# *Plasmodium falciparum*: Characterization of a late asexual stage Golgi protein containing both ankyrin and DHHC domains

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## Abstract

Proteins containing the DHHC motif have been shown to function as palmitoyl transferases. The palmitoylation of proteins has been shown to play an important role in the trafficking of proteins to the proper subcellular location. Herein, we describe a protein containing both ankyrin domains and a DHHC domain that is present in the Golgi of late schizonts of *P. falciparum*. The timing of expression as well as the location of this protein suggests that it may play an important role in the sorting of proteins to the apical organelles during the development of the asexual stage of the parasite.
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## 1. Introduction

The Apicomplexan parasites, that include the medically important *Plasmodium* species, are characterized by the presence of three novel asymmetrically oriented organelles. These organelles, located in the region of the parasite that first enters the erythrocyte, are termed apical organelles. The complicated process of erythrocyte invasion is mediated, in part, by proteins that are stored in these three organelles (rhoptries, micronemes, and dense granules) and released at the time of invasion. The organelles are formed late in the asexual erythrocytic cycle, at the time of formation of merozoites, the invasive form of the parasite. The trafficking processes that lead to the sorting of these proteins from the Golgi to the correct apical organelle remain to be defined. Unlike the closely related *Toxoplasma*, where a tyrosine-based cytoplasmic motif for sorting to rhoptries exists (Hoppe et al., 2000), no such motif has been identified in *Plasmodium*. We describe a molecule that, because of its structure, localization, and temporal expression might catalyze key lipid modifications of proteins that could play an important role in the sorting of proteins to apical organelles in *Plasmodium*.

In the course of the preliminary analysis of the *Plasmodium falciparum* genome (Gardner et al., 2002), it was noted that several proteins contained a novel conserved domain of approximately 48 amino acids which contains seven conserved cysteines and a characteristic DHHC (Asp-His-His-Cys) motif (Fig. 1). Subsequent studies on the yeast endoplasmic reticulum protein Erf2, which also contains a DHHC domain, showed that it was required for the appropriate localization of the yeast RAS protein to the internal leaflet of the plasma membrane (Bateman et al., 2000; Putilina et al., 1999). This failure of proper trafficking of RAS was shown to arise due to a lack of palmitoylation of the RAS protein. Further studies on

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                    Ank
                 Domains  TM
P.falciparum PFC0160w   5      4    CVTCNIIKPPRVHHCAECFHCIVHQDHHCVWVDNCIGIKNQRCFYMFI
P.vivax H14090          5      4    CVTCNIVKPPRVHQRAECFHCIVHQDHHCVWVDNCIGIKNQRAFYLFI
H.sapien HIP14          6      6    CSTCLIRKPVRSKHCGVCNRCIAKFDHHCPWVGNCVGAGNHRYFMGYL
L.major Q9BLUO          0      4    CMTCHIYRPSQSGHCRRCNNCVALFDHHCRLLGCCIGELNRRYFLLFL
D.melanogaster CG6618   6      4    CHSCRCLRPLRAKHCRVCNRCVSYFDHHCPFIYNCVGLRNRMWFFLFV
S.cerevisiae ERF2       0      4    CPSCRIWRPPRSSHCSTCNVCVMHVDHHCIWVNNCIGKRNYRFFLIFL
A.thaliana AF013294     0      4    CHHCSVCKAK-SDLFILCQRCVLKMDHHCVWIVNCVGARNYKFFLLFL
                                     *   *   :.       *  *:   ****  :  *:*  *   *  ::
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Fig. 1. Multiple alignment of DHHC domains. The alignment was constructed using the ClustalW program.

DHHC-containing proteins have shown that many of them function as membrane-associated enzymes that transfer a 16-carbon palmitate moiety to the sulfur on a cysteine residue (Roth et al., 2002). This palmitoylation is widespread in eukaryotes and results in soluble proteins becoming associated with membranes or altering the behavior of transmembrane proteins by modification of their cytoplamic tails (Linder and Deschenes, 2003).

## 2. Materials and methods

### 2.1. Parasite culture

The 3D7 strain of *P. falciparum* was cultured essentially as described by Trager and Jensen, with the modification of the addition of Albumax II (Invitrogen) rather than serum (Trager and Jensen, 1976). Parasites were synchronized by the treatment of cultures with 5% sorbitol, resulting in the lysis of trophozoite stages and survival of ring stage parasites.

### 2.2. Antisera production

An 800 bp portion of the DHHC-containing gene was amplified using the following primers: 5′ GAGAG ATCTAATTCTATAAACATCTTACATTGGGC and 3′ GAGACGCGTATAAACCATATTTAAACAGCC ATAATCACA. The resulting product was cut with *Mlu*I and *Bgl*II and ligated into a modified VR1020 vector (gift of Stephen Hoffman). Five hundred micrograms of this purified vector was injected intradermally into Sprague–Dawley rats three times at three week intervals. Animals were exsanguinated 10 days after the final injection and sera were collected.

### 2.3. Western blotting

Late stage parasites were saponin lysed and subsequently resuspended in PBS with 2% SDS and protease inhibitors (complete protease inhibitor cocktail (Roche) plus 1 $\mu$M pepstatin A). Extract equivalent to $5 \times 10^5$ parasites was run on a 3–8% Tris–acetate gel and transferred to PVDF (Invitrogen). The membrane was probed with a 1:500 dilution of primary sera and a 1:25,000 dilution of goat anti-rat sera conjugated to HRPO (Jackson Immunoresearch). Bands were visualized using ECL (Amersham).

### 2.4. Immunofluorescence

Late stage parasites were smeared and fixed for 10 min in 1% formaldehyde. Slides were subsequently blocked for 1 h at room temperature in blocking buffer (PBS, 1% normal goat serum (Jackson Immunoresearch), 0.1% Triton X-100). Primary antibodies were diluted in blocking buffer at the following concentrations: rat anti-DHHC 1:200, anti-RAP 1:500, anti-subtilisin 2 (a kind gift of Jean-Christophe Barale) 1:250, and rabbit anti-ERD2 (a kind gift of John Adams) 1:200. Slides were allowed to incubate with primary antibodies at room temperature for 1 h and were subsequently washed in PBS and stained with the appropriate secondary (Jackson Immunoresearch) diluted 1:500 for 30 min at room temperature. Slides were then washed in PBS, mounted in anti-fade reagent (Molecular Probes) and visualized using a Leica TSC-NT/SP confocal microscope under $63\times$ oil immersion objective. Images were subsequently deconvoluted using Imaris software.

## 3. Results and discussion

Completion of the *Plasmodium* genome has revealed the presence of 13 distinct proteins containing the DHHC domain, suggesting an active palmitoylation system in the organism (Aravind et al., 2003). We have analyzed one of
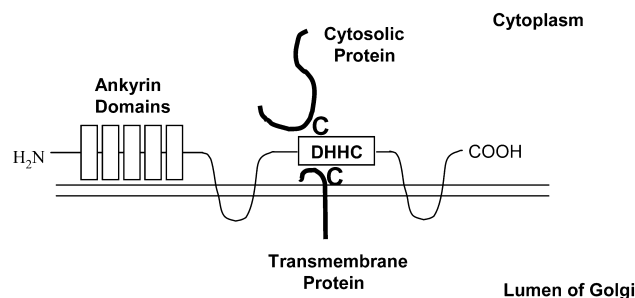


Fig. 2. Proposed model of topology of PfAnkDHHC in the Golgi membrane. The DHHC domain is exposed to the cytosol to serve as a palmitoyl transferase to cytosoloic proteins or transmembrane proteins with cysteines.

these proteins (NP473165). This gene encodes a protein containing five ankyrin domains in the N-terminus and a DHHC domain at the C-terminus with four transmembrane domains, two on either side of the DHHC domain (Fig. 2). This 75-kDa protein was accordingly named PfAnkDHHC, and its predicted eight-exon structure was
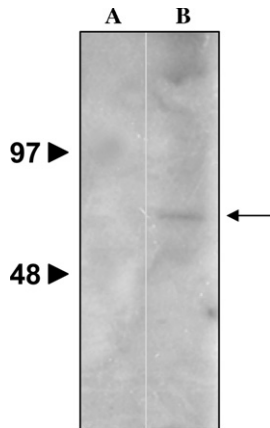


Fig. 3. Western blot analysis performed on 3D7 asexual parasite extract, 46 h post-invasion. Lane A was probed with rat pre-immune sera, lane B with sera from a rat immunized with the vector expressing PfAnkDHHC. Arrow indicates position of PfAnkDHHC protein.

confirmed by RT-PCR (data not shown). Orthologous proteins with similar domain architecture are seen in fungi (e.g., *S. cerevisiae* Akr1), plants, and animals (e.g., *Homo sapiens* HIP14) and are Golgi-localized proteins that have been implicated in intracellular trafficking and endocytosis (Feng and Davis, 2000; Singaraja et al., 2002). This suggests that PfAnkDHHC, like its orthologs in other eukaryotes, may participate in Golgi-associated modifications of proteins and thereby regulate their localization. For PfAnkDHHC to be located on the membrane of the Golgi and function in palmitoylation of the cytoplasmic tails of transmembrane proteins, it must adopt a membrane topology with the ankyrin domains on the cytosolic side of the membrane (model in Fig. 2). The 33-amino-acid ankyrin repeats form a helical superstructure and are believed to mediate protein-protein interaction in a range of biologic functions. Hence, they could serve as a scaffold for binding substrates in the case of the PfAnkDHHC protein.

Antiserum was produced against the C-terminal region of the protein that contains the DHHC domain. Although there are 13 DHHC domains containing proteins in the *Plasmodium* genome, none show greater than 22% similarity in the region against which the antiserum was raised. This antiserum was subsequently used to characterize PfAnk DHHC. The antiserum was used in a
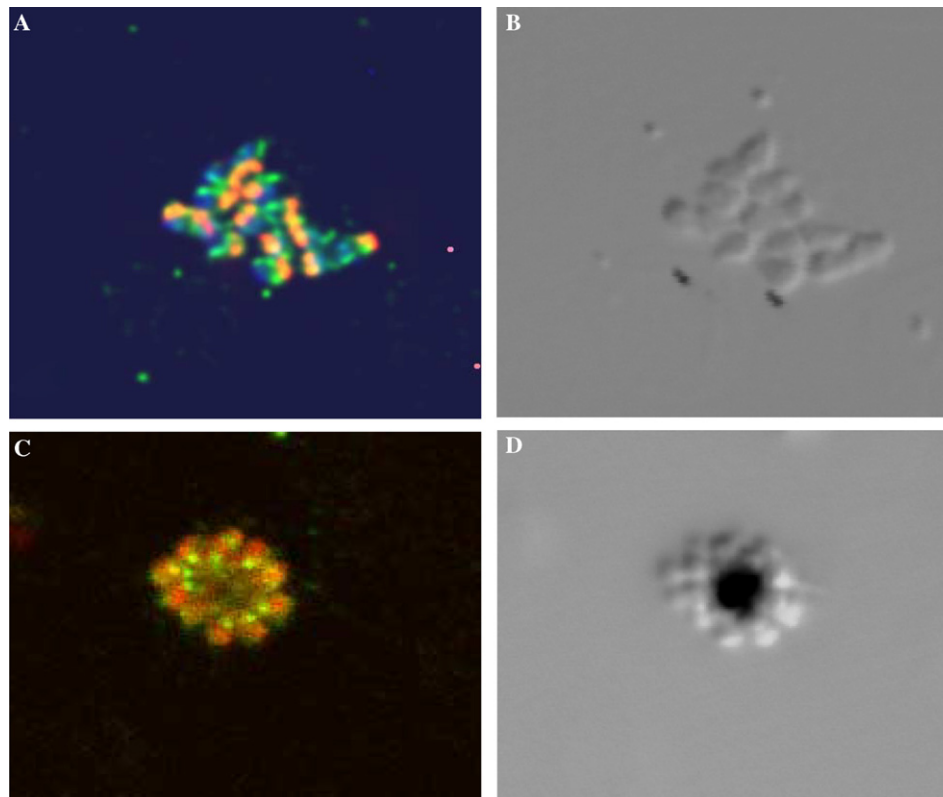


Fig. 4. Confocal microscopy demonstrates the location of PfAnkDHHC posterior to apical organelles. (A) 3D7 merozoites were labeled with rat-antiPfAnkDHHC and Alexa-488 conjugated anti-rat IgG (green) and with anti-RAP-1 and Alexa-594 conjugated anti-mouse IgG (red). Nuclei were stained with DAPI and appear blue. (B) DIC image corresponding to Fig. 2A. (C) 3D7 merozoites in schizonts labeled with anti-PfAnkDHHC (green) and a mouse antisera recognizing subtilisin-2 (red). Merozoites attached to the pigment containing residual bodies have their apical end oriented opposite to the residual body. (D) DIC image corresponding to Fig. 2C. All images 630×.

Western blot of parasite extract and found to recognize a protein of 75-kDa as predicted (Fig. 3). This serum was also used in immunofluorescence assays to localize the gene product. Neither rings nor trophozoites showed any staining, with staining only appearing at the late schizont stage. This expression profile would correlate with the RNA expression profiles from both of the recent global transcriptome analyses of *Plasmodium* (Bozdech et al., 2003; Le Roch et al., 2003). Fig. 4 shows the localization of the DHHC-containing protein within schizonts. The staining pattern indicates that PfAnkDHHC is located in each individual merozoite but was not located at the apical end in any of the apical organelles. Instead, it has a V-shaped distribution and appeared adjacent to the nucleus (Fig. 4A and data not shown). PfAnkDHHC is located separate from the dense granules, from immunofluorescence co-localization studies using antisera to subtilisin-2, a dense granule protein (Fig. 4C). The dense granules are an apical organelle located between the rhoptries and the nucleus (Bannister et al., 2000). Further confocal studies show significant staining overlap with the known Golgi marker ERD2 (Elmendorf and Haldar, 1993) confirming the localization of PfAnkDHHC to the Golgi (Fig. 5).

The palmitoylation of both transmembrane and cytosolic proteins has been shown to increase the likelihood of their association with lipid rafts. In the case of the T cell proteins Lck, LAT, and Lyn, it has been shown that the absence of palmitoylation can lead to the failure of association of these proteins with lipid rafts and subsequent loss of function (Kabouridis et al., 1997; Zhang et al., 1998). A recent report on a novel *P. falciparum* rhoptry protein, RAMA, speculates that lipid raft transport may be the method of delivery of this protein from the Golgi to the rhoptries (Topolska et al., 2003). The use of lipid rafts as a means of trafficking GPI-anchored and palmitoylated proteins to rhoptries is proposed.

The sorting of various proteins during the process of parasite maturation is an area that remains primarily unexplored. One of the unique problems presented in *Plasmodium* and other Apicomplexa is the sorting of proteins to the apical organelles. These organelles—the rhoptries, micronemes, and dense granules—are unique organelles that appear late in the development of the formation of merozoites, the invasive form of the parasite. The organelles play an important role in the invasion process, with many of the adhesive proteins thought to be responsible for initial binding and attachment to
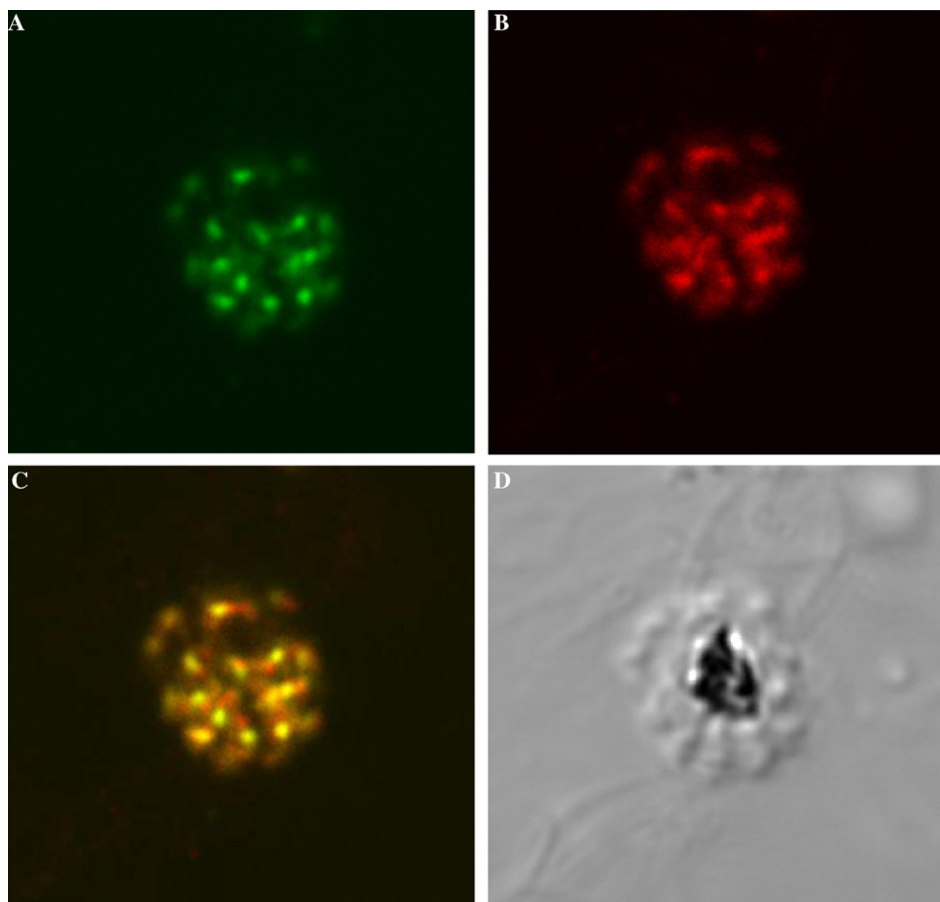


Fig. 5. Confocal microscopy localizes PfAnkDHHC to Golgi. 3D7 schizonts were labeled with (A) rat anti-PfAnkDHHC, and (B) rabbit anti-ERD2, a known Golgi marker. (C) Merged image of the two stains shows extensive co-localization. (D) Corresponding DIC image. All images 630×.

| Gene | Cytoplasmic Tail |
|------|------------------|
| PfNBP | KNNKQEYDKEQEKQQQNDFV**C**DNNKMDDKSTQKYGRNQEEVMEIFFDNDYI |
| PfRh2a | KTNSGDNNSNEINEAFEPNDDVLFKEKDEIIEITFNDNDSTI |
| PfRh2b | DRSNKDE**C**DFDM**C**EEVNNNDHLSNYADKEEIIEIVFDENEEKYF |
| PfRh4 | NEPHHIFQKEFSEADNAHSEEKEEYLPVYFDEVEDEVEDEVEDEDENENEVENENEDFNDI |
| PvRBP-1 | KKDDQEELNNGVEDDKVFEVKKSMHPENKEEIIDDSFVDIEY |
| PvRBP-2 | KDKDEEKDHNEHGYNEAFGEHDEYNMHDKEEVIEV**C**FNEED |
| PfEBA-140 | RMGKSNEEYDIGESNIEATFEENNYLNKLSRIFNQEVQETNISDYSEYNYNEKNMY |
| PfEBA-180 | RKNLDDEKGFYDSNLNDSAFEYNNNKYNKLPYMFDQQINVVNSDLYSEGIYDDTTTF |
| PfEBA-175 | QAKYQSSEGVMNENNENNFLFEVTDNLDKLSNMFNQQVQETNINDFSEYHEDINDINFKK |
| PvDBP | KNVASNDYEEEATFDEFVEYSDDIHRTPLMPNHIEHMQQFTPLDYS |
| PfAMA-1 | KRKGNAEKYDKMDEPQDYGKSNSRNDEMLDPEASFWGEEKRASHTTPVLMEKPYY |

Fig. 6. Table of cytoplasmic domains of invasion-related, apically located transmembrane proteins. A subset found in rhoptries has a cysteine available for possible palmitoylation.

erythrocytes being found in these organelles. These binding proteins include the Duffy-binding-like family, apical membrane antigen-1, and the reticulocyte-binding-like proteins. Unlike the case in *Toxoplasma* (Hoppe et al., 2000), no conserved motif has been found in the cytoplasmic tails of these proteins that might "mark" them for an apical organelle destination. Clearly, an alternate method for sorting must be in use. Acyl modification of proteins through cysteines has been shown to play a role in trafficking in other organisms, and we have shown here the presence of a protein containing a palmitoyl transferase motif in the Golgi of the parasite at a time when such acyl modification would need to take place. Some of the reticulocyte-binding-like proteins of *P. falciparum* and *P. vivax* contain a cysteine in the cytoplasmic region and could serve as targets for this palmitoylation (Fig. 6). The above preliminary characterization of PfAnkDHHC provides a handle for further investigation of the potential palmitoylation targets of this enzyme and its role in organellar trafficking.

## References

Aravind, L., Iyer, L.M., Wellems, T.E., Miller, L.H., 2003. *Plasmodium* biology: genomic gleanings. Cell 115, 771–785.

Bannister, L.H., Hopkins, J.M., Fowler, R.E., Krishna, S., Mitchell, G.H., 2000. A brief illustrated guide to the ultrastructure of *Plasmodium falciparum* asexual blood stages. Parasitology Today 16, 427–433.

Bateman, A., Birney, E., Durbin, R., Eddy, S.R., Howe, K.L., Sonnhammer, E.L., 2000. The Pfam protein families database. Nucleic Acids Research 28, 263–266.

Bozdech, Z., Llinas, M., Pulliam, B.L., Wong, E.D., Zhu, J., DeRisi, J.L., 2003. The transcriptome of the intraerythrocytic developmental cycle of *Plasmodium falciparum*. PLoS Biology 1, E5.

Elmendorf, H.G., Haldar, K., 1993. Identification and localization of ERD2 in the malaria parasite *Plasmodium falciparum*: separation from sites of sphingomyelin synthesis and implications for organization of the Golgi. EMBO Journal 12, 4763–4773.

Feng, Y., Davis, N.G., 2000. Akr1p and the type I casein kinases act prior to the ubiquitination step of yeast endocytosis: Akr1p is required for kinase localization to the plasma membrane. Molecular and Cellular Biology 20, 5350–5359.

Gardner, M.J., Hall, N., Fung, E., White, O., Berriman, M., Hyman, R.W., Carlton, J.M., Pain, A., Nelson, K.E., Bowman, S., Paulsen, I.T., James, K., Eisen, J.A., Rutherford, K., Salzberg, S.L., Craig, A., Kyes, S., Chan, M.S., Nene, V., Shallom, S.J., Suh, B., Peterson, J., Angiuoli, S., Pertea, M., Allen, J., Selengut, J., Haft, D., Mather, M.W., Vaidya, A.B., Martin, D.M., Fairlamb, A.H., Fraunholz, M.J., Roos, D.S., Ralph, S.A., McFadden, G.I., Cummings, L.M., Subramanian, G.M., Mungall, C., Venter, J.C., Carucci, D.J., Hoffman, S.L., Newbold, C., Davis, R.W., Fraser, C.M., Barrell, B., 2002. Genome sequence of the human malaria parasite *Plasmodium falciparum*. Nature 419, 498–511.

Hoppe, H.C., Ngo, H.M., Yang, M., Joiner, K.A., 2000. Targeting to rhoptry organelles of *Toxoplasma gondii* involves evolutionarily conserved mechanisms. Nature Cell Biology 2, 449–456.

Kabouridis, P.S., Magee, A.I., Ley, S.C., 1997. S-acylation of LCK protein tyrosine kinase is essential for its signalling function in T lymphocytes. EMBO Journal 16, 4983–4998.

Le Roch, K.G., Zhou, Y., Blair, P.L., Grainger, M., Moch, J.K., Haynes, J.D., De LaVega, P., Holder, A.A., Batalov, S., Carucci, D.J., Winzeler, E.A., 2003. Discovery of gene function by expression profiling of the malaria parasite life cycle. Science 301, 1503–1508.

Linder, M.E., Deschenes, R.J., 2003. New insights into the mechanisms of protein palmitoylation. Biochemistry 42, 4311–4320.

Putilina, T., Wong, P., Gentleman, S., 1999. The DHHC domain: a new highly conserved cysteine-rich motif. Molecular and Cellular Biochemistry 195, 219–226.

Roth, A.F., Feng, Y., Chen, L., Davis, N.G., 2002. The yeast DHHC cysteine-rich domain protein Akr1p is a palmitoyl transferase. Journal of Cell Biology 159, 23–28.

Singaraja, R.R., Hadano, S., Metzler, M., Givan, S., Wellington, C.L., Warby, S., Yanai, A., Gutekunst, C.A., Leavitt, B.R., Yi, H., Fichter, K., Gan, L., McCutcheon, K., Chopra, V., Michel, J., Hersch, S.M., Ikeda, J.E., Hayden, M.R., 2002. HIP14, a novel ankyrin domain-containing protein, links huntingtin to intracellular trafficking and endocytosis. Human Molecular Genetics 11, 2815–2828.

Topolska, A.E., Lidgett, A., Truman, D., Fujioka, H., Coppel, R.L., 2003. Characterisation of a membrane-associated rhoptry protein of *Plasmodium falciparum*. Journal of Biological Chemistry.

Trager, W., Jensen, J.B., 1976. Human malaria parasites in continuous culture. Science 193, 673–675.

Zhang, W., Trible, R.P., Samelson, L.E., 1998. LAT palmitoylation: its essential role in membrane microdomain targeting and tyrosine phosphorylation during T cell activation. Immunity 9, 239–246.