is indicated by "(x)."
[d] Annotated as a pseudogene (PG) in PlasmoDB.
[e] For proteins with a PEXEL motif, molecular mass and length were calculated for the PEXEL-cleaved form of the protein (the molecular mass for PEXEL-cleaved proteins was calculated by using the ExPASy Web tool [http://web.expasy.org/compute_pi/]).
[f] Existing viable knockout (KO) parasites or natural gene deletions are indicated by "x" (see reference 44 or the reference[s] indicated).
[g] Information or references referring to the corresponding gene or protein.

teins, 9 of which belong to the PHISTb/PHISTb-DnaJ subfamily (Tables 2 and 3). Each of these 9 PHIST proteins has a MEC motif in the C terminus downstream of the PHIST domain and in two cases is followed by a DnaJ domain. Three of these PHIST proteins also have been found to bind to inside-out vesicles (IOVs), suggesting an interaction between the MEC motif and a cytoskeleton interaction partner. Coprecipitation with the MEC motif of the PHISTb-DnaJ protein PF3D7_1038800 revealed band 4.1R as an interaction partner (39). Although these PHIST proteins were not further functionally characterized, the presence of a MEC motif, with its capacity to bind to band 4.1R, suggests their involvement in the remodeling of the iRBC cytoskeleton and, thus, a possible contribution to the pathology of malaria.

Seven PHISTb members (Table 3), including the well-known RESA, also comprise a DnaJ domain referred to as PHISTb-DnaJ. Proteins with a DnaJ domain belong to the HSP40 family. The J domains can act as cochaperones for proteins of the DnaK/HSP70 families and can associate with unfolded polypeptide chains to prevent their aggregation (18, 41). PHISTb and PHISTb-DnaJ differ in only one of the four conserved tryptophan positions (Fig. 4 and 5A), indicating their close relatedness. Another characteristic that sets the PHISTb-DnaJ proteins apart from the PHISTb subgroup is the extended length ranging from ~800 to 1,100 amino acids (15).

Several PHISTb proteins have been shown to localize at the host cell periphery, and solubility assays with green fluorescent



protein (GFP)-tagged PHISTb proteins suggested an interaction with components of the host cytoskeleton (20). The authors of this study also investigated sequence requirements for peripheral localization and showed that the PHIST domain or the N-terminal region of the PHIST domain alone is not sufficient for peripheral localization. The PHIST domain together with parts of the N-terminal region, however, conferred peripheral localization (20). This was shown for the PHISTb protein PF3D7_0401800 and the PHISTb-DnaJ protein PF3D7_0102200, and it remains to be confirmed whether this applies to all PHISTb proteins and the precise sequence or structural requirements that are necessary. It also needs to be shown whether PHIST proteins of the other subfamilies have similar requirements for correct localization. Although localization does not predict function, these observations in general confirm that PHISTb proteins tend to associate with the iRBC cytoskeleton.

PHISTc. Most information is available for the PHISTc subgroup, which is entirely shared with *P. vivax* and *P. knowlesi* (15), indicating that the expansion of this subgroup occurred before the lineage diverged. The PHISTc subgroup is also the most diverse group in length, with lengths varying from <200 to >1,200 amino acids. In most of the 18 PHISTc proteins (Table 4), the PHIST domain is found very near the C terminus of the protein (Fig. 3), similarly to the PHISTa subgroup. In contrast to PHISTb proteins, which are associated mostly with the iRBC cytoskeleton, several PHISTc proteins have been found at structures such as Maurer's clefts (19, 42) and exosome-like vesicles (43) and are thought to be involved in protein trafficking (44, 45). There is recent evidence for PF3D7_0936800 (PFI1780w) to also be localized at the host cell membrane (19).

Other PHIST proteins. PlasmoDB lists 14 additional proteins that are annotated as PHISTa-like or simply PHIST proteins but originally were not included in the PHIST family (Table 5). Some of the PHISTa-like proteins were included in the subtelomeric protein superfamily identified by Eksi et al. (27), most of which have now been included in the PHISTa subgroup. There seems to be a close relationship between the PHISTa and the PHISTa-like proteins, indicated by sequence alignment and the pattern of conserved tryptophan residues (Fig. 4 and 5A).

PHIST Gene Expression

Transcriptome analysis of the *P. falciparum* 3D7 strain showed that most *phist* genes were expressed at an early stage during the intraerythrocytic development cycle. Throughout the second half of the cycle, almost all *phist* genes were switched off, while some were upregulated again toward the very end of the cycle (Fig. 6) (46). Genes with similar expression patterns were described to be involved in parasite-specific processes such as host cell invasion (47). In *Plasmodium*, the presence of the protein is often delayed after transcription, and the appearance of PHIST proteins was delayed on average by ~11 h (48). Thus, most PHIST proteins are present and exported during the first half of the intraerythrocytic development cycle, again strongly suggesting an important role of PHIST proteins in host cell remodeling.

Some *phist* genes have been reported to be differentially expressed, for example, during gametocytogenesis (35), in pregnancy-associated malaria (29, 49), or in cerebral malaria (32). Variable expression generally has also been observed in field isolates (50).

Figure 6 presents expression data obtained only from asexual blood stage parasites, but a proteomic study revealed that a number of PHIST proteins are enriched in early gametocytes, the sexual blood stage of *P. falciparum*. Of 26 putatively exported proteins enriched during early gametocyte stages, 9 belong to the PHIST protein family (35) (Fig. 6, yellow boxes). Two of these proteins, namely, GEXP5 (PF3D7_0936600) and PfEMP1-trafficking protein 2 (PfPTP2) (PF3D7_0731100), have been shown by microscopy to localize to gametocytes, with the former having been shown to be expressed exclusively in gametocytes (51). A function during sexual development remains to be shown (43, 51). The presence of some PHIST proteins in gametocytes suggests transcriptional upregulation, and indeed, the expression of ~20 *phist* genes, including GEXP5 (PF3D7_0936600), was shown to be under PfHP1 regulation (52). HP1 is a negative regulator of AP2-G, a transcription factor needed for sexual conversion, which binds to a promoter motif common to several early gametocyte genes, including the *phist* gene PF3D7_1477300 (53, 54). This provides strong evidence that gene regulation by HP1 affects the expression of *phist* genes and their involvement in gametocytogenesis.

Almelli et al. (32) compared transcriptional differences of samples from patients with cerebral malaria and samples from asymptomatic malaria cases from the 3D7 reference strain. A number of *phist* genes from all subfamilies were differentially either up- or downregulated (Fig. 6).

Mackinnon et al. (50) compared gene expression levels of the *P. falciparum* 3D7 strain with those of field isolates and also showed that a number of genes were differentially expressed. Among the 20 most regulated genes were also 7 *phist* genes (PF3D7_0202100, PF3D7_0424000, PF3D7_0702100, PF3D7_0832200.1, PF3D7_0936600, PF3D7_0936800, and PF3D7_1477700), suggesting that some PHIST proteins might be dispensable in culture but not for *in vivo* growth.

It has also been shown that 14 *phist* genes were variably expressed in *P. falciparum* 3D7 (Fig. 6) (55), indicating an active role

FIG 3 Phylogenetic tree and protein domain prediction for PHIST proteins. The amino acid sequences of all 89 PHIST proteins were obtained from PlasmoDB. Of 19 PHIST proteins annotated as pseudogenes, 16 contained one or more premature stop codons in the amino acid sequence and were removed from the list. All those of the remaining 73 PHIST proteins with a PEXEL motif were PEXEL cleaved *in silico* by using the PEXEL motifs provided by Sargeant et al. (15), Boddey et al. (11), or Schulze et al. (12). The amino acid sequences were aligned with MUSCLE (163). The alignment is represented in a phylogenetic tree using the phylogenic tree tool built into MUSCLE at the EMBL-EBI website (164). The branch lengths are drawn in cladogram style and do not represent actual phylogenetic distances. The colored bars next to the gene identifications represent different PHIST subgroups, as indicated. The structure of the amino acid sequences was then analyzed with InterPro (https://www.ebi.ac.uk/interpro/) (165). A schematic representation of the results for each PHIST protein is shown next to its respective gene identification. The following different domains are highlighted: the PHIST domain, the DnaJ domain as defined by Pfam (family PF00226), the DnaJ-containing protein with an X domain as defined by InterPro (accession number IPR026894) or Pfam (family PF14308), the acyl coenzyme A (CoA) binding protein domain as defined by Pfam (family PF00887), coil domains as identified by InterPro, signal peptides as defined by SignalP or transmembrane domains, and the MEC motif as defined by Kilili and LaCount (39).

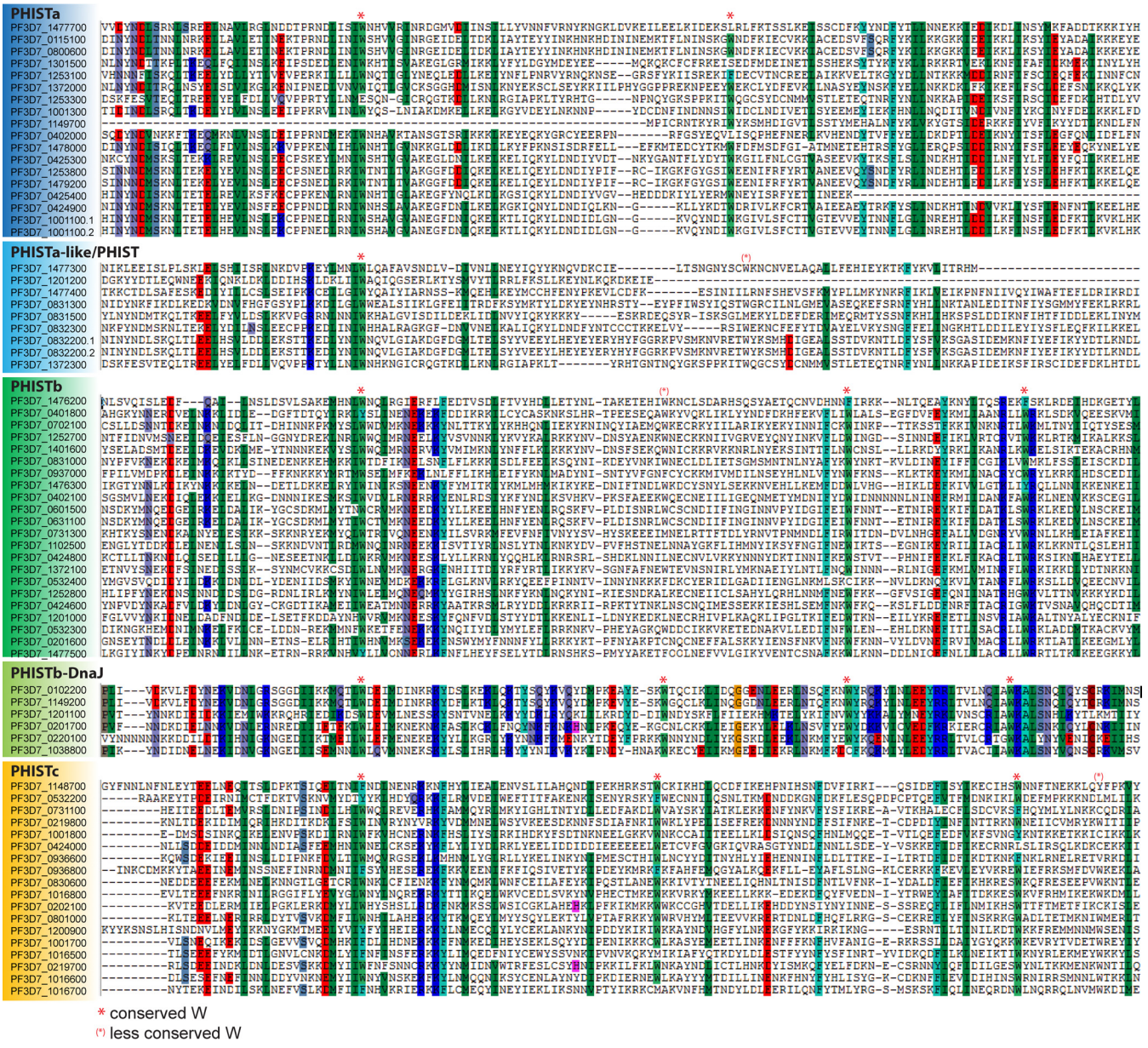


FIG 4 Sequence alignment of PHIST proteins. The processed amino acid sequences as described in the legend of Fig. 3 were sorted into PHIST subgroups, and individual alignments for each subgroup were obtained with CLUSTAL (166). The output was analyzed by using BioEdit (167). Conserved amino acids at a threshold of 83% are highlighted. Shown is the core of the alignment using the position of the first conserved tryptophan to align the different alignment blocks of the subgroups. Tryptophan residues conserved above or below the 83% threshold are marked as indicated.

in adaptation to changing environments such as heat shock (febrile illness) or nutrient depletion.

All of these studies were performed on the transcriptome or proteome level and reported general patterns and trends in gene expression or protein abundance. These studies repeatedly showed that *phist* genes or proteins are involved in different processes and support the notion that PHIST proteins play a central role in host cell remodeling. However, the functional and physical characterization of these proteins lags far behind. So far, we know that PHIST proteins are involved in cellular processes in iRBCs and in disease-associated functions, but our understanding of their actual function and interactions is very limited, at least for the large majority of them.

Below, we review and discuss individual PHIST proteins for which more detailed information is available, with most of them belonging to the PHISTb or PHISTc subgroup. Further information on other PHIST proteins is summarized in Tables 1 to 5.

PF3D7_0532400 (LyMP)

The PHISTb lysine-rich membrane-associated protein (LyMP) (PF3D7_0532400 or PFE1605w) is a PEXEL-containing PHISTb protein that is exported during the first half of the intraerythrocytic development cycle to the erythrocyte membrane, where it can localize at parasite-induced protrusions on the red blood cell membrane called knobs. Its transient localization at Maurer's clefts prior to its final destination correlates in space and time with

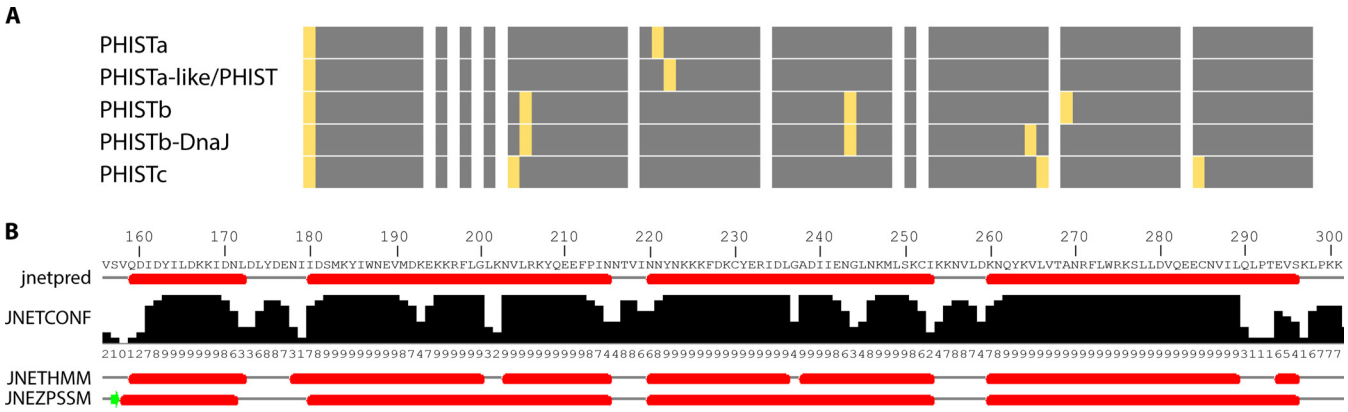


FIG 5 PHIST domain modeling. (A) Visual representation of the positions of the conserved tryptophan residues of the PHIST subgroups, treating PHISTb and PHISTb-DnaJ as well as PHISTa and PHISTa-like/PHIST domains as separate subgroups to show the slight variations between them. A consensus sequence was generated for each subgroup alignment from Fig. 4. The consensus sequence was then split into blocks of 10 residues represented by gray boxes (boxes containing no tryptophans were shortened). The positions of the tryptophan residues conserved within a subgroup are marked by yellow bars. (B) To exemplify the differences in structure prediction, the Jpred4 (168, 169) output is shown for the PHIST protein PF3D7_0532400. The top sequence is the consensus sequence used for PHIST identification by Sargeant et al. (15). JNetPRED displays the consensus prediction: helices are marked with red tubes. JNetCONF provides confidence estimates for the prediction. High values mean high confidence. For the JNetHMM profile-based prediction, the six predicted helices are marked as red tubes. For the JNETPSSM-based prediction, the four helices are marked as red tubes, and sheets are marked with a green arrow.

that of PfEMP1 (19). The PHIST domain of LyMP (amino acids 122 to 335) interacts with the intracellular acidic terminal segment (ATS) of PfEMP1. Conditional downregulation of LyMP reduced binding to CD36 by >60%, indicating that the interaction between the PHIST domain of LyMP and the ATS domain of PfEMP1 is important for the cytoadhesive properties of iRBCs (19, 38, 40, 56). A similar conditional downregulation of LyMP in iRBCs preselected for binding to different adhesion receptors displaying different PfEMP1 variants on the surface strongly differed in the reduction of cytoadherence (38), which led to the hypothesis that different PHIST proteins might be responsible for anchoring different PfEMP1 variants to the cytoskeleton.

It was further shown that the C-terminal segment of LyMP (amino acids 319 to 528) was able to bind IOVs, which are used to study protein interactions involving cytoskeletal proteins (40). Recently, we were able to show that this part of LyMP binds directly to human membrane protein band 3, which is linked to the cytoskeleton via ankyrin (38). Together with the ATS interaction mediated by the LyMP PHIST domain (19), it is evident that LyMP can act as a linker between the virulence complex of *P. falciparum* PfEMP1 and the host cytoskeleton.

PF3D7_0936800

PF3D7_0936800 (PFI1780w) is a PHISTc protein that was also shown to interact with the ATS domain of PfEMP1 albeit much more weakly than LyMP (56). A crystallographic structure of its PHIST domain (residues 85 to 247) has been obtained and is the first available structure for any PHIST protein. It confirms the predicted four-alpha-helix structure of the PHIST domain with a very short first alpha helix. The remaining three helices form a three-helix bundle with weak structural similarity to spectrin (19, 56).

PF3D7_0936800 has been classified as a noncanonical PEXEL protein with the first position of its PEXEL motif rendered from K to R, which was recently shown to be correctly cleaved and N-acetylated, confirming that this PHIST protein is correctly exported from parasites into iRBCs (12).

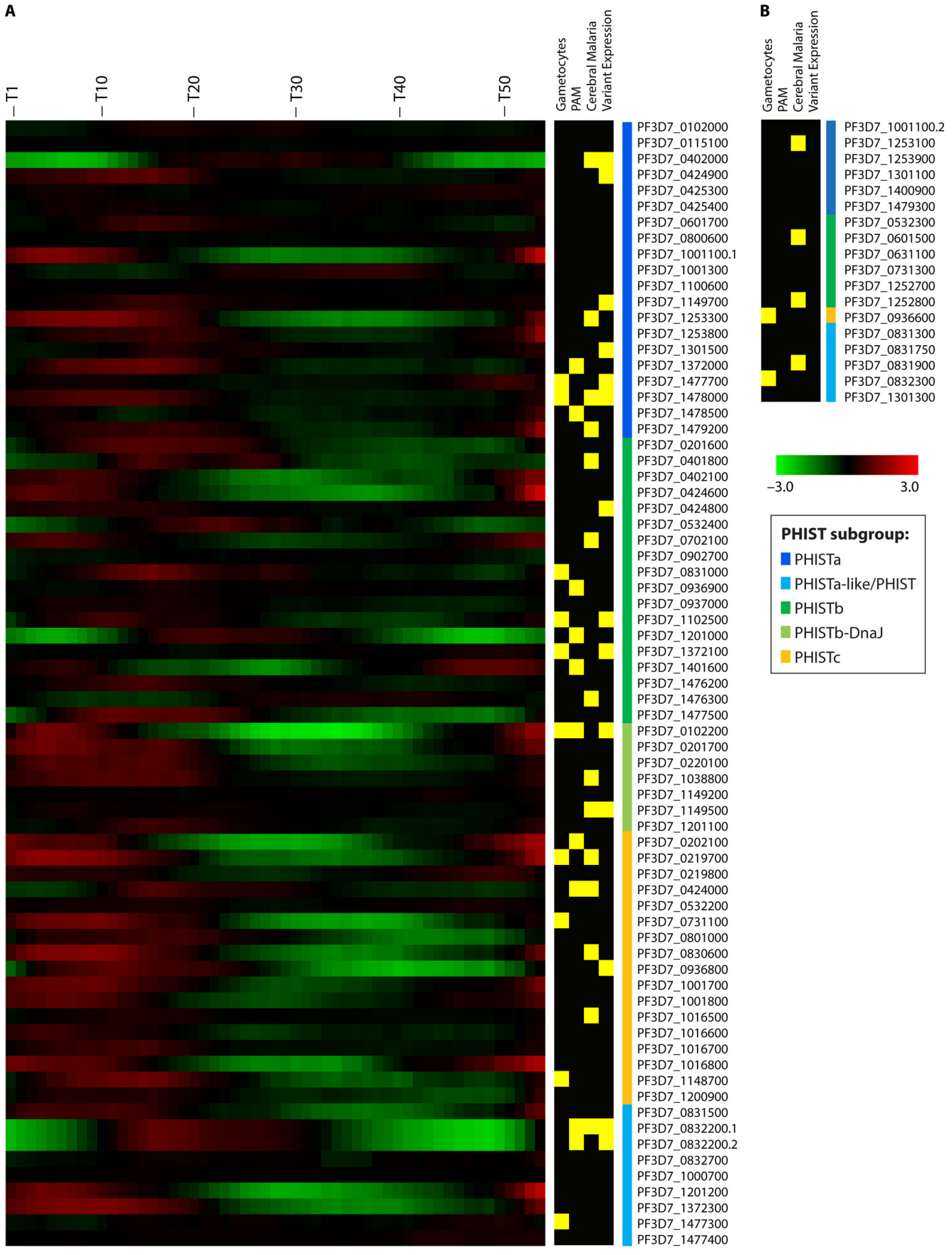
PF3D7_0936800 localizes underneath the iRBC membrane but is absent from knobs (19). It is speculated that it contains an interaction epitope for binding of iRBC membrane/cytoskeleton components (12). Here it is also noteworthy that PF3D7_0936800 has been found to be variantly expressed in 3D7 (55) and that there has been no reported PF3D7_0936800 knockout (PF3D7_0936800-KO) parasite.

PF3D7_0731100 (PfPTP2)

The PHISTc protein PF3D7_0731100 (PfEMP1 trafficking protein 2, PfPTP2) seems to play a role in PfEMP1 trafficking or surface display. Depletion of this protein leads to PfEMP1 accumulation in Maurer's clefts and its absence from knobs. Under flow conditions, PfPTP2-KO parasites do not adhere to chondroitin sulfate A (CSA), suggesting that the main role of PfPTP2 might be the trafficking of PfEMP1 from Maurer's clefts to knobs (42, 44).

Recently, PfPTP2 was also described to be located at exosome-like vesicles of ~70 nm that are involved in cell-cell communication and allow nucleic acid transfer between *Plasmodium* parasites. PfPTP2-deficient parasites showed reduced cell-cell communication, and Regev-Rudzki et al. (43) suggested that PfPTP2 is essential for cell-cell communication between *P. falciparum*-infected erythrocytes, leading to increased gametocytogenesis *in vitro*. PfPTP2 was the only protein involved in knob formation or PfEMP1 display (besides skeleton binding protein 1 [SBP1] [57]) but was also found to be enriched in early gametocytes (35, 43). In contrast, transcriptional analysis shows that PfPTP2 is downregulated in gametocytes; thus, PfPTP2 abundance in early gametocytes might be acquired through exosome-like vesicles, and this might in turn induce sexual conversion. Such a hypothetical model would accommodate the fact that PfPTP2 is upregulated in asexual stages but also enriched in gametocytes.

Immunoelectron microscopy and proteinase K assays revealed the localization of PfPTP2 on the cytosolic face of Maurer's clefts, on budding vesicles, and on the surface of exosome-like vesicles (43, 44).



PF3D7_0402000

The PHISTa protein PF3D7_0402000 was identified in a yeast two-hybrid assay to determine potential interaction partners of the human erythrocyte cytoskeleton protein band 4.1R. The protein interacted with the N and alpha lobes of the FERM (four-point-one, ezrin, radixin, and moesin) domain (Pfam family PF00373) of band 4.1R through helices 2 and 3 of the PHIST domain, but the entire domain was required for maximal interaction (58). The host cytoskeleton protein band 4.1R binds to actin filaments and links these to membrane proteins such as band 3 and glycophorin C, maintaining the biconcave shape (reviewed in references 58 and 59). In this context, it is noteworthy that other *P. falciparum* proteins, e.g., MESA (PF3D7_0500800), which is not a PHIST protein, bind to this host cell cytoskeleton interaction hub (60). In immunofluorescence imaging analyses, subpopulations of both band 4.1R and PF3D7_0402000 were shown to colocalize at the parasitophorous vacuole membrane (58). The role that PF3D7_0402000 plays in changes of membrane rigidity or host actin recruitment remains unclear.

PF3D7_0936600 (GEXP5)

GEXP5 (gametocyte exported protein 5) was originally classified as PHISTb (15) but is now grouped among the PHISTc proteins (16), which is confirmed by the position of its conserved tryptophan residues (Fig. 4). GEXP5 was the first PHIST protein detected in gametocytes. A GEXP5-GFP fusion protein, when episomally expressed under the control of the endogenous promoter in asexual stages, was not detected by fluorescence imaging (51) but could be detected when expressed under the *pfcam* promoter, confirming a cytosolic localization (20). This indicated that endogenous GEXP5 is expressed and exported only in sexual stages and is found in the iRBC cytosol of stage I to IV gametocytes. Its uniform distribution throughout the iRBC and its presence in the soluble protein fraction suggest that it is a soluble protein (51). GEXP5 is already found at 14 h postinvasion in ring stage parasites committed to gametocytogenesis and is now considered to be the earliest gametocyte marker (51). This early appearance of GEXP5 in committed parasites might explain the presence of transcripts in the expression data sets in PlasmoDB.

The expression and export of GEXP5 are independent of transcription factors normally associated with gametocytogenesis, such as PfAP2-G, but GEXP5 is not able to drive gametocyte maturation alone in the absence of these transcription factors (51). It is therefore assumed that GEXP5 is not involved in processes driving gametocytogenesis but is used to remodel or prepare the host cell to accommodate sexual development.

PF3D7_0102200 (RESA)

RESA (ring-infected erythrocyte surface antigen) contains both PHIST and DnaJ domains and is one of seven members of the PHISTb-DnaJ subgroup. Its name is a misnomer since RESA is an intracellular exported protein (20).

RESA is expressed in mature-stage parasites (61) and is stored in the dense granules of newly formed merozoites (62). Within minutes after the invasion of a merozoite into an erythrocyte, the contents of the dense granules, including RESA, are discharged into the parasitophorous vacuole (63–65). From there, RESA is then exported to the iRBC and localizes at the iRBC cytoskeleton, where it remains for ~24 h (64, 65). At the cytoskeleton, RESA is phosphorylated (66, 67) and interacts with spectrin (68). A fragment of 108 amino acids has been identified to interact with repeat 16 of the β-chain of spectrin (here it is important to note that residues 663 to 770 correspond to only a partial DnaJ X domain and not to the DnaJ J domain). As a result of this interaction, the spectrin tetramers are stabilized and rigidify the iRBC (69); however, it needs to be proven whether this interaction and the accompanying rigidification would also occur with the full-length domain or full-length RESA protein.

There is controversy regarding whether this interaction impairs the invasion of merozoites, since one study with erythrocytes prepacked with a recombinant form of RESA showed that the RESA-spectrin interaction had no significant effect on invasion (70). The recombinant RESA used in this study contained residues 322 to 1073 and lacked the entire PHIST domain (residues 174 to 294) (70) (Fig. 3). However, using a similar setup, Pei et al. showed that an even shorter fragment of RESA (residues 663 to 770) was able to reduce the invasion efficiency (69). Importantly, this recombinant peptide lacked both the PHIST domain and the DnaJ domain and hence might have created an artificial inhibitory interaction.

A domain of 70 amino acids found in RESA shares 39% homology with the DnaJ heat shock protein of *Escherichia coli* (41). Proteins with a DnaJ domain belong to the HSP40 family and act as cochaperones to the DnaK/HSP70 chaperones, which are involved in protein assembly and trafficking (18, 41). Proteins with a DnaJ domain have been described to be membrane associated (41), a feature shared by RESA. Initially, it was proposed that RESA destabilizes the RBC cytoskeleton (64), but now there is evidence that RESA in fact does the contrary, namely, stabilizing the cytoskeleton with the effect that it rigidifies ring stage infected parasites (65, 69, 71). It has been shown that the RESA-spectrin interaction protects iRBCs from thermal damage (65, 69, 70), and it has been speculated that the DnaJ domain of RESA might prevent spectrin from unfolding at elevated temperatures, or it might directly bind to hydrophobic regions of partially unfolded spectrin molecules (70). This rigidification of ring stage parasite-infected erythrocytes, potentially impairing passage through the spleen (72), is rather surprising, but this might simply be a side effect of the protective function of RESA against thermal damage to the host cell.

At the end of each 48-h cycle, the iRBC ruptures and releases

FIG 6 Heat maps. (A) Heat map of *phist* gene expression of the *P. falciparum* 3D7 strain over the course of 53 h. Microarray data on transcript abundance were reported previously (46) (accessed through PlasmoDB, release 28, 31 March 2016). The heat map was constructed by using TMeV4.9 (170). *phist* genes were clustered into their respective subgroups and were then ordered by gene identification. Genes annotated "*phista*-like" or as "*phist*" genes were grouped together as "*phist* others." Yellow boxes to the right of the heat map indicate PHIST proteins present in the early gametocyte proteome (35), differentially expressed *phist* genes in pregnancy-associated malaria (29, 49, 50, 84, 92, 112) and in cerebral malaria (32, 34), or if variant expression has been reported (55). Green to red colors represent fold changes (log fold changes from −3 to 3). (B) For a number of *phist* genes, no expression data were available in the data set, and these genes were grouped first by PHIST subgroup and then by gene identification. Additional information for selected PHIST proteins is provided in Tables 1 to 5. The colored bars next to the gene identifications indicate the PHIST subgroup.

new merozoites. This event is accompanied by fever peaks that last for a few hours (73, 74). During these fever episodes, the parasites are in ring stages, and this correlates with the time when RESA interacts with the cytoskeleton, protecting it from thermal damage caused by fever. After ~24 h, RESA dissociates from spectrin (61), and other proteins might be responsible for further rigidification of the cytoskeleton (71). This apparent functional contradiction might be an evolutionary tradeoff between the need for survival during fever and costs of increased cell rigidity to a level that still allows passage through the spleen. The fact that less deformable ring stage parasite-iRBCs are cleared by the spleen supports this idea of a tradeoff (72).

While the function of the DnaJ domain in RESA has been described in much detail, the function of the PHIST domain still remains enigmatic.

PVX_093680 (CVC-81_{95})

The only PHIST protein not from *P. falciparum* that has been further characterized is the *P. vivax* PHIST CVC-81_{95} (PVX_093680). Caveola-vesicle complexes (CVCs) are parasite-induced indentations in *P. vivax*-infected erythrocyte cell membranes. The exact structure, protein composition, and function are not yet fully understood, but CVC-81_{95} is exported, is a predominant protein in these CVCs, and is found on tubular extensions going inwards from the CVC (75, 76). Its orthologue in *P. cynomolgi* shows a similar localization (75). CVC-81_{95} was found by Acharya et al. (77) in *P. vivax*-infected peripheral blood, and another study showed that over 80% of patient sera reacted positively with CVC-81_{95} (78). Based on these findings, CVC-81_{95} was suggested to be involved in immune evasion (78), as the PHIST protein family was previously suggested to be involved in this process (24). Therefore, in *P. vivax*, PHIST proteins also seem to be localized to parasite-induced structures in iRBCs and might be essential for host-parasite interactions. However, more functional analyses are required to fully understand the role of this PHIST protein in the CVC of *P. vivax*.

phist Gene Knockout Study

In a large gene knockout study in *P. falciparum* to functionally characterize exported proteins, 17 *phist* genes were targeted for deletion (44). These genes included 1 *phista* gene, 7 *phistb* genes, 6 *phistb-dnaJ* genes, and 3 *phistc* genes. Of these, all but four (PF3D7_0936800, PF3D7_0401800, PF3D7_0402100, and PF3D7_1149000) were successfully disrupted, indicating that the majority was dispensable for *in vitro* growth. Except for PF3D7_0731100 (PfPTP2), none of the *phist* knockout parasites from this study were analyzed in greater detail. Some were reported to have an influence on altered knob morphology (PF3D7_0424600-KO) or reduced (PF3D7_0102200/RESA-KO) or increased (PF3D7_0220100-KO) iRBC rigidity (44). Further phenotypic characterization of these parasite lines is urgently needed to better understand the role of PHIST proteins in host cell remodeling.

Structure and Function

It is highly conceivable that PHIST proteins might fulfill similar roles as chaperones, since a number of chaperones also contain DnaJ domains. They may also be responsible for the close association with the host cytoskeleton and cell membrane due to the intrinsic positive charge of many PHIST proteins. The few studies on PHISTs have looked mostly at the PHIST domain only; however, Tarr et al. (20) suggested that the PHIST domain alone is not sufficient to correctly target the protein to its destination, at least for those PHIST proteins investigated. Mayer et al. (56) analyzed the binding affinities of various PHIST domains for a number of ATS domains, but only the PHIST protein PF3D7_0532400 has been studied in greater detail with respect to structure and function (19, 56). This is rather surprising for a protein family comprising a large proportion of the complete exportome of *P. falciparum*.

CONCLUSION

The three major PHIST subfamilies not only differ in sequence but also seem to differ in function. PHISTb proteins mostly interact with and localize to the iRBC cytoskeleton and seem to be involved in changes of cell properties such as rigidity. PHISTc proteins seem to be involved mostly in protein trafficking of other exported proteins, while the PHISTa subfamily remains enigmatic, and there is not yet sufficient information available to draw a conclusion on function.

The presence of PHIST proteins at various localizations in the host cell makes them highly interesting candidates as interaction partners for other exported proteins, and therefore, it would be important to understand what determines the destination of PHIST proteins. The identification of export requirements for PHIST proteins would increase our understanding of the interaction network of exported proteins.

It seems that some PHIST proteins have at least two binding domains, the PHIST domain and a second one toward the C terminus, e.g., the MEC motif, the spectrin binding site, the band 3 interaction epitope, or a DnaJ domain. This would make PHIST proteins ideal molecules for multifunctional interactions at the iRBC cytoskeleton or in protein export with the PHIST domain serving as a general adaptor, while the C-terminal domain could function as a highly specific binding site. However, only a few additional binding motifs have been identified in some PHIST proteins, and more PHIST proteins remain to be studied.

Our knowledge of PHIST proteins is still very limited and based mainly on studies of the 3D7 laboratory strain, which does not suffice to understand the full extent of PHIST protein functions for *in vivo* parasite survival and malaria-associated disease and pathology. PHIST proteins should be studied in various disease presentations from mild to severe malaria as well as in pregnancy-associated malaria. Furthermore, the role of PHIST proteins in gametocytogenesis and also in mosquito stages remains completely enigmatic. Since many PHIST proteins seem to be central for host cell remodeling, it is essential to understand their function in this crucial process.

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