Chapitre 4 : La protéine escorte sortiline joue un rôle centrale dans la voie de sécrétion régulée du parasite *Plasmodium falciparum.*

4.1 Avant-propos

Ce chapitre correspond à un article publié le 26 mars 2018 dans « Cellular Microbiology » (**Hallée, S., Counihan, N. A., Matthews, K., de Koning-Ward, T. F. & Richard, D. Cellular Microbiology e12844 (2018)** et dont je suis la première auteure. Cet article est présenté tel que publié. Pour cette publication, j'ai généré et caractérisé la lignée de parasites sortiline-3HA-glmS, effectué les essais de « knock-down » en Western-blot et en cytométrie en flux et procédé aux différentes expériences d'analyse du phénotype associé à la perte de la sortiline. J'ai aussi effectué les expériences de microscopie à fluorescence et de microscopie électronique pour le « knock-down » de la sortiline. De plus, j'ai établi le design expérimental, analysé les résultats et contribué à l'écriture du manuscrit. Natalie A. Counihan et Kathryn Matthews ont aussi généré une lignée de parasites sortiline-3HA-glmS. Elles ont procédé à l'analyse en microscopie à fluorescence des marqueurs PTEX150, RhopH3 et HSP101 en situation de « knock-down » de la sortiline. Elles ont aussi participé à l'analyse des résultats et révisé le manuscrit. Tania F. de Koning-Ward a contribué à l'analyse des résultats et à la révision du manuscrit. Dave Richard a supervisé mes travaux, participé au design expérimental et écrit l'article.

4.2 Résumé

Le complexe intra-membranaire et les organites sécrétoires qui constituent le complexe apical sont des composantes clés qui caractérisent les parasites de la famille *Apicomplexa*. Malgré leur rôle essentiel, les mécanismes derrière leur biogenèse chez le parasite de la malaria *Plasmodium falciparum* restent très mal définis. Les résultats présentés ici montrent qu'une diminution dans l'expression d'un homologue conservé de la protéine escorte du système endolysosomale sortiline prévient la formation du complexe intra-membranaire ainsi que la formation de nouveaux mérozoïtes. De plus, dans ces mêmes conditions, l'on observe un défaut dans le transport de protéines de rhoptries, de micronèmes et de granules denses menant à une accumulation de ces protéines apicales dans le réticulum endoplasmique ainsi que dans la vacuole parasitophore. Nous avons aussi démontré que le transport de protéines exportées vers le cytoplasme de l'érythrocyte et le transport via la voie de transport constitutive sont toujours fonctionels dans des conditions où l'expression de la sortiline est diminuée. Ensemble, les résultats présentés ici suggèrent que le parasite *P. falciparum* a réorienté le rôle d'escorte spécifique dans le transport endolysosomale de la protéine sortiline en un joueur central dans la voie de sécrétion régulée.

The malaria parasite Plasmodium falciparum Sortilin is essential for merozoite formation and apical complex biogenesis.

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4.3 Abstract

The inner membrane complex and the apical secretory organelles are defining features of apicomplexan parasites. Despite their critical roles, the mechanisms behind the biogenesis of these structures in the malaria parasite *Plasmodium falciparum* are still poorly defined. We here show that decreasing expression of the *P. falciparum* homologue of the conserved endolysomal escorter Sortilin-VPS10 prevents the formation of the inner membrane complex and abrogates the generation of new merozoites. Moreover, protein trafficking to the rhoptries, the micronemes and the dense granules is disrupted, which leads to the accumulation of apical complex proteins in the endoplasmic reticulum and the parasitophorous vacuole. We further show that protein export to the erythrocyte and transport through the constitutive secretory pathway are functional. Taken together our results suggest that the malaria parasite *P. falciparum* Sortilin has potentially broader functions than most of its other eukaryotic counterparts.

4.4 Introduction

The membrane-trafficking system is central to the subcompartmentalization of eukaryotic cells and its basic organization consists of the endoplasmic reticulum, the Golgi apparatus, a variety of endolysosomal organelles and finally the plasma membrane (Dacks *et al.*, 2007). Evolutionary reconstructions have led to a model for a highly complex last eukaryotic common ancestor containing a core molecular machinery essential for the various aspects of vesicular transport such as cargo selection, vesicle budding, and subsequent vesicle fusion once it reaches its target organelle (Bonifacino *et al.*, 2004). A central tenet of evolutionary cell biology is the assumption that orthologous proteins have retained comparable functions, a concept also known as functional homology (Koonin, 2005). However, recent experimental work on non-metazoan organisms and most notably apicomplexan parasites have revealed a number of examples of repurposing of effectors away from their canonical roles into parasite-specific adaptations (Sloves *et al.*, 2012, Klinger *et al.*, 2013, Kremer *et al.*, 2013, Klinger *et al.*, 2016, Sangare *et al.*, 2016, Venugopal *et al.*, 2017).

Apicomplexans are a group of obligate intracellular parasites relying on invasion and development inside of a host cell for survival. The inner membrane complex (IMC), flattened vesicles underlying the plasma membrane, and the apical complex, secretory organelles sequentially released during the invasion process, are defining structures of apicomplexan parasites (Morrissette *et al.*, 2002). These features are generated de novo and packaged into multiple daughter cells that, in the case of the malaria parasite *Plasmodium falciparum*, simultaneously bud from the mother cell plasma membrane (Margos *et al.*, 1999, Francia *et al.*, 2014). The IMC is used as a scaffold during the budding and assembly of daughter cells (Bannister *et al.*, 1995, Ferguson *et al.*, 2008, Francia *et al.*, 2014) and is critical for cell motility and invasion by providing an anchor point for the actin-myosin motor powering both processes (Mann *et al.*, 2001, Baum *et al.*, 2006, Frenal *et al.*, 2017). The organelles of the apical complex; the rhoptries, micronemes and dense granules, are sequentially secreted during invasion of an erythrocyte by the malaria merozoite where they play multiple roles such as host cell recognition, adhesins to provide movement and establishment of the parasitophorous vacuole (Cowman *et al.*, 2012).

The mechanisms governing the biogenesis of the *P. falciparum* IMC and apical complex are poorly defined and much of what we currently know is based on circumstantial evidence obtained from colocalization analyses with conserved effectors of vesicular trafficking (Agop-Nersesian *et al.*, 2009, Krai *et al.*, 2014, Kaderi Kibria *et al.*, 2015, Morse *et al.*, 2016). On the other hand, numerous functional analyses in the related model apicomplexan *Toxoplasma gondii* have revealed a critical

role for conserved members of the eukaryotic intracellular trafficking machinery. For example, members of the small G-proteins Rab- GTPases TgRab11a and TgRab11b control the assembly of the IMC (Agop-Nersesian *et al*., 2009, Agop-Nersesian *et al*., 2010, Harding *et al*., 2014) whilst TgRab5A and TgRab5C are required for the generation of the rhoptries and a subpopulation of micronemes (Kremer *et al*., 2013). Interestingly, knocking down the expression of the conserved VPS10/Sortilin endolysomal protein in *T. gondii* led to fully formed parasites lacking micronemes and rhoptries demonstating its critical role in their biogenesis (Sloves *et al*., 2012, Tomavo, 2013).

Sortilin proteins possess a conserved structure made up of an N-terminal pro-peptide, a VPS10 domain for binding to cargo proteins, a transmembrane domain and finally a cytoplasmic tail interacting with the intracellular trafficking machinery (Hermey, 2009). Evolutionary reconstruction has revealed that despite being relatively conserved in eukaryotic cells, Sortilin is absent from several lineages (Koumandou *et al*., 2011). Sortilins mediate specialized functions such as the trafficking of hydrolases to the yeast vacuole (Marcusson *et al*., 1994), the transport of cargo to the plasma membrane and endolysosomes and as a cell surface receptor in mammalian cells (Canuel e*t al*., 2009), and finally the biogenesis of secretory granules in the free-living ciliate *Tetrahymena* (Briguglio *et al*., 2013). Interestingly, polymorphisms in human Sortilin are potential risk factors in neurodegenerative diseases such as Alzheimer's and Parkinson's (Wilson *et al*., 2014, Cuccaro *et al*., 2016), in type 2 diabetes (Clee *et al*., 2006), in lipid metabolism disorders (Strong *et al*., 2014) and cancer (Wilson *et al*., 2016).

P. falciparum possesses a homologue of the Sortilin protein that localizes to the Golgi apparatus (Koumandou *et al*., 2011, Krai *et al*., 2014, Hallee *et al*., 2018) and we recently demonstrated that it interacted with regions of the GPI-anchored *P. falciparum* Rhoptry- associated membrane antigen (RAMA) that were sufficient to drive a fluorescent reporter to the rhoptries which suggested that PfSortilin could act as an escorter for the trafficking of RAMA to these organelles (Hallee *et al*., 2018).

Here, using parasite strains where the expression of PfSortilin can be conditionally regulated, we demonstrate that knockdown of PfSortilin prevents the formation of new merozoites and that this is potentially due to the disruption of the biogenesis of the IMC. Furthermore, we show that protein transport to the rhoptries, micronemes and dense granules is also abrogated while the constitutive secretory pathway remains functional. Taken together our data suggest that the malaria parasite *P. falciparum* Sortilin has potentially broader functions than most of its other eukaryotic counterparts.

4.5 Results

Knockdown of PfSortilin abrogates development of *P. falciparum* schizont parasites.

To investigate the role of PfSortilin in the asexual blood stages of *P. falciparum*, we engineered parasite lines where the endogenous locus was tagged with a triple HA tag and incorporated a glmS ribozyme in its 3' untranslated region (UTR), which has been successfully used in *P. falciparum* to conditionally regulate gene expression (Prommana *et al.*, 2013). PfSortilin-3HAglmS lines were generated in two different laboratories (Fig. S1) but results from only one clone will be presented for clarity. In most subsequent experiments, a previously generated PfSortilin-3HA line without glmS (Hallee *et al.*, 2018) was used as a control. Incubation of the PfSortilin-3HAglmS line with increasing concentrations of glucosamine (GlcN) showed a specific dose-dependent decrease in the amount of the protein expressed in parasites (Fig. 1A). Normalization of the signal revealed a decrease in expression of more than 50% compared to the aldolase control at 2.5 mM of GlcN (Fig. 1B) and this concentration was used for all subsequent analyses. To determine whether the decrease in expression had an effect on the asexual parasite lifecycle, growth curve analyses were performed. As expected, no effect of GlcN was seen for the control parasite line; however, a more than 50% decrease in parasitemia was obtained for the knockdown (KD) line after one cycle and up to 90% after two cycles, demonstrating that PfSortilin was essential for propagation through the asexual blood cycle of *P. falciparum* (Fig. 1C). To try to pinpoint at what stage of the cycle the PfSortilin KD line was affected, parasite smears taken at various time points were analyzed. The 50% knockdown level determined by Western blot is an average of a population of parasites and it is likely that individual cells have varying levels of residual Sortilin and this could lead to a range in the severity of the phenotypes and this is indeed what we observed. As seen in Figure 1D, the KD parasites progressed through ring and trophozoite stages normally, with hemozoin-containing food vacuoles clearly visible (Fig. 1D, arrowheads). However, once the schizont stage was reached, in around 50% of the parasites, no merozoites were formed even though nuclear division had occurred (Fig. 1E). To facilitate the interpretation of the data, we have decided to focus our analysis on the cells this most severe phenotype. Determination of the number of nuclei per schizont revealed a small but statistically significant decrease in the KD line (Fig. 1E, 17.8±0.4 vs 14.0±0.6, control vs KD, respectively). The reason for this small decrease in the number of nuclei could potentially be due to a general fitness problem and not a direct role for Sortilin in nuclear division. While control parasites transitioned into the second cycle and formed new rings, the KD parasites had still not produced merozoites and instead the cells had degenerated, with highly condensed nuclei (Fig. 1D, T60 and T66). Subsequent quantification of the number of merozoites produced per schizont revealed a significant decrease of

more than 70% in the KD line compared to the control. However, those merozoites that were produced in the KD line had no defect in their ability to invade new erythrocytes which suggests they had sufficient amounts of PfSortilin (Fig. 1F).

To more precisely define the timing of PfSortilin function we determined if the growth arrest of the KD line could be reversed by reinstating PfSortilin expression through removal of GlcN from the growth medium. When GlcN was removed at early trophozoite stages in cycle 1, the parasitaemia in the following cycle returned to control levels (Fig. S2, T1). However, if GlcN was removed when schizogony had already commenced, the parasites could not recover (Fig. S2, T2, T3, T4). These results suggest PfSortilin is essential at a point in the cell cycle between nuclear division and cytokinesis.

PfSortilin is critical for the trafficking of proteins to the apical complex organelles.

Our recent finding that PfSortilin interacted with regions of RAMA sufficient for localization to rhoptries provided support for Sortilin's role as a protein escorter to this organelle (Hallee *et al.,* 2018). To provide more direct evidence, IFAs were performed with antibodies to rhoptry proteins using the PfSortilin KD and control lines grown in the presence or absence of GlcN. A range of PfSortilin-3HA levels was observed but around 50% of the more than 100 cells investigated had no detectable PfSortilin-3HA by IFA. Apical complex proteins have a typical punctate pattern in schizont stage parasites as observed with the control line PfSortilin-3HA incubated with GlcN (Fig. 2Ai and iv) however, this was severely disrupted in the KD parasites for which no PfSortilin-3HA was detectable, in line with the number of schizonts that did not form merozoites as observed by Giemsa-stained smears. The remainder of the manuscript will focus on the phenotypic characterisation of the cells for which no PfSortilin-3HA was detectable by IFA. Strikingly, the fluorescence signal for the rhoptry bulb marker RAP1 in the KD line was found in the PV (Fig 2Aii), the default destination for soluble proteins entering the secretory pathway, and also sometimes around the DAPI-stained nuclei, reminiscent of the ER and this was confirmed by colocalization with Bip, an ER- resident protein (Fig. 2Bii). A similar pattern was seen for RhopH3, another rhoptry bulb protein (Fig. S3A). The rhoptries are subdivided into the bulb and the neck and the mechanisms behind this segregation are currently unknown. Labeling of the KD line with an antibody against RON4, a soluble rhoptry neck protein (Richard *et al*., 2010) showed labeling around the DAPI-stained nuclei and at the ER, (Fig. 2Av and Biv). As RON4 forms a tight complex with the membrane protein RON2, this may explain why, despite not containing any transmembrane domain, RON4 stayed trapped in the ER instead of going to the PV as observed sometimes for RAP1 (Collins *et al*., 2009).

A previous study showed TgSortilin was specifically required for formation of both rhoptries and micronemes in *T. gondii* (Sloves *et al.*, 2012). We therefore examined whether micronemal protein trafficking was affected in the PfSortilin KD line. Micronemes have been proposed to form a heterogeneous population based on the absence of colocalization between the micronemal proteins Apical merozoite antigen 1 (AMA1) and Erythrocyte binding antigen 175 (EBA175) (Healer *et al.*, 2002). When looking at their distribution in the PfSortilin KD line, both of these proteins remained trapped in the ER (Fig. 2Avii and viii, Bvi and viii). Taken together, the results reveal that PfSortilin is required for the trafficking of both rhoptry bulb and neck proteins and also for the different populations of micronemal proteins.

We next investigated whether dense granule protein trafficking was also disrupted in the PfSortilin KD line using antibodies against PTEX150 and HSP101, components of the translocon complex mediating protein export at the PVM (de Koning-Ward *et al.*, 2009, Beck *et al.*, 2014, Elsworth *et al.*, 2014). In the absence of PfSortilin, PTEX150 and HSP101 could be seen in the PV (Fig. 2C) or trapped in the ER (Fig. S3B). Therefore, PfSortilin is also required for protein targeting to the dense granules. Taken together, these results reveal that PfSortilin is essential for the transport of protein cargo to the rhoptries, micronemes and dense granules, organelles that form the apical complex.

To investigate whether the block in protein trafficking prevented the biogenesis of the apical complex organelles, the ultrastructure of the parasites was investigated. While the PfSortilin- 3HA control had clearly defined rhoptries (Fig. 2Di, arrow), around 50% of the KD parasites, similar to the proportion of parasites with disrupted rhoptry protein trafficking by IFA, had no discernable rhoptries (Fig. 2Dii and Fig. S4). This shows that PfSortilin and its transport of rhoptry proteins from the Golgi is essential for the generation of the rhoptry organelle. While we could never identify with confidence micronemes and dense granules in either the control or the KD line by electron microscopy to provide direct evidence that PfSortilin was also involved in their biogenesis, the mislocalization of AMA1, EBA175, PTEX150 and HSP101 in the KD line suggests that this is likely the case.

Globally, our data provide evidence that PfSortilin plays a critical role in the trafficking of proteins from the Golgi to the organelles of the apical complex and that, at least in the case of the rhoptries, absence of PfSortilin leads to the abrogation of their biogenesis. It is interesting to note that the KD schizonts that had properly segmented merozoites showed the typical punctate pattern associated with the apical complex by IFA suggesting that the structure had been properly formed and that these merozoites could potentially be infectious (results not shown). This would explain our results showing that the merozoites formed in the KD line were fully invasive (Fig. 1F).

Knockdown of PfSortilin expression disrupts IMC biogenesis and prevents cytokinesis and merozoite formation.

We next investigated the ultrastructural defects caused by the decreased expression of PfSortilin by electron microscopy. EM of control schizonts showed individual merozoites were readily discernable; however, in the KD line, although separate nuclei were visible, no merozoite and no membrane ingression were observed (Fig. 3A and Fig. S4). Plasmodium parasites divide by a process termed schizogony whereby a multinucleated schizont is first formed, after which biogenesis and assembly of the various organelles followed by cytokinesis takes place (Francia *et al*., 2014). In an attempt to understand why cell division was blocked in the absence of PfSortilin, we looked at markers of several structures involved in this process. The inner membrane complex (IMC) is one of the defining features of alveolates, a group of unicellular eukaryotes of which Plasmodium parasites are a member (Hu et al., 2002, Keeley et al., 2004, Gould et al., 2008, Kono et al., 2012). The IMC is formed by flattened vesicles underlying the plasma membrane and interconnecting with the cytoskeleton. The IMC has been proposed to act as a scaffold for plasma membrane ingression that encapsulates daughter merozoites (Ridzuan *et al*., 2012) and is formed by structurally diverse proteins. One of the best characterized is GAP45, a protein that forms part of the molecular motor involved in red blood cell invasion (Baum *et al*., 2006). GAP45 is a cytosolic protein that is found between the IMC and the plasma membrane through association with the transmembrane protein GAP50 and myristoylation and palmitoylation, respectively (Rees-Channer *et al*., 2006, Frénal *et al.,* 2010, Ridzuan *et al*., 2012). In the control line, GAP45 was found around the newly formed merozoites except at the apical tip, as expected (Fig. 3Bi). In the PfSortilin KD, identified by the disrupted RAP1 signal, the GAP45 pattern was completely disrupted such that the fluorescence signal was found in close proximity to the nuclei (Fig. 3Bii).

Alveolins are another component of the IMC where they form part of a subpellicular network (SPN) supporting the vesicular component of the IMC (Gould *et al*., 2008, Kono *et al*., 2012, Tremp *et al*., 2014). To look at the state of the SPN in the absence of PfSortilin, the PfSortilin-3HA-glmS line was transfected with a plasmid encoding the alveolin Alv5 fused to GFP (Kono *et al*., 2012, Kono *et al*., 2016). While the control parasites (incubated without GlcN) displayed the expected IMC labelling around the merozoites, the KD line showed several dots of fluorescence spread throughout the cell suggesting that there was no nucleation of the SPN (Fig. 3Biii and iv). It has previously been hypothesized that the small GTPase Rab11A, in addition to its conserved role in the delivery of vesicles to the expanding plasma membrane, could potentially traffic GAP45 to the IMC (Agop-Nersesian *et al*., 2009, McNamara *et al*., 2013). IFA with an anti-Rab11A antibody showed that in

the control line, spots of fluorescence in close apposition to the rhoptries could be seen (Fig. 3Bv), as previously described (Agop-Nersesian *et al.*, 2009). Intriguingly, in the PfSortilin KD line, the Rab11A signal colocalized extensively with ER-trapped RAP1 (Fig. 3Bvi). These combined results show that in the absence of PfSortilin, both the vesicular and the SPN components of the IMC are disrupted which suggests PfSortilin potentially acts as an escorter in the vesicular transport from the Golgi to the IMC.

We next investigated the status of various structures critical for the cell division process. The centrosome has been proposed to play a role in the initiation of merozoite budding and in the early stages of IMC biogenesis (Mahajan *et al.*, 2008, Kono *et al.*, 2012). To look at the appearance of centrosomes in absence of PfSortilin, IFA was performed with an antibody raised against the *Chlamydomonas reinhardtii* centrin that had previously been shown to recognize *P. falciparum* Centrin 3, a marker of the centrosome (Mahajan *et al.*, 2008). In both control and KD lines, several foci of fluorescence were seen which shows that centrosome duplication occurs properly without PfSortilin (Fig. S5A).

P. falciparum merozoites possess two or three subpellicular microtubules extending from the apical tip to the nucleus that form from the 8 nuclei stage in developing merozoites (Read *et al.*, 1993, Bannister *et al.*, 1995, Margos *et al.*, 1999) and treatment of schizonts with drugs interfering with microtubule polymerization has been shown to disrupt merozoite development (Pouvelle *et al.*, 1994, Schrével *et al.*, 1994). We were therefore interested in looking at the integrity of the microtubule cytoskeleton in our PfSortilin KD line. Using an anti-alpha tubulin antibody, foci of fluorescence and small filaments that sometimes ran from the apical tip to the basal region of developing merozoites were visualized in the control line, as previously described (Bannister 1995), as well as in the KD parasites (Fig. S5B, arrows). These results demonstrate that the organization of the microtubular cytoskeleton is not disrupted in absence of PfSortilin.

The basal complex marker BTP1 has recently been shown to mark the basal rim of the forming IMC which can be seen as a contractile ring that advances in synchrony with plasma membrane invagination during cell division (Kono *et al.*, 2016). Observation of the control parasites transfected with a BTP1-GFP construct showed a labelled basal complex in segmented schizonts (Fig. 3Ci). However, in the PfSortilin KD line, distinct BTP1-GFP foci were spread throughout the parasite, sometimes overlapping with ER-trapped RAP1, showing that the basal complex was not formed properly, in line with that absence of IMC formation (Fig. 3Cii).

The symbiont organelles mitochondria and apicoplast go through an expansion phase where they branch out throughout the cytoplasm before being segregated into the nascent merozoites (Waller *et al.*, 2000, van Dooren *et al.*, 2005). Based on the block in merozoite cytokinesis, we were interested in determining whether these organelles were properly expanded and segregated. In the control line, the apicoplast, as labelled with an anti-ACP antibody (Waller *et al.*, 2000, Gallagher *et al.*, 2011), displayed individual foci of fluorescence (Fig. 3Di). In contrast, while the apicoplast seemed to have expanded in the KD line, it did not look properly segregated. In addition, the ACP signal was sometimes overlapping with ER-trapped RAP1, which might mean that a portion of the ACP protein is also stuck in this structure (Fig. 3Dii). This observation fits with recent work proposing the existence of a transit peptide receptor in the Golgi that would divert apicoplast cargo to a specialized pathway, which would, based on our results, be independent of PfSortilin (Heiny *et al.*, 2014). In the case of the mitochondria, when labeled with Mitotracker extensive ribbons were observed in the KD line, indicating that like the apicoplast, this organelle could expand but segregation did not occur (Fig. 3Diii and iv). Overall, these data demonstrate that PfSortilin is most likely not involved in the trafficking of proteins to the apicoplast and the mitochondria but rather that the lack of proper organelle segregation was a consequence of the defect in cytokinesis, particularly since these processes have been proposed to be linked (van Dooren *et al.*, 2005).

PfSortilin is not essential for the constitutive secretory pathway

We next determined whether the defects in the PfSortilin KD line were caused by pleiotrophic effects due to the collapse of the parasite's secretory pathway, as observed in a *T. gondii* strain where Clathrin heavy chain 1 had been conditionally knocked down (Pieperhoff *et al.*, 2013), instead of a specific effect of the absence of PfSortilin on vesicular trafficking to the IMC. The trafficking of RAP1 to the PV in the PfSortilin KD line suggested that the default secretory pathway was at least partially functional in absence of the escorter (Fig. 4Aii). To investigate this in more detail, we looked at the localization of SERA5, a well characterized PV resident protein (Miller *et al.*, 2002). IFA showed a typical distribution of SERA5 around the forming merozoites in the control line (Fig. 4Ai). In the KD parasites, SERA5 was still trafficked to the PV (Fig. 4Aii, arrows), although clearly not as efficiently as the control as some of the signal looked like it remained stuck inside the parasite. The PV labelling was more easily discernable when we transfected the PfSortilin-3HA-glmS line with a plasmid expressing GFP targeted to the PV (Fig. 4Aiii and iv). We next examined whether the abundant plasma membrane protein MSP1 was properly trafficked. IFA revealed that the protein was distributed around the forming merozoites in the control line and at the plasma membrane of the KD line, although a significant fraction remained in the ER (Fig. 4Av and vi). To determine whether this

reduced efficiency could be due to a disruption of the Golgi apparatus structure, we looked at markers of the cis and trans-Golgi. In the control line, both ERD2 and GFP-Rab6 form tight spots, as previously described (Struck *et al.*, 2008) (Fig. 4Bi and iii). In the KD line, the foci labelled by ERD2 were similar to the control whilst the GFP- Rab6 foci looked somewhat fainter (Fig. 4Bii and iv). Altogether, this data demonstrates that the constitutive secretory pathway is still functional in absence of PfSortilin but with reduced efficiency, potentially due to some minor disruption of the trans-Golgi apparatus.

To survive inside its host cell, *P. falciparum* exports hundreds of effector proteins outside of the parasitophorous vacuole membrane (Hiller *et al.*, 2004, Marti *et al.*, 2004, Sargeant *et al.*, 2006). The mechanisms enabling protein export are slowly starting to be unraveled and the existence of an export specific escorter has been hypothesized (Boddey *et al.*, 2009, Marti *et al.*, 2013, Coffey *et al.*, 2016). We were thus interested to see whether PfSortilin could be involved in this process. To this end, the PfSortilin-3HA-glmS line was transfected with a plasmid expressing the nano-Glo luciferase fused to the *Plasmodium* export element (PEXEL), a motif sufficient to mediate protein export across the parasitophorous vacuole membrane (Marti *et al.*, 2004). This construct has been previously used as a high sensitivity marker for protein export (Azevedo *et al.*, 2014). Quantification of the luciferase signal revealed that both the control and KD line had similar levels of export of the reporter (Fig. 4C, blue lines). This suggests that PfSortilin is not involved in the export of PEXEL- containing proteins. Whether the same is true for exported PEXEL-negative proteins remains to be investigated.

Because of the potentially high amount of protein trapped in the ER upon PfSortilin KD, we hypothesized that ER poisoning might contribute to the death of parasites. To determine if this was the case, we investigated whether the KD line was more sensitive to ER stress by exposing parasites with various levels of PfSortilin expression to increasing concentrations of the reducing agent DTT. The results obtained varied widely from one experiment to another but in any case, the KD parasites were not more sensitive to DTT (Fig. S6). Globally, this suggests that the death of the PfSortilin KD line is not caused by ER toxicity.

4.6 Discussion

In this work, we have explored the role of the *P. falciparum* homologue of Sortilin and provided evidence it potentially has a broad role in the regulated secretory pathway of the human malaria parasite. A major difference between the PfSortilin KD generated herein and the previously reported *T. gondii* Sortilin KD (Sloves *et al.*, 2012) is that the latter only impacts the biogenesis of the micronemes and the rhoptries, resulting in fully formed tachyzoites without both organelles, whilst the PfSortilin KD parasites have a completely disrupted pellicle in addition to the apical complex phenotype. Upon first observing that the PfSortilin KD parasites did not undergo cytokinesis, we initially thought this was an off- target effect due to a fragmentation of the Golgi and thus a complete blockage of both the regulated and the constitutive secretory pathway, such as that seen in a *T. gondii* Clathrin heavy chain 1 (TgCHC1) knockdown (Pieperhoff *et al.*, 2015) or with the expression of a dominant negative mutant of TgCHC1 (Pieperhoff *et al.,* 2013). However, though we could clearly see a minor effect on the appearance of the Golgi apparatus, this did not lead to a complete block in trafficking of MSP1 to the plasma membrane, or of SERA5 and a GFP reporter to the PV. Normal levels of export of a PEXEL-containing reporter and the presence of hemozoin in the food vacuole further supports the presence of at least a partially functional secretory pathway, although we cannot at this stage exclude that both of these processes were completed by the trophozoite stage before PfSortilin was sufficiently knocked down. Thus, our data suggests that contrary to its other eukaryotic counterparts, PfSortilin is not a specialized escorter but perhaps a rather more general trafficking factor with roles in the biogenesis of structures as diverse as the inner membrane complex and the apical organelles. Taken together, this suggests that the malaria parasite possesses a highly minimized secretory pathway.

Our earlier work on rhoptry protein trafficking had led us to speculate that the differential trafficking of apical complex proteins was mediated by a clustering mechanism at the Golgi whereby escorter proteins specific for the different apical organelles would bind their respective cargo for packaging into transport vesicles (Richard *et al.*, 2009). Our results now demonstrate that whether the ultimate destination of the cargo is the micronemes, the rhoptries, the dense granules and even perhaps the IMC, PfSortilin likely acts as the escorter. This leads to the question as to how specificity is determined if a single escort protein is used for trafficking to all these destinations. Our proposition that the association of rhoptry and dense granule proteins with, and the exclusion of microneme proteins from, detergent resistant membranes could provide some level of specificity is certainly a possibility but is unlikely to be sufficient in itself (Richard *et al.*, 2009).

Differences in the timing of expression of proteins destined for different organelles is another way by which differential sorting could occur. Though the peak of expression of numerous IMC proteins like GAP50 and GAP45 and rhoptry proteins like RAP1, RON4 and RAMA is indeed earlier than micronemal and dense granule proteins, abundant transcripts are still detected in later stages which suggests that rhoptry proteins might still be synthesized and therefore need to be properly trafficked through the secretory pathway in later stage parasites (Jaikaria *et al*., 1993, Bozdech *et al*., 2003, Rovira-Graells *et al*., 2012). In other words, in late schizonts, concomitant expression of IMC, rhoptry, microneme and dense granule proteins might very well occur. Expression of rhoptry proteins under a micronemal promoter and vice-versa could help in answering this question; however, interpretation of the results might not be so straightforward since experiments where the micronemal protein PfSub2 was expressed under the AMA1 promoter, a microneme protein with a closely matching expression profile, revealed partially incorrect targeting (Child *et al*., 2013). This could mean that very minor differences in the timing of expression or perhaps in the level of expression could affect proper subcellular localization. That being said, our own earlier work showing that expressing RAP1 from the Merozoite surface protein 2 promoter results in proper trafficking to the rhoptry bulb suggests that perfect timing might not be as stringent for rhoptry proteins (Richard *et al*., 2009).

Work in the model apicomplexan *T. gondii* led to the proposal the parasite had repurposed conserved regulators of the endosomal system for trafficking of proteins to micronemes and rhoptries and the differential sorting could potentially occur in post-Golgi endosome-like compartments (Tomavo, 2013, Tomavo *et al*., 2013). Indeed, *T. gondii* rhoptry proteins and a sub-population of micronemal proteins require the early endosomal proteins Rab5A and C for their proper targeting whilst another population of MIC proteins is independent of them (Kremer *et al*., 2013). The *P. falciparum* homologue of Rab5A is restricted to apicomplexan parasites invading erythrocytes and its localization to hemoglobin containing vesicles suggested an involvement in its endocytosis (Elliott *et al*., 2008, Ezougou *et al*., 2014). However, a recent report in which PfRab5A was conditionally inactivated showed that parasites could successfully mature to the schizont stage but interestingly they were unable to egress. Unfortunately, the status of the apical complex and the IMC in these parasites was not investigated (Birnbaum *et al*., 2017). The late endosomal marker Rab7 and members of the retromer complex have also been localized in *P. falciparum* blood stages and shown to be distinct from the Golgi apparatus (Krai *et al*., 2014). However, contrary to results with the *Toxoplasma* homologue, we failed to show colocalization between PfSortilin and the Rab7- labelled structures (Hallee *et al*., 2018). Whether interaction between PfSortilin and the retromer complex is involved in the anterograde traffic of apical complex/IMC proteins and/or the retrograde transport of

the escorter back to the Golgi is thus unknown. Interestingly, despite Rab1 having a conserved role in trafficking between the ER and Golgi, a recent study reported that PfRab1A colocalized with the rhoptries, but only in late schizonts which is not consistent with a role for this small GTPase in the early steps of the biogenesis of the organelles (Bannykh *et al*., 2005, Morse *et al*., 2016). Phylogenetics analyses have shown that apicomplexan Rab1A is unique to chromalveolates which might explain its unusual location is *P. falciparum* and *T. gondii* (Quevillon *et al*., 2003, Elias *et al*., 2009, Kremer *et al*., 2013).

Another possibility is that transit of cargo through an endosome-like compartment is not required for proteins destined for the IMC/apical complex in *Plasmodium* parasites. Indeed, seminal ultrastructural studies by transmission electron microscopy suggested that the generation of the apical complex in *Plasmodium* occurs through a direct route from the Golgi to the secretory organelles (Langreth *et al*., 1978, Ward *et al*., 1997, Bannister, 2000, Bannister *et al*., 2003, Schrevel *et al*., 2008). Contrary to lysosome-related organelles that derive from the endosomal system, classic secretory granules form directly from the TGN (Raposo *et al*., 2007). Studies on the biogenesis of secretory granules in neuroendocrine cells and of β-cell insulin granules led to a proposal that some active sorting during maturation of the protein cargo was occurring post-TGN in a process called "sorting by retention" whereby missorted proteins are removed from the immature granules by clathrin coated vesicles and the clathrin adaptor protein AP1 (Arvan *et al*., 1998, Morvan *et al*., 2008, Tanguy *et al*., 2016). Perhaps a similar process could be involved in the sorting of proteins destined to the IMC/apical complex in *P. falciparum*. PfAP1 was indeed shown to overlap with the rhoptries in schizonts and to associate with RAP1, which might suggest a specific role for the adaptor protein in protein trafficking to the rhoptries (Kaderi Kibria *et al*., 2015). However, a recent report demonstrated the *T. gondii* AP1 was critical not only for rhoptry but also microneme biogenesis (Venugopal *et al*., 2017).

Budding of daughter cells in *Toxoplasma* and *Plasmodium* is initiated at the apical polar ring, one of two microtubule organizing centres (MTOC) in these parasites, the other being in the nuclear envelope (Morrissette *et al*., 2002). A recent landmark study in *T. gondii* identified a striated fibre emerging from the centriolar region, connecting the apical MTOC and extending as budding proceeds. Furthermore, preventing the formation of this fibre resulted in a block at one of the first steps in the cell division process i.e. the initiation of the apical MTOC and thus formation of the microtubule filaments and led to multinucleated cells, similar to the phenotype we observe in our PfSortilin KD line. Whether the rhoptries and micronemes were formed in the parasites lacking the striated fibre was unfortunately not investigated. (Francia *et al*., 2012). Striated fibre assembling

protein homologues are present in *P. falciparum* and many other apicomplexans (Lechtreck, 2003, Harper *et al*., 2009, Francia *et al*., 2014). Furthermore, structures potentially corresponding to the striated fibre described in *Toxoplasma* are present in *Eimeria* schizonts (Dubremetz, 1973) and have been proposed to be involved in vesicular trafficking from the Golgi to the rhoptries in *P. berghei* sporozoites (Schrevel *et al*., 2008). It will be interesting to explore what function the striated fiber plays in *P. falciparum* schizogony and whether its integrity is compromised in the PfSortilin KD line. Unlike *T. gondii* and ookinetes and sporozoites of certain species of *Plasmodium* (Patra *et al*., 2012, Wall *et al*., 2016), *Plasmodium* merozoites do not possess a conoid at their apical tip and the composition of their apical polar rings is not yet characterized so further understanding of the *Plasmodium* merozoite budding process will require the identification of markers for poorly defined structures such as the apical polar ring. The newly identified *P. falciparum* merozoite organizing protein, an apical protein that is required for proper merozoite segmentation could potentially represent such a marker (Absalon *et al*., 2016).

4.7 Conclusion

We have provided evidence that the malaria parasite *P. falciparum* uses the conserved lysosomal escorter Sortilin as a more general sorting factor with roles in vesicular transport from the Golgi apparatus to the secretory apical organelles and potentially the inner membrane complex (Fig. 5). This in turn suggests that the pathogen relies on an exquisitely simplified regulated secretory pathway compared to most other eukaryotic cell types studied to date. Our findings lead to some challenging questions such as how the parasite can use a single escorter protein to transport cargo from the Golgi apparatus to different organelles/subcellular structures and how a cell with such a paucity of conserved intracellular trafficking effectors manages to generate such a diversity of intracellular organelles.

4.8 Materials and Methods

Parasite culture

P. falciparum 3D7 were maintained in human O+ erythrocytes at a hematocrit of 4% with 0.5% (w/v) AlbumaxTM (Invitrogen) in RPMI medium (Life Tech). *P. falciparum* 3D7 parasite were originally obtained from David Walliker at Edinburgh University. Cultures were synchronized by incubation with 0.3 M alanine for 10 min (Braun-Breton *et al.*, 1988).

Cloning and transfection

All primers used are listed in Table S1 and all plasmids were sequenced and analyzed before transfection. The PfSortilin-3HA-glmS construct was made using the p3HA-glmS246-BSD (Gaumond *et al*, manuscript in preparation). A region of 979 bp of the 3' end of the PfSortilin gene was amplified without the stop codon using *P. falciparum* 3D7 genomic DNA and cloned into BglII-AvrII sites, upstream of the 3HA-glmS cassette. An additional PfSortilin- 3HA-glmS line was independently made by amplifying a fragment 1180 bp upstream of the stop codon of the PfSortilin gene using oligos 386 and 210. This fragment was cloned into the BglII and PstI sites of pRhopH2-HAglmS (Counihan *et al.*, 2017). *P. falciparum* 3D7 WT parasites were transfected with either PfSortilin-3HA-glmS plasmids and integrants selected and cloned as described previously (Gilberger *et al.*, 2003). Briefly *P. falciparum* 3D7 were transfected with 100 µg of purified plasmid DNA (Promega). Integrated parasites were selected using 2.5 mg/ml Blasticidin (Sigma-Aldrich).

The plasmid SP-GFP for expression of PV-targeted GFP was designed using a PCR fusion strategy. A primer containing the sequence of the acyl carrier protein signal peptide and the beginning of the GFP gene was designed and used to PCR amplify GFP. The resulting SP-GFP PCR product was cloned into AvrII-XhoI sites in pGFP-glmS(out) vector. The pGFP- glmS(out) vector was made from the pGFP-glmS plasmid (Prommana *et al.*, 2013) by removing the glmS ribozyme sequence and adding a multiple cloning site in front of the GFP sequence.

Southern Blot

Integration of Sortilin-3HA-glmS was confirmed by Southern blot according to standard procedure. Briefly, gDNA was extracted from parasites using the Blood genomic DNA extraction kit (Sigma). For each parasite line, 10 µg of gDNA was digested with XbaI (PfSortilin-3HA-glmS). Digested DNA fragments were separated on 0.7% (w/v) agarose gel then transferred on Hybond N+ membrane (GE) and hybridized.

Western blotting and protein processing

To verify the protein regulation with the glmS system, tightly synchronous young rings of PfSortilin-3HA and PfSortilin-3HA-glmS parasites were treated with increasing concentrations of glucosamine. Parasites (1×10^7) were harvested in the same cycle at the schizont stage, then saponin-lyzed and resuspended in SDS protein sample buffer. Membranes were probed with a mouse monoclonal anti-HA 1:200 (Cedarlane, clone HA.C5) and mouse monoclonal anti-Aldolase 1:1000 (Immunology Consultants Inc, MB720) as a loading control. The intensity of the signals was quantified using the Fiji sofware and a dose response curve was fit using Graph Prism v6.

Microscopy

Fluorescence microscopy acquisition was performed as previously described (Hallee *et al.*, 2015) and Counihan 2017 using a GE Applied Precision Deltavision Elite microscope and an Olympus IX71 microscope with 100x 1.4NA objective and with a sCMOS camera and deconvolved with the SoftWorx software. For immunofluorecence assays, parasites were fixed with cold MeOH (Dietz *et al.*, 2014), cold 90% acetone/10% MeOH or 4% paraformaldehyde (ProSciTech). After blocking in 3% bovine serum albumin (BSA fraction V, EMD), the slides were probed with combinations of antibodies: mouse anti-HA (Cedarlane, clone HA.C5, 1:1000); rabbit anti-HA (Abm, 1:1000); rabbit anti-ERD2 (MRA- 72, 1:1000) (Elmendorf *et al.*, 1993); rabbit anti-AMA1 ; 1:2000 (Healer *et al.*, 2005); mouse anti-RAP1 (1:3000) ; mouse anti-RON4 (1:2000) (Richard *et al.*, 2010); rabbit anti-EBA175 (1:1000) (O'Donnell *et al.*, 2006); rabbit anti-Bip (1:500) (Absalon and Dvorin, unpublished); mouse monoclonal anti-AMA1 (clone 1F9, 1:500) (Coley *et al.*, 2001); mouse anti-EBA175 (MRA711A, 1:500) (Sim *et al.*, 2011); rabbit anti-PTEX150 (1:300) and rabbit anti-HSP101 (1:300) (de Koning-Ward *et al.*, 2009); rabbit anti-SERA5 (1:1000) (Stallmach *et al.*, 2015); rabbit anti-MSP1 (1:1000) (Wilson *et al.,* 2011); rabbit anti-GAP45 (1:2000) (Jones *et al.*, 2006); rabbit anti-rab11a (1:200) (Agop-Nersesian *et al.*, 2009); rabbit anti-αtubulin II (MRA37, 1:1000); rabbit polyclonal anti-ACP (1:500) (Waller *et al.*, 2000, Gallagher *et al.*, 2011).

Primary antibodies were probed with Alexa Fluor 594 anti-rabbit IgG or anti-mouse IgG (Molecular Probes) and Alexa Fluor 488 anti-rabbit IgG or anti-mouse IgG (Cell Signaling). Slides were mounted with 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI, Invitrogen, 100ng/ul) in VectaShield (Vector Labs) or ProLong Gold anti-fade (Molecular Probes). For live imaging, Mitotracker Red (Molecular Probes, 10 nM) and ER tracker Red (Molecular Probes, 1 µM) were used with DAPI. Pearson's correlation coefficient between Alexa488 and Alexa594 channels were calculated on deconvolved regions of interests of image stacks, including zero-zero pixels and without thresholding

using the SoftWorx software (GE). Data were analyzed for statistical significance using one-way ANOVA followed by a Tukey multiple comparison test.

Conditional knockdown analysis

Transgenic parasites PfSortilin-3HA and PfSortilin-3HA-glmS were tightly synchronized and young rings were plated at 0.5% parasitemia and 4% hematocrit in a 12-well plate in 2 ml medium with or without 2.5 mM GlcN (Sigma). Each condition was done in triplicate. Parasites were harvested after 24, 72 and 120 hours in culture, stained with SYBRGold (Invitrogen-Molecular Probe) and fixed with 1% paraformaldehyde. Sample were then analysed by fluorescence activated cell sorting (FACS) using a BD FACSCanto and FACSDiva acquisition sofware and post-analyzed using FlowJo software. For each sample 100000 total events were recorded. The percentage of survival was obtained by normalizing to untreated parasites in the same experiment, which were taken as 100% survival. Uninfected red blood cells were used to determine the threshold for FITC signal.

To follow the development of the parasite throughout the erythrocytic cycle, smears of cells grown with or without GlcN were taken at various time points and stained with Giemsa.

To examine whether nuclear division was affected in the PfSortilin KD line, the number of nuclei was counted by IFA using paraformaldehyde fixation. Slides were stained with DAPI and with mouse anti-RAP1 to monitor proper knockdown of PfSortilin. Thirty schizonts were used for nuclei count for each condition (on or off GlcN). Data were analyzed for statistical significance using one-way ANOVA then a Tukey multiple comparison test.

Growth assay

For ER stress measurements, PfSortilin-3HA and PfSortilin-3HA-glmS parasites were incubated in the presence of 0 mM, 0.5 mM, 1 mM or 1.5 mM of GlcN. Rings were plated at 1% parasitemia and 4% hematocrit in a 24 well plate with 2.5 ml RPMI/well. They were then stressed using DTT at a final concentration of 0 mM, 0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM, 0.5 mM or 0.6 mM for 48h. Young rings from the following cycle were stained with SYBRGold (Invitrogen-Molecular Probe) and fixed with 1% paraformaldehyde. Samples were analyzed by FACS using a BD FACSCanto and FACSDiva acquisition sofware and post-analysed using FlowJo software. For each sample 100000 total events were recorded. Ratios were obtained by normalizing on 0 mM DTT for each GlcN condition. Experiments were performed in biological triplicates.

Invasion assay

The test the ability of the parasite to egress and to invade new red blood cells, PfSortilin-3HA and PfSortilin-3HA-glmS synchronized ring parasites were incubated in the presence or absence of 2.5 mM GlcN for 44 h. They were then treated with 10 mM of the cysteine protease inhibitor E64 (trans-Epoxysucci- nyl-L-leucylamido(4-guanidino)butane) to prevent schizont rupture for 4 h then an aliquot of the treated parasites was taken for FACS analysis. The parasites were filtered to liberate the merozoites and an aliquot of free merozoites was also harvested for FACS analysis. The free merozoites were immediately added to 25 µl of uninfected RBC and put back in culture under standard conditions in a 12 well plate with 3 ml of RPMI. After 24 hour in culture, parasitemia was measured by FACS analysis. Free merozoites and E64-treated parasites were counted by FACS using the Flow cytometry counting beads 123count eBeads (Invitrogen). Free merozoites were gated on SSC low, FITC high, E64-treated schizonts on SSC high, FITC high and beads FITC High, PE high. The number of free merozoites or E64-treated schizonts per µl was obtained knowing the beads dilution (1019 beads/µl). Merozoite production rate was calculated by normalizing the ratio merozoite/ E64-treated schizont of 2.5 mM GlcN treated parasites on the untreated 0 mM GlcN. The merozoite invasion efficiency was calculated by normalizing the ratio parasitemia/merozoite of 2.5 mM treated parasite on the untreated 0mM GlcN parasite.

Electron microscopy

For electron microscopy, PfSortilin-3HA and PfSortilin-3HA-glmS synchronized schizont stage parasites treated with 2.5 mM were purified by Variomacs magnetic separation (Mylteni Biotec). Purified schizonts were fixed in 1% glutaraldehyde (ProSciTech) on ice for 1h then washed twice in RPMI. RPMI was gradually replaced by water and the pellet was embedded in 1.2% DNA grade low melting temperature agarose. Solid agarose blocs were then dehydrated in ethanol and infiltrated with LR White resin (Ted Pella) followed by UV polymerization in LR White resin with 0.5% benzoyl peroxide. Samples were sectioned on a Leica Reichert Ultracut S ultramicrotome and observed on a Tecnai G2 spirit bioTWIN Transmission Electron Microsope.

Luciferase assay

PfSortilin-3HA-glmS parasites transfected with pEF-635-Nluc were tightly synchronized and young rings were incubated with or without 2.5mM GlcN until they reached the schizont stage after which luciferase activity was determined. Briefly parasites were diluted to 1% hematocrit in PBS and distributed in triplicate in a white microplate containing 4 volumes of PBS or 5% D-Sorbitol with Nano-Glo substrate (1/1000, Promega). Sorbitol lysis results in the disruption of the red blood cell

membrane and the release of the exported PEXEL- containing luciferase in the supernatant. After addition of luciferase, luminescence was measured at 5 min intervals over one hour using a Victor3 fluorometer (Perlin Elmer).

Rescue assay

PfSortilin-3HA-glmS tightly synchronized parasites were plated at the young ring stage at 1% parasitemia in 4% hematocrit with or without 2.5 mM GlcN. GlcN was removed after 24h, 28h, 42h or 46h. Parasites were put back in culture and at T60 and T90 an aliquot was taken to determine the parasitemia and the stage of the lifecycle by FACS. Stage determination was based on the intensity of FITC signal (ring FITC low, schizont FITC high).

4.9 Acknowledgments

4.10 References

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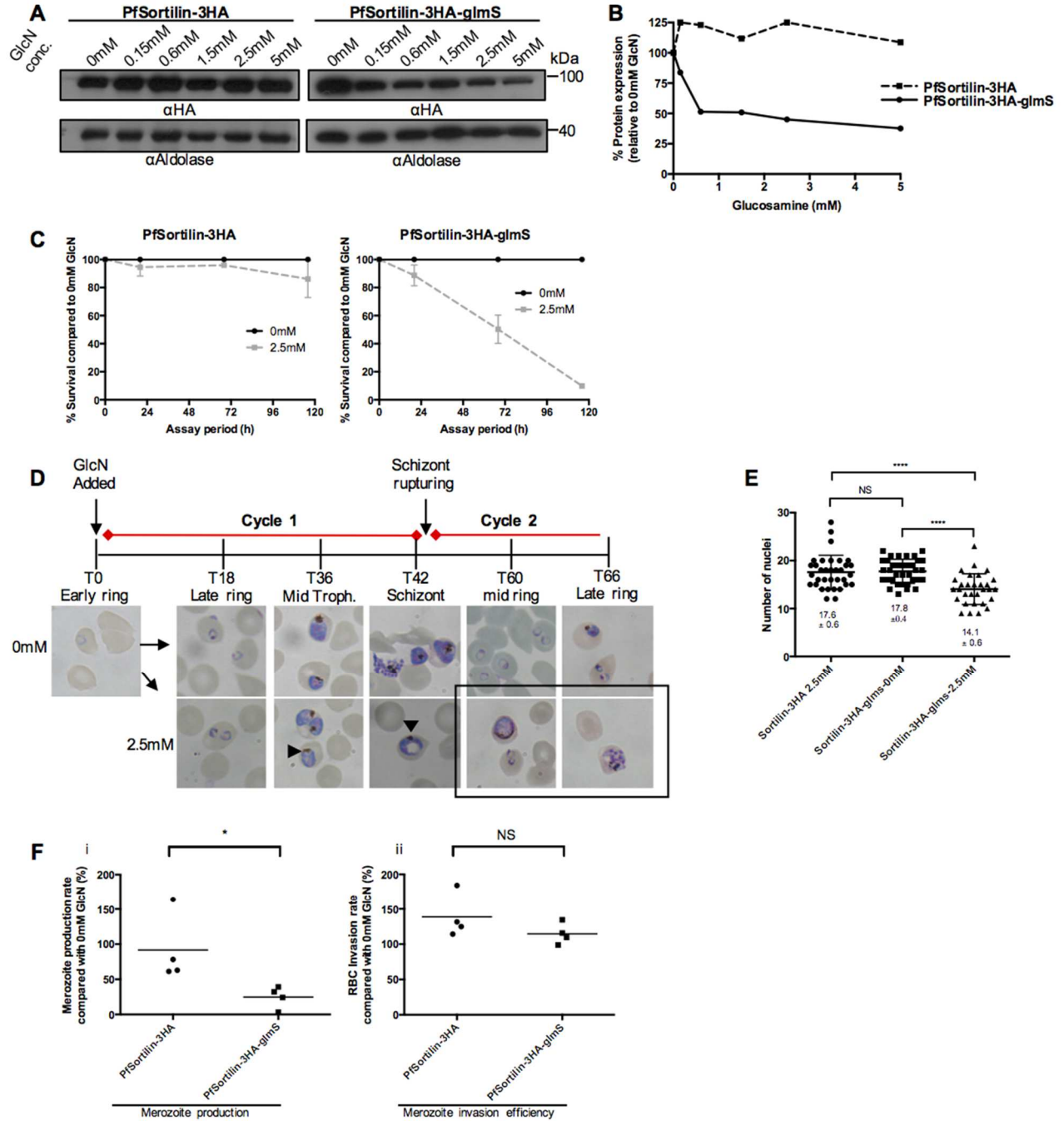
4.11 Figures

Figure 1. PfSortilin is essential for the *P. falciparum* asexual erythrocytic cycle. (A) Western blots showing a specific glucosamine dose-dependent decrease in the expression level of PfSortilin-3HA-glmS. GlnN: glucosamine (B) Quantification of the signals from (A) reveals a more than 50% decrease in PfSortilin-3HA levels in the glmS line. Values represent the % of growth compared to the 0 mM GlcN controls. (C) Growth curve analyses show that the PfSortilin-3HA–glmS line fails to proliferate in the presence of 2.5 mM whilst the PfSortilin-3HA control line is not inhibited (N=3 biological replicates) (D) Giemsa-stained parasite smears of the PfSortilin-3HA-glmS line shows that incubation with 2.5 mM GlcN leads to a block at the schizont stage in the first cycle (T42). The blocked parasites then go on to degenerate whilst the control has gone through the ring stage of the next cycle (T60 and T66, boxed panels) Arrowheads point to the food vacuole containing hemozoin. (E) Knockdown of PfSortilin-3HA results in a small but statistically significant decrease in the number of nuclei per schizont compared to controls. PfSortilin-3HA+2.5 mM GlnC, n= 34. PfSortilin-3HA-glmS+0 mM GlnC, n= 35. PfSortilin-3HA-glmS+2.5 mM GlnC, n= 30. ****= $p<0.0001$. (F) Knockdown of PfSortilin results in a decrease in the number of free merozoites produced but these are fully invasive. i: *= $p<0.05$. ii: NS= not significant. Mann- Whitney test.
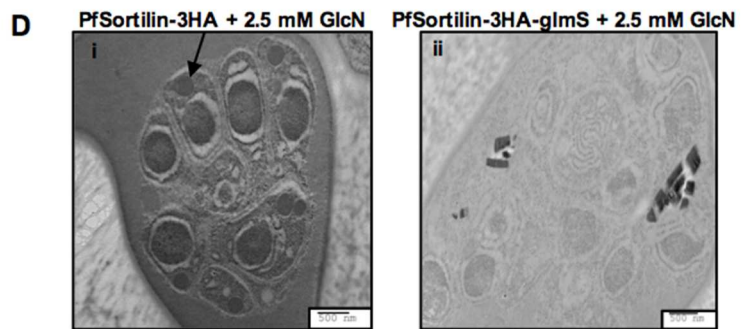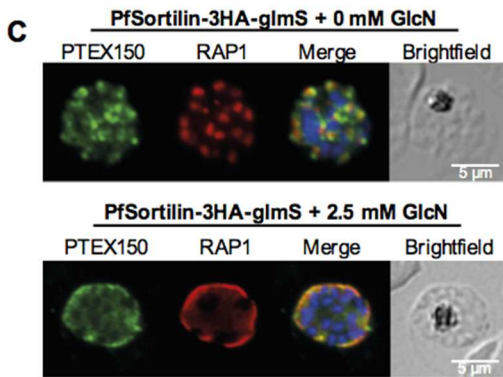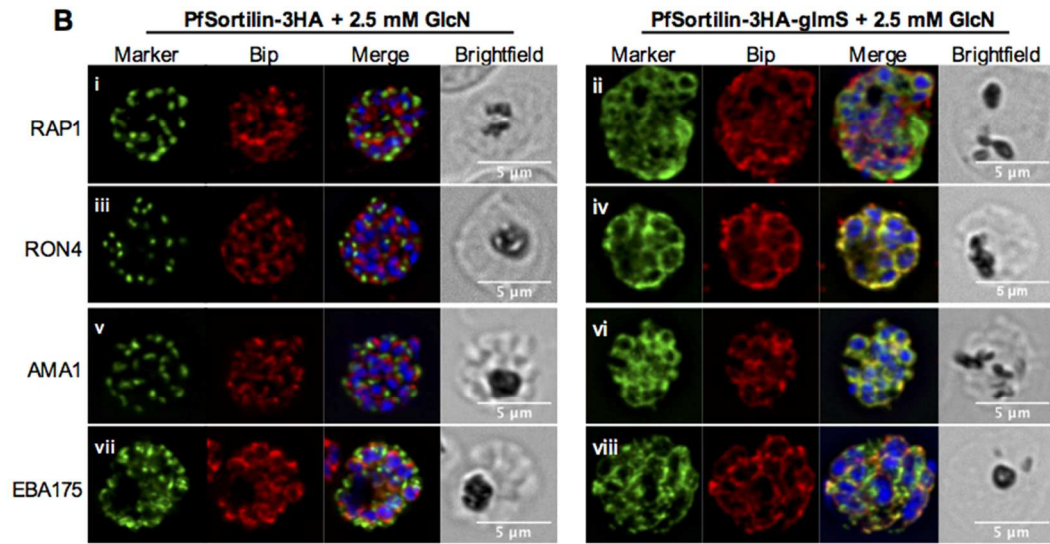
A

PfSortilin-3HA + 2.5 mM GlcN

HA | Marker | Merge | Brightfield

RAP1 — i

PfSortilin-3HA-glmS + 2.5 mM GlcN

HA | Marker | Merge | Brightfield

ii
iii

RON4 — iv / v
AMA1 — vi / vii
EBA175 — viii / ix

5 µm

B

PfSortilin-3HA + 2.5 mM GlcN

Marker | Bip | Merge | Brightfield

PfSortilin-3HA-glmS + 2.5 mM GlcN

Marker | Bip | Merge | Brightfield

RAP1 — i / ii
RON4 — iii / iv
AMA1 — v / vi
EBA175 — vii / viii

5 µm

C

PfSortilin-3HA-glmS + 0 mM GlcN

PTEX150 | RAP1 | Merge | Brightfield

PfSortilin-3HA-glmS + 2.5 mM GlcN

PTEX150 | RAP1 | Merge | Brightfield

5 µm

D

PfSortilin-3HA + 2.5 mM GlcN

i

PfSortilin-3HA-glmS + 2.5 mM GlcN
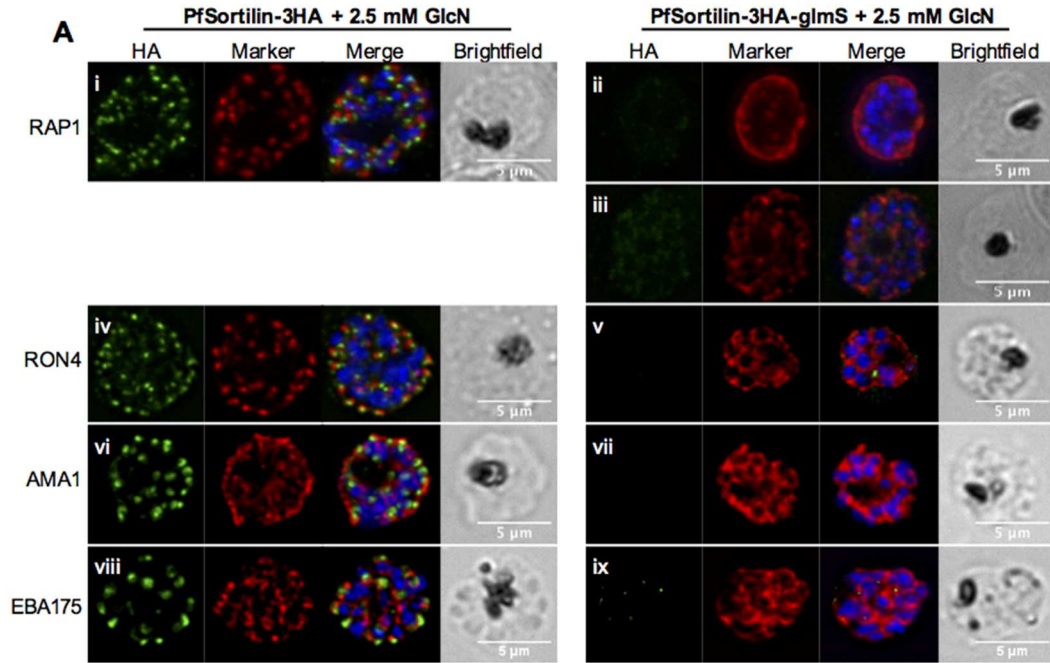
ii

500 nm

103

Figure 2. Knocking down PfSortilin abrogates trafficking to the apical organelles and prevents rhoptry formation. (A) IFA showing that the rhoptry bulb protein RAP1 traffics to the PV or remains trapped in the ER like the rhoptry neck protein RON4 and the micronemal proteins AMA1 and EBA175. (B) Colocalization with the ER marker Bip confirms that the apical proteins are trapped in the ER. (C) The dense granule protein PTEX150 is trafficked to the PV, like RAP1. (D) Electron microscopy reveals that rhoptries are not formed in absence of PfSortilin-3HA whilst they are easily seen in the control line (i, arrow). The marker label refers to the antibody used. Scale bars represent 5 µm for fluorescence images and 500 nm for electron microscopy.
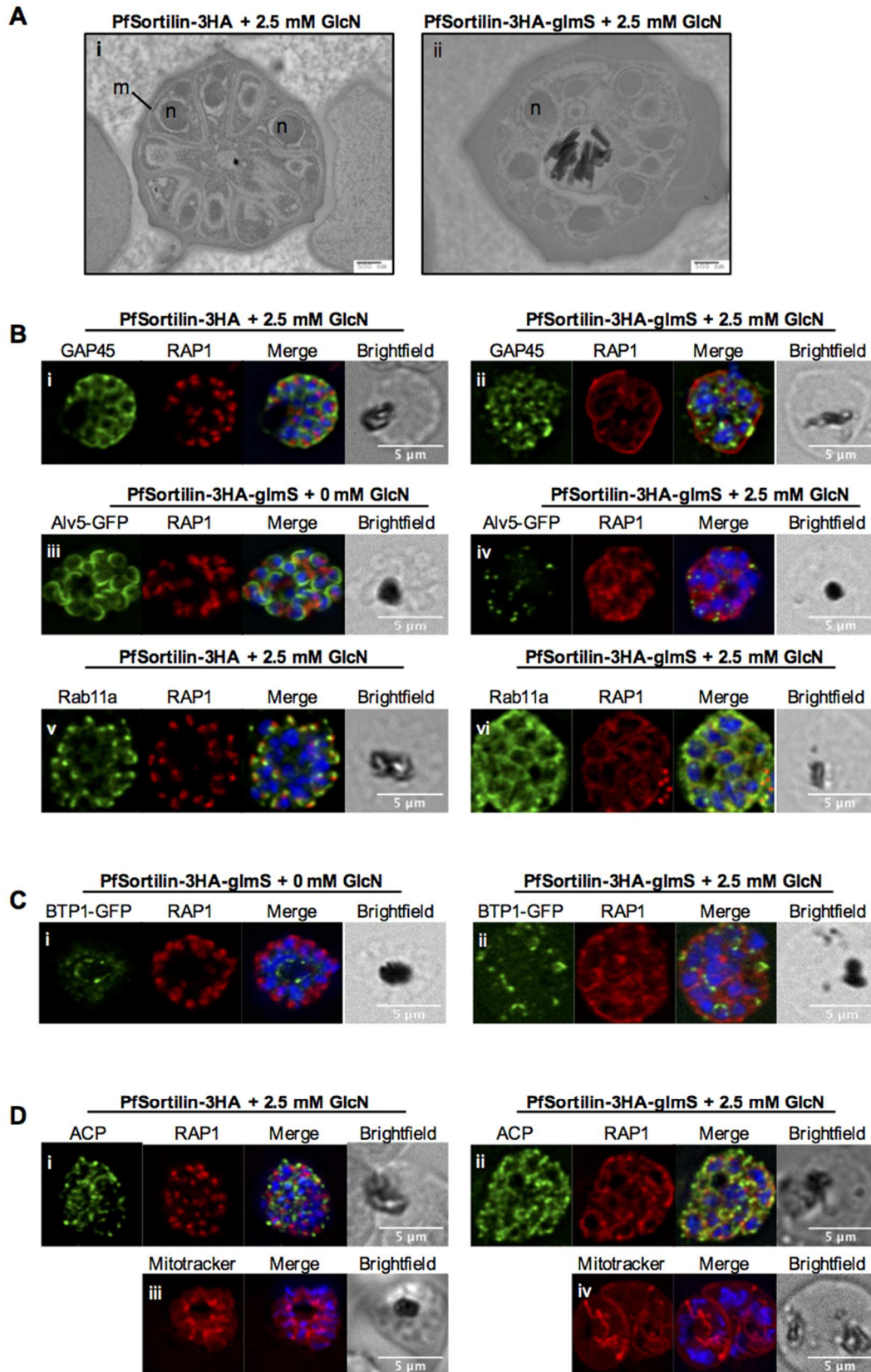
A

PfSortilin-3HA + 2.5 mM GlcN

PfSortilin-3HA-glmS + 2.5 mM GlcN

B

PfSortilin-3HA + 2.5 mM GlcN

PfSortilin-3HA-glmS + 2.5 mM GlcN

GAP45 RAP1 Merge Brightfield

GAP45 RAP1 Merge Brightfield

PfSortilin-3HA-glmS + 0 mM GlcN

PfSortilin-3HA-glmS + 2.5 mM GlcN

Alv5-GFP RAP1 Merge Brightfield

Alv5-GFP RAP1 Merge Brightfield

PfSortilin-3HA + 2.5 mM GlcN

PfSortilin-3HA-glmS + 2.5 mM GlcN

Rab11a RAP1 Merge Brightfield

Rab11a RAP1 Merge Brightfield

C

PfSortilin-3HA-glmS + 0 mM GlcN

PfSortilin-3HA-glmS + 2.5 mM GlcN

BTP1-GFP RAP1 Merge Brightfield

BTP1-GFP RAP1 Merge Brightfield

D

PfSortilin-3HA + 2.5 mM GlcN

PfSortilin-3HA-glmS + 2.5 mM GlcN

ACP RAP1 Merge Brightfield

ACP RAP1 Merge Brightfield

Mitotracker Merge Brightfield

Mitotracker Merge Brightfield

Figure 3. Knocking down PfSortilin prevents the biogenesis of the parasite pellicle and results in abrogated cytokinesis. (A) Subcellular localization of GAP45 (i and ii) and Alv5- GFP reveals that the IMC is not generated in the PfSortilin-3HA KD line. In addition, the small GTPase Rab11a colocalizes extensively with ER-trapped RAP1 (v and vi). (B) The basal complex marker BTP1-GFP shows a disrupted pattern in the KD line. (C) Electron microscopy shows that formation of merozoites is abrogated in the KD line. (D) The apicoplast and mitochondrion expand but fail to segregate in the KD line (ii and iv). GlcN: glucosamine. n: nucleus. m: merozoite. Scale bars represent 5 µm for fluorescence images and 500 nm for electron microscopy. For all IFAs, RAP1 is used as a control for proper knockdown. For the PfSortilin-3HA-glmS lines transfected with BTP1-GFP or Alv5-GFP, 0 mM GlcN was used as a control.
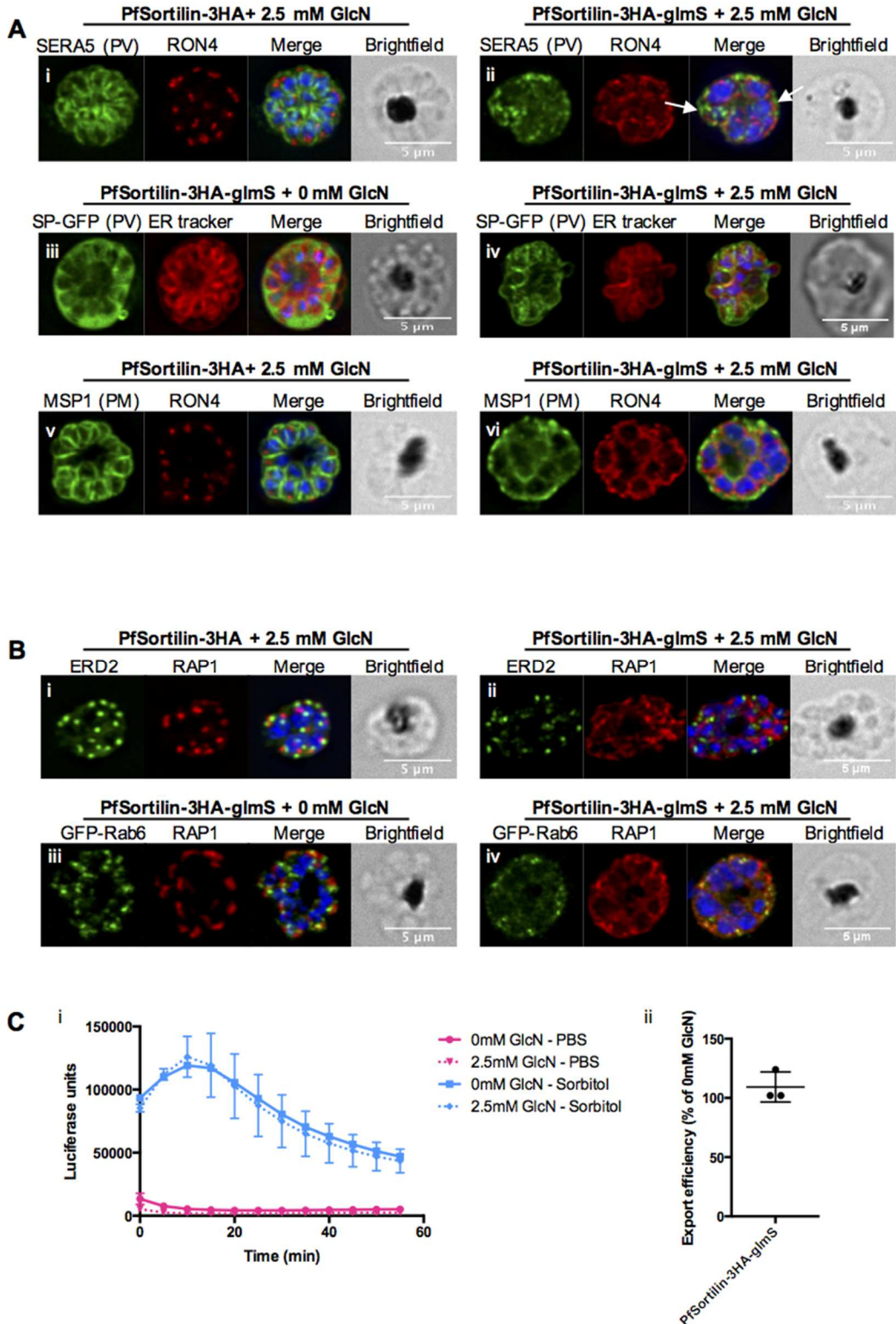
Figure 4. PfSortilin is not essential for the constitutive secretory pathway. (A) IFA showing that the PV resident protein SERA5 (i and ii), and even more clearly, a GFP reporter containing a signal peptide (iii and iv) can be trafficked to the PV. Whilst some of the parasite membrane protein MSP1

is trapped in the ER like RON4, a significant amount traffics to the parasite surface (v and vi). RON4 is used as a control for proper knockdown. (B) IFA using an antibody against the cis-Golgi marker ERD2 (i and ii) and an episomally expressed trans-Golgi marker GFP-Rab6 (iii and iv) show that the Golgi apparatus architecture is only slightly affected in the PfSortilin-3HA KD line. RAP1 is used as a control for proper knockdown. (C) Export of a PEXEL-containing luciferase reporter is not affected in the PfSortilin-3HA KD line (N=3). Scale bars represent 5 µm. For all IFAs, RAP1 or RON4 were used as a control for proper knockdown except for the SP- GFP line. For the PfSortilin-3HA-glmS lines transfected with SP-GFP or GFP-Rab6, 0 mM GlcN was used as a control.
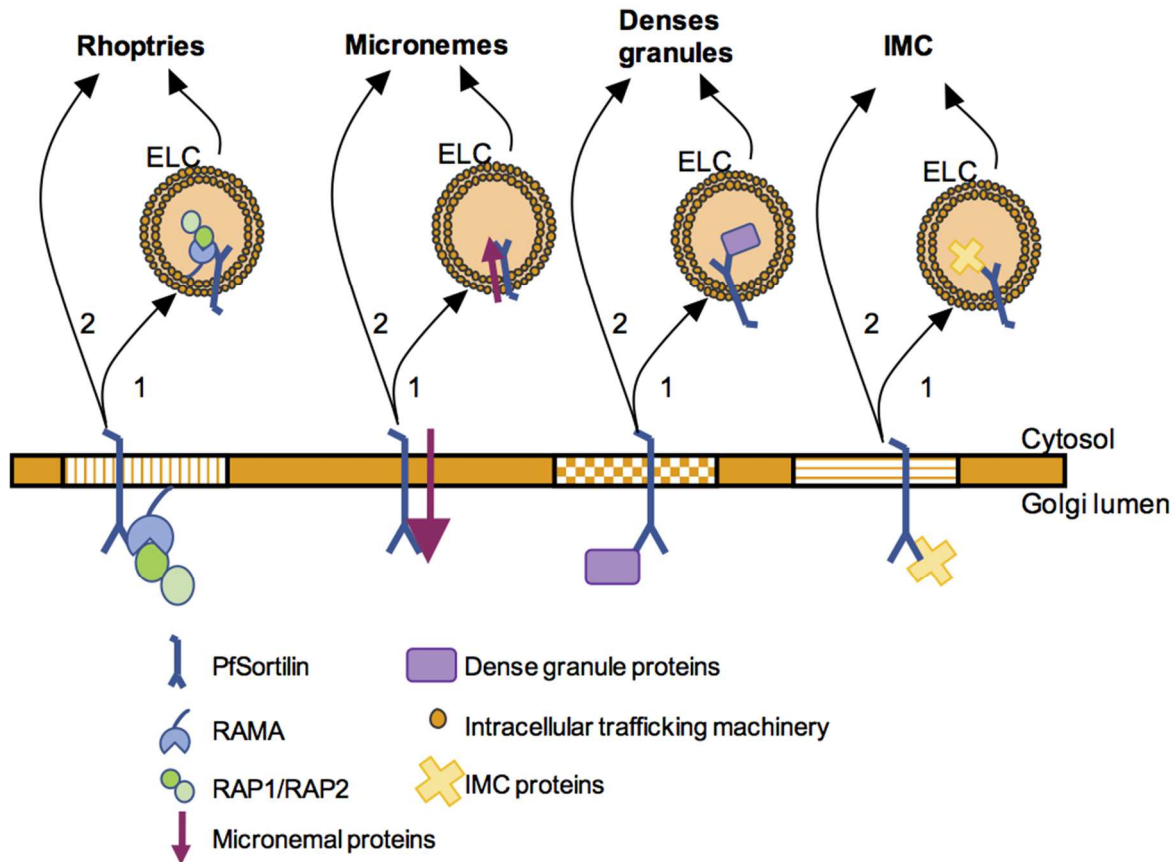
Figure 5. A model for the function of PfSortilin in the regulated secretory pathway. Within the Golgi apparatus, proteins destined to the IMC, rhoptries, dense granules and micronemes might cluster in distinct membrane microdomains, potentially through differential association with lipid rafts, where they interact with the escorter PfSortilin either directly or indirectly. Interaction of the cytoplasmic tail of PfSortilin with the intracellular trafficking machinery leads to vesicular budding from the respective Golgi microdomains. At this stage, the vesicles can potentially transit through an endosome-like compartment (ELC) for further cargo sorting (1) or traffic directly to their respective organelles (2). Additional specificity could be provided by organelle specific intracellular trafficking machinery (not added to the figure for clarity) and/or perhaps differences in the timing of expression of cargos destined to different organelles. Proteins destined for other locations such as the apicoplast and the plasma membrane have been omitted for clarity.
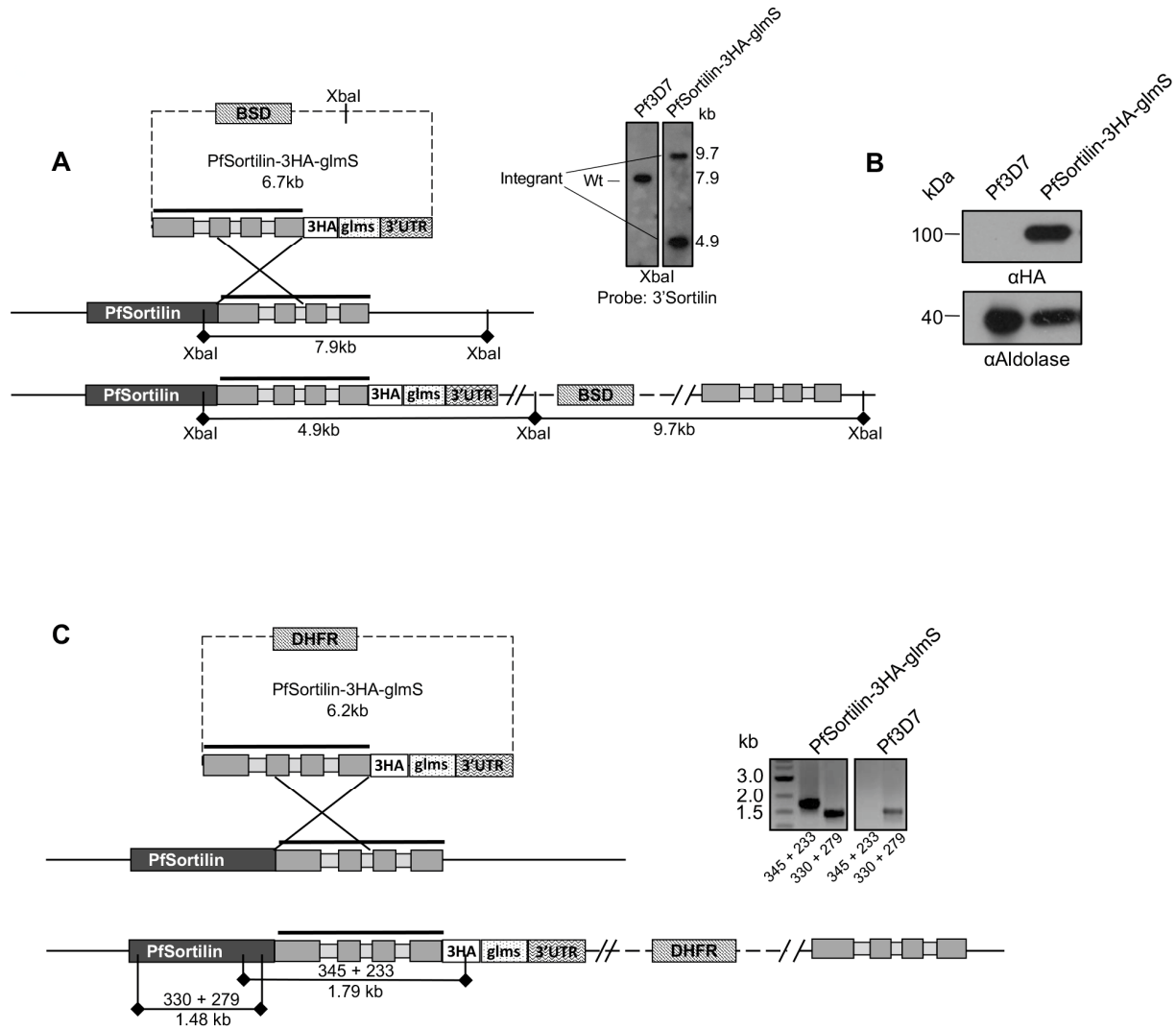
Figure S1. Generation of the PfSortilin-3HA-glmS lines. (A) Schematic of the knock-in strategy and Southern blot showing proper integration of the plasmid and the disappearance of the WT allele. (B) Western blot showing a specific band at the expected size of around 100 kDa for PfSortilin-3HA. (C) Schematic of the second knock-in strategy and PCR analysis showing proper integration of the plasmid of another independently generated PfSortilin-3HA-glmS line.
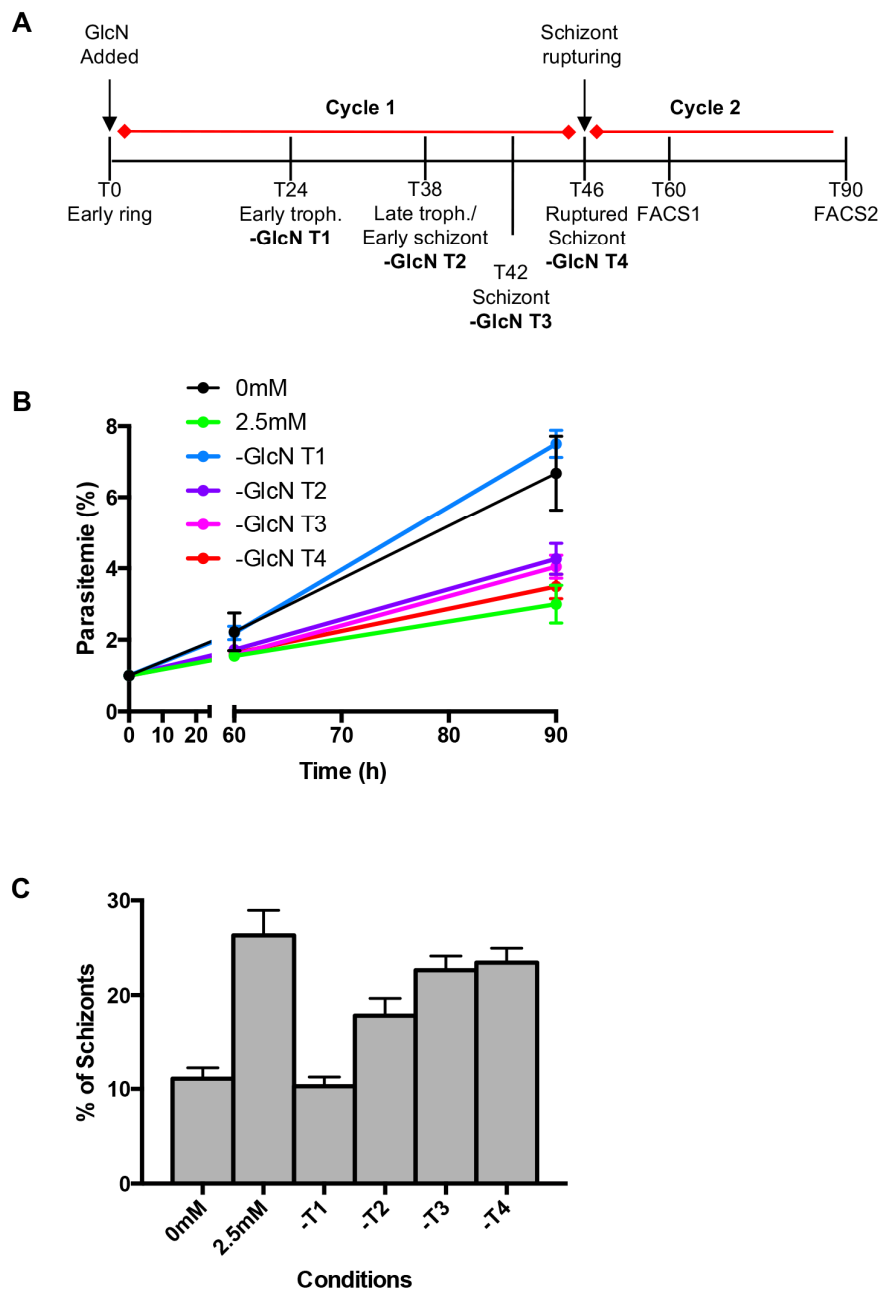
Figure S2. The effect of the PfSortilin knockdown is reversible up to the early schizont stage.
(A) Experimental schematic. T: Time at which GlcN was taken out of the growth medium. (B) Growth curve analysis showing that taking parasites off GlcN from the early schizont stage onwards does not allow recovery of parasites. (C) Quantification of the percentage of parasites trapped at the schizont stage in relation to when GlcN was taken off. GlcN: glucosamine.
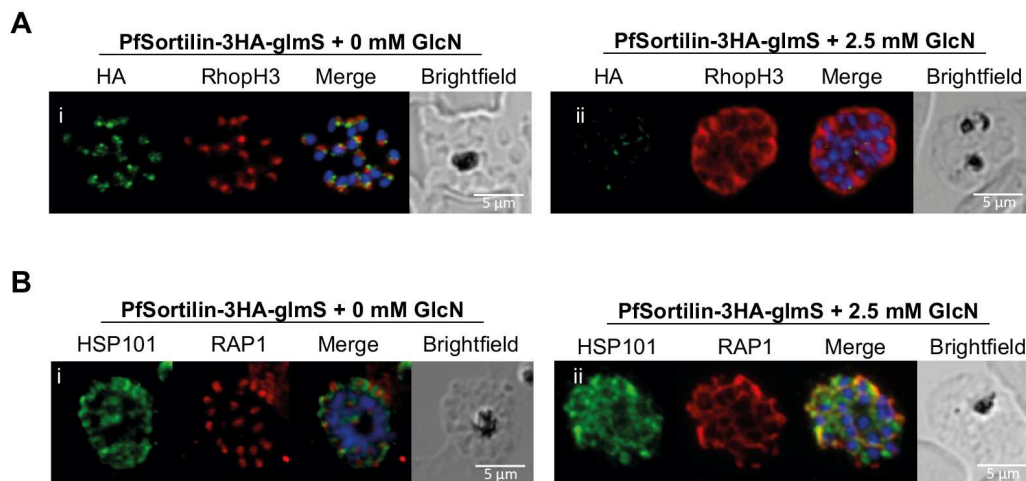
A

PfSortilin-3HA-glmS + 0 mM GlcN

HA RhopH3 Merge Brightfield

5 µm

PfSortilin-3HA-glmS + 2.5 mM GlcN

HA RhopH3 Merge Brightfield

5 µm

B

PfSortilin-3HA-glmS + 0 mM GlcN

HSP101 RAP1 Merge Brightfield

5 µm

PfSortilin-3HA-glmS + 2.5 mM GlcN

HSP101 RAP1 Merge Brightfield

5 µm

Figure S3. Knocking down PfSortilin disrupts the trafficking of the rhoptry bulb protein RhopH3 and the dense granule protein HSP101. (A) IFA showing that the rhoptry bulb protein RhopH3 remains trapped in the ER in the PfSortilin KD line. (B) The dense granule protein HSP101 remains trapped in the ER in the PfSortilin KD line. RAP1 is used as a control for proper KD. Scale bars represent 5 µm.
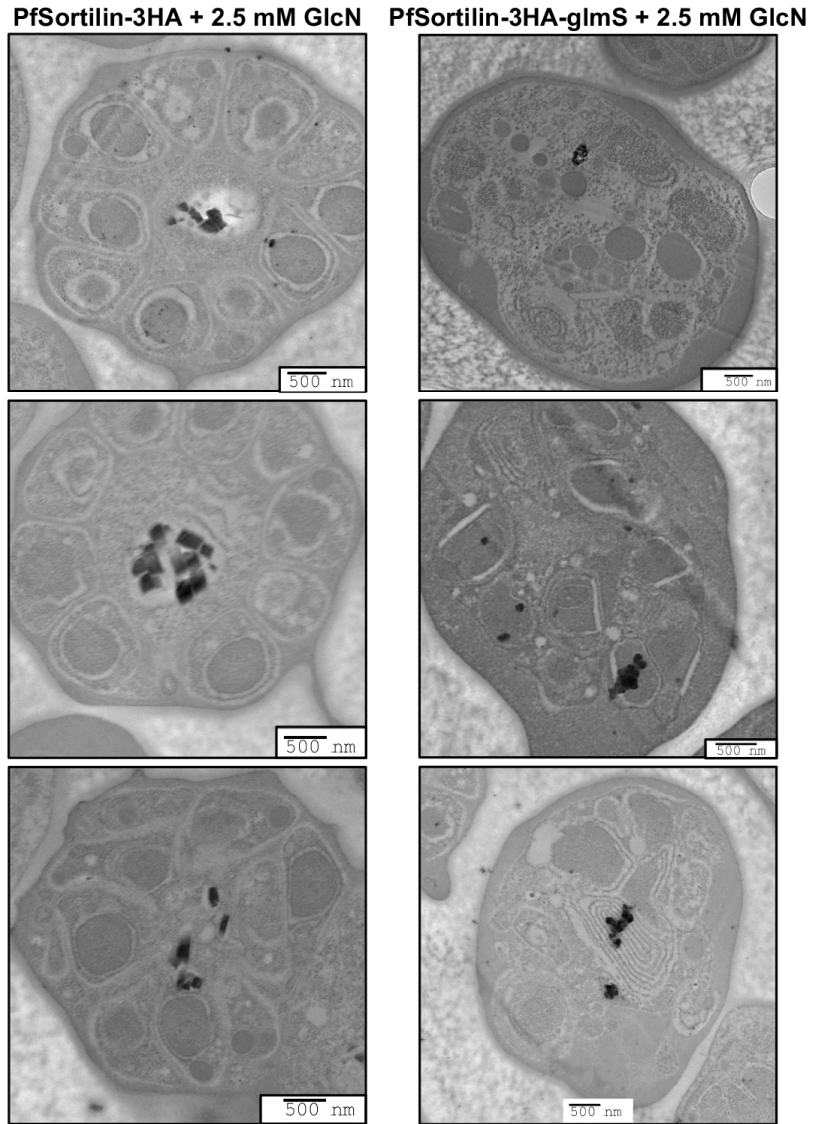
PfSortilin-3HA + 2.5 mM GlcN   **PfSortilin-3HA-glmS + 2.5 mM GlcN**

Figure S4. Additional electron microscopy images of the control and PfSortilin-3HA knockdown parasites. Scale bars represent 500 nm.
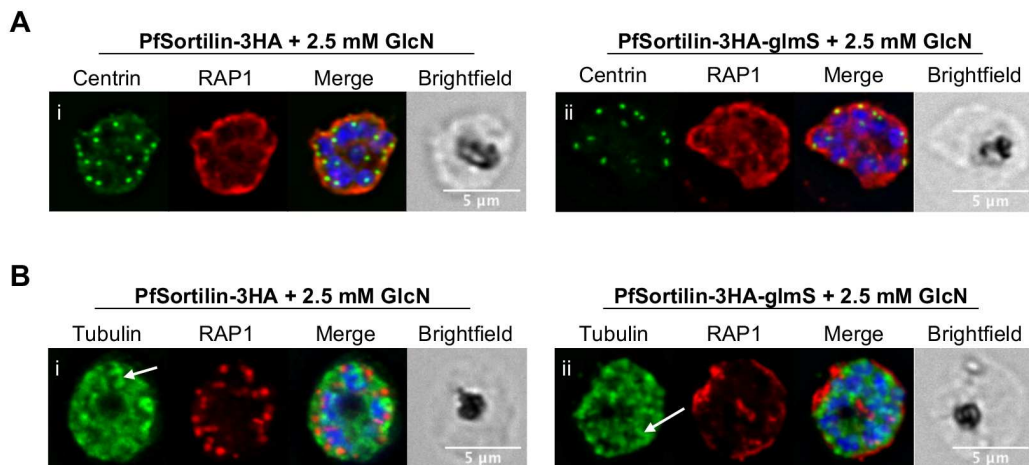
Figure S5. The centrosomes and microtubule cytoskeleton are not disrupted in the PfSortilin knockdown parasites. (A) IFA with an anti-Centrin antibody shows proper duplication of the centrosomes in absence of PfSortilin. (B) IFA with an anti-alpha tubulin antibody shows the same pattern of foci of fluorescence and sometimes filaments (arrows) in the control and KD line. Scale bars represent 5 µm
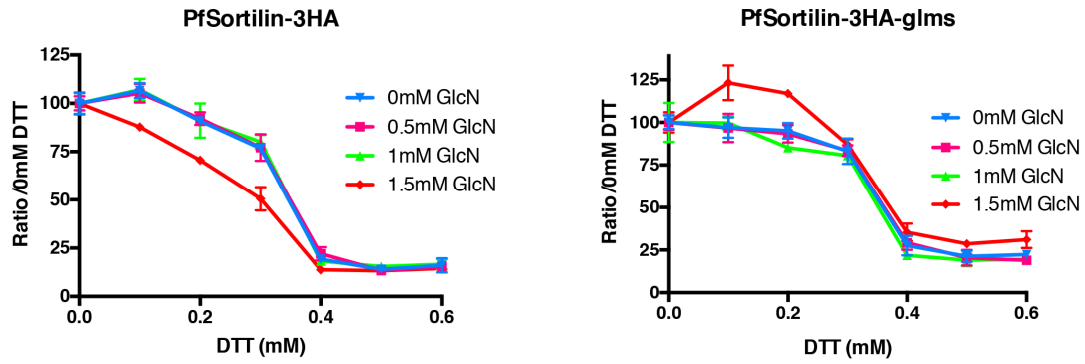
Figure S6. Decreasing PfSortilin expression does not lead to increased sensitivity to ER stress. Results displayed as percentage of growth compared to the parasitemia at 0 mM DTT. Results from one experiment representative of at least 3 independent biological replicates are shown. Error bars represent the standard deviation from 3 technical replicates.

Table S1. List of primers used in this study.

Sequences	Description	Restriction site
5' ata<u>agatct</u>gagacaaatacagaaaaaag 3'	Sortilin 1306 fw	BglII
5' ata<u>ctgcag</u>ctaataattcaatattatcagc 3'	Sortilin stopless rev	PstI
5' gc<u>agatct</u>gatccaacattaaccattgaagg 3'	Sortilin 2158 fw	BglII
5' gg<u>cctaggt</u>aataattcaatattatca gc 3'	Sortilin stopless rev	AvrII
5' ata<u>ggatcc</u>gaacaaataaaaaatggtataagc 3'	RamaA fw	BamHI
5' ata<u>ctcgag</u>gctatcatcgtattcgtcag 3'	RamaA rev	XhoI
5' ata<u>ggatcc</u>agcgaagaatatgattacgac 3'	RamaB fw	BamHI
5' ata<u>ctcgag</u>atatttcatttgttcgtctttcatctc 3'	RamaB rev	XhoI
5' ata<u>ggatcc</u>gtgatgaaagatgaagagatg 3'	RamaC fw	BamHI
5' ata<u>ctcgag</u>tttctcatcattttgtaagaaact 3'	RamaC rev	XhoI
5' ata<u>ggatcc</u>aaaaaaatggtcttttatgatttatacaagc 3'	RamaD fw	BamHI
5' ata<u>ctcgag</u>atcgaaaattttattattattttc 3'	RamaD rev	XhoI
5' ata<u>ggatcc</u>gataataaatttgtagcacataaa 3'	RamaE fw	BamHI
5' ata<u>ctcgag</u>gcttgacttatttccattttc 3'	RamaE rev	XhoI
5' ata<u>acgcgt</u>gaacaaataaaaaatggtataagc 3'	RamaA fw	MluI
5' ata<u>actagt</u>gctatcatcgtattcgtcag 3'	RamaAq rev	Spe1
5' ata<u>acgcgt</u>agcgaagaatatgattacgac 3'	RamaB fw	MluI
5' ata<u>actagt</u>atatttcatttgttcgtctttcatctc 3'	RamaB rev	Spe1
5' ata<u>acgcgt</u>gtgatgaaagatgaagagatg 3'	RamaC fw	MluI
5' ata<u>actagt</u>tttctcatcattttgtaagaaact 3'	RamaC rev	Spe1
5' ata<u>acgcgt</u>aaaaaaatggtcttttatgatttatacaagc 3'	RamaD fw	MluI
5' ata<u>actagt</u>atcgaaaattttattattattttc 3'	RamaD rev	Spe1
5' ata<u>acgcgt</u>gataataaatttgtagcacataaa 3'	RamaE fw	MluI
5' ata<u>actagt</u>gcttgacttatttccattttc 3'	RamaE rev	Spe1
5' gc<u>cctagg</u>atgtcaaataaaaaaagaaccatattaaaag 3'	Rab7 fw	AvrII
5' tac<u>ctcgag</u>ttaacaacaacgactttg 3'	Rab7 rev	Xho1
5' ccg<u>cctaggt</u>atatataatgaagatcttattactttgtataattttctatattatgtta acgctttaaaaatacaagtaaaggagaagaactttcactgg 3'	SP of ACP-GFP fusion PCR	AvrII
5' agtaaaggagaagaactttcactggag 3'	GFP-fw	
5' gg<u>ctcgag</u>ttatttgtatagttcatccatgccatgtg 3'	GFP-rev	XhoI

-Restriction sites are underlined.