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example is a Toxoplasma gondii gene encoding a protein with both cyclophilin and FKBP domains. Likely roles in protein folding and oligomerisation, RNA splicing and sexual differentiation have been suggested for parasite immunophilins. In addition, unexpected roles in parasite virulence (Mip FKBP of Trypanosoma cruzi) and host immuno-modulation (e.g. 18-kDa cyclophilin of Toxoplasma gondii) have been established. Furthermore, in view of the potent antiparasitic activities of cyclosporins, macrolactones and non-immunosuppressive derivatives of these compounds, immunophilins may mediate drug action and/or may themselves represent potential drug targets. Investigation of the mechanisms of action of these agents may lead to the design of potent and selective antimalarial and other antiparasitic drugs. This review discusses the properties of immunophilins in parasites and the 'animal model' Caenorhabditis elegans and relates these to our understanding of the roles of these proteins in cellular biochemistry, host-parasite interaction and the antiparasitic mechanisms of the drugs that bind to them.

Peptidyl-prolyl cis-trans isomerases (immunophilins) and their roles in parasite biochemistry, host–parasite interaction and antiparasitic drug action

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Abstract

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1. Introduction

The cyclophilin (CYP) and FK506-binding protein (FKBP) families, although unrelated in sequence, are often considered together because of their shared enzymic activities. Both cyclophilins and FKBPs, along with a smaller protein class, the parvulins, exhibit peptidyl-prolyl cis-trans isomerase (PPIase: EC 5.2.1.8) activity that plays a vital role in protein folding (Fischer and Aumüller, 2003). Although the peptide bonds of nascent polypeptides emerge from the ribosome in the trans-conformation, and the majority retains that energetically-favoured state in fully-folded proteins, there is a significant minority (~5–7% of the proteins with structures solved) of peptidyl-prolyl (Xaa-Pro) bonds that switch to the cis-conformation during folding, transport and assembly. The cis-trans isomerisation of Xaa-Pro bonds is one of the rate-limiting steps of protein folding. However, the influence of cyclophilins and FKBPs on the conformations, locations, oligomeric states and activities of various proteins in cells cannot be explained by PPIase activity alone. At least some cyclophilins and FKBPs can act as molecular chaperones in an analogous manner to certain members of stress protein families. The chaperone activity can be measured separately in vitro, for example via aggregation assays using model substrates, and may or may not be dependent on the presence of a functioning PPIase domain. An additional property common to most eukaryotic and prokaryotic members of each family is their interaction with certain immunosuppressive drugs: the cyclic undecapeptide cyclosporin A (CsA) binds to cyclophilins, and the macrolactones FK506 and rapamycin to FKBPs. For this reason, cyclophilins and FKBPs are known collectively as the immunophilins. Binding of any of these immunosuppressants inhibits the PPIase activity of its respective partner. Although this inhibition may have physiological consequences, it does not represent the mechanism of

immunosuppressive action as such. In T-lymphocyte suppression, CsA–cyclophilin or FK506–FKBP12 complexes form composite surfaces that strongly inhibit the protein phosphatase calcineurin, a crucial component of a Ca^{2+}-dependent signalling pathway (Matsuda and Koyasu, 2000). Calcineurin is also the relevant target in fungi, where inhibition of this phosphatase prevents recovery from pheromone-induced cell-cycle arrest (*Saccharomyces cerevisiae*) or growth at elevated temperatures relevant to virulence (*Cryptococcus neoformans*) (Wang and Heitman, 2005). In the case of rapamycin–FKBP, the relevant target is not calcineurin but the protein kinase TOR (target of rapamycin), and the downstream blockade is not on T-cell activation (G_0 to G_1 transition) but on proliferation (G_1 to S). Therefore immunophilins are not only involved in the folding, trafficking and activity of a range of cellular proteins, but also mediate the effects of certain pharmacologically-active small molecules. Aside from their roles in cellular biochemistry, immunophilins of parasites are particularly interesting for two additional reasons. First, there is evidence that some are involved in the pathogenesis of infections caused by protozoa and other microorganisms (Hacker and Fischer, 1993). Second, CsA, FK506, rapamycin and more excitingly, nonimmunosuppressive analogues of these compounds, have strong inhibitory effects on certain parasites in culture and in animal models of infection (Bell et al., 1996). This review considers the properties of the well-characterised immunophilin genes and their products, focussing on their roles in host–parasite interaction and the antiparasitic actions of certain drugs. The emphasis is on work published in the last ten years: for a fuller discussion of earlier work, see the reviews by Page et al. (1995b) and Bell et al. (1996).

2. Cyclophilins

2.1 *Genes and gene expression (mRNA level)*

It is common that a given genome contains more than one cyclophilin gene – eight in *S. cerevisiae* and at least 16 in humans (Galat, 2003) – and this may also be the case for most parasites. In this section we shall confine ourselves to those genes that are expressed and for which data of some functional relevance, e.g. PPIase or chaperone activity, cyclosporin binding, or specific distribution, have been obtained. Browsing of annotated genes in parasite genome databases reveals more putative cyclophilin or cyclophilin-like genes and more detailed data mining using conserved cyclophilin sequences can expose even more. We shall refrain from speculating on sequences that may or may not encode actual cyclophilins, except where it is likely to be informative, e.g. where a clear orthologue of a well-characterised gene in one parasite species is found in the genome of a closely-related species.

The properties of the protozoal cyclophilins for which there are firm, published expression and/or functional data are shown in Table 1. The nomenclature we shall use for protozoal cyclophilins is similar to the convention of Galat (2003): cyclophilin is abbreviated to CYP and a species-specific prefix and, especially if there is more than one type in one organism, a suffix representing the approximate molecular mass in kDa of the mature protein (if known), are added, e.g. hCYP18 (human 18-kDa cyclophilin, hCyPA), PfCYP19A (one of two *Plasmodium falciparum* cyclophilins of 19-kDa). In general, the known cyclophilins of protozoal parasites are closely related in sequence to each other and to hCYP18, the first cyclophilin to be discovered and the one with which other cyclophilins are usually compared. The residues known from three-dimensional structures to make close contact with CsA, especially the crucial tryptophan (position 121 in hCYP18), are for

the most part well conserved in the protozoal cyclophilins (Fig. 1). The appearance of the residues known to contact calcineurin in the presence of CsA is less consistent.

Three cyclophilin genes have been identified in *P. falciparum: Pfcyp19B* (formerly *PfCyP, PfCyP22:* Hirtzlin *et al.,* 1995), *Pfcyp24* (formerly *PFCyP*: Reddy, 1995) and *Pfcyp19A* (formerly *PfCyP19:* Berriman & Fairlamb, 1998). These genes are located on chromosomes 11, 8 and 3, respectively. Aside from the N-terminal extensions of PfCYP19B and PfCYP24, the major difference between the *P.falciparum* cyclophilins and hCYP18 sequences lies in 'insertions' of 4–6 amino acids situated around position 43-44 (hCYP18 numbering; Fig. 1), which lies in the linker region between helix α1 and strand β3 (Dornan et al., 2003). This corresponds to a region of substantial diversity among cyclophilins in general (Galat, 1999). All three *P. falciparum* cyclophilins have orthologues encoded in the *P. yoelii* and *P. berghei* genomes (Carlton et al., 2002; Hall et al., 2005), PfCYP19A and PfCYP19B in the *P. chabaudi* genome (Hall et al., 2005) and PfCYP19A in *P. vivax* (Cui et al., 2005). Of these non-*falciparum* cyclophilins, only the *P. berghei* PfCyP19A orthologue has been characterised (Nunes, J., 2003. Cyclophilins and the antimalarial activity of cyclosporin A. PhD thesis. University of Dundee).

All three *P. falciparum* cyclophilins are expressed at the mRNA level in erythrocytic parasites (Hirtzlin et al., 1995; Reddy, 1995; Bozdech et al., 2003; Le Roch et al., 2003): interestingly, *Pfcyp24* mRNA is highest in the immature (ring) stages, *Pfcyp19A* mRNA in the middle of the erythrocytic cycle and *Pfcyp19B* mRNA in the more mature (late trophozoite/schizont) stages. If these transcript levels are reflected in protein levels (which has been confirmed for PfCYP19B: Gavigan et al., 2003) then this may imply some functional redundancy coupled with stage-specificity.

High et al. (1994) isolated genes encoding two *Toxoplasma gondii* cyclophilins, TgCYP18 (TgCyP18.5) and TgCYP20 (Fig. 1). TgCYP20 was apparently part of a larger open-reading frame but the N-terminal amino acid of the mature protein corresponded to the beginning of a typical cyclophilin domain. Both were highly similar to hCYP18 in the core region but TgCYP20 differed in its 7-amino acid 'insertion' in the same region as in the *P. falciparum* cyclophilins. The 'dual family' FKBP–cyclophilin hybrid from *T. gondii* is discussed below. A newly-described cyclophilin of *Neospora caninum,* an apicomplexan pathogen of cattle, was highly similar to TgCYP18 (Tuo et al., 2005).

Cyclophilin genes have been found in *Leishmania major* (Rascher et al., 1998) and *L. donovani* (Dutta et al., 2001). Both had well-conserved CYP/CsA-binding domains. The *L.major* protein LmCYP19 included an unusual 11-amino acid 'extension' at the N-terminus and a 3-amino acid 'insertion' around residue 102, which is expected to fall in the linker between β-sheets 4 and 5 (Rascher et al., 1998). These features were conserved in the cyclophilins of various African trypanosomes (Dao-Thi et al., 1998; Pellé et al., 2002) and those of the South American trypanosome *Trypanosoma cruzi* (Búa et al., 2001) but not the signal-sequence-containing one of *L. donovani* (Dutta et al., 2001). *Giardia intestinalis* cyclophilin had an N-terminal 'extension' of similar length but unrelated sequence (Yu et al., 2002).

The last decade has witnessed an explosion in the database submissions of helminth immunophilin sequences and has been a direct output of ongoing expressed sequence tag (EST) and genomic sequence-based genome projects. This information explosion has been most dramatic for the free-living nematode *Caenorhabditis elegans,* which represents the first completed and annotated animal genome sequence

(*C. elegans* genome consortium 1998, www.wormbase.org). The *C. elegans* genome resource has permitted the global analysis of the cyclophilin and FKBP families. As a direct consequence, more immunophilins have been characterized to date in this single organism than in any other: 18 separate cyclophilin isoforms and 8 separate FKBP isoforms (Tables 2 and 3; www.wormbase.org). This large gene expansion of the metazoan immunophilins over the protozoal ones reflects the comparative increase in complexity of the organisms. Many of the *C. elegans* immunophilins have been characterized with regards to their expression, functional significance (Page, 1997; Page and Winter, 1998, 1999; Dornan et al., 1999; Ma et al., 2002; Picken et al., 2002; Table 1) and activity (Page et al., 1996; Dornan et al., 1999; Picken et al., 2002). Information available for other helminth species has predominantly focused on a small number of genes per species, but as genome projects progress and become annotated this situation is expected to change rapidly. In addition to being a well-established animal model, *C. elegans* clearly represents an ideal nematode model system (Gilleard, 2004). The greatest advantage of the model system however, is the fact that global gene function (Kamath et al., 2003) and transcript location experiments (Hope et al., 1998) can be carried out with relative ease. For these reasons, the *C. elegans* immunophilins will be used here as a framework into which the more limited information on parasitic helminth immunophilins will be organised. This complete *C. elegans* sequence data allows the subgrouping of the *C. elegans* CYPs (and FKBPs: see below) into four major classes; type A, conserved cytosolic forms; type B, secreted forms; type C, mitochondrial forms and type D, non-secreted multi-domain or divergent forms. The nomenclature used for helminth genes in this review follows the standard genetic nomenclature for *C. elegans*.

There are three type-A cyclophilin genes in the *C. elegans* genome: *cyp-2, -3* and *-7*. Global RNAi experiments (Kamath et al., 2003) reported wild type phenotypes for reduced expression of all three isoforms, but one single experiment with *cyp-7* reported some associated embryonic lethality (www.wormbase.org). From the parasitic helminths, direct orthologues of type-A cyclophilin genes have been cloned and sequenced from filarial nematodes (Ma et al., 1996), cestodes (Lightowlers et al., 1989) and trematodes (Argeat, 1992; Klinkert et al., 1996; Kiang et al., 1996). In the case of the filarial species *Onchocerca volvulus, Dirofilaria immitis* and *Brugia malayi*, these are highly homologous to the *C. elegans* type-A isoforms. In the trematode parasite *Schistosoma mansoni*, two separate type-A cyclophilins have been described, namely SmCypA (Klinkert et al., 1996) and Smp17.7 (Kiang et al., 1996).

The spatial expression patterns of the *C. elegans* A-type cyclophilins (at the transcript level) range from non-detectable for *cyp-2* (Table 1) to exclusive expression in the excretory cell and duct (*cyp-3*) (Dornan et al., 1999; Fig. 2B). In addition, *cyp-3* is expressed predominately in larval stages. The location in the excretory system is intriguing, and may indicate that CYP-3 is involved in the regulation of ion fluxes in analogy to the vertebrate renal system.

The type-B secreted cyclophilins *cyp-5* and *-6* are well characterized in *C. elegans* and parasitic helminth type-B isoforms are also well represented in the database and literature, being found in *B. malayi* (accession number Q6ynz2), *S. mansoni* and *S. japonicum* (Klinkert et al., 1995, 1996). The RNAi studies on the secreted *C. elegans* cyclophilins failed to uncover any associated phenotype, either singly or in combination (Picken et al., 2002). *C. elegans cyp-5* and *-6* are both predominately expressed in the embryo, with expression also detected in larval and adult stages (Picken et al., 2002). Both isoforms are also expressed in the nematode

gut (Picken et al., 2002; Table 2; Fig. 2A, F). A type-C cyclophilin has been described in *C. elegans* (Page et al., 1996) but not yet in any parasitic helminth.

The type D, divergent or multi-domain cyclophilins represent the largest and possibly most interesting class of CYPs in *C. elegans*. These isoforms characteristically have divergent CYP/CsA-binding domains and/or may also be flanked by additional non-cyclophilin domains. The divergent cyclophilins have reported or predicted roles as diverse as muscle protein folding, sexual differentiation of the germline, collagen folding and RNA splicing. The divergent 3-domain isoform CYP-4 (Page and Winter, 1998), or cyp60, has orthologues in the filarial species *B. malayi, O. volvulus* (Page and Winter, 1998) and *D. immitis* (Hong et al., 1998). CYP-4 is most abundant in the L1 stage and has strong somatic muscle cell expression pattern in *C. elegans* (Page and Winter, 1998; Table 2; Fig. 2D), with additional somatic and germline expression also being reported (Belfiore et al., 2004). This isoform has been hypothesized to be involved in proper muscle protein folding (Page and Winter, 1998) and is involved in the sexual differentiation of the hermaphrodite germ-line (Belfiore et al., 2004). The *cyp-8* isoform represents a non-essential gene in *C. elegans*, the transcript of which encodes two distinct domains; a divergent, but active, CYP domain (Page et al., 1996) and a large, C-terminal, charged, serine-rich domain that contains several functional nuclear location signals (Page and Winter, 1999). The transcript is expressed in all life-cycle stages but is confined spatially to the gut cell nuclei (Page and Winter, 1999; Table 1; Fig. 2C). Direct homologues of *cyp-8* are to be found in the filarial nematode *B. malayi* (Page et al., 1995a), *D. immitis* and *O. volvulus* (Hong et al., 1998). CYP-9 represents a two-domain cyclophilin with an unusual transcriptional organization, being found in an operon with a second protein-folding enzyme, protein disulphide isomerase (*pdi-1*)

(Page, 1997). This conserved transcriptional organization is also found in the closely related nematode *Caenorhabditis briggsae* (Page, 1999). This arrangement of operons is characterized by downstream genes being *trans*-spiced to a distinctive SL2 leader sequence, and is found in 15% of all *C. elegans* transcripts (Blumenthal and Stewart, 1997). Interestingly, 50% of the *C. elegans* cyclophilins are found in such operons (Blumenthal and Gleason, 2003). The enzymes encoded by *cyp-9* and *pdi-1* are hypothesized to cooperate in protein folding events, such as collagen trimerization. Both transcripts share a single promoter and are expressed in the cuticle-collagen synthesizing hypodermal tissues (Page, 1997). No direct homologue of *cyp-9* has to date been described in the parasitic helminths. CYP-13 is the first of the divergent isoforms predicted to play a role in RNA splicing. CYP-13 has an N-terminal RNA recognition motif (RRM) followed by a conserved CYP domain (Zorio and Blumenthal, 1999). This non-essential divergent cyclophilin gene is in an operon with an essential RNA-splicing factor, *uaf-2* (Zorio and Blumenthal 1999). A direct orthologue of *cyp-13* was recently cloned from the parasitic nematode *Haemonchus contortus* (Valle et al., 2005). Likewise a *cyp-13* orthologue is present in the trematode parasites *S. japonicum* (Zorio and Blumenthal, 1999) and *S. mansoni* (Valle et al., 2005) indicating an evolutionarily conserved role for this predicted splicing factor. Information regarding the final members of the divergent cyclophilins is limited to unpublished expression patterns and genome-wide RNAi studies (Table 2). From the global RNAi studies (Kamath et al., 2003) and the published studies summarized in Table 2, redundancy of function in this large gene family may contribute to the apparent lack of phenotype in knock-down parasites. In *Saccharomyces cerevisae,* combined knock-outs of the cyclophilins and FKBPs revealed a high degree of functional redundancy (Dolinski et al., 1997).

2.2 *Protein expression and locations*

In most eukaryotes there is a small, one-domain cyclophilin, like hCYP18, that is abundant in the cytosol. In the case of *P. falciparum,* PfCYP19A is the likeliest candidate for the hCYP18 homologue (see Table 1). It was found at ~1.2% of cellular protein in erythrocytic parasites and the available evidence suggests that it is cytosolic (Berriman and Fairlamb, 1998; Nunes, J., 2003. PhD thesis. University of Dundee; Gavigan et al., 2003). The location of PfCYP19B is more problematic. There was a cleaved signal sequence (Gavigan *et al.,* 2003) but no obvious endoplasmic reticulum-retention signal (Hirtzlin et al., 1995). Unexpectedly, the protein appeared to be located predominantly in the cytosol, according to immunofluorescence microscopy and cell fractionation/western blotting (Gavigan et al., 2003). PfCYP19B was also abundant in erythrocytic parasites at up to 0.5% of cellular protein and its highest copy number was in the mature, schizont stages (Gavigan et al., 2003). The conundrum of PfCYP19B's location has yet to be explained. The long, asparagine-rich, N-terminal extension of PfCYP24 (Reddy, 1995; Fig. 1) is an intriguing feature that appears not to be a typical signal sequence and is not closely related to any known non-*Plasmodium* sequences. It is not known whether this extension is cleaved and the evidence for production of significant quantities of the protein in blood-stage parasites is still limited. In *Toxoplasma,* TgCYP18 contained a signal sequence (High et al., 1994) and was secreted by tachyzoites to up to 2.4% of total protein in cell culture supernatant fluid (Aliberti et al., 2003). Similarly, the related *N. caninum* cyclophilin, which also has a presumed signal sequence, was detected in both tachyzoite lysates and culture supernatants using antibody to TgCYP18 (Tuo et al., 2005).

The *L. donovani* cyclophilin LdCYP had a cleaved signal peptide and was found predominantly or exclusively in the particulate fraction (Dutta et al., 2001). It was secreted in the presence of CsA, suggesting that CsA-sensitive binding to ER-resident proteins might normally anchor it in the cell. The cyclophilin of African trypanosomes, while predominantly cytosolic, was also found in culture supernatants (Pellé et al., 2002). Although its role in host–parasite interaction is unknown, it is a major band on western blots probed with immune bovine sera and a major constituent of an immunosuppressive fraction of *T. congolense* parasites.

The tissue-specific expression patterns of helminth cyclophilins have been determined mainly at the mRNA transcript level (see above and Fig. 2). In the case of *C. elegans cyp-5* gut expression, this reporter transcript result was confirmed using specific antibodies (Picken et al., 2002). *S. mansoni* P17.7 protein has been detected in the adult tegument and gut tissues (Kiang et al., 1996). Likewise, the expression of the *S. mansoni* CYP B protein was also restricted to the adult tegument and associated tubercles (Klinkert et al., 1995).

2.3 *Protein properties and functions*

PPIase activity has been demonstrated in vitro for the native and/or recombinant forms of several cyclophilins of protozoal parasites (Table 1). In *P. falciparum,* all three cyclophilins exhibited PPIase activity. Their substrates and/or binding partners are as yet unknown; however, PfCYP19A and PfCYP19B, but not PfCYP24, in complex with CsA were potent inhibitors of *P. falciparum* calcineurin *in vitro* (Dobson et al., 1999; Kumar et al., 2005b). The three-dimensional structures of wild-type and a mutant PfCYP19A in complex with CsA were solved to 2.1Å resolution by Peterson et al. (2000). The overall fold and interaction with CsA were

virtually identical to those of hCYP18 except for an extension in the linker between helix α1 and strand β3 as mentioned above.

LdCYP is a typical single-domain cyclophilin with PPIase activity (Dutta et al., 2001). However, it also possessed an unusual chaperone action, in that it could rescue functional monomers of *L. donovani* adenosine kinase from non-functional, soluble aggregates or protect urea-denatured, refolding forms of this enzyme from aggregation in vitro (Chakraborty et al., 2002, 2004). Adenosine kinase is unusual in forming soluble, non-functional aggregates in the absence of stress. The activity was ATP-independent, unaffected by CsA, independent of PPIase activity and was associated with direct binding of LdCYP to the kinase. Remarkably, the chaperone activity was maintained and even enhanced by deletion of the first 88 amino acids of the 166-amino acid (mature) LdCYP, indicating that the C-terminal part alone was sufficient. Molecular modelling studies suggested that the explanation could lie in the higher exposure of crucial hydrophobic residues in the truncated form. The effect of LdCYP on adenosine kinase was confirmed under more physiological conditions using an *Escherichia coli* co-expression system. Its relevance to *Leishmania* biology is not yet known.

The conserved A-type cyclophilins of *C. elegans* tested had significant PPIase activity against a defined synthetic substrate (Page et al., 1996). The CYP-3 enzyme has been crystallized and its structure solved to 1.8Å resolution. This structure shows remarkable similarity to hCYP18 with the exception of an additional divergent exposed loop structure (Dornan et al., 1999). Its PPIase activity and inhibition by CsA have also been demonstrated (Dornan et al., 1999). The recombinant *B. malayi* CYP A (Ma et al., 1996) and *S. mansoni* smp17.7 (Kiang et al., 1996) were also found to possess significant PPIase activity. Among the type-B secreted cyclophilins, CYP-

5 and -6 of *C. elegans* displayed potent PPIase activity (Page et al., 1996; Picken et al., 2002). In addition, CYP-5 has been structurally solved to 1.75Å and the structure of CYP-6 has been modeled on the CYP-5 structure (Picken et al., 2002) revealing high similarity but a significant negative charge difference on the CYP-6 surface. The PPIase activity of the C-type *C. elegans* cyclophilin CYP-1 has likewise been confirmed (Page et al., 1996). Among the D-type cyclophilins, CYP-4 has relatively low activity in the standard PPIase assay and this was a consistent observation for the remaining type D forms that have been analysed (CYP-8, -9, -10 and -11: Page et al., 1996; Page and Winter 1998). The divergent cyclophilin domain of the *B. malayi* orthologue of the CYP-8 enzyme, BmCYP-1, is an active PPIase that is relatively insensitive to CsA (IC_{50} = 860 nM: Page et al., 1995). This filarial parasite enzyme has been crystallized and solved to a resolution of 1.9Å (Taylor et al., 1998) and 2.15Å (Mikol et al., 1998). These structural studies uncovered the molecular nature of the insensitivity of this divergent isoform to CsA, confirming that a mere two amino acid changes in the CsA-binding pocket relative to hCYP18 had a profound effect on its conformation and subsequent ability to bind to this drug (Page et al., 1995; Taylor et al., 1998). Finally, the CYP-13 two-domain RRM cyclophilin orthologue from *H. contortus* has been expressed and characterized in both PPIase and RNA-binding assays (Valle et al., 2005).

2.4 *Roles in host-parasite interaction and pathogenesis*

T. gondii affects pro- and anti-inflammatory host cell signalling in such as way as to maximise parasite multiplication and spread while maintaining host survival (Denkers, 2003). One aspect of this manipulation is the up-regulation of interleukin (IL)-12-dependent production of interferon (IFN)-γ that is critical to host survival of acute toxoplasmosis. This effect appears to occur by two distinct pathways, one of

which is unique to *T. gondii* and involves triggering of the cysteine–cysteine chemokine receptor CCR5 in dendritic cells and macrophages by secreted *T. gondii* CYP18 (C-18: Aliberti et al., 2003). TgCYP18, but not hCYP18 nor PfCYP19A, appears to induce IL-12 production by interacting directly with CCR5. The effect was blocked by addition of CsA. These observations implied that structural determinants related to CsA binding but peculiar to TgCYP18 were responsible for induction of IL-12 synthesis. This idea was confirmed by modelling of the TgCYP18 structure on that of PfCYP19A and site-directed mutagenesis of putatively surface-exposed residues that were absent in PfCYP19A (Yarovinsky et al., 2004). Two of the TgCYP18 mutants had reduced interaction with CCR5 and reduced *IL-12* induction but four separate mutants with reduced PPIase activity did not, further suggesting that PPIase activity was not required for the effect. TgCYP18 appears to act as a structural mimic of CCR5-binding ligands, albeit one with no sequence similarity to the known host ligands for this receptor. There is also evidence that the *Neospora* cyclophilin NcCYP plays a role in stimulating IFN-γ production by bovine peripheral blood mononuclear cells and *N. caninum*-specific $CD4^+$ T-cells (Tuo et al., 2005). This effect is also blocked by CsA. IFN-γ production induced by *N. caninum* tachyzoites is thought to be critical in controlling the acute phase of neosporosis.

A potentially useful application of the findings on TgCYP18 results from the fact that CCR5 is, along with CXCR4, an important co-receptor for HIV-1 entry into host cells (Golding et al., 2003). TgCYP18 blocked HIV-1-envelope-dependent cell fusion and HIV-1 infectivity in that majority (R5 type) of viruses that use CCR5. This effect was presumably caused by competition of TgCYP18 with the HIV-1 envelope glycoprotein gp120 for binding to CCR5. Since this antiviral effect was also sensitive to the mutations in TgCYP18 that affected CCR5 binding, these data provide

a basis for design of new antiviral agents based on TgCYP18 (Yarovinsky et al., 2004). The effect of TgCYP18 on HIV is distinct from that of human CYP18, which involves interaction with the capsid protein and incorporation into virions during assembly and occurs in virus strains that use both CCR5 and CXCR4 co-receptors. Any consequences of the immunomodulatory effect of TgCYP18 in *T. gondii*/HIV-1 co-infections are at present unknown.

2.5 *Roles in antiparasitic drug action*

The potent activities of CsA and other cyclosporins against certain parasites have been widely noted (reviewed in Chappell & Wastling, 1992; Page et al., 1995b; Bell et al., 1996). In most cases, it has been assumed that the mechanism of antiparasitic action of cyclosporins is analogous to what is believed to be the principal mechanism of immunosuppressive action, namely binding to one or more cyclophilins followed by inhibition of calcineurin by the drug–cyclophilin complex. However, observations from some parasites, as discussed below, are not consistent with this model. Moreover, in no case has either a clear functional role for calcineurin been identified or the essentiality of this phosphatase established (although this could be accounted for by the relative genetic intractability of most parasites). It should at least be considered that the antiparasitic action of cyclosporins in some or all parasites might result from some other consequence of binding to cyclophilin that is unrelated to calcineurin, or from interaction with different molecules altogether. The demonstrated effects of cyclosporins on P-glycoproteins and on the mitochondrial permeability transition pore in other cell types and the physiological consequences of these interactions (Borel et al., 1996) should caution us against assuming that calcineurin is necessarily the target of cyclosporins in parasites.

Cyclophilins of malarial parasites have been of particular interest in view of the especially potent and selective antimalarial activity of cyclosporins (reviewed in Bell et al., 1996) and of the cyclophilin-binding, linseed peptide cyclolinopeptide A and analogues (Bell et al., 2000). Although cyclosporins themselves are unlikely to be developed as antimalarial agents, understanding their mechanisms of action might contribute to the development of non-cyclosporin agents that act in a similar way. The PPIase activity of extracts of *P. falciparum* was first demonstrated by Bell et al. (1994). However, no correlation was found between PPIase inhibition and inhibitory potency against cultured parasites among a group of naturally-occurring and semi-synthetic cyclosporins. This observation was later confirmed using recombinant PfCYP19A (Berriman and Fairlamb, 1998) and PfCYP19B (Gavigan et al., 2003) instead of crude parasite extract. Specifically, a non-immunosuppressive derivative of cyclosporin D, valspodar ([3'-keto-MeBmt]^{1} [Val]^{2}-cyclosporin; SDZ PSC 833) was the most potent antimalarial agent (IC_{50} = 32 nM) but had low affinity for cyclophilins (Bell et al., 1994). Since valspodar was a particularly good inhibitor of mammalian P-glycoprotein, and was more potent than CsA in this respect, it was suggested that a *P. falciparum* P-glycoprotein homologue might be the relevant molecular target (Bell et al., 1996). However, it has not been shown that any such protein is directly affected by cyclosporins. Gavigan and Bell (2003) have recently shown that CsA susceptibility of *P. falciparum* can be influenced by the genotype and expression level of *pfmdr1,* which encodes P-glycoprotein homologue 1 (Pgh1) – but this effect may be indirect.

A similar situation exists in *T. gondii* except that this parasite is not as susceptible to cyclosporins as *Plasmodium*. Silverman et al. (1997) found no correlation between growth inhibition and cyclophilin binding among a series of

cyclosporins. The most potent inhibitor, SDZ 215-918, did not bind to cyclophilins but inhibited rhodamine efflux, which may be a function of P-glycoprotein activity. It was hypothesised that a *T. gondii* P-glycoprotein was the likely molecular target of CsA. However, isolation of cyclosporin-binding proteins on affinity columns yielded only cyclophilins TgCYP18 and TgCYP20 (High et al., 1994). It would be relevant to ask whether the cyclophilin-bound, or only free, CsA inhibits the P-glycoprotein, otherwise the role of cyclophilins might be to reduce the concentration of free CsA available and thus antagonise the action of the drug.

Returning to malaria, three recent findings have put the spotlight back on cyclophilins (and calcineurin) as possible mediators of the action of CsA. The first study identified cyclophilins PfCYP19A and PfCYP19B (but not P-glycoprotein) as the major cyclosporin-binding proteins of *P. falciparum* (Gavigan *et al.,* 2003). The second was the demonstration in parasite fractions and as a recombinant protein of a *P. falciparum* calcineurin that was able to dephosphorylate proteins and was inhibitable by CsA in combination with PfCYP19A or PfCYP19B (Dobson et al, 1999; Kumar et al., 2004, 2005b). The third was the demonstration that, of nine independently-isolated CsA-resistant mutants of *P. falciparum*, two had mutations in *Pfcyp19A,* one in *Pfcyp19B* and one in each calcineurin subunit-encoding gene (Kumar et al., 2005b). A recombinant form of one of the altered PfCYP19A's (W128C) was bound by CsA but the combination was a poor inhibitor of calcineurin. Both calcineurin lesions were associated with CsA-resistant phosphatase activity. It would be informative to observe the effects of CsA and valspodar on transgenic parasites with overexpression, mutation or knock-out of the cyclophilin or calcineurin genes, if such parasites were viable.

The *Leishmania* species so far investigated are relatively resistant to CsA although they possess cyclophilin and calcineurin (Rascher et al., 1998; Banerjee et al., 1999; Dutta et al., 2001). Some authors have felt it necessary to explain this low susceptibility in terms of inability of the CsA–cyclophilin complex to bind to calcineurin (Rascher et al., 1998) or lack of abundance of cyclophilin in the cytosol (Dutta et al., 2001).

There has been little discussion of the idea that cyclophilins *per se* could be an antiprotozoal drug target. The low human toxicity of certain cyclosporins (Bell et al., 1996) and the observation that the homologue of hCYP18 is not required for viability of mice (Colgan et al., 2004) are some indication of the potential for selective toxicity to parasites. In malaria, the activity of another non-immunosuppressive cyclosporin, [MeVal^{4}]-CsA, which is a strong cyclophilin binder but whose complex with cyclophilins has very low affinity for calcineurin (Bell et al., 1994), suggests that cyclophilin might be a valid target in *Plasmodium*. The anti-nematode effects of CsA include strong and consistent moulting and structural defects in *C. elegans* when this drug is applied at relatively high concentrations (Page et al., 1995b). This effect is consistent with this compound inhibiting an endogenous PPIase that may be involved in gut or cuticle structural protein folding events. However, it is well established that *C. elegans* routinely requires drug concentration up to one thousand-fold higher than those effective against mammalian cells (Rand and Johnson, 1995). In view of the negative RNAi studies of cyclophilin function described above, it is difficult to identify a specific *C. elegans* cyclophilin that is involved in this effect. The possible functional redundancy in the cyclophilin gene family could be a confounding factor; for example the effect of CsA may be elicited by binding to a combination of the gut-expressed secreted isoforms CYP-5 and CYP-6 and the hypodermally-expressed form CYP-9.

Until stable double and triple mutants are available for these genes, the nature of the CsA target will remain elusive. Filarial parasites also express large mutigene cyclophilin families (see above) that may also be functionally redundant. Elucidating the role they play in biological processes, including the synthesis of the cuticle, will be further complicated by the fact that reproducible RNAi techniques have not yet been widely adopted in these parasites. Likewise, a hallmark of CsA-induced toxicity to cestode and trematode parasites is tegumental surface damage (Chappell et al., 1993; Page et al., 1995b; McLauchlan et al., 2000). It may be hypothesized that the phenotypes are elicited via inhibition of structural protein folding events but there was no correlation between anti-schistosomal properties and PPIase inhibition in a series of cyclosporins (Khattab et al., 1998). In light of these observations, it would be worthwhile investigating the antiparasitic activities of a range of cyclophilin ligands that do not form calcineurin-inhibitory complexes.

3. FK506-binding proteins (FKBPs)

3.1 *Genes and gene expression (mRNA level)*

As is the case for cyclophilins, several isoforms of FKBPs are usually found within the same organism. The first FKBP to be identified in the Apicomplexa was a 35-kDa FKBP in *P. falciparum* (Braun et al., 2003). Analysis of the *P. falciparum* genome database revealed PfFKBP35 to be the only obvious FKBP gene (Monaghan and Bell, 2005; Kumar et al, 2005a). PfFKBP35 contained not only a conventional FKBP domain but also an additional domain containing three tetratricopeptide repeat (TPR) regions (Monaghan & Bell, 2005; Fig. 3). TPR regions are rich in α-helix and have been associated with protein–protein interactions. Analysis of the genomes (Carlton et al., 2002; Cui et al., 2005; Hall et al., 2005) of other *Plasmodium* species

including *P. vivax* indicates the presence of a single *FKBP* gene with remarkable similarity to *PfFKBP35,* though these genes are not yet known to be expressed.

Likewise, only a single *FKBP* gene has been found in *T. gondii.* This gene encoded not a PfFKBP35 orthologue but a protein belonging to an intriguing new family of immunophilins (Adams et al., 2005) that contained both an FKBP domain and a CYP domain separated by TPR regions (Fig. 3). Similar 'dual-family' immunophilin genes could only be found in two bacterial species. The authors of this study proposed the name FCBP (FK506- and cyclosporin-binding protein) for these novel proteins. TgFCBP57 appears to be essential for growth of *T. gondii*, as judged by RNA interference (Adams et al., 2005).

Trypanosoma cruzi contains an FKBP homologue belonging to the Mip sub-family (Moro, 1995). This particular sub-family was first identified in the obligate intracellular bacterium *Legionella pneumophila*, where it was shown to play a role in survival within human macrophages, leading to the designation macrophage infectivity potentiator (Mip – Cianciotto et al., 1989). Mips have also been identified in other intracellular pathogens, but TcMip is the only protozoal one identified to date.

Experimental data on FKBPs from *C. elegans* (*fkb* genes) and the parasitic helminths are also relatively sparse in comparison to the cyclophilins. Information is summarized for *C. elegans* and used as a comparative system for the parasitic nematodes in table 3. The FKBP family also comprises cytosolic (A forms), secretory pathway (B forms) and divergent, multi-domain forms (D forms).

Two separate genes encode the conserved cytosolic isoforms in *C. elegans*, namely the single FKBP-domain isoform gene *fkb-2* and the dual FKBP-domain protein gene *fkb-8*. These genes are closely arranged (approximately 1 kb apart) on

chromosome I. *fkb-8* probably arose through a recent gene duplication event, a theory supported by its absence from the genome of the close relative *C. briggsae* (Table 3). FKB-2 has direct orthologues in *B. malayi* (accession number Q9U8J7) and *S. mansoni* (Rossi et al., 2002; Knobloch et al., 2004) but orthologues of FKB-8 have yet to be detected. The SmFKBP12 isoform is expressed in all life-cycle stages (Rossi et al., 2002) and its transcript was detected by in situ hybridization in the female gonad and in the tegument of both sexes (Knobloch et al., 2004).

There are numerous (50% of total) secreted (B-) isoforms of FKBP encoded in the *C. elegans* genome, all with a secretory signal peptide and a conserved ER retention signal (FKB-1, -3, -4 and -5). FKB-1 (a.k.a. FKBP-13) is a small single-domain isoform having direct orthologues in the filarial species *B. malayi*, *D. immitis* and *O. volvulus* (Ma et al., 1999). The remaining ER-resident forms FKB-3, -4 and -5 all have two FKBP domains. Direct orthologues of FKB-3, -4 and -5 have not yet been described in any parasitic helminth species.

The divergent (D-) isoforms characteristically have additional non-FKBP domains and two members are expressed in *C. elegans*. FKB-6 is the *C. elegans* equivalent of the functionally significant FKBP51/52, a steroid-receptor-associated, Hsp-90 binding co-chaperone (Riggs et al., 2003). This isoform possesses the characteristic TPR domains (Table 3). Direct orthologues of this isoform exist in *B. malayi* (accession number Q86M29) and *S. mansoni* (Osman et al., 1995). The final *C. elegans* type-D isoform, FKB-7, has a signal peptide, a single FKBP domain and two EF-hand domains (Table 3). No parasite orthologues have thus far been described for FKB-7. From the FBKP family, only *fkb-6* has been attributed an RNAi phenotype from the genome-wide screens, being variably embryonic lethal in *C. elegans* (Table 3; www.wormbase.org).

3.2 *Protein expression and locations*

PfFKBP35 is expressed throughout the intraerythrocytic life cycle (Kumar et al, 2005a). Immunofluorescent studies showed that, during the ring stage, PfFKBP35 is predominantly cytosolic, but as the parasites mature into trophozoites and schizonts, a significant amount of the protein is observed in the nucleus. The bacterial Mip homologues so far studied are all surface bound proteins, whereas the *T. cruzi* homologue TcMip is secreted (Moro et al., 1995). The processed form of this protein is 18.8 kDa in size and is secreted predominantly by the invasive trypomastigote form.

3.3 *Protein properties and functions*

The modular structure of PfFKBP35 comprises an N-terminal FKBP domain followed by a tripartite TPR domain. With the exception of an additional C-terminal cyclophilin domain, the domain architecture of TgFCBP57 is similar to that of PfFKBP35 (Adams et al., 2005; Fig. 3). The modular structures of these proteins, and in particular the presence of TPR motifs, suggest that their primary functions may be in protein trafficking as part of large hetero-oligomeric complexes. Human FKBP51 and hFKBP52 are known to form part of oligomeric complexes comprising various chaperones and co-chaperones (Pratt and Toft, 2003). Genome analysis suggests that *P. falciparum* contains the complete set of chaperone (namely, Hsp40, Hsp70 and Hsp90) and co-chaperone components (namely, Hop and p23) necessary for such complexes (Monaghan, P., 2004. Identification and characterisation of an FK506-binding protein from *Plasmodium falciparum*. PhD thesis, University of Dublin). PfHsp90 and PfHsp70 were recently shown to be present as part of large complexes of up to 300 kDa (Banumathy et al., 2003) and an interaction between PfFKBP35 and PfHsp90 was recently reported (Kumar et al, 2005b). The finding that PfFKBP35

translocates to the nucleus from the cytoplasm lends further support for such a role (Kumar et al, 2005a). Alternatively, or perhaps additionally, PfFKBP35 could serve an important role in the folding of proteins, either as a folding catalyst, a chaperone, or both. Studies of recombinant PfFKBP35 showed that PPIase activity was conferred by the FKBP domain and chaperone activity by both FKBP and TPR domains (Monaghan and Bell, 2005). The finding that PfFKBP35 can inhibit calcineurin in the absence of FK506 is highly unusual (Monaghan and Bell, 2005; Kumar et al, 2005a). Human FKBP38, which is suggested to regulate important cellular processes by modulating the activity of calcineurin, is the only other FKBP for which this phenomenon has been reported (Shirane and Nakayama, 2003), though this has recently been disputed (Weiwad et al., 2005). The domain architecture of hFKBP38 and PfFKBP35 is strikingly similar.

Analysis of the crystal structure of a mercaptopyruvate sulphurtransferase from *Leishmania major* has revealed an intriguing new FKBP-like protein. Sulphurtransferases are widely distributed throughout biology, but those of *L. major, L. mexicana, T. cruzi* and *T. brucei* posses unusual C-terminal extensions not known in any other sulphurtransferases (Williams et al., 2003) and the C-terminal extension of the *L. major* protein (LmMST) was recently shown to have remarkable structural similarity to an FKBP domain (Alphey et al., 2003). The primary sequence similarity between hFKBP12 and this C-terminal extension is quite low, and no PPIase activity was detected in a recombinant form of LmMST. However, the finding that LmMST folds independently, unlike most other sulphurtransferases which require molecular chaperones, suggests that this FKBP-like extension acts as an in-built chaperone, stabilizing the overall folding of the protein. Indeed, truncated forms of LmMST,

devoid of the FKBP-like region, lose their inherent ability to fold in the absence of exogenous chaperones (Williams et al., 2003).

The crystal structure of TcMip, solved at 1.7 Å, showed that with the exception of an extra β-strand, the overall fold was typical of FKBPs. The FKBP domain is flanked at either end by exposed α-helices. An N-terminal α-helix is also present in the *L. pneumophila* Mip but the C-terminal one is unique to TcMip (Pereira et al., 2002).

The *S. mansoni* SmFKB12 isoform is enzymically active and is inhibited by rapamycin, but *S. mansoni* parasites are resistant to this drug (Rossi et al., 2002). An enzymically active form of the *C. elegans* FKB-6 called p50 has also been characterized in *S. mansoni* (Osman et al., 1995).

3.4 *Roles in host-parasite interaction and pathogenesis*

Unlike the *L. pneumophila* Mip, which is involved in the ability of the bacterium to survive within its host cell, TcMip appears to function in the process of host cell penetration. The addition of a recombinant form of TcMip, produced heterologously in *E. coli*, was shown to enhance invasion of cultured simian epithelial or HeLa cells by trypomastigotes (Moro et al., 1995). Infectivity was greatly reduced by antibodies to TcMip or by either FK506 or its non-immunosuppressive derivative, L685,818. The latter compound was also able to protect mice from lethal infection by *T. cruzi* (Oz et al., 2002).

3.5 *Roles in antiparasitic drug action*

FK506 and/or rapamycin inhibit the growth of *P. falciparum, T. gondii* and *T. cruzi* in culture (Bell et al., 1994; Moro et al., 1995; Monaghan and Bell, 2005; Adams et al., 2005). From the point of view of correlating the anti-parasitic activity of FK506 and related compounds with effects on the activity of these proteins, each of

the protozoal FKBPs characterised so far shows significant conservation of the fourteen residues that have been shown in hFKBP12, the archetypal FKBP, physically to contact FK506 (Kay, 1996). There are at least two possible models, analogous to those discussed for cyclosporins above, by which FK506 and rapamycin could exert growth inhibitory effects through parasite FKBPs. The first model, by analogy with the current models of immunosuppressive action of these drugs, is that the compounds combine with the FKBP, and together the drug–receptor complexes inhibit an essential parasite target. For FK506, as for CsA, the target in T-lymphocytes is calcineurin (Matsuda & Koyasu, 2000), while the hFKBP12-rapamycin complex inhibits the protein serine/threonine kinase TOR (Lorberg and Hall, 2004). However, the effects of non-immunosuppressive analogues of FK506 on the growth of *P. falciparum* in culture (Monaghan et al., 2005) and the finding that PfFKBP35 inhibited the phosphatase activity of both bovine and *P. falciparum* calcineurin in the absence of FK506 (Monaghan and Bell, 2005; Kumar et al., 2005a), suggest that calcineurin is not involved in mediating the anti-malarial effects of FK506 and its analogues. Likewise, the lack of any obvious *P. falciparum* TOR homologue suggests that the anti-malarial actions of rapamycin are distinct from its immunosuppressive actions. A second possible model by which these inhibitors could exert their antimalarial effects is through direct inhibition of the biochemical activity of the FKBPs. Although the PPIase activity of recombinant PfFKBP35 is inhibited by FK506 (Monaghan and Bell, 2005), a study of FK520 (a compound identical to FK506 except for a change at C-21 from allyl to ethyl) and a number of synthetic non-immunosuppressive analogues suggested that anti-chaperone activity correlated better with antimalarial activity (Monaghan et al., 2005). This finding leads us to consider whether the PPIase activity of PfFKBP35 has any physiological significance. It is

known that all detectable PPIase activity in lysates of *P. falciparum* is inhibited by CsA (Bell et al., 1994) suggesting that the PPIase activity of parasite cyclophilins is more physiologically relevant. This was also shown for *S. mansoni* extracts (Khattab et al, 1998). The FKBP-like region of LmMST is devoid of enzymic activity yet appears to play an important role as a chaperone (Alphey et al., 2003). Indeed, the biological significance of the PPIase activity of certain other FKBPs remains controversial as their functions appear to be independent of PPIase activity (Galat, 2003; Shirane and Nakayama, 2003).

4. Conclusions and prospects

The number of known immunophilins in parasites has expanded enormously since this topic was last reviewed (Page et al., 1995b; Bell et al., 1996). Genome sequencing and expression sequence tag projects have played a major part in that expansion, a point well illustrated by the completed and annotated genome of the model nematode, *C. elegans* and several parasitic protozoa. However, many more are expected to be discovered in the incompletely sequenced genomes. Furthermore, the detailed assembly, annotation and characterisation of the gene products encoded by many of the sequences of interest has lagged behind their discovery. Nonetheless, recombinant production and biochemical characterisation of parasite immunophilins is relatively straightforward, so in several cases we know of the PPIase activities and the effects of ligand binding, and in some cases chaperone activity has been demonstrated. Studies of temporal expression and subcellular and tissue location have also been possible. The detailed structural characterizaton of certain members of the protozoal, filarial and *C. elegans* cyclophilin family members has uncovered many unusual features; including exposed divergent loops on the conserved forms, exposed

charge difference in the secreted forms and amino acid changes that clearly affect the binding of CsA and thus its inhibitory properties. Overall the data from helminth parasites indicates an important role for these enzymes in structural protein folding but understanding of the functions of protozoal cyclophilins is still fragmentary. For the FKBPs, proposed roles in protein folding, steroid receptor function, signal transduction and co-chaperone functions can all be envisaged and are awaiting experimental confirmation. Screening for protein–protein interactions and genetic manipulation can in the near future be expected to help expose the potentially manifold roles of parasite immunophilins.

Certain immunophilins have been shown to play crucial roles in host–parasite interaction. In the case of TgCYP18 this has enhanced understanding of, and perhaps the ability to manipulate, host immune signalling; in the case of TcMip, a virulence factor and possible target for therapeutic intervention has been found. It seems likely too that immunophilins will be crucial to the correct folding and transport of other virulence factors, for example the adhesive proteins exported to the host erythrocyte by *P. falciparum*.

Findings of potent and selective antiparasitic activity of non-immunosuppressive derivatives of the immunophilin-binding drugs CsA and FK506/FK520 have led to much interest in immunophilins as possible drug targets. Whether the immunophilins themselves are the relevant targets or are components of drug–receptor complexes that inhibit other target molecules such as calcineurin has not yet been clearly established for any parasite, and may vary from species to species. These non-immunosuppressive derivatives are clearly promising antiparasitic leads but they are large molecules with M_r of ~800-1200. Investigation of the antiparasitic properties of lower-M_r immunophilin ligands would seem to be

justified, as would further target validation studies of the immunophilins discussed here.

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Legends to figures

Fig. 1. Multiple amino acid sequence alignment of protozoal cyclophilin domains with human CYP18 performed using ClustalW 1.8.2 (www.ebi.ac.uk). The N-terminal end of the TgCYP20 open-reading frame and the FKBP and tetratricopeptide repeat domains of TgFCBP57 have been left out. Regions not found in the mature proteins, e.g. known signal peptides, are shown in bold. Blocks of identical amino acids are shaded in black and conserved amino acids in grey. The amino acids that contact CsA in the hCYP18–CsA complex are marked * and those that contact calcineurin in the hCYP18–CsA–calcineurin complex, # (Ke and Huai, 2004). For accession numbers and gene product names, see Table 1.

Fig. 2. Specific tissue location of a subset of *Caenorhabditis elegans* cyclophilins. A, Secretory pathway cyclophilin, CYP-6, located in the embryonic gut. B, Cyclophilin A homologue, CYP-3, is located in the excretory cell and duct in adult stage worms. C, Adult gut location of divergent multi-domain cyclophilin isoform, CYP-8. D, Divergent multi-domain cyclophilin, CYP-4, located in adult somatic muscle cells. E, DAPI-stained adult worm highlighting all nuclei. F, β-galactosidase stained worm in E revealing the gut cell location of the secreted isoform CYP-5. All images depict transgenic *C. elegans* worms, transformed with promoter::*lac*Z reporter constructs. Constructs encode a nuclear location signal to aid cell identification. Worms were fixed and stained with β-galactosidase and viewed microscopically via differential interference contrast optics. The lengths of adult worms and embryos are

typically 1 mm and ~70 μm, respectively. See Dornan et al., 1999; Page and Winter 1998, 1999; Picken et al., 2002 for more detail.

Fig. 3. The modular structures of the FKBPs from *Plasmodium falciparum* and *Toxoplasma gondii* are strikingly similar to those of certain well-characterized human FKBPs. Accession numbers are given in parentheses.

Table 1. Properties of cyclophilins of protozoal parasites

Protein name[a]	Gene accession number	Additional sequence motifs	Expression (mRNA)[b]	Expression (protein)[c]	Subcellular location	PPIase activity[d]	CsA binding	References
PfCYP19B	X85956, PF11_0164	signal sequence	C, N, M	W, F	mainly cytosolic	R (k_{cat}/K_m = 2.3 $\mu M^{-1}s^{-1}$)	IC_{50} = 10 nM	Hirtzlin et al., 1995 Gavigan et al., 2003 Bozdech et al., 2003 Le Roch et al., 2003
PfCYP24	PF08_0121	N-terminal extension	N, M	F	nd[e]	R[f]	Yes[e]	Reddy, 1995 Bozdech et al., 2003 Le Roch et al., 2003 Florens et al., 2002
PfCYP19A	U33869, PFC0975c	–	C, M	W, F	probably cytosolic	R (k_{cat}/K_m = 12 $\mu M^{-1}s^{-1}$)	K_i = 6.9 nM	Berriman and Fairlamb, 1998 Gavigan et al., 2003 Bozdech et al., 2003 Le Roch et al., 2003
PbCYP19	CAH98501	–	C	F	nd	R (k_{cat}/K_m =	nd	Nunes, 2003[g]

[a] Species abbreviations in protein names are as follows: Pf, *Plasmodium falciparum;* Pb, *P. berghei;* Tg, *Toxoplasma gondii;* Nc, *Neospora caninum;* Lm, *Leishmania major;* Ld, *L. donovani;* Tb, *Trypanosoma brucei;* Tc, *T. cruzi;* Gi, *Giardia intestinalis;* Eh, *Entamoeba histolytica*.
[b] C, cDNA clone; N, northern blot; M, microarray; R, reverse transcriptase PCR
[c] P, purified protein; W, western blot; F, peptide mass fingerprint
[d] N, native protein isolated from parasite; R, recombinant protein
[e] nd, not determined
[f] Impure protein preparation
[g] Nunes, J., 2003. PhD thesis. University of Dundee.

						1.93 $\mu M^{-1}s^{-1}$)		Hall et al., 2005
TgCYP18	U04633	signal sequence	C	P, F	secreted	N, R (k_{cat}/K_m = 14 $\mu M^{-1}s^{-1}$)	IC_{50} = 32 nM	High et al., 1994 Aliberti et al., 2003 Cohen et al., 2002
TgCYP20	U04634	–	C	P, F	nd	N	IC_{50} = 5 nM	High et al., 1994 Cohen et al., 2002
TgFCBP57	AAX51680	FKBP domain; TPR	C	nd	nd	R	IC_{50} = 750 nM[h]	Adams et al., 2005
NcCYP	CF422590	signal sequence	C	F, W	secreted	nd	nd	Tuo et al., 2005
LmCYP19	Y13576	–	C	W	nd	R (k_{cat}/K_m = 1.5 $\mu M^{-1}s^{-1}$)	K_i = 5.2 nM	Rascher et al., 1998
LdCYP	AF158368	signal sequence	R	W	particulate fraction	R (k_{cat}/K_m = 6.3 $\mu M^{-1}s^{-1}$)	K_d = 135 nM	Dutta et al., 2001
TbCYP19	Tb11.03.0250	–	C, N	W	cytosol, flagellum, secreted	nd	nd	Pellé et al., 2002
TcCYP19	AF191832	–	C	nd	nd	R	IC_{50} = 18.4 nM	Búa et al., 2001

[h] Isolated CYP domain

GiCYP	–	–	C, N	nd	nd	R	Yes	Yu et al., 2002
EhCYP	AF017993	–	C, N	nd	nd	R	Yes	Ostoa-Saloma et al., 2000

1 Table 2. Properties of *Caenorhabditis elegans* cyclophilins and their homologues in parasitic helminths.
2

Gene	Cosmid (chromosome)	Class	*C. briggsae* homologue	Phenotype (RNAi)	Expression pattern	Parasitic helminth homologue
cyp-1	Y49A3A.5 (V)	C	Y	Wt	All cells[a]	-
cyp-2	ZK520.5 (III)	A	Y	Wt	No pattern[a]	*Onchocerca volvulus cyp-2*, *Dirofilaria immitis cyp-2*, *Echinococus granulosus cypA*
cyp-3	Y75B12B.5 (V)	A	Y	Wt	Excretory system	*O. volvulus cyp-2*, *D. immitis cyp-2*, *Brugia malayi cyp-2*
cyp-4	F59E10.2 (II)	D	Y	Wt/Mog/Dpy	Body wall muscle Somatic and gonad	*B. malayi cyp-4*, *D. immitis cyp-3*, *O. volvulus cyp-4*
cyp-5	F31C3.1 (I)	B	Y	Wt	Gut, pharynx & body wall muscle	*B. malayi SM7* (Q6ynz2)
cyp-6	F42G9.2 (III)	B	Y	Wt	Gut	*Schistosoma mansoni cyp-B*, *S. japonicum cypB* (Q5d8j4), *B. malayi SM7*
cyp-7	Y75B12B.2 (V)	A	Y	Wt/Emb	nd	*O.volvulus cyp-2*, *D. immitis cyp-2*, *B. malayi cyp-2*
cyp-8	D1009.2 (X)	D	Y	Wt	Gut	*B.malayi cyp-1*, *O.volvulus cyp-1*, *D.immitis cyp-1*
cyp-9	T27D1.1 (III)	D	Y	Wt	Hypodermis	-
cyp-10	B0252.4 (II)	D	Y	Wt	Excretory cell[a]	-
cyp-11	T01B7.4 (II)	D	Y	Wt	Somatic muscle[a]	-
cyp-12	C34D4.12 (IV)	D	Y	Wt	Gut[a]	-
cyp-13	Y116A8C.34 (IV)	D	Y	Wt	nd	*Haemonchus contortus Cyp*, *S. japonicum (Q5der2)*
cyp-14	F39H2.2 (I)	D	Y	Wt/Emb/Let	nd	-
cyp-15	Y87G2A.6 (I)	D	Y	Wt	nd	-

cyp-16	Y17G7B.9 (II)	D	Y	Wt	Gut	*O. volvulus cyp-16*
cyp-17	ZC250.1 (V)	D	Y	Wt	nd	-
cyp-18	Y17G9B.4 (IV)	D	N	Wt	nd	-

Information based on *C. elegans* Wormbase version 145. Phenotypes: Wt = wild-type; Mog = maternalization of germline; Dpy = dumpy; Emb = embryonic lethal; Let = larval lethal. Y=yes; N=no; nd = not determined.

[a]Page, A.P. (unpublished data).

Table 3. Properties of *Caenorhabditis elegans* FKBPs and their homologues in parasitic helminths.

Gene	Cosmid (chromosome)	Class	*C. briggsae* homologues	Phenotypes (RNAi)	Expression pattern	Parasitic helminth homologues
fkb-1	F36H1.1 (IV)	B	Y	Wt	Gut	*Brugia malayi fkbp13, Dirofilaria immitis fkbp13, Onchocerca volvulus fkbp13*
fkb-2	Y18D10A.19.1(I)	A	Y	Wt	All cells	*B. malayi fkb12*
fkb-3	C05C8.3 (V)	B	Y	Wt	Hypodermis	*B. malayi fkb18 fragment* (Q9u8j8)
fkb-4	ZC455.10 (V)	B	Y	Wt	No expression	-
fkb-5	C50F2.6 (I)	B	Y	Wt	Hypodermis	-
fkb-6	F31D4.3 (V)	D	Y	Emb/Wt	Neuronal	*B. malayi fkb59; Schistosoma mansoni p50*
fkb-7	B0511.1 (I)	D	Y	Wt	Neuronal	-
fkb-8	Y18D10A.25 (I)	A	N	Wt	Gut	-

Information based on *C. elegans* Wormbase version 145. Expression patterns are from Page, A.P. (unpublished data).
Phenotypes: Wt = wild-type; Emb = embryonic lethal. Y=yes; N=no.

Figure 1

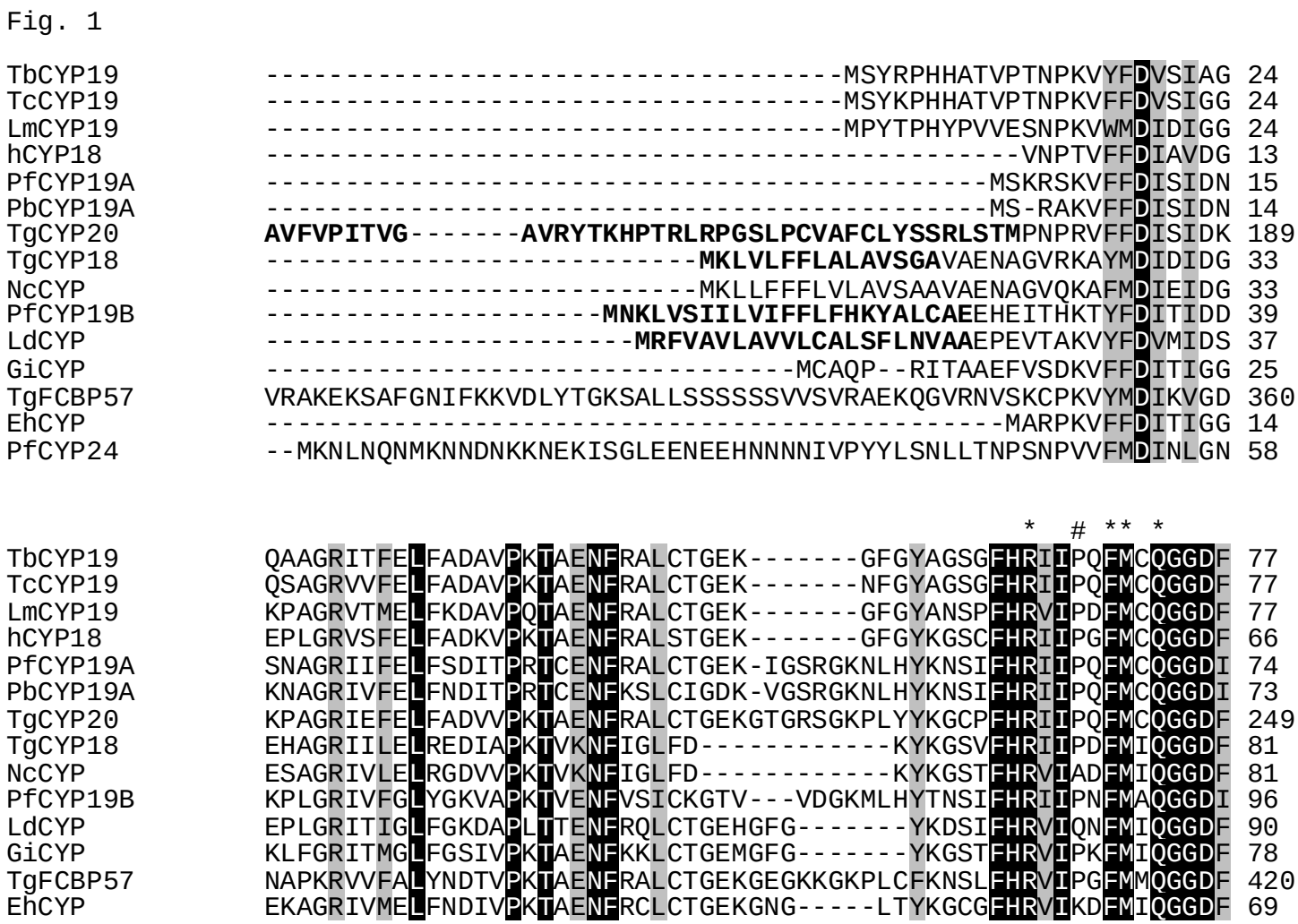
Fig. 1
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hCYP18 ----------VNPTVFFDIAVDG 13
PfCYP19A ----------MSKRSKVFFDISIDN 15
PbCYP19A ----------MS-RAKVFFDISIDN 14
TgCYP20 AVFVPITVG------AVRYTKHPTRLRPGSLPCVAFCLYSSRLSTMPNPRVFFDISIDK 189
TgCYP18 ----------MKLVLFFLALAVSGAVAENAGVRKAYMDIDIDG 33
NcCYP ----------MKLLFFFLVLAVSAAVAENAGVQKAFMDIEIDG 33
PfCYP19B ----------MNKLVSIILVIFFLFHKYALCAEEHEITHKTYFDITIDD 39
LdCYP ----------MRFVAVLAVVLCALSFLNVAAEPEVTAKVYFDVMIDS 37
GiCYP ----------MCAQP--RITAAEFVSDKVFFDITIGG 25
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EhCYP ----------MARPKVFFDITIGG 14
PfCYP24 --MKNLNQNMKNNDNKKNEKISGLEENEEHNNNNIVPYYLSNLLTNPSNPVVFMDINLGN 58
* # ** *
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TcCYP19 QSAGRVVFELFADAVPKTAENFRALCTGEK-------NFGYAGSGFHRIIPQFMCQGGDF 77
LmCYP19 KPAGRVTMELFKDAVPQTAENFRALCTGEK-------GFGYANSPFHRVIPDFMCQGGDF 77
hCYP18 EPLGRVSFELFADKVPKTAENFRALSTGEK-------GFGYKGSCFHRIIPGFMCQGGDF 66
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PbCYP19A KNAGRIVFELFNDITPRTCENFKSLCIGDK-VGSRGKNLHYKNSIFHRIIPQFMCQGGDI 73
TgCYP20 KPAGRIEFELFADVVPKTAENFRALCTGEKGTGRSGKPLYYKGCPFHRIIPQFMCQGGDF 249
TgCYP18 EHAGRIILELREDIAPKTVKNFIGLFD------------KYKGSVFHRIIPDFMIQGGDF 81
NcCYP ESAGRIVLELRGDVVPKTVKNFIGLFD------------KYKGSTFHRVIADFMIQGGDF 81
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LdCYP EPLGRITIGLFGKDAPLTTENFRQLCTGEHGFG-------YKDSIFHRVIQNFMIQGGDF 90
GiCYP KLFGRITMGLFGSIVPKTAENFKKLCTGEMGFG-------YKGSTFHRVIPKFMIQGGDF 78
TgFCBP57 NAPKRVVFALYNDTVPKTAENFRALCTGEKGEGKKGKPLCFKNSLFHRVIPGFMMQGGDF 420
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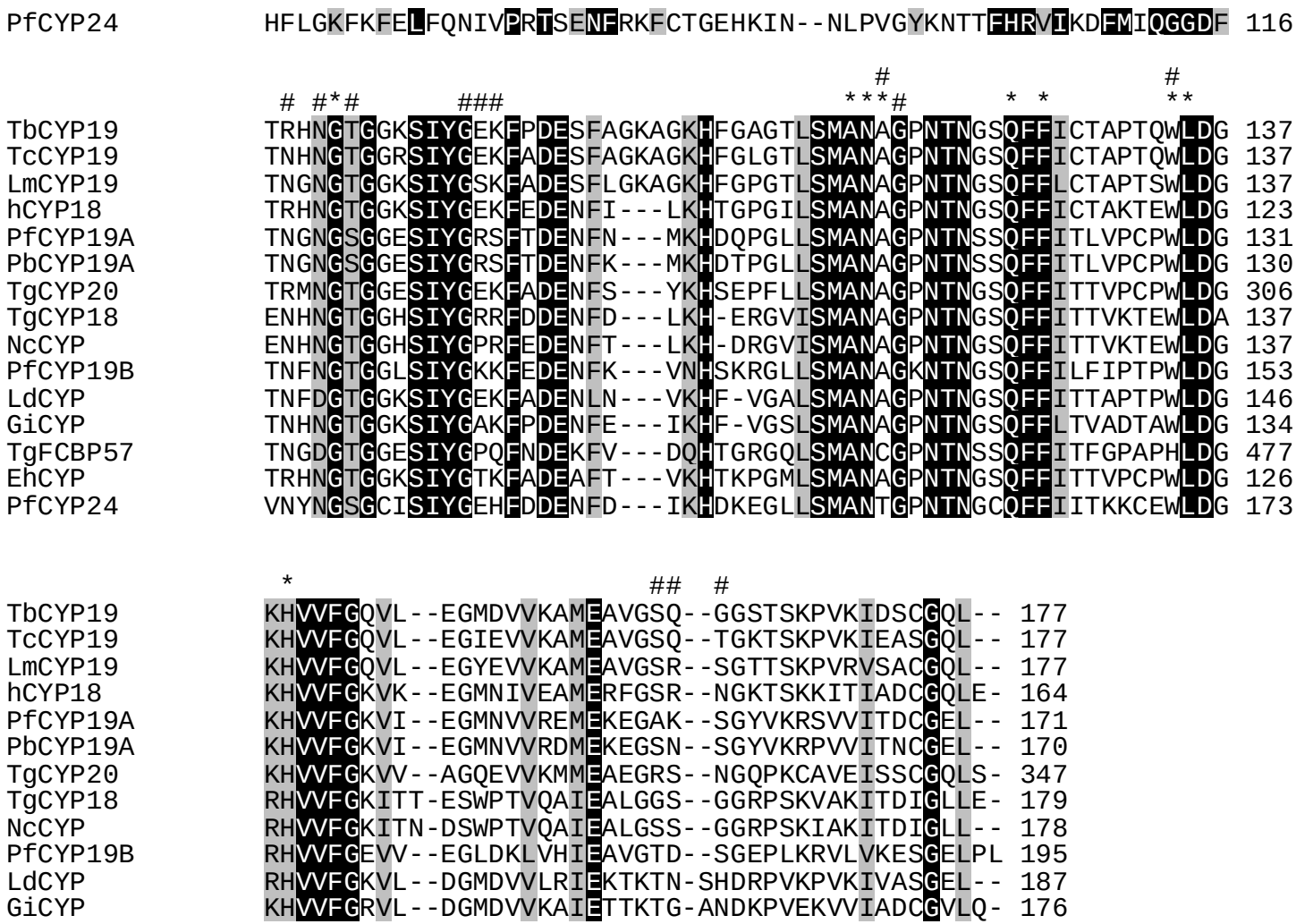
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hCYP18 TRHNGTGGKSIYGEKFEDENFI---LKHTGPGILSMANAGPNTNGSQFFICTAKTEWLDG 123
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PbCYP19A TNGNGSGGESIYGRSFTDENFK---MKHDTPGLLSMANAGPNTNSSQFFITLVPCPWLDG 130
TgCYP20 TRMNGTGGESIYGEKFADENFS---YKHSEPFLLSMANAGPNTNGSQFFITTVPCPWLDG 306
TgCYP18 ENHNGTGGHSIYGRRFDDENFD---LKH-ERGVISMANAGPNTNGSQFFITTVKTEWLDA 137
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PbCYP19A KHVVFGKVI--EGMNVVRDMEKEGSN--SGYVKRPVVITNCGEL-- 170
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NcCYP RHVVFGKITN-DSWPTVQAIEALGSS--GGRPSKIAKITDIGLL-- 178
PfCYP19B RHVVFGEVV--EGLDKLVHIEAVGTD--SGEPLKRVLVKESGELPL 195
LdCYP RHVVFGKVL--DGMDVVLRIEKTKTN-SHDRPVKPVKIVASGEL-- 187
GiCYP KHVVFGRVL--DGMDVVKAIETTKTG-ANDKPVEKVVIADCGVLQ- 176

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EhCYP           KHVVFGQVV--EGYDVVKMIENNPTG-AQDKPKKAVVIADCGQL-- 167
PfCYP24         KNVVFGRIIDNDSLILLKKIENVSVTPYIYKPKIAINIVECGEL-- 217
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Figure 2

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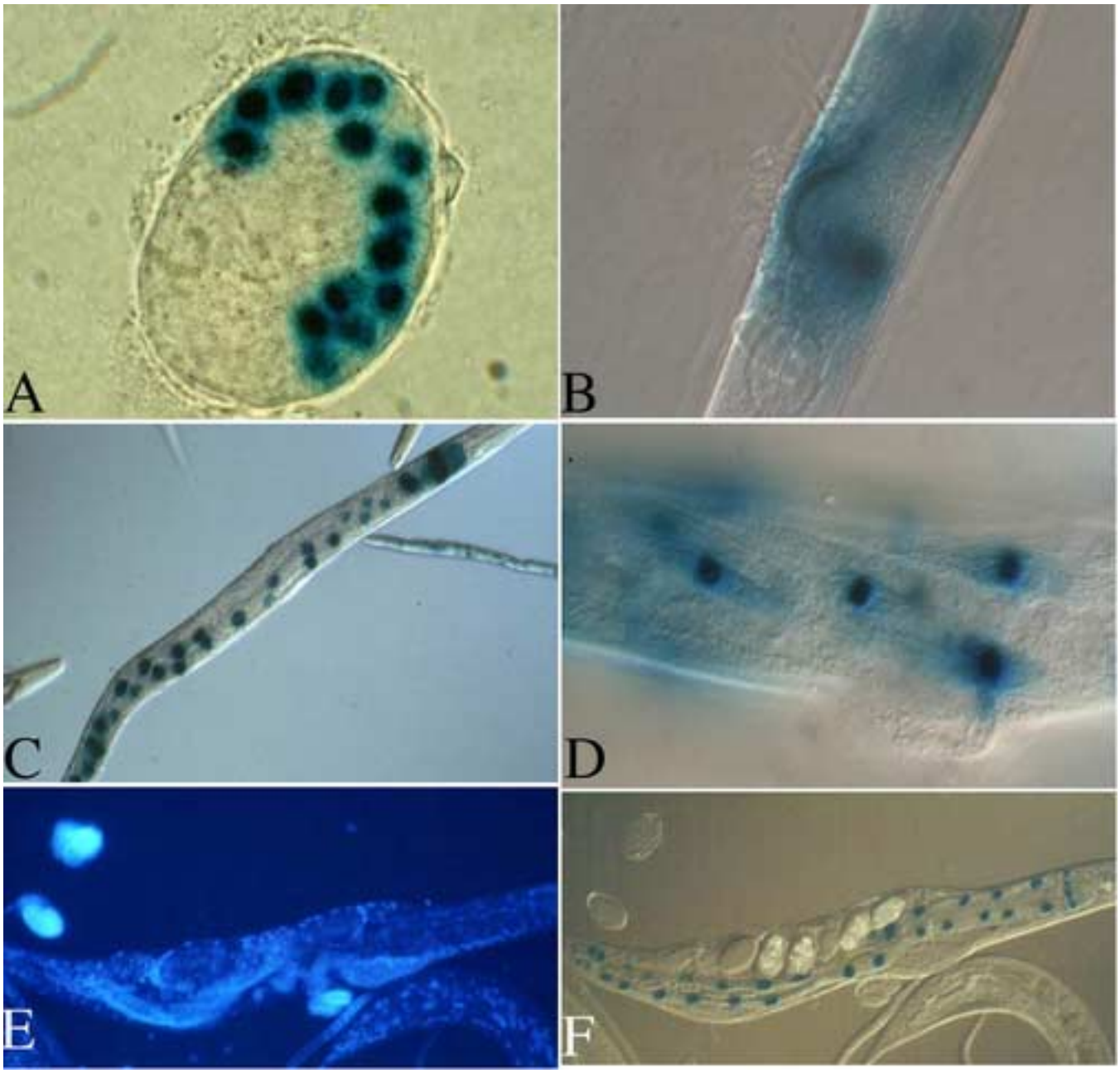
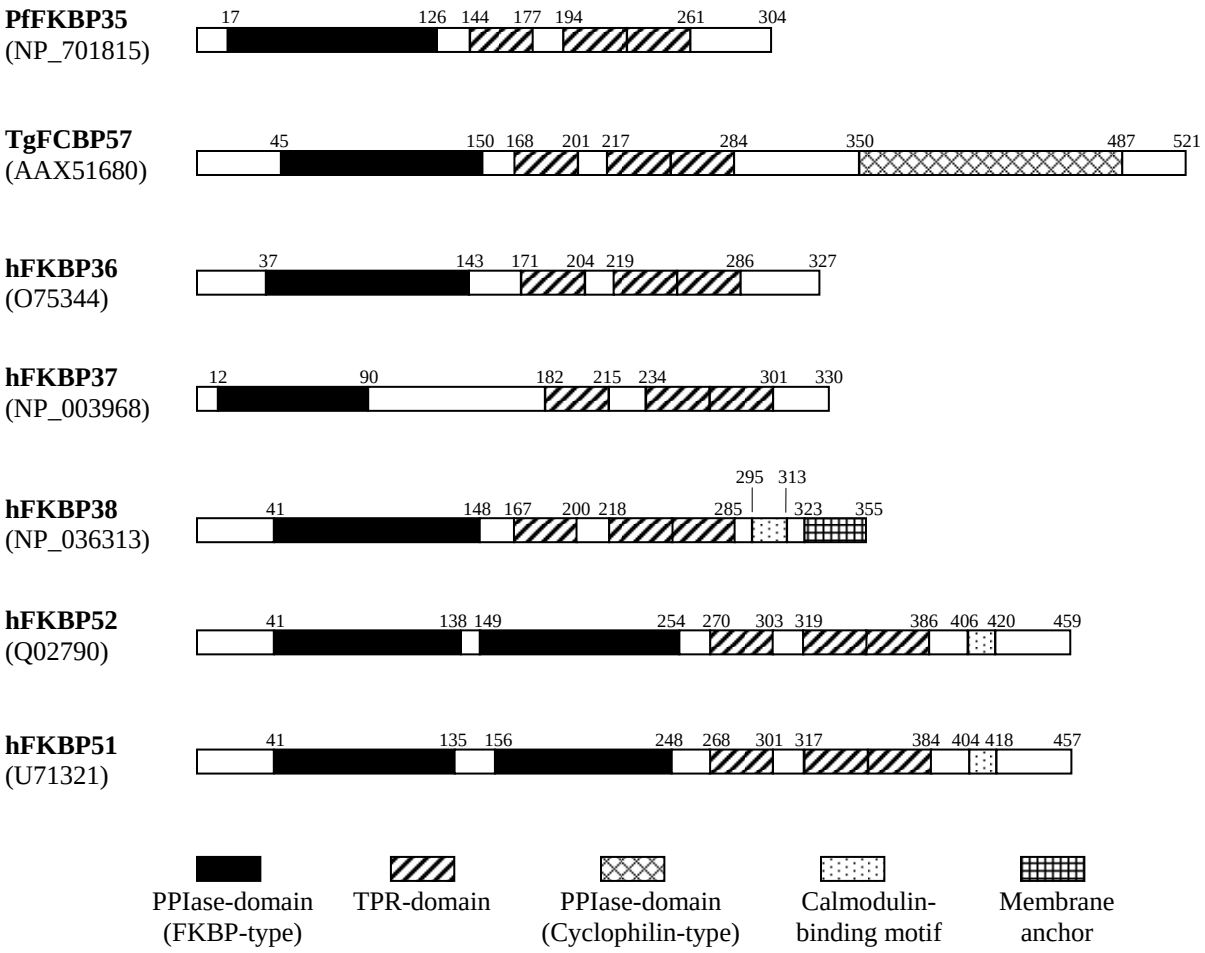


Figure 3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218
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323
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41
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270
303
319
386
406
420
459
hFKBP51
(U71321)
41
135
156
248
268
301
317
384
404
418
457
PPIase-domain (FKBP-type)
TPR-domain
PPIase-domain (Cyclophilin-type)
Calmodulin-binding motif
Membrane anchor

Replies to Editor-in Chief's comments

p12. The reference is correct. The sequence of the *S. mansoni* cyp-13 orthologue is depicted in Valle et al., 2005. The accession number goes back to another reference (Davis,R.E., Hardwick,C., Tavernier,P., Hodgson,S., Singh,H., 1995. RNA trans-splicing in flatworms. Analysis of trans-spliced mRNAs and genes in the human parasite, *Schistosoma mansoni*. J. Biol. Chem. 270 (37), 21813-21819) but in this article the sequence was not shown.

p 12 and 21-22: We agree that this modification was perhaps too vague. We have defined our points more precisely now (p 12 21-25; p21 line 22 – p 22 line 6).

p 22: We have replaced the reference to the Sanger web-site with the relevant literature citations.

p 39: The Nunes (p 7 line 16; p 13 line 6; Table 1) and Monaghan (p 25 line 21) theses have been cited fully in the text and removed from the references.

All other corrections have been made as requested.

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