*falcipain* 1 
(1710 bp)  
T7 SS Pro Mature SP6  
(234 bp) (762 bp) (714 bp)  

*falcipain* 2  
(1455 bp)  
T7 Pro Mature SP6  
(729 bp) (726 bp)  

Fig. 1 The vector map of pGEMT with *falcipain* gene cloned in the multiple cloning site, which is flanked on either sides with T7 and SP6 promoter sequences. The insert shows the schematic representation of *falcipain* genes. SS, signal sequence; Pro, pro region.
Fig. 2 Agarose gel electrophoresis of ssRNAs and dsRNAs of *falcipain 1&2* synthesized using T7 and SP6 polymerase. Sense strand (ss) RNA and anti sense strand (as) RNA were synthesized using polymerases and were mixed, the mixture was incubated at 65°C and cooled slowly to obtain double stranded RNA (dsRNA). *falcipain 1* dsRNA (Lane 1), *falcipain 1* ssRNA (Lane 2), *falcipain 1* asRNA (Lane 3), *falcipain 2* dsRNA (Lane 4), *falcipain 2* ssRNA (Lane 5), *falcipain 2* asRNA (Lane 6), 1kb marker (M).
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<th>Treatment</th>
<th>Concentration</th>
<th>Food vacuole abnormality</th>
<th>% ring parasites</th>
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<tr>
<td>aminopeptidase-N</td>
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*(Spodoptera litura) dsRNA*

**Table 1:** Effects of Falcipains dsRNAs and E-64 on the morphology and viability of cultured parasite
Fig. 3 Effects of falcipains dsRNA on *P. falciparum* parasite morphology; *P. falciparum* cultures were treated with *falcipains* dsRNA and the smears were made from the treated cultures. Smears were Giemsa stained and visualized for morphological effects. Treated parasites (panel B) at trophozoite stage showed swollen food vacuole where as untreated parasites (panel A) were normal.
Fig. 4 Inhibition of $^3$H-hypoxanthine uptake in cultured parasites by falcipains dsRNA. *P. falciparum* culture of volume (100µl) at ring stage were treated with *falcipains* dsRNA for 24 h. Approximately, 1µCi of $^3$H-hypoxanthine was added to each well and cultures were maintained for additional 24 h. The cells were harvested and $^3$H-hypoxanthine was quantified using scintillation counter. *Aminopeptidase-N* dsRNA (*Spodoptera litura*) was used as a control.
Fig. 5 Block in hemoglobin hydrolysis in *P. falciparum* parasite after treatment with *falcipains* dsRNA. Cultured parasites were incubated for 48 h with control (DEPC water) and *falcipains* dsRNA. After treatment, parasitized erythrocytes were lysed with saponin and washed thoroughly to remove erythrocyte cytosolic components. Parasite pellet were solubilised in reducing SDS-PAGE sample buffer and parasite proteins were resolved on 15% SDS-PAGE. Negative control (Lane 1), dsRNA from *falcipain 1&2* (Lane 2), dsRNA *falcipain 1* (Lane 3), dsRNA from *falcipain 2* (Lane 4), hemoglobin control (Lane 5), E-64 treated parasite (Lane 6). The arrow indicates the hemoglobin accumulation.
Fig. 6 Northern blot analysis demonstrates the effects of falcipains dsRNA treatment on levels of endogenous RNA transcripts. Total RNA was extracted from control parasites (Lane 1), aminopeptidase dsRNA treated parasites (Lane 2), falcipain 1 dsRNA treated parasites (Lane 3) and falcipain 2 dsRNA treated parasites (Lane 4) and separated on 1.2% agarose gel for Northern blotting. Blots were hybridized with random primer labeled falcipain 1 DNA probe (A) and falcipain 2 DNA probe (B).
Fig. 7 Generation of 25nt small RNA species, a characteristic feature of RNAi, upon incubation with *falcipain* dsRNAs in parasite culture. (A) Schematic representation of mechanism of RNAi. (B) $^{32}$P labeled dsRNAs made from each *falcipain* were added to *P. falciparum* cultures and incubated for 40 h. As a control, labeled dsRNA was added to normal RBCs for the same period. Total RNA was isolated from the parasites and low molecular weight RNA was purified from total RNA. *falcipain 1* dsRNA treated parasite (Lane 1), *falcipain 2* dsRNA treated parasites (Lane 2), untreated parasites (Lane 3), *falcipain* dsRNA added to the RBC (Lane 4).
Fig. 8 Denaturing agarose gel showing time dependent degradation of radio labeled mRNA by parasite extract. *P. falciparum* cultures were treated with dsRNA. To the extract made from the treated cultures, $^{32}$P labeled mRNA was added and incubated for different time periods and analyzed on agarose gel. *falcipain 2 dsRNA* treated parasites (panel A) and untreated parasites (panel B).
**falcipain 1 (M81341)**

Target sequence : 5' CGA AGA AAA CAG GAAGAG GTT 3'

  Sense strand: 5' CGA AGA AAA CAG GAA AGA GUU 3'
  Anti sense strand: 3' UU GCU UCU UUU GUC CUU UCU C 5'

**falcipain 2 (AF239801)**

Target sequence : 5' GTA GAA TCA CAA TAT GCT AT 3'

  Sense strand: 5' GUA GAA UCA CAA UAU GCU AU T T 3'
  Anti sense strand: 3' T T CAU CUU AGU GUU AUA CGA UA 5'

**Aminopeptidase-N (AF320769)**

Target sequence : 5' GAG CTC ATG AAG CGT TCA C 3'

  Sense strand: 5' GAG CUC AUG AAG CGU UCA CUU 3'
  Anti sense strand: 3' UU CUC GAG UAC UUC GCA AGU G 5'

**Fig.9** Target sequences identified from *falcipain* genes and their corresponding siRNA. The gene accession numbers are indicated next to the gene names mentioned above.
Fig. 10 Graph showing the effect of *falcipains* siRNA on the uptake of $^3$H hypoxanthine. *P. falciparum* cultures were incubated with *falcipains* siRNA for 24 h. Approximately 1μCi of $^3$H hypoxanthine was added to the cultures and incubated further for 24 h. *Aminopeptidase* siRNA (*Spodoptera litura*) was used as a negative control.
Fig. 11 Graphs showing the effect of siRNAs on total parasitemia as well as on different stages of parasite. *P. alciparum* cultures were incubated with 50 µg of *falcipain* 1&2 siRNA separately and after 56 h of incubation total parasitaemia (panel A), parasites at new rings stage (panel B) and at schizont stage (panel C) were counted.
Fig. 12 Effect of falcipain 2 siRNA and E-64 on the morphology of *P. falciparum* parasites at schizont stage. Morphological examination of *P. falciparum* cultures treated with falcipain2 siRNA and E-64 under light microscopy. Overview of siRNA treated parasites (panel A). Spherical clusters of merozoites were surrounded by a delicate membrane in case of E-64 and siRNA treated samples while schizonts in the control were normal (panel B). Arrow indicates abnormal parasites.
Fig. 13. Western blot analysis showing reduction in the amount of Falcipain 2 protein in treated parasites. *P. falciparum* cultures were treated with falcipain 2 siRNA and Western blot analysis of treated and untreated parasite extract was carried out using Falcipain 2 antibody. Control (untreated parasites) (Lane 1), *Aminopeptidase* siRNA treated parasites (Lane 2), *falcipain* 2 siRNA treated parasites (Lane 3). HRPII antibody was used to show loading control.
Fig.14 *Rupture of erythrocyte membrane is blocked in incompletely ruptured schizonts.* Fluorescent microscopic images of incompletely ruptured schizonts in 3D7+His parasites after 56 h siRNA treatment, showing the localization of GFP fluorescence immediately surrounding the daughter merozoites (panel A). Immuno-fluorescence images of incompletely ruptured schizonts using PfEMP1 antibodies (red) (panel B). Immuno-fluorescence images of incompletely ruptured schizonts using Band3 antibody (Erythrocyte surface protein) (panel C). Parasite nuclei were stained with DAPI.
Fig. 15 Confocal microscopic images showing the localization of Falcipain 2 in different stages of the parasite growth in erythrocytes. Trophozoite (Panel A), early and late schizont (panel B and C) and free merozoites (panel D). Falcipain 2 is indicated by red, DAPI by blue and MSP-1 by green staining respectively. Green staining represents PfERC in panel A.
Fig. 16 Localization *falcipain* 2 in different parasite fractions. Parasitized erythrocytes at schizont stage were treated with saponin and SLO separately. Both the supernatant and the pellet fractions were mixed with SDS-PAGE dye and run on 10% SDS-PAGE under reducing conditions. The gel was transferred on to the nitrocellulose membrane and incubated with mice anti falcipain2 antibody. The bands were developed by using ECL kit. Panel A shows saponin treated parasitized erythrocyte, where falcipain 2 protein was localized in both supernatant and pellet, panel B represents SLO treated parasitized erythrocytes, where falcipain 2 was localized only in pellet but not in the supernatant.
Fig. 17 Immuno fluorescence images showing the absence of fluorescence in *falcipain* 2 siRNA treated parasites. *Falcipain*2 siRNA treated parasites were stained with mice anti *falcipain* 2 antibody and rabbit anti MSP1 antibody (green). DAPI was used to show nuclear staining (blue staining). MSP staining was used as a positive control.
Fig. 18 Trafficking of falcipain 2 through brefaldin A sensitive vesicle mediated secretory system. Confocal microscope image showing immuno localization of falcipain 2 protein in brefeldin A treated parasites. Synchronous parasites were treated with brefaldin A for 16 h, smears of these parasites were immuno-stained with falcipain 2 antibodies (red) and anti ERC antibodies (green) and visualised using confocal microscope.
Fig. 19 Immuno electron microscopic image showing localization of falcipain 2 protein in trophozoite. The trophozoite stages were isolated by percoll gradient centrifugation, fixed in 1% glutaraldehyde and stained with anti falcipain 2 antibody followed by secondary antibody conjugated to gold particle. Falcipain 2 protein at trophozoite stage was localized in food vacuole, ER and the parasitophorous vacuole. Less amount of protein was also localized to the surface of the RBC. Arrow marks point out the gold labeled particle. Bar 100μm
Fig. 20 Immuno electron microscopic image showing localization of falcipain 2 protein in schizont stage. The schizont stages were isolated by percoll gradient centrifugation, fixed in 1% glutaraldehyde and stained with anti falcipain 2 antibody followed by secondary antibody conjugated to gold particle. Localization of Falcipain 2 protein in the parasitophorous vacuole and the vesicular net work outside the parasite in the erythrocyte cytosol (panel A). The localization of falcipain2 protein in the vesicular net work in the erythrocyte cytosol and close to the surface of the RBC membrane (panel B). Bar 100 μm.
Fig. 21 Immuno electron microscopic image showing localization of falcipain 2 protein in schizont stage. The schizont stages were isolated by percoll gradient centrifugation, fixed in 1% glutaraldehyde and stained with anti falcipain 2 antibody followed by secondary antibody conjugated to gold particle. falcipain2 protein was localized in the maurer’s cleft and close to the surface of the RBC membrane. Bar 100µm.
**bergheipain 1 (987bp)**

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**bergheipain 2 (1448bp)**

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**GFP (727bp)**

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Fig.22 Target sequences identified from berghepains’ and GFP genes and their corresponding siRNA.
10^7 Parasites injected intraperitoneally

labeled siRNA injected intravenously after 24 h

Blood was collected after 48 h

Small RNA species was isolated and dot blotted on Nylon membrane

Presence of radio-labeled siRNA was detected by autoradiography

Fig. 23 Autoradiograph of low molecular weight (LMW) RNAs from parasites to show uptake of labeled siRNA by the parasites: Labeled BP1-siRNA (spot 1), labeled BP2-siRNA (spot 2), LMW-RNAs from parasite treated with labeled BP1 siRNA (spot 3), and with labeled BP2 siRNA (spot 4).
Fig. 24 Morphological abnormalities in *berghepain’s* siRNA treated parasites: Micrographs of Geimsa stained *berghepains’* siRNA treated parasites. Overview of siRNA treated parasites, abnormal parasites are indicated (panel A). Magnified view of control parasites (panel B) and magnified view of parasites showing an abnormally enlarged food vacuole after siRNA treatment (panel C).
Fig. 25 Coomassie stained gel showing accumulation of hemoglobin in parasites treated with berghepain siRNAs. Total proteins from treated parasites (Lane 2) and from untreated parasites (Lane 3) was resolved on SDS-PAGE, showing accumulation of hemoglobin in treated parasites. Lane 1 indicates control hemoglobin and M stands for marker lane. Arrow mark indicates hemoglobin accumulation.
Fig. 26 Northern blot analysis showing the degradation of mRNA in *berghepain* siRNA treated *P. berghei* parasites. Northern blot analysis of total RNA isolated from untreated (Lane 1) GFP siRNA treated (Lane 2), and *berghepains’* siRNA treated (Lane 3) parasites. The membranes were probed with *bergheipain1* (panel A) and *bergheipain2* (panel B) DNA probes. Equal loading in all the wells was confirmed by ethidium bromide staining of rRNA (panel C).
Fig. 27 Schematic representation of mechanism of RNAi (A). Generation of small interfering RNA (approx 25) corresponding to berghepains by RNAi (B): Total LMW RNAs isolated from berghepains’ siRNA treated (Lane 1), GFP siRNA treated (Lane 2), and untreated (Lane 3) parasites were separated on PAGE and probed with berghepian 2 RNA probe. LMW RNA bands detected are marked. Lane 4 indicates the 21nt BP2 siRNA as a marker.