

1 **Function and essentiality of *Plasmodium falciparum* plasmepsin V**

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27 Running title: *Plasmodium falciparum* plasmepsin V is essential for parasite viability

29 **Abstract**

30 The malaria parasite replicates within erythrocytes. The pathogenesis of clinical malaria is in large part
31 due to the capacity of the parasite to remodel its host cell. To do this, intraerythrocytic stages of
32 *Plasmodium falciparum* export more than 300 proteins that dramatically alter the morphology of the
33 infected erythrocyte as well as its mechanical and adhesive properties. *P. falciparum* plasmepsin V
34 (PfPMV) is an aspartic protease that processes proteins for export into the host erythrocyte and is thought
35 to play a key role in parasite virulence and survival. However, although standard techniques for gene
36 disruption as well as conditional protein knockdown have been previously attempted with the *pfpmv* gene,
37 complete gene removal or knockdown was not achieved so direct genetic proof that PMV is an essential
38 protein has not yet been established. Here we have used a conditional gene excision approach combining
39 CRISPR-Cas9 gene editing and DiCre-mediated recombination to functionally inactivate the *pfpmv* gene.
40 The resulting mutant parasites displayed a severe growth defect. Detailed phenotypic analysis showed
41 that development of the mutant parasites was arrested at the ring-to-trophozoite transition in the
42 erythrocytic cycle following gene excision, likely due to a defect in protein export. Our findings are the
43 first to elucidate the effects of PMV gene disruption, showing that it is essential for parasite viability in
44 asexual blood stages. The mutant parasites can now be used as a platform to further dissect the
45 *Plasmodium* protein export pathway.

46

47 Introduction

48 Malaria was responsible for approximately 445,000 deaths and 216 million clinical cases in 2016,
49 an increase of ~5 million cases over the previous year [1]. Vital to the growth and pathogenicity of the
50 parasite is host cell remodelling in which the parasite modifies the host erythrocyte by the synthesis and
51 export of over 300 parasite proteins beyond the bounds of the parasitophorous vacuole (PV) within which
52 it replicates (for reviews, see [2,3]). The exported proteins are trafficked from the parasite to the host
53 erythrocyte via a putative parasite-derived protein complex known as the translocon of exported proteins
54 (PTEX), located within the PV membrane (PVM) [4,5]. The exported proteins extensively alter the
55 mechanical and adhesive properties of infected erythrocytes, resulting in vascular sequestration of the
56 infected cells and eventual destruction of the host erythrocyte [6]. Protein export in *Plasmodium* has been
57 most intensively studied in *P. falciparum* (reviewed in [7,8]), where the majority of known exported
58 proteins contain the pentameric localisation motif RxLxE/Q/D, termed *Plasmodium* export element
59 (PEXEL), typically located downstream of the N-terminal secretory signal sequence that regulates entry
60 into the ER [9,10]. Some proteins lacking a PEXEL motif, referred to as PEXEL-negative exported
61 proteins (PNEPs), can also be exported [11]. PNEPs do not contain a typical secretory signal sequence so
62 are unlikely to be N-terminally processed, but the first 20 amino acid residues of PNEPs are sufficient to
63 mediate their export. The unfolding of soluble PNEPs is then required for trafficking into the host cell
64 [12].

65 *P. falciparum* plasmepsin V (PfPMV) is an ER-located aspartic protease comprising 590 amino acid
66 residues (~68 kDa) [13]. PfPMV expression levels in the parasite progressively increase throughout
67 schizogony [13]. Numerous studies have now established that PMV is directly responsible for cleavage of
68 the PEXEL motif within exported proteins. Cleavage occurs on the C-terminal side of the conserved Leu
69 residue (RxL↓), revealing a new N-terminus that is rapidly acetylated (^{Ac}-xE/Q/D) [14]. A recent x-ray

70 crystal structure of *Plasmodium vivax* PMV (PvPMV) revealed a canonical aspartyl protease fold with
71 several important features [15]. These include a nepenthesin (NAP)-like insertion within the N-terminal
72 part of the enzyme, which may control substrate entry into the active site and influence enzyme
73 specificity, as well as a helix-turn-helix (HTH) motif near the C-terminus of the enzyme, which is
74 conserved in PMV from other *Plasmodium* species but not found in other parasite plasmepsins involved
75 in haemoglobin digestion. PMV also possesses an unpaired Cys residue (C140 in PvPMV, equivalent to
76 C178 in the *P. falciparum* enzyme) which is located in the flap of the structure and is restricted to
77 *Plasmodium* species. In attempts to establish the druggability and function of PMV, several
78 peptidomimetic inhibitors based on the PEXEL motif have been developed [16-18]. A co-crystal structure
79 of PMV bound to one of these compounds, the PEXEL-mimetic WEHI-842, showed that although the
80 unpaired Cys points into the active site of the enzyme, it does not appear to make contact with the
81 inhibitor [15,19]. Another inhibitor, WEHI-916, inhibits the activity of purified PMV isolated from both
82 *P. falciparum* and *P. vivax*, and higher concentrations of WEHI-916 were required to kill parasites
83 engineered to over-express PfPMV indicating on-target efficacy [16,18]. Compound 1, a hydroxyl-
84 ethylamine PEXEL-mimetic, inhibited PfPMV activity in vitro with picomolar potency but failed to
85 block parasite growth due to poor stability and membrane permeability [17]. Collectively, these findings
86 indicate that PMV has a number of distinguishing features that could be exploited in drug design, and can
87 potentially be targeted with suitable inhibitory compounds.

88 An important component in the validation of any enzyme as a drug target is genetic ablation of
89 enzyme expression. Unfortunately, previous attempts to genetically delete or knock-down PMV
90 expression have been only partially successful. Work by Boddey et al [20] failed to disrupt the *P. berghei*
91 PMV (PbPMV), suggesting (but not proving) that the gene is essential. Russo et al [21] similarly
92 attempted to disrupt the *pfpmv* gene using an allelic replacement approach. Single crossover homologous

93 recombination into the endogenous PMV locus was possible only when the catalytic dyad aspartate codon
94 was preserved, whilst four separate transfection experiments with constructs designed for non-
95 synonymous alteration of the active site codon were not successful. Conditional knockdown of PfPMV
96 expression was also attempted using the RNA-degrading *glmS* ribozyme system [18]. Although this
97 resulted in around 75-90% knockdown of PMV expression, the remaining PMV levels were presumably
98 sufficient to enable export and sustain parasite development. Gambini et al [17] generated an inducible
99 PMV knock-down by fusing PMV with a destabilising domain (DD), but only a 4 to 10-fold knock-down
100 of PMV cellular levels was achieved using this approach, which did not affect parasite viability. In
101 summary, whilst some of these data are consistent with an essential role for PMV, in the absence of any
102 reports of complete ablation of PfPMV expression, direct genetic proof that PMV is an essential protein is
103 lacking.

104 Here we have used a robust conditional genetic approach to truncate the *pfpmv* gene. Our findings
105 are the first to confirm genetically that deletion of PfPMV has a significant effect on ring-to-trophozoite
106 development and ultimately results in parasite death.

107

108 **Results**

109 **Generation of modified *pfpmv* parasites using CRISPR-Cas9**

110 Previous attempts to disrupt the *pfpmv* gene using conventional genetic techniques were
111 unsuccessful [20,21], and conditional knockdown approaches did not significantly affect PEXEL
112 processing or parasite viability [17,18], presumably due to relatively low levels of PfPMV expression
113 being sufficient to sustain parasite viability. To explore the consequences of complete functional
114 inactivation of PMV, we therefore took advantage of the DiCre conditional recombinase system, recently
115 adapted to *P. falciparum* [22]. Using Cas9-mediated genome editing [23] we first introduced synthetic
116 introns containing *loxP* sites [24] into the endogenous *pfpmv* locus such that they flanked (floxed) an
117 internal segment of the gene encoding Asp133 to Thr590. At the same time, the modified gene was fused
118 to a C-terminal HA3 epitope tag, as well as a 2A sequence followed by the aminoglycoside 3'-
119 phosphotransferase (*neo-R*) gene sequence conferring neomycin resistance activity to enable selection for
120 integration events (Fig 1A). Importantly, one of the PfPMV catalytic dyad residues (Asp365) is included
121 within the floxed region. The repair plasmid pT2A-5'UTR-3'-PMV- ΔDHFR was based on pT2A-DDI-
122 1cKO, which contains a modified selection-linked integration (SLI) region [25]. The genomic
123 modification was made in the DiCre-expressing *P. falciparum* B11 parasite clone [26] such that excision
124 of the floxed sequence could be induced by treatment of the transgenic parasites with rapamycin (RAP).
125 DiCre-mediated excision was predicted to generate an internally-truncated mutant form of PMV lacking
126 one of the catalytic dyad residues. Excision would also remove the *neo-R* gene and induce expression of
127 GFP as a fluorescence reporter indicative of parasites expressing the truncated *pfpmv* gene (Fig 2A).

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130

131 **Fig 1. Generation of *P. falciparum* parasite lines expressing PfPMV-HA.** (A) Using Cas9-mediated
132 recombination, the region of the *pfpmv* gene encoding Asp133 to Thr590 was replaced with two *loxP*
133 (black arrowhead)-containing *P. falciparum* SERA2 introns (yellow box) flanking a recondonised *pfpmv*
134 (blue box) fused to a *3xHA* epitope tag (purple box), a *2A* sequence (pink box), and a *neo-R* gene (white
135 box) and stop codon (red hexagon). The second *loxPint* was also fused with *2A* and *gfp* gene sequences.
136 The *gfp* gene is translated only following site-specific recombination of the *loxP* sites by DiCre
137 recombinase. Positions of hybridisation of primers used for confirmation of the integration event by
138 diagnostic PCR are shown as coloured arrows. (B) Diagnostic PCR analysis of genomic DNA of the
139 control parental B11 and integrant *P. falciparum* clones, confirming the predicted homologous
140 recombination event.

141

142 **Fig 2. DiCre-mediated conditional disruption of *P. falciparum* PMV expression.** (A) Strategy for
143 conditional truncation of the *pfpmv* gene. Positions of hybridisation of primers used for diagnostic PCR
144 analysis of the integration and excision events are shown as coloured arrows. (B) Diagnostic PCR
145 analysis of genomic DNA from transgenic *P. falciparum* PMV-C5 line at 24 h in cycle 0 (~20 h post
146 RAP- or DMSO-treatment), confirming the predicted DiCre-mediated excision events. Expected sizes of
147 the PCR amplicons specific for the intact or excised locus are indicated. (C) Live GFP fluorescence assay
148 of cycle 0 PMV-C5 parasites ~42 h following treatment at ring stage with DMSO or RAP. Parasite nuclei
149 were stained with Hoechst 33342. (D) Giemsa-stained blood smears, showing the morphology of treated
150 PMV-C5 parasites. The time-course of treatment and subsequent monitoring of the cultures is indicated
151 (top).

152

153

154

155 Successful modification of the *pfpmv* gene in the transfected parasite population following the
156 introduction of the targeting vector was confirmed by diagnostic PCR (data not shown). Limiting dilution
157 cloning of the modified parasites resulted in the isolation of parasite clones PMV-C5 and PMV-F9, which
158 were derived from independent transfections using different guide RNAs. Modification of the native
159 *pfpmv* locus was confirmed in both transgenic parasite lines by diagnostic PCR (Fig 1B). Both clones
160 replicated at a rate indistinguishable from that of the parental B11 parasites, suggesting that the
161 modifications did not affect parasite viability. Transgenic parasite clone PMV-C5 was used for all
162 subsequent experiments.

163

164 **Conditional truncation of the *pfpmv* gene leads to a developmental arrest**

165 Expression of the recodonised *pfpmv* gene in transgenic parasite clone PMV-C5 was expected to
166 produce an epitope-tagged PMV product (called PMV-HA), as well as expression of the *neo-R* gene
167 product. Note that in this event GFP is not produced because of the presence of a translational stop codon
168 directly downstream of the *neo-R* gene. Upon RAP-treatment, site-specific recombination between the
169 introduced *loxP* sites in the modified *pfpmv* locus of the PMV-C5 parasites was anticipated to reconstitute
170 a functional, albeit chimeric, intron. Splicing of this chimeric intron results in a truncated form of PMV
171 lacking the HA epitope tag, as well as allowing expression of free GFP. DNA extracts of RAP-treated and
172 mock-treated (DMSO-treated control) PMV-C5 parasites were analysed by diagnostic PCR (Fig 2A). To
173 confirm whether RAP-induced DiCre-mediated excision took place as expected, we monitored the
174 appearance of GFP-positive parasites following treatment of synchronous ring-stage parasites with
175 DMSO or RAP. As expected, GFP fluorescence was only detected in the RAP-treated parasites (Fig 2C).
176 Indeed, no parasites lacking GFP fluorescence could be detected upon microscopic examination of the

177 RAP-treated cultures, indicating highly efficient excision of the floxed *pfpmv* sequence. This was
178 confirmed by FACS analysis (Fig 3B), showing that 98.86 % of the RAP-treated parasites displayed GFP
179 fluorescence. These results confirmed the PCR-derived excision data and demonstrated essentially
180 complete conditional truncation of PMV within a single erythrocytic cycle in the PMV-C5 parasite clone.

181

182 **Fig 3. Functional disruption of PfPMV results in developmental arrest at the ring-to-trophozoite**
183 **transition.** (A) Growth assay showing relative replication rates over the two erythrocytic cycles
184 following treatment of PMV-C5 ring stages with DMSO or RAP. Parasite number values were
185 determined by FACS as described in Materials and Methods. (B) GFP fluorescence at ~42 h (schizonts)
186 of the PMV-C5 line treated at ring stage with DMSO (black) or RAP (green) as determined by flow
187 cytometry of fixed cultures. The percentage of GFP-positive RAP-treated parasites (i.e. the excision rate)
188 was 98.86%. (C) Western blot analysis of clone PMV-C5 following mock-treatment (-RAP) or RAP-
189 treatment (+RAP). Schizont extracts (~42 h post-treatment) were probed with antibodies to detect HA-
190 tagged PfPMV (anti-HA), or antibodies to GFP, MSP1, KAHRP, or HRP2.

191

192 To initially explore the effects of PMV truncation on parasite viability, we examined growth and
193 development of the Δ PMV mutant by microscopic examination of Giemsa-stained cultures. This revealed
194 that although RAP-treated PMV-C5 parasites appeared morphologically normal throughout the
195 erythrocytic growth cycle in which the parasites were treated (henceforward referred to as cycle 0),
196 development of the mutant parasites stalled at ring stage in the following cycle (cycle 1; Fig 2D). By the
197 end of cycle 1, only arrested pycnotic forms were observed. Collectively, these results suggested that
198 ablation of PMV expression causes a severe growth defect and developmental arrest between ring and
199 trophozoite stage.

200

201 **Egress, Invasion and Protein export are not affected by disruption of the**
202 ***pfpmv* gene in cycle 0**

203 Whilst the above results showed that truncation of PfPMV affected parasite development in cycle
204 1, it did not rule out the possibility of defects in egress and invasion of fresh erythrocytes at the end of
205 cycle 0 (the cycle of RAP-treatment). To evaluate and quantify this, we used flow cytometry to monitor
206 increases in parasitaemia at the transition between cycle 0 schizonts and cycle 1 rings. As shown in Fig
207 3A, the parasitaemia of PMV-C5 cultures at the end of cycle 0 as well as at the early ring stage of cycle 1
208 was unaffected by RAP-treatment, indicating no effects of PMV ablation on schizont development in
209 cycle 0, or the egress and invasion capacity of the released merozoites. However, upon further
210 development of the new parasite generation in cycle 1, the parasitaemia in the RAP-treated culture
211 reached plateau after the ring-trophozoite transition. This confirmed that the truncation of PfPMV did not
212 cause any effects on egress and invasion and that the enzyme is essential for the ring to trophozoite
213 developmental transition of the intracellular parasite. Confirmation of the high excision rate of the PMV-
214 C5 clone following RAP-treatment was also confirmed by FACS analysis (Fig 3B).

215 To further explore the PMV-null phenotype, we examined the expression of PMV-HA as well as
216 other non-exported and exported parasite proteins. Extracts of DMSO and RAP-treated PMV-C5
217 parasites were analysed by immunoblot ~42 h following treatment, using antibodies specific for the HA
218 epitope tag as well as GFP, the merozoite plasma membrane surface protein MSP1, and the exported
219 proteins KAHRP and HRP2. As shown in Fig 3C, only a faint HA signal was detected in the RAP-treated
220 sample, whilst a correspondingly strong GFP signal was observed only in this sample. These data were
221 in good agreement with the diagnostic PCR results, indicating high efficiency of conditional excision of
222 the floxed sequence in the PMV-C5 clone. In contrast, no differences between DMSO- and RAP-treated

223 parasites were detectable in expression levels of MSP1 (thought to play an important role in egress and
224 invasion [27]) Similarly, there were no significant differences in expression of KAHRP and HRP2, both
225 of which are examples of PEXEL-containing proteins that are cleaved by PMV [20,21,28]. This is
226 consistent with the notion that protein export remains unaffected in cycle 0 in the RAP-treated parasites.

227 We next determined the effects of PfPMV truncation on its subcellular localisation within the
228 parasite, as well as on the trafficking of other exported proteins in cycle 0. Immunofluorescence analysis
229 (IFA) showed that, as expected, PMV-HA showed a perinuclear localisation in schizonts of control PMV-
230 C5 parasites but the signal was lost in RAP-treated parasites (Fig 4). To determine the effects of PMV
231 ablation on protein export, mock- and RAP-treated PMV-C5 parasites were probed with anti-KAHRP2
232 and anti-HRP2 antibodies. The localisation and intensity of the MSP1 signal and the KHARP signal did
233 not alter upon RAP-treatment, correlating well with the western blot analysis. The HRP2 signal remained
234 detectable in both control and the RAP-treated parasites, but in contrast to the situation with the other
235 proteins examined, the fluorescence intensity of the HRP2 signal was substantially decreased in RAP-
236 treated parasites, suggesting a partial effect of PMV depletion on HRP2 trafficking. Together, these data
237 confirmed the loss of PMV-HA in RAP-treated parasites whilst indicating that both exported proteins
238 (KAHRP and HRP2) and merozoite surface proteins still localise correctly in cycle 0. However, the
239 decreased HRP2 signal intensity was consistent with a partial impact on protein export in cycle 0.

240

241 **Fig 4. IFA of schizonts of control (DMSO-treated) and RAP-treated integrant clone PMV-C5 ~42 h**
242 **following treatment (cycle 0).** The PMV-HA signal was lost following RAP treatment. Localization of
243 MSP1, KAHRP and HRP2 was unaffected; however, the HRP2 signal was significantly decreased in
244 intensity. Parasite nuclei were visualised by staining with DAPI.

245

246 Discussion

247 Malarial proteolytic enzymes play regulatory and effector roles in multiple key biological
248 processes in this important pathogen and have long been of interest as potential drug targets. In this study,
249 we have shown for the first time that functional ablation of the *pfpmv* gene leads to a block at the ring-to-
250 trophozoite transition and death of the parasite. To achieve this, we employed the robust conditional
251 DiCre approach in combination with Cas9-induced gene modification. This system provides a rapid and
252 efficient means of generating transgenic parasites as well as enabling complete disruption of the gene of
253 interest [29,30]. Our study demonstrates that DiCre-mediated conditional knockout is a powerful tool to
254 study essential genes in the human malaria parasite. Our genetic strategy also incorporated a method to
255 permit the selection of parasites in which genomic integration of the input constructs had taken place,
256 termed selection-linked integration (SLI), previously developed by Birnbaum *et al* [25]. For this, our
257 rescue plasmid contained an SLI-resistance marker (neomycin phosphotransferase), which was linked to
258 the modified *pfpmv* gene separated by a 2A 'skip' peptide [31,32]. This approach allows selection for
259 correct integration by one to two weeks of neomycin selection. In our case, with the assistance of Cas9-
260 mediated integration we in fact did not need to use neomycin selection in our experiments to select for
261 correct integration. However, our plasmids could be useful for future genetic complementation studies.

262 When the PMV-modified parasite clone PMV-C5 was RAP-treated at early ring stage for just 3
263 h, followed by removal of RAP, parasite development proceeded normally in the first erythrocytic cycle
264 (cycle 0). Whilst expression of most of the parasite proteins examined appeared unaffected, we did
265 observe some impact on expression of the exported parasite protein HRP2. This is reminiscent of the
266 observation by Russo *et al* [21] that the levels of HRP2 and RESA (ring-infected erythrocyte surface
267 antigen) were reduced by 30-50% in PMV mutants. We suspect that this may be due to low levels of
268 PMV expression early in cycle 0, perhaps due to transcription early in the erythrocytic cycle prior to gene

269 excision. That low levels of PMV are sufficient to sustain development has also been indicated by
270 previous conditional protein knockdown experiments [18]. Egress and invasion at the end of cycle 0 was
271 not affected in the RAP-treated PMV-C5 parasites; however, parasite development in the next cycle
272 stalled at the ring-to-trophozoite transition. This phenotype is very similar to that observed upon
273 treatment with the PMV inhibitor WEHI-916 [18], where the drug-treated parasites showed a growth
274 defect at the ring-to-trophozoite transition from approximately 20 h post-invasion. A similar parasite
275 developmental arrest at ring stage was also observed following conditional ablation of the PTEX
276 component HSP101 [33,34]. This implies that PMV function may be associated with PTEX, and may be
277 essential for ring-to-trophozoite development.

278 In summary, using a DiCre-mediated conditional gene editing approach to selectively disrupt the
279 *pfpmv* gene, we have shown that the gene is essential for the ring-to-trophozoite transition of intracellular
280 growth. This engineered platform will be useful for further study of PEXEL-protein export as well as for
281 dissection of PMV domain interactions, providing further impetus for focusing on PMV as a new
282 potential antimalarial drug target.

283

284

285 **Materials and Methods**

286

287 ***P. falciparum* culture, transfection and limiting dilution cloning**

288 Parasites (wild type clone 3D7 and the DiCre-expressing clone B11 [26]) were routinely cultured
289 at 37°C in human erythrocytes at 1-4% haematocrit in RPMI 1640 (Life Technologies) supplemented
290 with 2.3 gL⁻¹ sodium bicarbonate, 4 gL⁻¹ dextrose, 5.957 gL⁻¹ HEPES, 0.05 gL⁻¹ hypoxanthine, 0.5%
291 (w/v) Albumax II, 0.025 gL⁻¹ gentamycin sulphate, and 0.292 gL⁻¹ L-glutamine (complete medium) in an
292 atmosphere of 90% nitrogen, 5% carbon dioxide and 5% oxygen [35,36]. Routine microscopic
293 examination of parasite growth was performed by fixing air-dried thin blood films with 100% methanol
294 before staining with 10% Giemsa stain (VWR international) in 6.7 mM phosphate buffer, pH 7.1. For
295 synchronization, mature schizont stage parasites were isolated on cushions of 70% (v/v) isotonic Percoll
296 (GE Healthcare) as previously described [37,38]. Enrichment for ring stages following invasion was
297 performed using 5% (w/v) D-sorbitol [38,39].

298

299 **Cloning of repair plasmid pT2A-5'UTR-3'-PMV- ΔDHFR**

300 pT2A-5'UTR-3'-PMV- ΔDHFR plasmid is based on vector pT2A-DDI-1cKO (a kind gift of Dr.
301 Edgar Deu, the Francis Crick Institute). The plasmid comprised 5'UTR of *pfpmv* and nucleotides 1-397 of
302 *pfpmv* (Met1 to Lys132), followed by synthetic heterologous *loxP*-containing *sera2* and *sub2* introns
303 (*loxPint*) [24] flanking recodonised *pfpmv* sequence encoding residues Asp133 to Thr590 (GeneArt)
304 fused to a 3xHA epitope tag sequence, a 2A 'skip' peptide (2A) [40], and the neomycin resistance gene
305 (*neo*). The second *loxPint* site was immediately followed by another 2A sequence, a *gfp* gene and
306 nucleotides 1333 to 1773 of *pfpmv* which encode Lys445 to Thr590, as a 3'- targeting sequence.

307 Overlapping PCR was used to generate the *loxPint-2A-gfp* fragment from the template pT2A-DDI-1-
308 cKO-complement (obtained from Dr. Edgar Deu, the Francis Crick Institute). The resulting PCR product
309 was digested with *XhoI* and ligated into pT2A-DDI-1cKO pre-digested with the same restriction
310 enzymes, yielding plasmid pT2A-DDI-1-cKO-modified GFP (S1 Fig). The 5'-targeting sequence (903
311 bp) was PCR amplified from 3D7 genomic DNA using Phusion HF DNA polymerase (NEB) with
312 forward primer P3.1-*BglIII*_5'UTR_F-mod and reverse primer P2-Int_PMV_R. The recodonised fragment
313 (1,602 bp) containing *loxPint*, recodonised *pfpmv*, 3xHA, and 2A was amplified from the plasmid
314 17ACRILIP_2177297_endoPMV (GeneArt) with primers P4-PMV_int_F and P5-T2A_HA_R. PCR
315 products of 5'-target region and the recodonised fragment were ligated with pT2A-DDI-1-cKO-modified
316 GFP pre-digested with *BglIII* and *SalI* using In-fusion[®] HD cloning kit (Clontech, Mountain View, CA),
317 producing the plasmid pT2A-5'UTR-PMV-cKO. The 3'-target region (441 bp) was amplified from 3D7
318 genomic DNA using Phusion HF DNA polymerase (NEB) with forward primer P16-EcoRV-PMV-F and
319 reverse primer P17-EcoRV-PMV-R. This fragment was then ligated into pT2A-5'UTR-PMV-cKO pre-
320 digested with *EcoRV*, yielding the plasmid pT2A-5'UTR-3'PMV-cKO. The *hdhfr* gene was removed
321 from pT2A-5'UTR-3'PMV-cKO by digesting the plasmid with *BamHI* and *EcoRI*. The plasmid
322 backbone was blunt-end using T4 DNA polymerase (NEB) then religated using T4 DNA ligase (NEB),
323 giving rise to the repair plasmid pT2A-5'UTR-3'-PMV- ΔDHFR.

324

325 **Insertion of guide RNA sequences into CRISPR/Cas9 plasmids**

326 Potential guide RNA sequences specifically targeting *pfpmv* were identified using Benchling
327 (<https://benchling.com/crispr>). Two sets of guide RNA sequences were selected. Two pairs of
328 complementary oligonucleotides (P18-sgPMV-1F and P19-sgPMV-1R; P20-sgPMV-2F, and P21-
329 sgPMV-2R) corresponding to the 19 nucleotides adjacent to the identified PAM sequences were

330 phosphorylated using T4 polynucleotide kinase, annealed and ligated into pL-AJP_004 [41] predigested
331 with *BbsI*, resulting in the two guide vectors pSgRNA1 and pSgRNA2.

332

333 **Generation of *pmv-loxPint* parasites and conditional PMV truncation**

334 The repair plasmid pT2A-5'UTR-3'-PMV- Δ DHFR was linearized with *ScaI* prior to
335 electroporation. Percoll-enriched mature schizonts of *P. falciparum* clone B11 were electroporated with
336 20 μ g of pSgRNA1 or pSgRNA2 and 60 μ g of linearized pT2A-5'UTR-3'-PMV- Δ DHFR using an
337 Amaxa P3 primary cell 4D Nucleofector X Kit L (Lonza) as described [22]. Twenty-four hours post-
338 transfection, the electroporated parasites were treated with 2.5 nM WR99210 for 96 h to select for
339 transfectants harbouring pSgRNA plasmids before returning the cultures to medium without drug.
340 Integrant parasites generally reached parasitaemia levels suitable for cryopreservation within 2-5 weeks.
341 Detection of the *pfpmv-loxPint* modified locus was carried out by diagnostic PCR using primer pairs P6-
342 5'UTR_Screen_F and P23-rcPMV-5'integr-R, P6-5'UTR_Screen_F and 198_GFP_start_seq_R, and P24-
343 GFP-3'integr-F and P25-3'UTR-PMV-R. The wild-type *pfpmv* locus was detected by diagnostic PCR
344 using primers P6-5'UTR_Screen_F and P22-PMV-endo-R. Transgenic parasite clones were obtained by
345 limiting dilution cloning by plating a calculated 0.3 parasite per well in flat-bottomed 96-well microplate
346 wells as described [42]. Wells containing single plaques were subsequently expanded into round-
347 bottomed wells. Transgenic parasite clones (PMV-C5 and PMV-F9) were finally checked by diagnostic
348 PCR for integration and modification of the endogenous *pfpmv* gene. Once established, all transgenic
349 clones were maintained in medium without any drug.

350 Recombination between the *loxPint* sites was induced in tightly synchronised ring-stages of
351 parasite clone PMV-C5 by incubation for 3 h in the presence of 100 nM RAP in 1% (v/v) DMSO; mock
352 treatment was with 1% (v/v) DMSO only. DiCre-mediated excision of the floxed *pfpmv* was detected by

353 PCR analysis of parasite genomic DNA using primers P6-5'UTR_Screen_F and P23-rcPMV-5'integr-R,
354 and P6-5'UTR_Screen_F and Deu198_GFP_start_seq_R. Truncation of *PfPMV* was evaluated by
355 immunoblot analysis of SDS extracts of mature Percoll-enriched schizonts, probing with the anti-HA
356 antibody 3F10 (Roche), followed by horseradish peroxidase-conjugated secondary antibodies.

357

358 **Nucleic acid extraction and polymerase chain reaction**

359 For DNA extraction, total cell pellets were first treated with 0.15% saponin in PBS for 10 min,
360 then washed with PBS before DNA was extracted using a DNeasy[®] Blood & Tissue Kit (QIAGEN). For
361 diagnostic PCR amplification, GoTaq[®] (Promega) DNA master mix was used. Amplification of
362 fragments used in construct design was carried out using Phusion[®] high fidelity DNA polymerase (NEB).

363

364 **Indirect immunofluorescence Analysis (IFA) and Western blots**

365 For IFA, thin blood films were prepared from synchronous *P. falciparum* cultures enriched in
366 mature schizonts. The air-dried thin films were fixed in 4% (w/v) paraformaldehyde for 30 min,
367 permeabilised with 0.1% (v/v) Triton X-100 for 10 min, and blocked overnight in 3% (w/v) bovine serum
368 albumin (BSA) in PBS. Slides were probed with rat anti-HA 3F10 (1:100) to detect HA-tagged proteins,
369 human anti-MSP1 monoclonal antibody (mAb) X509 (1:500) to detect MSP1, mAb 89 (1:100) to detect
370 KAHRP, and mAb 2G12 (1:100) to detect HRP2. Primary antibodies were detected using Alexa Fluor
371 594-conjugated anti-human or anti-mouse secondary antibodies (Life Technologies), and Alexa Fluor
372 488-conjugated streptavidin (Life Technologies), diluted 1:2000. Slides were stained with 4,6-diamidino-
373 2-phenylindole (DAPI), mounted in Citifluor (Citifluor Ltd., UK). Images were visualised using a Nikon
374 Eclipse Ni microscope with LED-illumination with a 63x Plan Apo λ NA 1.4 objective. Images were

375 taken using an Orca Flash 4 digital camera controlled by Nikon NIS Element AR 4.30.02 software. All
376 images were subsequently analysed using FIJI software.

377 For Western blots, Percoll-enriched schizonts were pelleted, then resuspended into 10 volumes of
378 PBS. Samples were solubilised into SDS sample buffer, boiled, sonicated and centrifuged. The extracts
379 were subjected to SDS-PAGE under reducing conditions followed by transfer to nitrocellulose
380 membrane. Membranes were probed with rat anti-HA 3F10 (1:1000), human anti-MSP1 mAb X509
381 (1:1000), anti-GFP (1:1000), mAb 89 (1:1000) or mAb 2G12 (1:1000), followed by horseradish
382 peroxidase-conjugated secondary antibodies. Antigen-antibody interactions were visualised by enhanced
383 chemiluminescence (SuperSignal West Pico chemiluminescent substrate, Pierce).

384

385 **Parasite growth assay**

386 Parasitaemia measurement by FACS was as described previously [43]. Briefly, parasites
387 recovered at various time-points were fixed in 8% paraformaldehyde 0.04% glutaraldehyde, pH 7.4 and
388 stained with 2 μ M Hoechst 33342 (Invitrogen, Waltham, MA). Parasitaemia was calculated using the
389 FACS BD Fortessa flow cytometer (BD Biosciences). Briefly, cultures to be analysed were initially
390 screened using forward and side scatter parameters and gated for erythrocytes. From this gated
391 population, the proportion of Hoechst-stained cells in 100,000 cells was determined using ultraviolet light
392 with a violet filter (450/50 nm). Samples were analysed using FlowJo software.

393

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395

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399

400 **Author Contributions**

401 Conceived and designed the experiments: NB, CRC, FH, CWM, MJB. Performed the experiments:
402 NB, CRC, FH. Analysed the data: NB, CRC, MJB. Wrote the paper: NB, CRC, MJB.

403

404 **References**

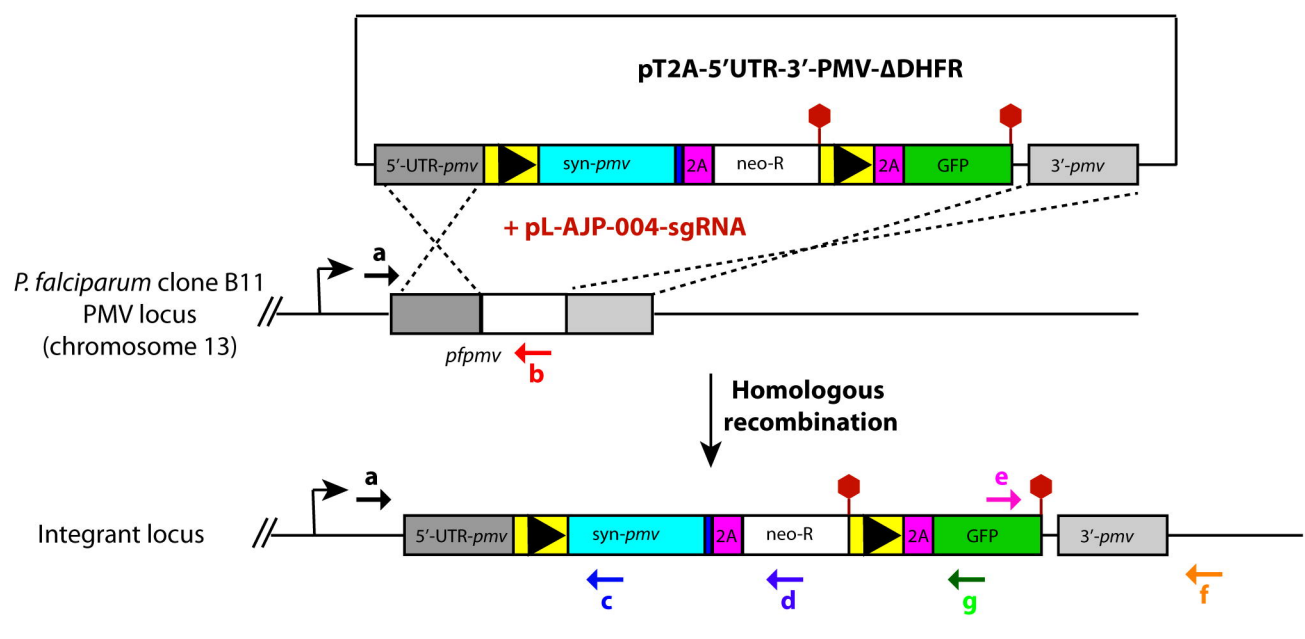
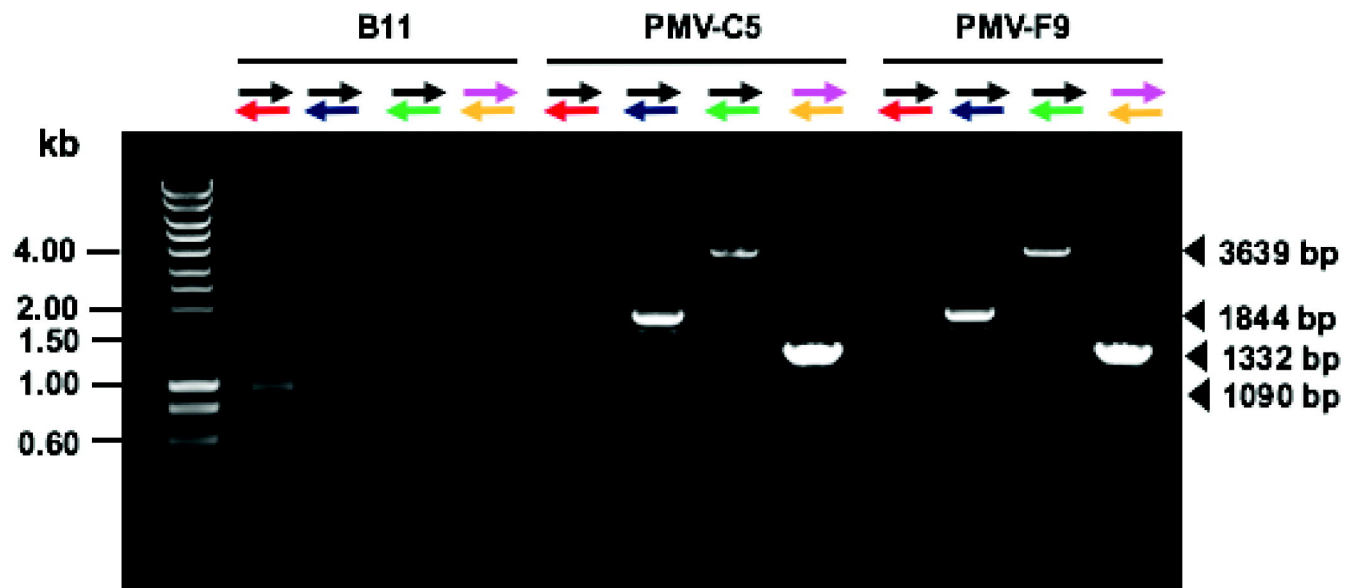
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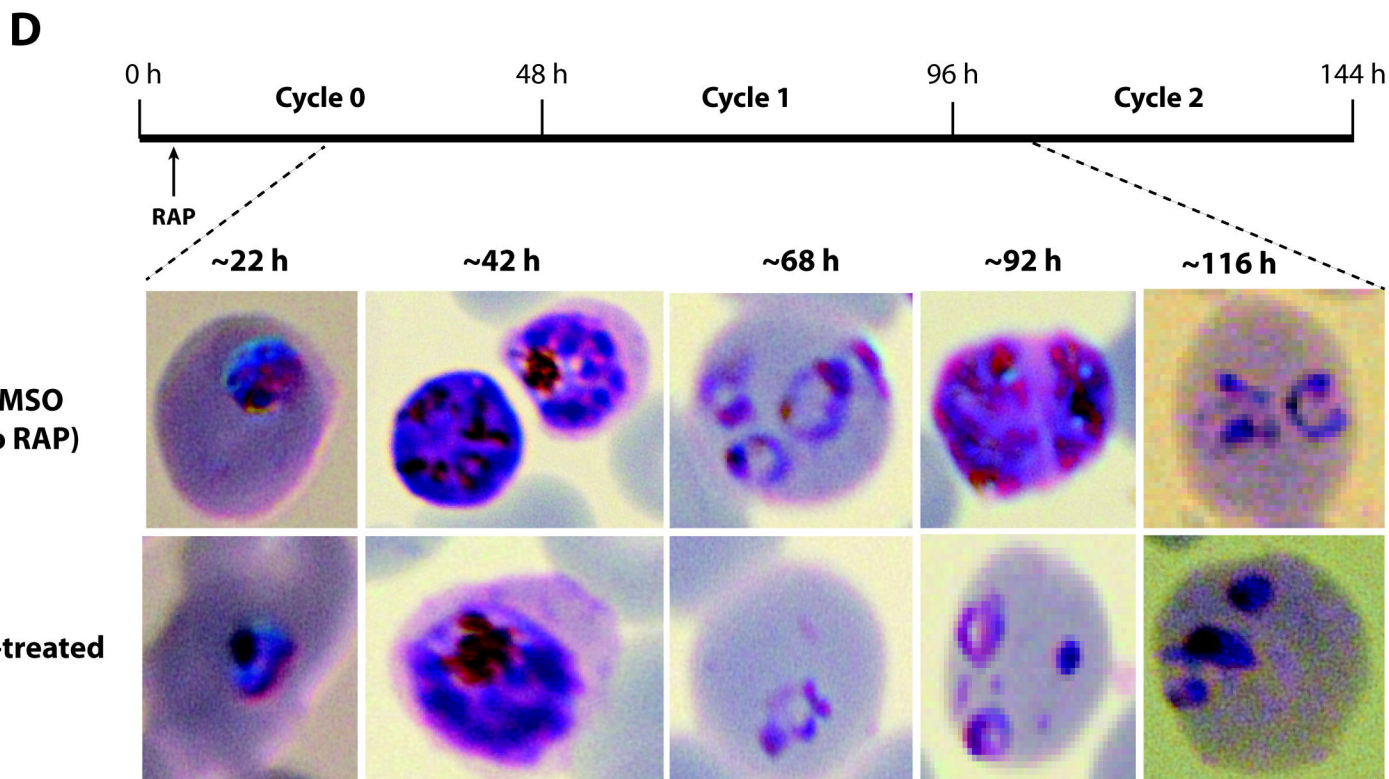
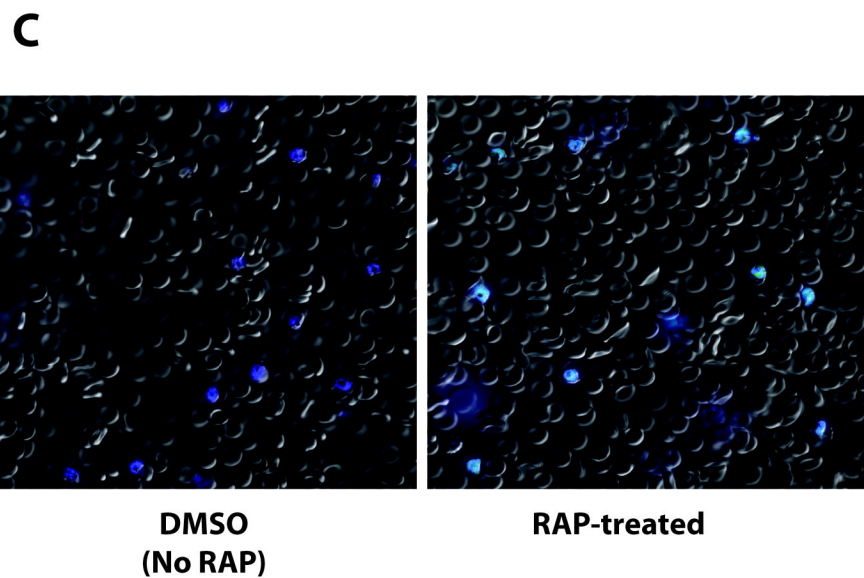
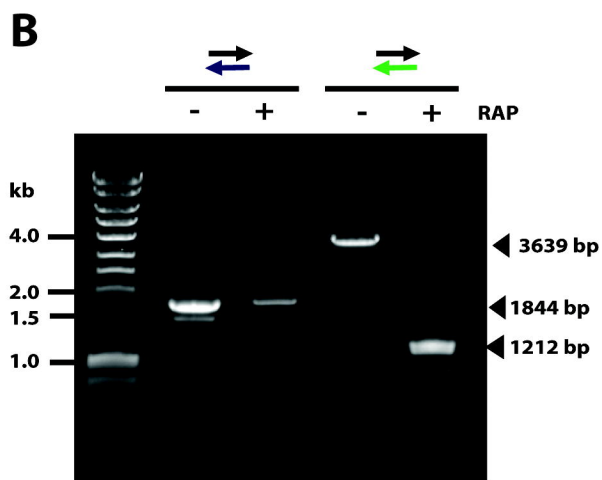
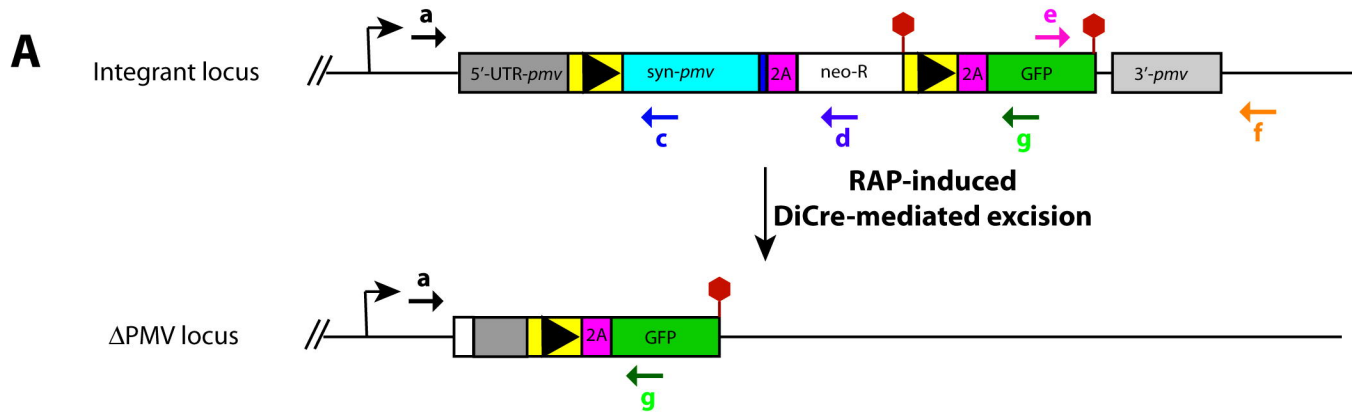
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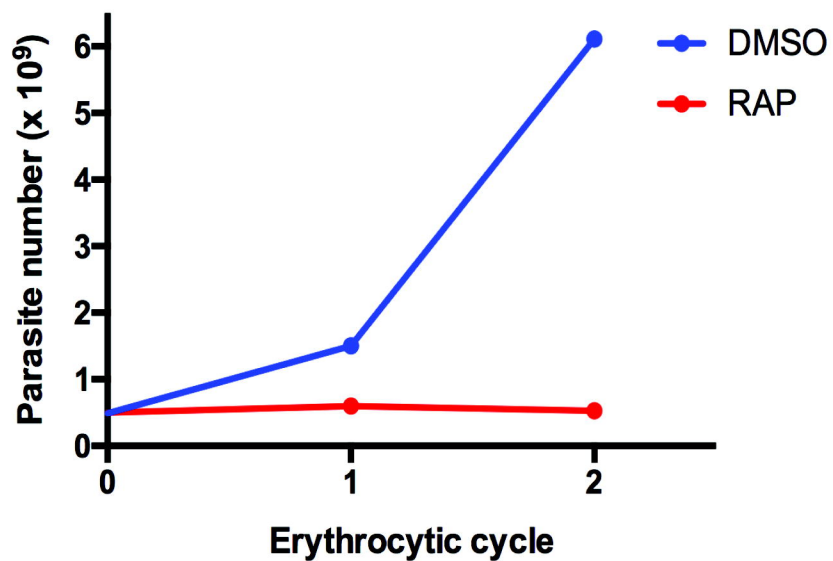
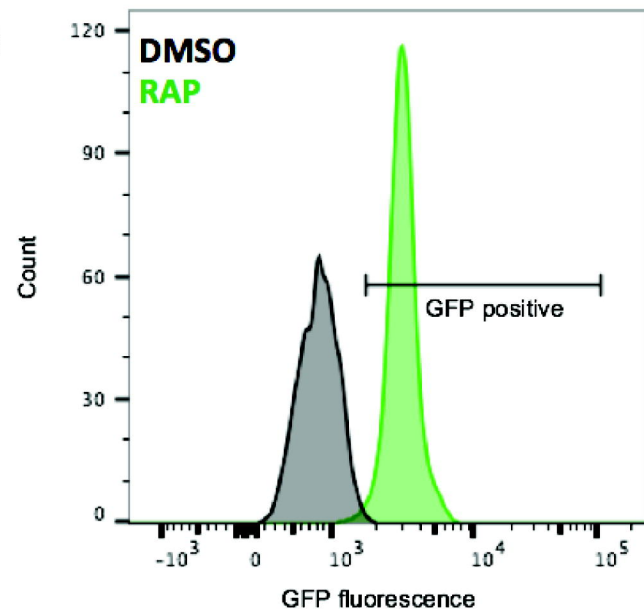
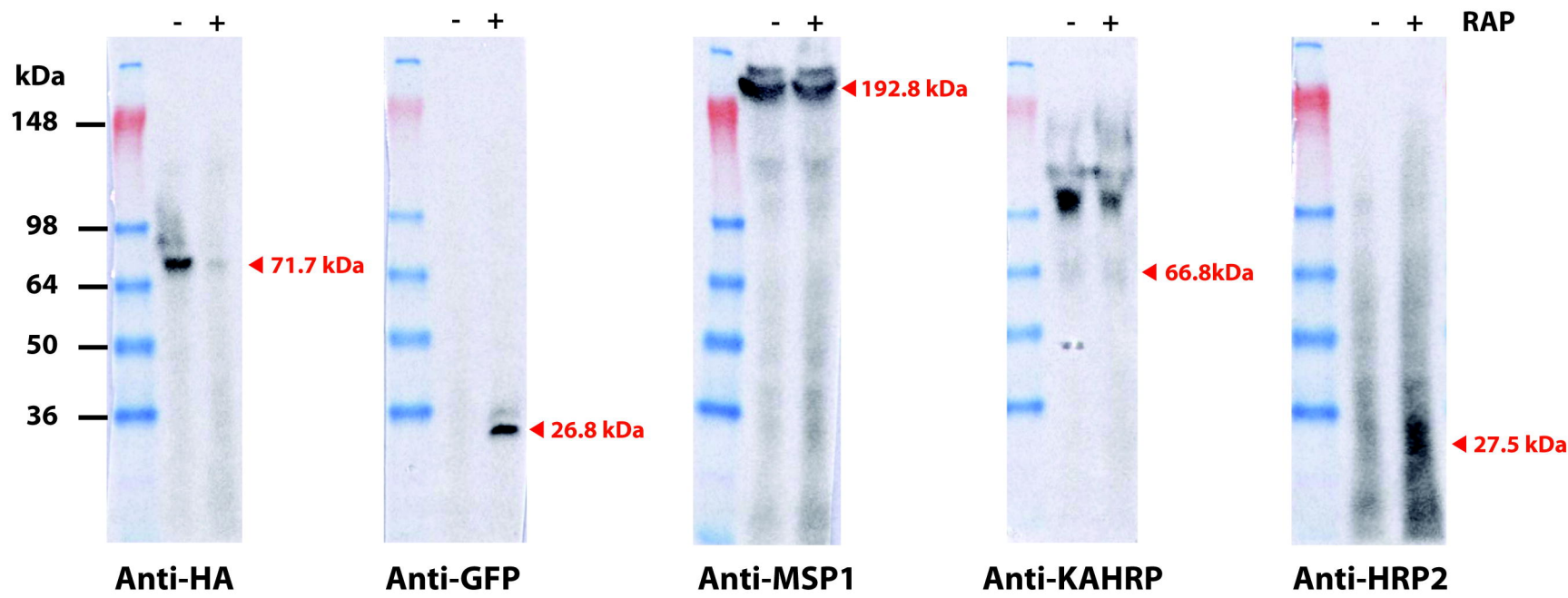
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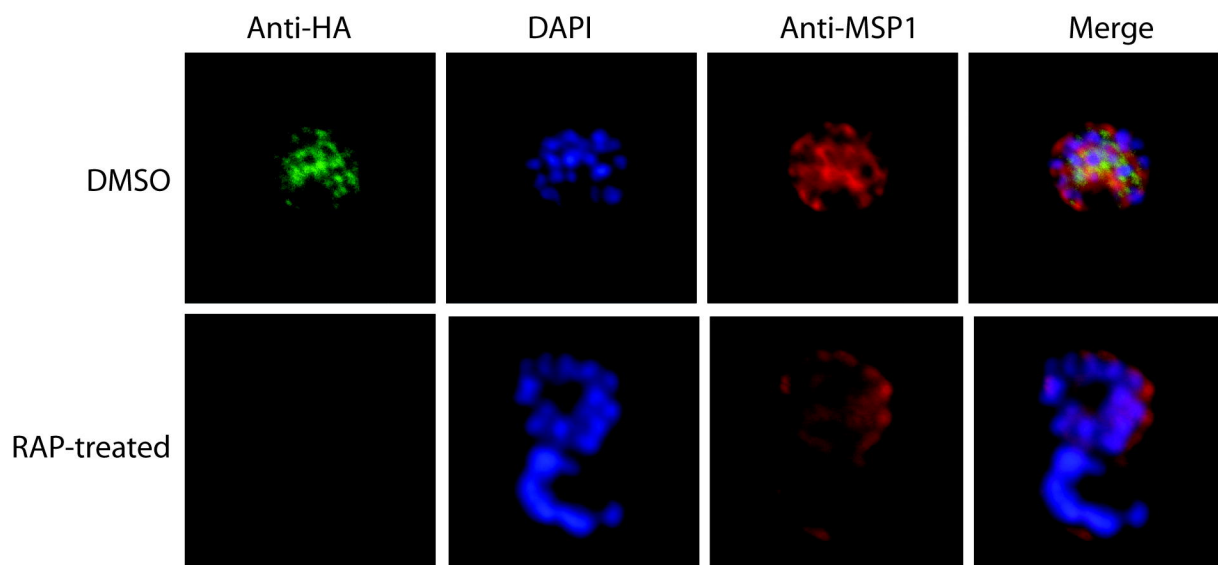
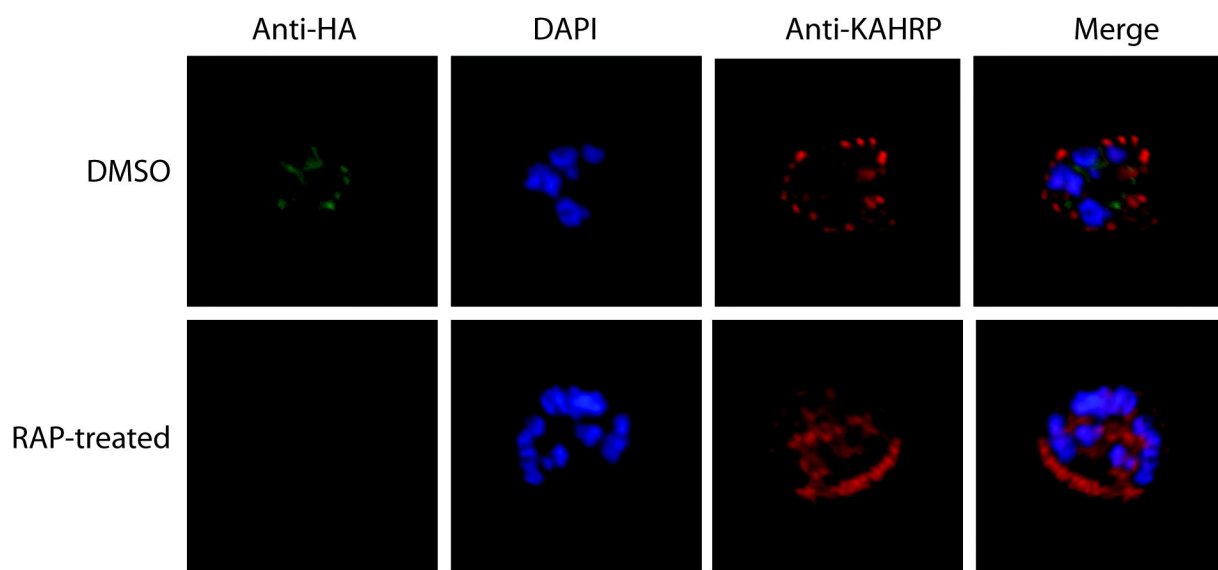
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