

# Molecular Determinants for Subcellular Trafficking of the Malarial Sheddase PfSUB2

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**The malaria merozoite invades erythrocytes in the vertebrate host. Iterative rounds of asexual intraerythrocytic replication result in disease. Proteases play pivotal roles in erythrocyte invasion, but little is understood about their mode of action. The *Plasmodium falciparum* malaria merozoite surface sheddase, PfSUB2, is one such poorly characterized example. We have examined the molecular determinants that underlie the mechanisms by which PfSUB2 is trafficked initially to invasion-associated apical organelles (micronemes) and then across the surface of the free merozoite. We show that authentic promoter activity is important for correct localization of PfSUB2, likely requiring canonical features within the intergenic region 5' of the *pfsub2* locus. We further demonstrate that trafficking of PfSUB2 beyond an early compartment in the secretory pathway requires autocatalytic protease activity. Finally, we show that the PfSUB2 transmembrane domain is required for microneme targeting, while the cytoplasmic domain is essential for surface translocation of the protease to the parasite posterior following discharge from micronemes. The interplay of pre- and post-translational regulatory elements that coordinate subcellular trafficking of PfSUB2 provides the parasite with exquisite control over enzyme–substrate interactions.**

**Key words:** malaria, microneme, *Plasmodium*, protease, sheddase, SUB2

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Malaria is a debilitating disease that threatens at least 1 billion people, infects around 500 million and is responsible for approximately 1 million deaths each year (1). The etiologic agent is the protozoan parasite *Plasmodium spp.*

which is transmitted through the bite of an infected female Anopheline mosquito. Replication of the asexual blood-stages of the parasite's complex life cycle causes the pathology of the disease. The asexual blood-stage life cycle of the parasite divides into three distinct phases: (i) invasion of the host erythrocyte by a merozoite; (ii) growth and multiplication of the intracellular parasite by schizogony to form new daughter merozoites; (iii) merozoite release, through a process known as egress, and subsequent reiteration of these events.

Erythrocyte invasion is driven by an actinomyosin motor which, in association with other molecular partners, is referred to as the glideosome (2–4). The merozoite attaches to an erythrocyte, then as the motor drives the parasite into the cell, components of the parasite surface coat are shed through the action of at least two serine proteases: a rhomboid called ROM4 (5) and the merozoite surface sheddase, a member of the subtilisin-like superfamily called PfSUB2 (6). Early electron microscopic studies of erythrocyte invasion suggested that the enzymes responsible for the proteolysis of surface proteins localize to a region of close association between the parasite and the host cell called the moving junction (7), though a recent study has questioned this supposition (8). The attachment of parasite proteins at the leading edge of the junction is thought to drag the erythrocyte membrane around the parasite, before being disengaged at the parasite posterior pole by proteolysis (9). The trafficking and compartmentalisation of substrates and other components of this shedding machinery therefore likely regulate invasion (10).

PfSUB2 is one of just three subtilisin-like proteases encoded within the *Plasmodium falciparum* genome (11). Like most subtilisins, PfSUB2 is expressed as a precursor zymogen, which undergoes maturation from a 150 kDa precursor (PfSUB2<sub>P</sub>), to a 75 kDa intermediate (PfSUB2<sub>I</sub>) and then to the terminal 70 kDa mature form (PfSUB2<sub>T</sub>) (12). For many subtilisins, maturation is a prerequisite for progression through the secretory transport system (13,14). On the basis of sensitivity to brefeldin A treatment, the primary maturation step of PfSUB2 is thought to take place in the endoplasmic reticulum (ER), with the second occurring beyond this compartment (11).

PfSUB2 is targeted to two distinct subcellular locations in the parasite. Initially PfSUB2 accumulates in a set of apical secretory organelles called micronemes, which are known to be the destination of other invasion factors (15,16). Following merozoite egress, PfSUB2 is discharged from these organelles and traffics across the surface of the free merozoite in an actin-dependent fashion that can be

disrupted by the actin destabilizing agent, latrunculin A (6). At least one other microneme protein, apical membrane antigen-1 (PfAMA1), is also discharged onto the surface of the merozoite at egress. Interestingly, once released the distribution of these two proteins, which were initially in the same micronemal location becomes very different; PfSUB2 caps as a tight focus at the posterior of the parasite, whereas PfAMA1 distributes circumferentially across the surface of the parasite via an apparently actin-independent mechanism (17,18). These observations are consistent with distinct mechanisms for the surface trafficking of these two proteins.

PfAMA1, as well as two other surface proteins called MSP1 and PTRAMP, are shed during invasion through the action of PfSUB2 (6,19). Given the apparently essential nature of this shedding (20–23), an understanding of the trafficking of PfSUB2 to the micronemes and parasite surface would enable dissection of the means by which the parasite is able to package key invasion factors and the protease responsible for their eventual release from the parasite surface to the same subcellular compartment. Knowledge of how PfSUB2 is brought into productive contact with its substrates may offer new avenues for the development of small molecules to interfere with this process.

Here we have investigated the factors governing the trafficking of PfSUB2. We provide evidence that trafficking of PfSUB2 may be dependent upon canonical regulatory features 5' of the gene locus. An extended fragment of the *pfsb2* 5' intergenic region is required to reproduce an authentic pattern of trafficking for an episomally maintained transgene product. Trafficking of PfSUB2 was also found to be dependent upon autocatalytic maturation, while an intact transmembrane domain and the highly conserved juxtamembrane region are required for correct microneme targeting. The cytoplasmic domain (CD) was found to be required for actin-dependent surface translocation of the protease. The results demonstrate that the trafficking of PfSUB2 is governed by a range of molecular determinants, requiring both pre- and post-translational regulation.

## Results

### ***PfSUB2* expressed under the control of the *pfama1* promoter is aberrantly trafficked**

In previous work we epitope-tagged PfSUB2 in the parasite by using single-crossover homologous recombination to fuse a triple haemagglutinin tag (HA3) to the extreme 3' end of the *pfsb2* coding sequence (6). However, repeated attempts to disrupt the *pfsb2* gene or introduce a GFP-tag proved unsuccessful. PfSUB2 is therefore likely essential for the intracellular survival of the blood-stage parasite. To study the factors involved in trafficking of the protease we decided to establish a transgene expression system using epitope-tagged PfSUB2 (PfSUB2-HA)

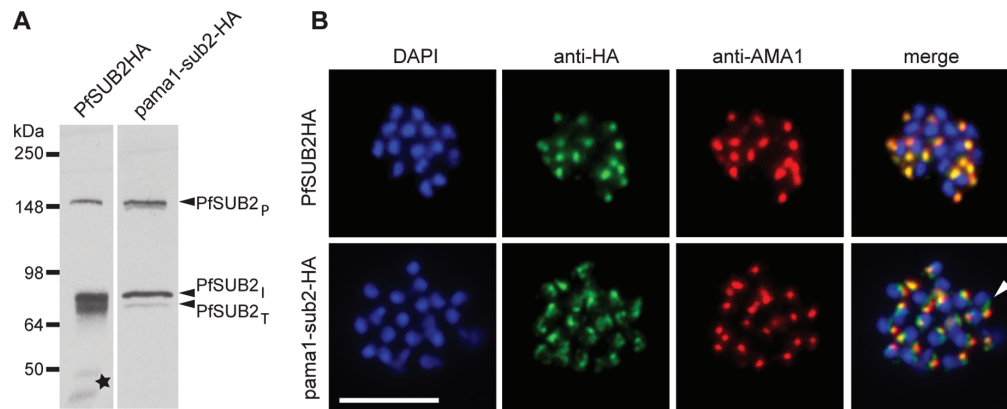
transgenes carried on episomally maintained plasmids. These constructs allow for the expression and study of PfSUB2 mutants in the parasite on a background of expression of the endogenous gene.

The transcriptional profile of *pfsb2* closely matches that of *pfama1*; transcription of both begins at around 43–45 h following erythrocyte invasion and peaks around 48 h post-infection (24,25). The *pfama1* promoter is well characterized and has been successfully used for the transgenic expression of other late-stage *P. falciparum* genes (23). We therefore initially attempted to express PfSUB2-HA from an episomal transgene driven by the *pfama1* promoter. As can be seen from Figure 1A, the transgene was successfully expressed and underwent the expected maturation events involving conversion of the precursor zymogen through an intermediate to the terminal form. However, indirect immunofluorescence assay (IFA) with HA-specific antibodies showed that the transgene product only partially colocalized with the endogenous microneme marker PfAMA1, with most of the signal distributed between the parasite cytosol and an undefined compartment between the nucleus and the apically disposed micronemes (indicated by the white arrowhead on the merged image for *pfama1*-sub2-HA in Figure 1B). This could be due to retention of a proportion of the transgene product in an early secretory compartment such as the ER, or the *Plasmodium* equivalent of the late endosome (LE) recently described for *Toxoplasma* and thought to be a transitory organelle for microneme proteins (26). This result suggested that although the transcriptional profiles of *pfama1* and *pfsb2* are almost superimposable, the corresponding promoters are not functionally equivalent.

### **Canonical features 5' of the *pfsb2* locus may affect gene expression and product localization**

Correct trafficking of other *Plasmodium* gene products has previously been shown to be dependent on the promoter used to drive expression of the transgene (27–29). We therefore investigated whether use of the native *pfsb2* promoter in our episomal system could correctly reproduce the authentic pattern of PfSUB2 trafficking to the micronemes.

The extreme AT bias of the *Plasmodium* genome, along with a paucity of identifiable transcription factors has made characterization of genomic regulatory elements difficult (30). In spite of this, a consensus is beginning to emerge; this is of a promoter structure similar to that of the canonical eukaryotic type, with the standard features of a major 5' transcriptional start site, an enhancer region and a transcription factor binding site. In light of this we performed an *in silico* examination of the region 5' of the 3D7 *pfsb2* locus. The major 5' transcription start site has been previously mapped by us to position –1168 bp upstream of the start ATG (12). Further upstream of the transcription start site there are multiple extended poly A/T tracts, known to influence the position of transcription



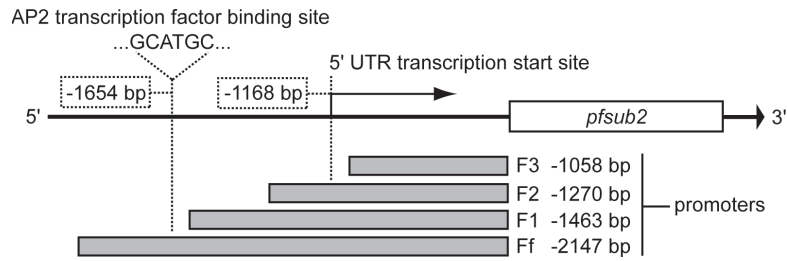
**Figure 1: Mislocalization of PfSUB2 expressed from an episomally maintained transgene driven by the *pfama1* promoter.** Synchronous late-stage schizonts were examined by western blot and indirect IFA. A) Schizont extracts were fractionated by SDS-PAGE, transferred to nitrocellulose and probed with anti-HA mAb 3F10. Lanes (left to right): PfSUB2HA positive control; episomally expressed HA3-tagged PfSUB2 transgene driven by the *pfama1* promoter. The results show that the *pfama1* promoter drives expression of an episomally maintained *pfsub2* transgene. Positions of the 150 kDa precursor (PfSUB2<sub>p</sub>), 75 kDa intermediate (PfSUB2<sub>i</sub>) and terminal 70 kDa form (PfSUB2<sub>t</sub>) are indicated. The filled star indicates a previously observed small HA-reactive product often present in the PfSUB2HA parasite extracts (6). B) Fixed thin-blood films were probed with the anti-HA mAb (green) and an anti-PfAMA1 rabbit polyclonal antibody (red). Parasite nuclei were counterstained with DAPI (blue). PfAMA1 localizes to micronemes at the apical end of the parasite. Most of the signal intensity for the tagged endogenous gene product (PfSUB2HA) overlaps with that of PfAMA1 as previously observed (6) (Pearson's coefficient 0.927). In contrast, although a proportion of the episomally expressed PfSUB2 (pama1-sub2-HA) signal overlaps with PfAMA1, a significant proportion localizes to an undefined cytosolic compartment (white arrowhead) (Pearson's coefficient 0.628, indicating the change in correlation of the signal intensities). Note that fluorescence intensities in IFA were not noticeably different between the PfSUB2HA and pama1-sub2-HA lines, indicating similar transgene expression levels (data not shown). Scale bar, 8 μm.

initiation (31). A recent study determined the recognition sites for two members of the apicomplexan AP2 (ApiAP2) family of transcription factors in *P. falciparum* (32). The presence of these binding motifs in the region 5' of a gene was found to correlate with their expression profile during the erythrocytic life cycle. However, the authors noted that the transcription profiles of the AP2 transcription factors do not necessarily match the transcription profiles of target genes. Interestingly, the transcription profiles of putative targets for the PF14\_0633 ApiAP2 transcription factor show a similar late-schizont expression profile to that exhibited by *pfsub2* (32). An examination of the *pfsub2* 5' flanking region identified a site that matched the published consensus binding motif for PF14\_0633 (GCATGC) (32), locating to position -1654 bp (including the six residues of the motif) upstream of the start ATG.

To investigate the relative contributions of each of these features and any others that might have been overlooked, a 2147-bp fragment (called Ff) of the *pfsub2* 5' flanking region was amplified from genomic parasite DNA and cloned into the episomal PfSUB2-HA expression vector in place of the *pfama1* promoter. In addition three different truncated forms of the same region were similarly inserted into the vector. The resulting four constructs were then transfected separately into the parasite and assessed in parallel for their capacity to drive expression and correct trafficking of the PfSUB2-HA transgene product. Figure 2 shows a schematic representation of this strategy, and highlights the features identified from the *in silico* examination detailed above. As shown in Figure 3A, all

four putative promoter sequences (F3, F2, F1 and Ff) successfully drove expression of the *pfsub2* transgene, as well as the correct pattern of post-translational maturation of the transgene product. However, IFA with the anti-HA antibody showed that in 3 of 4 cases the subcellular localization differed significantly from the PfSUB2-HA control line in which the endogenous gene has been HA-tagged (Figure 3B). Expression from the F3 and F2 promoter fragments resulted in the majority of the IFA signal being diffusely distributed throughout the schizont (Figure 3B). This suggested that the inclusion in the promoter of the previously mapped major 5' transcription start site was not sufficient to correctly target the expressed product to the micronemes. The IFA signal resulting from expression from the F1 fragment was more punctate (Figure 3B), with the majority of the signal correctly colocalizing with the microneme marker PfAMA1. However, it was not as tightly restricted to this apical compartment as observed for the endogenous gene product, and indeed was similar to the localization pattern observed for transgene expression driven by the *pfama1* promoter (Figure 1B). In contrast, expression of the PfSUB2-HA transgene under control of the Ff fragment matched the localization pattern seen for the tagged endogenous gene product (Figure 3B); expression was tightly restricted to the apical end of the developing intracellular merozoites, with the product predominantly colocalizing with PfAMA1.

From these data, we conclude that correct subcellular localization of an episomally maintained *pfsub2* transgene



**Figure 2: Examination of the *pfsub2* 5' flanking region.** Schematic representation (not to scale) of the intergenic sequence lying 5' to the *pfsub2* locus, and the four different regions examined for promoter activity (Ff, F1, F2 and F3). The position of the previously mapped major 5' transcriptional start site at  $-1168$  bp and the putative ApiAP2 transcription factor binding site at  $-1654$  bp are indicated in relation to the different promoter fragments and their distance from the ATG start codon of the *pfsub2* coding sequence (position 0).

product is strictly dependent upon the promoter used to drive its expression. The observation that the Ff promoter but not the truncated fragments enables correct targeting of an ectopically expressed protease suggests that critical regulatory elements for appropriate expression lie between  $-1463$  and  $-1654$  bp upstream of the PfSUB2 start codon. However, further mutagenesis studies will be required to confirm and precisely define the nature of those elements. The Ff-containing episomal construct was therefore used for all subsequent trafficking studies described unless otherwise stated.

#### **Primary proteolytic maturation of PfSUB2 is autocatalytic and is required for trafficking beyond an early secretory compartment**

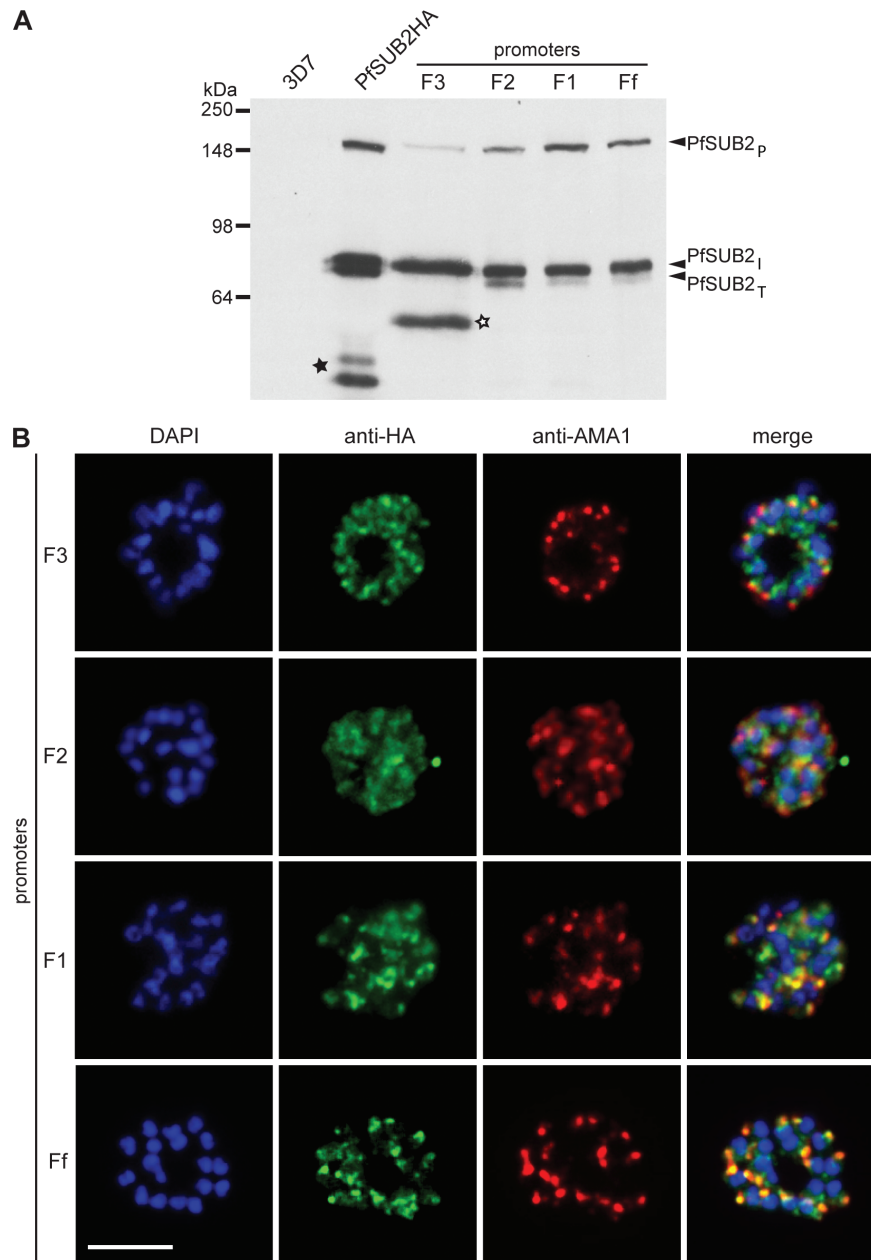
In common with the majority of subtilisin-like proteases, PfSUB2 undergoes proteolytic maturation (12). By analogy with other subtilisins, it is likely that the primary step of this maturation is autocatalytic, but this has yet to be confirmed. The autocatalytic maturation of several secreted eukaryotic subtilisins is associated with progression through the secretory transport system (14,33). However, the relationship between proteolytic maturation of PfSUB2 and its trafficking is unknown.

To address both of these points, we exploited the PfSUB2-HA episomal expression system described above to examine the consequences of substitution of the putative catalytic Ser residue, S960, with an Ala residue (S960A). This mutation has been shown to render subtilisin-like proteases catalytically inactive, but to have little effect upon the 3-dimensional architecture of the active site cleft (34). As shown in Figure 4A, the transgenic PfSUB2-HA S960A mutant but did not undergo detectable maturation beyond the 150 kDa zymogen precursor, strongly suggesting that the primary maturation step is autocatalytic. Since expression of this mutant was on the background of normal expression of endogenous PfSUB2, this observation additionally suggests that the primary autocatalytic maturation step is an intramolecular event that cannot efficiently occur *in trans*. Examination of the PfSUB2-HA S960A-expressing line confirmed that the mutant transgene product did not localize to the micronemes, instead appearing to be restricted

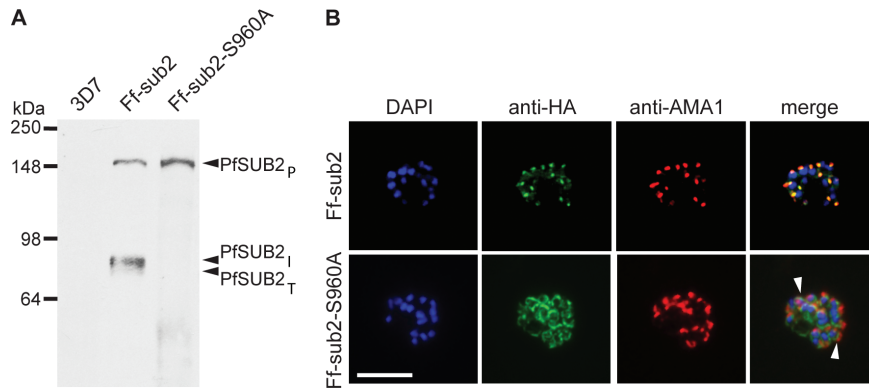
to a perinuclear location likely corresponding to an early secretory compartment (indicated by the white arrowheads in Figure 4B). We have previously shown that brefeldin A, a fungal metabolite that interferes with anterograde protein transport from the ER to the Golgi, induces accumulation of the 75 kDa PfSUB2<sub>1</sub> intermediate, indicating that primary processing of PfSUB2 occurs in the ER. These new data are consistent with that, although efforts to colocalize the aberrantly trafficked PfSUB2-S960A with antibodies to the ER marker PfBip were unsuccessful (data not shown). Collectively, these data suggest that firstly, primary maturation of PfSUB2 is autocatalytic and occurs in a non-micronemal secretory compartment (that is likely the ER), and secondly that proteolytic maturation of the protease is required for trafficking beyond this compartment to the micronemes. Autocatalytic processing of PfSUB2 may serve as a quality control checkpoint for transit of the protease beyond the ER.

#### **The transmembrane and CDs of PfSUB2 are not required for enzyme activity**

The PfSUB2 primary translation product comprises a secretory signal, an extended N-terminal prodomain, a putative catalytic domain and a highly conserved juxtamembrane structure. This is followed by a putative membrane-spanning sequence typical of type I integral membrane proteins, and finally a C-terminal CD which varies somewhat in length between different SUB2 orthologues (Figure S1). Having established that catalytic activity of the protease is important for trafficking, we investigated the role of the different putative domains for activity and trafficking of the protease. Since the prodomain of many subtilisins is required for folding (14), we decided to evaluate a series of episomal PfSUB2-HA expression constructs in which the gene had been progressively truncated from the C-terminus. These deletion mutants were expressed episomally in the parasite under the control of the Ff promoter sequence. As shown in Figure 5A, the constructs tested consisted of deletions of the cytoplasmic domain ( $\Delta$ CD), both the cytoplasmic and transmembrane domains ( $\Delta$ Tm + CD), or the entire conserved juxtamembrane, transmembrane and cytoplasmic domains ( $\Delta$ Jx + Tm + CD). Since we had



**Figure 3: Episomal expression of PfSUB2 driven by *pfsub2* 5' flanking sequences.** western blot and IFA analysis of PfSUB2 expressed under the control of different promoter lengths. A) Extracts of segmented schizonts were fractionated by SDS-PAGE, transferred to nitrocellulose and probed with anti-HA mAb 3F10. Lanes (from left to right): parental wild-type 3D7 (negative control); PfSUB2HA positive control; episomal HA3-tagged PfSUB2 transgene driven by the F3 promoter; episomal HA3-tagged PfSUB2 transgene driven by the F2 promoter; episomal HA3-tagged PfSUB2 transgene driven by the F1 promoter; episomal HA3-tagged PfSUB2 transgene driven by the Ff promoter. The filled star highlights the previously observed small anti-HA-reactive product in extracts of the control PfSUB2HA clone, and the open star highlights an additional anti-HA reactive species in the F3 transgenic line. B) Formaldehyde-fixed, thin-blood films of segmented schizonts were probed with the anti-HA mAb (green) or an anti-PfAMA1 rabbit polyclonal antibody (red). Nuclei were counterstained with DAPI (blue). PfAMA1 localizes to the micronemes at the apical end of the parasite. The majority of the PfSUB2 signal when expressed under the control of the F3 and F2 promoter fragments does not overlap with the PfAMA1 signal (Pearson's coefficient 0.183 and 0.518, respectively). While a significant proportion of the product expressed under the control of the F1 promoter correctly localizes to the micronemes (Pearson's coefficient 0.637), the Ff promoter results in greater overlap between the PfSUB2 and PfAMA1 signals, and is the only one that produces an authentic micronemal pattern of localization (Pearson's coefficient 0.682). Note that fluorescence intensities in IFA were not noticeably different between the various parasite lines, indicating similar transgene expression levels (data not shown). Scale bar, 8  $\mu$ m.



**Figure 4: Proteolytic maturation of PfSUB2 is autocatalytic and required for trafficking to micronemes.** Western blot and IFA analyses of wild-type and catalytically inactive PfSUB2. A) Extracts of segmented schizonts were fractionated by SDS-PAGE, transferred to nitrocellulose and probed with anti-HA mAb 3F10. Lanes (from left to right): parental wild-type 3D7 (negative control); Ff-sub2, episomal HA3-tagged PfSUB2 transgene driven by the Ff promoter (positive control); Ff-sub2-S960A, PfSUB2 S960A catalytic mutant driven by the Ff promoter. The PfSUB2-S960A mutant is expressed, but does not undergo post-translational proteolytic maturation. B) IFA examination of the subcellular localization of the PfSUB2-S960A mutant. Correct microneme targeting was assessed as before through colocalization with the microneme marker protein PfAMA1, and generation of Pearson's coefficients to indicate correlation of signal intensities. Formaldehyde-fixed parasites were probed with the anti-HA mAb 3F10 (green) and anti-PfAMA1 rabbit polyclonal antibody (red), with the nuclei counterstained with DAPI (blue). Controls: PfSUB2HA, Ff-sub2 (wild-type episome) (Pearson's coefficient 0.723). The unprocessed PfSUB2-S960A mutant remains restricted to a perinuclear location between the nucleus and the apical end of the parasite (indicated by white arrowheads), with greatly reduced overlap between the signals (Pearson's coefficient 0.141). Scale bar, 8  $\mu$ m.

already demonstrated that catalytically inactive protease could not progress beyond the zymogen precursor, we initially assessed the different regions for their contribution to protease activity. Parasites harbouring each transgene expression construct were established by transfection and drug selection, and then mature schizont stage parasites isolated and examined by western blot. As shown in Figure 5B, all the deletion mutants were successfully expressed in the parasite, and all underwent maturation beyond the zymogen precursor to a smaller truncated mature form. Interestingly, the  $\Delta$ CD zymogen precursor could not be visualised perhaps indicating that autocatalytic maturation is more efficient in the absence of the CD. Although it remains a formal possibility that other proteases within the secretory transport system could be responsible for the proteolytic cleavage of the truncated products, these data strongly suggest that neither the cytoplasmic, transmembrane nor juxtamembrane domains of PfSUB2 are required for autocatalytic activity.

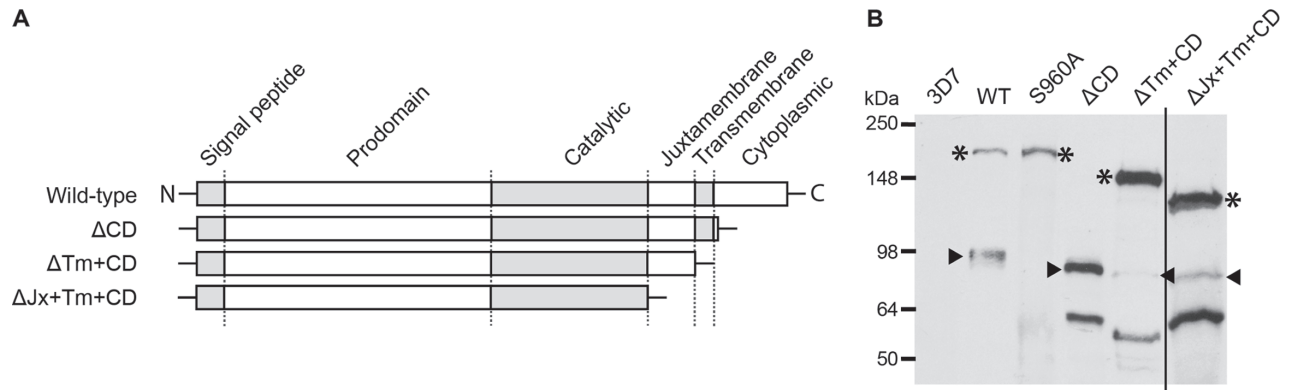
#### ***PfSUB2 catalytic activity is not sufficient for trafficking beyond an early secretory compartment***

Following the observation that none of the above truncations affected the catalytic activity of PfSUB2, we next investigated the effects of the truncations upon trafficking. Wild-type PfSUB2 is stored in micronemes in mature schizonts, and is then discharged to translocate across the surface of free merozoites following egress and around the time of reinvasion (6). In the first instance we therefore investigated whether the truncated proteins were correctly targeted to micronemes. The  $\Delta$ CD mutant was correctly targeted to micronemes (Figure 6),

indicating that the CD of the protease is not required for sorting and trafficking beyond the ER. In contrast, IFA analysis of parasites expressing the  $\Delta$ Tm + CD mutant revealed that, similar to the S960A mutant, trafficking was aberrant with the transgene product localizing to a site between the nuclear body and the apical end of the parasite (Figure 6). This finding demonstrates that catalytic activity alone (as determined by a capacity to undergo the primary step of maturation) is not sufficient to promote progression of the  $\Delta$ Tm + CD mutant through the secretory pathway. The mutant  $\Delta$ Jx + Tm + CD protein lacking the conserved juxtamembrane region similarly did not progress beyond the putative early secretory compartment (Figure 6). Given the inability of the  $\Delta$ Tm + CD mutant to progress beyond a compartment that was similar in appearance, the precise role of the juxtamembrane region in PfSUB2 trafficking could not be further assessed.

#### ***The CD of PfSUB2 is required for correct surface trafficking***

Upon merozoite egress, PfSUB2 is discharged from micronemes to traffic across the free merozoite surface and cap as an intense focus at the posterior pole of the parasite. Previous studies have shown that this surface translocation can be inhibited by the actin-destabilising agent latrunculin A (6). The only truncation mutant that correctly localized to micronemes was the  $\Delta$ CD mutant in which the CD had been removed. Therefore, we next examined the trafficking of the  $\Delta$ CD protease across the surface of merozoites following egress.



**Figure 5: The cytoplasmic, transmembrane and juxtamembrane domains of PfSUB2 are not required for autocatalytic maturation.** Schematic of progressive C-terminal gene truncation strategy and western blot of expressed truncated products. A) Schematic representation of truncated episomal expression constructs made through progressive C-terminal deletions of the *pfsb2* gene (not to scale). B) Extracts of segmented schizonts were fractionated by SDS-PAGE, transferred to nitrocellulose and probed with anti-HA mAb 3F10. Lanes (from left to right): parental wild-type 3D7 negative control; HA3-tagged full-length PfSUB2 episomal transgene (WT); Ff-sub2-S960A, PfSUB2 catalytic mutant (S960A); HA3-tagged PfSUB2 transgene lacking the cytoplasmic domain ( $\Delta$ CD); HA3-tagged PfSUB2 transgene lacking the transmembrane and cytoplasmic domains ( $\Delta$ Tm + CD); HA3-tagged PfSUB2 transgene lacking the juxtamembrane, cytoplasmic and transmembrane domains ( $\Delta$ Jx + Tm + CD). Truncated gene products are expressed as appropriately truncated precursors (asterisked) that undergo autocatalytic maturation to smaller mature protease species (arrowed).

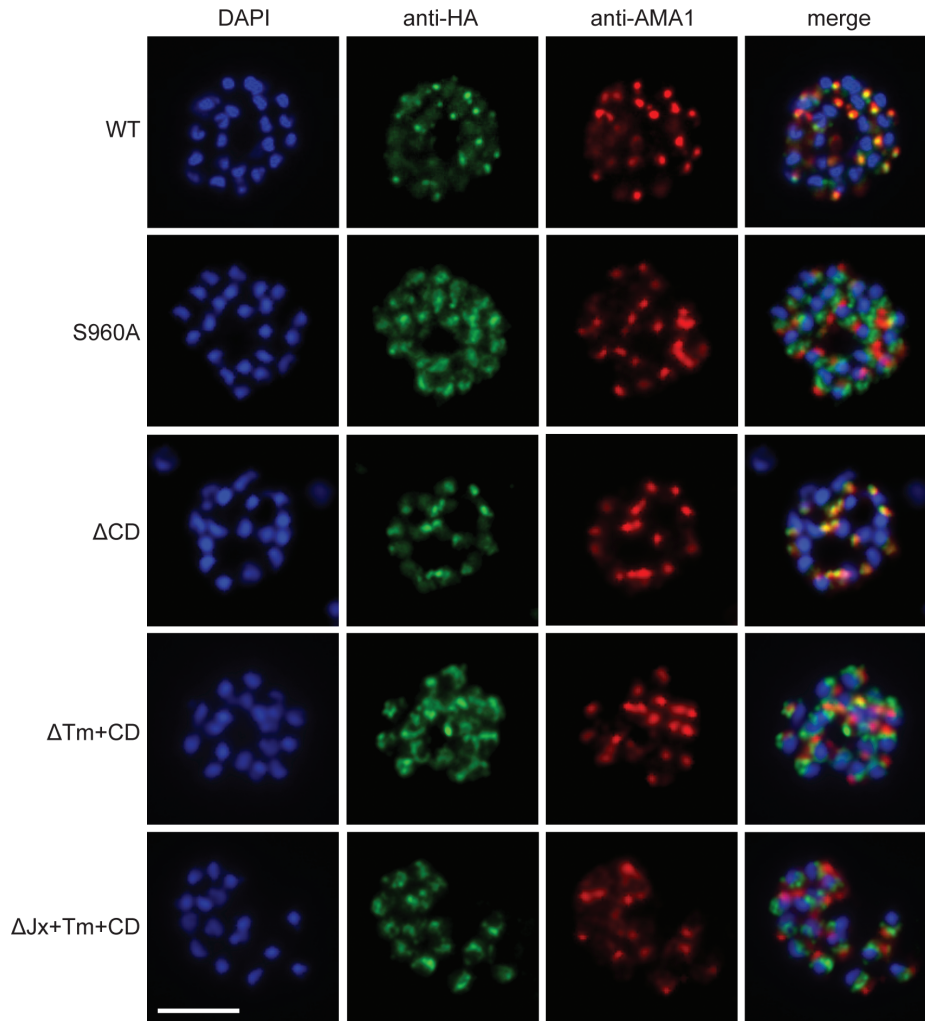
As shown in Figure 7, the IFA signal for the  $\Delta$ CD mutant presented a strong reaction with the periphery of the parasite, with the signal apparently uniformly distributed across the surface of merozoites. It completely colocalized with the PfAMA1 signal. The pattern clearly differed from that observed for the wild-type protease (Figure 7), and was reminiscent of the pattern of PfSUB2 distribution in parasites treated with latrunculin A (6). The aberrant localization of the  $\Delta$ CD mutant protease suggests that although the CD is not required for targeting of PfSUB2 to the micronemes, it is necessary for the subsequent actin-dependent surface translocation of the protease across the merozoite surface, likely essential for efficient cleavage of its membrane-bound substrates.

## Discussion

Following egress, many components of the merozoite surface coat are shed into the extracellular milieu. Some of these proteins, such as MSP1 and PfAMA1 are essential for viability of the parasite. PfSUB2 is regarded as the principal merozoite surface sheddase (6). The trafficking and compartmentalisation of substrates and other components of the shedding machinery may provide spatial and temporal control of PfSUB2 functions, so an understanding of how PfSUB2 is trafficked is necessary for understanding the regulation of its interaction with substrates. PfSUB2 is trafficked initially to micronemes, and then across the surface of the parasite in an actin-dependent manner, but the mechanisms employed for this targeting are unknown (6). The data presented here have demonstrated that this trafficking is dependent upon a variety of pre- and post-translational regulatory elements, and have begun to elucidate some of the molecular features involved.

### *The pfsb2* promoter affects trafficking of the gene product

Combining an *in silico* examination of the PfSUB2 5' UTR with a series of promoter constructs we determined that the *pfsb2* promoter plays a key role in correct trafficking of the protein to micronemes. In general, eukaryotic promoters comprise the following canonical features: a major 5' transcription start site, enhancer/repressor regions and putative transcription factor binding sites. Many of these features appear to be conserved in *Plasmodium* promoters (35,36). The exact nature of the elements present in the region 5' of the *pfsb2* locus required for trafficking remains undetermined. Although the 5' major transcription start site was absent from the F3 construct, there are typically multiple sites within the 5' region of *Plasmodium* genes that can be used for initiation of transcription (31,37). Therefore, any role played by the inclusion of this mapped site within the promoter is likely minor. As for other *Plasmodium* genes, it is possible that the inclusion of an enhancer or repressor region may also be important (38). However, given the difficulty of characterizing these elements and the highly repetitive nature of the 5' intergenic regions, definitive confirmation of this would require considerable further work. A putative ApiAP2 transcription factor binding site was identified 1654 bp upstream of the start methionine. This was particularly intriguing as the transcription profiles of putative target genes for this transcription factor match that of *pfsb2* (32), and so this site was included in the longest promoter fragment examined. The ApiAP2 family of transcription factors has received considerable attention (30,39), since the stage-specific transcription profile of this family of transcription factors mirrors the general pattern of transcription initiated upon invasion of the host erythrocyte (24,40). Mislocalization of PfSUB2



**Figure 6: The PfSUB2 transmembrane domain is required for correct trafficking to micronemes.** IFA analysis of schizonts expressing truncated episomal PfSUB2 constructs. Fixed thin-blood films were probed with the anti-HA mAb 3F10 (green), and anti-PfAMA1 rabbit polyclonal antibody (red). Nuclei were counterstained with DAPI (blue). PfSUB2 lacking the cytoplasmic domain ( $\Delta$ CD) correctly localizes to micronemes, with a high degree of correlation of the signal intensities (Pearson's coefficient 0.734). PfSUB2 mutants further truncated of the transmembrane or juxtamembrane domains ( $\Delta$ Tm + CD and  $\Delta$ Jx + Tm + CD) do not localize to the micronemes but are restricted to a compartment similar to the inactive gene product, with greatly reduced overlap between the signal intensities (Pearson's coefficient 0.254 and 0.177, respectively). Scale bar, 8  $\mu$ m.

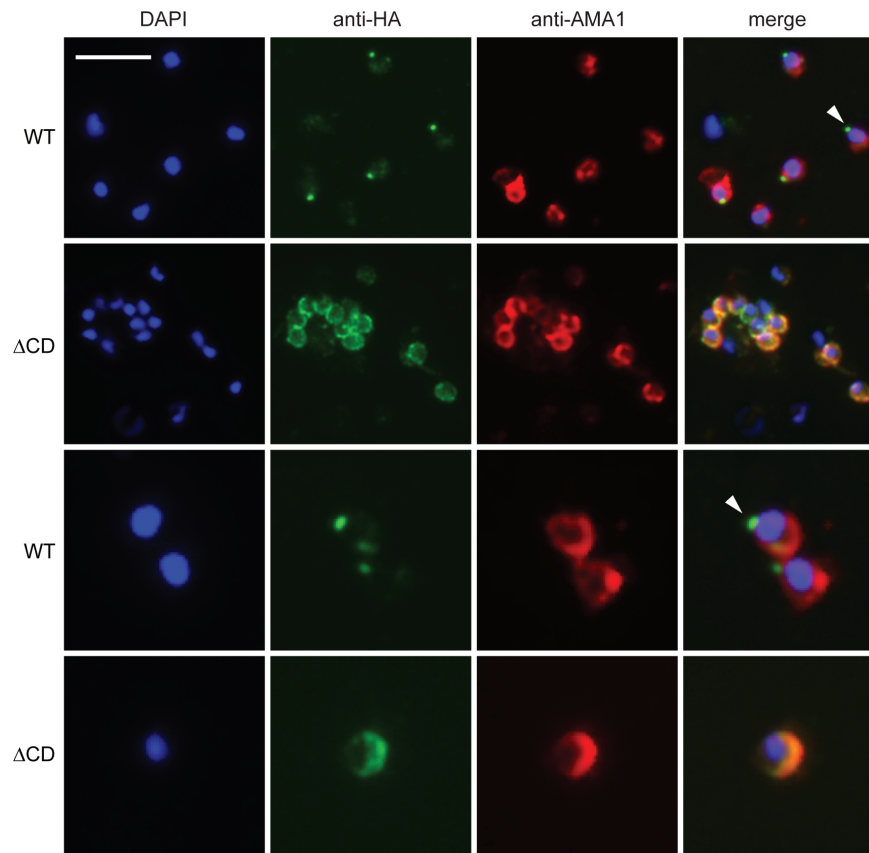
when expressed under the wrong promoter element(s) is likely due to incorrect timing and/or levels of expression. Our data add to a growing body of work examining the promoters of other *Plasmodium* genes such as *pfresa*, *pfama1*, *pfmsp2* and *pfeba175* (28,41–43), and support the conclusion that in order to recapitulate correct trafficking of a transgene product, the complete native promoter for the gene should be used.

#### **Proteolytic maturation of PfSUB2 is required for trafficking**

Our results show that autocatalytic processing of PfSUB2 is linked to its progression through the secretory pathway (13). Earlier work demonstrated that treatment of

the parasites with brefeldin A, which interferes with anterograde protein transport from the ER to the Golgi, did not block the primary processing of PfSUB2 (11). This suggested that primary processing occurs in the ER. Our observation here that the catalytically dead PfSUB2 mutant, PfSUB2-S960A, did not progress beyond an ER-like compartment suggests that processing may serve as a quality control for progression through the secretory pathway. Retention of PfSUB2-S960A in the ER was not conclusively demonstrated, but the weight of evidence would suggest that the unprocessed protease is retained within this compartment. Alternatively, it is possible that the retained PfSUB2 is located within the *Plasmodium* equivalent of the recently described *Toxoplasma gondii* LE, thought to be an intermediate





**Figure 7: The cytoplasmic domain of PfSUB2 is required for correct surface translocation and posterior capping.** IFA analysis of free merozoites expressing WT PfSUB2HA or the  $\Delta$ CD mutant. Formaldehyde-fixed parasites were probed with the anti-HA mAb 3F10 (green) and anti-PfAMA1 rabbit polyclonal antibody (red). Nuclei were counterstained with DAPI (blue). Wild-type PfSUB2 is secreted from the micronemes and trafficked across the surface of free merozoites in an actin-dependent fashion before capping as a posterior focus (white arrowheads) PfAMA1 is secreted from the micronemes and distributes to a uniform circumferential pattern. There is almost no overlap of the WT PfSUB2 and PfAMA1 signals (Pearson's coefficient 0.008). In contrast, the surface distribution of  $\Delta$ CD PfSUB2 matches that of PfAMA1, with a high degree of correlation between the signals (Pearson's coefficient 0.74). Scale bar, 8  $\mu$ m (upper 2 rows of images). The lower set of eight panels have been enlarged to better illustrate the differences in distribution of the markers used (Pearson's coefficients  $-0.048$  for WT PfSUB2 and  $0.824$  for  $\Delta$ CD PfSUB2).

compartment situated between the ER/Golgi and the apical organelles in the secretory transport system of this related apicomplexan (26).

#### **Truncation of PfSUB2 affects its localization**

Similar to previous data from studies of PfAMA1, PfEBA175, PfEBA181 and PfEBA140 (41), we found that PfSUB2 lacking its CD was correctly trafficked to micronemes. Surprisingly, despite multiple attempts, the PfSUB2  $\Delta$ CD precursor was never detectable by western blot in schizont extracts. One possible interpretation of this finding is that removal of the CD increases the rate of autocatalytic maturation of the zymogen precursor. The CD could play a regulatory role in this event, ensuring that activation and further trafficking only occur following further translocation through the ER.

The  $\Delta$ CD mutant was correctly trafficked to micronemes, and it is intriguing to consider how this would occur. It is

possible that PfSUB2 could interact with a chaperone-type protein in the ER that would regulate further downstream trafficking. This would be analogous to the situation seen in *T. gondii* for MIC2 and M2AP, where membrane-bound MIC2 interacts and cotraffics with the soluble M2AP (44,45). Upon merozoite egress, the  $\Delta$ CD protein was discharged from micronemes, showing that release of PfSUB2 from the micronemes is not dependent upon the CD. However, its subsequent distribution on the merozoite surface was atypical, matching that of PfAMA1, translocation of which is not actin-dependent (46). The PfSUB2 CD may be responsible for mediating interactions with components of the actinomyosin motor. To further explore this possibility, we made a more detailed examination of the CDs of all the *Plasmodium* SUB2 orthologues (Figure S1). Alignments of the CDs highlight the lack of conservation. A recent study implicated phosphorylation of the CD of another microneme protein, PfAMA1, as playing a key

role in the function of the molecule (42). Examination of a recently published *P. falciparum* phosphoproteome identified a phosphorylation site within the cytoplasmic tail of PfSUB2 (47). Further studies are underway to determine whether phosphorylation of the PfSUB2 cytoplasmic tail represents a post-translational modification required for PfSUB2 trafficking and activity.

PfSUB2 catalytic activity was shown to be independent of its membrane-associated conformation, since the  $\Delta$ Tm + CD truncation did not apparently affect autocatalytic maturation. The fact that the  $\Delta$ Tm + CD mutant was active in a fully soluble form makes it ideally suitable for renewed attempts at recombinant expression of active enzyme, which has not yet been achieved. Although the  $\Delta$ Tm + CD mutant was active, it was retained in an ER-like compartment. Coupled with the data from the PfSUB2-S960A mutant, this shows that catalytic activity and autocatalytic processing are not sufficient for trafficking beyond this compartment.

The juxtamembrane region of PfSUB2 is the most highly conserved portion of the molecule. The  $\Delta$ Jx + Tm + CD mutant exhibited autocatalytic processing but was retained in the ER-like compartment, in accord with our previous observation that the transmembrane domain of the protease is required for its progression through the secretory pathway. It is difficult to draw any further conclusions as to the functions of the juxtamembrane region of PfSUB2, and in future experiments it may be necessary to selectively delete it in order to fully examine the role that it plays in trafficking of the molecule. From the results of the  $\Delta$ CD truncation, it would appear that the cytoplasmic tail is necessary only for the surface trafficking of the protease, and therefore the juxtamembrane region may have a role in microneme trafficking, similar to the cysteine-rich region VI of the EBA family (41,48).

In conclusion, we have demonstrated that a variety of pre- and post-translational regulatory features govern the trafficking of PfSUB2. Future work will focus upon the role of the highly conserved juxtamembrane region and the CD, which may provide evidence of trafficking partners and potential roles in signalling.

## Materials and Methods

### Parasite maintenance, synchronization and transfection

Asexual blood-stages of *P. falciparum* clone 3D7 were maintained in RPMI-1640 Albumax medium (GIBCO™), with the addition of 2 mM L-glutamine, at 2–4% haematocrit (49). Mature schizont stage parasites were isolated on a 70% (v/v) Percoll (GE Healthcare) density gradient as described (50). Further synchronization using 5% D-sorbitol was performed as previously described (51). For transfections, ring stage parasites at 5–10% parasitaemia were electroporated as previously described (52).

### Transfection constructs

Parasite vectors were all based on the pHH1 transfection plasmid (53). This vector contains the *P. berghei* dihydrofolate reductase 3' UTR (54), and the

human dihydrofolate reductase (*dhfr*) selection cassette, which confers resistance to the antifolate drug WR99210 (Jacobus Pharmaceuticals).

### *pfsub2* promoter constructs for episomal expression

Promoter fragments were amplified directly from 3D7 genomic DNA with primers designed to incorporate 5' Hpa I and 3' Spe I restriction enzyme sites. The PCR products were digested with Hpa I, Spe I and cloned into identical sites in the pHH1 vector containing the *Pfama1* promoter (54) and the *sub2* synthetic gene (*pfsub2<sub>synth</sub>*) (6), to create constructs psub2-sub2wHA3-PEX, psub2-sub2wHA3-F1, psub2-sub2wHA3-F2 and psub2-sub2wHA3-F3.

### *pfsub2* catalytic mutant

The codon in *pfsub2<sub>synth</sub>* encoding putative catalytic nucleophile Ser960 was mutated to an Ala codon (S960A) by site-directed mutagenesis (SDM) using the QuikChange® Site-Directed Mutagenesis Kit (Stratagene), and verified by nucleotide sequencing.

### *pfsub2* truncation constructs

The Xho I/Not I restriction enzyme fragment of *pfsub2<sub>synth</sub>* was excised from psub2x-sub2wHA3-PEX and subcloned into identical sites in pGEX-6P-1-HA3 to create pGEX-sub2HA3-Xho I/Not I. SDM was used to introduce an additional Cla I restriction enzyme site at the 5' limit of the fragment that was to be truncated, such that the region to be truncated would be flanked by Cla I sites. Subsequent digestion with Cla I and religation generated the different truncation fragments. The Xho I/Not I fragment was then excised from this vector and subcloned back into psub2x-sub2wHA3-PEX to create all the truncation constructs described.

### Western blots

Western blots were performed as previously described (20). Briefly, 40 mL cultures of Percoll-enriched schizonts were centrifuged and pelleted parasites treated with 0.15% (w/v) saponin for 3 min at 4°C. The released parasites were pelleted by centrifugation, washed and resuspended in 100  $\mu$ L 2 $\times$  sodium dodecyl sulphate (SDS) sample buffer. Protein samples were electrophoresed on 7.5–12.5% SDS polyacrylamide gels (SDS-PAGE) and transferred to Hybond-C extra nitrocellulose membrane (GE Healthcare). Membranes were blocked and probed with the antibody described.

### Immunofluorescence assay

IFA was performed as described (20). Briefly, thin films of *P. falciparum* schizonts or naturally egressed merozoites were air-dried and fixed in 4% paraformaldehyde for 30 min, then permeabilized using 0.1% (v/v) Triton-X 100 (Sigma Pharmaceuticals) for 10 min, washed twice with PBS for 5 min and then blocked overnight at 4°C in 3% (w/v) bovine serum albumin (BSA) in PBS. Samples were then probed with the antibodies specified. Fluorescence images were acquired using a Zeiss Axioplan 2 Imaging system (Carl Zeiss) and AxioVISION 3.1 software.

### Image analysis for colocalization

Images were processed for the degree of colocalization of signals obtained with different antibody probes using IMAGEJ and the JACoP plugin as described by Bolte and Cordelières (55). Costes' automatic threshold function within the plugin was used to generate the Pearson's correlation coefficient values for the degree of colocalization. Briefly, this outputs a numerical figure between –1 and 1, with 1 representing perfect correlation, –1 being negative correlation (mutual exclusion), and 0 being no correlation of signal intensities.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

### Figure S1: Alignment of *Plasmodium* spp. SUB2 orthologues showing a heat map of amino-acid conservation (associated with Figure 5).

The comparison was performed using the PRALINE alignment software (<http://www.ibi.vu.nl/programs/praline/www/>). *falciparum*, *P. falciparum* clone 3D7; *vivax*, *P. vivax* Salvador I isolate; *knowlesi*, *P. knowlesi* H strain; *yoelii*, *P. yoelii yoelii*; *berghei*, *P. berghei* ANKA; *P. cynomolgi*. Features of note, including the highly conserved juxtamembrane region and the relatively poorly conserved cytoplasmic domain, are annotated below the alignment.

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