Structural insights into histone chaperone Asf1 and its characterization from *Plasmodium falciparum*

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Abstract

Asf1 is a highly-conserved histone chaperone that regulates tightly-coupled nucleosome assembly/disassembly process. We observed that *Plasmodium falciparum* Asf1 (PfAsf1) is ubiquitously expressed in different stages of the life cycle of the parasite. To gain further insight into its biological activity, we solved the structure of N-terminal histone chaperone domain of PfAsf1 (1-159 amino acids) by X-ray crystallography to a resolution of 2.4 Å. The structure is composed of two beta-sheet to form a beta-sandwich, which resembles an immunoglobulin-like fold. The surface-charge distribution of PfAsf1 is distinct from yAsf1 and hAsf1 although the core-structure shows significant similarity. The crystal-structure indicated that PfAsf1 may exist in a dimeric-state which was further confirmed by solution cross-linking experiment. PfAsf1 was found to specifically interact with *Plasmodium* histone H3 and H4 and was able to deposit H3/H4 dimer onto DNA-template to form disomes, showing its characteristic histone chaperone activity. We mapped the critical residues of PfAsf1 involved in histone H3/H4 interaction and confirmed by site directed mutagenesis. Further analysis indicates that histone interacting surface of Asf1 is highly conserved while the dimerization interface is variable. Our results identify the role of PfAsf1 as mediator of chromatin assembly in *Plasmodium falciparum*, which is the causative agent of malignant malaria in humans.
Introduction

Malaria is a mosquito-borne severe disease which is caused by a unicellular protozoa of the Plasmodium genus. According to the WHO Report, more than 200 million new cases of the disease are reported every year[1]. Among the five Plasmodium species, *Plasmodium falciparum* is the most virulent one causing major death world-wide[2,3]. Although malaria is a treatable disease, in the present scenario there is a surge in resistance to the available anti-malarial drugs[4,5]. This necessitates the identification of new generation anti-malarial targets. Chromatin assembly/disassembly is tightly regulated by various macromolecular machinery including ATP-dependent chromatin remodelling enzymes and histone chaperones which act in a well-coordinated manner in depositing either the canonical or variant histones onto DNA.

Histone chaperones are a ubiquitous class of acidic proteins which are involved in interacting with histones thereby preventing their non-specific aggregation and faithful deposition onto DNA[6–8]. Chromatin assembly is a multi-tiered process from histone synthesis to its deposition where histone chaperones are involved in multiple stages[9]. Post-synthesis of histones, the histone chaperones are involved in histone storage followed by transport from cytosol to nucleus. Within the nucleus the histone chaperones assists in assembly of histones in a step-wise manner. The assembly of H3/H4 tetramer to form tetrasome happens first. Subsequently two H2A/H2B dimer assembles to form the nucleosomes[10,11]. Chaperoning of H2A-H2B dimer is mediated by Nucleosome assembly proteins (such as Nap1)[12,13] in concert with chromatin-remodeling enzymes (such as ACF and CHD1)[14,15]. It has been shown that yNap1 can exchange H2A/H2B dimer thereby having significant role in chromatin transcription[16,17]. The assembly of two H3/H4 dimers onto DNA to form the tetrasome is mediated by different chaperones, either in a replication-dependent (mediated by CAF1)[18–20] or independent (mediated by HIRA) manner[21–23]. Chaperone-mediated chromatin assembly clearly has a preference for either canonical or variant histones. The H3 variant CENP-A assembly is mediated by HJURP[24,25] and the crystal structure of CENP-A harbouring nucleosomes has been shown to be more rigid than the canonical nucleosomes[26,27]. The variant histone H3.3 assembly onto chromatin mediated by HIRA or DAXX triggers transcription programs
in cells[28–30]. H2AZ, another transcription favouring variant of histone H2A, is assembled by ANP32E[31,32].

An upstream crucial chaperone implicated in both the replication-coupled and independent H3/H4 assembly onto chromatin is Anti-silencing function 1 (Asf1). Histone H3.1/H4 gets siphoned by Asf1 to CAF1 which deposits into DNA in replication-dependent manner[33,34]. Acetylated histones show a tight association to CAF1 complex. Asf1 plays a crucial role in H3K56 acetylation[35,36] mediated by Histone acetyltransferase, Rtt109 in yeast[37,38] and p300/CBP in human[39,40]. Acetylated H3K56 (H3K56Ac), is one of the first reported globular domain modification of histone H3, which plays crucial role in DNA damage response. The deposition of H3.3/H4 to DNA is also mediated by Asf1 which transfers the complex to HIRA or DAXX[22,41]. The structure of Asf1/HIRA complex suggest that the beta-sandwich domain of Asf1 interact perpendicularly to the B domain of HIRA[22,42]. Further, interaction of Asf1 with HIRA and CAF1 (through p60 subunit) is mutually exclusive. The crystal structure of yeast and human Asf1-H3/H4 complex elucidates how Asf1 stabilizes H3/H4 dimer and prevents formation of tetramer assembly[43,44]. Further these complex structures provide insights on how the histone H3 C-terminal tail is involved in interaction with Asf1. Although the Asf1 has been reported from Chordates to Euglenozoans, no significant information is available from the Apicomplexans genus. There are two isoforms of Asf1 in vertebrates, plants and C.elegans, however in case of yeast and Drosophila only single gene for Asf1 has been reported.

Characterization of histone chaperone Asf1 from *Trypanosoma brucei*, a protozoan parasite, which causes sleeping sickness, has been reported[45,46]. Two isoforms of Asf1 from *T. brucei* with distinct localization and function have been described. On the other hand, Asf1 from *Leishmania major* has been shown to play an important role in DNA damage response in association with chromatin remodelling machinery[47]. It is interesting to note that although the structural insights of Asf1 is available from yeast and human, no such report is available yet from that of any of the unicellular pathogenic microorganisms.

The genome of *Plasmodium falciparum* is unique among eukaryotes in having extremely high AT content[48–50]. Because of its unique genome architecture and proteome signature, many of its
proteins have been found to be structurally divergent, which accounts for its adaptive survival and propagation strategies to complete its life cycle spanning across human and mosquito hosts. Many of the chromatin interacting proteins of *Plasmodium falciparum* have been structurally and functionally characterized[51–55]. One well characterized group of histone chaperone proteins from *P. falciparum* are nucleosome assembly proteins, PfNAPS and PfNAPL[56]. Their histone binding and histone chaperoning activity has been established structurally and functionally and these proteins were shown to bind H3-H4 tetramer preferably [57–59].

Identification and biochemical characterization of Asf1 from *P. falciparum* has remained elusive. Database search yielded existence of only one isoform of putative Asf1 in *Plasmodium falciparum* (PF3D7_1224500). We report here that PfAsf1 is expressed in all the stages of the Plasmodium lifecycle. Further, it interacts specifically with histone H3 and H4 from *Plasmodium falciparum* and is able to deposit histones onto DNA template to form disomes thereby acting as a histone chaperone. We have subsequently cloned, expressed and purified PfAsf1 N terminal domain (1-159), which has been referred to as PfAsf1-NTD. We then crystallized and solved the structure of PfAsf1-NTD which elucidate a conserved beta-sandwich fold. Interestingly, the structure suggest that PfAsf1-NTD may exist as a dimer. Comprehensively, our study showed the chromatin assembly and disassembly process mediated by the histone chaperone Asf1 from the uniquely evolved malarial parasite *Plasmodium falciparum*.

**Materials and methods**

**Phylogenetic analysis**

Amino acid sequence of Asf1/putative Asf1 protein from different organisms representing different phyla was retrieved from Uniprot database (accession codes enlisted) in FASTA format. Multiple sequence alignment using MUSCLE programme in MEGAX [60] software package were performed and this aligned sequences were used to generate the phylogenetic tree. The tree depicting evolutionary history was generated by Maximum Likelihood method and JTT-matrix based model.
by applying a total of 250 bootstrap iterations. Matrix of pairwise distances was estimated using the JTT model. Using this estimated matrix initial tree(s) for heuristic search were automatically obtained using Neighbour-Join and BioNJ algorithms and then choosing the topology with superior log likelihood value. The generated Newick tree was exported and the figure for phylogentic tree was prepared using Figtree.

Cloning, protein expression and purification

The nucleotide sequence corresponding to amino acid 1-273 of putative histone chaperone Asf1 from *Plasmodium falciparum* 3D7 (PF3D7_1224500) was PCR amplified from cDNA and cloned using NdeI and XhoI restriction sites in MCS of pCold-II (Clontech, Takara) bacterial expression vector containing an N-terminal hexa-histidine tag. The gene encoding the stretch of amino acid residues (1-159) encompassing the putative histone chaperone domain of Asf1 from *Plasmodium falciparum* 3D7 was codon optimized and synthesized for optimal recombinant protein expression in bacteria. PfAsf1-NTD was cloned into pDEST-17 and pDEST-15 vectors using Gateway technology containing the poly-histidine and GST tags respectively. DH5 alpha cell was transformed for propagation of the PfAsf1-NTD clone. Rosetta P-Lys strain of *Escherichia coli* were transformed and plated on LB agar plate containing ampicillin (100µg/ml) and chloramphenicol (34µg/ml). 2 litre culture was grown in LB media containing ampicillin and chloramphenicol by vigorous shaking at 200rpm @ 37°C till the culture reached an OD$_{600nm}$ value between 0.6 and 0.8. Afterwards protein expression was induced for a period of 16 hrs by adding 1mM IPTG and shaking at 200rpm @ 20°C. For his-tagged PfAsf1-NTD, the cells were harvested by centrifuging @ 6000rpm at 4°C and resuspended on ice in lysis buffer (20mM Tris-Cl pH 8.0, 200mM KCl, 50mM Immidazole, 5% Glycerol, and 1 mM DTT, 0.05% NP-40). The cell lysate was sonicated and then clarified by centrifuging at 18000 rpm @ 4°C for 30mins for 2 times. The supernatant was collected and filtered with a 0.22 µ filter. The filtered supernatant was incubated with pre-equilibrated Ni-Sepharose beads (equilibrated in lysis buffer without adding glycerol and NP-40) at 4°C for 1.5 hrs. After incubation, the beads were washed with wash buffer (20mM Tris-Cl pH 8.0, 300mM KCl, 50mM Immidazole, 5% Glycerol, 1 mM DTT, 0.05% NP-40)
thoroughly. The washed beads were then incubated with precision protease enzyme for 40 hrs @ 4°C to cleave the hexa-histidine tag form the protein in precision protease buffer (20mM Tris-Cl pH 7.5, 150mM KCl and 1mM DTT). All the samples from each step were then analyzed on SDS-PAGE gel. The cleaved protein was then eluted using the same precision protease buffer and then passed through a gel filtration column (Superdex 75 16/600 pg from GE healthcare) to remove impurities. SEC-MALS (Multiple Angle Light Scattering) analysis was performed by injecting PfAsf1-NTD into Superdex 200 increase 10/300 GL column mounted on an HPLC system. Corresponding light scattering (LS) and refractive index (RI) was monitored by DAWN 8 and Optilab detector systems from Wyatt Technologies. The SEC-MALS data was analysed and plotted using Astra Software. GST-tagged PfAsf1-NTD was also purified using similar method with a different lysis buffer (20mM Tris-Cl pH 8.0, 200mM NaCl, 5% Glycerol, 1 mM DTT, 0.05% NP-40) and wash buffer (20mM Tris-Cl pH 8.0, 300mM NaCl, 5% Glycerol, 1 mM DTT) composition. When required, GST and histagged PfAsf1 were eluted after purification using linear gradient of glutathione and imidazole respectively. PfAsf1 full length (1-273) was purified under denaturing condition by refolding since it got expressed mainly in inclusion bodies. Briefly, the inclusion bodies containing PfAsf1 (1-273) protein was solubilised in denaturing condition in 6M Urea, 20mM Tris-Cl pH 7.5, 200mM NaCl, 1mM beta-merceptoethanol. The solubilised protein was affinity purified using Ni-NTA resin and eluted by imidizaole gradient. Urea was gradually removed by step dialysis at 4°C using a dialysis membrane with a 3.5 kDa molecular weight cut off. Urea concentration was reduced in the dialysis buffer (20mM Tris-Cl pH 8.0, 200mM NaCl, 2mM beta-merceptaethanol) in successive steps (6M, 4M, 2M, 1M, 0.5 M, 0.25 M and finally no urea) to allow refolding of denatured PfAsf1-FL. After dialysis precipitants were removed by high speed centrifugation (12000 rpm for 10 mins) under cold condition. The folded nature of protein was ascertained using circular dichroism spectroscopy. Protein concentration was estimated by Bradford assay.

Crystallization and Data collection

Purified PfAsf1-NTD protein was concentrated to 10mg/ml using an amicon concentrator from Millipore of 10kDa cut-off. Concentrated protein was used to set up several crystallization screens
using Emerald Biosciences Classic Screen using sitting drop method in 96 wells crystallization trays. For each condition in the trial 1μl of protein solution and 1μl of well solution was mixed and the drops were equilibrated at 20°C against a well solution volume of 100μl. Initial hits obtained from selected drops were optimized and a crystal of considerable size was obtained in a crystallization condition containing 200mM Sodium thiocyanate and 30% PEG3350. The crystals diffracted poorly and thus after several rounds of optimization we obtained good quality protein crystals in condition containing 100mM Sodium thiocyanate, 30% PEG3350 and 0.1M MES pH 6.5. The crystals were soaked in cryoprotectant solution containing 30% ethylene-glycol in mother liquor followed by loop mounting and flash cooling in liquid nitrogen. The diffraction data was collected using our in house rotating anode X-Ray Diffraction facility (Rigaku) with Mar345 image plate detector mounted on it. PfAsf1 crystallized in tetragonal space group P4₁ with unit cell parameters a=62.733 Å, b=62.733 Å, c=98.725 Å. The diffraction data was processed using XDS[62], merged and scaled respectively using Pointless and Aimless programs from CCP4 software suites[63–65].

**Structure determination**

The structure of PfAsf1-NTD was solved by molecular replacement using Yeast Asf1 (PDB ID: 2HUE) as search model. Molecular replacement was carried out using Phaser from Phenix suite[66]. The initial structure solution generated was refined using iterative cycle of refinement till a solution was obtained with acceptable statistics. Refinement and model building were done using PHENIX and COOT. The model was validated using MolProbidity. Graphic figures of the PfAsf1 structural models were prepared using Pymol graphics software[67] and Chimera [68]. The statistics pertaining to data collection, processing and refinement are presented in Table 1. The final PfAsf1 structure has been deposited in Protein Data Bank (PDB ID: 6A6Y).

**Antibody generation**

Polyclonal anti-PfAsf1 antibody was raised commercially using purified recombinant PfAsf1-NTD protein. 200ug of purified recombinant PfAsf1-NTD was injected subcutaneously into NZ white rabbit using Freund complete adjuvant in 1:1 ratio. This was followed by five booster doses at every 2-week interval after the first injection. The booster doses were given using Freund’s incomplete
adjuvant. Serum was obtained from blood collected from 6th bleed and finally purified PfAsf1 antibody was obtained by affinity chromatography. The specificity of the generated anti-PfAsf1 antibody was checked by assessing their binding to various concentration of recombinant PfAsf1 protein by western blot.

**Histone deposition EMSA**

EMSA based histone deposition assay on DNA was carried out following standard method[69] with some modifications. Human H3.1/H4 tetramer was purchased from New England Biolabs (catalogue no. M2509S) and 80 bp DNA fragments corresponding to nucleosome positioning sequence was prepared by PCR amplification using the 601_80 bp Forward (GTCGTAGACAGCTCTAGCA) and 601_80bp Reverse (TAGGGAGTAATCCCCTTG) set of primers as detailed in Scorgie et al., 2012 [69] using widom 601 plasmid DNA as template. The PCR product obtained was checked for purity by running on agarose gel and finally gel purified in water. Various concentrations of GST-PfAsf1-NTD (2-6μM) and 0.25 μM of histones H3/H4 were incubated in a reaction buffer containing 20mM Hepes pH 7.5, 200mM NaCl and 6% glycerol at room temperature for 15 minutes followed by addition of 80 bp DNA at a final concentration of around 100nM. After addition of 80bp DNA, the total reaction mix was further incubated at room temperature for approximately 1 hr. The reaction mix was then directly loaded onto pre-run 7% PAGE and resolved in 1X TAE buffer at 100 Volts for 90 minutes. The gel was stained with sybr green (invitrogen) and visualized using SYBR green filter in Gel-doc system. Afterwards the gel was transferred onto nitrocellulose membrane and processed using routine western blot method to probe for the presence of histone proteins in the newly formed species as evident in the gel stained with SYBR green.

**Pull down assays**

GST pull down assays was performed following a standard protocol[70] with some modifications. Briefly, purified GST-PfAsf1-NTD and its mutant proteins were incubated with Plasmodium histones (PfH2A, PfH2B, PfH3 and PfH4) at equimolar concentration at 4°C overnight in pull down assay buffer (50mM Tris-Cl pH 7.5, 150mM NaCl, 2mM DTT, 0.1% NP40 & 1mM PMSF). Glutathione agarose resin was pre-blocked in parallel with 5% BSA in pull down buffer. Then pre-blocked
glutathione-agarose resins was added to the reaction mix and incubated for further 2 hrs at 4°C. The bound resin was washed for four times and then boiled upon addition of laemmlı buffer. The samples and 10% input were then resolved on SDS-PAGE, transferred onto nitrocellulose membrane and blocked in 5% BSA solution. The membrane was further probed with anti-H3 (ab10799), anti-H4 (ab10158) and anti-GST antibodies (5475S).

**Cross-linking assay:**

PfAsf1 was purified in a buffer containing 20mM Hepes and 200mM NaCl. Stocks of sulpho-EGS and was made as per manufacturer's protocol (Thermo Scientific). Then diluted stocks of cross-linker of different concentrations (0.25 to 2.5mM) was incubated with 1mg/ml of PfAsf1-NTD in the above-mentioned buffer and incubated at room temperature for 1 hr followed by quenching of the reaction by addition of 50mM of Tris pH 7.5. After addition of laemmlı buffer the samples were loaded onto SDS-PAGE gel and resolved and stained with Coomassie blue and photographed using Bio-Rad gel documentation system.

**Parasite culture**

*P. falciparum*, 3D7 strain was cultured in fresh human erythrocytes at 1% hematocrit in complete RPMI-1640 (HEPES modified) medium (Sigma) supplemented with 0.5% AlbuMaxII, 0.2% glucose, 0.2% NaHCO3 and 100µM hypoxanthine. The culture methodology is based on a published protocol[71]. Parasite growth rate and stage was determined by the examination of acridine orange stained thin smears of the infected RBCs (iRBCs). To perform stage specific studies, parasites were synchronized by serial treatment with 5% D-sorbitol at ring stage and percoll gradient to isolate schizont stage.

**Localization of ASF-1 in Plasmodium**

iRBCs were washed twice with 1X PBS and cells were fixed with 4% paraformaldehyde containing 0.0075% gluteraldehyde for 30 min followed by permeabilization using 0.1% Triton-X for 15 min. Further cells were incubated with anti-*PfAsf-1* antibody at 4°C for overnight. Cells were washed with blocking buffer and incubated with secondary anti rabbit antibody labelled with alexa flour-567 for 1h and then cell were washed and mounted with antifade mounting media containing DAPI for 10 min.
Cells were analyzed under 63x objective of Zeiss confocal microscope.

**Stage specific protein expression of PfAsf1**

To obtain cell free parasite from iRBCs, pellet were incubated with 0.15% saponin for 6 min at room temperature followed by centrifugation at 1000g for 5 min. Pellet were washed twice with PBS and 4 volume of lysis buffer (100Mm Tris-HCl pH 7.4, 100Mm NaCl, 2.5Mm MgCl₂, 0.5% NP-40, 0.5% Triton-X-100) was added and incubated for 10 min at 4°C and the suspension was titrated through 26 guaze syringe three times followed by centrifugation at 10000 rpm for 10 min at 4°C and the supernatant (parasite lysate) was collected. The protein samples were resolved on 12% SDS-page followed by transfer to PVDF membrane. The membranes were blocked with 1% milk for 1h at room temp and incubated with anti-PfAsf-1 antibody for overnight at 4°C. Blot were washed and further probed with secondary anti rabbit antibody for 2h at room temperature. Signals were developed with SuperSignal west dura extended duration substrate kit.

**Surface plasmon resonance**

The SPR experiment was performed using Biacore 3000 from GE healthcare following a reported method[72] with modifications. Briefly, individual human histones H3 (M2503S) and H4 (M2504S) as well as H3-H4 tetramer (M2509S) from New England Biolabs (were immobilized on C1 sensor chip (GE healthcare) based on non-covalent interaction of histones with the sensor surface. The running buffer contained 10mM Tris-Cl pH 7.8, 100mM Nacl, 0.5mM DTT, 50uM EDTA pH 8.0) and immobilization was done at a flow rate of 10uL per minute with a total injection duration of 2.5 minutes. After stabilization time of 5 mins, different concentration of PfAsf1 (100nM-1250nM) were injected at a flow rate of 20ul/min and association was recorded for 3 minutes followed by monitoring of dissociation for 8 mins. After each cycle of analyte injection, the surface was regenerated and the ligand was immobilized again for further cycles of kinetic experiment. The surface was regenerated by two injections of 0.3% SDS of 30 seconds duration at a flow rate of 50ul/min followed by a stabilization time of 10 min. The instrument temperature was maintained at 20°C for all the SPR experiments. All the binding kinetics experiments were performed in duplicate and the obtained SPR curves were fitted using a 1:1 local fitting model using BIAevaluation software.
Site directed mutagenesis

All the mutations in plasmids were introduced using QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Mutation was confirmed by sequencing the whole gene.

Results

Evolutionary analysis of PfAsf1

Asf1 exists from unicellular protozoan to higher eukaryotes. Although the structure and function of Asf1 protein is well characterized in human and yeast, its existence in Apicomplexans has remained enigmatic. Plasmodium histone chaperones are known to play crucial role in chromatin organization and gene expression. However, no specific information on the biological function of Asf1 in Plasmodium species was available. A blastp search in the PlasmoDB using the *Saccharomyces cerevisiae* Asf1 sequence revealed existence of a putative chromatin assembly protein Asf1 (PF3D7_1224500) as a probable homologue. The putative Asf1 gene in *Plasmodium falciparum* is located in Chromosome 20 and the protein is comprised of 273 amino acids. The N terminal region of Asf1 protein contains the conserved histone chaperone domain (Figure 1A). In all of the *Plasmodium* sp. and two fungi species i.e., *Saccharomyces cerevisiae* and *Pyronema omphalodes*, Asf1 has an extended C terminal tail. Interestingly among the protozans, only apicomplexan species harbour the C terminal tail whereas in the euglenozoans species such as *Trypanosoma brucei* and *Leishmania major*, C terminal stretch is not significant. The C-terminal portion of ScAsf1 contains disordered segment, which is evident by presence of poly glutamic acid stretch, which promotes the histone H3/H4 interaction. Likewise, PfAsf1 also contains a poly-asparagine rich C-terminal tail, although its function is yet unknown. To understand the similarity of PfAsf1 across different species we generated a phylogenetic tree using a maximum likelihood approach, which revealed extensive divergence of PfAsf1 among the eukaryotic organisms (Figure 1B). Interestingly there is considerable divergence of PfAsf1 compared to its closely related protozoan parasites belonging to genus *Trypanosoma*, although they share the same node. Remarkably, trypanosomes harbour two isoforms of Asf1[46], which is a characteristic of higher vertebrates, whereas PfAsf1, like its yeast homologue (yAsf1) harbours a
single Asf1 isoform. This might indicate a distinct mode of evolution of Asf1 in Plasmodia and Trypanosome parasites, which can impact their histone chaperone function and consequent chromatin assembly.

**PfAsf1 is expressed in all stages of the erythrocytic life cycle of *P. falciparum***

In order to characterize the biological function of PfAsf1, we cloned, expressed and purified full length and N-terminal histone chaperone domain (1-159). The recombinant protein harbouring the histone chaperone domain was used to generate specific antibody. Western blotting analysis could detect both the full length and the histone chaperone domain confirming the specificity of the antibody (Figure 2A). In order to understand the stage-specific abundance and distribution of PfAsf1, *P. falciparum* cultures were synchronized and parasite cells were collected from each of the asexual erythrocytic stages (Ring, Trophozoite & Schizont). Immunoblotting experiments with whole cell lysates prepared from each of these asexual blood stages revealed differential expression of PfAsf1 at protein level (Figure 2B). PfAsf1 protein expression was found to be higher in schizont stage compared to ring and trophozoite stage. Since, in Schizont stage, *Plasmodium falciparum* goes through multiple rounds of DNA replication, the abundance of Asf1 at this stage could be instrumental in histone deposition onto the newly synthesised DNA mediating proper chromatin assembly. In order determine the subcellular localization of this protein, we performed immunofluorescence microscopy which indicated the presence of PfAsf1 mainly in the nucleus in all the erythrocytic asexual life cycle stages (Figure 2C). These results indicate that PfAsf1 is ubiquitously present in different stages of Plasmodium life cycle and its expression reaches a peak at the Schizont stage.

**Crystal structure of PfAsf1**

In order to determine the crystal structure of PfAsf1-NTD, we first purified the soluble protein from E.coli. We further performed size exclusion chromatography to obtain high purity protein which was used for crystallization. The purified protein till homogeneity was further confirmed through SDS PAGE (Fig.3A) which was very stable and did not precipitate upon concentration. We crystallized...
and solved the structure of PfAsf1 at 2.4 Å resolution. The structure elucidated that PfAsf1-NTD consists of ten anti-parallel beta strands forming two beta sheets (Fig. 3B). The top beta sheet is formed by three beta strands viz. β1, β3 and β6, whereas the bottom beta sheet is formed by five beta strands viz. β5, β4, β7, β8 and β10. These two beta sheets form the beta sandwich and are held together by tight hydrophobic core which is formed by F24, I26, F28, W40, I42, L66, M73, F75 and Y77 residues. There is a small beta sheet (β2, β9 and part of β8) and three small alpha helix (α1, α2 and α3) which is located at the top of the beta sandwich. The structure is elongated along the y-axis with an overall dimension of 57 Å X 20 Å X 12 Å (L x W x D) which is observed upon 90° rotation (Fig. 3C). The overall topology of the protein is similar to “switched” immunoglobulin family of proteins.

In order to identify how Plasmodium Asf1 is similar to yeast and human histone chaperone counterparts, we performed sequence alignment between PfAsf1, ScAsf1 and HsAsf1. Interestingly, it was observed that the sequence conservation was more on the secondary structure constituted by the beta strands, as compared to the the loop regions connecting the beta strands (Fig. 4A). In order to understand the overall conservation in 3D structure of Asf1 between yeast (colour coded brown), human (colour coded cyan) and Plasmodium (colour coded pink) species, we performed structural superposition which clearly indicated a conserved fold between all the three proteins (Fig. 4B). Root Mean Square Deviation (RMSD) upon Cα atom superposition indicated that there is a structural deviation between Plasmodium and human Asf1 of 1.2 Å and that between Plasmodium and yeast of 1.25 Å. The residue-wise deviation plot shows that the loops connecting the β strand show higher deviation compared to the core structure (Fig. 4C). We next performed surface charge distribution of Plasmodium falciparum Asf1 and compared it to yeast and human Asf1. We structurally aligned all the three Asf1 in similar orientation to compare the charge distribution difference among them. As observed there are distinct surface charge distribution pattern amongst all the three Asf1 (Fig. 4D), indicating that even though they are structurally similar, there could be alteration in its interacting partner of choice depending upon the nature of its surface residues. Taken together our results indicate that PfAsf1 has distinct structural features from that of ScAsf1 and HsAsf1, indicating its uniqueness
PfAsf1 exists as a dimer in the crystal structure

The oligomeric status of Asf1 identified in other species is monomer. On the other hand, our crystal structure indicates that PfAsf1-NTD exist as a dimer in the asymmetric unit (Fig. 5A). For prediction of PfAsf1-NTD oligomeric state in solution we did interface analysis using PISA server [73]. PISA analysis indicates that the stable oligomeric species of PfAsf1-NTD is a dimer. The total surface area of PfAsf1 dimer is 15640 Å², out of which 2820 Å² is buried upon dimerization. The free energy change of dimeric association is -23.1 kcal/mol, which also suggest that the dimer is stable. From the contact analysis between the two monomers, we observed that 16 out of 146 residues (10.6%), are critically involved in dimerization. We also observed three salt bridge interaction mediated through Chain A and Chain B. The residues involved in saltbridge interaction are between residues GLU-145 of chain A and HIS-141 of chain B as well as between LYS-8 of chain A and ASP-122 of Chain B (Fig. 5B). In addition to salt bridge interaction, we observed that there are several hydrophobic residues located in the dimer interface (ILE-26, LEU-38, TRP-40, ILE-42, ALA-102 and ILE-138). These residues become inaccessible to solvent upon dimerization. This indicates that hydrophobic interactions plays a major role in PfAsf1 dimerization. To further confirm the oligomeric status of PfAsf1 in solution, we performed cross-linking experiments employing Sulfo-EGS as a cross-linker. Our results clearly indicated that PfAsf1 formed dimer with an increasing concentration of the cross-linker (Fig. 5c). We subsequently performed size exclusion chromatography where we observed a concentration-dependent peak shift from monomer to dimer (Supplementary Fig. 1a). In order to further confirm the oligomeric status of PfAsf1 we performed SEC-MALS which indicated that in a lower concentration it exist predominantly as a monomer species (Supplementary Fig. 1b). Our results indicate that there is a dynamic equilibrium between monomer and dimer of PfAsf1 in vitro. This dynamicity shift towards monomer at a lower concentration and dimer at a higher concentration.
**PfAsf1 interacts with histone H3-H4 and deposits the same onto DNA forming disomes**

To understand the specificity of interaction of GST-PfAsf1 with recombinant histones from plasmodium, GST pull down assays were performed. GST-PfAsf1-NTD was found to interact with histone H3 and H4 (Fig. 6A) similar to its yeast and human homologue. We also observed similar association with recombinant Xenopus histones H3/H4. Consistent with its functioning as H3-H4 specific histone chaperone, we didn't find any interaction of PfAsf1-NTD with histone H2A and H2B.

In order to further ascertain the strength of interaction between PfAsf1 with H3 and H4, we performed the interaction studies with increasing salt concentration. We observed a reduced association of PfAsf1-NTD with histone H3 and H4 at a higher salt concentration indicating ionic interaction plays a major role (Supplementary Fig. 2). We further substantiated our results by biophysical characterization of the interaction by means of surface plasmon resonance (SPR). Both H3 and H4 interacted strongly with PfAsf1 as evident by high dissociation constant values (Fig. 6B) values obtained in kinetic measurements by SPR. Binding of PfAsf1 with histone H3 was comparatively more than that with histone H4. A tight binding with histone H3-H4 dimers is also inferred based on slow dissociation of the bound PfAsf1-H3-H4 complex as evident in the SPR sensograms. So the characteristic H3-H4 interaction of Asf1 is conserved in *Plasmodium falciparum*.

H3-H4 dimers and 80bp of nucleosome positioning DNA sequence do interact to form disome and tetrasmome species in vitro. However, histone chaperones by virtue of their specific mechanism of interaction and deposition of H3-H4 dimers into DNA do shift the equilibrium towards enhanced formation of either disomes or tetrasmomes. It has been shown that Asf1 specifically shifts the equilibrium towards formation of more disome species[74] while CAF1 induces formation of tetrasmomes[20]. Based on this we thought of determining whether the putative H3-H4 chaperone PfAsf1 also shows same pattern of formation of more disomes. Interestingly in our in vitro histone deposition experiments, we found PfAsf1 do enhanced formation of more of disome species than tetrasmomes (Fig. 6c). Our results indicate that PfAsf1 is a histone chaperone which can specifically deposit H3/H4 onto DNA template to form higher order chromatin structure.
Interaction with H3-H4 is mediated by certain critical residues in PfAsf1

Next we intended to identify the critical residues of PfAsf1 involved in interaction with histone H3 and H4. Co-crystal structure of yeast and human Asf1-H3/H4 complex shows that there is a conserved mechanism of interaction between Asf1 and histone H3/H4 dimer. To elucidate the critical residues involved in interaction between PfAsf1 and histone H3/H4 dimer, we structurally superimposed PfAsf1 on yeast Asf1-H3/H4 complex (PDB : 2HUE) and energy minimised the modelled structure (Fig. 7A). We mapped the critical residues involved in association of PfAsf1 with histone H3/H4 from the modelled structure and observed that Ala97, Asp57, Tyr115, Arg148 and Val150 of PfAsf1 mediates the interaction with histone H3 and H4. To determine the effect of these residues on histone interaction ability of PfAsf1, we subsequently generated disruptive mutations of these residues and performed GST-pull down experiments. We observed that the double mutant (R148E/V150A) of PfAsf1 showed reduced interaction with histone H3 and H4 indicating the critical importance of these residues for mediating the interaction of PfAsf1 with histone H3 and H4 (Fig. 7B). While the histone H3 binding was also compromised in A97R, D57R, minimal effect was scored with Y115A mutant. On the other hand histone H4 binding was critically affected in A97R, D57R and Y115A mutant. Thus the critical residues of PfAsf1 (R148, V150, A97, D57 and Y115) important for histone interaction as predicted from our modelled structure was also validated by GST-pull down assays. This indicates that PfAsf1 utilizes the same histone interacting surface like yeast. As we observed PfAsf1 exists as a dimer in crystal, we interrogated whether dimerization plays any role in histone interaction. As observed from the modelled structure (Fig. 7A) the histone interacting surface and dimerization surface are mutually exclusive. This prompted us to infer that dimerization does not influence histone binding by Asf1.

We subsequently mapped the surface residue conservation of Asf1 across different species by employing ConSurf server [75] where highly conserved residues are denoted in red, while the less conserved in cyan. Interestingly, the ability of PfAsf1 to dimerize was unique as observed from the conservation of residue analysis, indicating that the phenomenon was likely to be restricted to Plasmodium species (Fig. 7C). In higher eukaryotes, it has been shown that Asf1 interacts with other
downstream histone chaperones like Hip1, Cac2[76], Hira [22]. All this chaperones utilize β5 and β6 strands of Asf1 and interestingly the ConSurf analysis indicates that this surface is also conserved. We observed that the histone interacting surface of Asf1 also showed a major conservation, indicating that the role of Asf1 histone chaperone to recognize the histone H3/H4 substrates was evolutionarily conserved from api-complexan species to yeast and finally to human.

Discussion

Histone synthesis to deposition onto DNA is a multi-step process. Histone chaperone plays a crucial role in depositing the histone cargo onto DNA to form nucleosomes. In addition, Asf1 has also established function in chromatin disassembly following DNA damage and post-repair chromatin re-assembly. Thus Asf1 plays a crucial role in genome dynamics from yeast to higher eukaryotes, although its role in unicellular protozoan has remained elusive. The present work reflects the first structural and functional elucidation of Asf1 in Plasmodium falciparum, which is a causative agent of malignant malaria, that leads to around 0.4 million deaths worldwide.

All of the Asf1 harbour a conserved globular histone chaperone domain at the N-terminus[43,44,77], which is involved in histone interaction and its deposition. Additionally, there is a divergent C-terminal tail, which is present in yeast and apicomplexans. In yeast the C-terminal stretch is constituted of poly-glutamic acid, whereas Plasmodium harbours a poly-asparagine repeat. Although the C-terminal tail in yeast has been reported to enhance the histone interaction[78], its role in Plasmodium is yet to be identified. Phylogenetic analysis reveals that Plasmodium Asf1 is evolutionarily distant compared to its homologs in higher eukaryotes.

In the human host Plasmodium goes through a hepatic cycle (sporozoite stage) and aerythrocytic cycle (merozoite stage). Plasmodium Erythrocytic cycle consists of three stages: ring, trophozoite, and schizont. The merozoites multiply several times within the erythrocytic cycle thereby increasing the parasite burden as high as $10^{13}$ per host[79]. This clearly indicates that the parasite goes through several rounds of replication. Plasmodium contains well-defined chromatin structure and prior to replication the structure needs to be accessible to the replication machinery. This necessitates the
disassembly of the chromatin template prior to replication followed by its reassembly. In this context the histone chaperone plays a crucial role, which is instrumental in depositing histones to the newly synthesized DNA. Interestingly, we have observed that Asf1 is present ubiquitously in different erythrocyte stages of Plasmodium lifecycle, which indicates that PfAsf1 play a critical role in chromatin assembly/disassembly process in parasite.

Our structural studies of Asf1 from Plasmodium have revealed a similar immunoglobulin like beta sandwich fold, as that of yeast and human[44,77], indicating that histone chaperone function across species is evolutionarily conserved. We observed that PfAsf1 can interact with Plasmodium as well as Xenopus histones and can deposit histone H3/H4 to DNA forming disomes. However, Plasmodium Asf1 shows dimeric organization in crystal structure as well as in solution study. Interestingly, the histone interacting residues and the overall surface area of Asf1 showed conservation across species. On the other hand, the dimerization interface of Asf1 was observed to be quite unique for Plasmodium species. Asf1 can transfer histones H3.1/H4 to CAF1 in a replication dependent manner[33], while it transfers H3.3/H4 to Hira in a replication independent manner[21,42]. Interestingly although Plasmodium genome harbours CAF1 homologs[80], the histone chaperone Hira homolog is absent. This indicates that there are other proteins in Plasmodium, which may be involved in Asf1-mediated variant histone deposition during transcription.

The present study highlights the role of Asf1 from pathogenic *Plasmodium falciparum* species. Further, the detailed structural insights using X ray crystallography has led to elucidation of unique features distinctly different from yeast and human, which shows that this protein may function differently by its ability to associate with its interacting partner of choice. Our structure/function analysis of PfAsf1 suggests that it could be a potential target to develop anti-malarial drugs.

**Data Availability**

The atomic coordinates and structure factors for the crystal structure of PfAsf1-NTD have been deposited in Protein Data Bank having accession no 6A6Y.

**Competing interest**
The authors declare no conflict of interest.

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**Author Contributions**

SR and DKS conceived the study, designed experiments, analysed data and wrote the manuscript. CD and VS designed experiments and analysed the data. DKS and SG performed experiments. All authors reviewed the results and approved the final version of the manuscript.

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Figure legends

Figure 1. Domain organization and evolutionary analysis of PfAsf1. (A) Domain organization of PfAsf1 showing the conserved histone chaperone domain (residue 1-159) to its N terminal part. The C terminal part of the protein contain a disordered stretch. Illustration was prepared using IBS version 1.0.3 [81] (B) The phylogenetic tree depicting evolutionary relationship was reconstructed using MEGA X software package. The tree was created using Maximum Likelihood method and JTT substitution model with 250 rounds of bootstrap iteration of the aligned Asf1 sequences from different organisms. Branch lengths are measure of per site substitution nos. Scale bar as shown in figure.

Figure 2. Stage-specific distribution of PfAsf1 in Plasmodium falciparum. (A) Detection of recombinant PfAsf1-NTD & PfAsf1-FL by polyclonal PfAsf1 antibody by western blotting analysis. (B) Stage specific expression of PfAsf1 at protein level in different asexual blood stages of the parasites. Whole cell lysate from each of Ring, Trophozoite and Schizont stage cultures of P. falciparum was immunoblotted and probed with polyclonal anti-PfAsf1 antibody. The blots were probed with anti-Actin antibody for normalization. Intensity plot from average of three biological replicates are shown. (C) Immunofluorescence based sub-cellular localization of PfAsf1 in different asexual blood stages (Ring, Trophozoite & Schizont) of P. falciparum. Shown in the panel are DIC (Differential Interference Contrast) images of the different stages of Plasmodium life cycle. DAPI
(blue) was used as a nuclear marker and PfAsf1 was detected using anti-PfAsf1 antibody followed by secondary antibody conjugated to Alexa Flour 567(red). DIC, DAPI, PfAsf1 are merged in the fourth panel.

**Figure 3. Crystal structure of PfAsf1-NTD.** (A) SDS-PAGE profile of purified PfAsf1 histone chaperone domain corresponding to its theoretical molecular weight of 18.7 kDa. (B) Ribbon representation of PfAsf1 with beta strand forming three beta sheets denoted by pink, blue and green. The helices are coloured by golden yellow and the loop connecting beta strand are coloured off-white. (C) Front and side view of the crystal structure with a 90° rotation.

**Figure 4. Structural comparison of PfAsf1 with other eukaryotic Asf1 proteins.** (A) Structure based sequence alignment of PfAsf1 with yAsf1 and HsAsf1 using Espript [68]. (B) Structural superposition of PfAsf1 (magenta) with that of ScAsf1 (*Saccharomyces cerevisiae*; PDB ID 1ROC, brown) and HsAsf1 (*Homo sapiens*; PDB ID 1TEY, cyan). RMSD (Root Mean Square Deviation) (in Å) upon pairwise Cα atom superposition are indicated in the right panel. (C) Comparison of residue wise variation in RMSD value of PfAsf1 vs HsAsf1 and PfAsf1 vs ScAsf1 structures using 3d-SS [82]. (D) Comparison of electrostatic surface potential of PfAsf1 with that of ScAsf1 and HsAsf1. As shown in the figure, blue, red and grey indicates positive, negative and hydrophobic surface potentials respectively. The electrostatic map was generated using APBS [83] programme in Pymol.

**Figure 5. Oligomeric status of PfAsf1-NTD.** (A) Surface representation of dimeric PfAsf1 exhibiting the extensively buried surface along the interface between the two chains of PfAsf1. (B) The zoomed view of the dimeric interface showing residues (in stick representation) involved in H-bonding. The two monomers in the asymmetric unit are coloured in cyan and green. (C) Cross-linking of PfAsf1 with amine reactive crosslinker (Sulfo-EGS). PfAsf1-NTD was incubated with increasing concentration of each of this cross-linker and reaction mix was resolved on SDS-PAGE and stained with Coomassie blue. ● indicates a degradation band that arise during cross-linking, which also
undergoes dimerization.

**Figure 6. Role of PfAsf1 in Histone interaction and chromatin assembly.** (A) In vitro GST pull down assay using recombinant proteins showing specific interaction of GST-PfAsf1-NTD with Plasmodium histone H2A, H2B, H3 and H4. (B) Surface plasmon resonance (SPR) based biophysical analysis of interaction of PfAsf1-NTD with histones H3, H4 and H3-H4 dimer. Histones were immobilized on sensor chip C1 and a range of concentrations (0.1 uM to 1.25 uM) of PfAsf1 as indicated was passed as analyte for measurement of binding kinetics. The sensogram were analyzed by 1:1 local fitting and $K_d$ values obtained are as indicated. (C) Histone deposition EMSA showing the histone chaperone activity of PfAsf1-NTD. 100 nM of DNA was added to pre-incubated 0.25 uM of H3-H4 dimer and different concentrations (2-6 uM) of GST-PfAsf1. The assembly reaction was incubated at room temperature for 1 hr and resolved on 7% PAGE and stained with SYBR green. The representative gel showing the relative migration of different species (free DNA, disomes and tetrasomes). The immunoblot probed with anti-H3 showing presence of histones in the formed complex species. The intensity plot represents the data from average of five independent replicates.

**Figure 7. Molecular characterization of PfAsf1 with histone H3 and H4.** (A) Histone H3/H4 dimer (shown in cartoon) modelled onto PfAsf1 using yAsf1-H3/H4 (PDB ID: 2HUE). PfAsf1-NTD is depicted as a dimer with histone interacting monomer shown as surface representation, while the other monomer is shown in cartoon. (B) GST pull down assay of PfAsf1-NTD mutants (residues indicated) with histone H3 and histone H4. The comparative interaction was assessed by probing with anti-H3 and anti-H4 antibody in respective pull down experiments. (C) Surface representation of PfAsf1 showing level of conservation. The histone and other histone chaperones (Cac2/Hira/Hip1) interacting surfaces and dimerization interface along with the conservation scale (below) is highlighted.
Table 1. Data collection and refinement statistics.

|                          |     |
|--------------------------|-----|
| **Space group**          | P 43|
| **Cell constants**       |     |
| a, b, c, α, β, γ         | 62.73 Å 62.73 Å 98.72 Å         |
|                          | 90.00° 90.00° 90.00°             |
| **Resolution (Å)**       | 44.36 - 2.49 (2.58 - 2.49)      |
| **Total reflections**    | 98376 (9169)                     |
| **Unique reflections**   | 13132 (1258)                     |
| **Multiplicity**         | 7.5 (7.3)                        |
| **Completeness (%)**     | 97.80 (95.66)                    |
| **Mean I/σ(I)**          | 13.86 (2.55)                     |
| **Wilson B-factor**      | 41.8                              |
| **R-merge**              | 0.1232 (0.9359)                  |
| **R-meas**               | 0.1324 (1.007)                   |
| **R-pim**                | 0.04838 (0.3696)                 |
| **CC1/2**                | 0.997 (0.831)                    |
| **CC**                   | 0.999 (0.953)                    |
| **R-work**               | 0.192 (0.280)                    |
| **R-free**               | 0.215 (0.287)                    |
| **RMS(bonds)**           | 0.004                             |
| **RMS(angles)**          | 0.97                              |
| Description                     | Value |
|--------------------------------|-------|
| Ramachandran favored (%)       | 98.98 |
| Ramachandran allowed (%)       | 1.02  |
| Ramachandran outliers (%)      | 0.00  |
| Average B-factor               | 47.29 |
Figure 2

A. Image showing the protein bands for PfAsf1 and PfAsf1-FL in [kDa].

B. Western blot analysis showing the expression of α-PfAsf1 and α-Actin in Schizont, Trophozoite, and Ring stages.

C. Fluorescence images showing the localization of PfAsf1 and DAPI in Ring, Trophozoite, and Schizont stages.
Figure 3

A

SDS-PAGE

B

α1

α2

α3

β1

β2

β3

β4

β5

β6

β7

β8

β9

β10

C

Front View

Side View

90°
Figure 4

A

| PfAsf1 | HsAsf1 | ScAsf1 |
|--------|--------|--------|
| 1      | 1      | 1      |
| 2      | 2      | 2      |
| 3      | 3      | 3      |
| 4      | 4      | 4      |
| 5      | 5      | 5      |

B

C

Deviation (Å)

Residue

D

PfAsf1

ScAsf1

HsAsf1
Figure 5

A

Chain A

Chain B

B

Chain A

Chain B

C

![Chemical structure and gel analysis](image)

| Sulfo-EGS (mM) | Input |
|---------------|-------|
| 0.5           |       |
| 1.0           |       |
| 2.0           |       |
| 2.5           |       |

- Dimer
- Monomer

[kDa]
Figure 6

A

| GST – Pull down | GST – Pull down | GST – Pull down | GST – Pull down |
|-----------------|-----------------|-----------------|-----------------|
| Input x e PfAsf1-NTD | Input x e PfAsf1-NTD | Input x e PfAsf1-NTD | Input x e PfAsf1-NTD |
| α-H2A | α-H2B | α-H3 | α-H4 |
| α-PfAsf1 | α-PfAsf1 | α-PfAsf1 | α-PfAsf1 |

B

- H3 – PfAsf1-NTD
  - $K_d = 14.3 \text{ nM}$

- H4 – PfAsf1-NTD
  - $K_d = 35.2 \text{ nM}$

- H3-H4 – PfAsf1-NTD
  - $K_d = 10 \text{ nM}$

C

| GST-PfAsf1-NTD | H3/H4 | 80bp DNA |
|----------------|-------|----------|
| - | - | + |
| + | + | + |

Relative Disosome formation

- control
- GST-PfAsf1-NTD (μM)

α-H3
Figure 7

A

B

GST – Pull down

Input

Wild Type

A9T

D57R

R146E/V159A

Y115A

α – H3

α – GST

GST – Pull down

Input

ye

Wild Type

A9T

D57R

R146E/V159A

Y115A

α – H4

α – GST

C

Histones Interacting surface

Cac2/Hira/Hip1-interacting surface

Dimerization surface

The conservation scale:

1 2 3 4 5 6 7 8 9

Variable Average Conserved

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