An ER CREC family protein is a novel regulator of the egress proteolytic cascade in malaria parasites

Manuel A. Fierro[1,2], Miryam A. HortuaTriana[1], Carrie F. Brooks[1], Beejan Asady[1], Catherine Li[1], Silvia N.J. Moreno[1,2], and Vasant Muralidharan[1,2]

[1]Center for Tropical and Emerging Global Diseases

[2]Department of Cellular Biology

University of Georgia, Athens, GA, USA

Address correspondence to Vasant Muralidharan, vasant@uga.edu

Abstract

The role of the endoplasmic reticulum (ER) during egress of malaria parasites from erythrocytes is undefined. The parasite ER is thought to enable egress by acting as the main calcium (Ca^{2+}) signaling hub in these divergent eukaryotic parasites. However, no proteins involved in Ca^{2+}-signaling have been identified in the ER of these deadly human parasites. In this study, we explored the role of the Endoplasmic Reticulum-resident Calcium-binding protein (ERC), a member of the CREC family, in the lifecycle of two apicomplexan parasites, *Toxoplasma gondii* and *Plasmodium falciparum*. We find that the *Toxoplasma* ortholog (TgERC) plays a role in the storage of Ca^{2+} in the ER but is dispensable for the asexual lytic cycle. On the other hand, the *Plasmodium* ortholog (PfERC) is essential for asexual growth but not required for Ca^{2+} storage, organelle biogenesis, or protein trafficking. Instead, knockdown of PfERC inhibits parasite egress and invasion. Our results show that PfERC is critical for the rupture of the parasitophorous vacuole membrane, which is the first step in the egress of malaria parasites from erythrocytes. Surprisingly, PfERC knockdown does not affect the Ca^{2+}-dependent autoprocessing of the key egress protease, SUB1, but knockdown inhibits the second proteolytic maturation of SUB1 that occurs during its secretion from exoneme vesicles. We have therefore identified the first ER protein in *Plasmodium* that regulates egress of malaria parasites.

Introduction

Members of the phylum *Apicomplexa* are responsible for severe human diseases such as malaria, toxoplasmosis, and cryptosporidiosis. Together, this group of obligate intracellular parasites causes several hundred million infections every year and remains one of the major drivers of infant mortality (1-4). In fact, malaria results in nearly half a million deaths each year and most of the mortality is attributed to one species, *Plasmodium falciparum*. All the clinical symptoms of malaria are directly correlated to the invasion and lysis of the host red blood cells by these parasites. Malaria remains a vast health and economic burden in susceptible parts of the world and, despite continuous attempts for eradication and control, there is no effective vaccine and resistance has emerged to all existing drugs (5-8). This highlights the importance of understanding the factors that control the intracellular growth and development of the parasite, which will lead to the identification of key targets for antimalarial drug development.

Egress and invasion of apicomplexan parasites are ordered and essential processes which are regulated by signaling pathways dependent upon the second messengers, cGMP and Ca^{2+} (9-13). Upon invading the host cell, the parasites create and reside within a host-derived vacuole called the parasitophorous vacuole (PV). Within this vacuole, the parasites grow and divide into daughter cells, called merozoites in *Plasmodium*, which must egress from the host cell to complete the life cycle. This event is triggered by the activation of a cGMP dependent protein kinase (PKG) and the inhibition of PKG activity blocks egress (10, 14, 15). Ca^{2+}-signaling also induces egress although it is uncertain whether this pathway works downstream (11) or synergistically with cGMP signaling (10, 16, 17). For example, studies have shown that blocking the release of Ca^{2+} from intracellular stores using cell permeable Ca^{2+} chelators blocks egress in malaria parasites (17-19). It has been suggested that this release of Ca^{2+} into the cytoplasm comes from the parasite ER; however, the parasite genome lacks identifiable orthologs of ligand-gated Ca^{2+} channels such as the inositol 1,4,5,-triphosphate or ryanodine receptors (20). Increase in cytoplasmic Ca^{2+} is thought to

activate calcium dependent protein kinases (CDPKs) resulting in release of egress-related vesicles (16, 21).

In malaria parasites, these egress-related vesicles contain specific proteases that are proteolytically processed and activated, perhaps upon release into the PV (22-24). For example, one such pivotal enzyme is the serine protease, Subtilisin 1 (SUB1), which undergoes two cleavage events. First, the zymogen undergoes Ca^{2+} dependent autoprocessing in the ER (25, 26) and then, it is cleaved again by the recently identified aspartic protease, Plasmepsin X (PMX), probably during its secretion into the PV (23, 24). The proteolytic cleavage and activation of SUB1 is understood to be the point of no-return as the release of SUB1 into the PV results in the rapid (~10 minutes) breakdown of the parasitophorous vacuole membrane (PVM) (22, 27). Then, substrates of SUB1 such as merozoite surface protein 1 (MSP1) and serine-repeat antigen 6 (SERA6) help breakdown the RBC cytoskeleton and the RBC membrane (RBCM) (28, 29). Once egress is completed, the merozoites subsequently invade fresh RBCs to start the cycle again. Like egress, invasion requires specific secretory events such as fusion of micronemes to the merozoite membrane and secretion of rhoptry contents into the host cell, which provides the ligand-receptor pair essential for driving the parasite into the host cell (30, 31).

The parasite endoplasmic reticulum (ER) is thought to play a key role in egress and invasion of daughter merozoites. The putative functions of the parasite ER during these lifecycle stages, include biogenesis of the specific egress and invasion related organelles, transporting proteins to these organelles, and propagating Ca^{2+} signals essential for egress and invasion (32, 33). However, none of the proteins responsible for these ER-related functions during egress and invasion of apicomplexan parasites have been identified. We hypothesized that an ER-resident calcium binding protein (ERC) that is conserved in both *P. falciparum* (PfERC: PF3D7_1108600) and *T. gondii* (TgERC: TGME49_229480) plays a role in the Ca^{2+} signaling function of the parasite ER. In malaria parasites, this protein has been shown to bind calcium (34). However, the biological function of ERC is unknown in either apicomplexan parasite.

To address this, we used a ribozyme-based conditional knockdown system to study the role of PfERC in the asexual lifecycle *P. falciparum*, and we used a knock-out

approach to study the role of TgERC in the biology of *T. gondii*. We characterized the role of both Ca^{2+} binding proteins, PfERC and TgERC, and propose a model for their potential roles in Ca^{2+} signaling and in the life cycle of both parasites. This is the first ER resident protein identified in *P. falciparum* that is critical for the egress of malaria parasites from human red blood cells.

Results

PfERC and TgERC are EF-hand containing proteins localized in the ER

PfERC (PF3D7_1108600) and TgERC (TgME49_229480) are proteins related to the CREC (Calumenin, Reticulocalbin 1, ERC-55, Cab-45) family of proteins, which are characterized by the presence of multiple EF-hands and localize in various parts of the secretory pathway (35) (Figure 1A and Supplementary Figure 1). Like CREC members in other organisms, both PfERC and TgERC contain a signal peptide, multiple EF-hands, and an ER-retention signal (Figure 1A). However, TgERC differs from its *Plasmodium* homolog in that it only contains 5 EF-hands while PfERC contains and extended C-terminus that may contain a 6th, though degenerate, EF-hand (residues 314-325) (Supplementary Figure 1) (34). Since both proteins have an ER-retention signal they are predicted to reside within the lumen of the ER.

Generating conditional mutants of PfERC

In order to determine the biological role of PfERC, we used CRISPR/Cas9 gene editing to generate mutant parasite lines where the endogenous locus of PfERC was tagged with the conditional knockdown system employing the inducible ribozyme *glmS* or the inactive version of the ribozyme, *M9* (termed PfERC-*glmS* and PfERC-*M9* respectively) (Figure 1B and 1C) (36). Western blot and PCR analysis of PfERC-*glmS* and PfERC-*M9* parasite clones from two independent transfections demonstrate the correct insertion of the HA tag and the *glmS/M9* ribozymes at the PfERC locus (Figure 1D and 1E). Co-localization of PfERC with an ER marker also confirmed its sub-cellular localization to this organelle (Figure 1F).

PfERC is essential for asexual growth and for schizont to ring transition

To determine if PfERC was essential for intraerythrocytic survival, we grew asynchronous PfERC-*glmS* and PfERC-*M9* parasites in the presence of glucosamine (GlcN), which activates the *glmS* ribozyme leading to mRNA cleavage (Figure 1B). Western blot analysis shows that this leads to a reproducible reduction of PfERC expression in PfERC-*glmS* parasites while there is no reduction of protein levels in PfERC-*M9* parasites grown under identical conditions (Figure 2A-B). Importantly, this reduction in PfERC levels inhibited the asexual expansion of PfERC-*glmS* parasites, while there was no effect on the growth of PfERC-*M9* parasites (Figure 2C). The response of PfERC-*glmS* parasites was dose-dependent upon GlcN (Supplementary figure 2A)

Since our data show that PfERC was essential for growth within the host RBC, we used synchronous parasites to determine which asexual stage was affected by knockdown. We added GlcN to synchronized schizonts and took blood smears at regular intervals during the intraerythrocytic life cycle to detect morphological development of the asexual stages (Figure 2D). Our data show that all intracellular stages developed normally in both PfERC-*glmS* and PfERC-*M9* parasites grown with GlcN (Figure 2D and Supplementary Figure 2B). However, 55hrs after addition of GlcN, the PfERC-*glmS* parasites remained either as morphologically normal schizonts or were observed as daughter merozoites in the extracellular space as well as some that were attached to RBCs (Figure 2D). On the other hand, PfERC-*M9* parasites were able to egress and re-invade fresh RBCs and developed into ring stage parasites (Figure 2D and Supplementary figure 2B).

These data suggest that the knockdown of PfERC resulted in a defect in the conversion from schizonts to rings. Therefore, we induced knockdown and observed the conversion of schizonts into rings via flow cytometry at 44, 48, and 56 h post-addition of GlcN. The data show that over the course of 12 hours, PfERC-*M9* parasites transitioned from schizonts to rings as determined by the ring:schizont ratio while PfERC-*glmS* parasites were unable to convert from schizonts into rings resulting in a drastically reduced ratio (Figure 2E). These data show that there was a delay in the disappearance of the morphologically normal PfERC-*glmS* schizonts over the final few hours of the asexual life cycle compared to PfERC-*M9* schizonts, suggesting that knockdown of

PfERC led to a defect in egress (Figure 2F and 2G). Consequently, the delayed egress may lead to reduced numbers of ring stage parasites in PfERC-*glmS* parasites unlike PfERC-*M9* parasites (Figure 2F and 2H).

PfERC is required for PVM breakdown

Egress of daughter merozoites from the infected RBC is an ordered and rapid process where the PVM breakdown precedes the disruption of RBCM (Figure 3A). Therefore, we analyzed how egress of PfERC-*glmS* parasites was failing during knockdown. We took synchronized PfERC-*glmS* and PfERC-*M9* schizonts and initiated knockdown with addition of GlcN. These schizonts were allowed to reinvade fresh RBCs and proceed through the asexual stages for 48 hours until they developed into schizonts again. Then, these second cycle schizonts were incubated with inhibitors that block key steps during egress of *P. falciparum* (Figure 3A). To ensure synchronized egress, we used reversible inhibitors of PKG, Compound 1 (C1) or Compound 2 (C2), because inhibition of PKG allows merozoites to develop normally but prevents them from initiating egress (Figure 3A) (10, 15). We used flow cytometry to observe PfERC-*glmS* and PfERC-*M9* schizonts after washing off C1 and saw that there was a delay in the egress of PfERC-*glmS* schizonts while the majority (>60%) of the PfERC-*M9* schizonts were able to complete egress within two hours after washout of C1 (Figure 3B). Removal of C1 initiates the breakdown of the PVM followed by RBCM rupture (Figure 3A), suggesting that PfERC-*glmS* parasites fail to breach one of these membranes down despite removal of the PKG inhibitor.

Therefore, we tested whether PfERC knockdown prevented rupture of PVM or if PfERC functioned during RBCM breakdown (Figure 3A). PfERC-*glmS* and PfERC-*M9* schizonts (where knockdown had been initiated in the previous cycle) were incubated with C2 (10, 15) and observed by scanning electron microscopy (SEM) (Figure 3A and 3C). We observed that parasites treated with C2 were morphologically identical and had developed into mature schizonts within the PVM inside the RBC (Figure 3C). Then, we washed C2 from the parasites and observed these schizonts after 30 mins by SEM (Figure 3C). During this time period, PfERC-*M9* schizonts were able to initiate egress after removal of C2 and we observed free merozoites attached to the RBC as well as

clusters of merozoites that had broken out of the PVM but were contained by a collapsed RBCM wrapped around them (Figure 3C). In contrast, the PfERC-*glmS* schizonts were still stuck within the RBC and looked identical to the C2 arrested schizonts, suggesting that they had not initiated egress even though PKG was no longer inhibited (Figure 3C). These data suggest that knockdown of PfERC blocks egress at an early step, perhaps blocking the rupture of the PVM (Figure 3C).

We directly observed if breakdown of the PVM was impacted by knockdown of PfERC using transmission electron microscopy (TEM) (Figure 3D). Knockdown was induced by adding GlcN to PfERC-*glmS* and PfERC-*M9* schizonts and these parasites were allowed to go through one asexual cycle and develop into schizonts again 48hrs later. These schizonts were prevented from completing egress using the irreversible cysteine protease inhibitor, E-64 (Figure 3A). This inhibitor blocks the breakdown of the RBCM but allows both the breakdown of PVM and poration of the RBCM, which results in the loss of the electron dense contents of the infected RBC (Figure 3A) (15, 29, 37). Our results show that the PfERC-*M9* schizonts were able to break down the PVM as well as proceed with the poration of the RBCM after an 8-hour incubation with E-64, while the PfERC-*glmS* mutants were unable to proceed through the first step of egress and failed to rupture the PVM (Figure 3D). Overall, these data demonstrate that PfERC function is critical for the breakdown of the PVM (Figure 3C and 3D).

<u>Generating knockouts of TgERC</u>

We hypothesized that the ortholog of PfERC in the related apicomplexan parasite, *T. gondii,* may play a similar role in the egress of this parasite from its host cell. Therefore, we studied the biological role of TgERC by disrupting the transcription of the *tgerc* gene using CRISPR/Cas9 gene editing to insert the selection marker gene, dihydrofolate reductase (DHFR), in the *tgerc* locus (38, 39) (Figure 4A). Insertion of this gene into the *tgerc* locus interfered with the synthesis of the correct transcript and allowed the selection of clonal populations with pyrimethamine. The mutant *Δtgerc* was isolated by limiting dilution, and a PCR product of 6.1 kb obtained from the mutant parasites DNA indicated the insertion of the marker in the predicted locus (Figure 4B).

This fragment was only amplified from DNA of mutants while a fragment of 2.9kb was amplified from parental cells, which correspond to the intact locus of *tgerc* (Figure 4B).

In order to verify the localization of TgERC, we cloned and expressed TgERC in *E. coli* and the purified recombinant protein to immunize mice for the generation of specific antibodies. Western blot analysis using this antibody showed the presence of a ~38kDa band corresponding to TgERC in the parental (RH) parasites but absent in the Δ*tgerc* mutants (Figure 4C). Furthermore, IFAs using this antibody showed that TgERC localized predominantly to the endoplasmic reticulum in both extracellular and intracellular tachyzoites (Figure 4D).

<u>TgERC is required for Ca^{2+} storage but PfERC is not</u>

To determine whether TgERC was essential for the lytic cycle of the parasites, we performed plaque assays comparing growth of RH wild type with *Δtgerc* (Figure 4E). Our data show that the mutant parasites were able to form plaques about the same size as the controls (Figure 4F). These data suggest that unlike PfERC, TgERC was not required for egress from the host cell nor was it essential for the lytic cycle of *T. gondii*.

We next tested the if TgERC had any role in the storage of Ca^{2+} by testing for increases in cytoplasmic Ca^{2+} in response to thapsigargin. This inhibits the SERCA-Ca^{2+} ATPase, exposing leakage of Ca^{2+} from the ER into the cytoplasm as detected by fluorescence changes in parasites loaded with the Ca^{2+} indicator Fura-2AM (Figure 4G-J) (40, 41). We observed that the cytoplasmic Ca^{2+} increase in the *Δtgerc* mutants after adding thapsigargin was not significantly different than the response obtained under identical conditions when using wild type parasites (Figure 4G). However, when we performed the same experiment in the presence of extracellular Ca^{2+} (Figure 4I), the response to thapsigargin in *Δtgerc* tachyzoites was significantly reduced. The reduced response in the *Δtgerc* parasites indicates that the ER of these parasites is unable to store Ca^{2+} as efficiently as the wild type. This indicates that TgERC is part of the ER machinery involved in storing Ca^{2+}. However, it is evident that other mechanisms are in play since the parasites are still able to grow normally (Figure 4E-F).

Since knockout of TgERC resulted in defects in Ca^{2+} storage in the ER of *T. gondii* (Figure 4I and 4J), we hypothesized that PfERC is required for egress because it

plays a similar role in *P. falciparum* and does not have redundant mechanisms like *T. gondii* that compensate for the lack of TgERC. To test this hypothesis, synchronized PfERC-*glmS* and PfERC-*M9* schizonts were incubated with GlcN and allowed to proceed through one asexual cycle until they formed schizonts again. The second cycle schizonts were isolated using saponin lysis and loaded with Fluo-4AM to measure cytosolic Ca^{2+} (Supplementary Figure 3A). To assess if the storage of Ca^{2+} in the ER of the parasite was affected by knockdown of PfERC, we added the SERCA-Ca^{2+} ATPase inhibitor Cyclopiazoic acid (CPA) to these saponin-isolated parasites (Supplementary Figure 3A and 3B) (42). Inhibiting the SERCA-Ca^{2+} ATPase allows Ca^{2+} stored in the ER to leak into the cytoplasm, which results in a detectable change in the fluorescence of Fluo-4AM (Supplementary Figure 3B). Our measurements show that there was no difference in the amount of Ca^{2+} that leaked from the parasite ER, upon SERCA-Ca^{2+} ATPase inhibition, between PfERC-*glmS* and PfERC-*M9* schizonts (Supplementary Figure 3B).

To test if there was a defect in Ca^{2+} storage in neutral stores, we used the ionophore, Ionomycin, which releases Ca^{2+} from all neutral stores in the cell and measured the release of Ca^{2+} into the cytoplasm of PfERC-*glmS* and PfERC-*M9* schizonts. The parasites were isolated as described above and the changes in cytoplasmic Ca^{2+} were measured using Fluo-4AM (Supplementary Figure 3A and 3C). Again, we did not observe any difference in the amount of Ca^{2+} released into the cytoplasm of PfERC-*glmS* and PfERC-*M9* schizonts treated with ionomycin (Supplementary Figure 3C). These data suggest that the availability of free Ca^{2+} in the ER of *P. falciparum* is not affected by knockdown of PfERC. Furthermore, these data suggest that the observed egress defect upon PfERC knockdown was not a result of disequilibrium of Ca^{2+} in the parasite ER.

<u>Efficient SUB1 activation requires PfERC</u>

Electron microscopy data show that knockdown of PfERC prevents the breakdown of the PVM (Figure 3). A key event required for PVM breakdown is the proteolytic processing of SUB1, which starts a cascade that ends in the release of merozoites from the infected RBC (22, 29). Therefore, we tested if knockdown of PfERC

affects processing of PfSUB1. This protease is processed twice, once in the ER, where it undergoes a Ca^{2+}-dependent autocatalytic processing from its zymogen form (83-kDa) into a 54-kDa semi-proenzyme form (p54) (25, 26, 43). From the ER, SUB1 is transported to the egress-related secretory vesicles, the exonemes, which are secreted into the PV to initiate breakdown of the PV membrane. It is proposed that during secretion of SUB1, it is processed by PMX from its semi-proenzyme form (p54) to its mature form (p47) (23, 24). The secretion of mature p47 form of SUB1 initiates the breakdown of the PVM (22, 43). We hypothesized that PfERC is required for the first Ca^{2+}-dependent autoprocessing of SUB1, in the ER, from the 82-kDa zymogen into the p54 semi-proenzyme.

To test this hypothesis, PfERC-*glmS* and PfERC-*M9* schizonts were incubated with GlcN and allowed to progress through one asexual growth cycle (48 hours) to develop into schizonts again. Lysates from these synchronized schizonts were separated on a Western blot and probed with antibodies against SUB1 (Figure 5A and Supplementary Figure 4A). No change was observed in the Ca^{2+}-dependent autoprocessing of the zymogen-form of SUB1 into the semi-proenzyme (p54) form (Figure 5A and Supplementary Figure 4A). Surprisingly, we observed a reproducible and significant decrease in the processing of SUB1 from p54 to the p47 form in PfERC-*glmS* parasites (Figure 5A and 5B). Compared to PfERC-*M9* parasites, there was a >50% decrease in the amount of processed SUB1 (p47) in PfERC-*glmS* parasites (Figure 5B). These data suggest that PfERC is required for the secretion-dependent processing of SUB1 in exoneme vesicles.

Since we observed the presence of some mature SUB1 in PfERC-*glmS* parasites (Figure 5A), we tested if the activity of SUB1 was affected upon knockdown of PfERC by assaying for the processing of a known substrate of SUB1, the merozoite surface protein 1 (MSP1). MSP1 is required for the initial attachment of merozoites onto RBCs and it has been shown that correct processing of MSP1, by SUB1, is also required for efficient egress as it plays a role in breakdown of the RBC cytoskeleton after release from the PVM (28, 44-46). Lysates from synchronized second-cycle PfERC-*glmS* and PfERC-*M9* schizonts, treated as above, were separated on a Western blot and probed using anti-MSP1 antibodies (Figure 5C and Supplementary Figure 4B). Our data show

that the there was a ~60% reduction in MSP1 processing in our PfERC-*glmS* parasites as compared to PfERC-*M9* parasites after knockdown (Figure 5C). In fact, we observed that PfERC-*glmS* schizonts had 3 times more unprocessed MSP1 in contrast to PfERC-*M9* schizonts (Figure 5D). These data reveal that knockdown of PfERC leads to defects in SUB1 processing and activity, and consequently, MSP1 processing (Figure 5).

<u>Knockdown of PfERC does not affect protein trafficking or organelle biogenesis</u>

MSP1 is a GPI-anchored merozoite membrane protein that is presumably processed by SUB1 once the protease is secreted into the PV (28, 29). Therefore, we wanted to verify that knockdown of PfERC led to a specific defect in the egress cascade and is not due to a block in protein trafficking via the ER or defects in the biogenesis of egress and invasion related organelles. To address this, we used super resolution structured illumination microscopy (SR-SIM) to observe if there was a difference in the surface expression of MSP1 between PfERC-*glmS* and PfERC-*M9* schizonts upon knockdown of PfERC (Figure 5E). As before, knockdown was initiated in synchronized PfERC-*glmS* and PfERC-*M9* schizonts and after 48 hours, these schizonts were stained with anti-MSP1 antibodies. Our data shows that there was no difference in the trafficking of MSP1 to the surface of developing PfERC-*glmS* or PfERC-*M9* merozoites after knockdown (Figure 5E). In addition, we also tested whether the localization of other egress-and invasion-related proteins that traffic through the ER where affected after knockdown by staining similarly treated schizonts for the micronemal protein AMA1 and the rhoptry-bulb protein RAP1. Our data show that, again, there was no difference in the localization of these proteins in schizonts between PfERC-*glmS* and PfERC-*M9* parasites suggesting that the knockdown of PfERC does not cause a generalized defect in the secretory pathway (Figure 5F&G).

As the ER generates the lipid membranes required for generating organelles essential for egress and invasion, we wanted to see if their biogenesis was inhibited upon knockdown. If true, this could explain the processing defects that we observe for SUB1 and MSP1. We observed E64-treated schizonts (Figure 3A) by transmission electron microscopy and these data show that both micronemes and rhoptries remain morphologically intact in both PfERC-*glmS* and PfERC-*M9* parasites after knockdown

was induced (Figure 5H). Successful knockdown was confirmed by the lack of PVM membrane fragments in PfERC-*glmS* parasites, which are present in the PfERC-*M9* parasites, suggesting that PVM rupture occurred normally in the presence of PfERC but not when the protein is knocked down (Figure 5H). These data suggest that the knockdown of PfERC does not lead to defects in organelle biogenesis (Figure 3D and Figure 5H).

PfERC is required for invasion of merozoites

The synchronized growth assays suggest that knockdown of PfERC inhibits the invasion of merozoites into RBCs (Figure 2D, H and Figure 5C, D). To assess if invasion was reduced upon knockdown, PfERC-*glmS* and PfERC-*M9* schizonts in the second cycle after 48 hours in GlcN, were incubated with the PKG inhibitor, C1, for four hours (Figure 3A). The inhibitor was then washed off and the formation of ring stages was observed over two hours by flow cytometry (Figure 6A). We observed that there was a delay in the formation of ring stages as well as a drastic decrease in the numbers of ring stage parasites formed in PfERC-*glmS* parasites compared to the PfERC-*M9* control (Figure 6A). This could be due to loss of egress or could be a combination of effects on egress and invasion due to PfERC knockdown.

To decouple the egress and invasion phenotypes, we directly measured the efficiency of merozoite invasion (Figure 6B). This was accomplished by incubating second cycle PfERC-*glmS* and PfERC-*M9* schizonts with E-64 and then mechanically releasing the merozoites (Figure 6B) (47). Incubation with E-64 for 8 hours allows for the completion of schizogony and formation of invasion-competent merozoites. These purified merozoites were then allowed to invade fresh RBCs and the invasion rate was quantified using flow cytometry as described previously (Figure 6C) (47). These data show that there was a drastic reduction in the invasion efficiency of PfERC-*glmS* merozoites as compared to the control PfERC-*M9* merozoites, thus demonstrating that knockdown of PfERC led to a defect in invasion as well (Figure 6C).

PfERC knockdown affects proteolytic processing of AMA1

The reduced invasion of PfERC-*glmS* merozoites could be explained by the reduction in processing of MSP1, which is known to be required for the initial attachment of merozoites to the RBC (44-46, 48). Invasion of RBCs by *P. falciparum* merozoites requires secretion of contents from another apical organelle, the rhoptries, into the RBC (49-51). Proteins in the rhoptries, like the rhotry-bulb protein, RAP1, require proteolytic processing for their activity (23, 24). Once in the rhoptry, RAP1 is processed by the aspartic protease, Plasmepsin IX (PMIX), from a pro-form (p83) to a mature form (p67) (23, 24, 52, 53). Therefore, we tested if RAP1 processing was affected by knockdown of PfERC using Western blot analysis (Figure 6F and Supplementary Figure 4C). Our data show that the proteolytic processing of RAP1 was not affected by the knockdown of PfERC (Figure 6G), showing that knockdown does not lead to a generalized defect in the processing of all proteins that traverse through the secretory pathway.

Another key and essential step in invasion of merozoites is the formation of a tight junction between the parasite and the RBC and AMA1 is critical for the formation of this tight junction (30, 31). AMA1 is trafficked from micronemes to the merozoite surface and there it is processed from its pro-form (p83) to its mature form (p66) by an unknown protease (54-56). The proteolytic processing of AMA1 is critical for its function during invasion (57). Therefore, we tested if the processing of the AMA1 was affected upon knockdown of PfERC. As before, after initiating knockdown in synchronized schizonts, we separated lysates from second cycle PfERC-*glmS* and PfERC-*M9* schizonts on a Western blot and probed it with anti-AMA1 antibodies (Figure 6D and Supplementary Figure 4D). We observed a significant reduction in the proteolytic processing of AMA1 upon knockdown (Figure 6D). Indeed, there was a >40% decrease in the processing of AMA1 in PfERC-*glmS* mutants compared to the PfERC-*M9* control (Figure 6E). These data suggest that PfERC is required for the correct processing of AMA1 and therefore, essential for invasion of merozoites into the host RBC.

Discussion

In this study, we addressed the biological role of a conserved Ca^{2+}-binding protein that resides in the lumen of the ER of apicomplexan parasites. We have shown

that the *Toxoplasma* homolog, TgERC, is dispensable for *in vitro* growth, and the *Plasmodium* homolog, PfERC, is essential for asexual replication (Figures 2 and 3). Our data show that knockdown of PfERC did not affect the ring and trophozoite development but had a clear effect on the subsequent schizont-to-ring transition (Figures 2D-H, 3B, 6A, and Supplementary Figure 2). Specifically, these data show that PfERC is required for both egress from infected RBCs (Figures 2D, 2G, and 3) and invasion into host erythrocytes (Figures 2D, 2H and 6A-C). This is consistent with data that suggest PfERC may be transcriptionally controlled by the invasion-specific transcription factor PfAP2-I (58). Our data show that knockdown of PfERC leads to defects in the processing of proteins critical for invasion of merozoites into the host RBC, namely, MSP1 and AMA1 (Figures 5C-D and 6 D-E). However, the observed invasion defect is likely a secondary effect because several proteins critical for invasion are processed during egress (23, 24, 28, 29). Given the limitations of the conditional knockdown system, we cannot tease out a specific role for PfERC in invasion. As invasion occurs rapidly (<2mins), a potential specific invasion-related function of PfERC could be tested using a small molecule that specifically targets PfERC (59). Overall, these data show that PfERC is essential for egress of merozoites from the infected RBC and for invasion of merozoites into the host erythrocyte.

It has been observed previously that the mechanisms at play during egress of daughter parasites from the host cells differs between *Toxoplasma* and *Plasmodium.* This is highlighted by studies showing that orthologs of apicomplexan proteins can serve different functions in their respective organisms. For example, TgCDPK3 is required for egress of *T. gondii* tachyzoites while its *Plasmodium* ortholog, PfCDPK1, is essential for both egress and invasion (60). Similarly, another calcium dependent protein kinase, TgCDPK1, plays a vital role in egress and invasion of *Toxoplasma* tachyzoites but the *Plasmodium* ortholog, PfCDPK4, was shown to be required for the development of sexual stages (60). Additionally, TgSUB1 localizes to micronemes and is used for processing of micronemal proteins that are required for invasion of *T. gondii* into host cells (61). However, PfSUB1 is critical for egress of *Plasmodium* merozoites, localizes to exonemes, and upon secretion into the PV, processes both micronemal and non-micronemal proteins (22, 62). These differences could be related to their

development within different host cells. *Plasmodium* prefers the erythrocyte for its asexual expansion and *Toxoplasma* is able to make its home within any nucleated cell. Another factor is the difference in the primary structure of TgERC and PfERC, which possesses an additional putative EF-hand domain compared to its homolog in *T. gondii.* Therefore, it is not surprising that the biological role of TgERC may be different from the function of PfERC. This was evident in the data that suggest that TgERC plays a role in ER Ca^{2+} storage but PfERC may not (Figures 4I, 4J and Supplementary Figure 3). It is interesting to note that there are no orthologues of many Ca^{2+} entry channels such as store-operated channels (ORAI), or their partners such as the ER sensor protein stromal interaction molecule (STIM) in the genome of apicomplexan parasites (13, 20). Future work on identifying the interacting partners of TgERC may provide some clues as to the identity of putative ER Ca^{2+} sensors that could regulate the function Ca^{2+} entry channels in these divergent parasites.

During the formation of daughter merozoites in schizonts, several key egress and invasion related organelles essential for propagation of the infection are generated. The ER is thought to play a key role in the biogenesis of these organelles and the ER is responsible for transporting the essential proteins to these organelles (32, 33). As an ER-resident protein, knockdown of PfERC could affect several ER functions such as protein trafficking, organellar biogenesis, and Ca^{2+} signaling. Therefore, we tested if PfERC functions in the trafficking of proteins required for schizont to ring transition such as MSP1, AMA1, and RAP1. A defect in the secretory pathway would explain the observed deficiencies in the proteolytic processing of SUB1, MSP1 and AMA1, as transport out of the ER is required for their maturation (10, 26, 52, 63). However, super-resolution and electron microscopy experiments show that proteins on the merozoite surface, micronemes, and rhoptries are trafficked normally and biogenesis of egress and invasion organelles is normal (Figures 3D and 5E-5H). Likewise, Western blot analysis showed that the proteolytic processing of a rhoptry protein, RAP1, which is processed after transport to the organelle, occurs normally upon knockdown of PfERC (Figures 5G, and 6F-G) (23, 24). These data show that knockdown of PfERC does not result in a generalized defect in protein trafficking via the ER, or in organelle biogenesis.

Instead, our data show that PfERC knockdown inhibits the proteolytic maturation of a subset of proteins essential for egress and invasion.

A key enzyme that is required for initiating egress is the protease SUB1 as this serine protease plays a critical role in disruption of both the PVM and RBCM. It is produced as an 82-kDa zymogen in the ER, where it rapidly self-processes into a 54-kDa semi-proenzyme in the ER (43). If PfERC was needed for this autoprocessing event then this would explain the observed knockdown phenotypes as they are similar to that seen when SUB1 is conditionally knocked out (29). However, our data show that only the second processing step of SUB1 that produces the mature, active form of the protease (p54 to p47), is inhibited upon PfERC knockdown (Figure 5A-B and Supplementary Figure 4A). The processing of SUB1 from p54 to p47 occurs once it is trafficked out of the ER and putatively during secretion of exoneme vesicles (23-25). One possibility is that PfERC is required for the maturation of an unknown protease in this pathway that works upstream of SUB1. The recently discovered aspartic protease, PMX, is responsible for the maturation of SUB1 from p54 to p47 (23, 24). In turn, PMX itself is proteolytically matured from a 67kDa zymogen to a 45kDa active protease (23, 24). But unlike most aspartyl proteases, PMX does not autoprocess because inhibitors that block PMX activity do not inhibit its maturation (23). The maturase responsible for PMX cleavage is unknown and therefore, if this unknown protease requires PfERC for its activity, then knockdown of PfERC would lead to the observed defects in egress.

Immunofluorescence microscopy, electron microscopy, and Ca^{2+} measurements ruled out a role for PfERC in Ca^{2+} storage, protein trafficking, or organelle biogenesis (Figures 3D, 5E-G, 6F, 6G and Supplementary Figure 3). Another interesting possibility is that PfERC may play a role the signal-dependent release of Ca^{2+} from the ER. This is difficult to test in *Plasmodium* since there are no clear orthologs for a ligand-dependent Ca^{2+} channel in its genome (20). Such a putative role for PfERC is supported by data which show that knockdown of PfERC inhibits egress by preventing the breakdown of the PVM and by the defective processing of MSP1 by SUB1 (Figure 3C-D and 5A-D). The processing of MSP1 happens after the secretion of SUB1 into the PV as MSP1 is a GPI-anchored protein on the membrane of merozoites (28). Exocytosis of vesicles, such as those carrying SUB1, during egress is thought to be Ca^{2+} dependent (13, 19).

Therefore, these data are consistent with a model where PfERC functions in the signaling pathway that releases ER Ca^{2+} required for exocytosis of vesicles during egress.

PfERC is a member of the CREC family of proteins and other members of this family are known to play key roles in the function of ligand-gated Ca^{2+} channels. For example, calumenin and reticulocalbin 1 have been shown to interact with the Ryanodine and IP_3 receptors, respectively, and regulate their function (64, 65). It has also been suggested that some CREC members serve as Ca^{2+} sensors in the ER as they exhibit calcium-dependent structural changes (66, 67). Therefore, PfERC may play a role in signal-dependent release of Ca^{2+} from the ER by regulating the function of the unknown ligand-dependent Ca^{2+} channel in *P. falciparum.* Such a model would predict that PfERC binds to the putative ligand-dependent Ca^{2+} channel for its function such that absence of PfERC leads to a non-functional channel. Ongoing experiments to identify the binding partners of PfERC will help distinguish between these possibilities.

A principal finding of these studies is the identification of the first protein in the ER of *P. falciparum* with a specific role in egress of malaria parasites from RBCs and potentially in the invasion of parasites into the RBC (Figures 2, 3, 5 and 6). These studies lay the foundation for understanding the vital and key role that ER-resident proteins play in the egress of human malaria parasites from the infected RBC and their re-entry into the host cell. Some studies have suggested that a key class of antimalarials containing endoperoxides, which includes the frontline antimalarial artemisinin, may target PfERC (59) and one of the transcriptomic responses of artemisinin-resistant parasites is the overexpression of PfERC (68). These data suggest that targeting PfERC, and thus egress, is a viable strategy for antimalarial drug development.

Material and Methods

Cell culture and transfections. *Plasmodium* parasites were cultured in RPMI 1640 medium supplemented with Albumax I (Gibco) and transfected as described earlier (69-72). *T. gondii* tachyzoites were maintained in vitro by serial passage in Dulbecco's modified minimal essential media (DMEM) with 1% FBS, 2.5 µg/ml amphotericin B, and

100 µg/ml streptomycin. Host cells were human telomerase reverse transcriptase immortalized foreskin fibroblasts (hTERT) (73).

To generate PfERC-*glmS* and PfERC-*M9* parasites, a mix of two plasmids (50µg of each) was transfected in duplicate into 3D7 parasites. The plasmid mix contained pUF1-Cas9-guide (74) which contains the DHOD resistance gene, and pPfERC-HA-SDEL-*glmS* or pPfERC-HA-SDEL-*M9*, which are marker-free. Drug pressure was applied 48hrs after transfection, using 1µM (DSM1) (75), selecting only for Cas9 expression. DSM1 was then removed from the culturing medium once the parasites were detected in the culture, around 3 weeks post-transfection.

The *Δtgerc* knock-out line was generated by inserting the dihydrofolate reductase (DHFR) gene into the first ~300 nucleotides of the *tgerc* gene to disrupt the coding sequence. The CRISPR/Cas9 gene knockout method previously reported was used (39). A fragment corresponding to the single guide RNA (sgRNA) using primers 1+2 was cloned into pSAG1:CAS9::U6:sgUPRT (Addgene #54467) using the Q5 site-directed mutagenesis kit (NEB). The resulting pSAG1:CAS9::U6:sgTgERC was co-transfected with the DHFR cassette (in proportion 3:1) using primers 3+4 to tachyzoites of the RH strain. After pyrimethamine selection (1 µM), parasites were sub-cloned by limiting dilution and screened for positive clones by PCR using primers 5+6 shown in Table S1.

Construction of PfERC plasmids. Genomic DNA was isolated from *P. falciparum* cultures using the QIAamp DNA blood kit (Qiagen). Constructs utilized in this study were confirmed by sequencing. PCR products were inserted into the respective plasmids using the In-Fusion cloning system (Clontech), the sequence- and ligation-independent cloning (SLIC) method (71, 72), T4-ligation (New England BioLabs), or site-directed mutagenesis using QuickChange (Agilent). To generate the pHA-SDEL-*glmS*/*M9* plasmid, primers 7+8 were used to add an SDEL sequence at the end of the HA tag in pHA-*glmS* and pHA-*M9* plasmids (71, 72).

For the generation of the PfERC-*glmS*/*M9* conditional mutants, pHA-SDEL-*glmS*/*M9* plasmid was used as a donor DNA template consisting of two homology regions flanking the HA-SDEL tag and the *glmS* or *M9* sequence. To allow efficient

genomic integration of the pHA-SDEL-*glmS* and pHA-SDEL-*M9* donor plasmids, 800-bp sequences were used for each homology region. The C-terminus of the *pferc* coding region was PCR amplified from genomic DNA using primers 9+10 (containing the shield mutation) and was inserted into pHA-SDEL-*glmS* and pHA-SDEL-*M9* using restriction sites SacII and AfeI. The 3'UTR of *pferc* was PCR amplified from genomic DNA using primers 11+12 and was inserted into pHA-SDEL-*glmS* and pHA-SDEL-*M9* (already containing the C-terminus region) using restriction sites HindIII and NheI. For expression of PfERC guide RNA, oligos 13+14 were inserted into pUF1-Cas9-guide as previously described (74, 76). Briefly, pUF1-Cas9-guide was digested with BtgZI and annealed oligos were inserted using the SLIC method. Primers 9+12 and primers 9+15 (which recognizes the *glmS*/*M9* sequence) were used for clone verification.

***Plasmodium* growth assays**. Asynchronous growth assays were done as described previously (71, 72). Briefly, 5mM glucosamine (GlcN) (Sigma) was added to the growth medium and parasitemia was monitored every 24hrs via flow cytometry using a CyAn ADP (Beckman Coulter) or CytoFLEX (Beckman Coulter) instrument and analyzed by FlowJo software (Treestar, Inc.). As required, parasites were subcultured to avoid high parasite density, and relative parasitemia at each time point was back-calculated based on actual parasitemia multiplied by the relevant dilution factors. One hundred percent parasitemia was determined as the highest relative parasitemia and was used to normalize parasite growth. Data were fit to exponential growth equations using Prism (GraphPad Software, Inc.).

To determine the ring:schizont ratio of PfERC-*glmS* and PfERC-*M9* parasites, 7.5mM GlcN was added to percoll isolated schizont-stage parasites and parasites were allowed to egress and reinvade fresh RBCs. Two hours later, 5% sorbitol +7.5mM GlcN was added to the invaded culture to lyse any remaining schizonts and isolate two-hour rings. The ring-stage parasites were grown again in media supplemented with GlcN. Then samples were taken at 44hrs, 48hrs, and 56hrs, and read by flow cytometry to determine the population of rings and schizonts present at those times using FlowJo software (Treestar, Inc.). To determine the development of each life cycle stage during the asexual lifecycle of PfERC-*glmS* and PfERC-*M9* parasites, 7.5mM was added to

percoll isolated schizont-stage parasites and parasites were allowed to egress and reinvade fresh RBCs. Time points were then taken by blood smears and the percentage of the specific lifecycle stage was calculated as: $\% \, of \, stage = \frac{number \, of \, specific \, stage}{total \, number \, of \, parasites}$. Time 0hr is when GlcN was added. To determine the % amount of egressing or invading parasites, we took the % values of schizonts or rings at 44hrs and subtracted that number from % values of schizonts or rings at 12hrs later.

For growth assays using Compound 1 (4-[2-(4-fluorophenyl)-5-(1-methylpiperidine-4-yl)-1H-pyrrol-3-yl] pyridine), synchronized schizonts were grown with 7.5mM GlcN for about 48hrs. Then, schizonts were percoll isolated and incubated with Compound 1 for 4hrs and then removed by gently washing parasites twice with 1mL of warm, complete RPMI. Parasites were resuspended with fresh media and RBCs and samples were taken and read by flow cytometry. DNA content was determined using Hoechst staining (ThermoFisher).

Plaque assays. Plaque assays were done as previously described (77). Two hundred freshly egressed tachyzoites were used to infect a confluent monolayer of hTERT fibroblasts followed by 7 days of growth. Monolayers were fixed and stained with 5x crystal violet. Plaque sizes were analyzed with FIJI by measuring the area of fifteen plaques per biological replicate. At least 3 biological experiments were done for wild type control RH and TgERC knockout.

Generation of TgERC antibody. The *tgerc* gene (TgGT1_229480) was identified from ToxoDB. Total RNA of the wild type strain (RH) was extracted from freshly lysed parasites using Trizol® reagent (Sigma) following the manufacturer's instructions. The RNA sample was further treated with DNAse I for 10 min at 37 °C (New England Biolabs) to remove contaminating DNA. Four micrograms of purified mRNA were used to synthesize cDNA using the superscript III first-strand synthesis system according to the manufacturer's protocol (ThermoFisher Scientific-Life Technologies). *T. gondii* cDNA was used as a template for amplifying the *tgerc* gene (~ 1 Kb) with the primers

16+17 (Table S1). The PCR product was gel purified and cloned using the pET32 Ek/LIC vector system (Novagen). The construct was sequenced and transformed into *E. coli* BL21(DE3)-CodonPlus. Expression of the TgERC protein was induced for 3 h at 37°C with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were pelleted and resuspended in equilibration/binding buffer (50 mM Na_3PO4, 300 mM NaCl, 10 mM Imidazole, and 1:500 protease inhibitor cocktail P-8849). Cells were then sonicated on ice for 80 seconds total with amplitude set to 30% (Branson Digital Sonifier Model 250) and centrifuged at 12,000 rpm at 4^oC for 20 min. The supernatant was filtered through a 0.45 µm cellulose acetate filter (VWR) and the protein was purified using HisPur Ni-NTA chromatography following instructions from the manufacturer. Unbound proteins were washed off with a total volume of 12 ml of wash buffer (50 mM Na_3PO_4, 300 mM NaCl and 20 mM Imidazole) and the TgERC recombinant protein was eluted with 5 ml of elution buffer (50 mM Na_3PO_4 ,300 mM NaCl and 250 mM Imidizole). Elution fractions were verified by SDS-PAGE.

Antibodies against the TgERC recombinant protein were generated in mice. Six CD-1 mice (Charles River, Inc.) were intraperitoneally injected with 100 µg of purified TgERC recombinant protein mixed 1:1 with complete Freund adjuvant. Mice were boosted twice with 50 µg of antigen and the final serum was collected by cardiac puncture after CO_2 euthanasia (IACUC protocol A2018 02-021-Y1-A0)

Western blotting. Western blotting for *Plasmodium* parasites was performed as described previously (71, 72). Briefly, parasites were permeabilized selectively by treatment with ice-cold 0.04% saponin in PBS for 10 min and pellets were collected for detection of proteins with the parasite. For detection of MSP1, schizonts were isolated on a Percoll gradient (Genesee Scientific) and whole-cell lysates were generated by sonication. The antibodies used in this study were rat anti-HA (3F10; Roche, 1:3,000), rabbit anti-HA (715500; Invitrogen, 1:100), rabbit anti-PfEF1α (from D. Goldberg, 1:2,000), mouse anti-plasmepsin V (from D. Goldberg, 1:400), rabbit anti-SUB1 (from Z. Dou and M. Blackman, 1:10,000), rat anti-AMA1 (28G2; Alan Thomas via BEI Resources, NIAID, NIH 1:500), mouse anti-MSP1 (12.4; European Malaria Reagent Repository, 1:500) and mouse anti-RAP1 (2.29; European Malaria Reagent Repository,

1:500). The secondary antibodies that were used are IRDye 680CW goat anti-rabbit IgG and IRDye 800CW goat anti-mouse IgG (LICOR Biosciences) (1:20,000). The Western blot images were processed using the Odyssey Clx LICOR infrared imaging system software (LICOR Biosciences). Calculation of knockdown and processing ratios was determined by both the Odyssey infrared imaging system software and ImageJ 1.8 (NIH).

For *T. gondii*, lysates were prepared by resuspending a pellet of 1 x 10^8 tachyzoites in 50 µl of Cell Lytic^M lysis buffer containing 12.5 U benzonase and 1X protease cocktail inhibitor (P8340 Sigma). The reaction was stopped with one volume of 2% SDS and 1 mM EDTA. Total lysates were boiled in Laemmli sample buffer (BioRad) (78). Immunoblots were done following established protocols using polyclonal mouse anti-TgERC serum (1:1,000). The Odyssey Clx LICOR system was used for detection, and the secondary antibody was goat anti-mouse IRDye 800WC (1:10,000).

Immunofluorescence microscopy. For IFAs, cells were fixed as described previously (71, 72). The antibodies used for IFA were: rat anti-HA antibody (clone 3F10; Roche, 1:100), rat anti-PfGRP78 (MRA-1247; BEI Resources, NIAID, NIH 1:100), mouse anti-MSP1 (12.4; European Malaria Reagent Repository, 1:500), rat anti-AMA1 (28G2; Alan Thomas via BEI Resources, NIAID, NIH 1:500), and mouse anti-RAP1 (2.29; European Malaria Reagent Repository, 1:500). Secondary antibodies used were anti-rat antibody conjugated to Alexa Fluor 488 or 546 and anti-rabbit antibody conjugated to Alexa Fluor 488, (Life Technologies, 1:100). Cells were mounted on ProLong diamond with 4',6'-diamidino-2-phenylindole (DAPI) (Invitrogen) and imaged using a Delta-Vision II microscope system with an Olympus IX-71 inverted microscope using a 100x objective or an Elyra S1 SR-SIM microscope (Zeiss, Jena, Germany). Image processing, analysis, and display were performed using SoftWorx or Axiovision and Adobe Photoshop. Adjustments to brightness and contrast were made for display purposes.

For *T. gondii* IFAs, extracellular parasites were collected and purified as previously described (77). Parasites were washed once with buffer A with glucose (BAG, 116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 5.5 mM glucose and 50 mM HEPES, pH 7.4) and an aliquot with 2 x 10^4 parasites was overlaid on a coverslip

previously treated with poly-L-Lysine. Intracellular tachyzoites were grown on hTERT cells on coverslips previously infected with freshly lysed parasites. Both extracellular and intracellular parasites preparations were fixed with 2.5% paraformaldehyde for 20 min at room temperature (RT), permeabilized with 0.25% Triton X-100 and blocked with 3% bovine serum albumin (BSA). For co-localization studies parasites were exposed to primary antibodies mouse anti-TgERC 1:1000 and rabbit anti-GFP 1:800 after transfection with the P30-GFP-HDEL plasmid (79). The secondary antibodies were Alexa-Fluor488 goat-anti mouse and Alexa-Flour594 goat-anti rabbit at 1:1,000. Slides were examined using an Olympus IX-71 inverted fluorescence microscope with a photometric CoolSNAP HQ charge-coupled device (CCD) camera driven by DeltaVision software (Applied Precision).

Invasion Rate Quantification. To calculate the invasion rate, parasites were treated as described previously (47). Briefly, 7.5mM GlcN was added to percoll isolated schizont-stage parasites and parasites were allowed to egress and reinvade fresh RBCs. 48hrs later, schizonts were percoll purified and incubated with 20µM E-64 (Sigma) at 37°C in an incubator for 7-8hrs. Once incubation was done, merozoites were isolated by gently resuspending and passing the schizonts through a 1.2µm Acrodisic Syringe Filter (PALL). Merozoites were spun at 2000xg for 5min, and then resuspended in 100µL of complete RMPI medium and added to a 1mL culture of uninfected RBCs at 2% hematocrit. Cultures were grown in a FluoroDish cell culture dish (World Precision Instruments) and gassed in a chamber for 20-24hrs. Invasion rate was then measured by the following equation: $IR = iRBC \cdot \left[\frac{RBC/\mu L}{Mz/\mu L}\right]$ where "*iRBC*" is the parasitemia 20-24hrs later, "*RBC/µL*" are the free RBCs used before addition of merozoites and "*Mz/µL*" are the merozoites found in the 100µL suspension used before adding to fresh RBCs. Values for these variables were acquired by flow cytometry (CytoFLEX Beckman Coulter) with cells stained with acridine orange. The data were normalized using the IR values for PfERC-*M9* merozoites as 100%.

Transmission Electron Microscopy. 7.5mM GlcN was added to percoll isolated schizont-stage parasites and parasites were allowed to egress and reinvade fresh

RBCs. 48hrs later, parasites were percoll-isolated and then incubated with 20µM E-64 for 8hrs. After incubation, parasites were washed with 1X PBS and gently resuspended in 2.5% glutaraldehyde in 0.1M sodium cacodylate-HCl (Sigma) buffer pH 7.2 for 1hr at room temperature. Parasites were then rinsed in 0.1M Cacodylate-HCl buffer before agar-enrobing the cells in 3% Noble agar. Parasites were post fixed in 1% osmium tetroxide/0.1M Cacodylate-HCl buffer for 1 hour and rinsed in buffer and deionized water. Dehydration of the parasite samples was done with an ethanol series and then exposed to Propylene oxide before infiltration with Epon-Araldite. The blocks of parasites were trimmed, and sections were obtained using a Reichert Ultracut S ultramicrotome (Leica, Inc., Deerfield, IL). 60-70nm sections were placed on 200-mesh copper grids and post-stained with ethanolic uranyl acetate and Reynolds Lead Citrate. Grids were viewed with a JEOL JEM-1011 Transmission Electron Microscope (JEOL USA, Inc., Peabody, MA) using an accelerating voltage of 80 KeV. Images were acquired using an AMT XR80M Wide-Angle Multi-Discipline Mid-Mount CCD Camera (Advanced Microscopy Techniques, Woburn, MA).

Scanning Electron Microscopy. 7.5mM GlcN was added to percoll isolated schizont-stage parasites and parasites were allowed to egress and reinvade fresh RBCs. 48hrs later, parasites were percoll-isolated and then incubated with 2µM Compound 2 (4-[7-[(dimethylamino)methyl]-2-(4-fluorphenyl)imidazo[1,2-a]pyridine-3-yl]pyrimidin-2-amine) for 4 hours without shaking at 37°C in an incubator. After incubation, parasites were washed twice with warm, complete RPMI + 7.5mM GlcN. Samples were taken immediately after washing off Compound 2 and then 30min after and fixed as with TEM samples. Parasites were rinsed with 0.1M Cacodylate-HCl buffer before placing on glass coverslips prepared with 0.1% Poly-L-lysine. Parasites were allowed to settle onto the glass coverslips in a moist chamber overnight and then post fixed in 1% osmium tetroxide/0.1M Cacodylate-HCl buffer for 30 minutes. Cells on coverslips were rinsed three times in deionized water and then dehydrated with an ethanol series. The glass coverslips were critical point dried in an Autosamdri-814 Critical Point Dryer (Tousimis Research Corporation, Rockville, MD), mounted onto aluminum pin stubs

with colloidal paint, and sputter coated with gold-palladium with a Leica EM ACE600 Coater (Leica Microsystems Inc., Buffalo Grove, IL). Stubs were examined with the FE-SEM FEI Teneo (FEI, Inc., Hillsboro, OR) using the secondary electron detector to obtain digital images.

Calcium Measurements. To measure Ca^{2+} in PfERC mutants, knockdown was induced on synchronized schizonts. After 48hrs, schizonts were percoll purified and permeabilized selectively by treatment with ice-cold 0.04% saponin in PBS for 10 min. Isolated parasites were then washed 2X with BAG Buffer (116mM NaCl, 5.4mM KCl, 0.8mM $MgSO_4 \cdot 7H_2O$, 50mM HEPES, 5.5mM Glucose) + 7.5mM GlcN and incubated with 10µM Fluo-4AM (ThermoFisher) for 45min while rocking at 37°C. After incubation, cells were washed 2X with BAG buffer + 7.5mM GlcN and immediately taken for fluorimetric measurements. Fluorescence measurements were carried out in a cuvette (Sarstedt) containing parasites suspended in 2.5 ml of BAG buffer and 100uM EGTA (Sigma). The cuvette was placed in a Hitachi F-4500 fluorescence spectrophotometer and Fluo-4AM excitation was done at 505 nm with emission read at 530 nm (80). Drugs and reagents were added via a Hamilton syringe. Final concentration of CPA (Sigma) was 3 µM, and Ionomycin (Sigma) at 2µM.

Loading of *Toxoplasma gondii* tachyzoites with Fura-2AM (ThermoFisher) was done as previously described (40). Briefly, freshly collected parasites were washed twice with BAG by centrifugation (706 g for 10 min) and resuspended at a final density of 1 x 10^9 parasites/ml in loading buffer (BAG plus 1.5% sucrose, and 5µM Fura-2AM). The suspension was incubated for 26 min at 26 °C with mild agitation. Subsequently, parasites were washed twice with BAG to remove extracellular dye, re-suspended to a final density of 1 x 10^9/ml in BAG and kept on ice. For fluorescence measurements, 2 x 10^7 parasites/ml were placed in a cuvette with 2.5 ml of BAG buffer. Fluorescence measurements were done in a Hitachi F-7000 fluorescence spectrophotometer using the Fura-2AM conditions for excitation (340 and 380 nm) and emission (510 nm). The Fura-2AM fluorescence response to Ca^{2+} was calibrated from the ratio of 340/380 nm fluorescence values after subtraction of the background fluorescence of the cells at 340

and 380 nm as described previously (81). The Ca^{2+} release rate is the change in Ca^{2+} concentration during the initial 20 s after compound addition (41).

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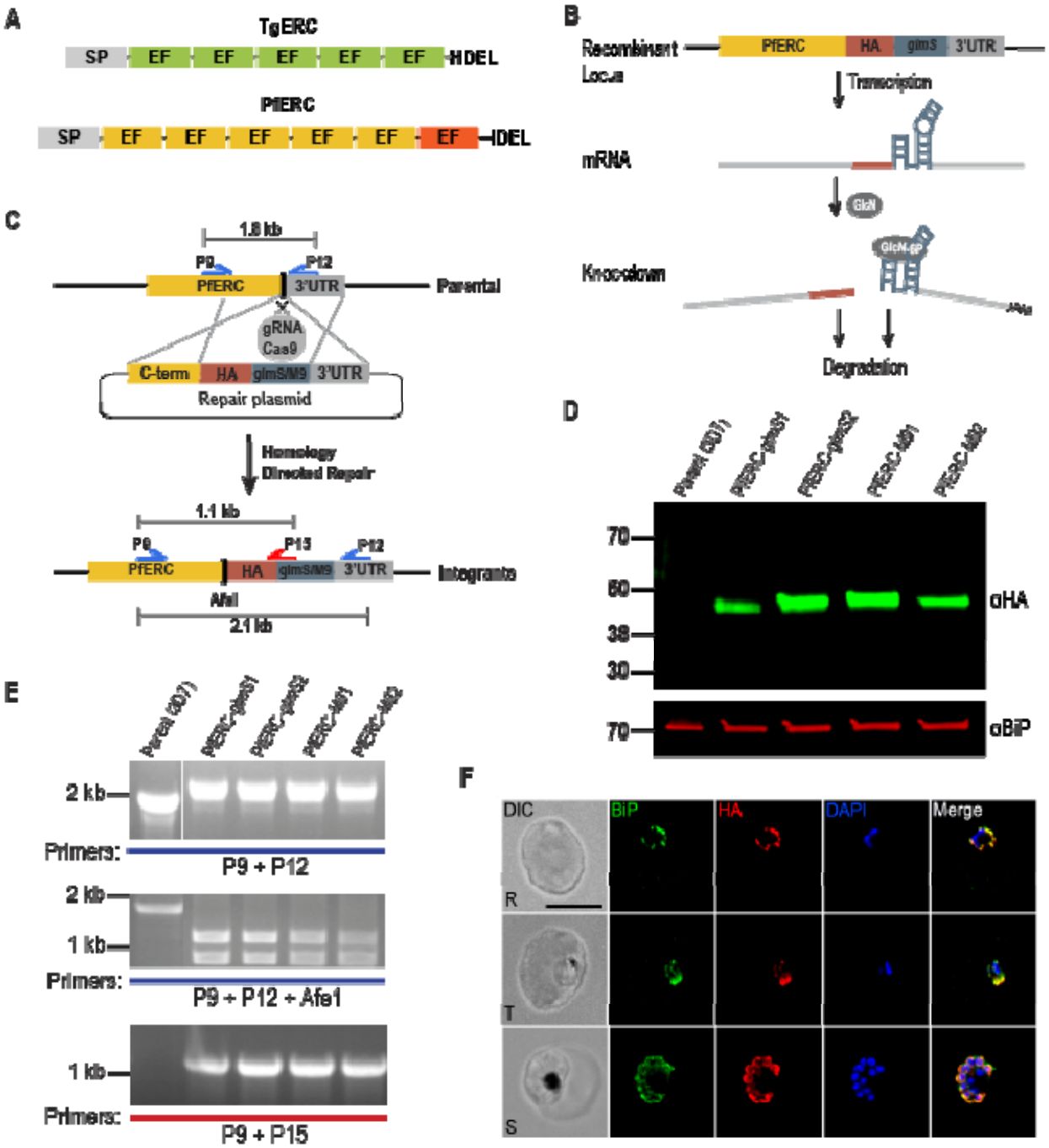

Figure 1: Generating PfERC-*glmS/M9* mutant parasites. (A) Schematic representation of the domain structure of PfERC and TgERC. Both proteins contain a signal peptide, 5 (TgERC) or 6 (PfERC) EF hands, and an ER-retention signal. (B) Mechanism of the conditional expression of PfERC using the *glmS* ribozyme system. This is an inactive ribozyme that is transcribed, but not translated, with the mRNA of a protein of interest. Addition of glucosamine (GlcN) leads to its phosphorylation within the

cell to glucosamine-6-phosphate (GlcN-6P). GlcN-6P binds to the transcribed PfERC-*glmS* mRNA and the *glmS* ribozyme is activated and cleaves itself from the mRNA. This leads to disassociation of the mRNA from its poly-A tail and leads to the degradation of target specific mRNA. The resulting decline in mRNA levels leads to reduced protein levels and, thus, loss of gene expression. As a control, we generated parasite lines containing a mutated version of the *glmS* ribozyme, called *M9*, which cannot cleave itself upon binding of GlcN. (C) Using the CRISPR/Cas9 system, we induced a double-stranded break in the PfERC locus that was repaired by a donor plasmid containing homology templates to the PfERC locus and appended a C-terminal 3XHA tag and a stop codon followed by the *glmS* or *M9* sequence to the targeted gene. The location of diagnostic primers used to demonstrate the repair of the locus via double cross-over homologous integration are also shown (P5, P8 and P11). (D) Western blot of lysates isolated from two independent clones and the parental line (3D7) probed with anti-HA antibodies show that the PfERC gene was tagged with HA in the mutants but not the parental line. PfBiP was the loading control. (E) PCR analysis of the generated mutants using specific primers (P5+P8; Table S1) in the C-terminus and 3'UTR of PfERC shows integration of the HA tag and *glms*/*M9* ribozymes into the PfERC locus. Modification of PfERC gene introduces an AfeI restriction enzyme site in this locus that is absent in the parental line. Digesting the PCR products (using AfeI) resulting from amplification using primers P5+P8 shows that AfeI is able to digest the PCR products from our mutants but not the parental line. PCR analysis using another primer pair (P5+P11) that sits on the *glmS*/*M9* sequence shows that amplification only occurs in the mutants but not in the parental line. (F) Representative IFA of PfERC-*M9* parasites showing that tagged PfERC localizes to the ER as shown with co-localization with the ER chaperone BiP in all asexual stages of the parasite. From left to right, the images are phase-contrast micrographs of parasites, parasites stained with anti-BiP antibody (green), parasites stained with anti-HA antibody (red), parasite nucleus stained with DAPI (blue), and fluorescence merge images of the parasites. Abbreviations: R, rings; T, trophozoites; S, schizonts. Scale bar, 5µm.

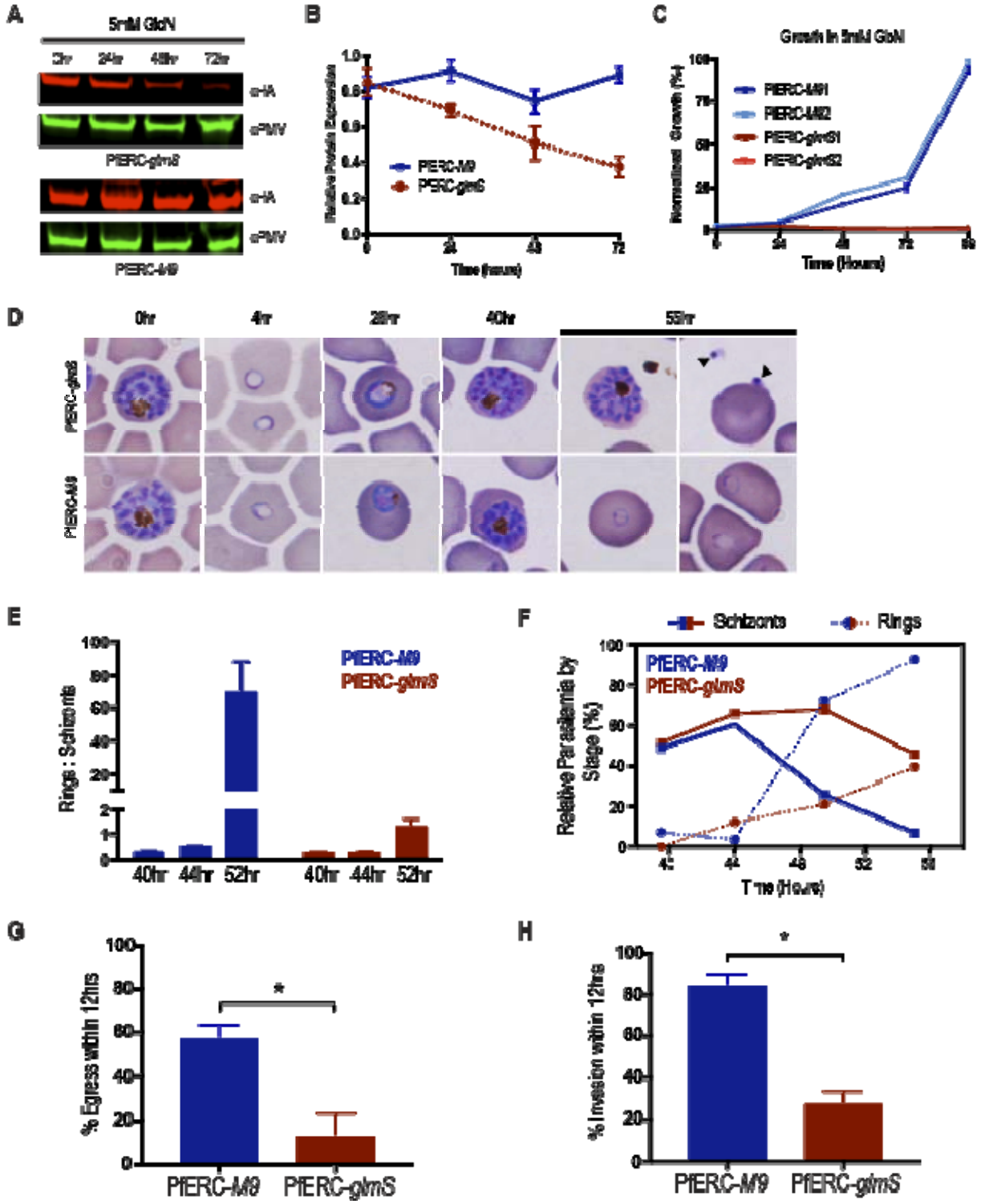

Figure 2: PfERC mutants fail to transition from schizonts to rings. (A) Western blot of parasite lysates isolated from PfERC-*glmS* and PfERC-*M9* parasites grown in the presence of 7.5 mM GlcN and probed with antibodies against HA (red) and against the ER-resident protease Plasmepsin V (PMV; green). One representative experiment out

of four is shown. (B) Quantification of the overall reduction in PfERC in PfERC-*glmS* and PfERC-*M9* parasites over time after addition of GlcN, as shown in (A). Data were normalized to the loading control (PMV) and are represented as mean ± SEM (n=4 biological replicates). (C) Growth curve of asynchronous PfERC-*glmS* and PfERC-*M9* clones grown in the presence of GlcN shows that PfERC is essential for asexual reproduction. Data are normalized to parasites grown without GlcN and are represented as the mean ± SEM (n=3 biological replicates). (D) Representative blood smears of synchronous PfERC-*glmS* and PfERC-*M9* parasites grown in the presence of GlcN (n=2 biological replicates). (E) Ring:schizont ratio of synchronous PfERC-*glmS* and PfERC-*M9* parasites taken at 40-52hrs post-addition of GlcN. Data are represented as the mean ± SEM (n=3 biological replicates). (F) The parasites shown in blood smears (D) were counted. The amount of each lifecycle stage (ring, trophozoite, schizont) was determined as a percentage of the total number of parasites for each time point. (G) The egress of PfERC-*glmS* or PfERC-*M9* parasites was quantified by determining the amount of schizonts in blood smears as in (D) present at 44hrs and at 56hrs. Egress was quantified using the following equation: (# of schizonts at 56h / # schizonts at 44h) x100. Data are represented as mean ± SEM (n=2 biological replicates; *P<0.05, unpaired *t*-test). (H) Invasion of PfERC-*glmS* or PfERC-*M9* parasites was quantified by determining the amount of ring stages in blood smears as in (D) present at 44hrs and at 56hrs. This was quantified using the following equation: (# of rings at 44h / # rings at 56h) x100. Data are represented as mean ± SEM (n=2 biological replicates; *P<0.05, unpaired *t*-test).

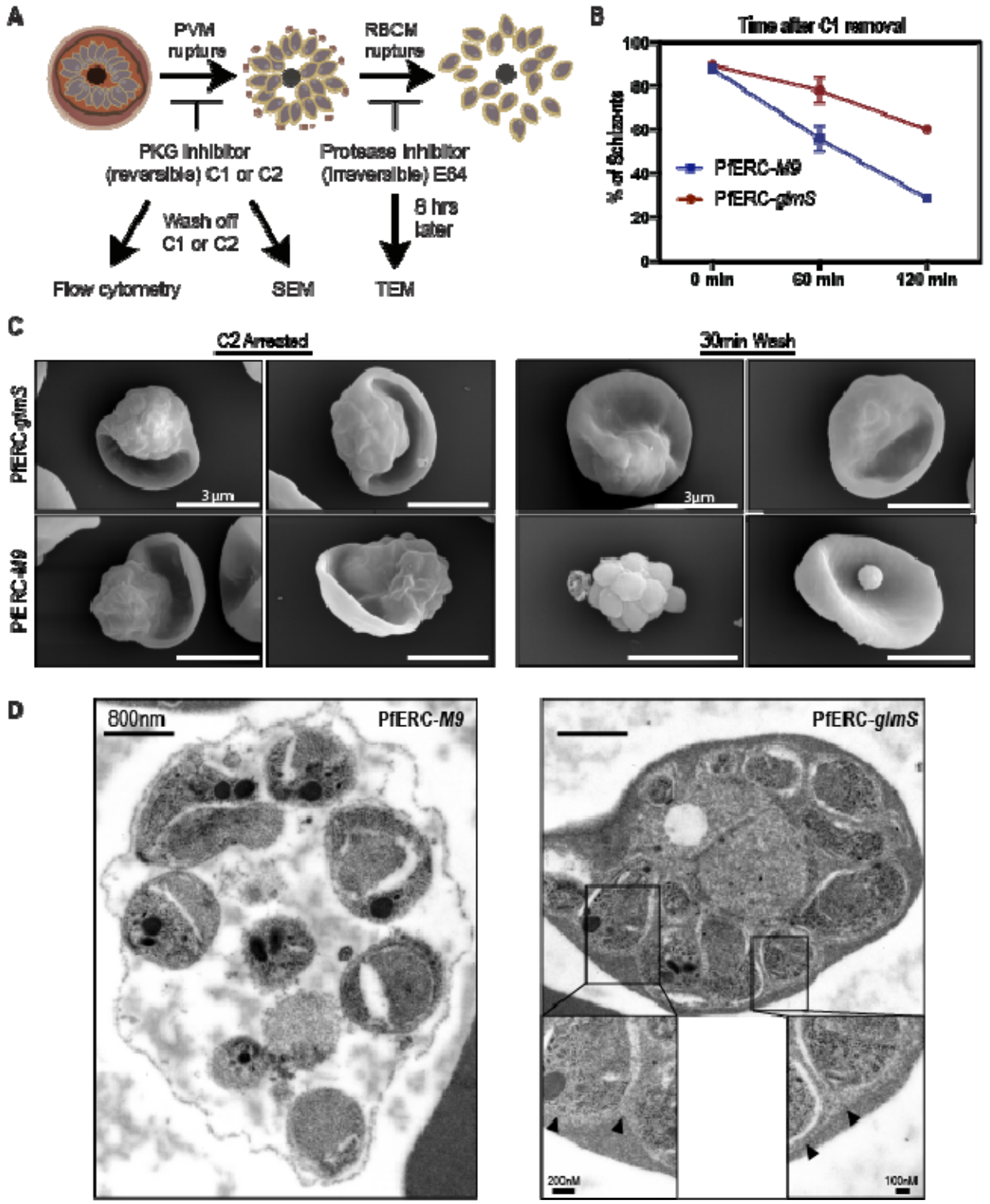

Figure 3: PfERC knockdown prevents PVM breakdown. (A) Schematic showing the experimental layout to study the effect of PfERC knockdown using compounds that inhibit egress in parasites. Abbreviations: C1/2- PKG inhibitors, compound 1 or

compound 2; SEM- Scanning Electron Microscopy; TEM- Transmission Electron Microscopy. (B) As shown in (A), synchronized PfERC-*glmS* and PfERC-*M9* schizonts were observed by flow cytometry after removal of C1 (time 0hr). Schizonts were quantified as a percentage of the total amount of parasites as determined by flow cytometry. Data are represented as the mean ± SEM (n=3 biological replicates). (C) Representative SEM images of C2 arrested PfERC-*glmS* (n=2 biological replicates) and PfERC-*M9* (n=2 biological replicates) mutants fixed immediately after washing off C2 and after 30mins, as shown in (A). Scale bar, 800nm. (D) Representative TEM images of PfERC-*glmS* (n=2 biological replicates) and PfERC-*M9* (n=2 biological replicates) schizonts incubated with E-64, as shown in (A). PVM is marked by black arrowheads.

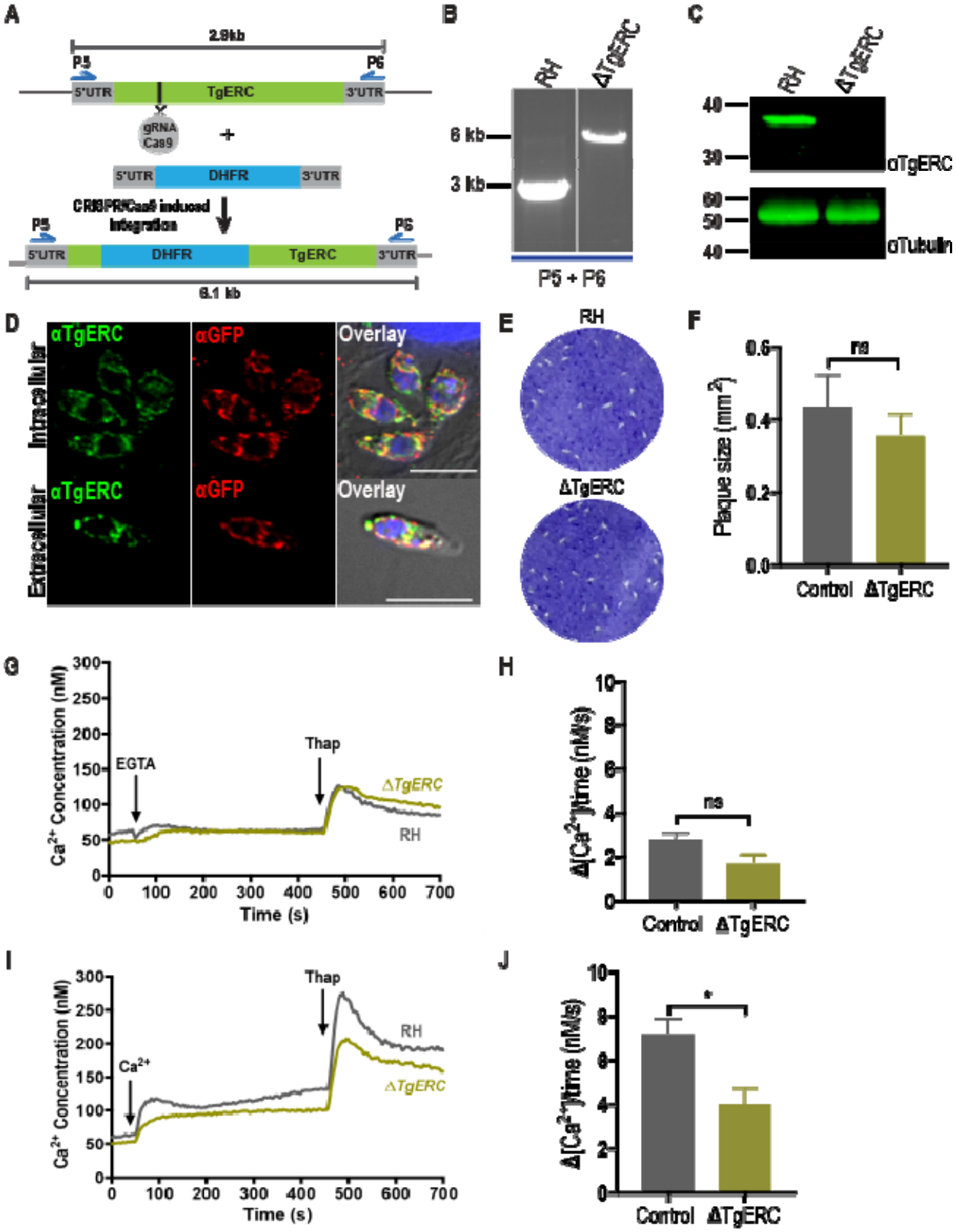

Figure 4: Generation and characterization of TgERC mutants. (A) Using CRISPR/Cas9, we generated *Δtgerc* mutants by introducing a DHFR cassette that

disrupted the open-reading frame of TgERC. (B) PCR analysis using primers that sit on the homology regions used for insertion showing amplification of the WT band (~3Kb) and insertion of the DHRF cassette as demonstrated by a higher product (~6Kb) in the mutants. (C) Western blot using generated antibodies against TgERC showing the presence of TgERC in the RH (WT) parasites but not in the *Δtgerc* mutants. αTubulin was used as the loading control. (D) IFAs of either extracellular or intracellular *Δtgerc* mutants transiently transfected with the P30-GFP-HDEL plasmid (79) which contains an ER-localized GFP. Left panel shows parasites probed with anti-TgERC antibodies, middle panel shows presence of transfected GFP, and right panel shows overlay of staining with DIC. Scale bar, 5µm. (E) Representative image of one plaque assay out of three biological replicates. (F) Quantification of plaque sizes between RH and *Δtgerc* parasites (n=3 biological replicates; n.s=non-significant, unpaired *t*-test). (G) Representative Ca^{2+} tracings of RH and *Δtgerc* parasites treated with the extracellular Ca^{2+} chelator EGTA and the SERCA pump inhibitor thapsigargin. (H) Quantification of the rate of Ca^{2+} release of RH and *Δtgerc* parasites from (G) (n=3 biological replicates; n.s=non-significant, unpaired *t*-test). (I) Representative Ca^{2+} tracings of RH and *Δtgerc* parasites treated with the extracellular Ca^{2+} and thapsigargin. (J) Quantification of the rate of Ca^{2+} release of RH and *Δtgerc* from (I) (n= 3 biological replicates, $*P<0.05$, unpaired *t*-test).

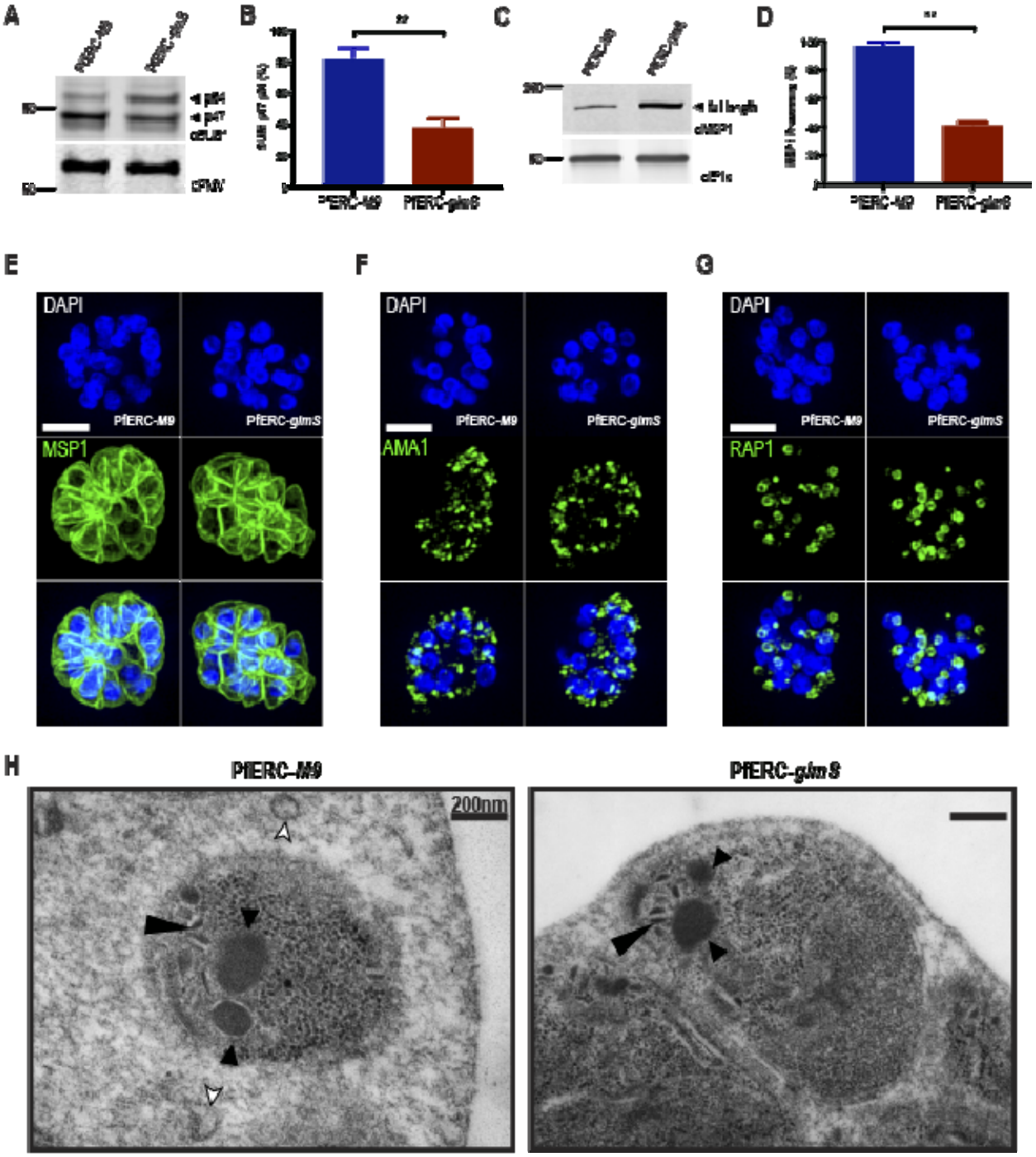

Figure 5: PfERC knockdown inhibits SUB1 and MSP1 processing. (A) Western blot of parasite lysates isolated from PfERC-*glmS* and PfERC-*M9* schizonts grown in the presence of GlcN for 48 hours and probed with anti-SUB1 antibodies (top panel) and against the ER-resident protease PMV (loading control, bottom panel). One representative experiment out of four is shown. The protein marker sizes that co-

migrated with the probed protein are shown on the left. (B) Quantification of the overall reduction in processing of SUB1 in PfERC-*glmS* and PfERC-*M9* parasites over time after addition of GlcN, as shown in (A). Data were normalized to the total SUB1 (processed + unprocessed) and are represented as mean ± SEM (n=4 biological replicates; ***P*<0.005 unpaired *t*-test). (C) Western blot of parasite lysates isolated from PfERC-*glmS* and PfERC-*M9* schizonts grown in the presence of GlcN for 48 hours and probed with anti-MSP1 antibodies (top panel) and against the anti-EF1α (loading control, bottom panel). One representative experiment out of two is shown. The protein marker sizes that co-migrated with the probed protein are shown on the left. (D) Quantification of the overall reduction in processing of MSP1 in PfERC-*glmS* and PfERC-*M9* parasites over time after addition of GlcN. Quantification of the overall reduction in MSP1 in PfERC-*glmS* and PfERC-*M9* parasites over time after addition of GlcN, as shown in (B). Data were normalized to the loading control (EF1α) and are represented as mean ± SEM (n=2 biological replicates; ***P*<0.005 unpaired *t*-test). (E-G) Representative Super-Resolution SIM images of PfERC-*glmS* and PfERC-*M9* schizonts stained with antibodies against MSP1, AMA1, and RAP1. From top to bottom, the images are parasites stained with anti-MSP1 (E), anti-AMA1 (F), anti-RAP1 (G) antibodies (green), parasite nucleus stained with DAPI (blue), and fluorescence merge images of the schizonts incubated with GlcN for 48hrs (n=2 biological replicates). Scale bar 2µm. (H) Representative TEM images of PfERC-*glmS* and PfERC-*M9* schizonts grown for 48hrs with GlcN and incubated with E-64 for 8 hours, as shown in Figure 3A (n=2 biological replicates). Small arrowheads point to rhoptries, large arrowheads to micronemes, and white arrowheads to PVM fragments (15). Scale bar, 200nm.

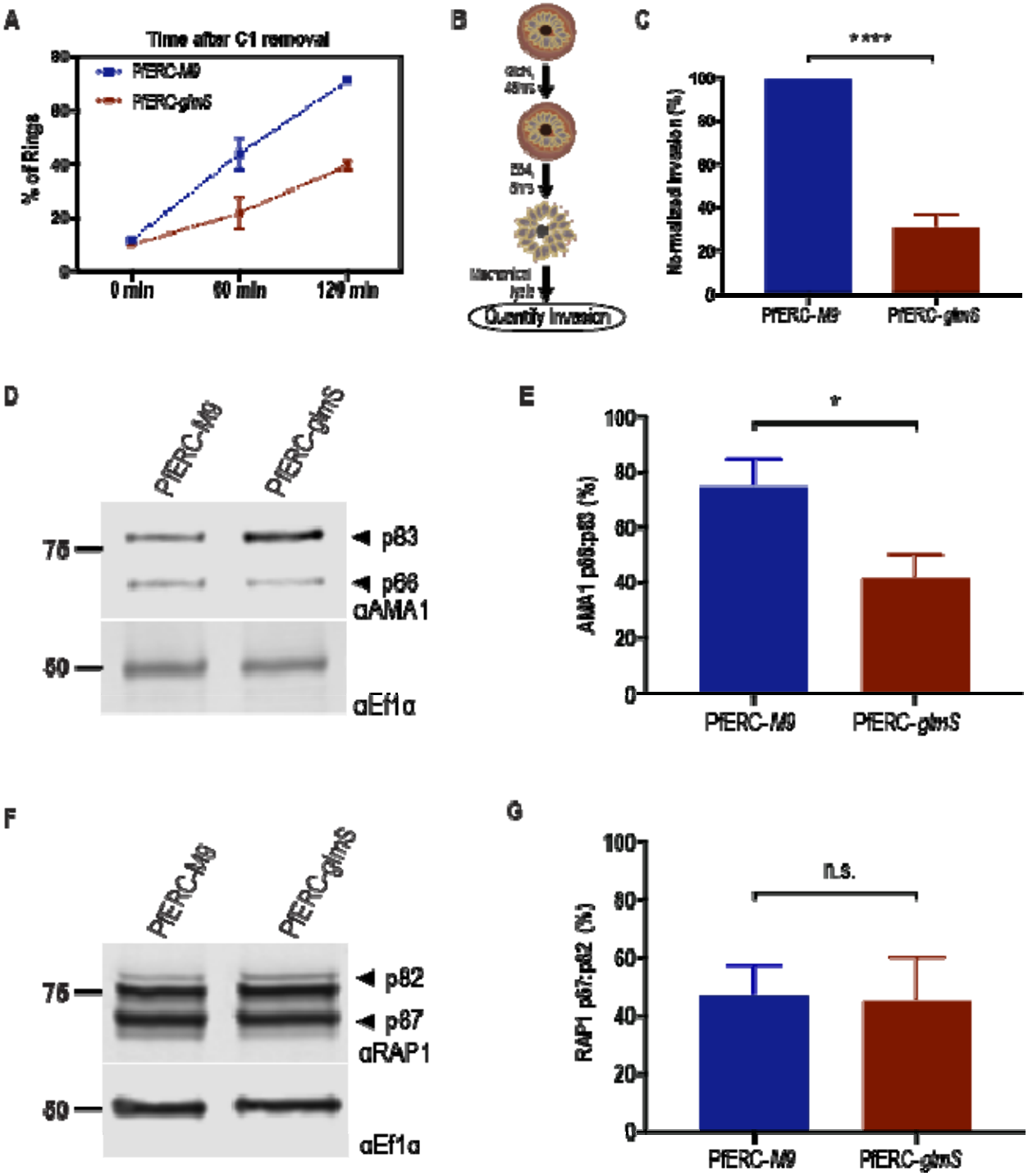

Figure 6: PfERC knockdown prevents invasion of RBCs. (A) As shown in Figure 4A, synchronized PfERC-*glmS* and PfERC-*M9* parasites were observed by flow cytometry after removal of C1 (time 0hr). Rings were quantified as a percentage of the total amount of parasites as determined by flow cytometry. Data are represented as the mean ± SEM (n=3 biological replicates). (B) Schematic showing the experimental layout

to study the effect of PfERC knockdown on invasion of merozoites into host RBCs. Merozoites were purified using mechanical lysis after 8hr incubation with E64. (C) Invasion rates of mechanically purified merozoites from PfERC-*glmS* and PfERC-*M9* parasites, as shown in (B), were quantified using flow cytometry (47). All replicates were normalized to PfERC-*M9* merozoites. Data are represented as mean ± SEM (n=7 biological replicates; ****$P<0.0001$, unpaired *t*-test). (D) Western blot of parasite lysates isolated from PfERC-*glmS* and PfERC-*M9* schizonts grown in the presence of GlcN for 48 hours and probed with anti-AMA1 antibodies (top panel) and anti- EF1αantibodies (loading control, bottom panel). One representative experiment out of eight is shown. The protein marker sizes that co-migrated with the probed protein are shown on the left. (E) Quantification of the overall reduction in processing of AMA1 in PfERC-*glmS* and PfERC-*M9* parasites over time after addition of GlcN, as shown in (D). Data were normalized to the total AMA1 (processed + unprocessed) and are represented as mean ± SEM (n=8 biological replicates; *$P<0.05$ unpaired *t*-test). (F) Western blot of parasite lysates isolated from PfERC-*glmS* and PfERC-*M9* schizonts grown in the presence of GlcN for 48 hours and probed with anti-RAP1 antibodies (top panel) and anti- EF1α antibodies (loading control, bottom panel). One representative experiment out of five is shown. The protein marker sizes that co-migrated with the probed protein are shown on the left. (G) Quantification of the overall reduction in processing of RAP1 in PfERC-*glmS* and PfERC-*M9* parasites over time after addition of GlcN, as shown in (F). Data were normalized to the total RAP1 (processed + unprocessed) and are represented as mean ± SEM (n=5 biological replicates; n.s=non-significant, unpaired *t*-test).

```
Cab45        1 -----------MVWPWVAMASRWGPLIGLAPCCLWLLGAVLLMDASARPANHSSTRERVA
ERC-55       1 ----------------MRLGPRTAALGLLL-LCAA---------------------AAGA
Calumenin    1 --------------------MDLRQFLMCLSLCTAFA------------------LSKPT
RCN1         1 ----------------MARGGRGRRLGLALGLLLALV------------LAPRVLRAKPT
TgERC        1 MAAFADRPTCRVSLGAVFLARELHRLRKSLPLCVFSL------------------------
PfERC        1 -------------------MMKINLYKLLCFICVIFL------------------------

Cab45       50 NREENEILPPDHLNGVKLEMDGHLNRGEHQEVFLG--KDLGGFDEDAEPRRSRRKLMVIF
ERC-55      23 GKAEELHYP---LGERRS--------DYDREALLGVQEDVDEYVK-LGHEEQQKRLQAII
Calumenin   23 EKKDRVHHEPQ-LSDKVHNDAQ--SFDYDHDAFLG-AEEAKTFDQ-LTPEESKERLGKIV
RCN1        33 VRKERVVRPDSELGERPPEDNQ--SFQYDHEAFLG-KEDSKTFDQ-LTPDESKERLGKIV
TgERC       38 ---FLFSFAFSALSGAPSQFAE------AAMPKLS-GEKLAELMQ-MDVKDIKERMLALF
PfERC       19 ------LHKNVVRSGDNMKYND--MKGLDDLSKLN-DDQVKDILG-LKIDGAKERIEKLF

Cab45      108 SKVDVNTDRKISAKEMQRWIMEKTAEHFQEAMEESKTHFRAVDPDGDGHVSWDEYKVKFL
ERC-55      71 KKIDLDSDGFLTESELSSWIQMSFKHYA---MQEAKQQFVEYDKNSDDTVTWDEYNIQM-
Calumenin   78 SKIDGDKDGFVTVDELKDWIKFAQKRWI---YEDVERQWKGHDLNEDGLVSWEEYKNAT-
RCN1        89 DRIDNDGDGFVTTEELKTWIKRVQKRYI---FDNVAKVWKDYDRDKDDKISWEEYKQAT-
TgERC       87 DLIDTNQDNTIDTEEAKEWSAKLKNAMH---QHQVRMEFQAIDKDNDGKVSLSELEAT--
PfERC       69 HLIDKNNDKEITEEELNTWSSFLKNEIF---LKQVQAEMGQIDSDKDGFISLNELNDA--

Cab45      168 ASKGHSEKEVADAIRLNEELKVDEETQEVLENLK---DRWYQADSPPADLLLTEEEFLSF
ERC-55     127 ---------YDRVIDFDENTALDDAFFESFRKLHLKDKKRFEKANQDSGPGLSLEEFIAF
Calumenin  134 ---------YGYVLDDP-----DPDLGFNYKQMMVRDERRFKMADKDGDLIATKEEFTAF
RCN1       145 ---------YGYYLGNPAEFH-DSSLHHTFKKMLPRDERRFKAADLNGDLTATREEFTAF
TgERC      142 ---------YVDSL--------DQKQLEQHKKEV---EQRFKTVDKDNDGLLDLSEIRIL
PfERC      124 ---------FAQNL--------DAKEVEKHSEGL---LKRFQIVDKDKDGKLSINEVGLL

Cab45      225 LHPEHSRGMLRFMVKEIVRDLDQDGDKQLSVPEFISLPVGTVENQQGQDIDDNWVKDRKK
ERC-55     178 EHPEEVLYMTEFVIQEALEEHDKNGDGFVSLEEFL----GDYRWDPTANEDPEWILVEKD
Calumenin  180 LHPEEYLYMKDIVVQETMEDIDKNADGFIDLEEYI----GDMYSHDGNTDEPEWVKTERE
RCN1       195 LHPEEFEHMKEIVVLETLEDIDKNGDGFVDQDEYI----ADMFSHEENGPEPDWVLSERE
TgERC      182 MDPGKDEGLMKIEIEEILNAQDKNGDRKITVTEFI--------ETEGTGSLNDVEKTELE
PfERC      164 IDPMKDEELKELEINEILEHHDVNKDGKISLDEFK-----QTRSDESSGVKKD-DEMALD

Cab45      285 EFEELIDSNHDGIVTAEELESYMDPMNEYNALNEAKQMIAVADEN-QNH-HLEPEEVLKY
ERC-55     234 RFVNDYDKDNDGRLDPQELLPWVVPNNQCIAQEEALHLIDEMDLN-CDK-KLSEEEILEN
Calumenin  236 QFVEFRDKNRDGKMDKEETKDWILPSDYDHAEAEARHLVYESDQN-KDG-KLTKEEIVDK
RCN1       251 QFNEFRDLNKDGKLDKDEIRHWILPQDYDHAQAEARHLVYESDKN-KDE-KLTKEEILEN
TgERC      234 KEFKSYDLNADGAIDVEELQQII----KDPHSHEIRMLLEEFTKDLKDG-KVGREQWEKE
PfERC      218 DF-NFFDANKDGFIDKEEIIKVY----FDPAHESGAINVNEIKENIFEGKKITYDLWNEK

Cab45      343 SEFFTGSKLVDYARSV-------------------------------------H-----
ERC-55     292 PDLFLTSEATDYGRQLH--------------------------------DDYFYH-----
Calumenin  294 YDLFVGSQATDFGEALV-------------------------------------RH-----
RCN1       309 WNMFVGSQATNYGEDLT-----------------------------------KNH-----
TgERC      289 FESFAVSMLTDNGEVLRFPEDY-----SGIEFP------FKTAVPQVDLDDEDKH-----
PfERC      273 ALKIAVTSLTDYGDVIRYPEDFKLDIGKNVILPTARSRAFEDDDMDADNTEDDKDEADDA

Cab45      360 --------EEF
ERC-55     315 --------DEL
Calumenin  313 --------DEF
RCN1       329 --------DEL
TgERC      333 --------DEL
PfERC      333 SQQKSPAIDEL
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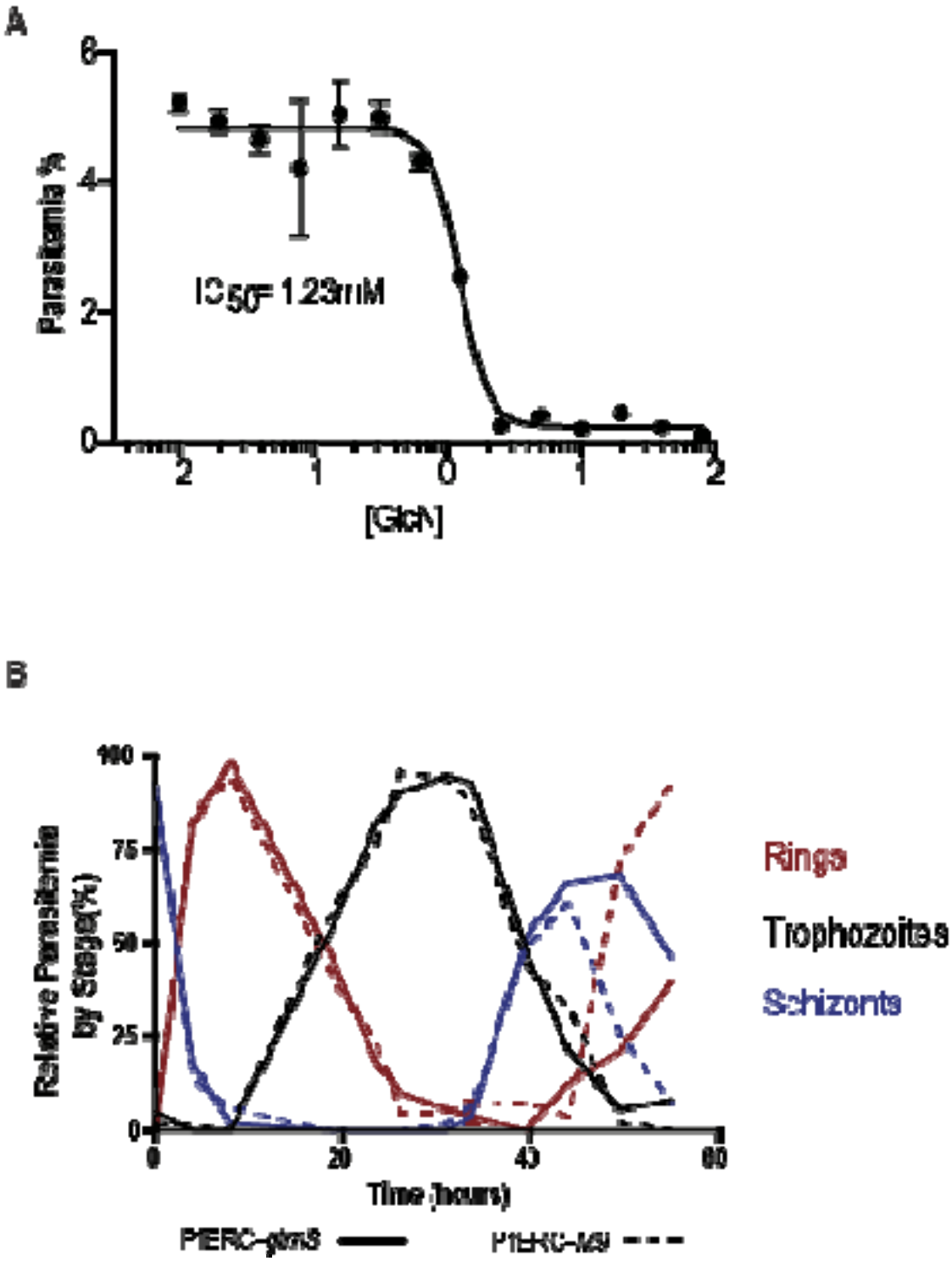
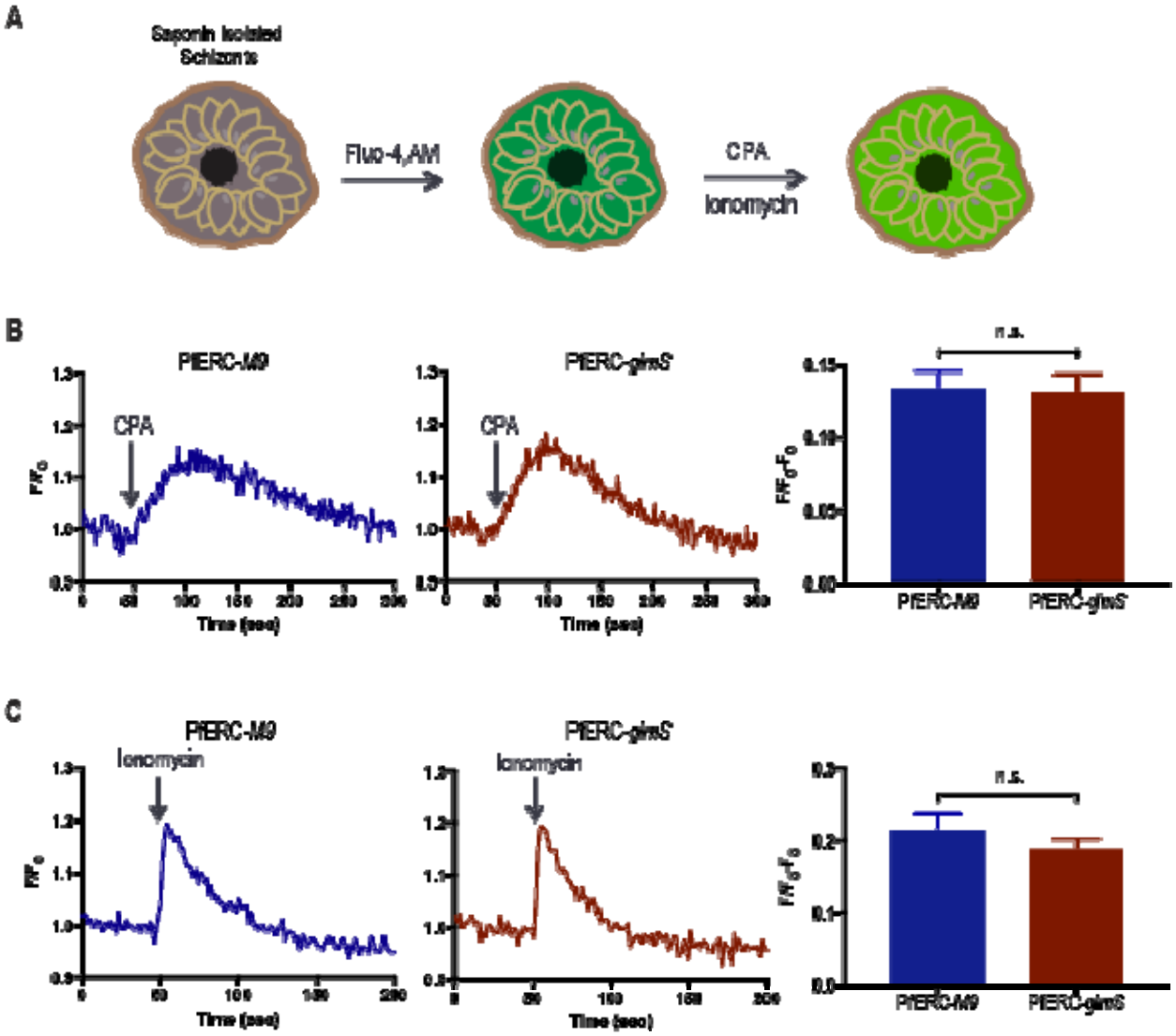
1197

Supplementary Figure 1: Sequence alignment of TgERC and PfERC to other members of the CREC family of proteins using MUSCLE alignment, viewed using JalView Software (http://www.jalview.org/) and BOXSHADE (82). Alignment was done using the human homologs: Cab-45, ERC-55 (RCN2), Calumenin, and Reticulocalbin1 (RCN1). Identical residues are shaded in black, similar residues are shaded in gray, and EF-hands are highlighted in red.



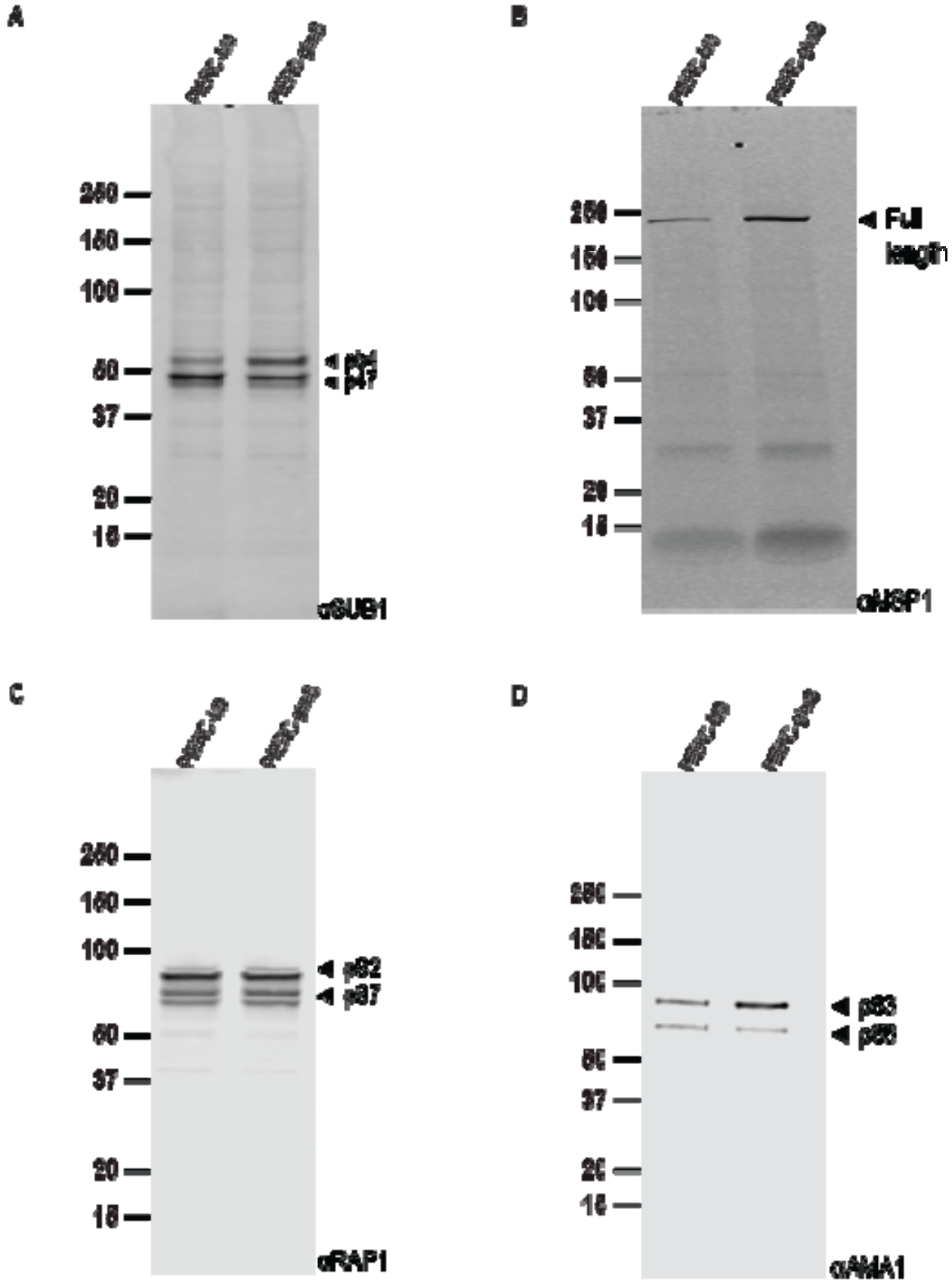
1221 **Supplementary Figure 2:** Effect of PfERC knockdown on parasite growth. (A)
1222 Representative graph (one out of three) showing dose-dependent response of PfERC-
1223 *glmS*2 to GlcN. Asynchronous PfERC-*glmS* parasites were incubated in different
1224 concentrations of GlcN and growth after three days was assessed by flow cytometry.

Data are represented as mean ± SEM of n=3 biological replicates. (B) Representative life cycle graph showing all stages from Figures 3D and 3F (n=2 biological replicates).



Supplementary Figure 3: Effect of PfERC knockdown on $Ca2^{2+}$ in the ER. (A) Experimental schematic showing how Ca^{2+} measurements were done in PfERC-*glmS* and PfERC-*M9* mutants. Synchronized PfERC-*glmS* and PfERC-*M9* schizonts were incubated with GlcN for 48 hours and isolated using saponin lysis, which lyses the RBC membrane but leaves the PV intact. Abbreviations: CPA- cyclopiazoic acid (B) Representative fluorescence tracings after CPA addition to PfERC-*glmS* and PfERC-*M9* schizonts, isolated as in (A). Quantification was done by calculating the difference in fluorescence between the basal to the highest peak of fluorescence. Data are represented as the combined mean ± SEM (PfERC-*glmS;* n=15 biological replicates; PfERC-*M9;* n=9 biological replicates; n.s- non-significant, unpaired *t*-test). (C)

Representative fluorescence tracings after Ionomycin addition to PfERC-*glmS* and PfERC-*M9* schizonts, isolated as in (A). Quantification was done by calculating the difference in fluorescence between the basal to the highest peak of fluorescence. Data are represented as the combined mean ± SEM (PfERC-*glmS;* n=9 biological replicates; PfERC-*M9;* n=5 biological replicates; n.s- non-significant, unpaired *t*-test).


A
250
150
100
50
37
20
15
p82
p37
αSUB1
B
250
150
100
50
37
20
15
Full length
αNSP1
C
250
150
100
50
37
20
15
p82
p37
αRAP1
D
250
150
100
50
37
20
15
p83
p68
αAMA1

1250

Supplementary Figure 4: Representative whole-blot images of Western blots of lysates from PfERC-*glmS* and PfERC-*M9* schizonts incubated with GlcN for 48 hours, probed with anti-SUB1, anti-MSP1, anti-AMA1, and anti-RAP1 antibodies from Figures 5 and 6. The protein marker sizes that co-migrated with the probed protein are shown on the left.

Table S1: Primers used in this study.

Primer	Primer Sequence
1	GCGGCGATGCCCAAGTTGTCGTTTTAGAGCTAGAAATAGC
2	AACTTGACATCCCCATTTAC
3	CGACTGTTCCCCCCAATCAATTCTACTTTCGTCGCTTATCTCGAGAAAACAAAGCTTCGCCAGGCTGTAAATCC
4	CTCCTGTCCGCAGAAGCTCTGTACGTGTTCTCAACAACTAGCTCTTCATGTTCATCCTGCAAGTGCATAGAAGGAAAGTTG
5	TCCGACTCTCCAACGTGGTTGC
6	GCTGCTACACAGCACTCCCTG
7	CTGCAGGTCTGGACATTTAAAGTTCATCACTAGCGTAATCTGGAACATCG
8	CGATGTTCCAGATTACGCTAGTGATGAACTTTAAATGTCCAGACCTGCAG
9	AATTCGCCCTTTCCGCGGAGAATAGAAAAATTATTTCATTTGATAGATAAAAACAATGAT
10	TGGGTAACTAGTAGCGCTTAATTCATCAATTGCTGGGGATTTTTGTTGCGATGCATCGTC
11	ATGATCTTGCCGGCAAGCTTTTTATATAAACATATTTTTTTTTTTTTTAACATAAAGGG
12	CCTTGAGCTCGCTAGCGACAAATTGGATAGATAATAGGGGGTACAAATATACATAC
13	AAGTATATAATATTCAATTGCTGGGGATTTTTGTGTTTTAGAGCTAGAA
14	TTCTAGCTCTAAAACACAAAAATCCCCAGCAATTGAATATTATATACTTA
15	GCAAGATCATGTGATTTCTCTTTGTTCAAGGAGTCACCCCC
16	GACGACGACAAGATAATGGCGGCCTTCGCAGACAG
17	GAGGAGAAGCCCGGTCAGTTCATCGTGTTTGTCTTCGTCGTCG