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Article

Mediator Complex of the Malaria Parasite *Plasmodium falciparum* Associates with Evolutionarily Novel Subunits

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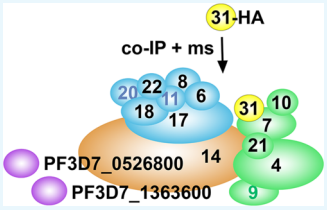
ABSTRACT: The eukaryotic Mediator is a large and conserved multisubunit protein complex that directly contacts RNA polymerase II and impinges on multiple aspects of gene expression. The genome of the human malaria parasite *Plasmodium falciparum* has been predicted to encode several Mediator subunits. We provide physical evidence for the presence of a Mediator complex in *P. falciparum* by using coimmunoprecipitation and mass spectrometry to identify interaction partners of the highly conserved Mediator subunit PfMed31. We identify 11 of 14 predicted Mediator subunits and the products of two uncharacterized genes, PF3D7_0526800 and PF3D7_1363600, which are strongly associated with PfMed31. As expected, several additional interaction partners have known roles in the transcriptional control of gene expression and mRNA processing. Intriguingly, multiple interaction partners are implicated in endoplasmic reticulum function and the ER stress (ERS) response, suggesting crosstalk between the ERS response and the transcriptional machinery. Our results establish for the first time the physical presence of the Mediator complex within *P. falciparum* and strongly suggest that it plays both conserved and unique roles in the control of gene expression. Data are available via ProteomeXchange with the identifier PXD027640.



INTRODUCTION

The parasite responsible for the most severe malaria in humans, *Plasmodium falciparum*, has a complex life cycle in the human host and mosquito vector. The clinical symptoms of malaria are due to the development of parasites within human red blood cells, wherein merozoite forms of the parasite invade RBCs and develop through the ring, trophozoite, and schizont stages over a span of 48 h. Genome-wide transcriptomic analyses have shown that the parasite presents a distinct pattern of gene expression such that more than 75% of genes display peak mRNA levels at a single timepoint during the intraerythrocytic developmental cycle, with different genes peaking at different stages.[1−3] Furthermore, for 30% of genes, a time interval has been observed between mRNA levels and protein expression.[4] Thus, the developmental stages of the parasite are accompanied by a unique coordinated pattern of gene expression.

In eukaryotes, tissue and cell-type-specific gene expression are orchestrated in part by positively acting gene-specific transcription factors, which recruit transcription initiation complexes to gene promoters or release a paused polymerase for transcriptional elongation. Relatively few gene-specific transcription factors have been identified in the *Plasmodium* genome, with the notable exception of the ApiAP2 family of proteins.[5]

By contrast, the parasite encodes most members of the basal or general transcriptional machinery. The Mediator is a multisubunit complex that acts as a molecular bridge between gene-specific transcription factors bound to enhancer sites and the general transcriptional machinery assembled at the promoter.[6,7] In higher eukaryotes, the entire complex has a molecular weight of ∼1 MDa and is composed of 25−30 different proteins. A combination of structural, genetic, and biochemical studies has shown that the Mediator is organized into four modules: head, middle, tail, and a dissociable cdk8 kinase module (CKM). Each module serves varied cellular functions. The tail module is known to contact activators and repressors to regulate transcription. The head and middle modules contact RNA polymerase II (RNAPII) and basal transcriptional factors, while the kinase module presumably has an inhibitory role in gene expression. Although Mediator was discovered as a protein complex required for activation of transcription, it has additional functions including transcription elongation[8] and termination,[9,10] mRNA processing,[11] and chromatin remodeling.[12]

A computational analysis of known and putative Mediator subunits from nearly 70 eukaryotes has shown the presence of a core Mediator complex composed of 17 subunits, conserved in all eukaryotes except kinetoplastids (trypanosomes and *Leishmania major*) in which the control of gene expression at the transcriptional level has been lost (or never acquired) in the course of evolution.[13,14] The Mediator is, therefore, presumed to play a near-universal role in gene expression. Fourteen Mediator subunits have been identified computa-

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tionally in *P. falciparum*.[13,14] All seven head subunits present in yeast are conserved in the parasite, as are most middle subunits apart from Med1 and Med19. Tail subunits are known to contact activators for specific gene regulatory pathways. Although transcription factors such as those of the ApiAP2 class have been identified in *Plasmodium* spp,[5] genes encoding conserved tail subunits have not been computationally detected in the parasite genome. Likewise, the dissociable CKM module has not been identified to date.

The study of Mediator function in *P. falciparum* will be indispensable to an understanding of gene regulation in the parasite and could open doors to novel therapeutic approaches. In the present study, we sought to undertake an initial characterization of the Mediator complex in *P. falciparum* by studying protein−protein interactions of the highly conserved Mediator subunit, Med31 (hereafter, PfMed31). Using coimmunoprecipitation and mass spectrometric analysis of episomally expressed PfMed31, we have, for the first time, shown the physical presence of the Mediator complex in *P. falciparum*. In addition to the identification and demonstration of the physical association of 11 out of 14 predicted Mediator subunits, we show that two previously uncharacterized proteins are strongly associated with PfMed31. These presumptive Mediator subunits are parasite-specific and may have evolved for specialized functions. Additional interaction partners implicate crosstalk between Mediator and the parasite endoplasmic reticulum (ER) stress (ERS) response.

MATERIALS AND METHODS

Ethics Approval and Informed Consent. This research involved the use of human blood drawn from healthy volunteers by trained healthcare professionals and was approved by the NTU Institutional Review Board and assigned the IRB approval number IRB-2013-07-020. Informed consent was obtained and documented by the signing of an approved consent form.

Parasite Strains, Culture, and Genetic Modification. *P. falciparum* T996 strain parasites were cultured at 2% hematocrit in RPMI 1640 medium containing 0.25% Albumax, 0.2% sodium bicarbonate, and 0.01 mg/mL gentamicin. Parasites were cultured in a sterile cell culture flask in a gaseous environment consisting of 5% CO_2, 3% O_2, and 92% N_2 and incubated at 37 °C. For episomal expression, the PfMed31 gene (PF3D7_1475000), excluding the stop codon was amplified from *P. falciparum* T996 genomic DNA with primers 5′-AATAGAAATATATCAGGATCATGGGAA-TAAGCCAAAAAAAG-3′ and 5′-GTACCTAAGCAC-CACGCTAGCTATTTGGTAACTAAAATATAAC-3′. The amplicon was inserted into the plasmid pBcamR_3XHA_X between restriction sites BamHI and NheI, placing the gene under the control of the calmodulin promoter and generating a C-terminal hemagglutinin tag fusion. Clones were generated by ligation-independent cloning. The resulting plasmid Med31-pBcamHA was transfected into the T996 parasite strain. Stable transfectants were selected using 2.5 μg/mL of blasticidin. The episomal PfMed31-HA gene was detected by PCR using the primers 5′-AGTCGGATCCATGGGAATAAGC-CAAAAAAAG-3′ and 5′-CATAAAGTTGTTAGAGCTCGG-CATAATCTGG-3′. Transcript expression was confirmed by reverse-transcription PCR using the primers 5′-ATGGGAA-TAAGCCAAAAAAAGTAC-3′ and 5′-TTAGAGCTCGGCA-TAATCTGGAACATCG-3′.

Coimmunoprecipitation and Western Blot Analysis. Asynchronous parasite cultures were lysed in 0.1% saponin,[15] and cell lysates were prepared with lysis buffer [50 mM Tris-Cl pH 7.5, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 1 mM PMSF, 1× protease inhibitor cocktail (Roche)]. The mixture was incubated at 4 °C on a rotating shaker for 1 h. The solution was centrifuged at 14,500*g* for 10 min at 4 °C. The resulting supernatant was transferred to a new tube, and 10% of the solution was taken as input for immunoprecipitation. The remaining solution was used for immunoprecipitation.

For immunoprecipitation using an anti-HA antibody, 1 μg of rat anti-HA antibody (Roche, clone 3F10) was added and incubated overnight on a rotating shaker at 4 °C. A nonspecific rabbit anti-IgG antibody was used as a negative control. 100 μL of SureBeads protein G magnetic beads (Bio-rad) were washed thrice with 1 mL of phosphate-buffered saline and once with 1 mL of lysis buffer [50 mM Tris-Cl pH 7.5, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 1 mM PMSF, 1× protease inhibitor cocktail (Roche)]. The sample containing the HA-tagged protein was added to the prewashed magnetic beads and incubated for 2 h on a rotating shaker at 4 °C. The beads were washed thrice with 1 mL of lysis buffer, with incubation on a rotating shaker at 4 °C for 10 min for each wash. The antibody-bound proteins were eluted with 40 μL of SDS-PAGE Laemmli buffer.

Coimmunoprecipitation for mass spectrometric analysis was performed using anti-HA magnetic beads (Pierce, Cat. no. 88836). 6 mL of infected blood (>10% parasitemia, 2% hematocrit) was harvested for the experiment. 25 μL of anti-HA magnetic beads were washed with 0.05% TBS-T (25 mM Tris pH 7.5, 0.15 M NaCl pH 7.5, 0.05% Tween-20). Approximately 500 μL of protein lysate was added to 25 μL of prewashed magnetic beads and incubated overnight on a rotating shaker at 4 °C. Four such reactions were carried out in parallel for samples from each of the T996 parental and PfMed31-HA-expressing strains and combined before the final elution step. The beads were washed twice with TBST, followed by a final wash with ultrapure water. The antibody-bound proteins were eluted with 50 μL of the SDS-PAGE Laemmli buffer.

For western blot analysis, parasites were released from infected erythrocytes by 0.1% saponin treatment and lysed using 50−200 μL of the parasite lysis buffer (4% SDS, 0.5% Triton X-100) in the presence of freshly added protease inhibitors. Proteins were resolved on a 12% resolving SDS-PAGE. Resolved proteins were transferred to a polyvinyl difluoride membrane. Western blotting was performed using a rat monoclonal anti-HA primary antibody (Roche, clone 3F10, 1:1000 dilution) followed by incubation with an anti-rat HRP secondary antibody (Abcam Cat. no. ab99655, 1:10,000 dilution). Chemiluminescence from HRP-conjugated secondary antibodies was detected using WesternBright ECL HRP substrate (Advansta Inc.) according to the manufacturer's instructions.

β-Actin was used as the loading control. Blots probed with anti-HA antibody were stripped and reprobed with mouse anti-actin antibody (Sigma) at 1:10,000 dilution. Anti-mouse HRP antibody at 1:10,000 dilution was used as a secondary antibody.

Mass Spectrometry. Eluate samples obtained after immunoprecipitation were separated using a 12% SDS-polyacrylamide gel and stained with Coomassie blue. The lanes corresponding to the samples of interest were cut using a

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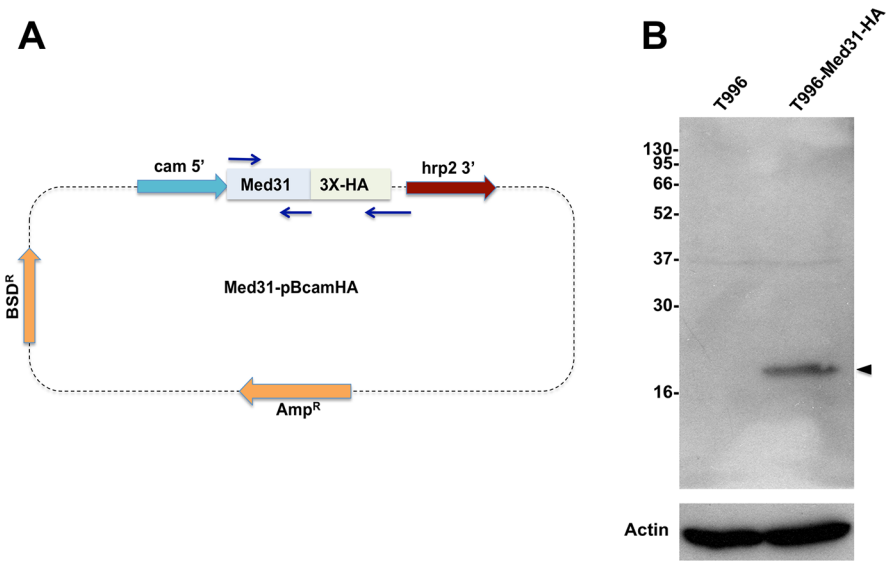

Figure 1. Generation of Med31-HA parasite strain. (A) Schematic representation of PfMed31-HA cloned into the pBcamHA vector. The calmodulin promoter (cam) drives transgene expression. The plasmid contains ampicillin-(Amp^R) and blasticidin-(BSD^R) resistance cassettes for selection in *Escherichia coli* and *P. falciparum*, respectively. The arrows (blue) indicate the locations of PCR primers designed to amplify endogenous PfMed31 and episomally expressed PfMed31-HA genes. (B) Use of an anti-HA antibody in a Western blot confirms the expression of the PfMed31-HA protein in the blasticidin-resistant strain but not in the wild-type T996 parent strain. Comparable loading was determined by stripping the same blot and reprobing for β-actin with appropriate antibodies. The position of molecular weight markers is shown to the left. The arrowhead to the right denotes the position of PfMed31-HA at ~19 kDa. The results of this Western blot were confirmed in a second independent experiment.

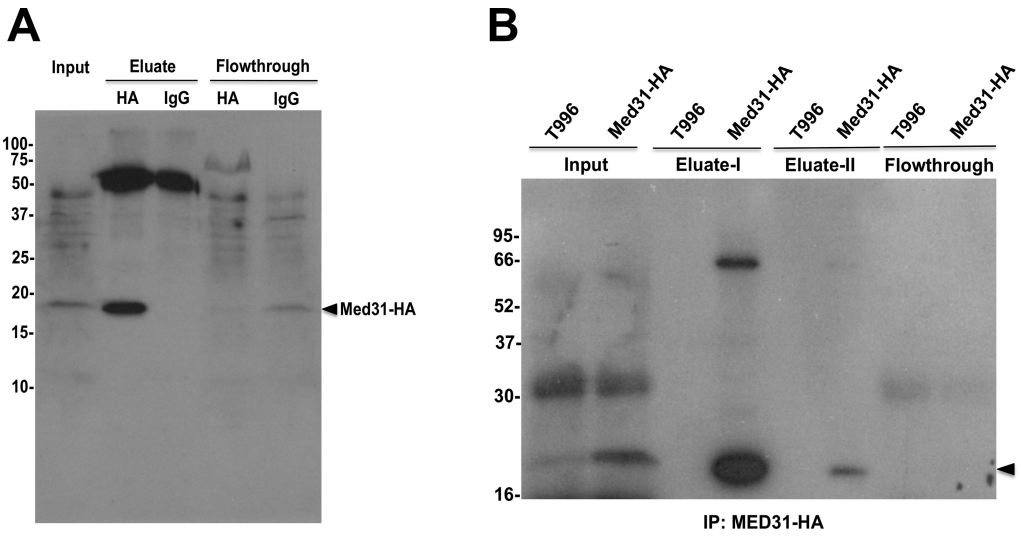

Figure 2. Immunoprecipitation of episomal PfMed31-HA protein. (A) PfMed31-HA protein was immunoprecipitated from Med31-HA parasites using the anti-HA antibody but not with a nonspecific IgG antibody (lanes labeled as Eluate). A heavy chain-specific secondary antibody was used for western blot, and so only the band corresponding to antibody heavy chain (~55 kDa) is observed in both eluate fractions using HA and IgG antibodies. (B) PfMed31-HA protein was immunoprecipitated from PfMed31-HA parasites and not from wild-type T996 parasite lysates. The arrowhead to the right denotes the position of PfMed31-HA at ~ 19 kDa. Highly similar results were obtained in a second independent experiment.

scalpel and subjected to an in-gel digestion protocol using trypsin for LC−MS/MS analysis, as described previously.[16] Briefly, the peptides were analyzed using a Dionex UltiMate 3000 RSLCnano system coupled to a Q Exactive instrument (Thermo Fisher Scientific, MA, USA). Separation was achieved with a Dionex EASY-Spray 75 μm × 10 cm column packed with PepMap C18 3 μm, 100 Å (Thermo Fisher Scientific) using solvent A (0.1% formic acid in 5% ACN) and solvent B (0.1% formic acid in 90% ACN) at a flow rate of 300 nL/min with a 60 min gradient. The Q Exactive apparatus with an EASY nanospray source (Thermo Fisher Scientific) was used to analyze peptides at an electrospray potential of 1.5 kV. A full MS scan (350−1600 m/z range) was obtained at a resolution of 70,000 at m/z 200 and a maximum ion accumulation time of 100 ms. Dynamic exclusion was set as 15 s, and the resolution of the higher energy collisional dissociation spectra was set to 17,500 at m/z 200. Single and unassigned charged ions were excluded from MS/MS. The MS/MS spectra of the raw data were processed and converted to the mascot generic file (mgf) format using Proteome Discoverer version 1.4 (Thermo Fisher Scientific). The mgf files were then used for protein sequence database search using

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Table 1. Presumptive Mediator Subunits of *P. falciparum* Identified by Co-IP and LC−MS/MS

s/ns/n	mediator subunit	module	gene ID	detected by MS	emPAI (Med31-HA)	emPAI (T996)[a]
1	Med6	head	PF3D7_1469700	yes	2.91	—
2	Med8	head	PF3D7_0411100	yes	1.40	—
3	Med11	head	PF3D7_0505900	no	NA	NA
4	Med17	head	PF3D7_0520200	yes	1.81	—
5	Med18	head	PF3D7_1213200	yes	2.57	—
6	Med20	head	PF3D7_1463000	no	NA	NA
7	Med22	head	PF3D7_1469800	yes	1.29	—
8	Med4	middle	PF3D7_1465200	yes	6.98	—
9	Med7	middle	PF3D7_0822100	yes	8.48	—
10	Med9	middle	PF3D7_1446700	no	NA	NA
11	Med10	middle	PF3D7_0707600	yes	2.20	—
12	Med21	middle	PF3D7_1126400	yes	5.85	—
13	Med31	middle	PF3D7_1475000	yes	0.65	—
14	Med14	scaffold	PF3D7_0709300	yes	1.49	—
15			PF3D7_0526800	yes	1.62	—
16			PF3D7_1363600	yes	3.67	—

[a]Proteins undetected in the control T996 co-IP sample are designated with a dash (−).

Table 2. Interaction Partners of PfMed31 Implicated in Transcription

s/n	protein	gene ID	emPAI (Med31-HA)	emPAI (T996)[a]
1	RPB2; DNA-directed RNA polymerase II subunit	PF3D7_0215700	0.09	—
2	RPB3; DNA-directed RNA polymerase II subunit	PF3D7_0923000	0.12	—
3	RPB9; DNA-directed RNA polymerase II subunit	PF3D7_0110400	0.15	—
4	GTF2H2; general transcription factor IIH subunit 2	PF3D7_1314900	0.04	—
5	ASF1; histone chaperone, putative	PF3D7_1224500	0.04	—
6	HP1; heterochromatin protein 1	PF3D7_1220900	0.15	—
7	BRD4; bromodomain protein 4, putative	PF3D7_1475600	0.05	—
8	AP2-O5; AP2 domain transcription factor, putative	PF3D7_1449500	0.05	—
9	zinc finger CCCH domain-containing protein, putative	PF3D7_1464200	0.03	—
10	TRA2B; alternative splicing factor	PF3D7_1002400	0.04	—
11	Snu13; spliceosome component	PF3D7_1123900	0.30	—
12	SF3B2; splicing factor 3B subunit 2	PF3D7_1461600	0.11	—
13	NCBP2/CBP20; nuclear cap-binding protein subunit 2	PF3D7_0415500	0.16	—

[a]Proteins undetected in the control T996 co-IP sample are designated with a dash (−).

Mascot algorithm version 2.41 to identify proteins using Uniprot *P. falciparum* (strain 3D7) protein sequence database with 4,314,641 residues, 5647 sequences (downloaded on 13 Aug 2014). Exponentially modified Protein Abundance Index (emPAI) was used for label-free protein quantification that was employed to determine the enrichment of proteins in each co-IP condition.

The significance of potential interaction partners was assessed by determining the ratio of emPAI scores obtained for experimental samples over that obtained for controls. Where no protein was detected in control samples, a value of 0.001 was initially assigned.

DATA AVAILABILITY

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE[17] partner repository with the dataset identifier PXD027640.

Username: reviewer_pxd027640@ebi.ac.uk Password: 6EfpRjNe. A summary of the results is presented in Table S1.

RESULTS

Coimmunoprecipitation and Mass Spectrometric Analysis. To detect the association of other Mediator proteins with PfMed31, we generated a parasite line, Med31-HA, that expresses hemagglutinin-tagged PfMed31 from an episomal vector. We chose PfMed31 as Med31 is the most conserved Mediator subunit across multiple species and is readily detected by simple bioinformatic searches of the *Plasmodium* spp. genome. The full-length PfMed31 open reading frame lacking the stop codon (399 bp) was cloned upstream of the hemagglutinin (3X-HA) tag already present in the pBcamR_3XHA-X vector (Figure 1A). Western blot of parasite protein lysates with anti-HA antibody revealed the expression of PfMed31-HA protein (∼19 kDa) in the Med31-HA strain and not in the wild-type T996 parent (Figure 1B).

To identify the protein-binding partners of PfMed31, we performed a coimmunoprecipitation (co-IP) experiment with the Med31-HA parasite strain using an anti-HA antibody. Western blot analysis showed that episomally expressed PfMed31-HA was successfully immunoprecipitated from parasite lysates with the anti-HA antibody and not with a nonspecific IgG antibody (Figure 2A). For subsequent mass spectrometric analysis, we scaled up the co-IP experiment and used the wild-type T996 parent strain as a negative control to rule out nonspecific interactions. Western blot analysis of the co-IP samples showed that the PfMed31-HA protein was immunoprecipitated from Med31-HA parasites (Figure 2B, Med31-HA Eluate I). The eluate from the wild-type T996

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Table 3. Interaction Partners of PfMed31 Implicated in the ER Function and ER Stress Response

s/n	protein	gene ID	function relating to ER and ER stress	detected in nucleus[23]	emPAI (Med31-HA)	emPAI (T996)[a]
1	Ramp4/Serp1	PF3D7_0219400	associated with Sec61 translocon complex gene target of XBP-1, a transcriptional effector of the UPR[21]	yes	0.04	—
2	Sec61g	PF3D7_0210000	one of the three proteins (a,b,g) forming the Sec61 protein-conducting channel of the ER translocon complex	no	0.54	—
3	DNAJ/Hsp40 (Pfj4)	PF3D7_1211400	mammalian genes encoding DNAJ/Hsp40 proteins such as ERdj4 are XBP-1 targets [21]	yes	0.57	—
4	Asna/Get3	PF3D7_0415000	required for tail anchoring of some proteins within the ER[37]	NA	0.10	—
5	TMED/p24 family	PF3D7_1314500	loss of TMED function in both yeast and *Arabidopsis* induces UPR	yes; exclusively	0.19	—
6	Rab5c	PF3D7_0106800	macroautophagy in other organisms (Ao 2014)	ND	0.19	—
7	RPN7	PF3D7_1129200	26S proteasome regulatory complex subunit	yes	0.31	—
8	RPN9	PF3D7_1030500	26S proteasome regulatory complex subunit	yes	0.20	—

[a]Proteins undetected in the control T996 co-IP sample are designated with a dash (−).

strain did not show any bands, supporting the specificity of the anti-HA antibody. A two-step elution was done using Laemmli buffer with most proteins extracted in the first elution step (Eluate I & II, Figure 2).

To detect protein partners of PfMed31, the IP eluate samples were further analyzed using LC-MS/MS (liquid chromatography coupled with tandem mass spectrometry). The peptide spectra were searched against the *P. falciparum* database to identify proteins associated with PfMed31. A total of 846 proteins were identified in the co-IP experiment (Table S1). Multiple proteins were exclusively detected in the HA-PfMed31 sample. Within this group, it is striking that those proteins with the highest emPAI scores are predicted components of the parasite Mediator complex[13] and include PfMed31 (Table 1).

These previously annotated *P. falciparum* Mediator subunits correspond to components of the head and middle Mediator modules along with the PfMed14 scaffold. These results, therefore, provide physical evidence for the presence of the *P. falciparum* Mediator complex, a feature that has only been computationally predicted to date. The predicted subunits PfMed9, PfMed11, and PfMed20 were not identified in our dataset. Importantly, two uncharacterized proteins, PF3D7_0526800 (368 amino acids) and PF3D7_1363600 (227 amino acids), were among the highest-scoring proteins detected, suggesting that they may be evolutionarily diverged Mediator components specific to the malaria parasite.

Within the group of presumptive PfMed31 interaction partners not detected in the negative control sample are additional proteins with functions in transcription (Table 2). These include two subunits of RNAPII (RPB2 and RPB3) and subunit 2 of the general transcription factor TFIIH. The subunit RPB9 of RNAPII was also found though with a low detectable presence in the negative control. Proteins with functions in chromatin modification and structure such as histone chaperone ASF1 (PF3D7_1224500), heterochromatin protein 1 (PF3D7_1220900), and a bromodomain protein 4 (PF3D7_1475600) were likewise identified. Presumptive transcription factors associated with PfMed31 include a putative ApiAP2-domain transcription factor (PF3D7_1449500) and a putative zinc finger protein (PF3D7_1464200). Interestingly, several proteins involved in mRNA processing and function were detected with moderate confidence. The association of PfMed31 with predicted Mediator subunits, RNAPII, and additional gene products implicated in transcriptional and post-transcriptional processes are all consistent with a conserved role for the Mediator complex in regulating gene expression in *P. falciparum*.

Among the presumptive partners showing strong to moderate interaction with PfMed31 are several proteins implicated in endoplasmic reticulum (ER) function and the ER stress (ERS) response (Table 3). The ERS response involves the deployment and coordination of the unfolded protein response (UPR), autophagy, and the ubiquitin-proteasome system (UPS), among other processes.[18] The initiation of a transcriptional program is key to the UPR; however, *P. falciparum* lacks homologs for the key transcriptional effectors of the UPR described in other eukaryotes, namely ATF4, ATF6, and XBP1.[19] Sec61 gamma is one of the three subunits forming the Sec61 ER translocon channel.[20] Serp1/Ramp4 associates with Sec61 and is upregulated under ER stress,[21,22] while DNAJ-type Hsp40 proteins, such as ERdj4, are XBP1 targets in mammals;[21] both proteins are detected in the parasite nucleus during the blood stage of infection.[23] Given the absence of transcriptional effectors of the ERS response in*P. falciparum*, our results suggest that Serp1/Ramp4, Sec61g, and Hsp40 could act through association with Mediator to regulate ER function and the ERS response. A role for the presumptive parasite Mediator complex in the ERS response is supported by the identification of additional proteins implicated in the UPR and 26S proteasome function, a number of which have been detected in the parasite nucleus.[23] This hypothesis is relevant to artemisinin resistance in the parasite, which has been proposed to depend upon activation of the ERS response.[19,24]

DISCUSSION

Computational analyses have identified 14 Mediator subunits encoded by the *P. falciparum* genome,[13,14] but biochemical evidence for the presence of a parasite Mediator complex has been lacking. Med31 is one of the most highly conserved Mediator subunits[25] and is a component of the Mediator middle module.[26] Using coimmunoprecipitation of tagged PfMed31 expressed in blood-stage parasites followed by mass spectrometric analysis, we now demonstrate for the first time the physical presence of the Mediator complex in *P. falciparum*. In total, 11 out of 14 putative Mediator subunits were among the highest-scoring proteins in our study. Yeast two-hybrid experiments, coimmunoprecipitation, and structural analyses have shown that Med31 specifically interacts with Med7, Med10, and Med21.[27−29] The parasite orthologs of each of

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these subunits are shown to interact with PfMed31 in the current study.

Med14, the core subunit of the Mediator complex in model eukaryotes, acts as a scaffold that stabilizes the head, middle, and tail modules[7] and would be expected to be indispensable for the Mediator function. *P. falciparum* has been computationally predicted to encode Med14,[13] and indeed this protein is identified in our study with high confidence.

Apart from Med30, all components of the yeast head module are encoded by the parasite.[13] Most of these predicted head module subunits except Med11 and Med20 have been physically identified as PfMed31 interaction partners in this study. The head and middle modules are connected through the Med17 (head)−Med21 (middle) interaction.[27] Our results reveal that these conserved subunits are indeed expressed in the parasite and associate with PfMed31. A 3D model of the middle module in yeast reveals a tetramer formed by heterodimeric subcomplexes of Med4/Med9 and Med7/Med21.[30] Three of these proteins have been identified as PfMed31 partners in the current study, while PfMed9 is predicted computationally. Since Med9 interacts with Med4, affinity purification of PfMed4 could lead to the isolation of PfMed9 from parasite protein lysates. The CKM module associates with the core Mediator only under certain conditions and is known to be involved in transcriptional repression. Computational studies of the *P. falciparum* genome have neither detected genes encoding members of the CKM nor have any been identified through our MS results. Interestingly, the CKM interaction with the core Mediator involves direct contact with Med19[26] which has not been identified in the *P. falciparum* genome.

The basic function of tail module subunits is to link Mediator to sequence-specific activators.[31] Structurally, Med14 spans the entire Mediator complex, bridging all the modules.[26] Apart from its architectural role, it is also required for Mediator to be transcriptionally active. Cevher et al.[32] observed that although a complex of 13 subunits derived from the head and middle modules was stable, Med14 was required for basal and coactivator functions of the complex under *in vitro* assay conditions.[32] That study and another by Plaschka et al.[33] have defined the minimum set of subunits required to assemble a functional Mediator complex under in vitro conditions. This minimal complex, termed the core Mediator, is composed of head and middle module subunits supported by the Med14 subunit. Med9 and Med19 have been identified as components of the core Mediator in yeast but not in humans, although these subunits are found in humans as well. Med30 is a component of the human core Mediator and has not been identified in yeast. Therefore, the composition of the functional core Mediator complex seems to vary among species. The predicted Mediator complex in *P. falciparum* is similar to the yeast core Mediator except for the Med19 subunit. It is unclear whether the parasite has only retained the minimal set of subunits, which are required for its basic functions, or if it contains other subunits required for specialized functions.

In the current study, two uncharacterized *P. falciparum* proteins, PF3D7_0526800 and PF3D7_1363600, were also identified as interacting partners of PfMed31. These two proteins have strong emPAI scores and are not detected in the negative control. To identify whether these protein sequences are related to known Mediator subunits, full-length protein sequences were analyzed by BLASTp and PSI-BLAST. However, these search strategies did not yield any matches with known Mediator subunits from other species nor were they detected in the careful bioinformatics approach of Bourbon.[13] Mediator subunits are known to contain intrinsically disordered regions (IDRs), which do not form any well-defined three-dimensional structures. These IDRs are thought to impart structural flexibility to the Mediator.[34] Disordered regions of 27−30 amino acids were found in both the proteins, one in the product of PF3D7_1363600 and two in that of PF3D7_0526800. Some Mediator subunits are species-specific, and it is therefore plausible that these two uncharacterized proteins are parasite-specific Mediator subunits with novel functions. For example, they could constitute a novel tail region required for interaction with transcription factors such as those of the ApiAP2 family.[35,36] Interestingly, an ApiAP2 factor is detected here as a potential PfMed31 interaction partner.

CONCLUSIONS

We report here the first physical evidence of the Mediator complex in *P. falciparum* using a proteomics approach (co-IP followed by LC−MS/MS). Our data confirm the presence of 11 out of 14 predicted *P. falciparum* Mediator subunits as strong interaction partners of PfMed31. Furthermore, our mass spectrometry analysis identified two uncharacterized proteins, PF3D7_0526800 and PF3D7_1363600, which are strongly associated with the Mediator complex and could constitute the parasite Mediator tail region. Additional interaction partners support the role of the Mediator in transcriptional control during the blood-stage infection and suggest a role for the complex in the coordination of the ERS response.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c00368.

Mass spectrometry results (XLSX)

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Notes

The authors declare no competing financial interest.

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