Dual role of an essential HtrA2/Omi protease in the human malaria parasite: Maintenance of mitochondrial homeostasis and induction of apoptosis-like cell death under cellular stress

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Abstract

Members of the HtrA family of serine proteases are known to play roles in mitochondrial homeostasis as well as in programmed cell death. Mitochondrial homeostasis and metabolism are crucial for the survival and propagation of the malaria parasite within the host. Here we have functionally characterized a Plasmodium falciparum HtrA2 (PfHtrA2) protein, which harbours trypsin-like protease activity that can be inhibited by its specific inhibitor, ucf-101. A transgenic parasite line was generated, using the HA-glmS C-terminal tagging approach, for localization as well as for inducible knock-down of PfHtrA2. The PfHtrA2 was localized in the parasite mitochondrion during the asexual life cycle. Genetic ablation of PfHtrA2 caused significant parasite growth inhibition, decreased replication of mtDNA, increased mitochondrial ROS production, caused mitochondrial fission/fragmentation, and hindered parasite development. However, the ucf-101 treatment did not affect the parasite growth, suggesting the non-protease/chaperone role of PfHtrA2 in the parasite. Under cellular stress conditions, inhibition of PfHtrA2 by ucf-101 reduced activation of the caspase-like protease as well as parasite cell death, suggesting the involvement of protease activity of PfHtrA2 in apoptosis-like cell death in the parasite. Under these cellular stress conditions, the PfHtrA2 gets processed but remains localized in the mitochondrion, suggesting that it acts within the mitochondrion by cleaving intra-mitochondrial substrate(s). This was further supported by trans-expression of PfHtrA2 protease domain in the parasite cytosol, which was unable to induce any cell death in the parasite. Overall, we show the specific roles of PfHtrA2 in maintaining mitochondrial homeostasis as well as in regulating stress-induced cell death.
Malaria remains to be one of the major life-threatening diseases, especially affecting children in tropical and subtropical regions. Identification of key metabolic pathways in the parasite is a prerequisite to develop new drugs. Malaria parasite harbours a single mitochondrion that harbour several metabolic pathways important for parasite survival. Further, mitochondrion also play role in programmed cell death (PCD) of the parasite under consensus stress conditions. Here we have identified a unique enzyme, HtrA2, localized in the parasite mitochondria; we show that this enzyme plays a dual role in maintenance of parasite mitochondria under normal conditions, as well as regulation of PCD under cellular stress conditions. We also show mechanism of activation of this enzyme under cellular stress conditions, which in turn activates Caspase like-enzymes in the parasite, key enzymes responsible for PCD. These results reveal important roles of the HtrA2 enzyme in the malaria parasite, and thus this enzyme can be targeted to design new anti-malarials.

Introduction

Malaria remains to be one of the major life-threatening diseases, especially affecting children in tropical and subtropical regions. In 2020, about 241 million cases have been reported causing 627,000 deaths worldwide [1,2]. Artemisinin-based combination therapies are mainly responsible for the decline in cases in the last few years; however, there is the rapid emergence of drug-resistant parasite strains against contemporary treatments including artemisinin combination therapies. Therefore, there is an urgent need to identify new drug targets and develop new antimalarials [3,4]. Identification of key metabolic pathways in the parasite as unique and specific targets is a prerequisite to develop new drugs. Two parasite organelles of prokaryotic origin, the apicoplast, and the mitochondrion are essential for parasite growth and segregation. Metabolic pathways in these organelles are shown to be potential drug targets to obstruct parasite development and segregation [5–9]. Therefore, understanding novel metabolic pathways in these organelles and understanding their functional role in the parasite may help us to design novel anti-malarial strategies.

Mitochondria are known to play diverse roles in various cellular activities including energy transduction and cell death. Any disruptions in mitochondrial function and its dynamics could be lethal for the cell. In addition, mitochondria produce free oxygen radicals (reactive oxygen species, ROS) as a result of intrinsic physiological pathways, which could be also harmful to the stability and functioning of mitochondrial proteins. Therefore, cells have developed molecular mechanisms to cope with diverse challenges imposed on mitochondrial integrity. In this direction, various heat shock proteins and chaperones ensure the proper folding of new proteins and help in refolding damaged proteins to maintain their optimal performance [10]. HtrA2 (high-temperature requirement A2)/Omi protein is among those heat shock proteins, which are present in human mitochondria, and are suggested to be important modulators of molecular quality control. This protein combines the advantage of functioning both as a chaperone and protease, based on requirements in the cell [10]. HtrA2/Omi belongs to a family of unique serine proteases, HtrA, which were first reported in E. coli [11]. HtrA proteins are shown to be vital for the survival of bacteria only at higher temperatures [12]. Therefore, it is suggested that HtrA proteins originated as a result of proteostatic stress in the cell, where they play a role in protein quality control by recognizing misfolded proteins or severely damaged proteins and their degradation if refolding is not possible [13]. In humans, there are four paralogs of HtrA proteases, HtrA 1, 2, 3, and 4. The most well-studied among these is HtrA2,
which has been also shown to play an important role in apoptosis. Human HtrA2/Omi harbours an N-terminal mitochondrial targeting sequence and gets localized to the mitochondria [14,15]. The HtrA2/Omi is shown to play a major role in protein quality control and maintaining the cellular homeostasis. A combination of the catalytic domain and at least one C-terminal PDZ domain allows HtrA2/Omi to rapidly and reversibly switch their actions according to the needs of the cell, which makes HtrA2/Omi a major quality control factor [10]. Protein structure analyses have shown that the HtrA2/Omi proteins from pyramid-shaped homotrimer with the N-termini at the top and the PDZ domains at the bottom; the catalytic triad located in an interior of the trimer, which gets buried in the tight interface between PDZ and protease domains to form a catalytically inactive complex [16]. The chaperone activity of HtrA2 is carried out by recognizing the hydrophobic features of unfolded polypeptides. The PDZ domains mediate initial substrate binding and subsequent translocation to the inner chamber. It has been also shown that HtrA2 activation as a protease requires the binding of an activating peptide with the hydrophobic groove of PDZ. The PDZ domains of HtrA2 display a high affinity for hydrophobic peptides having the sequence YYF(V) at the C-terminus. Upon ligand binding, the PDZ domain triggers conformational reorganizations that result in the formation of a functional and accessible protease active site. This model is supported by the observation that the proteolytic activity of HtrA2/Omi is significantly augmented in absence of its PDZ domain and by peptide binding [17].

The HtrA2/Omi activity is shown to be essential for cell survival. Mutation or deletion of HtrA2/Omi induces ROS (Reactive Oxygen Species) generation, and mitochondrial stress and triggers cell death pathway events [18]. In neural cells, the loss of HtrA2 has been shown to result in the accumulation of unfolded proteins in mitochondria, decreased mitochondrial respiration, increased ROS production, and ultimately neuronal cell death, as in the case of Parkinson’s disease [19,20]. HtrA2/Omi is shown to protect mitochondria from cellular stresses, as in the case of other homologous stress-adaptive proteins DegP and DegS in bacteria [13,21]. HtrA2/Omi is also shown to play role in apoptotic and autophagic signalling cell death pathways. Studies have shown that under stress conditions the activated HtrA2/Omi protease induces cell death in both a caspase-dependent as well as caspase-independent manner. HtrA2/Omi normally reside in mitochondrial intermembrane space, however, during stress conditions, like oxidative stress or heat stress, it breaches the inner membrane and migrates into the mitochondrial matrix [22–24]. In the mitochondrial matrix, HtrA2/Omi converts into mature protease form and starts its functions by regulating cell death pathways [24]. Similarly, in human neutrophils, the serine protease activity of HtrA2/Omi is required for a caspase-independent, non-classical cell death pathway, where the protease targets substrate(s) within the mitochondria. It was also shown that under apoptotic signals, HtrA2/Omi along with cytochrome c is relocated from the mitochondria to the cytoplasm [25]. The activated HtrA2/Omi protease translocated to the cytosol act as a proapoptotic protein, which inhibits the IAPs (Inhibitors of Apoptosis) and initiates activation of a cascade of caspases, leading to cell death [14,25–27]. In a brain ischemia rats model, the activated HtrA2/Omi gets translocated from mitochondria to the cytosol, which induces neuronal death [28], whereas its inhibition by a specific inhibitor, ucf-101, attenuated ischemia-induced activation of caspase-8 and caspase-3 [29]. In another process, HtrA2/Omi activates Beclin by acting on Hax-1 and disabling its activity resulting in the release of Beclin-1, a homolog of autophagy 6, which promotes autophagy in the cell [24]. In Plasmodium a related protease, DegP is shown to play role in thermoxidative stress and parasite growth [30].

In the present study, we have functionally characterized the homolog of trypsin-like serine protease HtrA2 in P. falciparum (PlasmoDB gene ID: PF3D7_0812200), PfHtrA2, intending to understand its role in mitochondrial homeostasis and parasite survival. We utilized the HA-
...ribozyme system to study the localization and transient knock-down of PfHtrA2 during the asexual stages of the parasite. PfHtrA2 was localized in the mitochondrion of the parasite; transient knock-down of PfHtrA2 confirmed its essentiality for parasite survival and its role in the development of functional mitochondria. We also show that the PDZ domain plays a role in regulating/blocking the protease activity of PfHtrA2. Further, we show that the protease activity of PfHtrA2 is required for apoptosis-like cell death in the parasite induced by cellular stress conditions, where the protease act within the mitochondrion likely by cleaving an intra-mitochondrial substrate(s). Overall, these results suggest a specific and essential role of PfHtrA2 in maintaining mitochondrial homeostasis and parasite survival.

**Results**

**Endogenous tagging of PfHtrA2 with C-terminal HA-glmS ribozyme-tag and localization in transgenic parasites**

The *P. falciparum* HtrA2 protein (PfHtrA2) contains the catalytic residues, histidine, aspartic acid, and serine (S1 Fig) and it is a conserved protein across *Plasmodium* spp. (S2 Fig) suggesting it plays an important role. To study the functional essentiality of PfHtrA2 (Fig 1A), a conditional knock-down using an endogenous tagging strategy was utilized. We used a single cross-over based homologous recombination method for C-terminal tagging of the native *pfhtrA2* gene with the HA-glmS ribozyme system [31], so that the fusion protein gets expressed under the control of the native promoter (Fig 1B). Integration of HA-glmS at the C-terminus of the *pfhtrA2* locus was confirmed by PCR-based integration analysis (Figs 1C and S3A). A clonal population of parasites having HA-glmS tagged *pfhtrA2* locus was obtained using serial dilution and clonal selection method. This clonally selected homogenous parasite line was then used for all further experiments. The expression of PfHtrA2-HA fusion protein (~43kDa) was confirmed specifically in transgenic parasites (Fig 1D) by western blot analysis using anti-HA antibodies; this band was not detected in wild-type 3D7 parasites (Fig 1D).

These transgenic parasites were studied for localization of the PfHtrA2-HA fusion protein in the asexual blood stages of the parasite by immuno-labelling and confocal microscopy. Anti-HA staining showed localization of the fusion protein in a cellular organelle that showed characteristic shape, structure, and division pattern of the parasite mitochondrion during the asexual blood-stage cycle. In the young stages of the parasite, the mitochondrion is present as a small round-shaped structure close to the nucleus, in trophozoite-stage parasites the mitochondrion is present initially as an elongated tubular structure and as a branched structure in the later stage (Fig 1E). Indeed, staining by MitoTracker Red CMXRos showed overlap with the anti-HA labelling in these parasites (Fig 1F). Further, co-localization studies were also carried out using MitoTracker staining and immunolabelling with anti-PfHtrA2 antibodies. The MitoTracker staining overlapped with anti-PfHtrA2 labelling (S3 Fig). Thus, these results confirm that PfHtrA2 is expressed in blood-stage parasites and localizes to the mitochondria in the parasites.

**Transient knock-down of PfHtrA2 inhibits parasite growth, disrupts the intra-erythrocytic parasite cycle, and affects mitochondria development**

To understand the functional significance of PfHtrA2 in parasite survival and its possible role in mitochondrion development, we utilized the transgenic PfHtrA2-glmS parasite line for inducible knock-down of PfHtrA2 expression. The glmS-ribozyme gets activated in presence of glucosamine (GlcN) to cleave itself, which in turn leads to the degradation of the associated PfHtrA2-mRNA. Transgenic parasites treated with different GlcN concentrations (0mM,
Fig 1. Generation of transgenic parasite expressing HA-glmS tag in the PfHtrA2 gene locus for localization transient knock-down. (A) Schematic showing domain organization of PfHtrA2; the N-terminal mitochondrial targeting sequence, a trypsin-like protease domain, a C-terminal PDZ domain, and active sites residues of protease domain are marked. (B) Schematic diagram showing the strategy used to incorporate the HA-glmS at the 3’ end of endogenous locus of pfHtrA2 through the single cross-over homologous recombination. (C) PCR-based analyses to confirm integration of plasmid in the target gene locus using total DNAs of the transgenic parasite culture (purified clonal parasite population) and wild type 3D7 parasite lines, locations of primers are marked in the schematic: lanes 1 and 4 (primers 1466A and 1380A) show amplification in both transgenic parasite with integrated plasmid and the wild-type line; lane 2 and 5 (primers 1466A and 1236A) showing amplification only in the parasites with integration; lane 3 and 6 (primers 1379A and 1380A) show amplification in both integrated or wild type parasites. (D) Western blot analysis of lysate of transgenic and wild-type parasites using an anti-HA antibody. The fusion protein band (~43kDa) was detected in the transgenic parasites only (lane 1) and not in the wild-type parasites (lane 2). Blot
2.5 mM, and 5 mM) were analyzed for selective knock-down of PfHtrA2 protein. Treated parasites showed GlcN concentration-dependent reduction in PfHtrA2 levels (70–90%) (Fig 2A). To avoid any potential GlcN-mediated toxicity observed at higher GlcN concentrations, all further experiments were carried out using 2.5 mM of GlcN. To study the effect of the inducible knock-down of PfHtrA2 (PfHtrA2-iKD) on parasite growth, total parasitemia was determined at different time points for three growth cycles (48 h, 96 h, 144 h) after GlcN treatment. The PfHtrA2-iKD set showed ~80% inhibition of parasite growth compared to the control set (Fig 2B); after 144 hpi insignificant growth was observed in the iKD set, showing ~90% of growth reduction as compared to the control. GlcN treatment showed no significant effect on wild-type parasite growth (S4A Fig).

The effect of selective knock-down of PfHtrA2 on parasite development and morphology was assessed at different time points (Fig 2C). As envisaged, in the control set, during each intra-erythrocytic cycle, parasites developed from ring to trophozoites to mature schizonts, and subsequently, merozoites were released from these schizonts, which invaded new erythrocytes; the intra-erythrocytic cycle effectively increased the total parasitemia for about 5-6-fold in every 48 h cell-cycle and about 15–20 folds in the assay set up for three cycles under the culture conditions used in the study (Fig 2B). In the PfHtrA2-iKD set, the parasite showed drastic reduction in multiplication (0.5–1.0-fold in every 48 h cell-cycle) and in three cell-cycles it could only grow two-three folds; these results show about 80% growth inhibition in the PfHtrA2-iKD set as compared to the control set (Fig 2B). Morphological observations of parasite intracellular development showed aberrant development of trophozoites and late-trophozoite stages (36 hpi) in the PfHtrA2-iKD set as compared to the control set (Fig 2C). A large number of trophozoites were observed as darkly stained, pyknotic stressed parasites in the PfHtrA2-iKD set; these parasites were not able to develop into schizonts at later time points. In the PfHtrA2-iKD set, at 48 hpi, a large number of stressed parasites were observed as compared to the control set which had fully formed schizont stages (Fig 2C).

**Downregulation of PfHtrA2 levels hinders the growth and segregation of the mitochondrion**

Given that the PfHtrA2 is a mitochondrial protease, we studied the effect of the downregulation of PfHtrA2 levels in the parasite on the development of its mitochondrion. The growth and morphology of parasite mitochondria, in control as well as in the PfHtrA2-iKD set, were observed at different time points during the erythrocytic cycle using the MitoTracker stain. As expected, the mitochondria in the control set showed normal growth and development: in the majority of parasites at the trophozoite stage (24–32 hpi) the mitochondria appeared as elongated structures; in the late-trophozoite/early-schizont stage (36–42 hpi) majority of parasites showed much elongated and/or branched mitochondria (Fig 3A–3C). In PfHtrA2-iKD set, a large proportion of parasites showed abnormal mitochondrial development starting from late-trophozoite stages (36 hpi); at the early-schizont stage (42 hpi), mitochondria were able to develop into branched structures only in ~20% of parasites. The majority of parasites (>80%) in the PfHtrA2-iKD set showed fragmented structure or diffused punctate staining of MitoTracker dye with no clear organelle structure (Fig 3A–3C).

To ascertain and quantitate the effect of iKD of PfHtrA2 on mitochondria development and segregation in the parasite, we assessed the replication of the mitochondrial genome as
Fig 2. Inducible knock-down of PfHtrA2 protein in the transgenic parasites and its effect on the growth and development of asexual stages of the parasites. (A) Western blot analysis using an anti-HA antibody, showing a reduction in the PfHtrA2-HA fusion protein levels in transgenic parasites grown in presence of 2.5 mM glucosamine (+GlcN) as compared to control (-GlcN); a blot ran in parallel with an equal amount of the same sample was probed with anti-Bip antibodies as a loading control. (B) Graph showing PfHtrA2-HA-glmS parasite growth in presence of 2.5 mM glucosamine (PfHtrA2-iKD) as compared to control. Tightly synchronized ring-stage parasite culture of transgenic parasites grown with or without glucosamine (control and PfHtrA2-iKD, respectively), and their growth was monitored as the formation of new rings determined at 48, 96, and 144hpi. All analyses were performed in triplicate or more (n = 3); the error bars indicate standard deviations. (C) Giemsa-stained images of parasites showing the effect on parasite morphology at different time points (Ring stages, 20-22hpi; trophozoite stages, 32-36hpi, schizont stages 44-48hpi) in parasite culture from control and PfHtrA2-iKD sets. In the PfHtrA2-iKD set, majority of trophozoites were observed as stressed parasites; these parasites were not able to develop into schizonts at later time points.

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compared to the replication of the nuclear genome in the late trophozoite stages (42 hpi, 84 hpi) of the \( \text{PfHtrA2-iKD} \) set. Quantitative PCR-based analysis was carried out to estimate the genomic equivalents of the \( \text{cox-3} \) gene localized on the mitochondrial genome; in addition, the genomic equivalence of the \( \text{tufA} \) gene localized on the apicoplast genome was also determined. The mitochondrial genomic equivalence in the \( \text{PfHtrA2-iKD} \) set showed a two-fold reduction as compared to the control set within the first growth cycle (Fig 3D); these effects were pronounced in the second cycle. There was no significant effect on the apicoplast genome as the genome equivalents for \( \text{tufA} \) were nearly similar in \( \text{PfHtrA2-iKD} \) and control sets (Fig 3D). These results clearly show that the downregulation of \( \text{PfHtrA2} \) disrupts mitochondrial development and segregation.
**Downregulation of PfHtrA2 disrupts mitochondrial function and induces oxidative stress**

Given that knock-down of the HtrA2 in mammalian cells leads to loss of mitochondrial function and induces oxidative stress [18], we investigated the effect of PfHtrA2 knock-down on the development of functional mitochondria and production of mitochondrial ROS in the parasite. The mitochondrial ROS production was assessed by using MitoSOX, which is an indicator for the specific detection of mitochondrial superoxide in live cells. In the control set, the trophozoite stage parasites showed faint mitochondrial staining using MitoSOX dye, whereas in the PfHtrA2-iKD set the mitochondria showed significantly enhanced fluorescence suggesting higher levels of ROS (Fig 4A). Quantitative levels of MitoSOX fluorescence in the mitochondrion for individual parasites were measured, which showed a significant increase of the mean normalized mean fluorescence intensity per parasite in the PfHtrA2-iKD set (Fig 4B). Further, the mitochondrial membrane potential was measured by staining with JC-1 dye. In the control set, the ratio of parasite population with mitochondrial red and cytosolic green staining was found to be ~1.0 (Fig 4C). However, in the PfHtrA2-iKD set, there was a significant decline in the ratio of JC-1 red- and green-stained population to ~0.5 (Fig 4C), which clearly suggested loss of mitochondrial membrane potential.

We assessed any activation of Caspase-like proteases in the PfHtrA2-iKD set; however, there was no significant labelling of CaspACE staining, and only background staining was detected as in the case of control sets (S4B Fig). Similarly, there was no significant TUNEL staining in the PfHtrA2-iKD set (S4C Fig). These results suggest non-apoptotic cell death in the parasites of the PfHtrA2-iKD set.

**Characterization of PfHtrA2 protease activity and identification of ucf-101 as its specific inhibitor**

To characterize the protease activity of PfHtrA2, the protease domain (135–290 aa; HtrA2-protease) was expressed as a histidine-tagged recombinant protein in E. coli and purified by affinity chromatography. The purified protein separated on SDS-PAGE (Figs 5A and S5A–S5C) as a single band of ~17 kDa, this protein was used to assess the protease activity using different small peptide substrates covalently linked to a fluorophore (AMC-amino-methyl-coumarin). These peptide substrates were specific for trypsin-like protease activity (N-Suc-AFK-AMC), serine proteases (Suc-LLVY-AMC, Z-GGL-AMC), and cysteine proteases (Z-FR-AMC, Z-LR-AMC, and Z-LRGG-AMC). The PfHtrA2-protease was able to significantly cleave the substrate-specific for trypsin-like protease (N-Suc-AFK-AMC) in a time-dependent manner; however, only weak activity was observed with any other substrate as compared to control wells (Fig 5A and 5B). The kinetic analysis of recombinant PfHtrA2-protease was determined by setting up reactions with varying concentrations of the substrate N-Succ-AFK-AMC (0–50 μM). The kinetic constant $K_m$ was calculated to be 12.97 μM (S5D Fig). The heterocyclic compound ucf-101 is a known inhibitor of the human HtrA2 protease activity [32]. The inhibitory potential of ucf-101 was assessed in the standardized protease activity assay of PfHtrA2. Ucf-101 was able to inhibit the protease activity of recombinant HtrA2-protease in a concentration-dependent manner and the IC$_{50}$ was calculated to be 8.1 μM (Fig 5C).

**PfHtrA2 protease activity is not essential for parasite survival**

The gene knock-down analysis showed that the PfHtrA2 protein is essential for parasite growth at the erythrocytic stages. To assess if the protease activity plays role in the functional essentiality of the protein, we analyzed parasiticidal efficacies of the PfHtrA2 protease activity...
inhibitor ucf-101. However, there was no effect on parasite growth for cultures treated with ucf-101 at concentrations ~IC\textsubscript{50} and ~IC\textsubscript{90} for the HtrA2-protease activity (Fig 5D). These results show that the protease activity of PfHtrA2 is not essential for parasite growth and development.

Interaction of PDZ-domain with protease-domain and inhibition of its activity

The PDZ domains are known to play role in protein-protein interaction and regulation of HtrA2 protease activity. To assess the possible role of the PDZ domain in maintaining the
inactive HtrA2 protease under normal conditions, we carried out protein-protein interaction analysis and suppression of protease activity by the PDZ domain. The PDZ domain of PfHtrA2 (316–404 aa) was expressed in with MBP-tag using an E. coli expression system and purified with affinity chromatography as a ~55kDa protein (Fig 6A). Interaction of purified recombinant HtrA2-PDZ protein with recombinant HtrA2-protease (Fig 6B), was confirmed by different strategies. In solution binding of both the recombinant proteins followed by immune pull-down with antibodies against the protease domain detected the HtrA2-PDZ in the eluates (Fig 6C); in parallel assays, immune pull-down with antibodies against the PDZ domain or with non-specific protein antibodies (anti-HDP) were used as positive and negative controls respectively. In another set of experiments, the interaction of HtrA2-PDZ with HtrA2-protease was assessed on a solid surface, followed by antibody-based detection; HtrA2-protease showed concentration-dependent interaction with HtrA2-PDZ in this assay (Fig 6D). A nonspecific recombinant PfHDP protein was used as a negative control, which showed no significant interaction with HtrA2-PDZ.

Further, to ascertain the specificity of interaction and to quantitatively assess the binding of HtrA2-PDZ with HtrA2-protease, a surface plasmon resonance-based biomolecular-interaction analysis was carried out. In this assay, the recombinant protein HtrA2-PDZ was immobilized permanently on the sensor surface (CM5 chip) as a ligand using EDC-NHS coupling,
Fig 6. Interaction of protease and PDZ domains of PfHtrA2, and its effect on its protease activity. (A-B) SDS-PAGE gels showing purified recombinant proteins PfHtrA2-PDZ (A) and PfHtrA2-protease (B). (C) Solution-binding assays of PfHtrA2-PDZ and PfHtrA2-protease recombinant proteins followed by in vitro pull-down of the protein complex using agarose beads labelled with anti- PfHtrA2-PDZ (lane 1), anti- PfHtrA2-protease (lane 2) or anti-PfHDP (lane 3) antibody; the presence of PfHtrA2-PDZ protein in the pulled down protein complex was detected by western blot analysis using an anti-PDZ antibody. The lower panel show western blot analysis to confirm the presence of PfHtrA2-PDZ as well as PfHtrA2-protease recombinant proteins in the reaction mixture of each set. (D) In vitro solid-phase interaction of PfHtrA2-protease recombinant proteins with increasing concentration of PfHtrA2-PDZ; protein-protein interaction was determined by ELISA using anti- PfHtrA2-PDZ antibody. Each assay was performed in triplicate and the error bar represents the standard deviation. (E) SPR-based analysis of the
interaction of PfHtrA2-protease proteins with PfHtrA2-PDZ; global fitting of the sensograms obtained by flowing increasing concentrations (6.25–400 μg/ml) of PfHtrA2-protease for 5 mins on PfHtrA2-PDZ immobilized on the CM5 chip and dissociation for 5 mins under similar flow conditions. Buffer alone was used as a control that showed no responses. Each interaction was analyzed in triplicate and the assay was repeated twice. (F) Interaction of PfHtrA2-protease proteins with PfHtrA2-PDZ suppresses its protease activity. Graph showing inhibition of PfHtrA2-protease in presence of PfHtrA2-PDZ; protease activity assay was carried out under different molar ratios of the two recombinant proteins, keeping the concentration of PfHtrA2-protease constant; the data is presented as compared to the activity of PfHtrA2-protease alone. Each assay was performed in triplicate and the error bar represents the standard deviations. The data sets were compared using an unpaired Student’s t-test (**p < 0.005, and ***p < 0.001).
negative effect in the parasite leading to mitochondrial stress-induced parasite cell death [8]. Localization studies in these transgenic parasites also showed PfHtrA2 majorly remains to be localized in the mitochondria after induction of mitochondrial stress (Fig 7D); further, western blot analysis of these parasites detected a ~25 kDa band as compared to ~43 kDa band of full-length PfHtrA2 protein, as in case of ER-stressed conditions (Fig 7E).

Fig 7. Localization and processing of PfHtrA2 under cellular stress conditions. (A) Schematic diagram showing domain organization of PfHtrA2 and indicating predicted sizes of full-length (~43kDa) and processed form (~25kDa). (B) Fluorescent microscopic images of parasites under ER stress after immuno-labelling using anti-PfHtrA2 antibody and staining with MitoTracker show localization of PfHtrA2 mainly in the mitochondria of the parasites as in the control set; parasite nuclei are stained with DAPI. (C) Western blot analysis of parasites under ER-stress shows the presence of processed form PfHtrA2 (~25 kDa) as compared to full PfHtrA2 protease (~43kDa) in the control set. (D) Fluorescent microscopic images of parasites under mitochondrial stress after immuno-labelling using anti-PfHtrA2 antibody and staining with MitoTracker show localization of PfHtrA2 mainly in the mitochondria of the parasites as in the control set; parasite nuclei are stained with DAPI. (E) Western blot analysis of parasites under mitochondrial stress shows the presence of processed form PfHtrA2 (~25 kDa) as compared to full PfHtrA2 protease (~43kDa) in the control set.

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PfHtrA2 protease activity plays role in cellular-stress induced apoptosis-like cell-death

In mammalian cells, the HtrA2 follows different pathways to regulate the activation of caspases, directly and indirectly [33,34]. Since cellular stresses are shown to induce activation of caspase-like cysteine proteases leading to apoptosis-like cell death in the parasite [7,9], we assessed any possible role of PfHtrA2 in this pathway. After induction of ER-stress, a large percentage of parasites (~50%) were found to be CaspACE positive, showing activation of caspase-like cysteine proteases (Figs 8A and S7A). The parasite cultures treated with ucf-101 (at IC\textsubscript{50} concentration) under ER-stress conditions, showed a significant reduction in CaspACE-positive parasites; in this set the CaspACE-stained population was only 30%, suggesting ~40%
reduction in caspase activation as compared to the ER-stressed set without ucf-101. Similarly, when parasites were treated with caspase inhibitor z-VAD-FMK under ER-stress conditions, there was a significant reduction in CaspACE stained population (Fig 8A). To further ascertain the role of PfHtrA2 in caspase-like proteases mediated cell death in ER-stressed parasites, we assessed the effect of ucf-101 and z-VAD-FMK on cell death among these parasites by TUNEL assay, using the same experimental setup as described above. Inhibition of PfHtrA2 by ucf-101 and inhibition of caspase-like proteases by z-VAD-FMK showed similar levels of effect on parasite cell-death in ER stressed parasites. Both the compounds showed ~40% reduction in cell death under the ER-stress condition (Fig 8B).

We further analyzed if PfHtrA2 mediates the activation of caspase-like proteases in the mitochondrial-stressed parasites. Induction of mitochondrial stress led to the activation of caspase-like cysteine proteases in a large percentage of parasites (~60%) as shown by CaspACE staining (Figs 8C, S7B and S7D). However, when parasites under mitochondrial stress were treated with ucf-101, there was a significant reduction in CaspACE stained population (~60% reduction) as compared to mitochondrial stress alone (Fig 8C). Treatment with z-VAD-FMK also resulted in ~80% reduction in CaspACE staining (Fig 8C). As in the case of ER stress, inhibition of PfHtrA2 by ucf-101 and caspase-like proteases by z-VAD-FMK showed a similar effect on mitochondrial stress-induced cell-death. In both sets, there was a 30–40% decrease in parasite cell-death under mitochondrial stress (Fig 8D).

Regulated trans-expression of PfHtrA2 protease-domain and its effect on cell-death

We further ascertained that the processed and activated PfHtrA2-protease plays role in initiating cell death cascade from within the mitochondria and not by getting released in the cytosol. For this, the protease domain of PfHtrA2 was overexpressed as a transgene in the parasites. Since the PDZ domain was found to be inhibitory to protease activity (Fig 6F), the PDZ domain was not included in the transgene. To avoid any deleterious effect of constitutive overexpression of the protease on the parasite, the protein levels in the parasite were regulated by tagging it with E. coli DHFR degradation domain (DDD) at the C-terminus along with the HA-tag. In addition, a transgenic parasite line overexpressing active site mutant of PfHtrA2-protease was also generated with the same strategy to use as a control. The parasite lines were labelled as PfHtrA2-P-DDD and PfHtrA2-P(mut)-DDD respectively (Fig 9A). In these transgenic parasites, the DDD system enables the folate analogue trimethoprim (TMP) to stabilize the fusion protein, whereas the removal of TMP results in the degradation of the fusion protein by proteasome machinery. These transgenic parasites showed expression of PfHtrA2-P-DDD and PfHtrA2-P(mut)-DDD fusion protein of expected size (~ 35 kDa) (Fig 9B) in respective +TMP sets, which was not detected in absence of TMP. Further, the immunofluorescence assay using anti-HA antibodies also detected the fusion proteins in the parasite cytosol for both the transgenic parasite lines after treatment with TMP (Fig 9C).

We next investigated the effect of the expression of PfHtrA2-protease in the parasite cytosol on its growth. However, no effect on parasite growth was found in PfHtrA2-P-DDD as compared to the control set, similarly, there was no significant difference in the growth of PfHtrA2-P-DDD and PfHtrA2-P(mut)-DDD parasites (Fig 9D). Further, any induction of cell death was assessed by measuring CaspACE and TUNEL staining after induction of expression of the transgene. However, no significant staining was observed in any of the two transgenic parasite lines; only background staining was detected as in the case of control sets (Fig 9E).
Fig 9. Trans-expression of PfHtrA2-protease in parasite cytosol is not detrimental to the parasite. (A) Schematics of plasmid constructs and transgene for trans-expression of PfHtrA2-protease and its mutant version in the parasite. The transgene corresponding to PfHtrA2-protease or its triple mutant version (PfHtrA2-P-DDD and PfHtrA2-P(mut)-DDD) was expressed under the hsp86 promoter, whereas levels of these proteins are regulated by E. coli DHFR-degradation domain (DDD) tag. The expression of fusion proteins was induced in presence of trimethoprim (TMP). (B) Western blot analysis of lysate of transgenic parasites grown in the presence (+TMP) or absence (-TMP) of trimethoprim, using an anti-HA antibody. Expression of fusion protein ~35 kDa, was not detected in the +TMP set for both the parasite lines; a parallel blot probed with anti-BiP antibodies as a loading control. (C) Fluorescent microscopic images of transgenic parasites grown in presence of trimethoprim, expressing PfHtrA2-P-DDD and PfHtrA2-P(mut)-DDD, and immune-stained with anti-HA antibody. The fusion proteins were present in the parasite cytosol in both cases. (D) Graph showing percentage growth of transgenic parasite cultures [PfHtrA2-P-DDD and PfHtrA2-P(mut)-DDD] in presence of trimethoprim as compared to control set grown in absence of trimethoprim. (E) Graph showing the percentage of CaspACE-labelled and TUNEL-positive parasites in the transgenic parasite cultures [PfHtrA2-P-DDD and PfHtrA2-P(mut)-DDD] grown in presence of
Discussion

A number of metabolic pathways in the mitochondrion and the apicoplast, two parasite organelles of prokaryotic origin, are considered potential drug targets in the parasite. HtrA2 is a serine protease that localizes in the mitochondrial membrane space and is known to play a role in mitochondrial protein quality control and homeostasis, as well as in the regulation of autophagy and cell-death [10,14,35–39]. Here, we have identified a homolog of HtrA2 in the human malaria parasite \textit{P. falciparum} (\textit{Pf}HtrA2) and carried out detailed localization, gene knock-down, and cellular functional analyses to understand its role in mitochondrial homeostasis.

The \textit{Pf}HtrA2 protein comprises an N-terminal mitochondrial targeting sequence (MTS), a trypsin-like protease domain, and a C-terminal PDZ domain, with conserved active site residues. Biochemical characterization of the recombinant \textit{Pf}HtrA2 protease domain confirmed that it is an active trypsin-like protease. Further, we confirmed the localization of \textit{Pf}HtrA2 in the parasite mitochondria, as predicted by the presence of a mitochondrial targeting sequence.

To understand its functional role, we utilized a transient gene knock-down strategy. A number of genetic tools have been developed for transient downregulation of target proteins in \textit{Plasmodium} by tagging native genes [40]. Here we have tagged the native gene at the C-terminus with a ribozyme system [31] for its regulatable knock-down. The ribozyme \textit{GlmS} strategy has been utilized in \textit{P. falciparum} to show the essentiality of the target protein by conditional knock-down of the expression levels [31,41]; these studies also utilized 2.5mM GlcN for partial knock-down, without causing any toxicity, showing 50–60% reduction in expression levels of the target gene. As shown in these studies, we also found that >80% reduction in gene expression levels can be achieved using a higher concentration of GlcN; however, at these concentrations, the GlcN shows a certain level of toxicity on the parasite; therefore, 2.5mM GlcN is most appropriated to assess the effect of transient knock-down of the target gene in the parasite.

Transient downregulation of \textit{Pf}HtrA2 caused severe parasite growth inhibition and disruption in the development of trophozoites into schizonts stages; further, this also caused disruption of mitochondrial development in the parasite, combined with the loss of mitochondrial membrane potential and induction of mitochondrial oxidative stress as shown by the increase in mitochondrial ROS levels. Loss of HtrA2 in neuronal cells is shown to cause increased accumulation of unfolded components of the respiratory machinery in mitochondria, defects in the electron transport chain, and enhanced production of ROS [20]; similarly, HtrA2 knock-down in embryonic fibroblast and human kidney cells are shown to disrupt the mitochondrial homeostasis leading to loss of mitochondrial membrane potential and increase in ROS production [42]. Our results for the first time suggest a key role of \textit{Pf}HtrA2 in regulating mitochondrial homeostasis and the development of functional mitochondrion in the parasite \textit{P. falciparum}. HtrA2 has been shown to perform a dual function in the eukaryotic cell, in addition to acting as a mitochondrial chaperone, as described above, it can also act as a protease to regulate cellular proteins. In neural cells, the loss of proteolytic function of HtrA2 results in the accumulation of its direct substrate, p66Shc, which cause ROS generation and induction of apoptosis [43–45]. Although a direct substrate of \textit{Pf}HtrA2 is not known in \textit{Plasmodium}, we show that \textit{Pf}HtrA2-iKD caused an increase in mitochondrial ROS levels and parasite death. However, there was no induction of Caspase-like-protease or nuclear DNA fragmentation in \textit{Pf}HtrA2-iKD, suggesting a non-apoptotic cell-death in these parasites. Indeed, there are several mechanisms of non-apoptotic regulated cell death including necroptosis, pyroptosis,
autophagic-cell death and ferroptosis [46,47]. We found that under the PfHtrA2-iKD induced mitochondrial reactive oxygen species (ROS) in the parasite combined with a loss in mitochondrial membrane potential, which ultimately results in non-apoptotic cell death without activation of Caspase-like protease. It has been shown that the production of mitochondrial ROS can trigger necroptosis and non-apoptotic cell death. Similarly, mitochondrial ROS production is shown to play role in autophagic-induced cell death [48]; further, the induction of ROS in cancer cells is shown to lead to loss of mitochondrial membrane potential, which triggers mitochondrial fission and ultimately causing mitophagy-related non-apoptotic cell death [49]. Similarly, in the present study, we found abnormal and fragmented mitochondria in the PfHtrA2-iKD set, which mimics the mitophagy-like response, ultimately leading to cell death.

A specific and cell-permeable inhibitor of HtrA2 protease, ucf-101, was identified from a high throughput screening of a combinatorial library [32]; the ucf-101 has been used in dissecting the role of HtrA2 in cell survival and cell-death. Since PfHtrA2 was found to be essential for parasite survival, we assessed if the protease activity of PfHtrA2 is critical for its functional role. The ucf-101 could effectively inhibit the protease activity of recombinant PfHtrA2; however, the ucf-101 showed no effect on the growth of parasites, which suggests that the PfHtrA2 protease activity is not essential for parasite survival. Indeed, the HtrA2 protein is also suggested to a play role in cellular homeostasis through the maintenance of mitochondrial proteins in the properly folded form [20], whereas the activated HtrA2 protease plays a pro-apoptotic role under cellular stress [14,50].

It has been shown that the PDZ domain of HtrA2 plays a key role in regulating the dual role of HtrA2 in human cells; during the normal cell cycle, the PDZ domain of HtrA2 blocks the protease active site and mediates protein-protein interactions, so that a large membrane-associated protein complex is formed that acts as a chaperone for protein quality control in the mitochondria [39]. The C-terminal PDZ domain is known to be involved in protein-protein interactions and regulation of the activity of the protease domain [10,51,52]. It has been shown that HtrA2 exists as an inactive trimer, where PDZ domains restrict substrate access to the protease domain [17,53,54]. Structural studies have also shown that the PDZ domain plays the role of the regulatory domain and it supervises the proteolytic activity of HtrA2 to avoid undesirable proteolysis [55–57]. However, under cellular stress/apoptotic stimuli, the N-terminal processing and phosphorylation lead to conformational change which removes the inhibitory effect of PDZ from the active site [35,58]. Indeed, our data confirmed the interaction of the PDZ-domain with the protease-domain of the PfHtrA2, this interaction inhibited the protease activity confirming the role of C-terminal PDZ in regulating the PfHtrA2 protease activity.

To understand the dynamic role of PfHtrA2, we utilized two cellular stress induction systems: we have earlier shown that proteasome inhibitor MG132 induces ER-stress in the parasite, which leads to apoptosis-like cell death [7]; in another study, we have shown that transient and regulated expression of an active-site mutant of an essential mitochondrial ClpQ protease, PfClpQ, disrupts this protease system, which causes mitochondrial dysfunction and leads to apoptosis-like cell-death [8]. Here we show that the cell-death induced by either of these cellular stresses is mediated by the activation of caspase-like VAD-FMK binding proteases. The activation of caspases is an important step toward the progression of apoptosis-like cell death. Plasmodium and Trypanosoma are shown to lack these conventional caspases; instead, they have meta-caspases (MCAs), which have a C14 domain with a cysteine and histidine caspase-like catalytic dyad [59], in addition, the pan-caspase inhibitor z-VAD-FMK is shown to inhibit cell-death in P. falciparum [60,61] suggesting a role of such caspase-like proteases in cell-death. In a previous study, it has been shown that VAD-FMK-binding proteases activation is responsible for the degradation of a highly conserved metacaspases substrate TSN (Tudor staphylococcal nuclease) in Plasmodium [62].
Using these two cellular stress systems we analyzed the possible role of PfHtrA2 in the activation of Caspase-like proteases and subsequent cell death. We observed a processing event of PfHtrA2, suggesting the generation of an activated protease domain under ER or mitochondrial-specific stress conditions. In mammalian cells under normal growth conditions, the Inhibitor of Apoptosis proteins (IAPs) are known to down-regulate the caspases by either obstructing their processing or by binding with their active site directly [63]; under apoptotic conditions, HtrA2 plays role in the removal of IAPs inhibitory effect by directly binding with IAPs through its N-terminal AVPS motif. This active form of HtrA2 also acts like a pro-apoptotic protein by virtue of its protease domain. Sequence analysis of PfHtrA2 showed that the motif AVPS is substituted for the motif ALPS, which is present just at the C-terminus of the protease domain. The presence of IAPs has not been reported in P. falciparum, however, activation of PfHtrA2 in stressed parasites may suggest its role in the regulation/activation of caspase-like proteases. In order to decipher the role of PfHtrA2 in the activation of caspase-like proteases, we used a specific inhibitor of HtrA2, ucf-101. Inhibition of PfHtrA2 by ucf-101 effectively reduced activation of the caspase-like protease as well as apoptosis cell-death, as evidenced by the reduction in TUNEL staining, in both ER-stressed and mitochondrial-stressed parasites. These effects were similar to the pan-caspase inhibitor z-VAD-FMK under both cellular stress conditions. Overall, these results suggest that PfHtrA2 gets activated as a protease under cellular stress conditions and plays role in the activation of caspase-like proteases which lead to subsequent apoptosis-like cell death. As described above, it is shown that the activated HtrA2 under apoptotic conditions relocates to the cytosol, degrades IAPs, and activates caspases [22,25]; in these systems, extra-mitochondrial expression of HtrA2 is also able to induce cell death [14]. However, under certain cellular stress conditions, the processed HtrA2 remains in the mitochondria to induce a pro-apoptotic effect; it is shown that hypoxia-induced cellular stress causes loss of membrane potential to promote translocation of HtrA2 into the mitochondrial matrix, where it cleaves protein substrates responsible for maintaining mitochondrial homeostasis and thus promotes cell-death [3,64,65]. An important distinction is that in our study the activated PfHtrA2 did not show mobilization into cytosol under cellular stress conditions and stayed in the mitochondria; which may suggest its role in cell death by acting within mitochondria. To further ascertain that the induction of cell death is not due to any cytosolic translocation of activated PfHtrA2-protease, a transgene corresponding to PfHtrA2-protease was transiently overexpressed in parasite cytosol under degradation-domain-based regulation. Since the PDZ domain was found to be inhibitory to protease activity, the PDZ domain was not included in the transgene. Transient expression of PfHtrA2-protease did not cause any induction of caspase-like protease or cell-death in the parasites. As summarized in Fig 10, these results suggest that the activated PfHtrA2 plays a role in targeting protein substrate in the parasite mitochondria, which disrupts mitochondrial homeostasis and causes activation of apoptosis cascade leading to parasite cell death. Overall, this study highlights the role of PfHtrA2 in mediating parasite organelle homeostasis and cell death, these results could be helpful to design a novel anti-malarial strategy.

Material and methods

Parasite culture, plasmid constructs, and parasite transfection

Plasmodium falciparum parasites, wild-type 3D7 strain and transgenic parasite lines, were cultured within human O+ erythrocytes maintaining 4% hematocrit in RPMI 1640 medium (Invitrogen Corp., San Diego, CA, USA) supplemented with Albumax I (0.5%) and hypoxanthine (0.001%) following the standard culture protocols [66,67]. Parasite culture was synchronized with repeated sorbitol treatment as previously described protocol [68]. To generate
The PfHtrA2-HA-glmS construct for endogenous tagging of the pfhtrA2 gene, the C-terminal fragment of PfHtrA2 (PF3D7_0812200, 743 bp except for the stop codon) was cloned into pHA-glmS vector [69] using the BglII and PstI restriction enzyme sites. Tightly synchronized ring-stage parasites were transfected with 100 μg of PfHtrA2-HA-glmS plasmid by electroporation (310V, 950 μF) [70]. Transfected parasites were selected on WR99210 drug pressure, and subsequently subjected to on and off drug cycles to get parasites with plasmid integrated into the main genome and further clonal selection was done to get pure integrant clones by limiting dilution method. Integration of HA-glmS at the C-terminus was confirmed by PCR using specific primers, as well as by western blot analysis using an anti-HA antibody.

To generate constructs for trans-expression of PfHtrA2-protease domain and its mutant form, a gene fragment corresponding to 138aa-284aa region of PfHtrA2 was fused with HA-DDD (Escherichia coli DHFR degradation domain) tag at its C-terminus. Transfected parasites were selected on WR99210 drug pressure, and subsequently subjected to on and off drug cycles to get parasites with plasmid integrated into the main genome and further clonal selection was done to get pure integrant clones by limiting dilution method. Integration of HA-glmS at the C-terminus was confirmed by PCR using specific primers, as well as by western blot analysis using an anti-HA antibody.

To generate constructs for trans-expression of PfHtrA2-protease domain and its mutant form, a gene fragment corresponding to 138aa-284aa region of PfHtrA2 was cloned into pSSF2 to generate constructs PfHtrA2-P-DDD. In another construct, the same fragment with triple mutations for the active site residues (His154Ala; Asp184Ala; and Ser263Ala) was cloned in a similar way to generate the construct PfHtrA2-P(mut)-DDD. The parasites were transfected with each of these constructs separately and selected over WR99210 drug pressure as described above. Expression of fusion proteins [PfHtrA2-P-DDD or PfHtrA2-P(mut)-DDD] was induced in presence of 5μM trimethoprim (TMP) in the culture.
Conditional knock-down, phenotypic analysis, and in-vitro growth assays

To assess the effect of PfHtrA2 HA-glmS transgenic parasites, we carried out glmS-mediated conditional knock-down. Tightly synchronized parasite cultures at the early ring stage (6–8 hpi) parasites were incubated with glucosamine (2.5 mM or 5 mM) or solvent alone and allowed to grow for three consecutive cycles. To assess the effect on the growth and morphology of the parasite, thin smears of parasite culture were made from each well at different time points (0, 24, 48, 72, 96, and 120 h) and stained with Giemsa stain for microscopic analysis. The parasitemia was determined in the next cycle using flow cytometry as well as by counting the parasites in Giemsa-stained smears. Flow cytometry analysis was carried out using the FACSCalibur flow cytometer and CellQuestPro software (Becton Dickinson, San Jose, CA, USA). The numbers of ring/trophozoite stage parasites per 5000 RBCs were determined in Giemsa-stained smears and percentage parasitemia [(number of infected erythrocytes/total number of erythrocytes) ×100] was calculated to assess the parasite growth inhibition. Each assay was performed three times separately on different days.

Induction of organelle stress in the parasites

ER stress was induced on parasites as described earlier [62]. Briefly, synchronized parasite cultures at early-stage trophozoites (24–28 h) with MG132 (Z-Leu-Leu-Leu-Al) at 50nM (~EC50) for 6h. Mitochondrial stress was induced in transgenic parasite line ClpQ(mut)-DD [8]; briefly, expression of active site mutant ClpQ protease was induced in synchronized parasites cultures at early-stage trophozoites (24–28 h) by treatment with Shld1 drug (1 μM). Each assay was performed in triplicate and the control set was treated with solvent DMSO alone.

Parasite fractionation and Western blotting

Western blot analysis was carried out to assess the expression of PfHtrA2-HA-glmS in the transgenic P. falciparum blood-stage parasites. Briefly, parasites were lysed by 0.15% saponin, the supernatant and washed pellets were separately suspended in Laemmli buffer, boiled, centrifuged, and the supernatant obtained was separated on 12% SDS-PAGE. The fractionated proteins were transferred from the gel onto the PVDF membrane (Amersham, Piscataway, NJ, USA) and blocked-in blocking buffer (1× PBS, 0.1% Tween-20, 5% skimmed milk powder) for 2 h. The blot was washed and incubated for 3 h with primary antibody [Rabbit anti-HtrA2 (1:500); rabbit anti-BiP (1:10000); rat anti-HA (1:1000)] diluted in dilution buffer (1× PBS, 0.1% Tween-20, and 1% milk powder). Subsequently, the blot was washed and incubated for 1 h with an appropriate secondary antibody (anti-rabbit, anti-rat, or anti-mouse, 1:20000) conjugated to HRP. Bands were visualized by using an ECL detection kit (Amersham) with Biorad ECL Chemidoc imaging system.

Organelle staining, immuno-labelling, and fluorescence microscopy

To visualize the mitochondria, the live transgenic parasites were stained with MitoTracker Red CMXRos (Invitrogen) at a final concentration of 50 nM for 20 mins at 37˚C. The nuclei of parasites were stained using 4′,6-diamidino-2-phenylindole (DAPI, Sigma) with a final concentration of 2 μg/ ml. For immunostaining, cells were washed with 1× PBS and subsequently fixed in 4% paraformaldehyde and permeabilized with 0.05% Triton-X. Immuno-labelling was done by incubation of fixed and permeabilized parasites with the primary antibody, anti-HA (1:500) for 3 hours and subsequently with the secondary antibody, Alexa488 goat anti-rat (1:500) for 1 h. The nuclei of parasites were stained with DAPI.

The stained parasites were visualized using a Nikon A1 confocal laser scanning microscope and images were analyzed using Nikon -NIS element software (version 4.1). The 3D images
were constructed by using a series of Z-stack images using IMARIS 7.0 (Bitplane Scientific) software.

**Mitochondria membrane potential assay, mitochondrial oxidative stress measurement, and analysis of activation of Caspase-like proteases**

The mitochondrial membrane potential was assessed by using JC-1 staining dye as described earlier [7]. Infected erythrocytes from parasite cultures in control and experimental sets were incubated with JC-1 (5,50,6,60 -tetrachloro-1,10,3,30 -tetraethylbenzimidazolyl-carbocyanine iodide) at a final concentration of 5 μM, for 30 mins at 37˚C. JC-1 dye remains in monomeric form in the cytoplasm and has a green fluorescence (525 nm); however, the membrane potential of functional mitochondria establishes a negative charge that allows the lipophilic dye to accumulate and form aggregates in the mitochondria, which have red fluorescence (590 nm). To determine the ratio of cell populations stained for JC-1(red) and JC-1(green), stained cells were washed with 1× PBS and examined by flow cytometry using FACSCalibur flow cytometer and CellQuestPro software (Becton Dickinson, San Jose, CA, USA), using green (488 nm) and red (635 nm) filters. The ratio of JC-1(red)/JC-1(green) was calculated to assess the loss of mitochondrial membrane potential. The JC-1-stained uninfected RBCs were used as background controls.

For mitochondrial oxidative stress measurement, PfHtrA2-HA-tagged parasites were grown in the presence and in absence of glucosamine and stained with MitoSOX Red (Mitochondrial Superoxide Indicator, Thermo Fisher) at a final concentration of 2 μM at 37˚C for 30 min in incomplete media. The stained parasites were washed with 1× PBS. Live MitoSox stained parasites were viewed using a Nikon A1 confocal laser scanning microscope as described above. Images were analyzed to determine the mean fluorescence intensities of individual parasites using Nikon -NIS element software (version 4.1).

For the Caspase-like cysteine protease activation assay, parasites from treated and control set of cultures were stained with CaspACE FITC-VAD-FMK (fluorescein isothiocyanate-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone) in situ Marker (Promega, Mannheim, Germany) as per manufacturer’s instructions. The parasites were incubated with 10 μM of CaspACE FITC-VAD-FMK for 30 min at 37˚C followed by washing with 1× PBS. The stained samples were analyzed by flow cytometry using FACS Calibur flow cytometer and CellQuestPro software (Becton Dickinson) to assess fluorescence staining (Em-525 nm/Ex-488 nm) of infected RBCs. Uninfected RBCs were used as background control.

**Isolation of total DNA, and quantitative real-time PCR**

Total genomic DNA was isolated from PfHtrA2-HA-glmS parasites grown in the presence or absence of glucosamine at different time points. Gene-specific primer sets were designed using Beacon Designer 4.0 software for each organelle and nuclear genome: tufA (apicoplast, PF3D7_1348300), cox3 (mitochondria, mal_mito_1), as well as for 18S ribosomal RNA (18S rRNA) housekeeping gene as control [71]; details of all the primers are given in S1 Table). The amplification reaction contained 10ng template genomic DNA, 2× Maxima SYBR Green qPCR Master Mix (Thermo Scientific), and 10 nM gene-specific primers. Real-time PCR was performed in MicroAmp optical 96 well plates in automated ABI Step one Plus Version. Threshold cycle (Ct) values were calculated using SDS 2.4 Software (Applied Biosystem). Standard curves were used to determine genome equivalents of Ct values for the respective gene and 18S rRNA gene for each sample. Genome equivalents of each gene were normalized using that of the 18S rRNA gene for all the samples; the organelle: nuclear genome ratio was calculated relative to that of the control sample as described earlier [9].
In vitro protein-protein interactions assays

In vitro protein-protein interaction was assessed by solution binding of the two proteins followed by immune pull-down of the protein complex, as described previously [7]. Briefly, 1μg of both *Pf*HtrA2-PDZ and *Pf*HtrA2-protease recombinant proteins were incubated in 100 μl of binding buffer (50mM phosphate buffer pH-7, 75mM NaCl, 2.5mM EDTA pH-8.0, 5mM MgCl₂, 0.1% NP-40 and 10mM DTT) for 2hr. Subsequently, the complex reaction mixture was incubated for 2hr at 4°C with protein A-Sepharose beads, having immobilized anti-HtrA2-PDZ or anti-HtrA2-protease domain antibody. Beads were washed extensively with binding buffer; bound proteins were solubilized in SDS-PAGE buffer and analyzed by immunoblotting using an anti-HtrA2-PDZ antibody. In control reactions, a non-specific anti-*PfHDP* antibody was immobilized on sepharose beads in place of the *Pf*HtrA2 antibody.

In vitro protein-protein interaction was also analyzed with a solid surface interaction-based technique, where bound proteins were detected by ELISA. Briefly, 96 well microtiter plate Maxi-Sorp micro-titer plate (Nunc International, Nunc, Langenselbold, Germany) was coated with 100 ng of *Pf*HtrA2-PDZ protein in each well. After washing three times with 1× PBS containing 0.05% Tween-20 (PBS-T), the wells were blocked with 1% BSA for 2hr. Recombinant protein *Pf*HtrA2-Protease was added in varying concentrations (25-200ng) into *Pf*HtrA2-PDZ coated wells and incubated for 3hr at room temperature. After repeated washing with PBS-T, the wells were sequentially incubated with rabbit anti-HtrA2-protease domain antibody (1:2000) and HRP conjugated anti-rabbit antibody (1:3000). After consecutive washes, enzyme reactions were developed with substrate o-phenylenediamine dihydrochloride-H₂O₂. The resulting absorbance was measured at 490nm using a SYNERGY HTX multi-mode microplate reader. A non-specific protein (*PfHDP*) was used in place of *Pf*HtrA2-Protease as the negative control.

Surface-plasmon resonance (SPR) based analysis of protein-protein interaction

The real-time protein-protein interaction of protease and PDZ domain proteins of *Pf*HtrA2- was analyzed by SPR using Biacore T200 instrument (GE Healthcare, Uppsala, Sweden) as described previously [7]. Briefly, the Sensor chip CM5 dextran matrix was activated with a mixture of 1-ethyl-3-[3-dimethylaminopropyl]-carbodiimide (EDC) and N-hydroxysuccinimide (NHS). Recombinant protein *Pf*HtrA2-PDZ (100μg/ml in HBS-EP Buffer) was immobilized over the activated surface of the sensor chip flow cell at a rate of 10μl/min and 14769 RU was achieved. Another sensor chip flow cell was blocked with 1M ethanolamine-HCl pH 8.5, to use as a control. Kinetic binding analysis was done by injecting an increasing concentration of *Pf*HtrA2-protease recombinant protein at a flow rate of 30 μl/min on an immobilized surface. The kinetic parameters were analyzed using Biacore evaluation software, version 4.1.1 (GE Healthcare).

Statistical analysis

The data were compared using unpaired Student’s t-test; the data sets were analyzed and the graphical presentations were made using GraphPad Prism ver 5.0.

Supporting information

S1 Method. Supplementary Method.

(DOCX)
S1 Table. List of primers used in the study.

S1 Fig. Clustal W alignment of amino acid sequences of PfHtrA2 with its homologs in organisms. The protease domain is underlined with green line, PDZ domain with blue line, and red asterisks indicate the putative catalytic triad. Amino acids that are identical in all the species are shown in red boxes and amino acids present in at least three of the species are shown in yellow boxes with consensus >70%. In consensus line upper case letters indicates identity and lower case indicates >70% consensus; I indicates I or V; % indicates F or Y; # indicates N, D, Q or E; $ indicates L or F.

S2 Fig. Clustal W alignment of amino acid sequence of Pf. falciparum HtrA2 with its homologs in other Plasmodium species. The protease domain is underlined with green line, and PDZ domain is marked with blue line. Amino acids that are identical in all the species are shown in red boxes and amino acids present in at least three of the species are shown in yellow boxes with consensus >70%. In consensus line upper case letters indicates identity and lower case indicates >70% consensus.

S3 Fig. (A) PCR-based analyses using total DNAs of the transgenic parasite culture (purified clonal parasite population) and wild type 3D7 parasite lines, to confirm that the transgenic parasite culture does not contain any wild-type parasites, locations of primers are marked in the schematic (1466A and 1788A). Amplicon of expected size is detected in 3D7 parasite lines but not in the transgenic parasite line. (B-C) Expression and Localization of PfHtrA2 in P. falciparum parasites. (B) Western blot analysis of P. falciparum parasite lysate using anti-PfHtrA antibody (raised against the protease domain) showing detection of full length PfHtrA2 protein of predicted size (~43kDa). (C) fluorescent images of P. falciparum parasite immune-stained using anti-PfHtrA2 antibody and co-stained with MitoTracker stain, PfHtrA2 labelling overlapped with mitochondrial staining; parasite nuclei were stained with DAPI.

S4 Fig. (A) Graph showing wild type 3D7 parasite growth in presence of 2.5 mM glucosamine as compared to solvent control. Tightly synchronized ring-stage parasite culture of parasites were grown with or without glucosamine and their growth was monitored as the formation of new rings determined at 48, 96, and 144hpi; no significant difference in growth was observed between the two sets. (B-C) Inducible knock-down (iKD) of PfHtrA2 (PfHtrA2-iKD) induce non-apoptotic like cell death. Graph showing percentage of CaspACE-labelled (B) and TUNEL positive parasites (C) in the transgenic parasite cultures grown in presence of 2.5 mM glucosamine (PfHtrA2-iKD) and control set; no significant induction of CaspACE or TUNEL labelling was observed in the PfHtrA2-iKD, only basal level of staining was observed as in case of control sets. Wild type 3D7 parasite treated with MG132 or solvent alone were used as positive and negative controls respectively.

S5 Fig. Expression and purification of recombinant protein PfHtrA2-protease. (A) SDS-PAGE gel of E. coli lysate showing induction of expression of recombinant PfHtrA2-protease. (Un: un-induced bacterial culture; In: induced with IPTG). (B) SDS-PAGE gel showing purified recombinant PfHtrA2-protease. (C) Western blot analysis of recombinant PfHtrA2-protease using anti-His antibodies. (D) Graph showing activity of reaction velocity of
PfHtrA2-protease activity at various concentrations of substrate used.

S6 Fig. SPR-based analysis of the interaction of non-specific his-tagged proteins (PfMSP1) with PfHtrA2-PDZ as control set; the sensorgrams were obtained by flowing increasing concentrations (6.25–50 μg/ml) of PfMSP1 for 5 mins on PfHtrA2-PDZ immobilized on the CM5 chip and dissociation for 5 mins under similar flow conditions. No significant association/dissociation values were obtained at any concentration

S7 Fig. Activation of z-VAD-FMK binding caspase like cysteine proteases in P. falciparum parasites under cellular stress conditions. Fluorescent microscopic images showing activation of CaspACE-tagged parasites under ER stress (A) and mitochondrial stress (B), as compared to control. CaspACE stained cells are marked with arrows.

S1 Data. Numerical data values for Figs 2B, 3C, 3D, 4B, 4C, 5A, 5B, 5C, 5D, 6D, 6F, 8A, 8B, 8C, 8D, 9D and 9E.

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