Unraveling the Human Malaria Parasite’s Ubiquitome

Ponts1,3 Nadia, Saraf Anita2,3, Chung1 Duk-Won D., Harris1 Alona, Prudhomme1 Jacques, Washburn2 Michael P., Florens2 Laurence, Le Roch1,* Karine G.

1Department of Cell Biology and Neuroscience, University of California, 900 University Avenue, Riverside, CA 92521, USA
2Stowers Institute for Medical Research, Kansas City, MO 64110, USA
3These authors contributed equally to this work.

*Corresponding author. E-mail: karine.leroch@ucr.edu. Tel: +1 (951)-827-5422. Fax: +1 (951)-827-3087

Running Title: Ubiquitylation in Plasmodium falciparum

Abstract
Malaria is one of the deadliest infectious diseases worldwide. The most severe form is caused by the eukaryotic protozoan parasite Plasmodium falciparum. Recent studies highlighted the importance of post-translational regulations for the parasite’s progression throughout its life cycle, protein ubiquitylation being certainly one of the most abundant. The specificity of its components and the wide range of biological processes in which it is involved make the ubiquitylation pathway a promising source of suitable targets for anti-malarial drug development. Here, we combined immunofluorescent microscopy, biochemical assays, in silico prediction and mass spectrometry analysis using the Multidimensional Protein Identification Technology, or MudPIT, to describe the P. falciparum ubiquitome. We found that ubiquitin conjugates are detected at every morphological stage of the parasite erythrocytic cycle. Furthermore, we detected that more than half of the parasite’s proteome represents possible targets for ubiquitylation, especially proteins found to be present at the most replicative stage of the asexual cycle; the trophozoite stage. A large proportion of ubiquitin conjugates were also detected at the schizont stage, consistent with a cell activity slow down to prepare for merozoite differentiation and invasion. Finally, for the first time in the human malaria parasite, our results strongly indicate the presence of heterologous mixed conjugations SUMO/UB. This discovery suggests that sumoylated proteins may be regulated by ubiquitylation in P. falciparum. All together, our results present the first stepstone towards a better understanding of ubiquitylation and its role(s) in the biology of the human malaria parasite.

Introduction
Malaria is one of the most lethal infectious diseases. Half of the world’s population is at risk leading to about 250 million malaria cases and one million deaths every year (source: WHO at http://www.who.int/malaria/en/). The most severe forms of malaria, leading to death, are caused by the protozoan parasite Plasmodium falciparum. Despite significant advances in the research against malaria, many aspects of the parasite’s biology remain obscure. Recent studies highlighted the importance of post-translational regulations for the parasite’s progression throughout its life cycle (reviewed in (1)). Among those, protein ubiquitylation is certainly one of the most abundant post-translational modifications of proteins. Ubiquitylation is classically involved in numerous crucial biological processes in eukaryotic cells in a proteasome-dependent or independent manner. The specificity of its components and the wide range of biological processes in which it is involved make the ubiquitylation pathway an important source of suitable targets for drug treatments (see (2) for a review). Proteasome inhibitors are indeed promising drugs to treat cancer and both autoimmune and infectious diseases (see (3) for a review). In the particular case of Plasmodium, the proteasome inhibitors salinosporamide-A (4), bortezomib (5), and MLN-273 (6) were shown to
Ubiquitylation in *Plasmodium falciparum*

be efficient antimalarials. In addition to their cellular roles, the components of the ubiquitin system are involved in many host-pathogen interactions. Pathogens often utilize the host ubiquitylation system to bypass the infected host immune system (see (7, 8) for reviews). Ubiquitylation consists in the attachment of one (mono-ubiquitylation) or more (poly-ubiquitylation i.e., the formation of a poly-ubiquitin chain) ubiquitin moieties to a target protein via the formation of an iso-peptide bond between the C-terminal di-glycine motif of ubiquitin and a lysine in the target protein. The entire process requires the sequential intervention of three families of enzymes, namely E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin ligases involved in specific substrate recognition (see (1, 9, 10) for reviews). In *P. falciparum*, previous in silico studies identified four predicted sources of ubiquitin moieties. The polyubiquitin gene PFL0585w contains five conserved ubiquitin repeats (9, 10). The two ubiquitin-fusion proteins PfUB527a and PfUB140 (PF14_0027 and PF13_0346, respectively) contain an ubiquitin moiety at their N-terminal side (10). More recently, one conserved ubiquitin domain was identified in PF08_0067 and is believed to be part of an Endoplasmic Reticulum-Associated protein Degradation (ERAD)-like pathway (11). In addition to these four genes encoding ubiquitins, more than 100 proteins involved in the ubiquitylation system were identified in silico (10), i.e., about 2% of *P. falciparum*’s total protein-coding genes. By contrast, only a few substrates for ubiquitylation have been identified in *P. falciparum*, including actin (12) and the histone protein H2B (13). The quickly reversible character of ubiquitylation (intervention of deubiquitinases) and the rapid degradation of poly-ubiquitylated proteins (proteasome) render the isolation and analysis of ubiquitylated proteins challenging. Various methods can be used to identify ubiquitylated proteins both in silico (14, 15) and experimentally (16-20), including immunoprecipitation of ubiquitylated proteins coupled to mass spectrometry analysis. In human, specific precipitation of ubiquitin-conjugated proteins identified 345 substrates for ubiquitylation (21). Here, we explore *P. falciparum*’s ubiquitome (i.e., all the proteins that are targets for ubiquitylation) using a combination of immunofluorescent localization of ubiquitin conjugates, detection of ubiquitylating activities of various parasite extracts, in silico prediction of ubiquitin targets genome-wide using the previously published algorithms *UbPred* (14) and *UbiPred* (15), and immunoprecipitation of ubiquitin conjugates followed by immunoprecipitates analysis using Multidimensional Protein Identification Technology (MudPIT). The application of these techniques to the analysis of *P. falciparum*’s ubiquitome during its asexual cell cycle permitted the discovery of unknown features. We found that more than half of the parasite’s proteome represents possible targets for ubiquitylation. Nearly 100 of these targets were confirmed by MudPIT analysis. The dataset contains a variety of parasite-specific proteins that are crucial for the parasite’s metabolism and survival, such as the haemoglobinase plasmepsin II. The schizont stage was found to contain the largest number of ubiquitylated proteins, whereas the ring stage contained the least. This observation is consistent with the parasite’s reduction in size and activity before maturation into invasive merozoites. Finally, we found evidences of heterologous mixed modifications SUMO/UB of the same substrate. Such modifications are believed to target sumoylated proteins to the proteasome for degradation. The biological implications of these findings are discussed.

**EXPERIMENTAL PROCEDURES**

**Parasite strain and culture conditions**
Sorbitol-synchronized *P. falciparum* parasite strain 3D7 was cultured in human erythrocytes according to previously described protocols (22-24). For each experiment, cultures were harvested 48 hours after the first sorbitol treatment (ring stage), and after 18 and 36 hours (respectively trophozoite and schizont morphological stages monitored by Giemsa staining).

**Ubiquitylation assays**
Parasite protein extracts were freshly prepared before each ubiquitylation assay. 2-5x10⁹ parasites
were extracted by 0.15% saponin lysis of red blood cells (15 min of incubation on ice) followed by three washes in ice-cold PBS supplemented with 2 mM PMSF. After 15 min of incubation on ice, the parasite pellet was resuspended in one milliliter of ice-cold cytoplasmic lysis buffer (20 mM HEPES pH 7.9, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.65% Igepal®, 0.5 mM PMSF, Roche Complete Mini EDTA-free Cocktail Protease Inhibitor) and left on ice for five more minutes. After 10 min of centrifugation at 4°C and 1500g the supernatant was collected (cytoplasmic protein extract) and the pelleted nuclei were lysed in 100 µL of nuclei lysis buffer (20 mM HEPES pH 7.9, 0.1 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 1.5 mM MgCl2, 1 mM DTT, 25% glycerol, 1 mM PMSF, Roche Complete Mini EDTA-free Cocktail Protease Inhibitor) for 20 min at 4°C under vigorous shaking. The nuclear protein extract was then cleared by centrifugation at 4°C and 6000g for 10 min and combined to the cytoplasmic extract. Protein concentrations were measured by Bradford assay (25).

Fresh (i.e., prepared the same day and never frozen) protein extracts were used to perform ubiquitylation assays according to a modified previously published protocol (26). 16.5 µL of protein extract were mixed on ice with 18.5 µL of ubiquitylation reaction mix (20 mM HEPES pH 7.9, 1.5 mM DTT, 1X Energy Regeneration Solution (BostonBiochem®), five micrograms of biotinylated ubiquitin (BostonBiochem ®), and 20 µg of native ubiquitin (BostonBiochem®), and incubated at 30°C under gentle agitation. After two hours of incubation, the reaction was mixed with Laemmli buffer (27), run on a 10% SDS-PAGE, and transferred onto a PVDF membrane. After two hours of incubation with streptavidin proteins coupled to horseradish peroxidase (Jackson ImmunoResearch Laboratories, Inc.), the presence of incorporated biotin-ubiquitin was revealed by electroluminescence (Pierce® ECL Western blotting substrate).

In silico identification of putative targets for ubiquitylation

The complete set of 5446 translated coding sequences for *P. falciparum* was downloaded from PlasmoDB v6.5 (www.plasmodb.org) and analyzed for the presence of putative ubiquitylation sites using *UbPred* (14) and *UbiPred* (15). Predictions with *UbPred* were classified by level of confidence, from low (score ranging from 0.62 to 0.69) to medium (score ranging from 0.70 to 0.84) to high (score equal to or higher than 0.85), according to the software instructions. With regard to the predictions made with *UbiPred*, proteins were considered potentially ubiquitylated with high confidence when prediction scores were equal to at least 0.85 (15).

Immunofluorescent localization of ubiquitylated proteins

Immunofluorescence detection of ubiquitylated proteins was performed according to a modified version of a previously described protocol (28). Infected red blood cells were washed once with PBS and an aliquot was resuspended in 300 to 400 volumes of PBS containing 1% BSA (w/v). 30 µL aliquots of cell suspension were spotted on the surface of a depression slide and completely air-dried. The cells were then fixed for 10 min at room temperature with 4% paraformaldehyde in PBS. After rinsing the slide once with PBS, the cells were blocked with 1% BSA in PBS (w/v) for 30 min at room temperature in a humidified chamber. The liquid was then removed and the slide was incubated with a 1:250 dilution of anti-conjugated ubiquitin antibody (clone FK2, Enzo® Life Sciences) in solution with 1% BSA/PBS (w/v) for two hours at room temperature in a humidified chamber. After liquid removal and three washes with PBS for five minutes, the slide was incubated with 1:250 of secondary antibody (Texas Red®-X goat anti-mouse IgG, Invitrogen™) in solution with 1% BSA/PBS (w/v) for one hour at room temperature in a humidified chamber. After liquid removal and three washes with PBS for five minutes, nuclei were stained with DAPI 100 ng/mL for five minutes. The slide was then carefully washed three times for five minutes with PBS. The slide was finally mounted with slow-fade mounting medium (Fluormount-G, Southern Biotech) and images were viewed with a fluorescence microscope within 72 hours of preparing the slide. Images were prepared and mounted using the software ImageJ (http://rsbweb.nih.gov/ij/).

*In silico identification of putative targets for ubiquitylation*

The complete set of 5446 translated coding sequences for *P. falciparum* was downloaded from PlasmoDB v6.5 (www.plasmodb.org) and analyzed for the presence of putative ubiquitylation sites using *UbPred* (14) and *UbiPred* (15). Predictions with *UbPred* were classified by level of confidence, from low (score ranging from 0.62 to 0.69) to medium (score ranging from 0.70 to 0.84) to high (score equal to or higher than 0.85), according to the software instructions. With regard to the predictions made with *UbiPred*, proteins were considered potentially ubiquitylated with high confidence when prediction scores were equal to at least 0.85 (15).
Protein ubiquitylation leads to the rapid degradation of the target protein by the proteasome, which is a major obstacle to the isolation and analysis of ubiquitylated proteins. To prevent the degradation of ubiquitylated proteins in the sample, parasite cultures were treated with 400 nM of the proteasome inhibitor MG132 (EMD Chemicals Inc.) six hours before harvest. In addition, ubiquitin moieties are rapidly removed in vivo by deubiquitylating enzymes (DUB). To ensure maximum detection of targets for ubiquitylation, all reagents were supplemented with 20 mM of N-ethylmaleimide (NEM), 2 mM PMSF, and complete mini EDTA-free protease inhibitor cocktail (Roche Diagnostics). Cultures were harvested by five minutes of centrifugation at 800g and 4°C, followed by three washes in PBS supplemented with NEM, PMSF and anti-protease cocktail. Parasites were extracted with 0.15% w/v saponin (in PBS) for 15 min on ice. Cells were pelleted by centrifugation for 10 min at 3200g and 4°C, and washed with PBS (supplemented with NEM, PMSF and anti-protease cocktail) until the supernatant came out clear. Parasite pellets were then flash frozen and stored at -80°C until immunoprecipitation.

Parasites were thawed in immunoprecipitation buffer (0.5% Triton X-100, 50 mM Tris-HCl pH 7.5, 300 mM NaCl, 20 mM NEM, 0.5 mM EDTA, 2 mM PMSF, Roche complete mini EDTA-free protease inhibitor cocktail) and lysed by six strokes of sonication (10s each) on a Fisher Dismembrator Model 100 set to power 2 1/2. Lysates were cleared by 15 min of centrifugation at 13000 g and 4°C, and incubated with washed agarose A beads (Invitrogen™) for one hour at 4°C under constant agitation. After 10 min of centrifugation at 13000g and 4°C, the supernatant was incubated with anti-conjugated ubiquitin mouse IgG (clone FK2 that does not react with free ubiquitin, Enzo® Life Sciences) overnight at 4°C. In parallel, negative control samples were left without antibody (beads only). The conjugates and the negative control samples were then incubated with washed agarose A beads for two hours at 4°C. The beads were collected by 30s of centrifugation at 4000g, washed three times in immunoprecipitation buffer, and eluted with Laemmli buffer. Immunoprecipitates were analyzed by 12% SDS-PAGE, transfer on PVDF membrane, incubation with a rabbit anti-ubiquitin IgG (Millipore) followed by immunodetection with a goat anti-rabbit IgG coupled to horseradish peroxidase (Millipore™) and revelation by electroluminescence (Pierce® ECL Western blotting substrate). Alternatively, immunoprecipitated proteins and negative controls were eluted three times from the beads with 50 µL of elution buffer (50 mM Tris-HCl pH 6.8, 100 mM DTT, 2% SDS) before MudPIT analysis.

**Immunoprecipitation of plasmepsin II, plasmepsin I, and plasmepsin IV, and anti-ubiquitin western blot**

Asynchronous cultures of parasites were treated with 400 nM of MG132 (EMD Chemicals Inc.) six hours before harvest. Cultures were harvested by five minutes of centrifugation at 800g and 4°C, followed by three washes in PBS supplemented with NEM 20 mM, PMSF 2 mM and complete mini EDTA-free protease inhibitor cocktail (Roche Diagnostics). Parasites were extracted with 0.15% w/v saponin for 15 min on ice. Cells were pelleted by centrifugation for 10 min at 3200g and 4°C, and washed with PBS supplemented with NEM, PMSF and anti-protease cocktail until the supernatant came out clear. Parasite pellets were then flash frozen and stored at -80°C until immunoprecipitation. Parasites were thawed in immunoprecipitation buffer and lysed by six strokes of sonication (10s each) on a Fisher Dismembrator Model 100 set to power 2 1/2. Lysates were cleared by 15 min of centrifugation at 13000 g and 4°C, and incubated with washed PureProteome™ Magnetic protein A beads (Millipore™) for two hours at 4°C under constant agitation. Samples were then settles on a magnetic rack and the supernatant was transferred to a fresh tube. The desired antibody was added to the tube and incubated overnight at 4°C under constant agitation. The conjugates were then incubated overnight with washed PureProteome™ Magnetic protein A beads at 4°C and constant agitation. Samples were then settles on a magnetic rack and the supernatant was removed. The beads were then washed three times in immunoprecipitation buffer, and eluted with Laemmli buffer. The immunoprecipitates were resolved by SDS-PAGE and transferred on PVDF membrane. Ubiquitylation was detetected using a rabbit anti-ubiquitin IgG (Millipore) followed by immunodetection with a goat anti-rabbit IgG.
Ubiquitylation in *Plasmodium falciparum*

coupled to horseradish peroxidase (Millipore™) and revelation by electroluminescence (ECL reagent, Pierce). Antibodies anti-plasmepsin I, II, IV were obtained from the MR4, deposited by D.E. Goldberg (30–32) (accession numbers MRA-66, MRA-813A, and MRA-814A respectively).

**MudPIT analysis of immunoprecipitated proteins**

Immunoprecipitated proteins were TCA-precipitated and the pellets were solubilized in 100 mM TRIS-HCl pH 8.5 and 8 M Urea; TCEP (Tris(2-Carboxylethyl)-Phosphine Hydrochloride, Pierce) and CAM (Chloroacetamide, Sigma) were added to a final concentration of 5 mM and 10 mM, respectively. Protein suspensions were digested overnight at 37°C using Endoproteinase Lys-C at 1:50 w/w (Roche). Samples were brought to a final concentration of 2 M urea and 2 mM CaCl₂ before performing a second overnight digestion at 37°C using Trypsin (Promega) at 1:100 w/w. Formic acid (5% final) was added to stop the reactions. Samples were loaded on split-triple-phase fused-silica micro-capillary columns (33) and placed in-line with an Eksigent NanoLC 2D system. Full MS spectra were recorded on the peptides over a 400 to 1,700 m/z range in the Orbitrap at 60K resolution, followed by fragmentation in the ion trap (at 35% collision energy) on the first to fifth most intense ions selected from the full MS spectrum with dynamic exclusion enabled for 90s (34). A total of 3, 3, 4 and 7 technical replicates were acquired for the ring, trophozoite, schizont, and negative controls samples, respectively.

**MudPIT data analysis**

RAW files were extracted into ms2 file format (35) using RAW_Xtract v.1.0 (36). MS/MS spectra were searched using SEQUEST v.27 (rev.9) (37) with a peptide mass tolerance of 50 ppm, and searched against a protein database combining non-redundant 5439 Plasmodium falciparum (PlasmoDB release 5.5) and 30552 human proteins (NCBI 2008-03-04 release), as well as 162 usual contaminants such as human keratins, IgGs, and proteolytic enzymes. To estimate false discovery rates (FDR), each protein sequence was randomized (keeping the same amino acid composition and length) and the resulting "shuffled" sequences were added to the database used for the SEQUEST searches, for a total search space of 72306 amino acid sequences. To account for alkylation by CAM, +57 Da were added statically to cysteine residues for all searches. Peptide/spectrum matches were sorted, selected and compared using DTASelect/CONTRAST (38). Peptides had to be full tryptic and at least 7 amino acid long, with a DeltCn cut-off of 0.08, and XCorr minima of 1.8, 2.0, and 3.0 for singly-, doubly-, and triply-charged spectra. Proteins had to be detected by two such peptides or 1 peptide with 2 spectra in each run. NSAF v7, an in-house developed software, was used to create the final report on all non-redundant proteins detected across the different MudPIT runs, and calculate FDRs, which were on average 0.4% ± 0.3 and 0.7% ± 0.4 at the spectral and protein levels, respectively. Protein abundance was evaluated according to their normalized spectral abundance factors (dNSAF) calculated as the spectral count relative to the length of a given protein normalized by the sum of the relative spectral counts in the sample (34). We used the open source software package "plgem" (written in R and maintained by the BioConductor project) to establish some statistical significance on this dataset (39). Briefly, the runs with the most replicates (negative controls) were used to fit a Power Law Global Error Model on the mean versus standard deviation plots. Then STN ratios against Control as a baseline were calculated using observed mean values and PLGEM-calculated standard deviation values:

\[
STN = \frac{\text{ObsMean}_{stage} - \text{ObsMean}_{control}}{\text{CalcSD}_{stage} + \text{CalcSD}_{control}}
\]

Finally a series of random re-sampled STN ratios were computed to calculate significance thresholds \((p\text{-values})\) of PLGEM-based STN values (Supplemental Table S2). Proteins not detected in negative controls or with \(p\text{-values} \leq 0.05\) in at least one of the three stages examined were considered potential ubiquitylated conjugates (Supplemental Table S2, first group of 73 *Plasmodium* proteins). The entire MS/MS dataset was queried for the presence of lysines modified by 114.04 Da (“ubiquitin marks”) on the 49 proteins with significant \(p\text{-values}\) as previously described (40).
RESULTS

Ubiquitylation in Plasmodium falciparum erythrocytic stages

*P. falciparum*’s genome contains more than 100 genes that are potentially encoding the components of the ubiquitylation system, including four sources of ubiquitin (PFL0585w, PF14_0027, PF13_0346, and PF08_0067), eight E1 enzymes (with various specificities for ubiquitin and ubiquitin-like moieties), 14 E2 enzymes, more than 50 E3 ubiquitin ligases, and about 30 deubiquitinases (10). To experimentally confirm the presence of an active ubiquitylation system during *P. falciparum*’s asexual cell cycle, we explored *in silico* identification of putative targets for ubiquitylation during the parasite’s asexual cell cycle, fresh (same day) protein extracts were prepared from parasites harvested at ring, trophozoite and schizont stages and tested for the presence of endogenous ubiquitylating activity (fig. 1A). Ubiquitylation activity was detected in all three morphological stages of the parasite.

We further investigated the localization of ubiquitin conjugates in intra-erythrocytic parasites by immunofluorescence microscopy using an anti-ubiquitin antibody that does not react with free ubiquitin (which minimizes eventual background due to the intra-cellular pool of free ubiquitin). Results are shown on fig. 1B. At all stages, ubiquitin-conjugates are detected in abundance in the entire cell. This pattern is consistent with the fact that ubiquitylation is usually found in both the cytoplasm and the nucleus. In addition, localized intense spots of fluorescence can be observed, consistent with the presence of ubiquitin-conjugates in the parasite’s vesicles. These results confirm the presence of a functional ubiquitylation system in all asexual stages of *P. falciparum*.

*In silico* identification of putative targets for ubiquitylation

We explored *in silico* the ubiquitome of *P. falciparum*. We used the algorithm *UbPred* to identify the proteins among the 5446 translated protein-coding sequences that contain favorable ubiquitylation sites (14). Three different cut-offs were used to classify the results: (i) all identified proteins (low to high confidence), (ii) proteins identified with medium to high confidence, and (iii) highly confident results only (see the experimental procedures for more details). Results are summarized in Table 1. A total of 5036 proteins were predicted to contain ubiquitylation sites, 3516 of them with a high confidence level (*i.e.*, ~65% of the input dataset, fig. 2A). This predicted abundance of targets for ubiquitylation is slightly higher that the predictions made for *Saccharomyces cerevisiae* for which we found 52% of its proteome identified by *UbPred* with high confidence (*S. cerevisiae* datasets were used to train and test the accuracy of the algorithm). When the same algorithm was applied to *Arabidopsis thaliana*’s proteome, only 39% of the total proteins were highly confident targets for ubiquitylation. In addition, the average number of ubiquitylation sites per protein is much higher in *P. falciparum* (Table 1).

Following the same trend, lysine is the second most abundant amino acid in *P. falciparum*’s proteome with a frequency close to 12%, about double the frequencies observed in *S. cerevisiae* and *A. thaliana*, i.e., 5% and 6% respectively (fig. 2B). Slightly different results were obtained using *UbiPred*: 2077 proteins were predicted as ubiquitylated with high confidence, i.e., about 38% of the total proteome. This discrepancy might find its origin in the different datasets used to train the predicting algorithms. Both *UbPred* and *UbiPred* were built using ubiquitylated proteins as positive trainers. The negative datasets (non-ubiquitylated sites), however, were chosen differently: verified non-ubiquitylated proteins were used for the training of *UbPred* whereas putative non-ubiquitylated sites were used to train *UbiPred*. As a consequence, the likelihood of false negatives is higher for results generated with *UbiPred*, consistent with a lower number of predicted targets for ubiquitylation.

The overlap between *UbPred* and *UbiPred* predictions consisted of 1332 proteins. All together, we identified a total of 4261 highly confident putative targets for ubiquitylation using *UbPred* and *UbiPred* in combination (~78% of the parasite’s proteome; see Supplemental Table S1 for a complete list). The high lysine content of the parasite’s proteins and the relative abundance of predicted ubiquitin targets may indicate major roles for ubiquitylation in *P. falciparum*’s biology. We examined in more details the cellular localization of the 4261 highly confident ubiquitin targets identified with *UbPred* and *UbiPred* (used in combination). We found 299 of them contain an
Ubiquitylation in *Plasmodium falciparum*

apicoplast-targeting signal according to the plasmoAP rules (41, 42), 233 and 154 contain a PEXEL (43, 44) or HT (45) signal, respectively, and are targeted to the human host erythrocyte membrane, and 1288 have one or more transmembrane domains (46) (data queries made on PlasmoDB v6.5). These values do not represent any significant enrichment of ubiquitylated proteins according to the cellular compartment.

*MudPIT identification of ubiquitin conjugates in* *P. falciparum* *asexual stages*

Protein extracts were prepared from *P. falciparum* cultures harvested at ring, trophozoite, or schizont stage. Ubiquitin conjugates were then immunoprecipitated with an anti-ubiquitin antibody that does not recognize free ubiquitin (see Materials and Methods), and visualized by anti-ubiquitin western blot. We tested the immunoprecipitation procedure on an asynchronous population and confirmed that the antibody selectively enriches the sample in ubiquitin conjugates and does not precipitate free ubiquitin (fig. 3A). The procedure was performed on stage-specific extract. We found that all asexual intra-erythrocytic stages contain ubiquitylated proteins (fig. 3B).

The immunoprecipitated ubiquitin-conjugates were further analyzed by MudPIT (see Materials and Methods). A total of 437 proteins were detected from the anti-ubiquitin immunoprecipitated samples (Supplemental Table S2). To increase the confidence of our analysis, we rejected all the proteins that were not significantly enriched in the ubiquitin immunoprecipitations compared to samples precipitated in the absence of specific antibody (see Materials and Methods). This method minimizes the possible number of false positives. The counterpart is nonetheless a higher rate of false negatives since proteins that precipitate non-specifically in the absence of anti-ubiquitin antibody can still be conjugated to ubiquitin. Using this filter, we find 73 *Plasmodium* proteins, ~1.3% of the translated genome, that were specifically detected in the ubiquitin immunoprecipitations, 24 of them with low spectral counts. For comparison, previous works using similar technique (same antibody and same denaturing conditions) identified 345 ubiquitin conjugates in human cells (21), ~ 1.7 % of the translated genome, and 200 ubiquitin conjugates in *Arabidopsis thaliana* (16), ~ 0.8 % of the translated genome).

Ubiquitin-conjugates were the most abundant in schizont stage, with 63 proteins identified (17 of which with low abundance) out of 73 (fig. 3C). A total of 33 proteins were specific to this stage (15 of them were found in the low abundance dataset) and 18 were present at all stages (with various abundances) such as ubiquitin itself (gene PFL0585w), various ribosomal proteins, and parasite-specific proteases such as plasmepsin II (gene PF14_0077). Among the 63 ubiquitin-conjugates found at schizont stage, 50 are present in the highly confident in silico-predicted dataset (see Supplemental Table S1 and S2), and 22 were predicted with low to medium confidence with at least one of the algorithms (including ubiquitin itself, gene PFL0585w; see Supplemental Table S3). The 40S ribosomal protein S15A (PFC0735w) was the only protein identified by neither UbPred nor UbiPred. Remarkably, ubiquitin (the polyubiquitin gene PFL0585w contains five ubiquitin repeats) was found in the low to medium confidence dataset (Supplemental Table S3). Ubiquitin contains seven lysine residues that can all serve for the extension of ubiquitin chains: K6, K11, K27, K29, K33, K48, and K63. UbPred identified K63 with low confidence, and UbiPred identified K33 (low confidence, score = 0.53) and K48 (medium/high confidence, score = 0.83). The scores for K6, K11, K27, and K29 were below threshold (data not shown). Our MudPIT analysis, nonetheless, found evidences of ubiquitylated K6 and K11 in the samples (Supplemental Fig. S1). Spectra matching such modified peptides from the ubiquitin proteins represented 1.3% and 13.3% of the total spectral count, respectively (Table 4). K48 and K63 were also detected as modified (80.4% and 4.9% of the spectra). K48 is the most abundant linkage usually found in eukaryotic cells and is involved in targeting ubiquitin-tagged substrates to the 26S proteasome for degradation. The roles of K6, K11, K27, K29, and K33 are still not understood (see (47) for a review). These results show that the linkages K6, K11, K48, and K63 (at least) exist in *P. falciparum*.

We found 24 and 36 ubiquitylated proteins in rings and trophozoites, respectively. The 60S ribosomal protein L27a (PFF0885w) was found to be ubiquitylated at ring stage exclusively (5 spectral
Ubiquitylation in *Plasmodium falciparum*

Counts), and seven ubiquitin-conjugates were trophozoite-specific (Table 2). Two of them are members of the ubiquitin/proteasome system in *P. falciparum* (10). UFD2 (PFD0265w) is a U-box ubiquitin ligase involved in the endoplasmic reticulum-associated protein degradation pathway (ERAD), and PFD0265w encodes a metallo-deubiquitinase (mov34) closely related to the PRP8 RNA splicing factor. PF10_0068, PFB0715w, and PF14_0096 are also involved in the metabolism of RNA.

**Alternative validation of plasmepsin II as a substrate for ubiquitylation**

The plasmepsin II is a cysteine protease that catalyzes the degradation of hemoglobin in the food vacuole of the human malaria parasite. After translation, the full-length protein (~51.4 kDa) is transported to the food vacuole where a 124 aa-long N-terminal pro-domain is cleaved off to leave a 329 aa-long mature and functional plasmepsin II (fig. 5A). We used the antibody Ab737 (MRA-66 (29)), targeted to the N-terminal portion of the mature protein, to immunoprecipitate both the full-length pre-protein and the mature plasmepsin II (fig. 4A). Ubiquitylations were revealed using an anti-ubiquitin antibody (fig. 4B). The characteristic smear of ubiquitylated proteins at high molecular weights was observed in the total protein extract (input) and in the flow through (FT) of proteins from the IP. This smear was replaced by a characteristic banding pattern that indicates the presence of poly/multi ubiquitylation on the pre- and mature plasmepsin II. The signal indicating ubiquitylation was particularly strong on the mature form of the protein (fig. 4C, lanes 1 through 3) in agreement with the predominance of mature plasmepsin II during the parasite’s asexual cycle. We examined the sequence of the plasmepsin II with regards to our bioinformatic predictions of ubiquitylation sites (Supplemental Table S4). We found 20 putative ubiquitylation sites when *UbPred* and *UbiPred* are used in combination. Eight of these sites are present on the mature plasmepsin II.

Similar experiments were performed with regards to the hemoglobinases plasmepsin I and IV. Specific antibodies were used to immunoprecipitate plasmepsin I and plasmepsin IV, respectively (see Materials and Methods). Based on our mass spectrometry analysis, these two plasmepsins were found at relatively low levels in our samples and were not significantly enriched compared to the control (see Supplemental Table S2). No evidence of specific ubiquitylation on the immunoprecipitated plasmepsins I and IV was detected using an anti-ubiquitin antibody (fig. 4C and data not shown). These results indicate that either these two plasmepsins are not ubiquitylated or their abundance is below detection threshold, consistently with our mass spectrometry analysis. All together, these observations confirm that the plasmepsin II is a genuine substrate for ubiquitylation and validate further our mass spectrometry analysis.

**Functional classification of the ubiquitin conjugates identified by MudPIT**

We examined in more details the characteristics of the MudPIT dataset. We classified the 73 proteins into 11 functional categories according to their annotation in PlasmoDB v6.5 (fig. 5). We find 12% of the dataset consist of chaperon and other proteins involved in folding, 11% of proteins involved in translation (mostly ribosomal proteins), RNA metabolism (such as splicing factors), and ubiquitin-dependant metabolic processes (see Supplemental Table S2). In human cells proteins involved in translation/protein synthesis represent 18 % of the immunoprecipitated ubiquitin-conjugates (21). This value drops down to 4.5 % in *A. thaliana* (16). Proteins involved in transcription represent 4% of the dataset, *i.e.*, twice as much as the proportion found in human cells (21), but half that of plant (16). This group contained a subunit of the RNA polymerase II (PFB0715w) and the putative ApiAP2 transcription factor PFF0200c that is mainly expressed and found ubiquitylated at schizont stage only (spectral count = 4). Proteins involved in parasite-specific processes (invasion, hemoglobin metabolism, liver stage-specific) represent 8% of the dataset. Remarkably, 11% of the dataset contained proteins of the ubiquitin-proteasome system, an observation consistent with the fact that ubiquitin is covalently and sequentially attached to the various components of the ubiquitin machinery before being transferred on the targeted substrates (Table 3). Among them are components of the 26S proteasome (PF08_0109, PF13_0063), the E2
Ubiquitylation in *Plasmodium falciparum*

enzymes PFC0255c homolog to MMS2, and two ubiquitin ligases (the “E4” ligase UFD2 PF08_0020, and the zinc “RING” finger, PF10_0046 homolog to the *A. thaliana* CIP8). Altogether, our observations showed that ubiquitin targets were involved in a wide array of biological processes in the parasite including pathogenicity.

Relative abundance of ubiquitin proteins in *P. falciparum*’s asexual stages

The abundance of each protein detected by MudPIT was estimated using the Normalized Spectral Abundance Factor (NSAF, (34)). As expected, ubiquitin originated from mono- and poly-ubiquitin chains was by far the most abundant protein found in all samples (Supplemental Table S2). The detected levels peaked at trophozoite stage (fig. 6, plain line). We compared these abundances to the ubiquitin levels detected in a previous whole proteome analysis (48). In this study, Le Roch and Colleagues showed that ubiquitin proteins are more abundant at ring and schizont stage than trophozoite stage (fig. 6 dashed line). The discrepancies between our IP data and Le Roch and Colleagues’ ubiquitin levels can be explained by the fact that the antibody used in our immunoprecipitations does not recognize free ubiquitin. With this regard, the increased levels of total ubiquitin at ring and schizont stage in the whole proteome dataset may be attributed to a higher pool of free ubiquitin at these stages. This hypothesis is consistent with a higher diversity of ubiquitin-conjugates at schizont stage that appears dramatically reduced at ring stage.

Total protein levels vs. ubiquitylated protein levels

We further compared our dataset to total protein levels measured by Le Roch and Colleagues in their previous whole proteome analysis (48). Out of the 73 ubiquitin-conjugates that we specifically identified in the present work, 42 were present in Le Roch and Colleagues’ dataset (Supplemental Table S5). In some instances, such as PFL1425w (γ subunit of T complex protein 1) at ring and schizont stages or PF11_0161 (falcipain 2b) at schizont stage for example, no protein had been detected during the proteomic analysis but ubiquitylated ones were identified in our dataset (see Supplemental Table S5). In these cases, since our samples were treated with the proteasome inhibitor MG132 for six hours before harvest, it is likely that the detected conjugates are targeted to the proteasome for degradation. This hypothesis is consistent with various discrepancies observed by Le Roch and Colleagues between mRNA protein levels in a given stage (48) and highlights the importance of post-transcriptional and post-translational regulations for the parasite’s biology.

DISCUSSION

The present study proposes more than 4200 putative ubiquitin substrates and validates ~2% of them for ubiquitylation during the intraerythrocytic cycle of the human malaria parasite *P. falciparum*. Ubiquitylated proteins were detected in all cellular compartments of the parasite, consistent with observations in other eukaryotes. Altogether, we found a high proportion of the proteome being possible targets for ubiquitylation across the various stages of the parasite’s life cycle. This observation is consistent with a particularly high lysine content in *P. falciparum*’s proteome that offers many possible anchor points for ubiquitin. In addition, these results represent other pieces of evidence that post-translational modifications in general and ubiquitylation in particular are major regulatory pathways in the malaria parasite. Most of the ubiquitin-conjugates that we identified were found during the schizont stage, consistent with a reduction in size and activity of the parasite for maturation into the invasive merozoite. We nonetheless showed that a smaller number of proteins which ubiquitin conjugates were detected in the other intra-erythrocytic stages. When coupled to previously published proteomic analysis (48), higher levels of
ubiquitin are expected at ring and trophozoite stages, revealing a higher pool of free ubiquitin in these stages than in schizont stage. This observation highlights the importance of ubiquitylation events at the schizont stage before differentiation into the invasive merozoite. Given these elements, ubiquitylation events may be of major importance for merozoite differentiation.

The results obtained in silico using the two distinct algorithms UbPred and UbiPred show good overlap. The discrepancies observed between the two algorithms probably originate from their differences of conception. Both UbPred and UbiPred were built using positive (ubiquitylated) and negative (non-ubiquitylated sites) datasets, with an important difference in the choice of the negative dataset. UbPred was trained to recognize non-ubiquitylated sites on experimentally verified non-ubiquitylated proteins. The negative dataset for UbiPred, on the other hand, consisted of putative non-ubiquitylated sites more likely to contain more false negatives that the experimentally verified one. As a consequence, the likelihood of false negatives is higher for results generated with UbiPred, consistent with less predicted targets for ubiquitylation. In addition, we found that the two algorithms perform differently depending on the type of linkage that is considered. These differences probably find roots within the nature of training datasets. Less abundant linkages are more difficult to detect. *P. falciparum* seems to contain a relatively high proportion of K11 linkages, 13.3 %. K11-linkages in humans are suspected to play a key role in mitotic protein degradation (49). In *Saccharomyces cerevisiae*, high levels of K11-linkages have been observed to play a key role in endoplasmic reticulum-associated degradation (ERAD) of proteins (50). *Plasmodium* possesses a conventional ERAD system. In addition, there are growing evidences of an ERAD-like pathway that is involved in protein translocation to the parasite-specific plastid, namely the apicoplast (11, 51), which essentiality for the parasite’s survival is clearly established (see (52) for a review). The potential importance of such linkages in *Plasmodium* (suggested by their abundance) could thus be major in terms of cell cycle progression, parasite survival, and virulence. When the levels of ubiquitylated proteins were compared to previously published levels of total proteins (48) we found that proteasome-dependant ubiquitylation may explain, in some instances, discrepancies between mRNA levels and protein levels (48). In other cases, it is likely that proteasome-independent ubiquitylations occur in the malaria parasite. Such post-translational modifications classically involved in protein targeting and regulation in eukaryotes.

From the technical point of view, our method is sensitive and accurate. Both the *Plasmodium* and the human databases were used to cross-match the detected peptides and specifically identify proteins based on their sequences. This step is indeed crucial given that the red blood cell contains a wide range of proteins independently from *Plasmodium* infection (reviewed in (53, 54)). Our analysis however highlights the limits of the approach that was used to identify ubiquitin-conjugates at a large scale. The main challenge is indeed to obtain a reliable negative control to exclude false positives while minimizing the number of false negatives: non-specific interactions do not necessarily invalidate the specific ones. As is, we identified 73 *Plasmodium* ubiquitin conjugates together with 364 potential additional candidates. The rejection of these 364 candidates is directly linked to the baseline drawn by the chosen negative control. The question of how to choose a good control to draw a blank baseline receives, indeed, no perfect answer. Using various experimental strategies, *e.g.* protein capture via ubiquitin-binding domains, could be a piece of solution by permitting a broader discovery of the various ubiquitin conjugates.

The 73 proteins in our dataset of confident ubiquitin conjugates are involved in a variety of biological processes, such as translation. Many ribosomal proteins are ubiquitylated during the red blood cell cycle of *P. falciparum*. In human cells, the ribosomal subunits S3, S18, L23a, L24, and L28 were previously shown to be ubiquitylated (21, 55). Our results are consistent with a function for ubiquitylation in the quality control of ribosome biogenesis. Ribosome assembly, indeed, is a highly complex association of numerous and various proteins thus prone to misfolding and other maturation errors. The high proportion of
Ubiquitylation in *Plasmodium falciparum*

ubiquitylated proteins in our dataset may reflect this propensity to errors and the subsequent targeting to the proteasome after ubiquitin tagging. Another hypothesis is that ubiquitylation of ribosomal proteins plays a role in the regulation of translation, as previously shown in human cells (55).

Our dataset also contained 11 proteins related to the ubiquitin/proteasome system itself. Since all interactions between ubiquitin and the enzymes catalyzing its transfer to various targets (including ultimately the proteasome) involve the formation of a covalent bond, the identification of a subset of these enzymes is a validation of our dataset. Most of the proteins that we identified are subunits of the proteasome. In addition, we also found one E2 ubiquitin-conjugating enzyme, two ubiquitin ligases, and the ubiquitin-like PISUMO (PFE0285c). The existence of heterologous ubiquitin chains mixed with SUMO has been previously observed in other organisms (see (56) for a review) but has never been reported in *Plasmodium*. The proposed function of heterologous SUMO-UB chains is to target sumoylated proteins to the proteasome for degradation. Various SUMO targets were previously reported and indicated an important role of sumoylation in the parasite’s biology (57). Among them, Issar and Colleagues (2008) identified several transcription and chromatin regulators including the histone protein H4 (PF11_0061) that we also identified in our dataset. Our results further suggest that the histone variant H2A.Z (and possibly H2A, H2B, and H3) is also a target for ubiquitylation. Such ubiquitylation have been previously observed in human and yeast and are possibly linked to gene activity (see (58) for a review). In addition, we also found a major subunit of the RNA polymerase II conjugated to ubiquitin at trophozoite stage (see Supplemental Table S2). Such ubiquitylation was previously associated with transcriptional arrest in yeast (reviewed in (59) and (60)) but was never observed in *Plasmodium*. Finally we found that at least one of the few specific ApiAP2 transcription factors that are known in *Plasmodium*, PFF0200c, is ubiquitylated at schizont stage. All together, these data strongly indicate that ubiquitylation play an important role in regulating gene expression at the transcriptional level. A few proteins involved in parasite’s specific processes, such as the hemoglobinase plasmepsin II, were also identified (the ubiquitylation of plasmepsin II was further validated by immunodetection).

Our results provide the first general view of the *P. falciparum*’s ubiquitome and represent an important step towards the characterization of the ubiquitylation system in the parasite. This study identifies various new targets for ubiquitylation and provides numerous leads for investigating the malaria parasite’s biology through post-translational modifications. On the whole, our observations strongly propose ubiquitination as a major regulator of various biological processes in *Plasmodium*, including gene expression and various parasite-specific metabolisms. The ubiquitin targets shown in the present work could therefore represent excellent objects of research for future development of anti-malarial drugs.

**ACKNOWLEDGEMENT**

The authors thank P. Radivojac for providing a stand-alone version of *UbPred* for Windows® 32 bits, and C-W. Tung for running *Ubipred*. The following reagent was obtained through the MR4 as part of the BEI Resources Repository, NIAID, NIH: *Plasmodium falciparum* 1C6-24, MRA-813A, *Plasmodium falciparum* Anti-plasmepsin II Rabbit Antiserum 737, MRA-66, *Plasmodium falciparum* 13.9.2, MRA-814A, deposited by DE Goldberg. The study was supported by UCR start-up funds (KLR).

**REFERENCES**

Ubiquitylation in *Plasmodium falciparum*

Ubiquitylation in \textit{Plasmodium falciparum}

Ubiquitylation in *Plasmodium falciparum*

**TABLES**

**Table 1. Ubiquitin target predictions by *UbPred* (14)**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Confidence Level</th>
<th>Number of proteins</th>
<th>Percentage of the total proteome</th>
<th>Average number of site per protein</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Plasmodium falciparum</em></td>
<td>Low to high</td>
<td>5036</td>
<td>92.5</td>
<td>18.7</td>
</tr>
<tr>
<td></td>
<td>Medium to high</td>
<td>4446</td>
<td>81.6</td>
<td>14.6</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>3516</td>
<td><strong>64.6</strong></td>
<td><strong>8.7</strong></td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td>Low to high</td>
<td>5258</td>
<td>89.3</td>
<td>7.9</td>
</tr>
<tr>
<td></td>
<td>Medium to high</td>
<td>4470</td>
<td>76.0</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>3059</td>
<td>52.0</td>
<td>4.1</td>
</tr>
<tr>
<td><em>Arabidopsis thaliana</em></td>
<td>Low to high</td>
<td>42953</td>
<td>82.9</td>
<td>5.6</td>
</tr>
<tr>
<td></td>
<td>Medium to high</td>
<td>34834</td>
<td>67.2</td>
<td>4.3</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>20214</td>
<td>39.0</td>
<td>3.1</td>
</tr>
</tbody>
</table>

**Table 2. Ubiquitin-conjugates specifically found in trophozoite stage†**

<table>
<thead>
<tr>
<th>Systematic name</th>
<th>Product description</th>
<th>Function within the ubiquitin/proteasome system††</th>
<th>Spectral count(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PF10_0068</td>
<td>RNA binding protein, putative</td>
<td>N/A†††</td>
<td>5</td>
</tr>
<tr>
<td>PFB0715w</td>
<td>DNA-directed RNA polymerase II second largest subunit, putative</td>
<td>N/A</td>
<td>2</td>
</tr>
<tr>
<td>PFD0265w</td>
<td>pre-mRNA splicing factor, putative</td>
<td>deubiquitinase (mov34)</td>
<td>3</td>
</tr>
<tr>
<td>PFF1415c</td>
<td>Heat shock DnaJ protein, putative</td>
<td>N/A</td>
<td>2</td>
</tr>
<tr>
<td>MAL8P1.103</td>
<td>conserved <em>Plasmodium</em> protein, unknown function</td>
<td>N/A</td>
<td>2</td>
</tr>
<tr>
<td>PF08_0020</td>
<td>ubiquitination-mediated degradation component, putative</td>
<td>UFD2 U-box ubiquitin ligase</td>
<td>6</td>
</tr>
<tr>
<td>PF14_0096</td>
<td>RNA binding protein Bruno, putative (HoBo)</td>
<td>N/A</td>
<td>2</td>
</tr>
</tbody>
</table>

†Gene names and product descriptions are given according to www.geneDB.org unless specified otherwise. ††Annotation proposed based on protein domain architecture (10). †††Not applicable.
Ubiquitylation in *Plasmodium falciparum*

Table 3. Components of the ubiquitin/proteasome system identified by immunoprecipitation coupled to MudPIT†

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Systematic name</th>
<th>Product description</th>
<th>Stage(s)</th>
<th>Spectral count(s)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyubiquitin</td>
<td>PFL0585w</td>
<td>polyubiquitin</td>
<td>Ring, Trophozoite, Schizont</td>
<td>266, 632, 265</td>
</tr>
<tr>
<td>Mms2††</td>
<td>PFC0255c</td>
<td>ubiquitin conjugating enzyme E2, putative</td>
<td>Trophozoite, Schizont</td>
<td>2, 8</td>
</tr>
<tr>
<td>Ufd2</td>
<td>PF08_0020</td>
<td>ubiquitination-mediated degradation component, putative (U-box ubiquitin ligase)</td>
<td>Trophozoite</td>
<td>6</td>
</tr>
<tr>
<td>CIP8</td>
<td>PF10_0046</td>
<td>E3 “RING” ubiquitin ligase, putative</td>
<td>Ring, Trophozoite, Schizont</td>
<td>2, 3, 2</td>
</tr>
<tr>
<td>N/A</td>
<td>PF11_0142</td>
<td>Ubiquitin domain-containing protein</td>
<td>Trophozoite, Schizont</td>
<td>6, 17</td>
</tr>
<tr>
<td>N/A</td>
<td>PF08_0109</td>
<td>Proteasome subunit alpha type 5, putative</td>
<td>Trophozoite, Schizont</td>
<td>2, 6</td>
</tr>
<tr>
<td>N/A</td>
<td>PF13_0063</td>
<td>26S proteasome regulatory subunit 7, putative</td>
<td>Trophozoite, Schizont</td>
<td>4, 4</td>
</tr>
<tr>
<td>Sumo</td>
<td>PFE0285c</td>
<td>Small ubiquitin-related modifier, putative</td>
<td>Ring, Trophozoite, Schizont</td>
<td>2, 3, 4</td>
</tr>
</tbody>
</table>

*Average spectral count among all replicates with a positive count.
†Gene names and product descriptions are given according to www.geneDB.org unless specified otherwise.
††According to BLASTP results performed on the NCBI refseq protein database.
†††Not available

Table 4. Ubiquitin marks on ubiquitin itself found in *P. falciparum*

<table>
<thead>
<tr>
<th>Lysine</th>
<th>Number of spectra found in MudPIT†</th>
<th>Score for <em>UbPred</em> predictions</th>
</tr>
</thead>
<tbody>
<tr>
<td>K6</td>
<td>8 (1.3%)</td>
<td>0.49</td>
</tr>
<tr>
<td>K9</td>
<td>0</td>
<td>0.38</td>
</tr>
<tr>
<td>K11</td>
<td>79 (13.3%)</td>
<td>0.54</td>
</tr>
<tr>
<td>K27</td>
<td>0</td>
<td>0.38</td>
</tr>
<tr>
<td>K29</td>
<td>0</td>
<td>0.56</td>
</tr>
<tr>
<td>K48</td>
<td>477 (80.4%)</td>
<td>0.54</td>
</tr>
<tr>
<td>K63</td>
<td>29 (4.9%)</td>
<td>0.62</td>
</tr>
</tbody>
</table>

†Out of 593 spectra obtained merging all runs
FIGURES AND FIGURE LEGENDS

Fig. 1. Ubiquitylation in *P. falciparum* at ring, trophozoite and schizont stages.

A. *In vitro* ubiquitylation activity of parasite’s protein extracts prepared at different morphological stages. Ubiquitylation assays were performed in the presence of biotin-ubiquitin and revealed by western-blot streptavidin-HRP with electrochemiluminescence detection. The presence of multiple bands reveals the presence of various biotin-ubiquitin-conjugates. **B.** Immunofluorescent *in situ* localization of ubiquitin conjugates in parasites at their ring, trophozoite (TROPH.), or schizont (SCHIZ.) stages. The parasites were visualized under transmitted (trans.) light, their nuclei stained with DAPI (blue), and the ubiquitin-conjugates visualized in the red. Ubiquitin conjugates are present at all investigated stages.
Fig. 2. Comparison of proteomes for *P. falciparum*, *S. cerevisiae*, and *A. thaliana*.

A. Portion of the total proteome that, according to the *UbPred* algorithm, is not a target for ubiquitylation (blue bars) or a target for ubiquitylation according to *UbPred* predictions made with low (red bars), medium (green bars), or high confidence (purple bars). The large majority of *P. falciparum*’s proteins are confident putative targets for ubiquitylation.

B. Amino acid content in *P. falciparum* (blue bars), *S. cerevisiae* (red bars), and *A. thaliana* (green bars). *P. falciparum*’s proteome contains a higher proportion of lysines than *S. cerevisiae* and *A. thaliana*. 
Fig. 3. Selective immunoprecipitation and MudPIT identification of ubiquitin-conjugates.
A. Antibody performances were tested on an asynchronous population of *P. falciparum* during its asexual cycle. The sample was first incubated with anti-ubiquitin antibody and then agarose beads. Proteins that were not captured by the antibody (unbound) were removed by centrifugation. The beads with antibodies-ubiquitylated proteins conjugates were washed three times (wash 1 to wash 3) before the elution of specifically bound ubiquitylated proteins (precipitate). Our results show a significant enrichment in ubiquitin-conjugates in the immunoprecipitate without the presence of free ubiquitin. B. Ubiquitin-conjugates content in 10 µL of immunoprecipitated proteins for rings, trophozoites, and schizonts. C. Venn diagram representing the number of different ubiquitin-conjugates identified by MudPIT in each morphological stage.
Ubiquitylation in *Plasmodium falciparum*

Fig. 4. Alternative validation of plasmepsin II as a substrate for ubiquitylation.

A. Plasmepsin II (PMII) is translated into its 51.4 kDa pro-form and transported to the parasite’s food vacuole where it is processed into a 36.9 kDa mature and functional protease. The antibody MRA-66 (Ab737) recognizes the N-terminal side of the mature PMII and was used to immunoprecipitate the full length and the mature PMII from protein extracts. B. Anti-ubiquitin western blot of the anti-PMII IP experiment (see Materials and Methods). Input = full protein extract; FT = flow through; P = precursor PMII; M = mature PMII. C. Anti-ubiquitin western blot of the anti-PMII IP experiment, with different exposure times, and of the anti-PMIV IP experiment. The ubiquitylated mature form of plasmepsin II is abundantly detected after 10 s of exposure. Ubiquitylated PMIV is however not detected after 10 min of exposure.
Fig. 5. Functions of the ubiquitin targets identified by MudPIT.
Ubiquitin conjugates were classified according to their known or suspected function and expressed as the percentage of the total ubiquitin conjugates.
Ubiquitylation in *Plasmodium falciparum*

**Fig. 6. Abundance of ubiquitin in IP samples vs. total ubiquitin pool.**

Ubiquitin abundance in immunoprecipitated samples were measured by the average NSAF value observed at each asexual stage (plain curve). The total pool of ubiquitin in each stage has been previously quantified (48) (dashed curve). The Pearson product-moment coefficient of correlation between the two dataset equals -0.998.