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The ubiquitin system: an essential component to unlocking the secrets of malaria

parasite biology.

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Abstract

Exploration of the ubiquitin system in eukaryotes has shown that the chemical modification of proteins by ubiquitin, known as ubiquitylation, is an incredibly important post-translational event that is crucial to numerous cellular processes. Ubiquitylation is carried out by a series of enzymes that specifically target proteins to either change their activity or their location or earmark them for degradation. Using a wide range of genome-wide approaches, the ubiquitin system has been shown to be of particular importance in the survival and propagation of the human malaria parasites. In this review, we highlight our current understanding of the ubiquitin system in *Plasmodium,* and discuss its possible role in the development of drug resistant malaria strains.

Introduction

The ubiquitin system is one of the principle pathways used by all eukaryotic cells to regulate protein abundance levels and protein activities. It encompasses a complex network of enzymes that add and remove ubiquitin from a protein substrate. The reversible covalent attachment of ubiquitin is one of the most pervasive post-translational modifications that occur in eukaryotic cells. Similar to phosphorylation, ubiquitylation is involved in a variety of cellular processes, such as protein turnover[1], transcriptional regulation[2], cell cycle progression[3], differentiation[4], and signal transduction[5]. While the ubiquitin system is commonly associated with the proteasome degradation pathway, it is also involved in many processes that are proteasome-independent.

Ubiquitin is a conserved 76-amino-acid polypeptide that is covalently attached to one or more lysine residues on cellular proteins through a complex enzymatic cascade, which includes an E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme and an E3 ubiquitin ligase[6] (Figure 1). Ubiquitin itself contains seven lysines that can be further conjugated to the carboxyl terminus of another ubiquitin to form a polyubiquitin chain. Ubiquitylation begins with the activation of ubiquitin via an E1 ubiquitin-activating enzyme. This initial reaction results in a C-terminal thioester linkage between the E1 ubiquitin-activating enzyme and ubiquitin. Subsequently, an E2 ubiquitin-conjugating enzyme catalyzes the transfer of ubiquitin from the E1 enzyme to a conserved cysteine on the E2 enzyme by a trans-esterification reaction. The final step in the ubiquitylation process is the targeted attachment of ubiquitin to a protein via an E3 ubiquitin ligase, creating an isopeptide bond between a lysine on the target protein and the C-terminal glycine on ubiquitin[6]. E3 proteins are categorized into three major classes of enzymes, Homologous to E6-associated protein C-terminus (HECT) ligases, Really Interesting New Gene (RING) fingers and U-box E3 ligases, which are the main determinants of substrate specificity. The formation of polyubiquitin chains linked through lysine at positions 11 and 48 on ubiquitin (Lys 11 and 48) target protein substrates to the proteasome for degradation; while alternative linkages, such as Lys 6, 9, 27 and 33 have unsubstantiated roles in eukaryotic cells[7, 8]. Moreover, linkages such as Lys 29 and 63, fulfill other cellular functions, including aiding in lysosomal degradation and many proteolysis-independent mechanisms, respectively[8, 9](Figure 1). Like

phosphorylation, ubiquitylation can be reversed by the activity of deubiquitylation enzymes (DUBs). It is the cooperative interplay between these enzymes that determines the prevalence of ubiquitylated proteins and their role in the cell.

Over of the last 30 years, our understanding of the components of the ubiquitin system in eukaryotic organisms has grown significantly due to its implications in human disease. Dysregulation of the ubiquitin system has been implicated in numerous human diseases, including neurological and infectious diseases and several different types of cancer[10-14]. In particular, the ubiquitin system has been demonstrated to be a contributing factor in the etiology of Parkinson's disease[12], in addition to other neurodegenerative disorders[13]. The ubiquitin ligase (*PARK2*) that was shown to be dysfunctional in Parkinson's disease also has a role in ubiquitin-mediated autophagy of intracellular pathogens, specifically *M. tuberculosis[14]*. Exploring proteins involved in the spindle assembly checkpoint has also revealed that ubiquitylation enzymes are crucial regulators of the cell cycle[15]. As a result of unchecked cell cycle progression, many of these mutated ubiquitin-proteasome system (UPS) related proteins are the source of different types of malignancies[16, 17]. These studies and the possibility of targeting the ubiquitin system as novel therapeutic strategies emphasize the importance of understanding this cellular mechanism in the context of human pathology.

Here we consider the significance of the ubiquitin system in the survival and propagation of the human malaria parasite, *Plasmodium falciparum*. *P. falciparum* is

the most virulent form of malaria and is responsible for approximately 650,000

deaths every year. It has been projected by the World Health Organization that half

of the worlds' population is still at risk for contracting the parasite[18]. The human

malaria parasite has a complex life cycle consisting of three major stages: the

mosquito stage and the human liver and blood stages[19]. An infected mosquito that

bites a human injects parasites called sporozoites into the bloodstream. The

sporozoites quickly travel to the liver to invade liver cells. Sporozoites differentiate

and divide to produce tens of thousands of merozoites. When the merozoites exit

the liver cells and re-enter the blood stream.   Subsequently, they invade

erythrocytes, and within 48 hours a parasite can differentiate and develop to

produce up to 32 new daughter cells that egress and reinvade new red blood cells.

This asexual cycle continues until the parasite is stressed in the human host.

Parasites then undergo sexual differentiation, which can require up to two weeks

for full maturation. When a female mosquito takes a blood meal, sexually mature

male and female gametocytes are taken up and undergo sexual reproduction in the

mosquito midgut. Sporozoites then migrate to the salivary glands of the mosquito

and are injected into a human host during another blood meal, thus completing the

cycle[19]. Throughout its life cycle, the parasite undergoes cell differentiation multiple

times, requiring changes in protein activity and degradation of unnecessary stage-

specific proteins[20, 21]. The central role of ubiquitylation in all of these processes as

well as the identification of parasite-specific components of the ubiquitin system

open up new opportunities for drug discovery. With a continued need for new

preventative and curative measures to combat the parasite infection, understanding

the mechanisms that control the parasite ubiquitylation pathways at the molecular level may open new doors for the development of novel therapeutic strategies. In the face of increased drug resistance to many of the most efficacious anti-malarials[22], the discovery of specific inhibitors targeting different components of the ubiquitin system in the malaria parasite have prompted investigations into this pathway.

In this review, we highlight the current knowledge of the ubiquitin system in *Plasmodium spp.*, focusing on the power of genome-wide approaches to discover the components of the system and its substrates. Additionally, we discuss the recent implications of the ubiquitin pathway in the acquisition of drug resistance.

Identifying components of the ubiquitin system using comparative proteomic analyses

Comparative genomic and proteomic analyses are essential tools for characterizing protein functions in different species. Comparative proteomics allows investigators to search and identify proteins and their potential function based on preexisting knowledge from other species. Functional characterization of proteins can be done computationally and/or experimentally[23]. Typically, this entails relying on specific protein features, such as functional or structural domains, to determine the function of a given protein in a different species[24]. This approach has been particularly useful with studying the ubiquitin system in *Plasmodium spp.* It was not until 2007 that the importance and the functional components of the ubiquitin system were

beginning to be understood in the malaria parasite[25, 26]. While still in its infancy, our understanding of the ubiquitin system in *Plasmodium spp.* has grown significantly over the last six years, with much of our knowledge attributed to the initial comparative analyses discussed below.

A comprehensive *in silico* proteomic analysis identified most, if not all components of the ubiquitin system in *Plasmodium*. A hidden markov model (HMM) analysis was used to discover proteins containing one of the 24 Pfam domains related to the ubiquitin system[26]. These domains included those found in ubiquitin, ubiquitin-like proteins, and the enzymes involved in activation, conjugation, ligation and deconjugation of ubiquitin to its target substrates. While the study focused on *Plasmodium spp.,* three other apicomplexan species and five non-apicomplexan eukaryotic organisms were included in the analysis to provide a comparative perspective and to evaluate the completeness of the HMM search. Over 100 UPS-related proteins were found in *Plasmodium spp.,* and similar numbers were identified in the other apicomplexan parasites. The number of UPS-related proteins found in the apicomplexans was comparable to the *S. cerevisiae* proteome suggesting that most of the components of the UPS are well conserved among lower eukaryotes. In contrast, significantly more UPS-related proteins were identified in the multicellular eukaryotic proteomes used in the analysis, *C. elegans*, *A. thaliana, D. melanogaster* and *H. sapiens*.

A total of 114 UPS-related proteins were identified in the *P. falciparum* genome: 9 were identified as ubiquitin or ubiquitin-like proteins; 8 were E1 or E1-like activating enzymes; 14 were E2 or E2-like conjugating enzymes; 54 were E3 or E3-like ligases and 29 were DUB or DUB-like proteins (DUBLps)[26]. In addition, three fusion-protein precursors for ubiquitin (PF3D7_1402500, PF3D7_1365900 and PF3D7_1211800) were identified in all of the apicomplexans, and all precursor proteins displayed a high level of conservation among the eukaryotic species examined (Figure 2). Ubiquitin-like modifiers were also identified in the HMM search among most of the *Plasmodium spp.*, which included: neural precursor-cell expressed developmentally and down-regulated 8 (NEDD8), small-ubiquitin-related modifier (SUMO), homologous to ubiquitin 1 (HUB1), ubiquitin-related modifier 1 (URM1) and autophagy 8 (ATG8). These results agree with an earlier study, exploring UBL modifiers in various protozoans species[25]. Moreover, later studies in *Plasmodium* have shown sumoylation to be an important regulator in the oxidative stress response [27], and ATG8 to be a key UBL modifier in autophagy and possibly in the biogenesis of the apicoplast organelle[28, 29]. There is no current understanding of the role of NEDD8 in the *P. falciparum;* however, it likely serves an essential role in cell cycle progression. Similarly, very little is known about the roles of URM1 and HUB1 in eukaryotes (Table 1). Other UBL modifiers, such as ISG15, FAT10, UFM1 and FUB1, were missing in apicomplexan proteomes. These findings are not surprising because some of these UBL modifiers appear to be limited to multicellular eukaryotes and are involved in immunological responses[30, 31].

To examine the degree of conservation among the ubiquitin system proteins in *Plasmodium spp.*, an all-against-all blast search was conducted for each of the proteins domains between the proteomes of 13 different eukaryotes[26]. The comparison of the E1 proteins showed relatively high conservation between the species, when examining the ubiquitin-activating (UBA) domain. The UBA domain is characteristic among all E1 enzymes and is responsible for activating ubiquitin or ubiquitin-like proteins and transferring them to their cognate E2 enzymes. Among the E1 proteins, homologs of UBA1-3 were discovered in *P. falciparum,* as well as in other apicomplexans. UBA1 is the first protein in the ubiquitylation cascade that is responsible for transferring an activated ubiquitin to E2 enzymes[32], while UBA2 and UBA3 are ubiquitin-like proteins responsible for activating and transferring SUMO and NEDD8, respectively[33, 34] (Table 1). Similar to the E1 proteins, the E2 ubiquitin-conjugating enzymes showed a high degree on conservation between the species[26]. A total of 14 E2 proteins were identified in *P. falciparum,* with the other apicomplexans exhibiting a similar number of E2 proteins. While all of the apicomplexans encoded the majority of E2 homologs found in other species, two atypical E2 proteins were discovered within the *Plasmodium* and *Toxoplasma* genera that may be unique among the apicomplexan phylum.

In contrast to the E1 and E2 proteins, E3 ubiquitin and E3 ubiquitin-like ligases were found to be very diverse between the species. Approximately 50% of the UPS-related proteins found within *P. falciparum* were E3 or E3-like proteins. Members from each of the three families of E3 ubiquitin ligases, RING, HECT and U-box, were

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discovered in all apicomplexans, of which the RING E3 ligases were the most abundant and divergent in *P. falciparum*[26]. While also implicated in proteasome-independent mechanisms, RING E3 ligases are key regulators of ubiquitylation and protein turnover in eukaryotes[35]. Among the E3 ligases identified in the HMM search were homologs of the well-studied Skp1-Cullin-Fbox (SCF)-type ligase and the Anaphase-Promoting Complex (APC). Interestingly, *Plasmodium spp.* differed from the other eukaryotes in that they lacked one of the homolog subunits of the APC. In other eukaryotes, the APC plays a vital role during mitosis. *Plasmodium* undergoes a specialized type of cellular division, known as schizogony, which involves four to five rounds of asynchronous multiplication of its genome before the daughter cells separate. Therefore, a divergent composition of the APC is not entirely unexpected. The differences observed in *Plasmodium* among the E3 and E3-like ligases compared to other eukaryotic organisms suggest that these proteins may represent potential targets for anti-malarials.

The discovery of DUBs in *Plasmodium* is of particular importance because, like the E3 ubiqutin ligases, DUBs and DUBLps are less conserved between species. The first DUB to be described in *P. falciparum* was UCH-54 (PF3D7_1117100, Table 1), which was demonstrated to remove ubiquitin and NEDD8 from conjugated proteins[36]. This finding was later confirmed in a BLASTP homology search that discovered a total of 11 DUB and DUBLps, representing four of the five subclasses[25]: JAB1/MPN/Mov34 metalloenzymes, ubiquitin C-terminal hydrolases (UCHs), ubiquitin-specific proteases (USPs) and Machado-Joseph disease (MJD) proteases.

Among the most divergent DUBs are the metalloproteases. Eight novel DUB and DUBLps were later identified in the HMM search, including the fifth subclass, known as the otubain proteases[26]. Interestingly, DUB inhibitors affecting subclasses, such as UCH and USP, have been shown to be effective anti-malarials, with 50% inhibitory concentrations (IC_{50}) in the low micromolar range (Chung, Hamilton, Lee, Prudhomme and Le Roch, unpublished data).

Altogether, these initial findings have shown that *Plasmodium* contains many of the same ubiquitylation components as other eukaryotes, with some noticeable characteristics. Moreover, differences found in apicomplexan parasites highlight key changes that have occurred over the course of evolution, and support the idea that the ubiquitin system is a prime target that can be exploited for therapeutic purposes.

Novel Targets for Anti-malarials

Among the proteins of the ubiquitin system identified in *Plasmodium* were components of the classical ER-associated degradation (ERAD) pathway[26]. ERAD is a highly conserved mechanism that uses the ubiquitin system machinery to selectively target misfolded proteins in the ER, and translocate them to the cytosol where the aberrant proteins are degraded by the proteasome[37]. With the use of *in vitro* biochemical assays and cellular localization using immunofluorescence, it was confirmed that *Pf*UBA1, *Pf*UBC7 and *Pf*HRD1 (PF3D7_1225800, PF3D7_1203900 and PF3D7_1422500, respectively, Table 1) were the corresponding homologs of the

major proteins of the ERAD pathway[38]. In two recent studies, the ERAD mechanism was shown to be essential for survival of the human malaria parasite, *P. falciparum*[38, 39]. By targeting a crucial protein of the ERAD machinery, known as the signal protein peptidase (SPP), with different inhibitors, it was shown that *P. falciparum* parasites lost their ability to degrade unstable and misfolded proteins[39]. While it is suspected that most of the regulation of gene expression in *P. falciparum* occurs at the transcriptional and post-transcriptional levels[40, 41], the malaria parasite may also be particularly sensitive to perturbations in protein abundance levels. Moreover, this suspicion is supported by reported IC_{50} values that shown inhibitors of the ERAD mechanism are effective antimalarials[38, 39]. Despite the relatively high conservation among the components in the ERAD pathway in eukaryotes, these proteins remain potential candidates for drug intervention due to their essentiality in parasite survival.

Similarly, proteins encompassing a parasite-specific ERAD-like system were also discovered in the HMM search in apicomplexan parasites[26]. The ERAD-like pathway contains all of the ubiquitylation enzymes needed for activation (PF3D7_1333200 and PF3D7_1365400), conjugation (PF3D7_1345500), ligation (PF3D7_0316900 and PF3D7_0312100) and deconjugation (PF3D7_1031400)[42-44]. However, in addition to containing the requisite ubiquitylation domains, ERAD-like proteins contain a signal peptide that is responsible for targeting the proteins to a specialized organelle, known as the apicoplast. The apicoplast is non-photosynthetic plastid that is the result of a secondary endosymbiosis event involving a red alga that created a four membrane-bound plastid. It is an essential organelle that is

responsible for fatty acid metabolism, and isoprenoid biosynthesis[45-47]. While the apicoplast contains a ~35kb-long circular genome encoding ~30 proteins[48], most of its function is fulfilled by ~500 nuclear-encoded proteins. The trafficking pathways involved in the import of these proteins through the four apicoplast membranes remains unclear. Apicoplast targeting involves the conventional secretory pathway through the endoplasmic reticulum (ER), and a bipartite leader peptide located at the N-terminus of transported proteins[49-51]. The bipartite domain consists of a signal peptide (cleaved off after passage into the ER) followed by a transit peptide that avoids secretion of the protein and is sufficient for apicoplast stromal targeting. The discovery of this ERAD-like system in Apicomplexa is similar to the symbiont-specific ERAD-like machinery (SELMA) found in Chromalveolates, a supergroup of plastid-containing unicellular eukaryotes related to red algae[44, 52]. The SELMA is implicated in pre-protein import to the periplastidal compartment (PPC)[53]. Similar to the SELMA system, the ERAD-like system found in *Plasmodium spp.* and *Toxoplasma gondii* has been showed to be critical for the import of nuclear-encoded proteins into the apicoplast[42]. While the ERAD-like system in apicomplexans is capable of ubiquitylating proteins *in vitro*[42], the role of ubiquitylation *in vivo* remains unknown. Despite uncertainties, the functional characterization of parasite-specific ubiquitylation components offers new avenues for antimalarial strategies.

Exploring the *Plasmodium* ubiquitome

Following the identification of the components of the ubiquitin system in *Plasmodium*, the next logical step in understanding the ubiquitylation process is identifying the proteins that are ubiquitylated. The addition and removal of ubiquitin and the degradation of ubiquitylated proteins can occur rapidly making identifying the ubiquitome, defined as all proteins that are subject to ubiquitylation, a challenging task. However, several methodologies have been adapted for ubiquitome identification. An *in silico* prediction model known as *UbPred* was developed based on experimental evidence in mutant yeast strains to identify similar sequence and structural biases that were common among ubiquitylation sites[54]. The *UbPred* model is based on identifying disordered regions within a substrate to predict ubiquitylation sites. While the evidence for this model shows it to be a better global predictor of ubiquitylated substrates, it does not take into account known degradation signals, such as the destruction box (D box) and the KEN-box, and it has been previously suggested to be only a weak signal for proteolysis[55]. An alternative prediction algorithm, known as *UbiPred*, that relies on physicochemical properties of the protein sequences has also been developed to predict ubiquitylation sites[56]. However, *UbiPred* did not perform as well as *UbPred* in predicting ubiquitylated substrates[54]. Moreover, there have been several studies that have identified ubiquitylated proteins experimentally at the proteome-wide level[57-64]. Many of these studies utilized techniques relying on isolating and identifying ubiquitylated proteins through immunoprecipitation followed by mass spectrometry.

To our knowledge, there has only been a single study exploring the ubiquitome in *Plasmodium spp.* In a recent study by Ponts *et al.*, a combinatorial approach was used to identify all ubiquitylated substrates[65]. First, both *UbPred* and *UbiPred* were used in an *in silico* approach to determine the ubiquitome in *P. falciparum.* From the 5,446 protein-coding sequences, *UbPred* identified 5,036 proteins predicted to contain ubiquitylation sites. In contrast, *UbiPred* predicted less than half the number of proteins (2,077 proteins) to contain ubiquitylation sites. The authors concluded that the discrepancy observed between the different prediction models was likely attributed to the differences in the ways the models were trained. While ~70% of the *P. falciparum* proteome was predicted to contain ubiquitylation sites, only 52% and 39% of the *S. cerevisiae* and *A. thaliana* proteomes, respectively, were shown to contain ubiquitylation sites[65]. Collectively, this initial study suggests that *P. falciparum* contains more proteins that are subject to ubiquitylation relative to these eukaryotic species, *A. thaliana* and *S. cerevisiae*; however, additional studies are needed to confirm this finding. One possible explanation for this observation could be the amino acid composition difference between the different species; lysine is the second most abundant amino acid in *P. falciparum,* and *P. falciparum* has about twice the amount of lysine relative to the other two eukaryotic species[65].

This *in silico* study was supplemented by a proteome-wide experimental approach that coupled immunoprecipitation to multidimensional protein identification technology (MudPIT). MudPIT is a "shotgun" approach that couples 2-D liquid chromatography to mass spectrometry to identify peptides contained with a

complex protein mixture[66]. Using an anti-ubiquitin antibody, a total of 437 ubiquitylated proteins were detected from the three asexual stages in *P. falciparum* erythrocytic cycle[65]. While only 73 of these *Plasmodium* proteins were detected with significant enrichment, similar percentages in ubiquitin conjugates were observed in human and *A. thaliana* cells using comparable techniques[58, 67]. The number of ubiquitylated proteins was highest in the schizont stage with 33 of the proteins being unique to this morphological stage. Among the ubiquitylated proteins discovered was a well-established transcription factor, known as ApiAP2 (PF3D7_0604100, Table 1). This finding strongly suggests that ubiquitylation of transcription machinery may play an important role in the regulation of gene expression in *P. falciparum*. Chaperone proteins, proteins involved in RNA metabolism and translation had the highest representation among the various detected ubiquitylated proteins. This latest finding also suggests a role for ubiquitylation in the regulation of gene expression at the translational level. Also characterized within this study were the different lysine linkages between ubiquitin and its protein substrates. Ubiquitin contains seven lysine residues at different positions that all can interact to form polyubiquitin chains. Linkages using Lys-6, Lys-9, Lys-11, Lys-27, Lys-33 and Lys-29 were not identified in the *in silico* analysis; however, Lys-6 and Lys11 were detected experimentally. Lys-48 and Lys-63 linkages were detected using both approaches[65]. These findings demonstrate that these four lysine linkages, Lys-6, Lys-11, Lys-48 and Lys-63, are likely the most abundant in *P. falciparum* (Figure 2).

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The large discrepancy observed between predicted and experimentally validated ubiquitylated proteins is likely the result of challenges in isolating ubiquitylated substrates. Additional experimental studies will need to be developed to provide a more accurate depiction of the number of ubiquitylated substrates in *P. falciparum.* Collectively, these studies suggest that a large proportion of the *Plasmodium* proteome is ubiquitylated and that ubiquitylated proteins are involved in many different cellular processes and are critical contributors of the parasite life cycle progression.

Drug resistance

One of the most exciting findings from recent genome-wide studies in *Plasmodium* is the recent implication of the ubiquitin system in mechanisms of drug resistance. Drugs like quinine and chloroquine were once the first choice in the treatment of malaria, but since the emergence of resistant strains, novel anti-malarials are continually in demand. Much of the current knowledge surrounding the development of resistant *Plasmodium* strains to chloroquine, mefloquine, pyrimethamine and artemether-lumefantrine is centered on a small number of genes that mostly encode transporter proteins, which include ATPase6, DHFR, CRT and MDR1[68-71].

The identification of novel genetic mutations involved in drug resistance employed the use of different mapping techniques, such as linkage analysis and genome-wide association studies (GWAS). Linkage analysis entails crossing organisms with each

other, and using genetic markers to calculate recombination rates to pinpoint the location of a causal mutation. Alternatively, GWAS relies on the use of custom genotyping arrays, typically using thousands of SNPs or next generation sequencing technology to sequence hundred of strains, to calculate allele frequencies between resistant and sensitive strains to identify a genetic location. Using these genome-wide methodologies, genes comprising components of ubiquitin system have been implicated in the acquisition of resistance to some of the current anti-malarial drugs, specifically pyrimethamine and artemisinin[72-76].

One of the first accounts of the role of the ubiquitin system in drug resistance was the discovery of a high prevalence of mutations in resistant strains in genes encoding components of the ubiquitin system[77]. In a more recent study, two novel mutations were identified in both an E2 ubiquitin-conjugating enzyme and an HECT E3 ubiquitin ligase[73] (Table 1). Using a sequence-based GWAS, 45 Senegalese *P. falciparum* parasites were sequenced, and the discovered SNPs were interrogated for association with resistance to 12 different anti-malarials. Using both a test for natural selection, known as cross-population extended haplotype homozygosity (XP-EHH), and a mixed model association test, known as EMMA, several of the previous loci conferring resistance were identified, including CRT, MDR1 and DHRF. However, one of the most interesting findings was the identification of an E2 enzyme, encoded by PF3D7_1243700 and an E3 ligase, encoded by PF3D7_0826100, both possibly involved in a mechanism of drug resistance to pyrimethamine.

Currently, artemisinin is the most effective anti-malarial, and over the last few years there has been an aggressive effort to identify the genes(s) responsible for the burgeoning of artemisinin-resistant strains. Using a genome-wide linkage group selection (LGS) analysis, missense mutations were discovered on chromosome 2 in a gene encoding a deubiquitylase (UBP-1) in the *P. Chabaudi* artemisinin-resistant strain AS-ATN. It was proposed that at least one of these mutations, specifically the V2728F mutation, was conferring artemisinin resistance[75, 76]. However, the mutation in UPB-1 has not been confirmed in *P. falciparum* artemisinin-resistant strains[78], and additional data will be needed to confirm its role in mechanisms of drug resistance.

While the role of UBP-1 in drug resistance has not yet been validated in *P. falciparum*, other genes involved in the ubiquitin system have been identified as possibly involved in artemisinin resistance. An E3 ubiquitin-ligase, known as RAD5 (Table 1), has been implicated in two independent genome-wide association studies (GWAS)[72, 74]. Both studies showed a significant association with resistance to artemisinin in a similar location on chromosome 13. No causal mutations were confirmed in the genes interrogated in the region in either study; however, artemisinin resistance may be multifactorial and identifying perfectly correlative SNPs could be arduous. Moreover, another gene discovered in these GWAS was DNA polymerase delta (Table 1). Both of these enzymes, DNA polymerase delta and RAD5 are part of a pathway known as postreplication repair. Interestingly, UBP1 is

also part of the postreplication repair pathway, and it is conceivable that genes involved in this particular pathway could drive artemisinin resistance.

Conclusion

Plasmodium spp. are incredibly pervasive and some of them are lethal human pathogens. Understanding the cellular components critical to parasite development is essential in the search for innovative therapeutic strategies. Similar to other eukaryotes, the ubiquitin system in *Plasmodium* is essential to parasite survival. The system is critical in numerous cellular processes, as such degradation of misfolded proteins, oxidative stress response, trafficking of proteins to the apicoplast, as well as mechanisms of drug resistance. Exploration of the ubiquitin system using genome-wide approaches has greatly advanced our understanding of this pathway in apicomplexan parasites in general and in particular *Plasmodium*. Moreover, these large-scale studies have demonstrated that ubiquitylation is a common post-translational modification in *P. falciparum*. Furthermore, the recent implications of the ubiquitin system in mechanisms of drug resistance highlight the importance of this particular pathway in parasite biology. Malaria research would greatly benefit from studies exploring the ubiquitin system that focused on components of the ubiquitin system that are parasite-specific. Successful identification of these targets will likely yield new and effective therapies to combat malaria infections. Additional in depth studies exploring the role of the ubiquitin system in the acquisition of drug resistance is another area within malaria research that strongly warrants further investigation. In the near future, we suspect more

researchers will explore ubiquitylation events in *Plasmodium*, as we are just now

scratching the surface of this overlooked pathway.

Figure legends:

Figure 1: Depiction of the ubiquitin-mediated pathways in *P. falciparum*.

Ubiquitin is initially activated by an E1 ubiquitin-activating enzyme and then

transferred to an E2 ubiquitin-conjugating enzyme. The E2 enzyme may either

complex with an E3 ubiquitin-ligase to directly ubiquitylate a target substrate or

transfer the ubiquitin to an E3 enzyme, which will subsequently ubiquitylate a

target substrate. Substrates polyubiquitinated at lysine K48 are targeted to the

proteasome for destruction. Ubiquitylation of substrates at other lysine residues

can modulate substrate activity in many cellular processes. Deubiquitylating

enzymes (DUBs) remove ubiquitin by cleaving the peptide bonds and replenish the

ubiquitin pool.

Figure 2: A comparative alignment of the ubiquitin protein sequences in different eukaryotes.

The ubiquitin protein sequences show a high level of conservation among different

organisms. Lysine residues that are known sites for ubiquitylation and extension

are highlighted. † In *P. falciparum* K6, K11, K48, and K63 have been detected as

sites for ubiquitylation and extension.

Table 1: Validated proteins of the ubiquitin system in *Plasmodium*

All discussed proteins of the ubiquitin system are listed in the table above along

with reference numbers and what is known about their respective functional role(s)

in *Plasmodium*.

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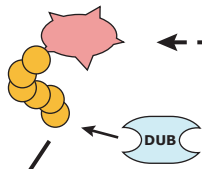
Molecular BioSystems
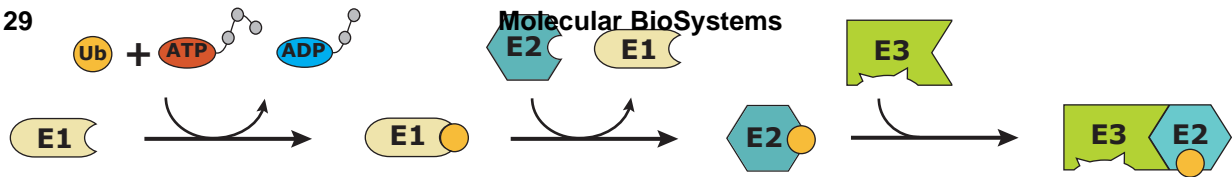
Ubiquitin-activating enzyme (E1)
*8 E1 or E1-like proteins identified in
P. falciparum

Ubiquitin-conjugating enzyme (E2)
*14 E2 or E2-like proteins identified in
P. falciparum

Ubiquitin Ligase (E3)
*54 E3 or E3-like proteins identified in
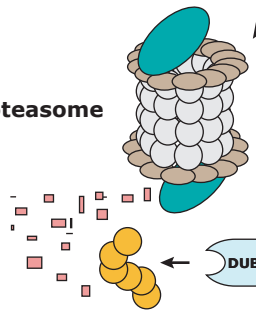*P. falciparum*
Types Include: Ring, HECT, UBox

Substrate
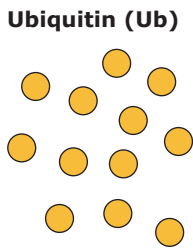
Ubiquitin (Ub)

Polyubiquitinated
*On K48 residues

Proteasome Dependent Pathway

Proteasome Independent Pathway

26S Proteasome

Deubiquitinating Enzyme (DUB)
*29 DUBs or DUB-like proteins
identified in *P. falciparum*

Mono-, Multi-, Polyubiquitylated
substrates

Protein Regulation

Cell Cycle

DNA Repair/
Transcription

Chromatin Remodeling

Cellular Stress Response

Signal Transduction

Differentiation/
Development

Trafficking/
Endocytosis

```
                                           †         †                                   †    †   †                         †                      †
                                           K6        K11                                K27  K29 K33                        K48                    K63
                                           |         |                                   |    |   |                         |                      |
       Plasmodium falciparum  MQIFVKTLTGKTITLDVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLRGG 76
              Homo sapiens    MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLRGG 76
           Toxoplasma gondii  MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLRGG 76
    Saccharomyces cerevisiae  MQIFVKTLTGKTITLEVESSDTIDNVKSKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLRGG 76
                  Consensus   ****************:**.****:***:********************************************
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	Plasmodium Gene ID	Enzyme/Protein Classification	Function/Putative Function	References
Ub/Ub-like				
Ubiquitin	PF3D7_1402500	Ub	Ubiquitylation of substrates	26, 38, 42, 44, 65
	PF3D7_1365900	Ub	Ubiquitylation of substrates	26, 38, 42, 44, 66
	PF3D7_1211800	Ub	Ubiquitylation of substrates	26, 38, 42, 44, 67
ATG8	Pf3D7_1019900	Ub-like protein	Autophagy	28, 29
NEDD8	Pf3D7_1313000	Ub-like protein	Unknown	-
SUMO	Pf3D7_0505800	Ub-like protein	Oxidative Stress Response	27
E1/E1-like				
UBA1	Pf3D7_1225800	E1	Activation of ubiquitin	38
UBA2	Pf3D7_1237000	E1-like protein	Activation of SUMO	27
UBA3	Pf3D7_0817000	E1-like protein	Activation of NEDD8	-
Unnamed	Pf3D7_1333200	E1-like protein	Part of the ERAD-like system	42, 44
Unnamed	Pf3D7_1365400	E1-like protein	Part of the ERAD-like system	42
E2/E2-like				
UBC7	Pf3D7_1203900	E2 Conjugating Enzyme	ERAD	38
Unnamed	Pf3D7_1243700	E2 Conjugating Enzyme	Implicated in drug resistance	73
Unnamed	Pf3D7_1345500	E2-like protein	Part of the ERAD-like system	42
E3/E3-like				
HRD1	Pf3D7_1422500	E3 Ligase	ERAD	38
RAD5	Pf3D7_1343400	E3 Ligase	Implicated in drug resistance	72, 74
Unnamed	Pf3D7_0826100	E3 Ligase	Implicated in drug resistance	73
Unnamed	Pf3D7_0316900	E3-like protein	Part of the ERAD-like system	42
Unnamed	Pf3D7_0312100	E3-like protein	Part of the ERAD-like system	42
DUB				
UCH54	Pf3D7_1117100	DUB	DUB with deNeddylating activity	36
UBP1	Pf3D7_0104300	DUB	Implicated in drug resistance	75, 76
OTU-like cysteine protease	Pf3D7_1031400	DUB	Targeted to the apicoplast	Unpublished