

Cell Host & Microbe

Supplemental Information

**Processing of *Plasmodium falciparum* Merozoite
Surface Protein MSP1 Activates a Spectrin-Binding
Function Enabling Parasite Egress from RBCs**

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Supplemental Figures and legends

Figure S1

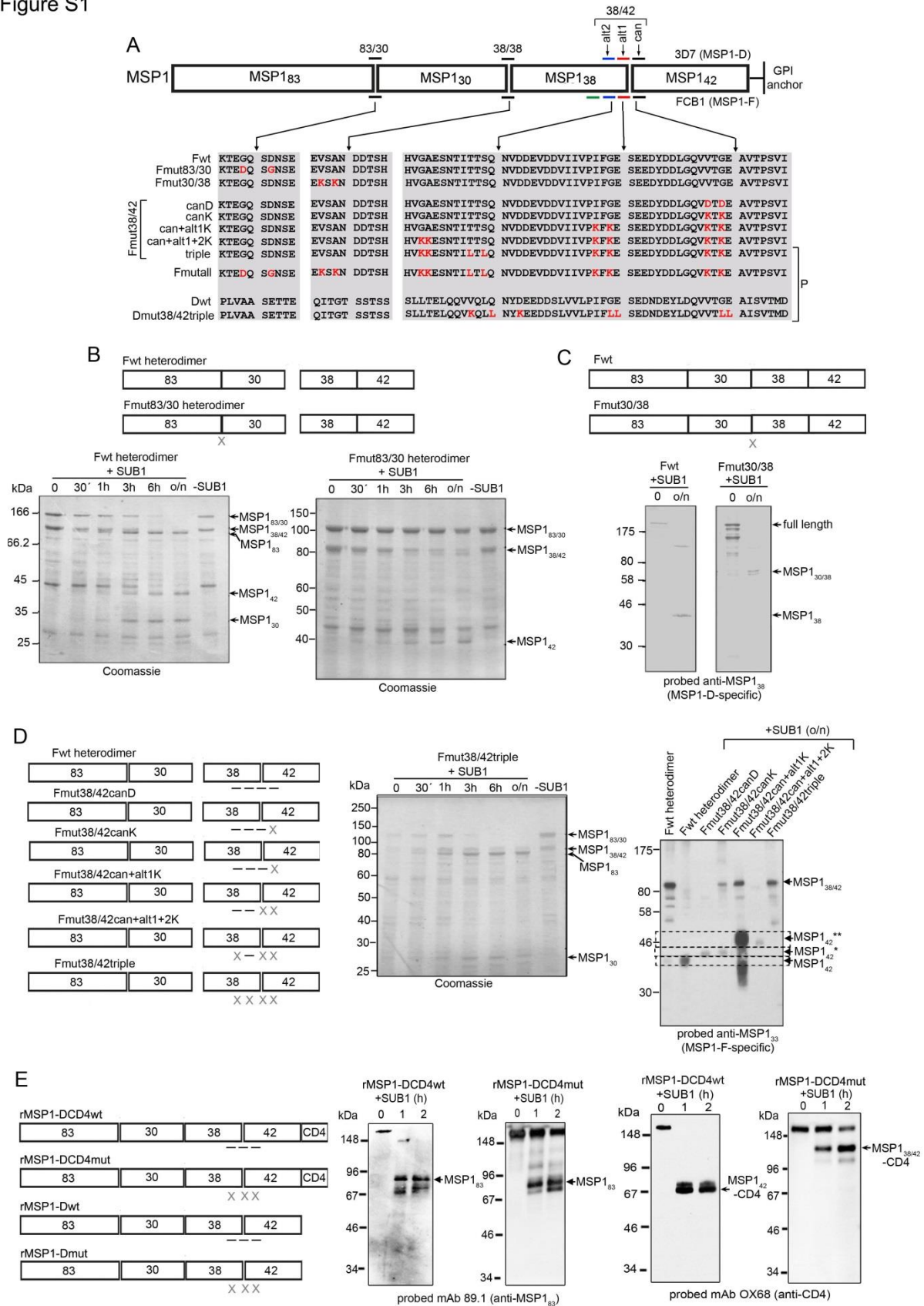


Figure S1, related to Figure 1. Mutagenesis of Processing Sites blocks rPfSUB1-mediated Cleavage of Recombinant MSP1

(A) Schematic of MSP1 processing products and primary processing sites, above a ClustalW2 alignment of flanking sequences in the recombinant wild-type proteins and mutants used in this study. Experimentally confirmed cleavage sites are arrowed and indicated by gaps. Substitutions to block cleavage (red) simultaneously replaced 2 residues at the P4, P2, P1, P2' or P3' positions important for recognition by PfSUB1. Mutations additionally studied for impact on parasite growth by transgene expression in the parasite are indicated on the right (P).

(B) Mutagenesis of the 83/30 site blocks PfSUB1-mediated cleavage. Top, schematic of the recombinant Fwt heterodimer and Fmut83/30 heterodimer mutant. Positions of mutations (see panel A) are indicated (grey cross). Below, typical time-courses of digestion of both proteins. For each, 50 µg of protein was incubated with rPfSUB1. Samples taken at intervals were analysed by SDS-PAGE and Coomassie staining alongside samples incubated overnight (o/n) in the absence of rPfSUB1. Positions of parental and product proteins are indicated. Note the lack of digestion of the MSP1_{83/30} fragment in the case of the Fmut83/30 heterodimer mutant.

(C) Mutagenesis of the 30/38 site blocks cleavage. Top, schematic of the Fwt recombinant and Fmut30/38 mutant. Positions of mutations (see panel A) are indicated (grey cross). Below, Western blot analysis of undigested (0) or digested (o/n) proteins. Positions of parental and product proteins are indicated. Note the appearance of a stable MSP1_{30/38} intermediate and absence of a MSP1₃₈ product in the case of Fmut83/30.

(D) Mutagenesis of at least three sites is required to block cleavage in the 38/42 region of MSP1-F. Left-hand side, schematics of the Fwt heterodimer and 38/42 region mutants. Positions of known or predicted 38/42 cleavage sites are shown by short horizontal lines, whilst positions of mutations (see panel A) are indicated (crosses). Middle, SDS-PAGE analysis of a time-course of digestion of the Fmut38/42triple mutant, performed as described in (C). Whilst cleavage took place normally at the

83/30 site, the MSP1_{38/42} fragment was completely stable, indicating complete blockade of cleavage in the 38/42 region by the introduced mutations. Right hand side, Western blot analysis of cleavage of the various proteins. Note the increase in the apparent mass of the MSP1₄₂ fragments produced when mutations were successively introduced along the 38/42 region. The MSP1₄₂, MSP1₄₂^{*}, and MSP1₄₂^{**} processing products are predicted to result from cleavage at the canonical, alt1, and alt2 38/42 sites of the Fwt heterodimer. Positions of migration of full-length Fwt heterodimer fragments are indicated (with arrows), as are the various processing products.

(E) Mutagenesis of the canonical, alt1 and alt2 38/42 sites in MSP1-D completely blocks cleavage in the 38/42 region. Left-hand side, schematics of rMSP1-DCD4wt and its mutant derivative rMSP1-DCD4mut, which contains mutations at the canonical, alt1, and alt2 38/42 sites (grey crosses; equivalent to mutant Dmut38/42triple, panel A). Proteins rMSP1-Dwt and rMSP1-Dmut were identical except that they lacked the C-terminal CD4 fusion partner. Right hand side, analysis of cleavage. The indicated proteins were incubated with rPfsUB1 for 0-2 h then analysed by Western blot, probing either with monoclonal antibody (mAb) 89.1 which recognises the MSP1₈₃ product, or mAb OX68 which recognises the CD4 fusion partner. Cleavage of both proteins (at the 83/30 site) to produce MSP1₈₃ occurred as expected. However, whereas cleavage to produce the C-terminal MSP1₄₂-CD4 product occurred as expected in rMSP1-DCD4wt, this was completely ablated in rMSP1-DCD4mut due to the mutant 38/42 site being completely refractory to cleavage; a much larger C-terminal fragment (MSP1_{38/42}-CD4) was instead produced, likely resulting from cleavage at the 30/38 junction.

Figure S2

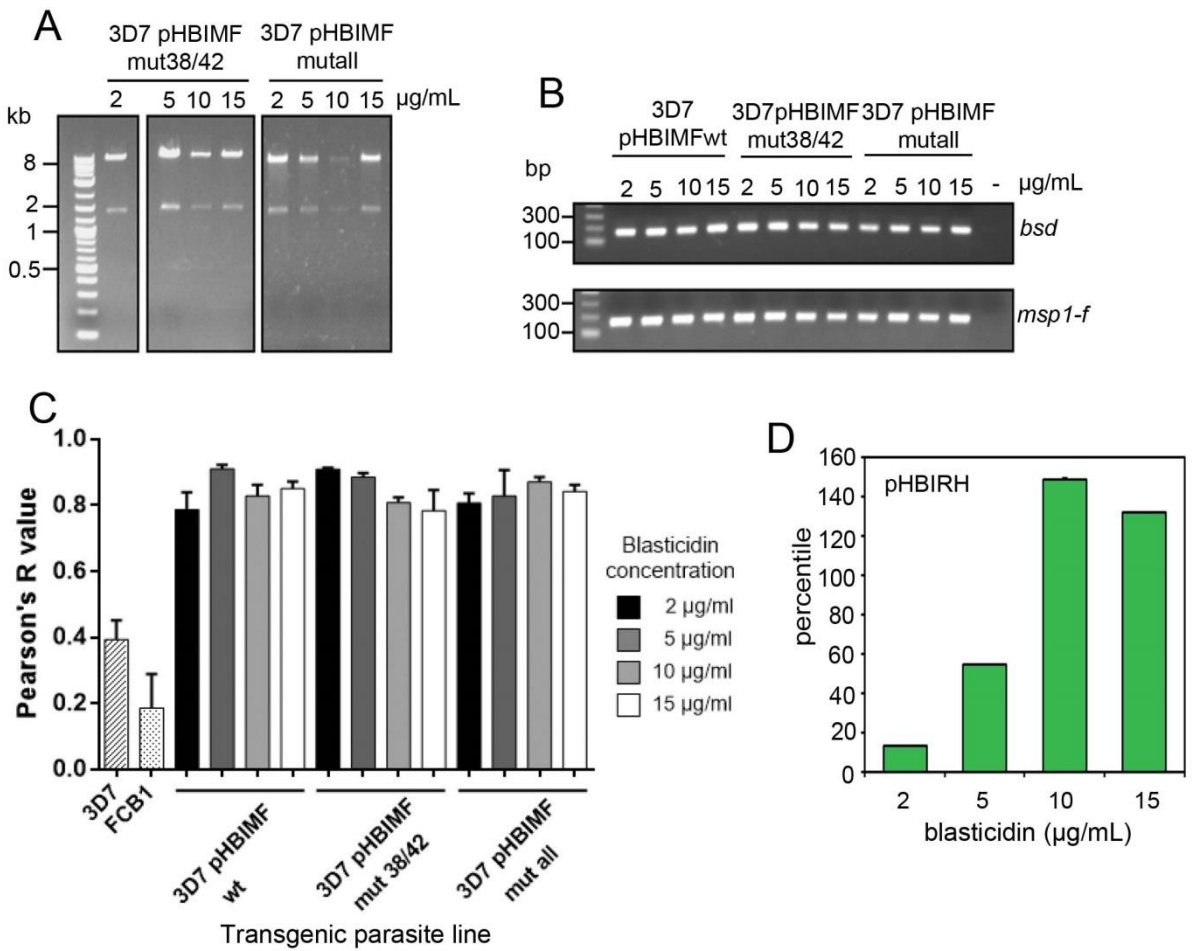


Figure S2, related to Figure 2. Stable Carriage of Episomal Constructs for Transgenic Expression of MSP1 in *P. falciparum*

(A) Plasmid rescue from *P. falciparum* 3D7 parasites stably transformed with pHBIMFmut38/42 or pHBIMFmutall detects no signs of plasmid rearrangement. Genomic DNA was prepared from parasite lines maintained in the presence of the indicated blasticidin concentrations, then episomal plasmids rescued by transformation of *E. coli*. Digestion of DNA from randomly-selected individual colonies with *Bgl II* and *Xho I* reproducibly yielded the expected diagnostic fragments of 8,494 bp and 1,874 bp.

(B) PCR analysis of genomic DNA isolated from the indicated parasite lines grown under varying blasticidin concentrations detected no signs of plasmid rearrangement following carriage in *P. falciparum*. The primer pairs used (bsd3up plus bsd3down, or f83up plus f83down) each specifically

amplify a ~180 bp fragment from the *bsd* and *msp1-f* genes (see *Supplementary Experimental Procedures* for a list of all primers used in this study). The extreme right-hand lane of each gel (labelled “-“) is a no DNA template control.

(C) Quantitative analysis shows correct co-localization of endogenous MSP-1D and transgenic MSP-1F. A minimum of ten schizont IFA images per parasite line (similar to those shown in Figure 2B) were analysed using the Coloc2 Plugin of ImageJ. Pearson’s R values are shown plotted as mean values. Error bars, SEM. Pearson’s R values close to 1.0 indicate co-localization of the MSP-1D and MSP-1F signals. The parental 3D7 and FCB1 parasites show very low Pearson’s R values indicating no co-localization, while all the transgenic parasite lines have a Pearson’s R value of ~0.8 indicating good co-localization of the endogenous MSP-1D and transgene-derived MSP-1F at the parasite plasma membrane, irrespective of the blasticidin concentration used for selection.

(D) Increasing blasticidin concentrations lead to increased expression of the *Renilla* luciferase gene from 3D7 parasites transfected with the control episome pHBIRH. Shown are transgene RNA transcript levels measured by qRT-PCR, as a percentile of endogenous *msp1-d* transcript levels (100%). SEM values in all cases were <0.1%.

Figure S3

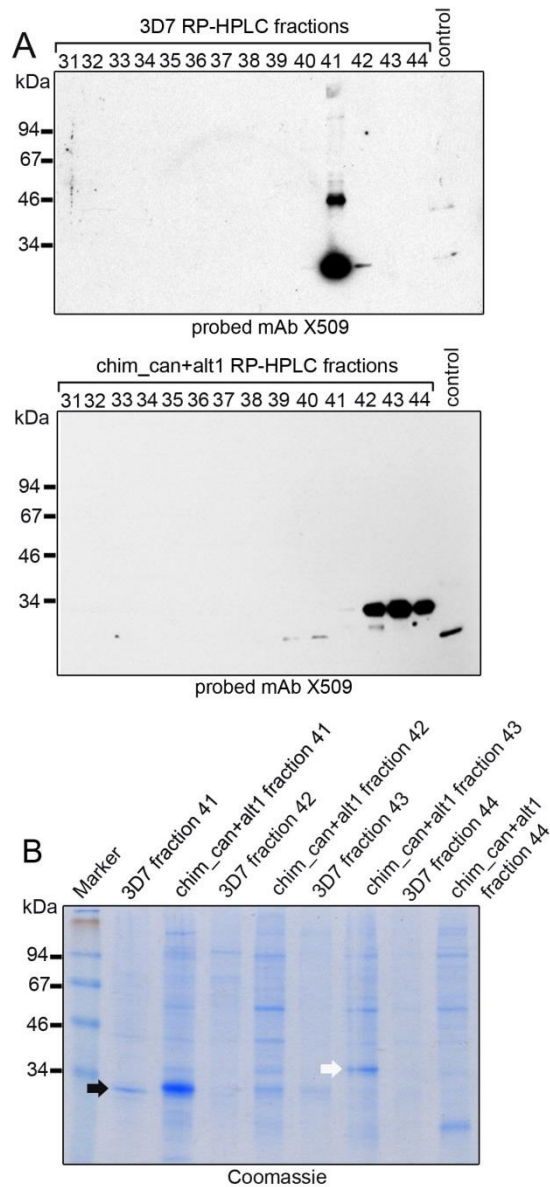


Figure S3, related to Figure 3. N-terminal Sequencing of the MSP1₃₃ Shedding Product from chim_can+alt1 clone B2 Parasites Confirms the 38/42alt2 Processing Site**

(A) Mature schizonts of parental 3D7 *P. falciparum* parasites or transgenic chim_can+alt1 clone B2 were cultured overnight with fresh erythrocytes in protein-free medium to allow merozoite egress and release of shed MSP1 fragments. Supernatants from the two cultures were harvested, concentrated, clarified, and separately fractionated on a C4 RP-HPLC column (Vydac) as described in *Supplemental Experimental Procedures*. Eluate fractions (1 ml) were dried, solubilised in 100 µl of

SDS sample buffer, and samples analysed by Western blot, probing with the MSP₁₃₃-specific mAb X509. The majority of the MSP₁₃₃ from parental 3D7 parasites was recovered in eluate fraction number 41 (upper blot). In contrast, the slightly slower-migrating modified MSP₁₃₃** fragment purified from the chim_can+alt1 clone B2 was recovered in fractions 42 to 44 (lower blot). The lane marked 'control' in both blots is a sample of parental 3D7 culture supernatant, showing the position of migration of wild-type MSP₁₃₃. Small amounts of MSP₁₄₂ are also evident in the 3D7-derived material.

(B) SDS-PAGE and Coomassie blue staining of RP-HPLC fractions 41-44 from both fractionation experiments. Identity of the MSP₁₃₃ fragment from wild type 3D7 culture supernatants (black arrow) was confirmed by Edman degradation, which identified its N-terminal sequence as AISVT. Edman degradation of the corresponding MSP₁₃₃** shed fragment purified from supernatants of chim_can+alt1 clone B2 (white arrow) identified its N-terminus as NYDEE, confirming that it resulted from cleavage at the 38/42alt2 site.

Figure S4

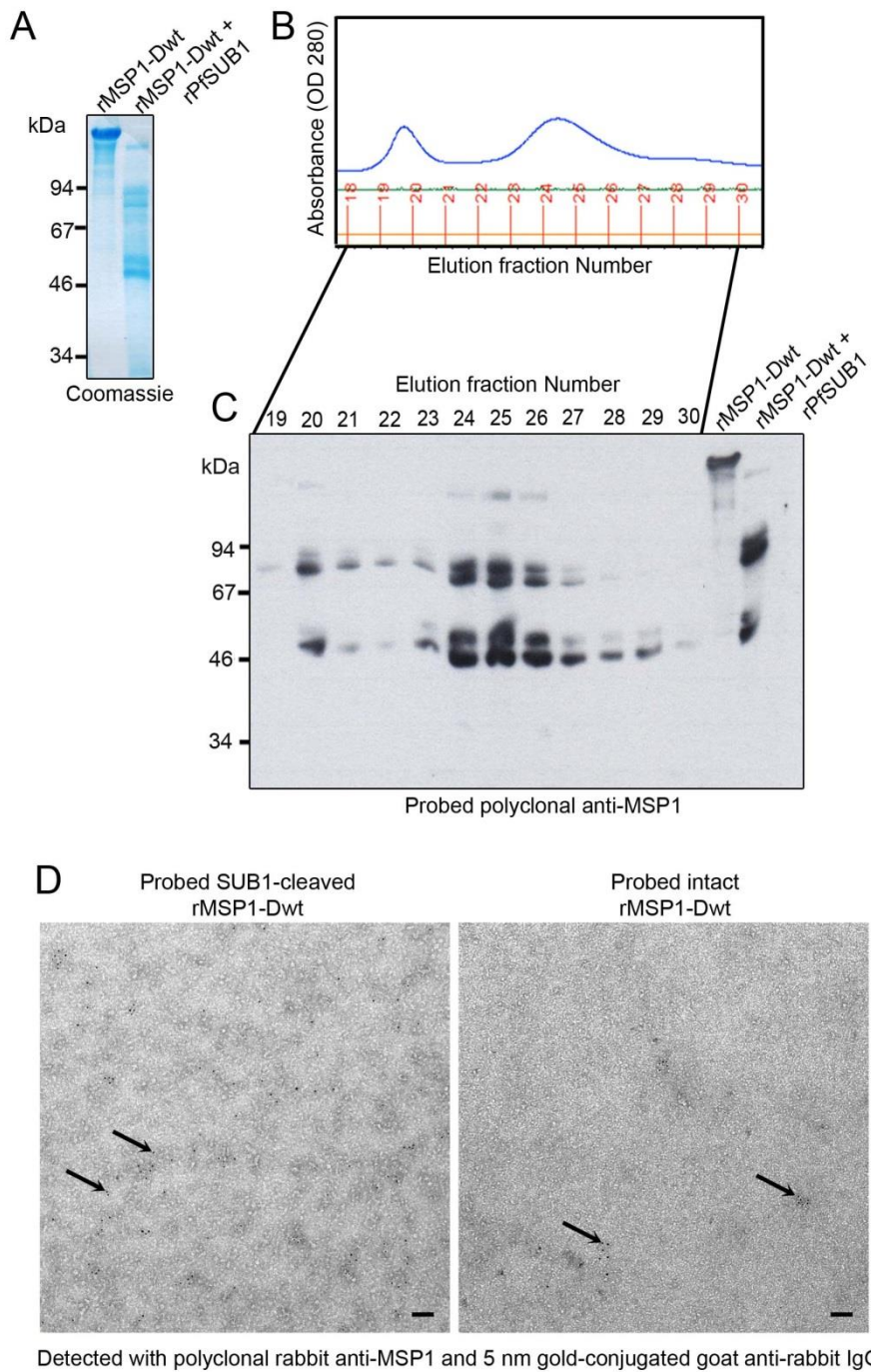


Figure S4, related to Figure 4. Proteolytic Processing *in vitro* of Recombinant MSP1-D with rPfSUB1 Produces a Non-covalently-associated Protein Complex that binds to the Erythrocyte Cytoskeleton

(A) SDS-PAGE and Coomassie blue-stained purified rMSP1-Dwt before and after *in vitro* digestion with rPfSUB1. The four dominant cleavage products are evident.

(B) Size-exclusion chromatography elution profile of rPfSUB1-digested rMSP1-Dwt (blue trace) fractionated on a Superdex 200 HR 10/30 column (GE Healthcare) equilibrated in 25 mM HEPES pH 7.4, 150 mM NaCl.

(C) Western blot analysis of the indicated elution fractions in parallel with intact protein and unfractionated total digestion products (two right-hand lanes), probing with a rabbit polyclonal anti-MSP1 antibody that detects all the processing products. Most of the cleavage products co-elute between fractions 24 to 26, indicating that they migrate predominantly as a single protein complex. The earlier eluting peak at fractions 19-20 likely represents protein aggregates. Similar results were obtained with rPfSUB1-digested rMSP1-DCD4wt (not shown).

(D) Immunolabelling of TX-100-extracted RBC cytoskeletons incubated with rPfSUB1-cleaved or intact rMSP1-Dwt. Shown are typical fields of view, with 5 nm gold beads visible as small black dots (some examples are arrowed). Scale bars, 100 nm.

Figure S5

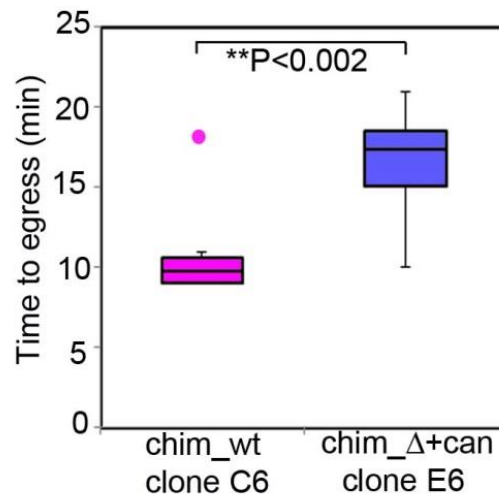


Figure S5, related to Figure 5. Egress of chim_Δ+can Parasites is Delayed relative to chim_wt Parasites

Graphical summary of egress data from Movie S2, in which egress of a 1:1 mixture of Hoechst-labelled chim_Δ+can clone E6 parasites and unlabelled chim_wt clone C6 parasites was observed by time-lapse DIC microscopy. Time to individual egress events was recorded by visual examination of movie frames. The box plot shows time to egress from the start of imaging. Horizontal lines, median. Whiskers indicate the range, with a single outlier point ($>1.5\times$ the interquartile range) indicated. The mean delay in time to egress for the chim_Δ+can schizonts in this experiment relative to the chim_wt clone was 5.7 min ($P<0.002$, Student's t-test), in agreement with the results presented in Figure 5B. A similar delay was observed in chim_Δ+can parasites in reciprocal experiments in which the chim_wt schizonts were labelled with Hoechst 33342 (not shown), showing that the delay in egress was not caused by the labelling. Separate analysis of DAPI-labelled segmented chim_Δ+can clone E6 and chim_wt clone C6 schizonts showed no significant differences in number of nuclei formed per schizont (mean values of 20.5 ± 4.0 and 21.0 ± 5.0 nuclei per schizont, respectively, $n=16$).

Figure S6

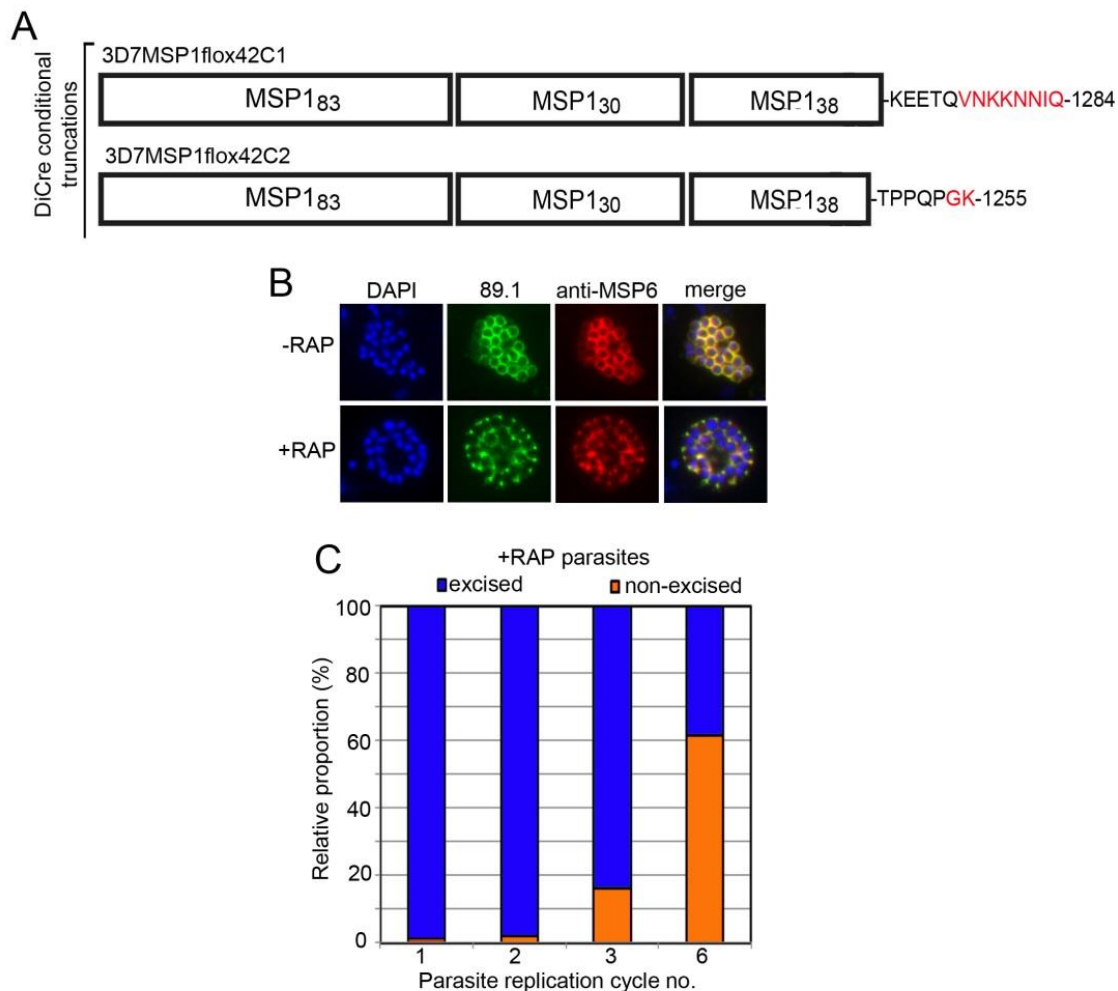


Figure S6, related to Figure 6. Truncation of MSP1 results in Loss of Merozoite-bound MSP1 complex and a Severe Fitness Defect

(A) Schematic of truncation mutants produced by DiCre-mediated conditional removal of the C-terminal segment of MSP1 and its GPI anchor. Full details of the method used to produce the two parasite lines, called 3D7MSP1floxed42C1 and 3D7MSP1floxed42C2 are provided in *Supplementary Experimental Procedures*. Two different strategies for truncation of the *mSP1-d* gene produced slightly different C-termini (red). The predicted amino acid length of each gene product is indicated.

(B) IFA showing co-localisation of MSP1 (recognised by mAb 89.1) and the partner protein MSP6 (recognised by polyclonal anti-MSP6 antibodies) in 3D7MSP1flox42C1 clone E3 parasites. The pattern seen in the control (-RAP) parasites is typical of a normal merozoite surface localisation, whereas that observed in the RAP-treated parasites is typical of a PV 'waggonwheel' localisation.

(C) Results of a single typical experiment monitoring relative proportions of parasites expressing truncated and full-length MSP1 in a population of RAP-treated 3D7MSP1flox42C2 clone B51 parasites over the course of the ensuing 6 erythrocytic cycles (12 days). Phenotype was determined by IFA, probing thin films of the culture with both mAb 89.1 and mAb X509. The numbers of parasites expressing full-length MSP1 (reactive with both mAb 89.1 and mAb X509; non-excised) or truncated MSP1 (reactive with mAb 89.1 but not with mAb X509; excised – see Figure 6) were determined by microscopic analysis of at least 100 schizonts in each sample. Relative proportions are represented as bar graphs. The non-excised population initially formed ~1% of the population, but rapidly out-competed the excised population by the end of cycle 6 (reaching 61%), indicating that truncation of MSP1 results in a severe loss of fitness. The replication rate per cycle of excised parasites was calculated to be 2.7 fold less than that of non-excised parasites.

Supplemental Movie legends

Movie S1, related to Figure 5. chim_Δ+can Parasites Display an Egress Delay compared to chim_wt parasites

Synchronous schizonts of chim_wt clone C10 (left) and chim_Δ+can clone D2 (right) were Percoll-enriched then returned to culture and allowed to mature for 4-5 h in the presence of the PKG inhibitor compound 1 (C1, 2 μM). The parasites were washed in fresh warm medium without C1 and observed by time-lapse DIC microscopy, taking images at 5 sec intervals. Imaging commenced precisely 4 min 20 sec following C1 removal. Time after start of microscopy is indicated (top left). The mean delay in time to egress for chim_Δ+can clone D2 schizonts relative to chim_wt clone C10 in this experiment was 5.2 min ($P < 0.001$, Student's t-test) (biological replicate no. 3 in Figure 5). Similar results were obtained for separate distinct clones (derived from a separate transfection) of each transgenic parasite line (chim_wt clone C6 and chim_Δ+can clone E6; not shown).

Movie S2, related to Figure 5. chim_Δ+can Parasites Display an Egress Delay compared to chim_wt parasites

Synchronous schizonts of chim_wt clone C6 and chim_Δ+can clone E6 were Percoll-enriched then returned to culture and allowed to mature for 4-5 h in the presence of C1. Just before use, the chim_Δ+can clone E6 parasites were treated for 5 min with Hoechst 33342 (1 μg mL⁻¹). The labelled parasites were then washed in warm medium containing C1 to remove excess dye prior to mixing at a 1:1 ratio with the chim_wt parasites. The schizont mixture was washed in fresh warm medium without C1 and observed by time-lapse DIC microscopy (left), taking images at 5 sec intervals. Imaging commenced exactly 5 min following C1 removal. A single fluorescence image together with a DIC image (right) was recorded just prior to starting the time-lapse imaging, enabling the chim_wt (red circles) and chim_Δ+can (blue circles) schizonts to be identified by overlaying the images. Time after start of microscopy is indicated (top left). The mean delay in time to egress for the chim_Δ+can clone E6 schizonts in this video relative to chim_wt clone C10 was 5.7 min ($P < 0.002$) (Figure S5). A

similar delay was observed in chim_Δ+can parasites in reciprocal experiments in which the chim_wt schizonts were instead labelled with Hoechst 33342 (not shown), showing that the delay in egress was not caused by the labelling.

Movie S3 and S4, related to Figure 6. Dysregulation of Egress in Parasites Expressing Truncated, non merozoite-bound MSP1

Synchronised, newly-invaded forms of 3D7MSP1flox42C1 clone E3 were treated with RAP (100 nM) or vehicle only (DMSO, 1% v/v) for 4 h, then washed and cultured for a further ~44 h to allow schizont development. The schizonts were Percoll-enriched, returned to culture and allowed to mature for 4-5 h in the presence of C1, then washed in warm medium without C1 and immediately observed by time-lapse DIC microscopy, taking images at 5 sec intervals. Imaging commenced precisely 4 min 20 sec following C1 removal. Control-treated parasites (left) underwent normal 'explosive' egress with rapid dissemination of daughter merozoites. In contrast, RAP-treated parasites (right) appeared to undergo normal PVM rupture (e.g. arrowed schizont in Movie S3) but abortive RBC membrane rupture, with inefficient release of merozoites. Identical results were observed for 3DMSP1flox42C2 clone B51 (not shown).

Movie S5, related to Figure 7. *P. falciparum* Egress May Involve Shear Forces Induced by Movement of Intracellular Merozoites

Time-lapse DIC video microscopy of wild-type 3D7 *P. falciparum* showing a single egress event selected because it clearly shows that egress is a two-step process. Rupture of the PVM at ~14.1 min is followed by transient intracellular movement of the merozoites. This allows repeated impingement of the free merozoites upon the inner face of the host erythrocyte membrane before final membrane rupture and egress. Images were taken at 5 sec intervals and the movie frame rate is 10 frames/second.

Supplemental Tables

Table S1 DNA oligonucleotide primers used in this study

Primer Name	Sequence (5'-3')
endo3D7-MSP1-BglII-targ-F	CCAACAAAGATCTGCATCCTCTACCAATACCC
endo3D7-MSP1-R	GGGTTTCTTCTTTTGTGGATCCTGAACTACCAC
syn3D7-MSP1-F	GTGGTAGTTCAGGATCCACAAAAGAAGAAACCC
syn3D7-MSP1+PstI-R	GCATGCCTGCAGCTTGCCCTCTATGAGCTTTGATATGATGG
SDM-PIFLLSEDND-FOR	GCCAACTTCTCCTATCAGAAGACAACGACG
SDM-PIFLLSEDND- REV	CGTCGTTGTCTTCTGATAGGAGGAAGATTGGC
SDM-VVTLLAISVT-FOR	GTGACACTAATCGCTAGCAGGGTGACCACTTGG
SDM-VVTLLAISV-REV	CCAAGTGGTACCCTGCTAGCGATTAGTGTCAC
SDM-FOR-NYDEE- 3rdsite	GCAGCAGGTCAAGCAACTCTAAATTATAAGGAGGAAGACG
SDM-REV-NYDEE- 3rdsite	CGTCTTCTCCTTATAATTTAGGAGTTGCTTGACCTGCTGC
Deletion-lowcomplexity-REV2	GACCACTTGGTCTAGAGTTACAAGTTGTAACCTTTTGCTTCTGGG
Deletion-lowcomplexity-FOR3	CTAGACCAAGTGGTCACCGGGGAAGCGATTAGTGTCAC
Deletion-lowcomplexity-VVTLL-FOR3	CTAGACCAAGTGGTCACCTGCTAGCGATTAGTGTCAC
nearREV3	GGGTAATACAATAAGGAATCATCTTCTTCG
Integration_UOT_FOR	GGAACATCATCTACATCCAGTCCTGG
REV2.5	GCATCAAATCCGACTCCAGTACGTCGAGG
NH1	GTGCGGCCGCGCTGTCACTCCTTCCGTG
NH2	GTGAGCTCCTATTAGATGAAGCTGTACAGGATCAG
NH3	GAGCGGCCGCATGAAAATCATTTTCTTCTCTGTTC
NH4	GTGAGCTCCTATTAGATGAAGCTGTACAGGATCAG
f8330-mut-DG-For	CATTAAAAGTGAAGACCAGTCAGGTAACCTCCGAGCC
f8330-mut-DG-Rev	GGCTCGGAGTTACCTGACTGGTCTTCAGTTTTAATG
f3038-KK-For	GACAAGCCCCGAAAAGAGCAAGAACGACGACACC
f3038-KK-Rev	GGTGTGCTCGTCTTGCTCTTTTCGGGCTTGTC
f3842can-mutDD-For	CTCGGCCAGGTGGACACCGATGAGGCTGTCACTCC
f3842can-mutDD-Rev	GGAGTGACAGCCTCATCGGTGTCCACCTGGCCGAG
f3842can-mutKK-For	CTCGGCCAGGTGAAGACCAAGGAGGCTGTCACTCC
f3842can-mutKK-Rev	GGAGTGACAGCCTCCTTGGTCTTCACCTGGCCGAG
f3842alt-mutKK-For	CATCATTGTGCCTAAGTTCAAGGAGAGCGAGGAG
f3842alt-mutKK-Rev	CTCCTCGCTCTCCTTGAAGTTAGGCACAATGATG
f3842-2ndalt-mutKK-For	CCTGCATCTACTCATGTCAAAAAAGAGTCCAATAC
f3842-2ndalt-mutKK-Rev	GTATTGGACTCTTTTTGACATGAGTAGATGCAGG
CB-mutLL-P2P4-For	GTCCAATACAATTCTCACATTGCAGAACGTCGACGATG
CB-mutLL-P2P4-Rev	CATCGTCGACGTTCTGCAATGTGAGAATTGTATTGGAC
bsd-3-up	TGGCAACCTGACTTGTATCG
bsd-3-down	AGCAATTCACGAATCCCAAC
f83-up	AACCAATCCATCTGACAAC
f83-down	ATTTGAAGCCATGAATGTT

Table S2 Primary antibodies used in this study

mAb X509 (human)	Monoclonal antibody recognising an epitope within the N-terminal 100 amino acid residues of the <i>P. falciparum</i> 3D7-type MSP1 ₄₂ . The antibody therefore recognises both MSP1 ₄₂ and MSP1 ₃₃ (Blackman et al., 1991).
mAb 89.1 (mouse)	Monoclonal antibody recognising an epitope within the repeat regions in 3D7-type <i>P. falciparum</i> MSP1 ₈₃ (Holder and Freeman, 1982).
mAb 111.4 (mouse)	Monoclonal antibody recognising an epitope within the C-terminal 100 amino acid residues of the FCB1-type MSP1 ₄₂ (Holder et al., 1985).
Polyclonal anti-AMA1 (mouse)	Polyclonal antiserum raised against recombinant <i>P. falciparum</i> 3D7 AMA1 (Collins et al., 2009)
Polyclonal anti-MSP1 (rabbit)	Rabbit polyclonal antiserum raised against parasite-derived full-length <i>P. falciparum</i> MSP1 (T9/94 clone) (M. Blackman, unpublished)
mAb OX68 (mouse)	Monoclonal antibody against rat CD4 (domains 3 and 4). (Santa Cruz Biotech)
Polyclonal anti-PfSUB1 (rabbit)	Rabbit antiserum raised against a recombinant fusion protein extending from residues Ser ³³⁰ to His ⁶⁹⁰ of PfSUB1 (Blackman et al., 1998).
Polyclonal anti-MSP1 ₃₃ , MSP1-F specific antiserum (rabbit)	Rabbit antiserum raised against a recombinant protein corresponding to a 93 amino acid residue region of the MSP1 ₃₃ fragment of the FCB1-type <i>P. falciparum</i> MSP1 (Blackman et al., 1993)
Polyclonal MSP1-D specific antiserum (rabbit)	Anti-3D7-MSP-1D _{42ΔEGF} rabbit serum. Kind gift of H. Bujard, ZMBH, Heidelberg, GER
Polyclonal anti-MSP1 ₃₈ , MSP-1D specific (rabbit)	Kind gift of Prof. H. Bujard, ZMBH, Heidelberg, Germany
Polyclonal anti-MSP6 (rabbit)	Kind gift of Prof. H. Bujard, ZMBH, Heidelberg, Germany

Supplemental Experimental Procedures

Peptide cleavage assays

N-terminally acetylated synthetic decapeptides were procured from Biomatik at a purity of at least 95%. Peptides were dissolved in DMSO and concentrated stock solutions (100 mM) stored at -20°C. For digestion assays, peptide stocks were diluted to 5 mM in a total volume of 100 µL in SUB1 digestion buffer (25 mM HEPES pH 7.4, 12 mM CaCl₂, 25 mM CHAPS). This was divided into two tubes, each containing 50 µL diluted peptide. To one tube, 5 µL recombinant PfSUB1 (rPfSUB1) (specific activity 1500 U/mL) was added. Both tubes were incubated at 37°C for 2 h. Cleavage of peptides was then assessed using gradient elution reversed phase high pressure liquid chromatography (RP-HPLC). 10 µL samples of digested or undigested peptides were fractionated on a 4.6 mm x 25 cm C18 RP column (Vydac) eluting at 1 mL/min with a 0-45% (v/v) gradient of acetonitrile in 0.1% trifluoroacetic acid (TFA) over 35 min. Digestion products were identified by electrospray mass spectrometry as previously described (Blackman et al., 2002; Withers-Martinez et al., 2002). Peptide products of digestion were identified as Ac-PIFGE (predicted m/z 604.298, observed 604.303), SEEDY (predicted m/z 642.225, observed 642.232), NYDEE (predicted m/z 628.367, observed 628.4) and Ac-VVQLQ (predicted m/z 669.236, observed 669.2).

Recombinant expression in *Escherichia coli* of wild-type and mutant MSP1-F proteins

Design of plasmid expression constructs. Expression constructs for full length MSP1-F (Fwt) or heterodimeric MSP1-F (Fwt heterodimer) were engineered using pZ vectors as the backbone and expressed as described previously (Kauth et al., 2003). Mutations were introduced into the synthetic *msp1-f* gene by QuikChange II site-directed mutagenesis (SDM). The construct to produce mutant Fmut83/30 (Figure S1) was generated by introducing mutations to create pZ-Fmut83/30 by using primers f8330-mut-DG-For and f8330-mut-DG-Rev. Construct pZ-Fmut30/38 was similarly created

using primer pair f3038-KK-For and f3038-KK-Rev, and pZ-Fmut38/42canD was created using primer pair f3842can-mutDD-For and f3842can-mutDD-Rev. Mutations at the canonical 38/42 site were introduced to create pZ-Fmut38/42canK using primers f3842can-mutKK-For and f3842can-mutKK-Rev. This plasmid was then modified to pZ-Fmut38/42can+alt1K by performing SDM PCR using primers f3842alt-mutKK-For and f3842alt-mutKK-Rev. The plasmid was further modified to create pZ-Fmut38/42can+alt1+2K using primers f3842-2ndalt-mutKK-For and f3842-2ndalt-mutKK-Rev. To introduce mutations at all 38/42 sites, construct pZ-Fmut38/42triple was created by SDM of pZ-FCB1mut38/42can+alt1+2K using primers CB-mutLL-P2P4-For and CB-mutLL-P2P4-Rev.

Recombinant protein purification and processing assays. Recombinant protein aggregated in inclusion bodies and was refolded by pulse renaturation according to (Rudolph and Lilie, 1996). The two separately produced halves of the Fwt heterodimeric protein and corresponding heterodimeric mutants called Fmut83/30 and Fmut38/42 were re-associated during refolding by addition in equal amounts (0.1 mg/mL) at each step. Purified Fwt, Fwt heterodimer and modified versions were digested *in vitro* by rPfsUB1 as described previously (Koussis et al., 2009) with some modifications to the protocol. The protein concentration was adjusted to 0.5 mg/mL with SUB1 digestion buffer along with addition of protease inhibitors E64 (10 μ M), leupeptin (10 μ g/mL) and Pefabloc (100 μ g/mL). 1.5 units of rPfsUB1 was added per 100 μ L of volume and the reaction incubated at 16°C. For time-course digests, seven reactions were set up and the reaction was stopped after 0, 30 min, 1 h, 3 h, 6 h and overnight by boiling in SDS sample buffer for 10 min at 80°C. The 16°C incubation temperature was used in order to reduce protein precipitation during the long incubation periods.

Transgenic expression of MSP1-F in 3D7 parasites

Creation of plasmid transfection constructs. The *Renilla luciferase* gene from expression plasmid pHBIRH (Epp et al., 2008) was replaced by the synthetic sequence encoding the entire *mSP1-f* gene from *P. falciparum* FCB-1 including sequences for the signal peptide and GPI anchor signal (Pan et al.,

1999) to yield pHBIMHFwt after several cloning steps. First, the *Renilla luciferase* gene of pHBIRH was replaced via restriction sites NotI (5') and SacI (3') by a region encoding MSP1-F₄₂, which was previously amplified via PCR using primers NH1 and NH2, resulting in plasmid pHBI-f42. Synthetic full-length *msp1-f* (Pan et al., 1999) was PCR amplified using primers NH3 and NH4 and inserted into pHBI-f42 using NotI and BstBI restriction sites. The *msp1-f* sequence in the resulting expression vector pHBIMHFwt was verified by nucleotide sequencing on both strands. Mutations Fmut38/42triple and Fmutall were further introduced by SDM into pHBIMFwt to create vectors pHBIMFmut38/42 and pHBIMFmutall respectively, which were also verified by nucleotide sequencing on both strands.

Transfection into P. falciparum 3D7 parasites. The created constructs were transfected into *P. falciparum* 3D7 parasites by DNA loading of red blood cells (RBC). 2 mL of RBC (50% haematocrit) were mixed with 6 mL incomplete cytomix and centrifuged for 2 min at 800 g. The supernatant was removed and the blood cell pellet was washed once. 400 µL of the RBC pellet was mixed with 400 µL incomplete cytomix and 100 µg of plasmid DNA. The mixture was transferred to two pre-chilled cuvettes and incubated on ice. Electroporation was performed at 0.31 KV and 960 µFD capacitance. The time constant was in the range of 10 to 13 ms. The cuvette was put back on ice for 5 min and the cells then transferred to a 15 mL tube containing 4 mL RPMI-Albumax medium. The cells were pelleted by centrifugation at 800 g for 2 min and supernatant containing lysed red blood cells was discarded. The transfected erythrocytes were then mixed with 100 µL schizont-infected red blood cells (1% parasitemia) to achieve a final parasitemia of 0.2–0.3%. Culture medium (10 mL) was added and the petri dish was returned to the incubator, with daily changes of culture medium. Selection with blasticidin was performed initially at 2 µg/mL concentration, which was subsequently increased when blasticidin resistant parasites reached a parasitemia of at least 1%.

Growth rate determination. Parasitaemia measurements were carried out using FACSCalibur (Becton-Dickinson). As negative controls, stained uninfected erythrocytes as well as unstained infected erythrocytes were used. For determination of parasitaemia, 10^5 erythrocytes per sample were counted. Results were displayed in a histogram plotting the fluorescence FL3 (x-axis) against the total cell count (y-axis). This representation allowed the discrimination of different parasite stages by their DNA content, which was proportional to fluorescence intensities. For the determination of relative parasite replication rates, expansion of parasitemia over one life cycle was measured. Mature schizonts were set to 0.5% parasitemia and measured by flow cytometry. After one growth cycle (approximately 44 h) the parasitemia was measured again. The growth factor was calculated by dividing the parasitemia at 44 h by the starting parasitemia at 0 h. Significance was determined using Kruskal-Wallis test.

Plasmid rescue. Parasite lines maintained in the presence of a range of blasticidin concentrations, were used for preparation of genomic DNA and episomes rescued by transformation of *E. coli*. Randomly-selected individual bacterial colonies were expanded in LB, plasmid DNA extracted and digested with BglIII and XhoI. Digestion products were analysed by agarose gel electrophoresis to visualise diagnostic bands.

Quantitative Real time PCR (qRT-PCR). First strand synthesis of cDNA was performed using the SuperScript® II First-Strand Synthesis Kit (Invitrogen) according to the manufacturer's instructions. Briefly, reverse transcription of 0.5 µg RNA was carried out in a thermal cycler by incubating the reaction for 10 min at 22°C followed by 50 min at 42°C and terminating the reaction by heating to 70°C for 15 min. The reverse transcription product was used directly in PCR reactions or stored at -20°C. qRT-PCR was performed with gDNA and cDNA preparations using the ABI 7500 sequence detection system and SensiFAST™SYBR® Lo-ROX Kit (Bioline) according to the manufacturer's

instructions. The concentration of gDNA templates was adjusted to 30 ng/ μ L while cDNA was diluted 1:5 prior to use as template. qRT-PCR was performed in triplicate. Data were analysed with SDS 1.3.1 software (Applied Biosystems). In order to determine the absolute copy number of genes or transcripts number, standard curves were generated. The relative gene copy or transcript number was determined by normalisation with the respective data for actin (p100). Transgene expression was displayed as a percentile of *msp1-d* expression (100%). Each gene used for absolute quantification was amplified using serial dilutions from either genomic DNA (*msp1-d*, *actin*) or from plasmid DNA (*msp1-d*, *msp1-f*, *bsd*, *Renilla luciferase*). The DNA concentration was determined by spectrophotometry and adjusted to 10^8 copies per 20 μ L solution. Serial 10-fold dilutions ranging from 10^7 to 10^{-1} copies per real-time PCR reaction were made in H₂O. Triplicate measurements were made for each dilution. The obtained Ct values were plotted against the copy number and a best-fit standard curve was generated. The standard curves were linear across a range of seven logs of DNA concentrations. The detection limit varied between the analysed genes but usually was 10 copies. Standard curve equations were used to calculate the copy numbers obtained by analysing the transgenic parasite lines.

Modification by homologous recombination of the *P. falciparum* 3D7 *msp1* gene

Design of targeting constructs. Construct pHH1-3D7wt was designed to integrate by single-crossover homologous recombination into the 3D7 *msp1-d* locus, reconstituting the coding sequence of the endogenous gene with a gene that expresses a chimeric MSP1 protein. This encodes the 3D7 type MSP1 sequence except for the C-terminal 19 kDa region which is of the FCB1/Wellcome type (wMSP1₁₉). To reliably introduce mutations at the 38/42 position, the DNA sequence in the entire C-terminal region (except that encoding the 19 kDa fragment), from the SUB2 cleavage site near the 3' end to 168 bp upstream of the 38/42 canonical cleavage site at the 5' end was recodoned in the resultant plasmid. Upstream of the recodoned sequence was 998 bp of endogenous 3D7 sequence (target sequence) to drive single cross-over homologous recombination at the *msp1-d* locus. The 998

bp targeting fragment was created by amplifying from *P. falciparum* 3D7 genomic DNA using primers endo3D7-MSP1-BglII-targ-F and endo3D7-MSP1-R. The recodonized fragment was created by amplifying recodonized sequence from plasmid pZ-3D7-MSP138/42 (Kauth et al., 2003; Pan et al., 1999) using primers syn3D7-MSP1-F and syn3D7-MSP1+PstI-R. The fragments were then joined together by overlapping PCR (using primers endo3D7-MSP1-BglII-targ-F and syn3D7-MSP1+PstI-R) to create a fragment with 5' BglII and 3' PstI restriction site overhangs. This was ligated into the pHH1-MSP1₁₉ backbone, which has the wMSP1₁₉ sequence at the 3' end (created by digesting plasmid pMSP1chimWT (Child et al., 2010) with BglII and PstI). The resultant plasmid construct was called pHH1-3D7wt, which was used for transfection into 3D7 parasites, or further modified to create mutations in the 38/42 cleavage region.

For creation of the mutant constructs, pHH1-3D7wt was digested with BglII and PstI and the insert sub-cloned into the pSL1180 vector. The coding sequence flanking the 38/42alt1 site was then mutagenized using the Quikchange[®] site-directed mutagenesis kit (Agilent) by SDM from PIFGESEDND to PIFLLEDND using primers SDM_PIFLLEDND_FOR and SDM_PIFLLEDND_REV, to create construct pHH1-3D7mut38/42alt1. The 38/42 canonical site was similarly mutagenized from VVTGESEDND to VVTLLEDND with primers SDM-VVTLLEDND-FOR and SDM-VVTLLEDND-REV, to create construct pHH1-3D7mut38/42can. The double mutant construct pHH1-3D7mut38/42double was created by introducing the PIFLLEDND mutation in the plasmid pHH1-3D7mut38/42can. The alt2 site mutant construct pHH1-3D7mut38/42alt2 and the triple mutant construct pHH1-3D7mut38/42triple were similarly created by introducing the VVQLQNYDEE→VKQLLNYKEE mutation in the plasmids pHH1-3D7wt and pHH1-3D7mut38/42double respectively, using primers SDM-FOR-NYDEE-3rdsite and SDM-REV-NYDEE-3rdsite. All final construct sequences were verified by nucleotide sequencing on both strands.

All three putative MSP1 38/42 cleavage sites are contained within a predicted unstructured region (not shown) in the 38/42 cleavage region of MSP1. It was reasoned that this region could be deleted so long as at least a single 38/42 cleavage site (the canonical 38/42 site VVTGE↓AISVT) was

left intact. In order to delete this region, plasmids pHH1-3D7mut38/42Δ+can (which encodes an MSP1 protein product with 69 amino acids deleted but retains the downstream canonical 38/42 site unmodified) and pHH1-3D7mut38/42Δ+mut (which combines the 69 amino acid deletion with the canonical site mutation VVTGE↓AISVT → VVTLLAISVT) were both designed to integrate into the *msp1-d* locus by single cross over homologous recombination. Using construct pHH1-MSP1wt as template, a targeting region for homologous recombination (fragment A) was amplified by PCR using primers endo-3D7-MSP1-BglII-targ-F and Deletion-lowcomplexity-REV2. In order to ensure integration upstream of the deletion to be introduced, the deletion was engineered into the 3D7 recodonized sequence. The recodonized fragment containing the deletion, “B_{VVTGE}”, was created by PCR using primers Deletion-lowcomplexity-FOR3 and syn-3D7-MSP1+PstI-R. Another recodonized fragment containing additional point mutations to render the canonical site uncleavable by PfSUB1 (VVTGE↓AISVT → VVTLLAISVT), called “B_{VVTLL}” was generated using primers Deletion-lowcomplexity-VVTLL-FOR3 and syn-3D7-MSP1+PstI-R. Fragments A and B_{VVTGE} (or B_{VVTLL}) were stitched together by a second step of overlapping PCR using the outside primers endo-3D7-MSP1 BglII-targ-F and syn-3D7-MSP1+PstIR. The resulting A+B_{VVTGE} and A+B_{VVTLL} fragments contained a 207 bp deletion encoding the following 69 amino acid residues (alt1 and alt2 SUB1 cleavage sites are shown in bold):

PPQPDVTPSPLSVRVSGSSGSTKEETQIPTSGSLLTELQQ**VVQLQNYDEE**DDSLVVL**PIFGESEDNDEY**

The 5' and 3' extremities of the two stitched fragments were digested with restriction enzymes BglII and PstI and ligated into the parental plasmid pHH1-MSP1wt, thus creating plasmid constructs pHH1-3D7mut38/42Δ+can and pHH1-3D7mut38/42Δ+mut. The presence of the deletion and mutations was confirmed by nucleotide sequencing on both strands.

Transfection of 3D7 P. falciparum for homologous integration into the msp1 locus. Approximately 10 μg of plasmid DNA was ethanol precipitated and resuspended in 10 μL sterile buffer TE (Qiagen). The Amaxa™ P3 primary cell 4D Nucleofector™ X Kit L (Lonza) was used for transfections. The input DNA was added to 100 μL P3 primary cell solution, mixed with 10-20 μL of packed synchronous mature

schizonts and added to the cuvette, which was electroporated in a 4D-Nucleofector machine (Lonza) using program FP158. The transfected schizonts were rapidly added to 2 mL of complete medium (RPMI Albumax supplemented with glutamine) containing erythrocytes at a haematocrit of 15%, and transferred to a shaking incubator at 37°C for 30 min. Finally the cultures were supplemented with 7 mL of complete RPMI medium to obtain a final haematocrit of 3% and incubated overnight at 37°C in a small angle-necked flask (Nunc™). The presence of the human dihydrofolate reductase (*dhfr*) selectable marker in the transfection plasmids allowed selection of integrants with the antifolate WR99210 (Jacobus Pharmaceuticals, New Jersey, USA), added to 2.5 nM 20 h after transfection. The culture medium was subsequently exchanged every day for the next 4 days to remove cell debris which accumulates during electroporation and then twice a week until parasites were detected by Giemsa smear. Drug-resistant parasites were generally detectable in thin blood films 2-3 weeks post transfection. After this, parasite stocks (at ~5% ring parasitaemia) were cryopreserved in liquid nitrogen and genomic DNA was prepared for parasites containing integration vectors. Integrants were selected by drug cycling as follows. Drug was removed from the medium and parasites cultured in its absence for 3-4 weeks, after which the drug was added back and the medium changed daily for 2 days. Once parasitaemia was re-established, parasites were cryopreserved in liquid nitrogen and genomic DNA was prepared. The above cycling process was repeated until integration was detectable by PCR analysis. Integration was confirmed by diagnostic PCR. Integrant lines were then cloned by limiting dilution. For this, the parasite culture was diluted in medium containing fresh red cells at a haematocrit of 2% in order to dispense 0.3 parasite per 100 µL per well in a 96-well microplate (Nunc™). The medium was changed every two or three days until growth was detectable by eye due to a change in the colour of the medium (about two weeks after cloning). Clones were expanded and cryopreserved.

Diagnostic PCR to detect integration of targeting constructs. To assess whether transfection constructs had integrated into the *mSP1-d* genomic locus, diagnostic PCR was performed using extracted genomic DNA as template. Primer pairs specific for detection of integration, namely Integration-UOT-FOR and REV3 (or REV2.5 for deletion constructs) were designed such that the forward primer hybridised in a genomic region upstream of the targeting region present in the plasmid and the second primer hybridised in a region unique to the introduced plasmid. Primer pairs Integration-UOT-FOR and nearREV3 were designed to detect presence of the unmodified *mSP1-d* locus.

Purification and N-terminal sequencing of the shed MSP₁₋₃₃ fragment

Culture supernatants (~50 ml) collected in Albumax-free medium from wild-type 3D7 or transgenic parasite schizonts allowed to undergo egress for 5 h were clarified, then concentrated to 1 mL using 10 kDa cut off ultrafiltration Centricon filters (Millipore). The concentrated culture supernatants were acidified by the addition of 20 μ L TFA and loaded onto a Vydac 4.6 mm x 150 mm 214TP C4 RP-HPLC column. Bound proteins were eluted with an acetonitrile gradient, eluting at 1 mL/min with a 0-40% (v/v) gradient of acetonitrile in 0.1% (v/v) TFA over 20 min, followed by 40-55% (v/v) gradient of acetonitrile in 0.1% (v/v) TFA over 30 min. Collected eluate fractions (1 mL each) were dried overnight in a Speedvac. Dried fractions were each resuspended in 100 μ L of 1x SDS reducing sample buffer. Small samples of each of these fractions were electrophoresed on 12.5% SDS PAGE gels and analysed by Western blot, probing with mAb X509 to determine the position of elution of the MSP₁₋₃₃ fragment or the equivalent fragments from the mutant clones. Proteins of interest were then subjected to N-terminal sequencing. For this, remaining samples of fraction of interest were transferred from a 8-16% precast gradient gel (Invitrogen) onto a PVDF membrane (Immobilon P, Millipore) by overnight transfer (150 mA current) using CAPS buffer (10 mM CAPS, 5 mM DTT, 10% methanol, pH 11). Transferred proteins were visualised with Coomassie brilliant blue. N-terminal

sequencing analysis was carried out by the Protein and Nucleic Acid Chemistry Facility (University of Cambridge, UK).

Purification of recombinant full length recombinant MSP1-D

Recombinant full length MSP1-D proteins (rMSP1-Dwt, rMSP1-DCD4wt and rMSP1-DCD4mut) with an N-terminal secretory signal and a C-terminal hexahistidine (His₆) tag was expressed in HEK293E cells and secreted into culture supernatant as described previously (Crosnier et al., 2013). For purification, ~1 litre of culture supernatant was buffered by the addition of 1 M Tris-HCl, pH 8.2 to 20 mM, then incubated with Ni-NTA agarose beads (15 mL of slurry), and washed with equilibration buffer (20 mM Tris-HCl pH 8.2, 300 mM NaCl). Unbound material was removed by passing the suspension through a sintered glass funnel and the beads were washed with 200 mL of equilibration buffer. The Ni-NTA agarose bound rPfMSP1 was eluted into 45 mL of elution buffer (250 mM imidazole in 20 mM Tris-HCl, 300 mM NaCl). The eluted material was concentrated to 250 µL using a Centricon™ -70 Plus (30 kDa cut off) tube then further purified by size exclusion chromatography using a HiLoad Superdex®200 26/60 column equilibrated in 25 mM HEPES pH 7.4, 150 mM NaCl, collecting 5 mL fractions. Purity and yield was assessed by subjecting 20-30 µL samples of the eluted fractions to SDS PAGE and staining with Coomassie brilliant blue. Purified proteins were stored at -80°C until required for further experiments.

Circular dichroism studies on recombinant MSP1

Full length rMSP1-Dwt or mutant forms at 0.156 mg/mL concentration in 500 µL buffer (25 mM HEPES, pH 7.4, 150 mM NaCl, 15 mM CaCl₂) was equilibrated at 37°C and monitored for 5 h for any structural changes on a Jasco J-715 spectropolarimeter. To examine the effects of digestion with PfSUB1, 6 µL of rPfSUB1 (0.84 mg/mL in the sample) was added to another identical sample and monitored for structural changes under identical conditions. The secondary structure composition was averaged using the algorithms CONTINLL and SELCON3 (Sreerama and Woody, 2004).

Heparin binding assays

Heparin-agarose beads (Sigma Aldrich) suspended in a 50% slurry in 20% ethanol were washed twice with distilled water and then three times with heparin assay buffer (25 mM HEPES, pH 7.4, 15 mM NaCl, 0.07% Tween® 20). The beads were resuspended in an equal volume of heparin assay buffer and 50 µL of the resuspended slurry dispensed into 1.4 mL Eppendorf tubes for the binding experiment. 50 µL of purified rMSP1-Dwt (cleaved with rPfsUB1 or uncleaved) was added to a final concentration of 0.1 µg/µL to the heparin beads. Control tubes were set up where binding was tested in the presence of 1 mg/mL heparin sodium salt solution (Sigma). The beads were incubated with proteins of interest for 20 min with shaking at room temperature. Unbound supernatant was collected and the required amount of SDS sample buffer (+DTT) was added. The beads were washed 5 times with 500 µL heparin assay buffer and bound proteins finally eluted into SDS sample buffer (containing DTT). All samples were heated for 5 min at 90°C then electrophoresed on a 4-16 % BioRad® MiniProtean™ TGX gradient gel. The gel was Coomassie stained and imaged on the BioRad® Chemidoc™ MP system. Total protein intensity in each lane was estimated using Image Lab software.

Overlay immunofluorescence for investigating MSP1 interactions with erythrocytes

Erythrocytes were washed, resuspended in RPMI-Albumax medium and smeared on a glass slide. The thin films were air dried, fixed in 4% paraformaldehyde (in PBS) for 30 min at RT and permeabilised in 0.1% (v/v) Triton X-100 (Sigma) for 10 min. Fixed slides were then washed three times with PBS for 10 min and blocked overnight at 4°C in 3% (w/v) bovine serum albumin (BSA) in PBS. The following day, slides were probed with purified rMSP1-Dwt (previously cleaved with rPfsUB1 or uncleaved) at 25 ng/µL in 1% BSA in PBS 0.005% (v/v) Tween 20 (PBST). The slides were then washed three times with PBS for 10 min. Next, the slides were probed with an anti-MSP1 primary antibody (purified mAb X509 at a dilution of 1:2000) for 30 min at 37°C and then washed three times for 10 min in PBS. Slides were then probed with Alexafluor conjugated goat anti-human

IgG (1:1000) and washed three times with PBS. Slides were mounted in PBS/Glycerol and images collected using AxioVision 3.1 software on an Axioplan 2 Imaging system (Zeiss) using a Plan-APOCHROMAT 100x/1.4 oil immersion objective (Harris et al., 2005). Mean pixel intensity was determined for the fluorescence images using the Histogram tool in Adobe Photoshop.

Overlay (Far Western) assay for investigating spectrin binding by MSP1

Erythrocyte ghosts were prepared and overlay assays performed as described previously (O'Donnell et al., 2006). Briefly, human RBC were washed in RPMI medium (without Albumax) and lysed in 5 mM ice cold sodium phosphate buffer, pH 7 (diluted from a 1 M stock prepared by mixing 57.7 mL of 1 M Na_2HPO_4 and 42.3 mL of 1 M NaH_2PO_4 solutions). Ghosts were centrifuged (13,000 rpm in a Heraeus Biofuge Fresco) and washed repeatedly until the supernatant appeared clear and the erythrocyte ghosts appeared a pale white colour. Ghosts were stored at -80°C until further use. The ghosts were thawed, solubilized in SDS-sample buffer and heated for 4-5 min at 95°C . Ghost preparations were run on a 4-16% gradient gel and transferred to a nitrocellulose membrane by overnight Western transfer. The blot was then blocked in 5% milk in PBST for 1 h. The blot was washed 3 x 5 min with PBST and probed with rMSP1-Dwt, rMSP1-DCD4wt, or rMSP1-DCD4mut protein (previously digested with rPfSUB1 or undigested). For this a 6 mg/mL solution of purified protein in 25 mM HEPES pH 7.4, 150 mM NaCl was diluted 1:400 into 2 mL of PBST containing 1% (w/v) BSA and incubated with the blot for 2 h. The blot was then washed 3 x 5 min in PBST before probing with a primary anti-MSP1 antibody (purified mAb X509 diluted 1:2000 in PBST 1% BSA, or mAb OX68 diluted 1:1000 in PBST 1% BSA) for 1 h. MAb OX68 was used if the recombinant MSP1 possessed a C-terminal CD4 tag. This was followed by washing and 1 h incubation in an appropriate HRP-conjugated secondary antibody (goat anti human IgG for mAb X509, goat anti mouse IgG for mAb OX68). Both secondary antibodies were used at 1:10,000 dilution in PBST 1% BSA. The blot was developed using ECL reagents and either visualised using photographic films or using a Biorad Chemidoc™ MP system.

Binding of rMSP1 to inside-out vesicles (IOVs)

Preparation of IOV. IOVs were prepared from human RBC as described previously (Kilili and LaCount, 2011). Briefly, RBC from 40 ml of whole blood were collected by centrifugation and washed at least three times in five volumes of cold RPMI medium without Albumax, calcium or magnesium. After each wash, cells were pelleted and the supernatant, along with any visible buffy coat, was removed. After the final wash, RBCs were resuspended in RPMI without Albumax at 50% hematocrit and stored at 4°C. RBC ghosts were prepared from 1 ml of packed RBCs by adding 30 ml of ice-cold lysis buffer (5 mM phosphate buffer, pH 8.0, protease inhibitor (PI) cocktail, and 0.1 mM EDTA), mixing several times by inversion, and incubating on ice for 10 min. Lysed cells were centrifuged at 48,000 x g for 10 min. After the removal of approximately 95% of the supernatant, the pellet was dislodged by slowly swirling in the remaining buffer and transferred to a fresh tube, leaving behind the contaminating intact RBC pellet. Fresh buffer was added, and the centrifugation and pellet transfer steps were repeated two to three times until the pellet appeared creamy white and the supernatant was free of haemoglobin.

To prepare IOVs, the pellet was resuspended in 30 ml of vesiculation buffer (0.5 mM phosphate buffer, pH 8.5), incubated on ice for 1 h, and centrifuged at 48,000 x g for 15 min. The pellet was resuspended in 1 ml of vesiculation buffer, and the suspension was passed through a 27-gauge needle at least 10 times. Vesiculation buffer was added to adjust the volume to 30 ml, and the suspension was subjected to centrifugation as described above. The pellet, which contained the IOVs, was resuspended in 1.5 to 2 ml of IOV storage buffer (138 mM NaCl, 5 mM KCl, 6.1 mM Na₂HPO₄, 1.4 mM NaH₂PO₄, 5 mM glucose) and stored at 4°C for less than 1 week until use.

IOV pull-down assays. IOVs (specific amounts used are indicated in the figure legends) were incubated overnight without mixing at 4°C in 250 µl of cold IOV blocking buffer (1x PBS, 2% w/v BSA, PI cocktail). The IOVs were collected by centrifugation at 16,000 x g for 10 min at 4°C, and the

supernatant was removed by aspiration. The pellet was resuspended in 250 μ l of freshly made ice-cold IOV binding buffer (1x PBS, 1% BSA, PI cocktail). Equal amounts of rPFSUB1-cleaved or uncleaved rMSP1-Dwt (see figure legends for details) were added to the IOVs, mixed briefly, and incubated on ice overnight at 4°C with occasional mixing during the first 2 to 3 h. The IOVs then were washed twice with cold IOV wash buffer (1x PBS, 0.25 mM KCl) by completely suspending the pellet and incubating it for 5 min on ice. After the last wash, the pellet was resuspended in 3x SDS sample loading buffer and processed for SDS-PAGE followed by Western blotting probing with the MSP1-specific mAb 89.1.

Immuno-electron microscopic analysis of binding of recombinant MSP1 to RBC cytoskeletons

Erythrocyte cytoskeletons were immobilised on carbon-coated gold grids and binding by recombinant MSP1 was assessed as follows. Cytoskeletons of uninfected erythrocytes were prepared *in situ* on grids as follows. Grids were glow-discharged, coated with 0.01% (w/v) poly-L-lysine (Sigma) for 30 seconds, washed once with water and blotted dry. Erythrocytes in PBS (~20% haematocrit) were applied to the grids, allowed to adhere for 1 minute, then blotted from the back and washed once with PBS. Grids carrying cells were dipped sequentially into low salt lysis buffer (1 mM Tris pH 4.7, 1 mM KCl, 0.2 mM MgCl₂), low salt lysis buffer containing 2% Triton X-100, and then low salt lysis buffer without detergent to wash, for 60 seconds in each solution, passing through the meniscus several times. The resulting cytoskeletons were then incubated for 30 minutes in blocking buffer (0.5% w/v cold water fish skin gelatine, 1% normal goat serum), followed by incubation for 30 min with rMSP1-Dwt (rPFSUB1-cleaved or uncleaved) in blocking buffer. The grids were then probed with a rabbit polyclonal anti-MSP1 antiserum diluted 1:250 or 1:2500 in blocking buffer, followed by probing with gold-conjugated secondary antibody (goat anti-rabbit Alexa488 conjugated to 5 nm gold) diluted 1:10 in blocking buffer. Grids were washed with PBS between incubation steps, and finally washed with lysis buffer and stained with 2% sodium silicotungstate. Controls included no-protein controls and no primary antibody controls. At least 8-10 separate RBC ghost skeletons and 8-

10 nearby similar areas of carbon support for each condition were imaged using a Tecnai 10 transmission microscope. Numbers of gold beads were counted and gold bead density was expressed as the average of the densities in collected images.

Time lapse video microscopy of *P. falciparum* egress

Video microscopy of *P. falciparum* schizont egress was performed as described previously (Collins et al., 2013b). Synchronised schizonts were Percoll-enriched and further cultured in the presence of the *P. falciparum* PKG inhibitor compound 1 (C1, 2 μ M) in RPMI Albumax medium for 4 h. Microscopy chambers (internal volume \sim 80 μ l) for observing live schizonts were constructed by adhering 22 \times 64 mm borosilicate glass coverslips to microscope slides with strips of double-sided tape, leaving \sim 4 mm gaps at each end. The schizonts were pelleted, washed once in warm (37°C) fresh RPMI Albumax without C1 then immediately resuspended into fresh warm medium and introduced by capillary action into the pre-warmed chamber. The chamber was immediately transferred to a temperature-controlled microscope stage at 37°C on an Axio Imager M1 microscope (Zeiss) equipped with an EC Plan-Neofluar 100 \times /1.3 oil immersion differential interference contrast (DIC) objective and an AxioCam MRM camera. Images were routinely collected at 5 s intervals; beginning 4 min 20 sec after washing off C1, over a total of 30 min. For each experiment, videos of the chim_ Δ +can and chim_ Δ +mut parasite populations were made alternately over the course of an afternoon in order to ensure that differences in rate of egress were not a result of variation in maturity of the parasite populations. The images were then annotated using Axiovision 3.1 software and exported as AVI movie files. Individual egress events were then annotated by detailed visual analysis of the movies using Microsoft PowerPoint and the delay to the time of egress was recorded for each schizont for subsequent statistical analysis. Mean time to egress was thus determined for each schizont population and plotted as bar graphs for the two different mutants.

In order to further reduce difference of conditions during video microscopy and for better comparison of the chim_ Δ +can and chim_ Δ +mut parasites, purified schizonts from both populations

were mixed together in the same microscopy chamber prior to imaging. To discriminate between the two transgenic parasite clones, the chim_Δ+mut population was labelled with the DNA labelling dye Hoechst (1 μg/mL) for 5 min prior to washing off the C1. An initial fluorescence image was collected (exposure of 10 ms) and this overlaid with a DIC image. Time lapse video microscopy and analysis of annotated videos were then performed as described earlier. The reciprocal experiment of labelling the chim_Δ+can parasites was then performed to rule out any effects of dye incorporation on time to egress.

Conditional truncation of *P. falciparum* MSP1 using the loxPint/DiCre system

*Generation of constructs and transfection of *P. falciparum* for conditional truncation of the *msp1* gene.* To obtain conditional truncation of the *msp1* gene we used an approach which uses silent loxP sites within a heterologous *P. falciparum* intron (called loxPint). The system is described in detail in a different manuscript (Matthew Jones, Sujaan Das, Michael Blackman and Moritz Treeck, in preparation). Briefly, the loxPint module was introduced by homologous recombination into the *P. falciparum* 3D7 genomic *msp1* gene in two different locations, allowing the creation of two slightly different truncations of MSP1-D (3D7MSP1flox42C1 and 3D7MSP1flox42C2; see Figure S6). For this, two ~400 bp sequences corresponding to the loxPint fragment flanked by targeting sequence at the 5' and 3' ends (-AGAAACCC**AG**-loxPint-**AT**CCCCACAT- and -CCTCAACC**AG**-loxPint-**AT**GTAACTCC-; letters in bold indicate a naturally occurring AGAT motif which effectively serves as the intron-exon boundary) were synthesised (Geneart®) and introduced into construct pHH1-3D7wt using restriction sites HpaI and BstEII to generate plasmid constructs pMSP1_loxPint-1 and pMSP1_loxPint-2. The structure of each construct was confirmed by nucleotide sequencing on both strands. The constructs were independently transfected into the DiCre-expressing *P. falciparum* 1G5DiCre clone (Collins et al., 2013a) and parasites selected in which the constructs had integrated into the *msp1* genomic locus by single cross-over homologous recombination. The parasite lines were cloned by limiting

dilution to obtain two distinct parasite clones corresponding to each of the different placements of the loxPint, called 3D7MSP1flox42C1 clone E3 and 3D7MSP1flox42C2 clone B51.

To obtain rapamycin-induced excision of the 3' end of the modified *mSP1* gene, deleting coding sequence for the MSP1₄₂ fragment and GPI anchoring sequence (Figure 6 and Figure S6), cultures containing synchronised, newly invaded ring forms of clones 3D7MSP1flox42C1 clone E3 and 3D7MSP1flox42C2 clone B51 were divided into two and treated with rapamycin (final concentration 100 nM, diluted 1:100 from a 10 µM stock in DMSO) or mock treated (1:100 dilution of DMSO) for 4 h, then washed to remove the rapamycin and returned to culture as described previously (Collins et al., 2013a). Schizonts purified from the cultures at the end of the same replicative cycle were used in PCR, IFA, or Western blot analysis or for time-lapse video microscopy.

IFA of free merozoites. Purified 3D7MSP1flox42C1 clone E3 or 3D7MSP1flox42C2 clone B51 schizonts from rapamycin-treated or mock-treated control cultures were incubated in medium containing 2 µM C1 for 4 h, then washed and resuspended in warm C1-free medium to allow egress (or abortive egress in the case of the rapamycin-treated mutants). The resulting preparations, which contained free merozoites, were used to prepare thin films for IFA as described above.

Growth assays to determine the effects of MSP1 truncation on parasite replication. Cultures (2% haematocrit) containing synchronous ring stages of clones 3D7MSP1flox42C1 clone E3 and 3D7MSP1flox42C2 clone B51 at 1% parasitaemia were divided into two flasks and either rapamycin treated (100 nM in DMSO) or mock treated with DMSO for 4 h. The parasites were then washed and returned to culture for a total of 4-6 erythrocytic cycles. Samples were collected at the end of every replicative cycle for IFA and parasitemia counts. At the end of the second replicative cycle (day 4) and the fourth cycle in the case of the longer experiments, each cultures was diluted 1:10 by diluting into a 2% haematocrit suspension of fresh erythrocytes in fresh medium. Parasitaemia was determined by microscopic counting of Giemsa-stained thin films and plotted after adjustment to

take dilution factors into account. The parasites in at least 1000 erythrocytes counted at each time point in three independent counts, and the mean parasitaemia values was plotted with SD as error bars. Exponential growth curves were fitted to the data and replication rates per erythrocytic cycle determined from the slopes. To assess relative fitness of the truncated mutants, IFA was performed probing with mAb X509 and mAb 89.1, which gave an estimate of the proportion of non-excised (mAb X509 reactive) parasites as a proportion of all MSP1-positive (mAb 89.1 reactive) parasites in the rapamycin-treated population over several growth cycles.

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