SUPPLEMENTARY MATERIAL

Identification and molecular characterization of an Alba-family protein from human malaria parasite *Plasmodium falciparum*

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METHODS

Molecular modeling and docking studies

The full-length sequence of gene encoding putative PfAlba3 in *P. falciparum* genome was predicted and annotated on chromosome 10 (Gene ID PF10_0063, http://www.PlasmoDB.org). For homology search, BLASTP program was used (www.ncbi.nlm.nih.gov). Multiple sequence alignment of PfAlba3 and Alba protein from other species was done by the MAFFT (68) and analyzed by jalview program (69). To envisage fold prediction and homology modeling of PfAlba3, protein sequence (PfAlba3) was subjected to PSI-PRED (70) and HHPRED (71) server for prediction of secondary and tertiary structure. Three dimensional (3D) coordinates of PfAlba3 monomer subunits were generated by MODELLER 9.8 (72) package using chain A and chain B of 1NFH as reference structures. Lowest energy minimized models were selected and further validated using PROCHECK (73) and Verify 3D (74) structure validation tools. Loops were remodeled using MODELLER loop modeling and LOBO (75) loop modeling server. Furthermore, dimer model of PfAlba3 was generated using the monomer models of chain A and chain B of PfAlba3. PATCHDOCK protein-protein docking program was used to generate 1000 docking decoys (76). Out of them, the best dimer orientation of PfAlba3 was selected using a combination manual judgment and docking parameters. Structural superposition of the Alba structures was performed using the MUSTANG (77) program and the pair wise root mean square deviation (RMSD) values were used to build the structure based distance tree. Structural distances between the model and reference structures were also calculated using the MUSTANG program. In order to identify the possible DNA binding mode of PfAlba3, modeled structure was docked with DNA (B form) using the protein-ligand mode of PATCHDOCK (76). Best DNA-PfAlba3 docking orientation was selected using a combination manual screening and docking parameters.

Parasite culture, synchronization and isolation of parasite from infected erythrocytes

P. falciparum (3D7) was cultured as described earlier (78) at a hematocrit level of 5% in complete RPMI medium (CRPMI; RPMI1640 medium, supplemented with 25 mM HEPES, 50 μg ml^{-1} gentamycin, 370 μM hypoxanthine and 0.5% (w/v) AlbuMax II) using tissue-culture flasks. In brief, ring stage rich parasite culture (60 ml, 5% haematocrit) with approximately 10% parasitemia was pelleted at 800 x g and the pellet (3 ml) was resuspended in 60 ml of 5% D-sorbitol and incubated for 10 mins at 30°C with occasional shaking. Intact cells were removed from lysed ones by centrifugation at 800 x g for 5 min. Cells were aseptically washed thrice in complete RPMI medium (CRPMI; RPMI1640 medium, supplemented with 25 mM HEPES, 50 μg ml^{-1} gentamycin, 370 μM hypoxanthine and 0.5% (w/v) AlbuMax II) and finally diluted to desired parasitemia with fresh uninfected human RBCs keeping final haematocrit level at 5%. Synchronization of the culture was confirmed by microscopic examination of Giemsa-stained thin smears. Parasite was isolated as described previously (79,80). In short, erythrocytes with ~10% parasitemia was centrifuged at 800×g for 5 min, washed and re-suspended in cold phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 5.3 mM Na_2HPO_4 and 1.8 mM KH_2PO_4). RBC was lysed by an equal volume of 0.5% saponin in PBS (final concentration 0.25%) to the erythrocyte suspension on ice for 15 min. It was then centrifuged at 1300×g for 5

min to pellet parasite, pellet was finally washed with PBS and stored at -80°C for further use. Total RNA was isolated as described above from each stage of parasite.

Total RNA isolation and PCR amplification

Freshly isolated parasites were immediately stored in RNA-*later* solution to isolate total RNA. Total RNA was extracted from the parasites using Trizol (Invitrogen) method as described previously (81). The full-length transcript of PfAlba3 (PF10_0063) and PfSir2A (PF13_0152) were reverse transcribed from total RNA and PCR amplified using specific forward and reverse primers in a total volume of 50 µl using Ready.To.Go™ RT-PCR beads. RT-PCR cycles were as follows: 42°C for 30 min, denaturation at 94°C for 3 min, then 35 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 45 s and extension at 68°C for 1.5 min; and final extension at 68°C for 10 min for PfAlba3. RT-PCR cycles for PfSir2A were as follows: 42°C for 30 min, denaturation at 94°C for 3 min, then 35 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 45 s and extension at 68°C for 2 min; and final extension at 68°C for 10 min. PCR product was analyzed through electrophoresis on a 1% agarose gel in Tris-acetate-EDTA at 10 V/cm. Primer sequence used for PCR amplification are as follows. Restriction site was shown by bold letters and underlined.

PfAlba3_pET28a_F-AG**CCATGG**CAAGCACAGAAGAAGTATCTCAG
PfAlba3_pET28a_R-CG**CTCGAG**GTTTGCTACAAAATCTGGGTG
PfSir2A-pET28a_F1- 5'CGC**GGATCC**ATGGGTAATTTAATGATTTCCTTTTTG3'
PfSir2A -pGEX5x-3_F2- 5'CGC**GGATCC**GAATGGGTAATTTAATGATTTCCTTTTTG3'
PfSir2A _ R 5'CCG**CTCGAG**CTACATTATTTTCTTATTTTTTCAC

Protein modification, circular dichroism and fluoremetric studies

Chemical modification of surface exposed amino terminus of recombinant PfAlba3 was done using chemical modifiers such as acetic anhydride and trinitro benzo sulfonic acid (TNBS). *In vitro* acetylation of the purified PfAlba3 was performed using acetic anhydride as described previously (82,83) with slight modification. In brief, PfAlba3 (1 mg) was incubated in 5 ml of 50 mM sodium phosphate buffer (pH 8.0) and continuously rotated at 4°C. Reaction was initiated by the addition of acetic anhydride in a molar ratio of 500: 1 with respect to amino groups of protein. Reaction was continuously maintained at pH 8.0 with a slight addition of 0. 2 N NaOH. To minimize acetylation of protein hydroxyl groups and improve protein stability during acetylation reaction, 20% glycerol was maintained in reaction mixture (84). Aliquots were removed from the reaction mixture at intervals of 15 (mild acetylation), 30 min (high acetylation) and protein solution was dialyzed against 10 mM sodium phosphate buffer (pH 7.4) and 20% glycerol at 4°C to remove excess salt and acetic anhydride. Modification of lysine residues by acetylation was checked by western immunoblotting using anti-acetyl lysine antibody.TNBS modification of PfAlba3 was performed in 50 mM potassium phosphate buffer, pH 7.4 containing 20% glycerol and the reaction was initiated by the addition of 0.1% TNBS and incubated for 15-30 min . The TNBS-modified protein was dialyzed against 500 ml of 10 mM sodium phosphate buffer (pH 7.4).

Alteration in conformational properties after modification of PfAlba3 was assessed by circular dichroism (CD), fluorescence studies of native and modified protein. CD spectra were collected for PfAlba3 (native) and modified PfAlba3 (by acetic anhydride and TNBS) in the far-

UV range in a J-810 CD spectrometer (Jasco Easton, MD, USA). A cylindrical cuvette with a total volume of 350 µl and a path length of 0.1 cm was used for each experiment. The CD spectra of PfAlba3 (native), mild / high acetylated PfAlba3 and TNBS- modified PfAlba3 in optically clear phosphate buffer (20 mM potassium phosphate, pH 7.2) containing a final concentration of 0.2 mg/ml.CD spectra were recorded from 200 to 250 nm at a scan rate of 50 nm/min with five accumulations. Raw data files were uploaded onto the DICHROWEB online server (http://www.cryst.bbk.ac.uk/cdweb/html/home.html) and analyzed using K2D algorithm, which was optimized for the analysis of data recorded in this range (85). Fluoremetric analysis of unmodified and modified PfAlba3 was performed as described earlier (86).

Antibody generation

Polyclonal antibody against the purified PfAlba3 and PfSir2A was generated in rabbit and mouse respectively. Antibodies were affinity-purified using Melon^{TM} gel IgG spin purification kit (pierce). Monoclonal anti acetyl lysine antibody was purchased from Novus biological. To confirm the specificity of anti-PfAlba3 and anti- PfSir2A antibody, western blotting was done using un-induced and induced bacterial cell lysate as well as purified protein. Each fraction was resolved in 15% SDS-PAGE and electroblotted onto the nitrocellulose membrane and subjected to western blotting.

***In vitro* transcription assay**

Transcription assay was carried out by using tRNA^{lysine} gene as a template. tRNA^{lysine} gene was PCR amplified using forward primer containing T7 promoter sequence at 5' end from *P. falciparum* genomic DNA. Primer used for RT-PCR was listed as tRNA^{lysine}-F-5' TAATACGACTCACTATAGCCTCTTTAGCTCAGTTG (T7 promoter sequence was underlined) and tRNA^{lysine}-R-AGCCTCCTAGGGGACTCGAACCCCTG. Prior to T7 transcription, PCR product was purified using PCR product purification kit (Qiagen). The reaction was performed in a final volume of 20 µl containing 500 ng of template DNA (t-RNA^{lysine} gene), 20 unit of T7 RNA polymerase (Fermentas), 1X transcription buffer (Fermentas), 0.5 mM of each NTP (Fermentas) (instead of CTP, which was used at 12 µm), 30 µci of alpha-^{32}P labeled CTP and 20 unit RiboLock™ RNase inhibitor (Fermentas). After incubation for 2 h at 37°C, reaction was terminated by the addition 10 mM EDTA and cooling at -20°C. To check the transcription silencing mediated by PfAlba3, *in vitro* transcription reaction was assembled in presence of both non-acetylated recombinants PfAlba3 as well as acetylated PfAlba3. Heat inactivated protein or buffer was used as control. Reaction products were extracted with phenol/chloroform method and transcripts were recovered by ethanol precipitation. An equal volume of 2X RNA loading dye (Fermentas) was added, heated at 70°C for 10 min and resolved in a 12% denaturing urea PAGE.

Protein-protein interaction studies

GST pull-down assay was carried out using 5 µg of each separately expressed and purified GST-fused PfSir2A and His-tagged PfAlba3. Both the protein were mixed and incubated in binding buffer for interaction studies. The bound protein complex was further pulled out using glutathione-sepharose 4B beads (Amersham), washed, and loaded on 15% SDS-PAGE. A

mixture of GST (26kDa) alone with His-tagged PfAlba3 (13kDa) was used as negative controls, whereas GST-PfSir2A (56 kDa) and His-PfMIF(~12.8kda) acted as control. For far western blotting recombinant GST tagged PfSir2A and control protein (GST alone, PfMIF) were subjected to SDS-PAGE and transferred onto nitrocellulose membranes (Amersham Biosciences). Proteins were visualized by staining with Ponceau-S (0.5% in 1% acetic acid). Membranes were blocked in blocking buffer (containing 15% glycerol, 150 mm NaCl, 25 mm Tris-HCl pH 7.5, 0.5 mm EDTA, 0.1% Tween 20, 5% skimmed milk powder) for 2 h at room temperature. Afterward, membranes were incubated for 2 h in blocking buffer containing purified (recombinant) acetylated/ non acetylated PfAlba3. Membrane was washed 5 times with wash buffer (15% glycerol, 150 mm NaCl, 25 mm Tris-HCl pH 7.5, 0.5 mm EDTA, 0.1% Tween 20) and subsequently allowed to react with anti- PfAlba3 antibody.

His tag pull-down assay was performed using the ProFound^{TM} pull-down poly-His protein-protein interaction kit (Pierce) according to manufacturer instruction. An immobilized cobalt chelate was added to the Handee^{TM} spin columns and incubated with the purified PfAlba3 for 3-4 h at room temperature to allow the binding of His tag fusion protein as bait. Subsequently, the unbound PfAlba3 was removed with the wash buffer. The parasite lysate was prepared as described and protein concentration of the lysate was analyzed using the Lowry method. The clear supernatant was added to a column containing the bound PfAlba3 and incubated overnight at 4°C with gentle shaking. After incubation, the suspensions were centrifuged at 8000 × g at 4°C and washed three times with cold TBS (tris buffered saline) in order to remove unbound material. The supernatant was removed after the third wash. Then the protein-protein complex was eluted using the elution buffer and analyzed in 15% SDS-PAGE, followed by western blotting using anti PfSir2A antibody. Purified His tag PfMIF was used as a control.

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SUPPLEMENTARY TABLES

Supplementary Table 1

Pairwise root square mean deviation (RMSD) values between PfAlba3 monomer models and known Alba 3D structures

	PfAlba3_A	PfAlba3_B	1NFH_A	1NFH_B	1NFJ_A	1NH9_A
PfAlba3_A	0.0	2.4	1.5	1.6	1.8	1.4
PfAlba3_B	2.4	0.0	2.6	2.0	2.3	1.9
1NFH_A	1.5	2.6	0.0	1.1	1.2	0.6
1NFH_B	1.6	2.0	1.1	0.0	0.9	0.8
1NFJ_A	1.8	2.3	1.2	0.9	0.0	0.7
1NH9_A	1.4	1.9	0.6	0.8	0.7	0.0

Supplementary Table 2.

CD analysis of secondary structure of native and modified PfAlba3.CD analysis of PfAlba3 was measured at a protein concentration of 0.2mg/ml in 20 mM phosphate buffer (pH 7.2). Secondary structure analysis was performed using Dichroweb server using the K2d algorithms. Secondary structure was also calculated from primary amino acid sequence by using HHpred software.All values are represented in %.

Secondary structure	CD Analysis using K2d				Predicted from primary amino acid sequence using HH Pred	Predicted from Model structure
	Native PfAlba3	Mild Ac-PfAlba3	High Ac-PfAlba3	TNBS modified PfAlba3		
α-Helix	26.00	26.54	26.32	25.12	26.17	26.16
β-sheet	32.51	32.95	31.64	31.16	29.90	29.80
Random coil and β turn	41.49	40.51	42.04	43.72	43.92	41.04

LEGENDS FOR SUPPLEMENTARY FIGURES

Supplementary Figure S1. (A) Multiple alignment of amino acid sequence of Alba homologue from diverse species. Species name and their respective gene bank accession number/swiss prot are as follows: *Aeropyrum pernix* (BAA80837.2), *Picrophilus torridus* (Q6KZR7.1), *Archaeoglobus fulgidus* (NP_070780.1), *Methanothermobacter thermautotrophicus* (NP_276597.1), *Methanosphaera stadtmanae* (YP_447258.1), *Thermococcus gammatolerans EJ3* (ACS34054.1), *Sulfolobus* (*Sulfolobus solfataricus*, NP_342446.1), *Pyrobaculum islandicum* (YP_930376.1), *Plasmodium berghei* (XP_675994.1), *Plasmodium vivax* (XP_001614369.1), *Plasmodium chabaudi* (XP_744187.1), *Plasmodium falciparum* (XP_001347348.1), *Plasmodium knowlesi* (XP_002258691.1), *Trypanosoma bruci i*(XP_828521.1), *Leishmania infantum* (AAK81869.1), *Arabidopsis thaliana* (NP_565781.1), *Nitrosopumilus maritimus* (YP_001581589.1) and *Pyrococcus furiosus (*NP_579610.1 *)*. Black and grey background represented identical and homologue residues respectively. Sequence alignment of PfAlba3 with conserved domain database (cdd_pfam_01918) sequence shows below the alignment. (B) Color coded matrix representation of the sequence identities, whereas side panel shows phylogenetic tree of PfAlba3 and other aligned Alba sequence (as shown in Figure 1A).

Supplementary Figure S2. (A) Western blotting of recombinant PfAlba3 using anti-His and anti- PfAlba3 antibodies. Lane 1; pre-induced fraction, lane 2; induced fraction and lane 3, 4 purified recombinant PfAlba3. (B) *In vitro* acetylation of PfAlba3 . Acetylation of PfAlba3 was performed by acetic anhydride under different condition and aliquots of samples were removed. Aliquotes after 15 minutes and 30 minute were further used for DNA binding studies of PfAlba3 and designated as mild and highly acetylated PfAlba3 samples. Confirmation of lysine acetylation of recombinant PfAlba3 by western blotting using anti-acetyl-lysine antibody. Lane 1; non acetylated PfAlba3, lane 2; acetylated PfAlba3 and lane 3; acetylated trypsin (Sigma) used as positive control. (C) Effect of lysine modification on DNA binding affinity of PfAlba3. PfAlba3 (2 μg) was subjected to mild and high acetylation and also by TNBS (Trinitro benzosulfonic acid) modification. The DNA binding affinity of modified PfAlba3 was checked by agarose gel shift assay. Lane P; ϕX174 dsDNA alone, lane 1; ϕX174 dsDNA plus PfAlba3 (2μg), lane 2; ϕX174 dsDNA plus mild acetylated PfAlba3 (2μg), lane 3; ϕX174 dsDNA plus highly acetylated PfAlba3 (2μg), lane 4; ϕX174 dsDNA plus TNBS modified PfAlba3 (2μg) respectively. (D) Gel retardation assay was executed with increasing concentrations of highly acetylated PfAlba3. ϕX174 dsDNA was incubated with highly acetylated PfAlba3 at 20^{o}C and reaction was incubated for 15 min followed by electrophoresis in 0.7% agarose /1XTBE gel. Lane P; ϕX174 dsDNA alone, lane 1-8; ϕX174 dsDNA plus increasing concentrations of acetylated PfAlba3 (40, 100, 200, 400, 1000, 2000, 3000, 4000 ng) respectively.

Supplementary Figure S3. Investigation of conformational changes of modified PfAlba3 as determined by CD (circular dichorism) and fluorescence analysis**.** (A) Far-UV CD spectrum of PfAlba3 is compared with the spectra of mild acetylated, high acetylated and TNBS modified PfAlba3. The mean residue ellipticity per residue [θ] (deg.cm^{2}.dmol^{-1}) is plotted against the wavelength. CD spectra were recorded in 20 mM phosphate buffer (pH 7.2) using the same amount of protein (0.2 mg). To account for background absorption, the absorption intensity measured from control solution, containing buffer only, was subtracted. (B) Tyrosine

fluorescence of PfAlba3 is compared with the mild acetylated, high acetylated and TNBS modified PfAlba3. An excitation wavelength of 274 nm was applied while emission spectra were recorded in the range between 280–400 nm. Equal concentration of each protein was used for fluorescence studies. Background fluorescence intensity was subtracted from that of each solution containing protein**.**

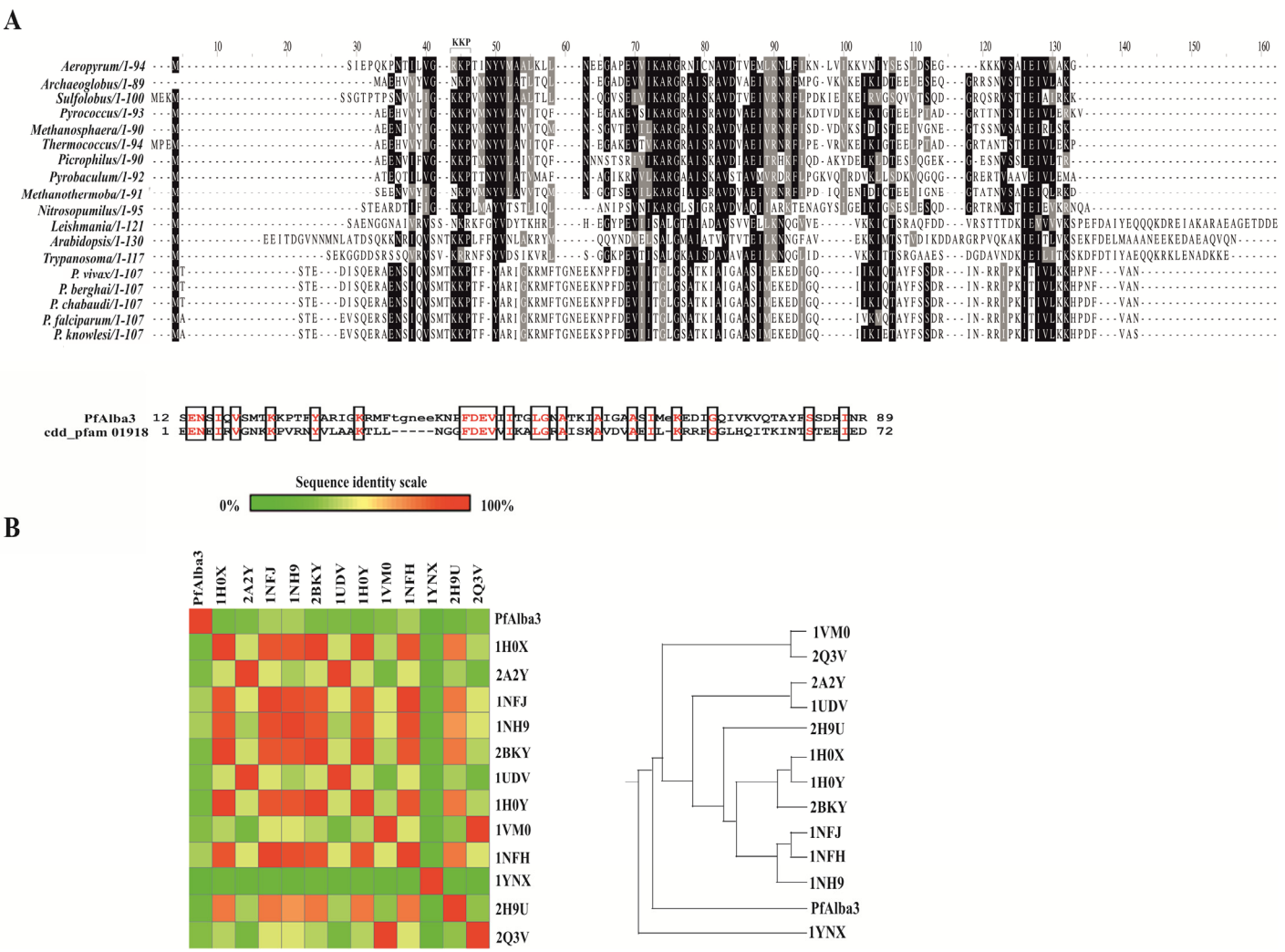
Supplementary Figure S4. *In vitro* transcription repression assay. *In vitro* transcription assay was followed on a template containing the T7 promoter upstream to $tRNA^{lys}$ gene. Transcription reactions were assembled in presence and absence of PfAlba3 (non-acetylated) (A) and mild acetylated PfAlba3 (B). Buffer alone or heat inactivated protein was used as a control. The reactions were conducted in presence of α-P^{32} labeled CTP as described under "Materials and Methods" and the resultant products were resolved in a 12% urea PAGE, gel was dried and subjected to autoradiography.

Supplementary Figure S5. (A) Stage specific expression of PfSir2A. Expression of PfSir2A in *P. falciparum* intra-erythrocytic stages. The sorbitol synchronized parasites lysate was resolved in 12% SDS-PAGE and subjected to western blotting using anti- PfSir2A antibody. To confirm the equal amount of protein in parasite lysate blot was reprobed with anti tubulin antibody. (B) Far western analysis. Purified recombinant PfSir2A (GST tag) was separated by SDS-PAGE and transferred onto nitrocellulose membranes. Proteins were denatured, renatured and then incubated with purified native PfAlba3 (nonacetylated). After washing, bound proteins were detected with rabbit anti PfAlba3 antibody. GST and MIF proteins were used as negative controls. GST, glutathione *S*-transferase. PfSir2A (GST tag) in each reaction were loaded in duplicate.

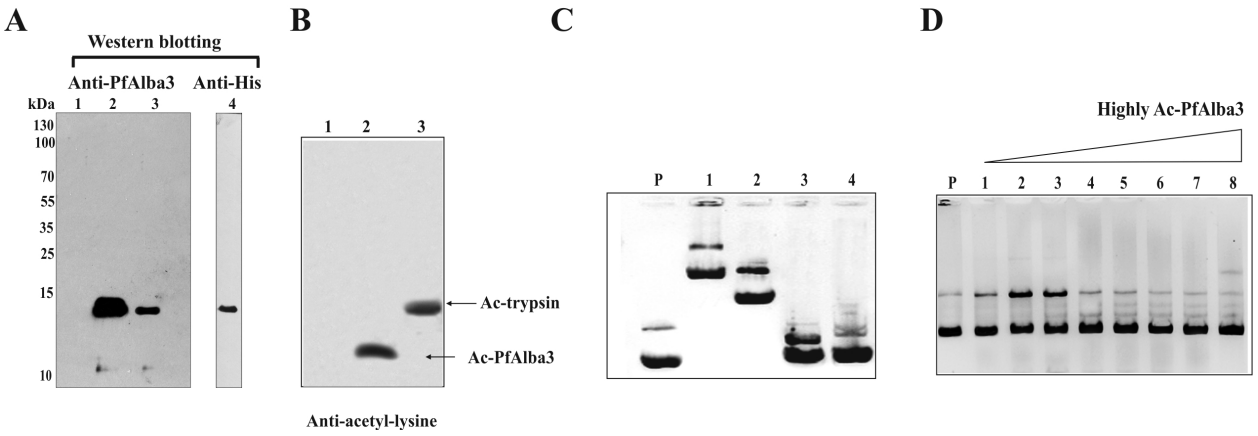
Supplementary Figure S6. Confirmation of deacetylated product from *in vitro* deacetylation reaction using N terminal peptide (specific to PfAlba3) by recombinant PfSir2A. Products of deacetylation reaction were collected and subjected to MALDI-mass analysis. (A) Non acetylated peptide, (B) MALDI mass analysis of reaction mixture of deacetylation reaction using $K22^{Ac}$ peptide in presence of PfSir2A and NAD^+ (500 μM), (C) MALDI mass analysis of reaction mixture of deacetylation reaction using $K23^{Ac}$ peptide in presence of PfSir2A NAD^+ (500 μM).

SUPPLEMENTARY FIGURES

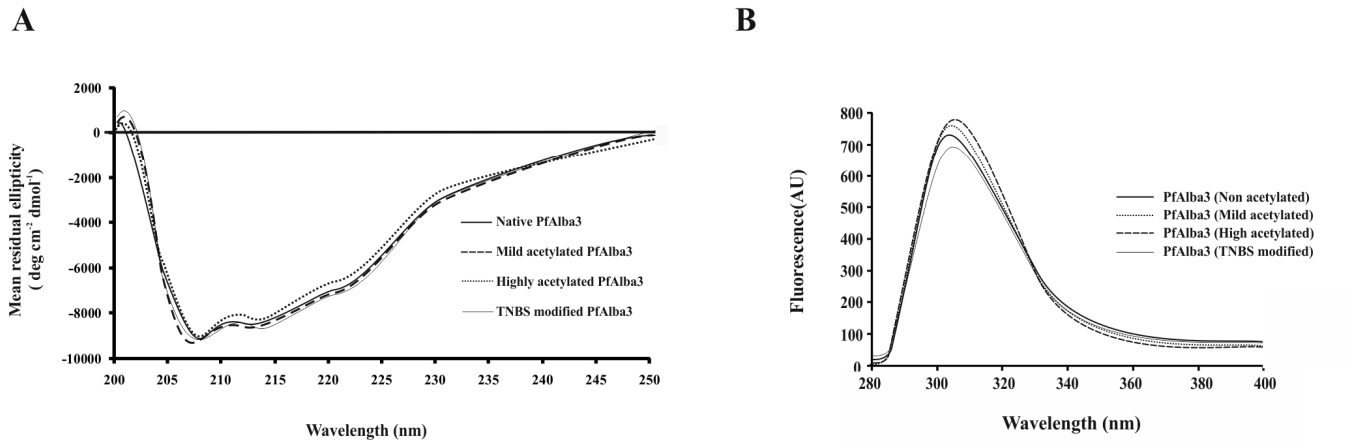
Supplementary Figure S1



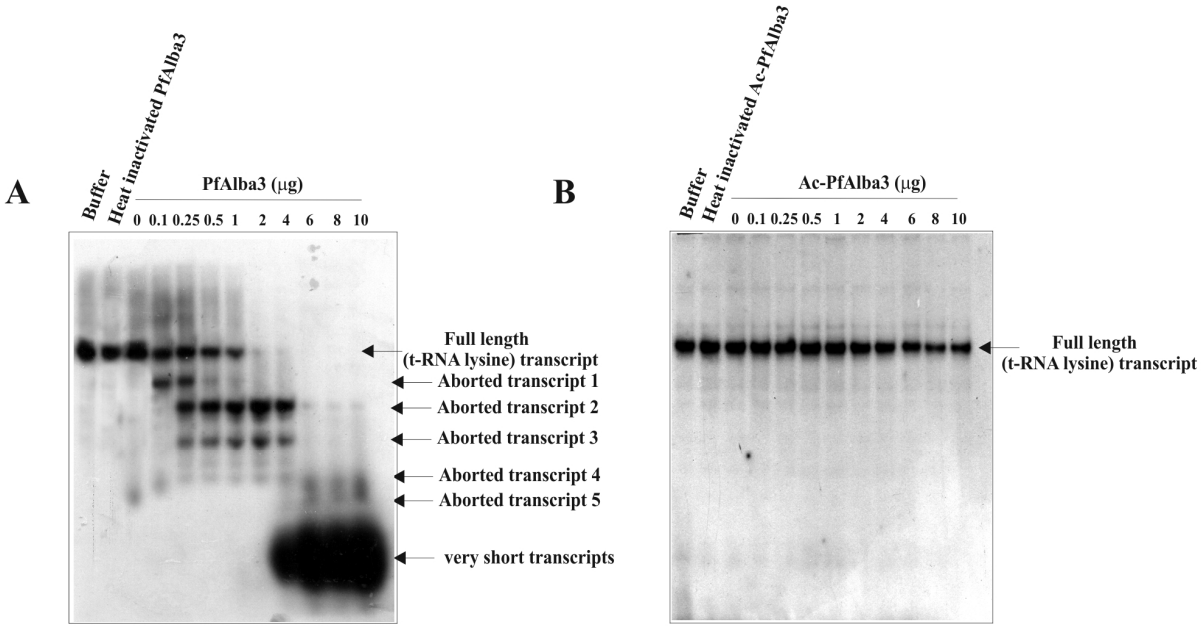
Supplementary Figure S2



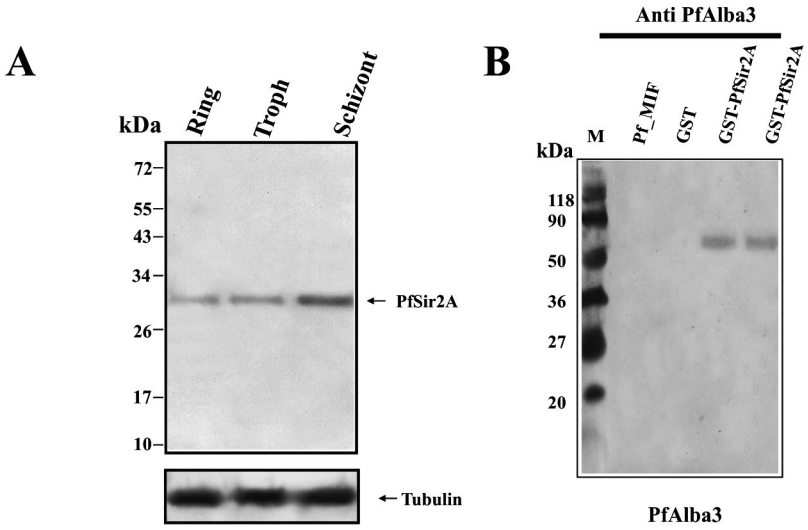
Supplementary Figure S3



Supplementary Figure S4



Supplementary Figure S5



Supplementary Figure S6