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Review

An evolutionary perspective on the kinome of malaria parasites

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Malaria parasites belong to an ancient lineage that diverged very early from the main branch of eukaryotes. The approximately 90-member plasmodial kinome includes a majority of eukaryotic protein kinases that clearly cluster within the AGC, CMGC, TKL, CaMK and CK1 groups found in yeast, plants and mammals, testifying to the ancient ancestry of these families. However, several hundred millions years of independent evolution, and the specific pressures brought about by first a photosynthetic and then a parasitic lifestyle, led to the emergence of unique features in the plasmodial kinome. These include taxon-restricted kinase families, and unique peculiarities of individual enzymes even when they have homologues in other eukaryotes. Here, we merge essential aspects of all three malaria-related communications that were presented at the Evolution of Protein Phosphorylation meeting, and propose an integrated discussion of the specific features of the parasite's kinome and phosphoproteome.

Keywords: malaria; kinome; evolution; phosphoproteomics; protein kinase; comparative genomics

1. INTRODUCTION

(a) *Malaria: disease status, need for new control agents*

Malaria has been a scourge of mankind since the emergence of our species, and has contributed to human evolution, being the strongest known selective pressure in the recent history of the human genome [1]. The disease still kills almost a million people each year, mostly young children in sub-Saharan Africa. There was an encouraging trend in the recent decades (the death toll owing to malaria was up to 3 million a year in the last quarter of the twentieth century [2]), to which the introduction of combination therapy (notably artemisinin combination therapy, ACT) contributed significantly [3,4]. There is, however, a very worrying development, with a number of occurrences of decreased sensitivity of malaria parasite field isolates to artemisinin [5]. It is of crucial importance to maintain an active drug discovery/development pipeline of new antimalarials with novel modes of action [3]. In view of the success encountered in targeting protein kinases in the context of cancer chemotherapy [5], the kinome of malaria parasites has been proposed as a possible target [6].

(b) *Plasmodium: phylogeny, evolutionary origin and life cycle*

Malaria parasites (genus *Plasmodium*) constitute a taxon within the Apicomplexa, a group that, together with Stramenopiles, ciliates and dinoflagellates, constitute the Chromalveolata (figure 1). This phylum diverged very early from the main eukaryotic lineage. In line with their phylogenetic relatedness to dinoflagellates, many of which are photosynthetic, malaria parasites and many Apicomplexa contain a remnant plastid and are presumed to descend from photosynthetic ancestors that arose by secondary endosymbiosis; loss of photosynthetic capability is thought to have accompanied the emergence of a parasitic lifestyle [11,12]. The vast phylogenetic distance between malaria parasites and classical eukaryotic model systems such as yeast and metazoans (which are phylogenetically closely related to each other within the Opisthokonta taxon; figure 1) is reflected by profound divergences even in fundamental aspects of biology. These include (to name just a few) an unprecedented mode of transcriptional regulation [13], the coexistence of several different ribosomal RNA sets in the genome [14], and the presence in the

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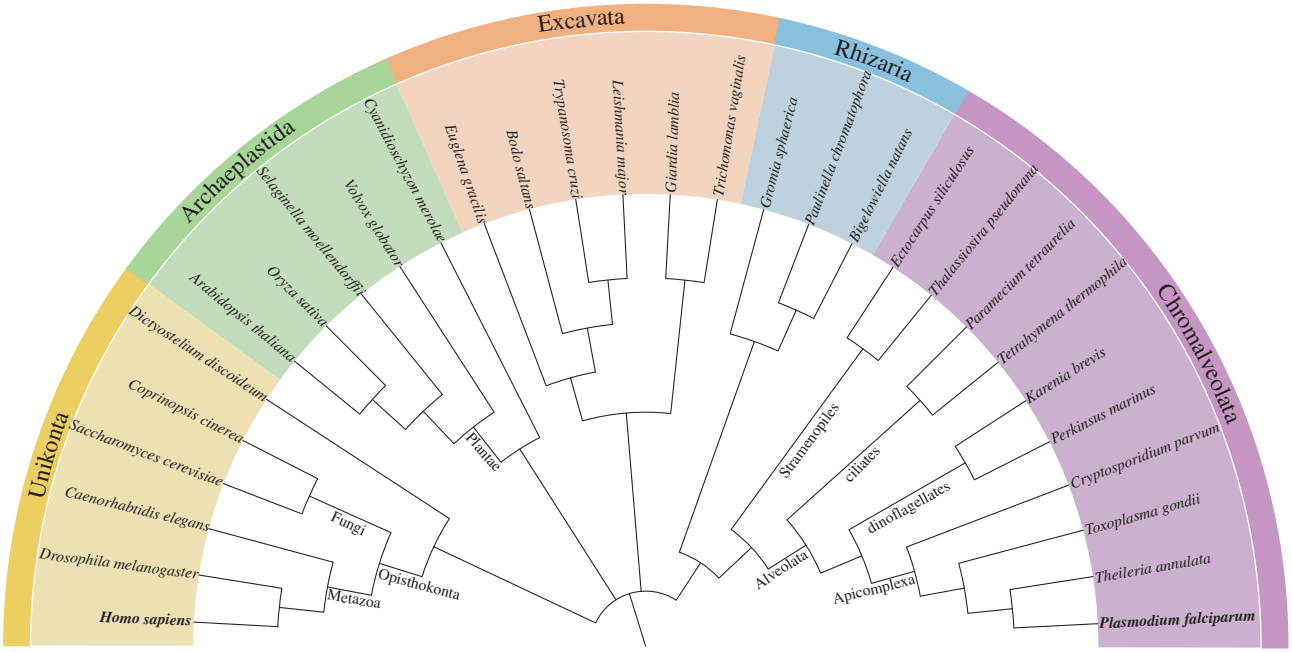

Figure 1. Taxonomic arrangement of *Plasmodium* and model organisms on the eukaryotic tree. Cladogram of representative species in Eukaryota, with *Plasmodium* and humans indicated in bold. Colours indicate the eukaryotic supergroups defined by Adl *et al.* [7]. Rhizaria and Chromalveolata are placed together per Hackett *et al.* [8], and the phylogeny of apicomplexan species is according to Kuo *et al.* [9]. The tree image was rendered with the Interactive Tree of Life server (iTOL) [10] and edited in INKSCAPE (http://inkscape.org).

nuclear genome of several genes (see Fast *et al.* [15] for an example) and gene families, such as the calcium-dependent kinases (CDPKs) [16] and the plant-like ApiAP2 transcription factors [17], that most presumably originated in the host cells that were colonized in the two successive rounds of endosymbiosis [12,15].

Plasmodium species are obligate parasites with a complex life cycle that requires a vertebrate host and a mosquito vector, of the genus *Anopheles* in the case of the five species that infect humans. Sporozoites are injected into the bloodstream during a blood meal, and reach the liver where a first (asymptomatic) round of schizogonic replication occurs, generating, in the case of the most virulent species, *Plasmodium falciparum*, up to 40 000 merozoites. These are geared to invade erythrocytes, where a second round of schizogony occurs; malaria pathogenesis is caused by the synchronous rupture of infected erythrocytes. Transmission to the mosquito requires the differentiated, cell-cycle-arrested sexual forms (male and female gametocytes) that develop in a fraction of the infected erythrocytes. Upon ingestion by the mosquito, asexual parasites are digested, but gametocytes undergo further development into gametes, zygotes and eventually oocysts where sporozoites are generated. These cells accumulate in the insect's salivary gland, where they are primed for infection of a novel human host. Stage transitions during this complex life cycle are likely to require efficient signalling pathways, and evidence for the role of protein phosphorylation in life-cycle progression is emerging [18–20].

2. THE *PLASMODIUM* KINOME

(a) In silico *characterization*

Soon after the *Plasmodium falciparum* genome sequence became available [21], *in silico* analyses were performed to identify the parasite's kinome, which was reported to encompass 84 [22] or 99 [23] genes encoding protein kinases, depending on the stringency applied for inclusion of borderline sequences; a more recent study [24] settled for a 91-sequence kinome, excluding atypical kinases (aPKs) such as RIOs and PIKKs (figure 2). Many of the sequences that constitute the *P. falciparum* kinome cluster within the familiar AGC, CMGC, CK1, TKL and CaMK groups found within the mammalian kinome, and behave as expected, in terms of their biochemical regulation, from their homologues in other organisms. For example, several predicted CDPKs (see below) are indeed stimulated by calcium [29–31], and the activity of at least two enzymes clustering within the cyclin-dependent kinase (CDK) family has been shown to be dependent on the binding of cyclins [32,33], four homologues of which have been identified in the *P. falciparum* proteome [34]. However, several malarial protein kinases do not cluster within the established eukaryotic protein kinase (ePK) groups that constitute the human kinome. These include the 21 so-called FIKKs, a novel family of atypical protein kinase-like enzymes named after a conserved Phe–Ile–Lys–Lys motif, and a family of CDPKs similar to calcium-regulated protein kinases found in plants but not in metazoans. Conversely, two clusters that are prominent in the human kinome are not represented in the kinome of malaria parasites: the tyrosine kinases and the MAPKK family in the STE group, a group that is itself dramatically reduced in size, with just one member (see below and figure 3 for a distribution of ePK groups among the major eukaryotic taxons).

In addition to taxon-restricted kinase families, many 'orphan' sequences do not cluster with ePK groups found in other eukaryotes. A good example is that of PfPK7, an orphan kinase that is involved, in

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Figure 2. Phylogenetic tree of the *Plasmodium falciparum* kinome. Circular tree of all 91 eukaryotic protein kinases (ePK) in *P. falciparum* as defined by Talevich *et al.* [24]. Representative genes from human (Hs), *Arabidopsis thaliana* (At) and *Plasmodium berghei* (Pb) are indicated with labels coloured gold, green and purple, respectively. Branch and arc colours indicate kinase classification by ePK major group [25,26], according to Talevich *et al.* [24], with minor modifications in group assignment according to the gene tree. To construct the tree, the sequences of 91 protein kinases were retrieved from GeneDB (http://genedb.org), *P. falciparum* 3D7 sequence v. 3. Conserved regions of the kinase domain were aligned with MAPGAPS [27] and unconserved sequence positions, as identified by MAPGAPS, were removed. A gene tree was then inferred from the resulting 245-column alignment using RAxML [28] with the rapid bootstrap analysis and maximum-likelihood tree search algorithm, LG amino acid substitution model, and gamma model of substitution rate heterogeneity. The tree image was rendered with the Interactive Tree of Life server (iTOL) [10] and edited in Inkscape (http://inkscape.org). A grey circle on a branch indicates bootstrap support greater than 50; larger circles indicate greater bootstrap values.

line with its absence from the yeast and mammalian kinomes, in a parasite-specific function, namely the regulation of the number of daughter merozoites generated by each schizont [36]. Furthermore, most individual plasmodial enzymes clustering within known ePK groups and families display peculiarities that distinguish them from their homologues in other eukaryotes. These include (sometimes very large) low complexity extensions and insertions in the catalytic domain, usually occurring at loops between secondary structure elements; this feature is not restricted to kinases, but occurs throughout the parasite's proteome, and its evolutionary function is still being debated [37–41]. Many protein kinases, called 'semi-orphans' in the initial kinome characterization [22], clearly belong to a specific kinase group or family, but do not have a clear orthologue in other organisms. A good example is the CDK-related kinase Pfcrk-5, which forms a distinct branch with the CDK cluster, and which displays an atypical and apicomplexan-specific motif in the activation loop [24]. Some *Plasmodium* kinases in a given family appear to contain domains/motifs that are characteristic of other kinase families; a striking example is that of the NIMA-related kinase Pfnek-1: despite overall homology and clear phylogenetic linkage to the NIMA family, Pfnek-1 possesses a MAPKK-like activation motif SMAHS, which is clearly reminiscent of the SMANS motif of mammalian MEK1 and distinct from the FXXT motif usually found at the corresponding position in other members of the NIMA family [42]. Interestingly, Pfnek-1 (and another NIMA-related kinase, Pfnek-3 [43]) has been tentatively implicated in the regulation of an atypical mitogen-activated protein kinase (MAPK), Pfmap-2 [42], which itself has an atypical activation motif

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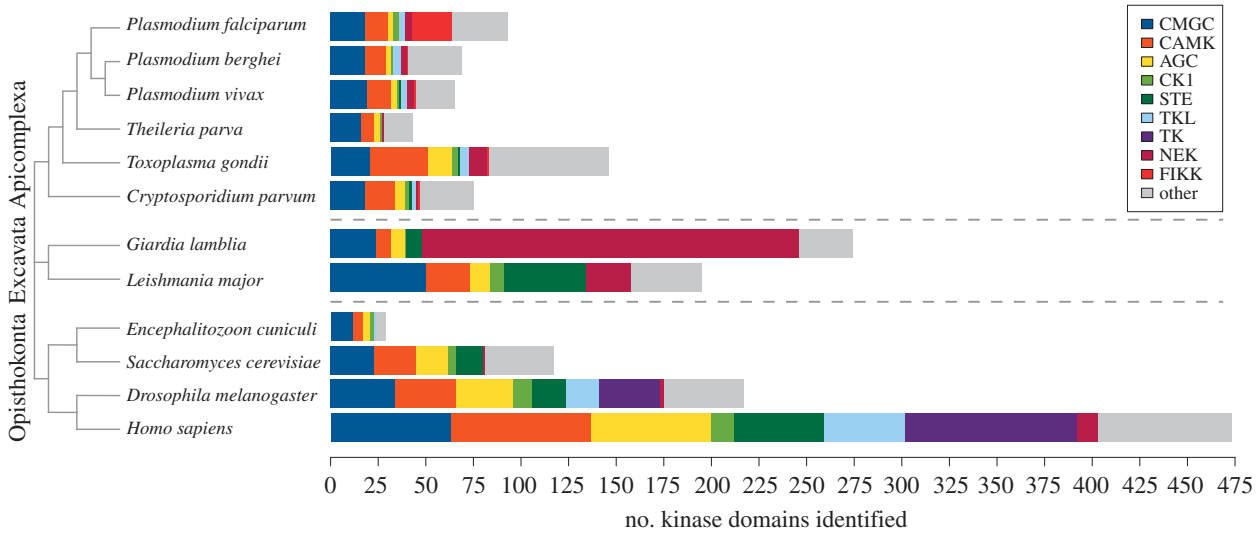

Figure 3. ePK group distribution across representative of the main eukaryotic taxons. Composition of protein kinase major groups and the apicomplexan-specific FIKK family in the genomes of three *Plasmodium* species and three other apicomplexans. The kinomes of the model organisms *Saccharomyces cerevisiae* (Baker's yeast), *Drosophila melanogaster* (fruitfly) and *Homo sapiens* (human), as well as the phylogenetically distant parasite *Giardia lamblia* (figure 1) and the microsporidium *Encephalitozoon cuniculi*, which has the smallest characterized eukaryotic kinome [35], are included for comparison. The cladogram along the left edge indicates the evolutionary relationships between species. In the stacked bar chart associated with each species, block width indicates number of genes found belonging to each major group of eukaryotic protein kinases; total bar width indicates total kinome size. Data are adapted from Talevich *et al.* [24] and KinBase (http://kinase.com/kinbase/).

[44]. In conjunction with the absence of MEK/MAPKK homologues in the plasmodial kinome [22–24], this might point to an unorthodox MAPK regulation pathway in the parasite (see below).

(b) *Functional analysis of the kinome*

Protocols for manipulation of the genome of malaria parasites have been available for a decade or so [45]. The roles of individual protein kinases were first investigated following a reverse genetics approach in the rodent malaria model *Plasmodium berghei*. This revealed essential functions of a MAPK (Pbmap-2) and a CDPK (PbCDPK4) in male gametogenesis [29,46,47], of two NIMA-related kinases in meiosis in the mosquito vector [48,49], and of a CDK in asexual proliferation in erythrocytes [50]. This approach culminated in a kinome-wide study demonstrating that 23 *P. berghei* ePKs are redundant for asexual erythrocytic parasite development in mice and identifying phenotypes in sexual development for a number of these 23 ePKs [51]. A similar strategy in *P. falciparum* identified roles for an orphan kinase in proliferation rate linked to the number of progeny merozoites per schizont [36], for an eIF2α kinase in response to starvation stress, similar to GCN2, its closest homologue in yeast [52], and for CDPKs in motility during invasion [53] or egress of merozoites from the erythrocyte [54], to cite a few specific studies; more recently, a kinome-wide approach [19] identified 36 ePKs as refractory to gene disruption, and thus as likely crucial players in asexual proliferation in erythrocytes.

3. *PLASMODIUM* PHOSPHOPROTEOMICS

The determination of the essential protein kinases in the blood stage of *P. falciparum* [19] not only reveals the protein kinases that might constitute targets for novel antimalarial therapy but also provides a framework to identify key phospho-signalling pathways [55]. Identifying kinase substrates would represent a significant advance in our understanding of such pathways. The huge advances achieved recently in mass spectrometry-based phosphoproteomic approaches allow large portions of the phosphoproteome to be identified in a single experiment [56–58]. Because *P. falciparum*-infected red blood cells at the schizont stage can be readily isolated from an *in vitro* culture, this stage in the parasite life cycle was the first to be interrogated by mass spectrometry techniques designed to investigate the global phosphoproteome. Two such studies have recently been published, one revealing 1117 [19] unique phosphor-acceptor sites on over 600 *P. falciparum* proteins, and another, using lower filter stringency, detecting over 5000 occupied phosphorylation sites [20]. Many of the identified phosphosites overlapped between the two studies.

(a) *Tyrosine phosphorylation in* Plasmodium falciparum

Among the unexpected findings from these studies was the identification of tyrosine phosphorylation of parasite proteins, because, as mentioned earlier, there are no members of the tyrosine kinase group in the *P. falciparum* genome. The strongest evidence for tyrosine phosphorylation of *P. falciparum* proteins was in the activation loop of two protein kinases: PfGSK3 (PFC0525c) and PfCLK3 (PF11_0156) [19]. Phosphorylation of PfGSK3 occurred at Y229, analogous to Y279 and Y216 on mammalian GSK3α/β, the activation loop auto-phosphorylation sites necessary for enzymatic activity [59,60]. The tyrosine phosphorylation of the CDK-like kinase PfCLK3 appears to follow similar lines: this kinase is a serine/threonine kinase related to the pre-mRNA-processing kinase hPRP4 found in higher

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eukaryotes [22]; both PfCLK3 and hPRP4 cluster within the dual-specificity tyrosine phosphorylated-regulated kinase (DYRK) family [22,61], members of which form an active transient intermediate during translation that is able to *cis*-auto-phosphorylate on tyrosine [60]. This process is essential for full catalytic activity of the mature enzyme, which then shows only serine/threonine kinase activity. In the case of PfCLK3, phosphorylation occurs on Y526 within a TSY motif in the activation loop [19]. In most DYRKs, however, the phosphotyrosine is contained within a YXY motif where the second Y is the site of co-translational auto-phosphorylation [60]. Mutation of Y526 in PfCLK3 significantly reduced the serine/threonine kinase activity of the recombinant enzyme [19], indicating that tyrosine phosphorylation of PfCLK3, as for other DYRKs, is essential for a fully active kinase.

The tyrosine phosphorylation of PfCLK3 and PfGSK3 is a somewhat special case. The question remains—are there parasite proteins that are phosphorylated in *trans* on tyrosine residues? Only a small number of tyrosine-phosphorylated peptides have been identified [19,20], and the confidence levels associated with these peptides are usually very low [19]. This may reflect the low abundance of phosphorylated tyrosines, and additional enrichment techniques such as immunoprecipitation with anti-phosphotyrosine antibodies may be necessary to draw firm conclusions regarding the extent of tyrosine phosphorylation in *P. falciparum*. It is relevant to mention here that SH2 domains, which are major phosphotyrosine-binding devices in eukaryotic cells [62], appear to be essentially absent from malaria parasites: a query of the plasmodial proteomes with the SH2 HMM profile on InterPro of Pfam (entry PF00017) yielded only one weak hit in *P. berghei*, in the hypothetical protein PBANKA_114210; although this protein has syntenic orthologues in all other *Plasmodium* species, the latter do not possess a recognizable SH2 motif. We therefore propose that this is a spurious hit, and that there are no SH2 domains in plasmodia. This is in line with the apparent low occurrence of phosphotyrosine discussed earlier, although we cannot exclude that taxon-specific, cryptic phosphotyrosine-binding domains have evolved in these organisms. It is noteworthy that yeast does not possess SH2 domains, and the only non-metazoan organism in which these domains have been shown to occur is the social free-living amoeba *Dictyostelium*, whose kinome also lacks typical tyrosine kinases [63].

One exception to the low-confidence phosphotyrosine sites in *Plasmodium* proteins, however, appears to be the protein PHISTB (PFE1600w). This *P. falciparum* protein is exported to the cytoplasm of the red blood cell [64], and was unequivocally identified as a tyrosine-phosphorylated protein [20]. What is not clear, however, is whether this tyrosine phosphorylation occurs only once the protein reaches the red blood cell cytoplasm. If this is the case, then it is possible that PHISTB is phosphorylated by a host rather than a parasite tyrosine kinase. The ability of host protein kinases to impact on the parasite erythrocytic life cycle has indeed been previously documented in the case of the parasite-dependent activation of an erythrocyte signalling pathway that is required for parasite survival [65].

(b) *Regulation of protein kinases by phosphorylation in* Plasmodium falciparum

Among the proteins determined to be phosphorylated in the schizont stage of *P. falciparum*, 23 kinases were determined to be phosphoproteins [19]. Many of these phosphorylations were on sites within their activation loops and therefore potentially associated with the regulation of catalytic activity [66]. These include, in addition to PfGSK3 and PfCLK3 already discussed, activation loop phosphorylation of the cyclic nucleotide-dependent kinases PfPKA (PFI1685w) and PfPKG (PF14_0346) [19], both of which have crucial functions in the erythrocytic cycle [54,67–70]. How are these kinases regulated? Preliminary studies using phospho-specific antibodies have shown that the activation loop phosphorylation of these protein kinases does not appear to change during the life cycle of the parasite (A. Tobin 2012, unpublished data). This is similar to the constitutively phosphorylated activation loop of mammalian PKA that is mediated by the phosphoinositide-dependent protein kinase PDK1 [71]. That activation loop phosphorylation of parasite protein kinases is dynamic for certain kinases is illustrated by the parasite kinases Pfcrk-1 (PFD0865c) [72] and Pfcrk-3 (PFD0740w) [73]. These kinases are related to the mammalian and fungal CDKs that require both cyclin binding and activation loop threonine phosphorylation for full activity [74]. The threonine activation loop phosphorylation observed for Pfcrk-1 and Pfcrk-3 may therefore be mediated by an upstream kinase in a phospho-signalling cascade analogous to that of the mammalian system. A possible candidate for the kinase acting upstream of Pfcrk-1 and Pfcrk-3 in *P. falciparum* would be Pfmrk (PF10_0141), as this enzyme displays closest homology to CDK7, a kinase that in mammalian cells acts upstream of the CDKs [75].

Overall, the *in silico* characterization of the *P. falciparum* kinome, the assessment of the function of the parasite's protein kinases by reverse genetics, and the global phosphoproteomics approach providing a glimpse into the substrate and effectors of these enzymes, all point to the importance of protein phosphorylation in malaria parasites, and illustrate the phylogenetic divergence between the parasite and its host. In §4, we will consider the specificities of the plasmodial kinome from an evolutionary angle.

4. EVOLUTIONARY PERSPECTIVES ON THE *PLASMODIUM* KINOME

One of the goals that initially motivated our analysis of ePKs in *P. falciparum* was to assess the potential of the parasite's kinome as a target for antimalarial drug discovery. In this purpose, we and others have sought to identify distinctive protein features that appear and are conserved in the parasite, but do not appear in the host. Comparative analysis of sequences and protein structures has revealed a number of such features conserved to varying degrees in *P. falciparum*, related *Plasmodium* species, and other apicomplexan parasites of medical and veterinary importance. Given the ancient evolutionary divergence between *Plasmodium* and most model organisms (figure 1), investigation of the cell signalling components in *Plasmodium* and its relatives

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provides scientific insights into the early evolution of eukaryotes, as well as a basis for comparison for the study of other protists. Earlier, we pointed out several distinctive features of the *P. falciparum* kinome and phospho-signalling transduction pathways, and briefly described how reverse genetics and phosphoproteomics can provide information on their involvement in the organism's biological functions. We will now compare the plasmodial kinome with those of other eukaryotes, to address the questions of how parasite-specific kinome features evolved, and whether some of its features are shared by other parasites. This has a direct impact on drug discovery and development: could kinase inhibitors that target parasite-specific features be developed? If so, could the same inhibitors be used to target similar features in related parasites?

(a) *Parasitic lifestyle correlates with kinase gene loss*

The total number of kinases, i.e. the kinome size, is markedly reduced in *P. falciparum* and other apicomplexans in comparison with other model eukaryotes. This reduction of the kinome is in line with an overall gene loss observed in the *Plasmodium* genome. Gene loss and general compaction of the genome (loss of introns, smaller intergenic regions) have been noted as the dominant mode of genomic evolution in obligate intracellular parasites such as the Apicomplexa [76] (see figure 3 for a comparison of ePK group counts across eukaryotes). From a superficial comparison, the kinome of *P. falciparum*, consisting of roughly 91 ePKs [24] plus at least five aPKs [22], appears to constitute a percentage of the total proteome (1.7% of 5228 protein-coding genes) that is similar to that found in other, non-parasitic eukaryotes: the kinome of the baker's yeast *Saccharomyces cerevisiae* comprises 117 ePKs (2% of 5770 genes; plus 14 or 10 aPKs, depending on the study) [77,78], the fruitfly *Drosophila melanogaster* has 223 ePKs (1.4% of approx. 15 000 genes) [79] (plus 15 aPks) and the human kinome 478 (1.9% of approx. 25 000 genes) [80,81] (plus 38 aPKs). However, *P. falciparum* is the only *Plasmodium* species in which the FIKK family is vastly expanded; if we consider only typical ePKs, the percentage of the kinases in the kinome decreases from 1.7 to 1.3, towards the lower limit found in eukaryotes (in an astounding comparison, the ciliate *Paramecium tetraurelia*, related to malaria parasites by the clade Alveolata, possesses 2606 protein kinases, which amounts to 6.6% of its proteome [82]!).

Analysis of the kinomes of several diverse eukaryotic parasitic and free-living species led to a hypothesized 'core' set of between 68 and 85 protein kinases found in the common ancestor of all extant eukaryotes [83]. This would suggest that evolutionary necessity has preserved these approximately 85 kinases as the 'minimal kinome' of extant free-living eukaryotes. However, obligate parasites may be freed from some of the evolutionary constraints that preserve certain ePK families in other species, and thus show reduction or loss of kinase families that appear essential in other species. An extreme example of such gene loss is the microsporidium *Encephalitozoon cuniculi*, an obligate parasite that contains only 29 typical ePKs [35]. A similar example in *Plasmodium* is the absence of key components of MAPK cascades. Typically, this signal transduction pathway is formed by three or four protein kinases associated with distinct ePK families: MAP4K or MEKKK (a tyrosine-like kinase such as Raf, or a member of the STE20 family), MAP3K or MEKK (a member of the STE11 family), MAPKK or MEK (a STE7 member) and MAPK family (best characterized in the ERK1/ERK2, p38 and JNK subfamilies) [84]. However, in *Plasmodium* and most other apicomplexans, the STE group of kinases is reduced to one or two distantly related members, or even missing entirely [85], although two clear (but atypical) members of the MAPK family are present [86]. One of these, Pfmap-1, has been demonstrated to be phosphorylated on the conserved TXY motif in its activation loop [20], while this otherwise highly conserved activation motif is substituted in the other MAPK (Pfmap-2) by TSH [44]; no evidence for *in vivo* phosphorylation of this TSH motif is available, but mutating either the Thr or the His (but not the Ser) dramatically affects kinase activity of the recombinant enzyme [44]. Absence of the classical upstream regulators of the MAPK cascade is rare in Eukaryota, but has also been observed in *E. cuniculi*; in the latter case, not only are the STEs that act as MAPK regulators missing but the microsporidial kinome does not even include MAPK homologues [35]; in view of the association of microsporidia with fungi, which have full MAPK cascades, this is most likely the result of evolutionary gene loss. Similarly, the presence of MAPKK homologues and other STEs in several species of chromalveolates (including the photosynthetic diatom *Thalassiosira pseudonana*, the parasitic dinoflagellate *Perkinsus marinus* and the free-living ciliate *Tetrahymena thermophila* [87]) suggests that the absence of such homologues in Apicomplexa results from gene loss, rather than from evolutionary divergence that preceded appearance of MAPK pathways in the main eukaryotic lineage. In any case, it appears that the parasitic lifestyle of *Plasmodium* and microsporidia has altered the evolutionary constraints that preserve the complete MAPK cascade found in most other eukaryotes. The functionality of similar cascades is likely to still be necessary in these organisms, but satisfied in other ways. For example, one of the two *Plasmodium* MAPKs (map-2) is required for the transition from gametocyte to gamete that occurs in response to the new environment met by the parasite upon ingestion by the mosquito; the mode of activation of the enzyme is unclear (as discussed earlier, it has an unusual putative activation motif, and there is no typical MAPKK in the *Plasmodium* kinome). It has nevertheless been established that in *map-2*$^{-}$ parasites, the ontogeny of eight flagellated microgametes from a single male gametocyte is blocked [46,47], and hence that MAPK signalling, albeit differing in important aspects, is still exploited by malaria parasites to generate adaptive responses to environmental changes as in higher eukaryotes.

(b) *Gene loss is offset by expansions of taxon-specific protein families*

The overall trend of gene loss in obligate parasites is occasionally offset by dramatic expansions of specific gene families, including novel kinase families [88].

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The kinome of *P. falciparum* includes a number of 'orphan' families that have no orthologues in other eukaryotic lineages [22,23]. The most notable of these is the FIKK family, a novel and rapidly evolving family of exported proteins, including the *P. falciparum* R45 trophozoite protein, and named after a conserved 'FIKK' motif in the second sub-domain of the protein kinase domain (the first 'K' corresponding to the conserved lysine in sub-domain II of ePKs) [22]. This family is expanded to 19 copies in *P. falciparum*, plus one or two pseudogenes, and at least six copies in *P. reichenowi*, a species closely related to *P. falciparum* [89]. As discussed earlier, the expansion of the FIKK family accounts for most of the difference in kinome size between *P. falciparum* and the other *Plasmodium* species [24]. Most of the FIKK genes in *P. falciparum* are located in sub-telomeric regions, including a cluster of tandem duplicates on chromosome 9 [89]. Synteny breaks between *Plasmodium* species frequently appear near FIKK genes. It is hypothesized that the transplantation of the FIKK gene to a sub-telomeric region in the common ancestor of *P. falciparum* and *P. reichenowi* placed this gene in a rapidly evolving chromosomal region, enabling rapid gene duplication and diversification along this evolutionary branch [90]. BLAST and HMM profile searches identified only weak similarity of the FIKK C-lobe to kinases in non-apicomplexans, and did not provide a clear signal as to which known kinase family the ancestor of FIKKs may have belonged to (E. Talevich 2012, unpublished data). The maximum-likelihood method we used to infer the *P. falciparum* kinome gene tree does not indicate the root of the tree, precluding insights into the kinase group at the origin of FIKKs; a more elaborate phylogenetic approach might provide some information in this respect.

The pattern of genome compaction offset by expansion and diversification appears in other obligate parasitic protists. In the coccidians, another sub-clade of Apicomplexa that includes *Toxoplasma gondii*, there is a novel family of ePKs targeted to the rhoptry (a secretory organelle found in most apicomplexans) [91,92]. This kinase family, dubbed ROPK, is amplified to several dozen copies in all of the coccidians for which the whole-genome annotation is currently available [24]. At least some these kinases are secreted into the host cell and modulate its transcriptional repertoire [91,92].

In *Giardia lamblia*, a parasitic species belonging to a separate supergroup of protists, Excavata, expansion occurred in another ePK family, that of the NIMA-related kinases (NEKs); again, this amplification is correlated with a lineage-specific feature, in this case, the elaborate cytostructure of the parasite cells [83]. The expansion of the NEK family is seen to a lesser extent in *Trichomonas vaginalis* [83] and trypanosomes [93], which are also parasitic excavates with elaborate flagellar systems. Three of the four *Plasmodium* NEK family members are expressed predominantly in sexual stages [48,49,94], which include the only flagellated cell form in the *Plasmodium* life cycle, the male gamete; however, only one Pfnek has been clearly shown to be male-specific [95,96], whereas two others are associated with female development and appear to be involved in meiosis [48,49].

(c) *Kinase gene architecture*

Another striking feature of *Plasmodium* evolution is the remarkably high rate of genomic rearrangement [97]. In protein kinase genes, this phenomenon contributes to variation in protein domain architecture. The 19 copies of the FIKK family in *P. falciparum* each contain a long amino-terminal extension in addition to the protein kinase domain; however, the sequence in this region, including possible functional accessory domains, is not conserved between gene copies, with the exception of export signals near the very N-terminal end [89]. This suggests rapid diversification of function, a view that is supported by the star-like shape, with branches of similar length, of the FIKK phylogenetic tree [22], and by the following biological observations: (i) different FIKKs are targeted to distinct sub-cellular compartments [90]; (ii) in the case of two FIKKs with the same sub-cellular locations, distinct substrates appear to be targeted by the enzymes [98]; and (iii) the same two FIKKs, the only ones so far to have been the subject of published reverse genetics investigations and for which viable knock-out lines were obtained, displayed phenotypes in the rigidity of the infected erythrocyte, despite phosphorylating distinct proteins [98].

Another multi-member kinase family in malaria parasites is that of the CDPK, which is essentially restricted to Plantae and Chromoalveolata (with a few distantly related members having been reported in trypanosomatids; [93]), and is characterized by a carboxyl-terminal tail containing two to four calcium-binding EF-hand domains. *Plasmodium falciparum* contains five CDPKs with this architecture (PfCDPK 1–5), but also a sixth sequence with a substantial amino-terminal tail that includes another pair of EF-hand domains [16]. The shuffling and duplication of EF-hand domains in apicomplexan CDPKs has been carefully chronicled with respect to *Cryptosporidium parvum* [99].

(d) *Structural variations within the kinase domain*

The kinase domain itself contains 11 conserved structural regions, called subdomains [25]. While these motifs are deeply conserved, as mentioned earlier the loops between subdomains are more variable and can be elaborated through insertions and deletions (indels), as well as amino acid substitutions [26]. This process may sometimes produce new functional features. Indels frequently appear in the catalytic domain of many *Plasmodium* kinases, and some are conserved across the *Plasmodium* genus or even more broadly in apicomplexans. Because they usually occur in the loops between secondary structure elements, they are thought not to disrupt the classical kinase fold (see Halbert *et al.* [73]). In addition to indels, there are significant sequence peculiarities in a number of *Plasmodium* kinases (especially the so-called orphans, see earlier text) that include small insertions not located in inter-domain loops or atypical activation motifs. It is difficult to predict the effect of such divergent features on the three-dimensional structure. However, the crystal structures made available by the Structural Genomics Consortium provide an opportunity to understand the structural/functional

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role of such divergent features [100]. There are several *Plasmodium* protein kinase structures currently available in the Protein Data Bank (PDB), produced by the Structural Genomics Consortium, for which no manuscript has yet been published (to our knowledge, only two PfPK three-dimensional structures had been reported [101,102] prior to the SGC initiative, both of which displayed unusual characteristics). These include three structures of the novel MAPK Pfmap-2 [PDB:3NIE] (see earlier) and its orthologues in *P. berghei* [PDB:3N9X] and *T. gondii* [PDB:3RP9]. We look forward to seeing these new structures described in future publications.

The kinome of another apicomplexan, *C. parvum*, was recently analysed from both evolutionary and structural perspectives, with particular focus on kinase inhibitors that may be developed to treat the disease cryptosporidiosis [99]. In particular, the authors characterized three CDPKs, CpCDPK1 [CryptoDB:cgd3_920; PDB:3DFA, 2WEI], CpCDPK2 [CryptoDB:cgd7_1840; PDB:2QG5, 3F3Z] and CpCDPK4 [CryptoDB:cgd7_40; PDB:3HKO], orthologous to *P. falciparum* PFB0815w, PFF0520w and PF07_0072, respectively. The authors found a unique insert in CpCDPK4 that remarkably includes a zinc finger between the N and C lobes of the kinase domain; this feature has not been observed in any other protein kinase solved till date, and illustrates the divergent evolutionary path followed by the kinomes of Apicomplexa, and the accompanying emergence of potential functions that are not associated with ePKs in other eukaryotes.

Because the structural coordinates were made available through PDB early on, another team was also able to analyse these structures and identify a possible MAPK-like mode of regulation in CpCDPK2 [24].

(e) *Functional roles of pseudokinases in intracellular parasites?*

Three highly conserved residues in the protein kinase domain (a lysine and two aspartates located respectively in subdomain II, the catalytic loop and adjacent to the amino-end of the activation segment) are directly involved in catalytic activity in typical ePKs, and the mutation of any of these generally results in loss of kinase activity. A protein that preserves the kinase fold but lacks residues thought to be essential for kinase catalytic activity is predicted to be a pseudokinase (although it must be kept in mind that some predicted pseudokinases have actually been shown to possess phosphotransfer catalytic activity; [103–105]). Pseudokinases can nonetheless still perform other important cellular functions, such as acting as a scaffold to provide substrates or binding partners to other kinases [104]. One putative pseudokinase in the *P. falciparum* kinome that may play such a role is PFE0170C, which we tentatively classify in the SCY1 family, homologues of the yeast gene Scy1p, a suppressor of Ras mutations [106]. In another lineage of apicomplexans, namely the coccidians such as *T. gondii*, the diversification of rhoptry kinases (ROPKs) has produced a number of apparent pseudokinases [91]. Interestingly, one of these ROPKs, ROP5, has been shown to play an essential role in the virulence of *T. gondii* strains [107,108].

5. CONCLUDING REMARKS

In our endeavour to understand the role of protein phosphorylation in the biology of malaria parasites, the surface of the underlying complexity has barely been scratched. Unlike organisms that are closely related to much better-understood systems such as yeast and mammalian cells [109], and for which cross-reacting tools and reagents can be used to address questions regarding the function of individual kinases, malaria parasites, because of their long independent evolutionary history and ensuing phylogenetic isolation, require de novo investigations. There is a need for 'reinventing the wheel', not only because tools must be generated to study the taxon-specific signalling machinery that evolved in Apicomplexa, but also because, to a large extent, sensible inferences cannot be made from knowledge gained in model systems. How could one predict the function of ePKs that do not have even remote homologues in yeast or mammalian cells? This makes research into *Plasmodium* kinomics more difficult and demanding, but all the more exciting too, as the biology that is and that will be uncovered is likely to shed light from a variant perspective on the biology of protein phosphorylation in other eukaryotes. Another silver lining to this cloud is that the peculiarities that make *Plasmodium* kinomics research difficult may well be the very features that will enable the discovery and development of new antimalarials based on protein kinase inhibition. The major message we have tried to convey in the preceding sections is that the phylogenetic distance between malaria parasites and their human host translates into important divergences in their respective kinomes, and that most *Plasmodium* kinases display atypical properties (when compared with mammalian protein kinases) that can be exploited towards selective inhibition.

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