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1 **The AMA1-RON complex drives *Plasmodium* sporozoite invasion in the mosquito and**
2 **mammalian hosts**

3

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24 Running head: AMA1-RON complex in *Plasmodium* sporozoites

25

26 Keywords: *Plasmodium*; malaria; sporozoites; conditional mutagenesis; AMA1; RONS.

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28

29 **Abstract**

30 *Plasmodium* sporozoites that are transmitted by blood-feeding female *Anopheles* mosquitoes
31 invade hepatocytes for an initial round of intracellular replication, leading to the release of
32 merozoites that invade and multiply within red blood cells. Sporozoites and merozoites share
33 a number of proteins that are expressed by both stages, including the Apical Membrane
34 Antigen 1 (AMA1) and the Rhoptry Neck Proteins (RONs). Although AMA1 and RONs are
35 essential for merozoite invasion of erythrocytes during asexual blood stage replication of the
36 parasite, their function in sporozoites was still unclear. Here we show that AMA1 interacts with
37 RONs in mature sporozoites. By using DiCre-mediated conditional gene deletion in *P. berghei*,
38 we demonstrate that loss of AMA1, RON2 or RON4 in sporozoites impairs colonization of the
39 mosquito salivary glands and invasion of mammalian hepatocytes, without affecting
40 transcellular parasite migration. Three-dimensional electron microscopy data showed that
41 sporozoites enter salivary gland cells through a ring-like structure and by forming a transient
42 vacuole. The absence of a functional AMA1-RON complex led to an altered morphology of the
43 entry junction, associated with epithelial cell damage. Our data establish that AMA1 and RONs
44 facilitate host cell invasion across *Plasmodium* invasive stages, and suggest that sporozoites
45 use the AMA1-RON complex to efficiently and safely enter the mosquito salivary glands to
46 ensure successful parasite transmission. These results open up the possibility of targeting the
47 AMA1-RON complex for transmission-blocking antimalarial strategies.

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49

50 **Author summary**

51 Malaria is caused by *Plasmodium* parasites, which are transmitted by mosquitoes. Infectious
52 stages of the parasite known as sporozoites colonize the mosquito salivary glands and are
53 injected into the host when the insect probes the skin for blood feeding. Sporozoites rapidly
54 migrate to the host liver, invade hepatocytes and differentiate into the next invasive forms, the
55 merozoites, which invade and replicate inside red blood cells. Merozoites invade cells through
56 a specialized structure, known as the moving junction, formed by proteins called AMA1 and
57 RONs. The role of these proteins in sporozoites remains unclear. Here we used conditional
58 genome editing in a rodent malaria model to generate AMA1- and RON-deficient sporozoites.
59 Phenotypic analysis of the mutants revealed that sporozoites use the AMA1-RON complex
60 twice, first in the mosquito to safely enter the salivary glands and ensure successful parasite
61 transmission, then in the mammalian host liver to establish a replicative niche. Our data
62 establish that AMA1 and RONs facilitate host cell invasion across *Plasmodium* invasive
63 stages, and might represent potential targets for transmission-blocking antimalarial strategies.

64

65

66 **Introduction**

67 Host cell invasion is an obligatory step in the *Plasmodium* life cycle. There are several
68 invasive stages of *Plasmodium*, each equipped with its own set of specialized secretory
69 organelles and proteins that facilitate invasion into or through host cells. Invasive stages of
70 Apicomplexa typically invade target host cells actively by gliding through a structure known as
71 the moving junction (MJ), which consists of a circumferential zone of close apposition of
72 parasite and host cell membranes. Studies with *Toxoplasma gondii* tachyzoites and
73 *Plasmodium falciparum* merozoites have shown that formation of the MJ involves the export
74 of rhoptry neck proteins RONs into the host cell, where RON2 is inserted into the host cell
75 membrane and serves as a receptor for the Apical Membrane Antigen 1 (AMA1), that is
76 secreted from the micronemes onto the surface of the parasite [1–3]. Formation of the MJ is
77 associated with active penetration inside the parasitophorous vacuole (PV), which is essential
78 for further development and replication of the parasite.

79 Although the AMA1-RON2 interaction seems to be conserved across the phylum of
80 Apicomplexa, its role in *Plasmodium* sporozoites is controversial. *Plasmodium* sporozoites
81 express AMA1 and the RON proteins RON2, RON4 and RON5 [4–10]. Two studies reported
82 that AMA1 is not essential for development in the mosquito and during hepatocyte invasion in
83 *P. berghei*, while RON4 in contrast was shown to be essential for hepatocyte invasion,
84 suggesting independent roles for AMA1 and RON proteins in sporozoites [7,11]. However,
85 both polyclonal antibodies against AMA1 [4] and the R1 peptide inhibitor of AMA1 [12],
86 effectively reduced hepatocyte invasion by *P. falciparum* sporozoites [13]. More recently, a
87 promoter swap strategy was employed to knockdown RONs in *P. berghei* sporozoites,
88 uncovering an unexpected role of these proteins during invasion of the mosquito salivary
89 glands [14,15]. Owing to these conflicting data, the precise role of AMA1 and RONs in
90 *Plasmodium* sporozoites is uncertain.

91 As conventional reverse genetics cannot be used to target AMA1 and RONs, due to their
92 essential nature in asexual blood stages, previous studies relied on conditional approaches
93 such as the Flippase (FLP)/Flp recombination target (FRT) system [7] or promoter swap

94 strategies [14] to target these genes. The rapamycin inducible DiCre recombinase system, first
95 introduced to apicomplexan research in *T. gondii* [16] and *P. falciparum* [17], has recently
96 emerged as a potent method of gene inactivation in different developmental stages of *P.*
97 *falciparum* [18] and *P. berghei* [19]. We recently described a fluorescent DiCre-expressing
98 parasite line in *P. berghei* and showed that efficient and complete gene excision can be
99 induced in asexual blood stages and also sporozoites [19]. In this study, we used the DiCre
100 system to achieve conditional deletion of *ama1*, *ron2* and *ron4* genes in *P. berghei* sporozoites.
101 Our data reveal that sporozoites rely on AMA1 and RONs to invade salivary glands in the
102 mosquito and hepatocytes in the mammalian host, implying a conserved feature of the invasion
103 process across invasive stages of *Plasmodium*.

104

105

106 **Results**

107 **Deletion of *ama1* 3'UTR is not sufficient to abrogate AMA1 expression in *P. berghei***

108 To ablate AMA1 protein expression in *P. berghei*, we first decided to conditionally delete
109 the 3' untranslated region (UTR) of *ama1* using the DiCre method, as previously reported with
110 the FLP/FRT system [7]. We floxed the 3'UTR of *ama1*, together with a GFP and an hDHFR
111 marker, to generate the *ama1* Δ utr parasite line in the mCherry-expressing PbDiCre parasite
112 background [19] (**Figs 1A** and **S1A**). To exclude any unspecific effects arising from
113 modification of the *ama1* locus, we also generated a control parasite line (*ama1*Con) where
114 we introduced the LoxN sites downstream of the 3' UTR (**Figs 1B** and **S2A**). After transfection
115 and selection with pyrimethamine, pure populations of recombinant parasites were sorted by
116 flow cytometry and genotyped by PCR to confirm correct genomic integration of the constructs
117 and to exclude the presence of any residual unmodified PbDiCre parasites (**S1B** and **S2B**
118 **Figs**).

119

120

121 **Fig 1. Deletion of the 3' UTR of *ama1* has no phenotypical impact in *P. berghei***

122 **A-B.** Strategy to generate *ama1* Δ utr (A) and *ama1*Con (B) parasites by modification of the wild
123 type *ama1* locus in PbDiCre parasites. **C-D.** Blood stage growth of untreated and rapamycin-
124 treated *ama1* Δ utr (C) or *ama1*Con (D) parasites. Rapamycin was administered at day 2. The
125 graphs represent the parasitaemia (mean +/- SEM) in groups of 3 mice. **E.**
126 Immunofluorescence staining of rapamycin-treated *ama1*Con and *ama1* Δ utr blood stage
127 schizonts with anti-AMA1 antibodies (blue). The right panels show mCherry (red), GFP (green)
128 and AMA1 (blue) merged images. Scale bar = 10 μ m. **F.** Immunofluorescence images of
129 rapamycin-treated *ama1*Con and *ama1* Δ utr sporozoites after staining with anti-AMA1
130 antibodies (magenta). The right panels show Hoechst (blue) and AMA1 (magenta) merged
131 images. Scale bar = 5 μ m.

132

133

134 We next analyzed the effects of rapamycin on *ama1Con* and *ama1Δutr* parasites during
135 blood stage growth (**Figs 1C** and **1D**), by quantifying the percentage of excised
136 (mCherry⁺/GFP⁻) and non-excised (mCherry⁺/GFP⁺) parasites by flow cytometry (**S1C** and
137 **S2C Figs**). In the *ama1Con* infected group, rapamycin treatment induced complete excision
138 of the floxed GFP cassette (**S2C Fig**), which, as expected, had no significant effect on parasite
139 growth and multiplication in the blood, which was comparable to the untreated group (**Fig 1D**).
140 Excision of the GFP cassette was also confirmed by genotyping PCR (**S2B Fig**). Surprisingly,
141 rapamycin treatment of the *ama1Δutr* infected group also had no effect on both parasite growth
142 and multiplication in the blood (**Fig 1C**), despite efficient DNA excision based on
143 disappearance of the GFP cassette after rapamycin treatment (**S1C Fig**). Genotyping of
144 mCherry⁺/GFP⁻ parasites by PCR and sequencing of the locus after excision confirmed that
145 the 3'UTR had been excised in rapamycin-treated *ama1Δutr* parasites, excluding any
146 contamination with parental PbDiCre (**S1B Fig**).

147 We next examined rapamycin-treated *ama1Con* and *ama1Δutr* blood-stage schizonts by
148 immunofluorescence staining with anti-AMA1 antibodies. Intriguingly, we observed AMA1
149 expression in both *ama1Con* and *ama1Δutr* merozoites after rapamycin exposure (**Fig 1E**),
150 implying that deletion of the *ama1* 3'UTR alone was not sufficient to abrogate expression of
151 the protein in merozoites. We further analyzed the impact of 3'UTR deletion on AMA1
152 expression in sporozoites. For this purpose, *ama1Con* and *ama1Δutr* parasites were treated
153 with rapamycin or left untreated and then transmitted to mosquitoes, as described previously
154 [19]. Deletion of the *ama1* 3'UTR in *ama1Δutr* parasites had no impact on oocyst formation in
155 the midgut or sporozoite invasion of salivary glands, which were comparable to untreated
156 *ama1Δutr* and both rapamycin-treated and untreated *ama1Con* parasites (**S3 Fig**). As
157 observed in merozoites, AMA1 protein was also detected in salivary gland sporozoites from
158 rapamycin-treated *ama1Δutr* by immunofluorescence, similar to *ama1Con* parasites (**Fig 1F**).
159 We conclude from these data that deletion of the 3'UTR of *ama1* is not sufficient to abrogate
160 AMA1 protein expression and cause phenotypical changes in *P. berghei* merozoites and
161 sporozoites.

162

163 **Complete conditional gene deletion of *ama1* in *P. berghei***

164 Since deletion of the 3'UTR was insufficient to deplete AMA1, we decided to delete the
165 full-length *ama1* gene, by placing LoxN sites both upstream and downstream of the gene (**Fig**
166 **2A**). One intrinsic feature of the Cre Lox system is the retention of a Lox site following
167 recombination. We therefore reused rapamycin-treated *ama1*Con parasites, which contained
168 a single LoxN site downstream of *ama1* 3'UTR and had excised the GFP-hDHFR marker (**Fig**
169 **1B**), and transfected these parasites with the *ama1*cKO construct designed to introduce a
170 second LoxN site upstream of the *ama1* gene, together with a GFP-hDHFR cassette (**Figs 2A**
171 and **S4A**). Following transfection, the resulting *ama1*cKO parasites were sorted by FACS and
172 genotyped to confirm correct integration of the construct into the genome and verify the
173 absence of any residual unmodified *ama1*Con parasites (**S4B Fig**). We then evaluated the
174 effect of rapamycin treatment on blood-stage growth of *ama1*cKO parasites, by injecting mice
175 with 10⁶ pRBCs and treating them with a single oral dose of rapamycin. In contrast to untreated
176 parasites, *ama1*cKO parasite growth was abrogated in mice upon rapamycin exposure (**Fig**
177 **2B**), thus confirming efficient gene deletion and the essential role of AMA1 in merozoite
178 invasion and parasite survival in the blood. Genotyping by PCR confirmed *ama1* gene excision
179 in rapamycin-exposed *ama1*cKO parasites, but also revealed the persistence of non-excised
180 parasites 2 and 6 days after rapamycin treatment (**S4C Fig**), which eventually outcompeted
181 the excised population.

182

183

184 **Fig 2. AMA1 is required during *P. berghei* invasion of mosquito salivary glands**

185 **A.** Strategy to generate *ama1*cKO parasites by modification of the *ama1* locus in rapamycin-
186 treated *ama1*Con parasites. **B.** Blood stage growth of rapamycin-treated and untreated
187 *ama1*cKO parasites. The graph represents the parasitaemia (mean +/- SEM) in groups of 3
188 mice. Rapamycin was administered at day 2. **, p < 0.01; ****, p < 0.0001 (Two-way ANOVA).
189 **C-E.** Quantification of midgut sporozoites (MG-SPZ, C), salivary gland sporozoites (SG-SPZ,

190 D) or haemolymph sporozoites (HL-SPZ, E) isolated from mosquitoes infected with untreated
191 or rapamycin-treated *ama1Con* and *ama1cKO* parasites. The graphs show the number of
192 sporozoites per female mosquito (mean +/- SEM). Each dot represents the mean value
193 obtained in independent experiments after dissection of 30-50 mosquitoes (MG, HL) or 50-70
194 mosquitoes (SG), respectively. Ns, non-significant; ****, $p < 0.0001$ (One-way ANOVA followed
195 by Tukey's multiple comparisons test). **F-H.** Quantification of excised (mCherry⁺/GFP⁻, red)
196 and non-excised (mCherry⁺/GFP⁺, green) midgut sporozoites (MG-SPZ, F), salivary gland
197 sporozoites (SG-SPZ, G) or haemolymph sporozoites (HL-SPZ, H) isolated from mosquitoes
198 infected with untreated or rapamycin-treated *ama1Con* and *ama1cKO* parasites. **I.**
199 Immunofluorescence imaging of untreated and rapamycin-treated *ama1cKO* salivary gland
200 sporozoites after staining with anti-AMA1 antibodies (magenta). The right panels show
201 Hoechst (blue) and AMA1 (magenta) merged images. Scale bar = 5 μ m. **J.** Quantification of
202 AMA1-positive and AMA1-negative sporozoites among untreated or rapamycin-exposed
203 *ama1Con*, *ama1 Δ utr* and *ama1cKO* sporozoites, as assessed by microscopy.

204

205

206 **AMA1 is required for sporozoite invasion of the mosquito salivary glands**

207 In order to determine the function of AMA1 in sporozoites, we transmitted rapamycin-
208 treated and untreated *ama1cKO* parasites to mosquitoes, 24 hours after rapamycin treatment.
209 In parallel, mosquitoes were fed with rapamycin-treated and untreated *ama1Con* parasites as
210 a reference line. Both rapamycin-treated and untreated *ama1cKO* parasites were capable of
211 colonising the mosquito midgut (**S5 Fig**), comparable to *ama1Con* parasites (**S3 Fig**). Despite
212 no difference in the levels of exflagellation between the parasite lines and treatment conditions,
213 we observed a slight reduction in the number of midgut sporozoites for rapamycin-exposed
214 *ama1cKO* parasites, which however was not statistically significant (**Fig 2C**). Importantly,
215 quantification of the percentage of excised (mCherry⁺/GFP⁻) and non-excised
216 (mCherry⁺/GFP⁺) parasites revealed close to 100% gene excision in sporozoites isolated from

217 the midguts of mosquitoes infected with rapamycin-treated *ama1Con* and *ama1cKO* parasites
218 **(Fig 2F)**.

219 In the next step, we quantified sporozoites isolated from the salivary glands of infected
220 mosquitoes and observed no difference between mosquitoes infected with untreated
221 *ama1Con* or *ama1cKO* parasites **(Fig 2D)**. In sharp contrast, the number of salivary gland
222 sporozoites isolated from rapamycin-treated *ama1cKO* infected mosquitoes was severely
223 reduced as compared to untreated parasites **(Fig 2D)**. As expected, we could only observe
224 mCherry⁺/GFP⁺ (non-excised) salivary gland sporozoites in untreated *ama1Con* and
225 *ama1cKO* parasites, while rapamycin-treated *ama1Con* and *ama1cKO* sporozoites were
226 mCherry⁺/GFP⁻ (excised) **(Figs 2G and S5B)**. Interestingly, a small proportion (<10%) of
227 *ama1cKO*^{rapa} salivary gland sporozoites were mCherry⁺/GFP⁺ (non-excised), suggesting an
228 enrichment of sporozoites harbouring an intact *ama1* gene, in the salivary glands of infected
229 mosquitoes **(Fig 2G)**.

230 In order to determine if a defect in egress from oocysts or invasion of the salivary glands
231 was the reason behind the reduction in *ama1cKO*^{rapa} salivary gland sporozoite numbers, we
232 quantified haemolymph sporozoites from infected mosquitoes at day 14 post infection. There
233 was no significant difference between the numbers of haemolymph sporozoites isolated from
234 *ama1Con* and *ama1cKO* infected mosquitoes with or without rapamycin treatment **(Fig 2E)**.
235 Using microscopy, we could only see non-excised (mCherry⁺/GFP⁺) haemolymph sporozoites
236 for untreated *ama1Con*- and *ama1cKO*-infected mosquitoes, while all rapamycin-treated
237 *ama1Con* and *ama1cKO* haemolymph sporozoites were excised (mCherry⁺/GFP⁻) **(Fig 2H)**.
238 The absence of a defect in egress from oocysts was also documented by microscopy imaging
239 of the abdomen of infected mosquitoes, where scavenging of circulating sporozoites following
240 egress results in bright red fluorescence of pericardial cellular structures **(S6 Fig)**. A similar
241 percentage of mosquitoes displayed mCherry-labelled pericardial cells between untreated and
242 rapamycin treated *ama1Con* and *ama1cKO* infected mosquitoes, confirming that loss of AMA1
243 expression in sporozoites does not affect sporozoite egress from oocysts **(S6 Fig)**.

244 Lastly, we verified the loss of AMA1 expression in sporozoites by immunofluorescence
245 imaging of salivary gland sporozoites using anti-AMA1 antibodies. AMA1 was detected in
246 untreated *ama1cKO* sporozoites and untreated and rapamycin-treated *ama1Con* sporozoites,
247 with a typical micronemal distribution (**Figs 1F and 2I**). However, no AMA1 was detected in
248 *ama1cKO* sporozoites after rapamycin treatment, indicating the loss of AMA1 (**Fig 2I**).
249 Quantification of AMA1 expression showed that all sporozoites from *ama1Con* and *ama1 Δ utr*
250 expressed AMA1, irrespective of rapamycin exposure, similar to untreated *ama1cKO*
251 sporozoites (**Fig 2J**). In contrast, >95% of the sporozoites isolated from mosquitoes infected
252 with rapamycin-treated *ama1cKO* parasites lacked AMA1 expression, confirming successful
253 gene deletion and protein depletion (**Fig 2J**). Overall, our results demonstrate that loss of
254 AMA1 expression in sporozoites impairs invasion of the mosquito salivary glands, without
255 affecting development or egress from oocysts.

256

257 **AMA1 is required for efficient sporozoite invasion of hepatocytes**

258 In the next step, we tested if AMA1-deficient salivary gland sporozoites could infect
259 hepatocytes. AMA1 was previously suggested to be implicated in cell traversal of *P. falciparum*
260 sporozoites [13]. Hence we first verified if *ama1* gene excision in *P. berghei* affected sporozoite
261 cell traversal *in vitro*, using a dextran assay as previously described [20]. Quantification of
262 dextran-positive cells indicated that cell traversal was comparable between *ama1Con* and
263 *ama1cKO* rapamycin-treated parasites, implying that both motility and cell traversal activity of
264 salivary gland sporozoites were unaffected by excision of *ama1* (**Fig 3A**).

265

266

267 **Fig 3. Sporozoite AMA1 is required for efficient infection of mammalian cells**

268 **A.** Quantification of sporozoite cell traversal activity (% of dextran-positive cells) in rapamycin-
269 treated *ama1Con* and *ama1cKO* parasites. The values for rapamycin-treated *ama1cKO*
270 parasites are represented as percentage of the rapamycin-treated *ama1Con* parasites (mean
271 +/- SEM of three independent experiments). Each data point is the mean of five technical

272 replicates. Ns, non-significant (Two-tailed ratio paired t test). **B.** Quantification of EEFs
273 development *in vitro*, done by flow cytometry or microscopy analysis of HepG2 cells infected
274 with sporozoites isolated from either untreated or rapamycin-treated *ama1Con* and *ama1cKO*
275 infected mosquitoes. The data for rapamycin-treated *ama1Con* and *ama1cKO* parasites are
276 represented as percentage of the respective untreated parasites (mean +/- SEM). Each data
277 point is the mean of three technical replicates in one experiment. Ns, non-significant; *, $p <$
278 0.05 (Two-tailed ratio paired t test). **C.** Quantification of excised (mCherry⁺/GFP⁻, red) and non-
279 excised (mCherry⁺/GFP⁺, green) EEF populations for untreated and treated *ama1Con* and
280 *ama1cKO* parasites. **D.** Fluorescence microscopy of EEF development (24h p.i.) *in vitro*, in
281 HepG2 cells infected with salivary gland sporozoites from untreated or rapamycin-treated
282 *ama1Con* and *ama1cKO* parasites. The right panels show Hoechst (blue), mCherry (red) and
283 GFP (green) merged images. Scale bar = 10 μ m. **E.** Immunofluorescence imaging of
284 mCherry⁺/GFP⁻ (excised) rapamycin-treated *ama1Con* and *ama1cKO* EEFs after staining with
285 anti-UIS4 antibodies (green). The right panels show Hoechst (blue), mCherry (red) and UIS4
286 (green) merged images. Scale bar = 10 μ m.

287

288

289 We then infected HepG2 cell cultures with sporozoites isolated from the salivary glands
290 of mosquitoes previously fed with rapamycin-treated or untreated *ama1Con* and *ama1cKO*
291 parasites. We quantified infected cells, containing exo-erythrocytic forms (EEFs), at 24 h post
292 infection by flow cytometry and fluorescence microscopy. We observed a minor but non-
293 significant reduction in the number of EEFs for rapamycin-treated *ama1Con* parasites
294 compared to untreated controls (**Fig 3B**). In contrast, the number of EEFs obtained from
295 hepatocytes infected with rapamycin-treated *ama1cKO* sporozoites was significantly reduced
296 as compared to untreated parasites (**Fig 3B**). As expected, non-excised (mCherry⁺/GFP⁺)
297 parasites comprised the majority of EEFs quantified for *ama1Con* and *ama1cKO* untreated
298 parasites (**Fig 3C**). Conversely, excised (mCherry⁺/GFP⁻) EEFs were predominantly observed
299 in hepatocytes infected with rapamycin-treated *ama1Con* and *ama1cKO* parasites. However,

300 a small enrichment of non-excised (mCherry⁺/GFP⁺) EEFs was observed with rapamycin-
301 treated *ama1cKO* (**Fig 3C**), as observed with salivary gland sporozoites (**Fig 2G**). Importantly,
302 we could not observe any obvious defect in developmental size or morphology in 24h EEFs
303 between treatment conditions with the two parasite lines, by fluorescence microscopy (**Fig 3D**).
304 Finally, UIS4 staining of the PV membrane confirmed that mCherry⁺/GFP⁻ excised *ama1cKO*
305 sporozoites could form a PV *in vitro*, similar to EEFs from rapamycin-treated *ama1Con* (**Fig**
306 **3E**), implying that in the absence of AMA1, sporozoites conserve a residual capacity to
307 productively invade host cells.

308

309 **RON2 and RON4 interact with AMA1 in sporozoites and are required for host cell** 310 **invasion**

311 Merozoite AMA1 interacts with RON proteins for invasion of erythrocytes [21–23]. In
312 order to investigate whether similar protein interactions also occur in sporozoites, we
313 performed immunoprecipitation experiments using lysates from transgenic sporozoites
314 expressing RON4 fused to mCherry and beads coupled to anti-red fluorescent protein (RFP)
315 nanobodies (RFP-trap). RON4, RON2, RON5 and AMA1 were the main proteins identified by
316 mass spectrometry among co-precipitated proteins, showing that AMA1-RON interactions are
317 conserved in salivary gland sporozoites (**S1 Table**). We decided to focus on RON2 and RON4
318 and generated conditional mutants, using a two-step strategy to introduce LoxN sites upstream
319 and downstream of the genes in PbDiCre parasites (**Figs 4A, S7 and S8**). Clonal populations
320 of *ron2cKO* and *ron4cKO* parasites were obtained after pyrimethamine selection and FACS
321 sorting, and verified by genotyping PCR (**S7 and S8 Figs**). In agreement with an essential role
322 for RON2 and RON4 in the blood, rapamycin-induced gene excision reduced blood-stage
323 growth in *ron2cKO* and *ron4cKO* infected mice (**Figs 4B and 4C**).

324

325

326 **Fig 4. RON2 and RON4 are required for sporozoite invasion in the mosquito and**
327 **mammalian hosts**

328 **A.** Strategy to generate *ron2cKO* and *ron4cKO* parasites in the PbDiCre line. **B-C.** Blood stage
329 growth of rapamycin-treated and untreated *ron2cKO* (B) and *ron4cKO* (C) parasites. The graph
330 represents the parasitaemia (mean +/- SEM) in groups of 5 mice. Rapamycin was administered
331 at day 1. **, $p < 0.01$; ****, $p < 0.0001$ (Two-way ANOVA). **D-F.** Quantification of midgut
332 sporozoites (MG-SPZ, D), haemolymph sporozoites (HL-SPZ, E) or salivary gland sporozoites
333 (SG-SPZ, F) isolated from mosquitoes infected with untreated or rapamycin treated *ron2cKO*
334 or *ron4cKO* parasites. The graphs show the number of sporozoites per infected female
335 mosquito (mean +/- SEM). Each dot represents the mean value obtained in independent
336 experiments after dissection of 30-50 mosquitoes (MG, HL) or 50-70 mosquitoes (SG),
337 respectively. Ns, non-significant; *, $p < 0.05$; **, $p < 0.01$ (Two-tailed ratio paired t test). **G-I.**
338 Quantification of excised (mCherry⁺/GFP⁻, red) and non-excised (mCherry⁺/GFP⁺, green)
339 midgut sporozoites (MG-SPZ, G), haemolymph sporozoites (HL-SPZ, H) or salivary gland
340 sporozoites (SG-SPZ, I) isolated from mosquitoes infected with untreated or rapamycin-treated
341 *ron2cKO* and *ron4cKO* parasites. **J.** Quantification of EEFs development *in vitro*, done by
342 microscopy analysis of HepG2 cells infected with sporozoites isolated from either untreated or
343 rapamycin-treated *ron2cKO* and *ron4cKO* infected mosquitoes. The data for rapamycin-
344 treated parasites are represented as percentage of the respective untreated parasites (mean
345 +/- SEM). Each data point is the mean of five technical replicates in one experiment. Ns, non-
346 significant; *, $p < 0.05$ (Two-tailed ratio paired t test). **K.** Quantification of sporozoite cell
347 traversal activity (% of dextran-positive cells) in untreated and rapamycin-treated *ron2cKO* and
348 *ron4cKO* parasites. The data for rapamycin-treated parasites are represented as percentage
349 of the respective untreated parasites (mean +/- SEM). Each data point is the mean of five
350 technical replicates from one experiment.

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353 We then transmitted *ron2cKO* and *ron4cKO* parasites to mosquitoes, with or without
354 rapamycin treatment. Both parasite lines could colonize the midgut of mosquitoes as
355 evidenced by microscopy imaging of midgut oocysts (**S9 Fig**). Rapamycin treatment of

356 *ron2cKO* and *ron4cKO* parasites before transmission led to a modest reduction of midgut and
357 haemolymph sporozoite numbers (**Figs 4D** and **4E**). However, there was no difference in the
358 percentage of mosquitoes displaying mCherry-labelled pericardial cells (**S10 Fig**), indicating
359 no defect in egress from oocysts for both *ron2cKO*^{rapa} and *ron4cKO*^{rapa} sporozoites. In contrast,
360 the numbers of salivary gland sporozoites were severely reduced for rapamycin-treated
361 *ron2cKO* and *ron4cKO* parasites (**Fig 4F**), as observed with the *ama1cKO* line (**Fig 2D**). As
362 expected, rapamycin treatment before transmission induced robust gene excision in both
363 *ron2cKO* and *ron4cKO* sporozoites (**Figs 4G-4I**). Despite reduced invasion after rapamycin
364 treatment we could recover sufficient numbers of *ron2cKO* and *ron4cKO* salivary gland
365 sporozoites to assess host cell invasion *in vitro*. As observed with *ama1cKO* parasites,
366 rapamycin-induced gene excision of *ron2* and *ron4* impaired invasion of HepG2 cells, as
367 shown by reduced EEF numbers (**Fig 4J**). As observed for AMA1-deficient sporozoites, cell
368 traversal activity was preserved in *ron2cKO* and *ron4cKO* sporozoites after rapamycin
369 treatment (**Fig 4K**). Overall, our data support an active role for RON2 and RON4 in invasion
370 of both mosquito salivary glands and hepatocytes, similar to AMA1.

371

372 **AMA1 and RON2 play a role at the entry site during invasion of mosquito salivary glands**

373 In order to get more insights into the colonization of the mosquito salivary glands by
374 sporozoites, we used serial block face-scanning electron microscopy (SBF-SEM) for three-
375 dimensional volume imaging of whole infected salivary glands. We first compared mosquitoes
376 infected with WT (PbGFP) or rapamycin-treated *ama1cKO* parasites at day 21 post-feeding.
377 SBF-SEM data confirmed the lower parasite density in glands infected with *ama1cKO* as
378 compared to WT (**S11 Fig**). WT sporozoites were observed inside acinar cells and in the apical
379 secretory cavities, where they clustered in bundles (**S11A Fig** and **Movie 1**). Despite reduced
380 numbers of sporozoites, we observed a similar distribution of *ama1cKO* parasites inside the
381 salivary glands, with both intracellular and intraluminal sporozoites (**S11B Fig** and **Movie 2**).
382 Most of the sporozoites were found lying in direct contact with the cytosol inside acinar cells,
383 without any visible vacuolar membrane (**S11 and S12 Figs**). Nevertheless, we also observed

384 some sporozoites surrounded by membranes (**S12 Fig**). However, careful examination of the
385 3D SBF-SEM images revealed that these structures may correspond to invaginations of
386 cellular membranes surrounding portions of intracellular sporozoites, rather than actual
387 vacuoles (**S12A-B Figs** and **Movie 3**). Similar to the WT, *ama1*ckO parasites surrounded by
388 membranes were found inside acinar cells (**S12C Fig**). We also observed sporozoites present
389 in the secretory cavity and surrounded by a cellular membrane, with both WT (**S12D Fig**) and
390 *ama1*ckO parasites (**S12E Fig**). These data thus confirmed the defect of colonization of the
391 mosquito salivary glands by AMA1-deficient sporozoites, but showed no difference in the
392 distribution of the parasites inside the infected glands or in transcellular migration toward the
393 secretory cavities, suggesting a defect at the entry step.

394 In an effort to capture sporozoite invasion events we analyzed infected salivary glands
395 by SBF-SEM at an earlier time point, 15 days post-feeding (**Fig 5**). We were able to visualize
396 three invasion events with untreated *ama1*ckO parasites (noted as wt) (**Figs 5A-F, S13**, and
397 **Movie 4**). The extracellular portion of all three sporozoites was lying underneath the basal
398 lamina (**Figs 5A** and **S13A-B**), tightly adhering to the acinar cell surface throughout the
399 parasite length (**Figs 5D-E** and **S13E-G**). In all three events, the entry site consisted in a flat
400 ring-like aperture in the host cell membrane, through which sporozoites were apparently
401 penetrating smoothly without any major alteration of their shape (**Figs 5C-D** and **S13E-H**). The
402 circular aperture was tilted from the cell surface plane, so sporozoites appeared to penetrate
403 the cells tangentially (**Figs S13D-E** and **S13J-K**). Although the resolution was not sufficient to
404 distinguish all the cellular membranes in detail, the intracellular portion of the invading
405 sporozoites appeared to be surrounded by a vacuole (**Figs 5A-B** and **S13**). Full rhoptries, as
406 evidenced by dense material, as well as empty vesicles, suggestive of discharged rhoptries,
407 were observed at the apical tip of invading parasites (**Figs 5B-C, S13J-K** and **Movie 5**). We
408 could also find fully internalized sporozoites containing seemingly full and empty rhoptries
409 (**S14A-B Fig**). Altogether these observations strongly support that sporozoite entry into acinar
410 cells is associated with rhoptry discharge and the formation of a vacuole.

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Fig 5. Capturing sporozoite entry into salivary glands with serial block face-scanning electron microscopy (SBF-SEM)

A-F. SBF-SEM images showing an untreated *ama1cKO* sporozoite (noted as wt) penetrating into a mosquito salivary gland cell. Panels A and B show the same parasite in two different sections. In A, the sporozoite is cut twice (black arrows), with one part located outside the cell, underneath the basal lamina (BL, white arrow), and the other one inside the cell, within a vacuole surrounded by a membrane (white arrowhead). In B, a tight vacuole can be seen surrounding the intracellular portion of the invading sporozoite (arrowhead), as well as a full rhoptry (white arrow). The volume segmentation in C shows full rhoptries (blue) and empty vesicles (green) in the apical portion of the parasite. In D, the extracellular and intracellular parts of the sporozoite are colored in purple and pink, respectively, while the cell appears is yellow. The volume image in E shows the host cell surface (yellow), revealing a deep imprint of the extracellular parasite segment (black arrow) and the circular aperture at the point of entry (black arrowhead). In F, the entry site is shown at higher magnification. An overview of the segmentation process corresponding to panels A-F is shown in Movie 4. Segmentation of the rhoptries is shown in Movie 5. **G-K.** SBF-SEM images showing a rapamycin-treated *ron2cKO* sporozoite penetrating into a mosquito salivary gland cell. In G, the sporozoite is caught in the process of entry through an elevated host cell structure (arrow) associated with a tight constriction of the parasite body. The intracellular portion of the parasite is surrounded by a vacuole (white arrowhead). A volume segmentation of the sporozoite is shown in H, superimposed on the same section as in G. In the volume representations in I and J, the extracellular and intracellular parts of the sporozoite are colored in purple and pink, respectively, while the cell appears is yellow. The entry site is marked with an arrowhead, and shown at higher magnification in K. An overview of the segmentation process corresponding to panels G-K is shown in Movie 6. Scale bars, 2 μm .

440 We also captured four invasion events with rapamycin-treated *ron2cKO* parasites (**Figs**
441 **5G-K, S15 and Movie 6**), revealing several notable differences as compared to control
442 sporozoites. The entry site consisted in an elevated cup-like structure, with host cell membrane
443 ruffling and protrusions surrounding the invading parasites (**Figs 5G-K and S15E-J**). Strikingly,
444 all four mutant sporozoites displayed a marked constriction at the entry point (**Figs 5G-I, S15A-**
445 **C and S15G-H**). We also noted differences in the parasite positioning as regard to the host
446 cell surface. While the extracellular portion of control parasites was intimately associated with
447 the host cell surface (**Figs 5D-E and S13E-G**), mutant sporozoites were captured in a more
448 upward position, with no adhesion of the parasite rear end to the salivary gland surface (**Figs**
449 **5G, S15B and S15G-H**). Most of the sporozoite body was internalized, with only a minor portion
450 localized outside the cell, the junction between the two regions being pinched by host cell
451 membrane structures (**Figs 5I, S15B-C and S15H**). As seen with control parasites, the
452 intracellular sporozoite portion was surrounded by a vacuole, which however was wider than
453 the one seen with WT parasites (**Figs 5G and S15A,D,G**). Also, we observed internalized
454 RON2-deficient sporozoites containing both full and seemingly empty rhoptries (**S14C and**
455 **S15D Figs**), indicating that the lack of RON2 does not impair rhoptry discharge. Although we
456 did not capture invading AMA1-deficient sporozoites, we could find intracellular sporozoites
457 displaying strong bending of their body (**S16A Fig**), similar to RON2 mutant parasites (**S16B**
458 **Fig**), possibly caused by a tight constriction inflicted during entry through a dysfunctional
459 junction. These observations strongly suggest that, in the absence of a functional AMA1-RON
460 complex, sporozoites are impaired during the invasion process.

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462 **Invasion by AMA1- or RON2-deficient sporozoites is associated with a loss of integrity** 463 **of the salivary gland epithelium**

464 Interestingly, passage of WT sporozoites from acinar cells to the secretory cavities could
465 be associated with an alteration of the apical cellular membrane integrity, with leakage of
466 cytoplasmic material in the secretory cavity (**S17A Fig**). However, the overall architecture of
467 the infected gland did not seem to be altered despite the presence of numerous sporozoites

468 **(Fig 6A)**. In contrast, salivary glands from mosquitoes infected with rapamycin-treated
469 *ama1cKO* parasites, despite low parasite loads, showed signs of epithelial damage, with
470 alteration of the basal membrane and cellular vacuolization (**Fig 6B**). Closer examination of
471 SBF-SEM data revealed sites where the basal lamina was ruptured and detached from the
472 underlying epithelium (**Fig 6C**). Of note, the basal lamina was not visible in either of the
473 *ron2cKO* invasion events (**Figs 5 and S15**), possibly as a result of a complete rupture or
474 detachment at the entry site. AMA1-deficient sporozoites found close to the surface,
475 presumably caught shortly after invasion, were sometimes observed inside large vacuoles (**Fig**
476 **6D**). In some instances, such large vacuoles were associated with a rupture of the cell plasma
477 membrane (**Fig 6E**). Similar cellular damage was also observed with *ron2cKO* mutants (**Fig**
478 **6F**).

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480

481 **Fig 6. Invasion by AMA1- and RON2-deficient sporozoites is associated with a loss of**
482 **integrity of the mosquito salivary gland epithelium**

483 **A-B.** SBF-SEM sections of salivary glands infected with WT (A) or rapamycin-treated
484 *ama1cKO* parasites (B), day 21 post-infection. The *ama1cKO*-infected gland shows signs of
485 cellular damage (black arrows) despite low parasite density. A single intracellular sporozoite
486 is indicated by a white arrow. Scale bars, 10 μm . **C-E.** SBF-SEM sections of salivary glands
487 infected with rapamycin-treated *ama1cKO* parasites, day 15 post-infection. Disruption of the
488 basal lamina is indicated by an arrow. In D, a large vacuole is visible around an intracellular
489 sporozoite and is indicated by an asterisk. In E, both the basal lamina and the cell plasma
490 membrane are ruptured (arrow), resulting in a large cellular vacuole that communicates with
491 the outside (asterisk). Scale bars, 2 μm . **F.** SBF-SEM sections of salivary glands infected with
492 rapamycin-treated *ron2cKO* parasites, day 15 post-infection. A large vacuole surrounding an
493 intracellular sporozoite is indicated by an arrow. Scale bar, 2 μm . **G.** Fluorescence microscopy
494 images of salivary glands infected with untreated (UT) or rapamycin-treated (+Rapa)
495 *ama1cKO* or *ron2cKO* parasites, day 16 post-infection. Samples were stained with Hoechst

496 77742 (Blue). The panels show mCherry (red), GFP (green) and Hoechst (blue) and
497 transmitted light merge images. Zones of retraction of the acinar epithelial cells are visible in
498 the lobes infected with AMA1- and RON2-deficient sporozoites (arrows). Scale bars, 50 μ m.
499 **H.** Quantification of salivary gland lobes showing retracted epithelium after infection with
500 untreated or rapamycin-treated *ama1cKO* and *ron2cKO* parasites. The data shown are from
501 two independent experiments (Fisher's exact test, $P = 0.0286$ for *ama1cKO* and $P < 0.0001$ for
502 *ron2cKO*).

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505 To corroborate SBF-SEM observations, we imaged entire salivary glands by
506 fluorescence microscopy (**Figs 6G** and **S18**). Upon examination of salivary glands infected
507 with rapamycin-treated *ama1cKO* or *ron2cKO*, we frequently observed zones where epithelial
508 cells were detached from the basal lamina and retracted, creating pockets suggestive of liquid
509 accumulation (**Fig 6G**). Such lesions were also observed in salivary glands collected from
510 mosquitoes fed with untreated *ama1cKO* or *ron2cKO*, albeit at significantly lower frequencies
511 despite much higher parasite loads (**Fig 6H**). However, heavily infected lobes showed signs of
512 internal remodeling of the actin cytoskeleton (**S17B Fig**), and were prone to rupture during
513 manipulation.

514 Collectively, our data support a role of AMA1 and RONs during sporozoite entry into
515 mosquito acinar cells through a junction, leading to the formation of a transient vacuole.
516 Dysfunction of the junction in the absence of the AMA1-RON complex impairs parasite entry
517 and may cause collateral host cell damage.

518

519

520 **Discussion**

521 AMA1 and RON proteins play an essential role in *Plasmodium* merozoites during
522 invasion of erythrocytes, where they participate in the formation of the MJ. In contrast, their
523 role in sporozoites was unclear so far. In this study, we exploited the DiCre recombinase
524 system to delete *ama1*, *ron2* or *ron4* genes in *P. berghei* prior to transmission to mosquitoes,
525 allowing subsequent functional investigations in sporozoites. We generated *ama1cKO*,
526 *ron2cKO* and *ron4cKO* parasites in a two-step approach by introducing Lox sites upstream
527 and downstream of the genes in mCherry-expressing PbDiCre parasites, together with a GFP
528 cassette to facilitate monitoring of gene excision. Rapamycin treatment of *ama1cKO*, *ron2cKO*
529 and *ron4cKO* parasites led to a major impairment in blood-stage growth, consistent with an
530 essential role for AMA1 and RONS in RBC invasion, but without affecting transmission to
531 mosquitoes. Remarkably, with all three conditional lines, we observed a dramatic (>10-fold)
532 reduction in the number of salivary gland sporozoites with rapamycin-exposed parasites as
533 compared to untreated parasites, despite comparable midgut and haemolymph sporozoite
534 numbers, showing that AMA1 and RONS are important for efficient invasion of the salivary
535 glands, but not egress from oocysts. AMA1- and RON-deficient sporozoites also displayed a
536 3-6 fold reduction of invasion of mammalian hepatocytes. The similar phenotype of *ama1cKO*,
537 *ron2cKO* and *ron4cKO* mutants, combined with mass spectrometry evidence of an interaction
538 between AMA1 and RON proteins, is consistent with AMA1 playing a role together with the
539 RON proteins during sporozoite host cell invasion. It thus appears that the function of AMA1
540 and RONS cannot be dissociated, unlike previously thought [7]. Our data are in line with those
541 from two studies where a promoter exchange strategy was used to knockdown *ron2*, *ron4* and
542 *ron5* in *P. berghei* sporozoites [14,15]. All three mutants shared a similar phenotype, with a
543 defect in salivary gland invasion and reduced infection of HepG2 cell cultures.

544 Our results differ from those of Giovannini *et al.*, who depleted AMA1 in *P. berghei*
545 sporozoites by targeting the 3'UTR of *ama1* gene using the FLP/FRT conditional system, and
546 observed no effect during mosquito or hepatocyte infection [7]. In this system, the FLP is under
547 the control of the *trap* promoter and mediates DNA excision during sporozoite development,

548 resulting in late depletion of AMA1 protein (beyond day 16 post-feeding), a time frame that
549 would not permit the observation of a salivary gland invasion phenotype. In contrast, with the
550 DiCre system as used here, excision occurs in blood stages prior to transmission to
551 mosquitoes, long before sporozoites are formed and produce AMA1 and RON proteins. The
552 presence of residual AMA1 protein in salivary gland sporozoites after FLP-mediated excision
553 of the 3'UTR could also explain why no defect in hepatocyte invasion was observed in the
554 previous study. Deletion of the 3'UTR of *ama1* using the DiCre system was not sufficient to
555 abrogate protein expression in our study, as reported before with other genes in *P. berghei*
556 and *P. falciparum* [17,24]. In the *ama1* Δ utr line, the downstream genomic sequence (used as
557 a 3' homology region) may be sufficient to stabilize the transcripts and compensate for the lack
558 of 3'UTR following rapamycin-induced excision. This could also contribute to the discrepancy
559 between our results and the previous report by Giovannini *et al.*, where upon recombination
560 the 3'UTR was replaced by a plasmid backbone sequence [7].

561 Invasion of salivary glands by *Plasmodium* sporozoites remains a poorly characterized
562 process. A previous electron microscopy analysis of the salivary glands of *Aedes aegypti*
563 mosquitoes infected with avian *P. gallinaceum* documented sporozoites entering the salivary
564 glands through an invagination of the basal lamina while forming a junctional area between
565 the anterior tip of the sporozoite and the plasma membrane of the acinar cells [25]. The same
566 study showed that newly invaded sporozoites were surrounded by a vacuole inside acinar
567 cells, while those that had entered the secretory cavities were either devoid of a vacuole or
568 present inside disintegrating vacuoles [25]. In another study, *P. falciparum* sporozoites were
569 observed penetrating salivary glands of *Anopheles stephensi* mosquitoes through holes in the
570 basal membrane without causing any obvious damage to the gland [26]. Here, using three-
571 dimensional volume electron microscopy, we could capture *P. berghei* sporozoites in the
572 process of entering acinar cells in *A. stephensi* mosquitoes. Our data support that haemolymph
573 sporozoites initially enter the salivary glands by forming a transient vacuole. During traversal
574 of mammalian cells, sporozoites use the perforin-like protein 1 (PLP1) to egress from transient
575 vacuoles [27]. Whether sporozoites use a similar machinery to exit the entry vacuole in the

576 mosquito salivary glands remains to be determined. Imaging of three invasion events with
577 control parasites showed sporozoites intimately adhering to the cell surface and penetrating
578 inside a nascent vacuole through a ring-like aperture, suggestive of a MJ. All three invading
579 WT sporozoites were located between the basal lamina and the epithelial cells. How
580 sporozoites cross the basal lamina remains unclear, but might involve the secretion of parasite
581 proteases. Our functional data combined with the SBF-SEM images suggest that RONs are
582 secreted from rhoptries prior to or during invasion of the salivary glands, where they could form
583 a complex with AMA1 at the entry junction. Consistent with a rhoptry discharge event
584 associated with salivary gland invasion, previous ultrastructural imaging studies of sporozoites
585 have reported the presence of four or more rhoptries in midgut-derived sporozoites, as
586 opposed to two in mature salivary gland sporozoites [8,28–30].

587 SBF-SEM also revealed morphological defects at the entry site of RON2-deficient
588 sporozoites, with intense host cell membrane ruffling associated with a tight constriction of the
589 parasite body at the entry site. These observations suggest that, despite the absence of a
590 functional AMA1-RON complex, mutant sporozoites are still capable of forming a junction.
591 Interestingly, while invading WT sporozoites were adhering to the host cell surface along their
592 body, the RON2 mutants entered cells in an upward position, as described before with AMA1-
593 deficient *T. gondii* tachyzoites [7,11]. While we cannot formally exclude a role of AMA1-RONs
594 in parasite attachment to the host cell, it is possible that blockage of the entry of RON2-deficient
595 sporozoites resulted in detachment of their rear end from the cell surface. These observations
596 strongly suggest that RON2-deficient sporozoites were halted during the process of entry
597 through a dysfunctional junction. Host cell invasion by apicomplexan zoites relies on a
598 balanced combination between host cell membrane dynamics and parasite motor function [32].
599 The membrane ruffling surrounding invading RON2-deficient sporozoites is reminiscent of
600 actin-driven host cell protrusions observed with myosin A-deficient *T. gondii* tachyzoites, which
601 are impaired during entry due to a motility defect [33]. Beyond participating in the assembly of
602 the junction, AMA1 and RONs could be required to ensure proper function of the junction

603 during invasion of mosquito acinar cells, possibly through interactions with host cell
604 cytoskeleton components as described with RONs in *T. gondii* [31].

605 Interestingly, infection of the mosquito salivary glands by AMA1- or RON2-deficient
606 sporozoites was associated with a loss of integrity of the epithelium, with rupture of the basal
607 lamina and cell vacuolization. This suggests that during sporozoite entry into the salivary gland,
608 AMA1-RONs may contribute to maintaining a sealed junction around the parasite, to allow
609 invasion without creating a leak, thus preventing cell damage. In line with this hypothesis,
610 erythrocyte lysis has been observed during invasion of AMA1-depleted *P. falciparum*
611 merozoites [34]. Our data thus provide a possible molecular basis to explain how thousands
612 of sporozoites can colonize the salivary glands of a single mosquito without causing overt
613 tissue damage. As sporozoites can remain in the salivary cavities for several days before they
614 are transmitted, harmless entry in the glands is likely essential to ensure parasite transmission.
615 Damage inflicted to the salivary gland epithelium during invasion of AMA1-RON mutants may
616 also have detrimental effects on mosquito feeding and survival.

617 Despite the significant reduction in numbers, a minor proportion of rapamycin-treated
618 *ama1cKO*, *ron2cKO* and *ron4cKO* sporozoites could still invade the salivary glands of infected
619 mosquitoes. While we cannot exclude the presence of residual non-excised parasites inside
620 infected glands in the SFB-SEM experiments, these parasites should only represent a minority
621 of salivary gland sporozoites after rapamycin exposure (<10%). Some mutant parasites may
622 succeed in penetrating the glands despite a dysfunctional junction, as suggested by our SFB-
623 SEM data. Alternatively, some degree of plasticity may allow sporozoites to use alternative
624 adhesion or invasion ligands, as observed in *T. gondii* where paralogs can compensate for the
625 lack of a functional AMA1-RON2 pair [35]. While there is no known paralog of RON2 in
626 *Plasmodium*, the Membrane Associated Erythrocyte Binding-Like protein (MAEBL) contains
627 two AMA1-like domains [36], and was in fact reported to be essential for invasion of the salivary
628 glands [37,38]. Interestingly, MAEBL was not identified by co-immunoprecipitation in the
629 RON2, RON4, RON5 complex in oocyst [15] or salivary gland (this study) derived sporozoites,

630 and AMA1-deficient sporozoites fail to invade the mosquito salivary glands, thus arguing
631 against a compensatory role for MAEBL in AMA1-deficient sporozoites.

632 When tested on hepatocyte cell cultures, only a minor proportion of AMA1, RON2 or
633 RON4-depleted salivary gland sporozoites productively invaded and developed into EEFs. The
634 defect in hepatocyte invasion was less pronounced in comparison to that observed for the
635 salivary glands. This differential dependency on AMA1-RONs during host cell invasion could
636 relate to different membrane properties impacting the junction [32]. Consistent with our results,
637 a previous study has shown that anti-AMA1 only partially inhibited *P. falciparum* infection of
638 human hepatocytes *in vitro* [4]. Interestingly, knockdown of RON2 in sporozoites was shown
639 to affect cell traversal and hepatocyte invasion, both *in vitro* and *in vivo*, with the authors
640 implying that loss of RON2 affected attachment to both the salivary glands and hepatocytes,
641 thereby influencing invasion [14]. An earlier report on *P. falciparum* sporozoites showed that
642 interfering with the AMA1-RON2 interaction affected host cell traversal [13]. However, in our
643 study, rapamycin-treated *ama1cKO*, *ron2cKO* and *ron4cKO* parasites showed no defect in
644 sporozoite cell traversal but were impaired in productive invasion. While these differences in
645 phenotypes could be attributed to differences between *P. falciparum* and *P. berghei*, it is
646 possible that the use of salivary gland sporozoites in our study versus those obtained from the
647 haemolymph by Ishino *et al.* accounted for the difference in observations for cell traversal
648 between experiments. We only assessed sporozoite infectivity in HepG2 cell cultures, showing
649 a 3-6 fold reduction in host cell invasion. It is possible that more severe defects would be
650 observed under *in vivo* conditions, but the low numbers of AMA1- and RON-deficient
651 sporozoites recovered from mosquito salivary glands precluded their analysis *in vivo* in mice.

652 Based on our findings, we propose a model where *Plasmodium* sporozoites use the
653 AMA1-RON complex twice, in the mosquito and mammalian hosts (**Fig 7**). First, AMA1 and
654 RONs could mediate the safe entry of sporozoites into the salivary glands via the formation of
655 a junction and a transient vacuole, in a cell-specific manner and without compromising the cell
656 membrane integrity, to ensure successful colonization of the glands and subsequent parasite
657 transmission. This model fits with previous reports showing that sporozoites can massively

658 infect salivary glands without causing cellular damage [39,40]. This crossing event would differ
659 from the cell traversal activity of mature sporozoites in the mammalian host, which is
660 associated with a loss of membrane integrity and cell death [41]. Following sporozoite
661 inoculation into the mammalian host, AMA1 and RONs facilitate productive invasion of
662 hepatocytes, presumably through the formation of a canonical MJ that leads to the formation
663 of the PV where the parasite can replicate into merozoites. Colonization of the salivary glands
664 and productive invasion of hepatocytes involve transcellular migration versus establishment of
665 a replicative vacuole, respectively. However, both events likely require tight membrane sealing
666 around the invading parasite and subversion of the host cortical cytoskeleton, a function that
667 could rely on the AMA1-RON complex. Our study reveals that the contribution of AMA1 and
668 RON proteins is conserved across *Plasmodium* invasive stages. Pre-clinical studies have
669 shown that vaccination with the AMA1-RON2 complex induces functional antibodies that better
670 recognize AMA1 as it appears complexed with RON2 during merozoite invasion, providing an
671 attractive vaccine strategy against *Plasmodium* blood stages [42,43]. Our results indicate that
672 the AMA1-RON complex might also be considered as a potential target to block malaria
673 transmission.

674

675

676 **Fig 7. Model of AMA1-RON function in *Plasmodium* sporozoites.** AMA1 and RON proteins
677 drive two distinct sporozoite invasion events in the mosquito and mammalian hosts. After
678 egress from oocysts, sporozoites first rely on AMA1 and RONs to enter the mosquito salivary
679 glands inside a transient vacuole, without causing epithelium damage, to eventually
680 accumulate in the secretory cavities after crossing the acinar cells. Then, following parasite
681 transmission to a mammalian host, AMA1 and RONs are required for efficient productive
682 invasion of hepatocytes inside a parasitophorous vacuole. Both events supposedly involve
683 rhoptry secretion and the formation of a junction, which however is uncoupled from the
684 formation of a canonical parasitophorous vacuole during colonization of the insect salivary
685 glands.

686 **Materials and methods**

687 **Mice**

688 Female Swiss mice (6–8 weeks old, from Janvier Labs) were used for all routine parasite
689 infections. All animal work was conducted in strict accordance with the Directive 2010/63/EU
690 of the European Parliament and Council ‘On the protection of animals used for scientific
691 purposes’. Protocols were approved by the Ethical Committee Charles Darwin N°005
692 (approval #7475-2016110315516522).

693

694 **Parasites**

695 Conditional genome editing was performed in the *P. berghei* (ANKA strain) PbDiCre line,
696 obtained after integration of mCherry and DiCre expression cassettes at the dispensable
697 *p230p* locus [19]. Two additional lines expressing RON4-mCherry (bioRxiv
698 2021.10.25.465731) and/or GFP [44] were used for immunoprecipitation and electron
699 microscopy experiments, respectively. Parasites were maintained in mice through
700 intraperitoneal injections of frozen parasite stocks. *Anopheles stephensi* mosquitoes were
701 reared at 24°C with 80 % humidity and permitted to feed on infected mice that were
702 anaesthetized, using standard methods of mosquito infection as previously described [45].
703 Post feeding, *P. berghei*-infected mosquitoes were kept at 21°C and fed daily on a 10%
704 sucrose solution.

705

706 **Host cell cultures**

707 HepG2 cells (ATCC HB-8065) were cultured in DMEM supplemented with 10% fetal calf
708 serum, 1% Penicillin-Streptomycin and 1% L-Glutamine as previously described [46], in culture
709 dishes coated with rat tail collagen I (Becton-Dickinson).

710

711 **Vector construction**

712 In order to target different genes of interest, we first generated a generic plasmid,
713 pDownstream1Lox (Addgene #164574), containing a GFP-2A-hDHFR cassette under the

714 control of a *P. yoelii* *hsp70* promoter and followed by the 3'UTR of *P. berghei* *calmodulin* (*cam*)
715 gene and a single LoxN site. The plasmid also contains a yFCU cassette to enable the
716 elimination of parasites carrying episomes by negative selection with 5-fluorocytosine.

717 The *ama1Con* plasmid was designed to excise only ~30 bp downstream of *P. berghei* *ama1*
718 3'UTR. Two fragments were inserted on each side of the GFP-2A-hDHFR cassette of the
719 pDownstream1Lox plasmid: a 5' homology region (HR) homologous to the terminal portion of
720 *ama1* (ORF and 3' UTR) followed by a single LoxN site, and a 3' HR homologous to a
721 sequence downstream of the 3' UTR of *ama1* gene. The *ama1*Δ_{utr} plasmid was assembled
722 similarly to the *ama1Con* construct except that the 5' HR consisted in the terminal portion of
723 *ama1* ORF followed by a LoxN site and the 3' UTR of *P. yoelii* *ama1*, to allow excision of the
724 3'UTR upon rapamycin activation of DiCre. The *ama1cKO* plasmid was designed to introduce
725 a single LoxN site upstream of *ama1* in the rapamycin-treated (excised) *ama1Con* parasites,
726 which already contained a residual LoxN site downstream of the gene. To generate the
727 *ama1cKO* plasmid, the pDownstream1Lox vector was first modified to remove the downstream
728 LoxN site. Then, a 5' HR and a 3' HR, both homologous to sequences located upstream of
729 *ama1* gene, were cloned into the modified plasmid on each side of the GFP-2A-hDHFR, with
730 a single LoxN site introduced upstream of the GFP-2A-hDHFR cassette.

731 To generate *ron2cKO* and *ron4cKO* constructs, two separate plasmids, P1 and P2, were
732 generated to insert a LoxN site upstream of the promoter and downstream of the gene of
733 interest, respectively, in two consecutive transfections. P1 plasmids were constructed by
734 insertion of 5' and 3' HR on each side of the GFP-2A-hDHFR cassette in the
735 pDownstream1Lox plasmid, with a second LoxN site introduced upstream of the GFP cassette.
736 The 5' HR and 3' HR correspond to consecutive fragments located in the promoter region of
737 the GOI. Because the intergenic sequence between *ron4* gene and its upstream gene is short,
738 and in order to maintain expression of the upstream gene and exclude any unwanted
739 duplication and spontaneous recombination events, we introduced the 5' HR of *ron4* in two
740 fragments, with fragment 1 corresponding to the region just upstream of the ORF while
741 fragment 2 corresponded to the 3' UTR from the *P. yoelii* ortholog of the upstream gene. P2

742 plasmids were constructed in a similar manner by insertion of a 5' HR and a 3'HR on each side
743 of the GFP-2A-hDHFR cassette in the pDownstream1Lox plasmid. The 3' HR regions
744 corresponded to the 3' UTR sequences of *RON2* or *RON4*, respectively. For both target genes,
745 the 5' HR was divided into two fragments, where fragment 1 corresponded to the end of the
746 ORF followed by a triple Flag tag, and fragment 2 corresponded to the 3' UTR from the *P.*
747 *yoelii* ortholog gene, in order to avoid duplication of the 3' UTR region and spontaneous
748 recombination.

749 All plasmid inserts were amplified by PCR using standard PCR conditions and the CloneAmp
750 HiFi PCR premix (Takara). Following a PCR purification step (QIAquick PCR purification kit),
751 the fragments were sequentially ligated into the target vector using the In-Fusion HD Cloning
752 Kit (Clontech). The resulting plasmid sequences were verified by Sanger sequencing (GATC
753 Biotech) and linearized before transfection. All the primers used for plasmid assembly are
754 listed in **Table S2**.

755

756 **Parasite transfection**

757 For parasite transfection, schizonts purified from an overnight culture of PbDiCre parasites
758 were transfected with 5–10 µg of linearized plasmid by electroporation using the AMAXA
759 Nucleofector device (Lonza, program U033), as previously described [47], and immediately
760 injected intravenously into the tail vein of Swiss mice. For selection of resistant transgenic
761 parasites, pyrimethamine (35 mg/L) and 5-fluorocytosine (0.5 mg/ml) were added to the drinking
762 water and administered to mice, one day after transfection. Transfected parasites were sorted
763 by flow cytometry on a FACSAria II (Becton-Dickinson), as described [44], and cloned by
764 limiting dilutions and injections into mice. The parasitaemia was monitored daily by flow
765 cytometry and the mice sacrificed at a parasitaemia of 2-3%. The mice were bled and the
766 infected blood collected for preparation of frozen stocks (1:1 ratio of fresh blood mixed with
767 10% Glycerol in Alsever's solution) and isolation of parasites for genomic DNA extraction,
768 using the DNA Easy Blood and Tissue Kit (Qiagen), according to the manufacturer's
769 instructions. Specific PCR primers were designed to check for wild-type and recombined loci

770 and are listed in **Table S2**. Genotyping PCR reactions were carried out using Recombinant
771 Taq DNA Polymerase (5U/μl from Thermo Scientific) and standard PCR cycling conditions.

772

773 ***In vivo* analysis of conditional mutants**

774 DiCre recombinase mediated excision of targeted DNA sequences *in vivo* was achieved by a
775 single oral administration of 200μg rapamycin (1mg/ml stock, Rapamune, Pfizer) to mice.

776 Excision of the GFP cassette in blood stage parasites was monitored by flow cytometry using
777 a Guava EasyCyte 6/2L bench cytometer equipped with 488 nm and 532 nm lasers (Millipore)

778 to detect GFP and mCherry, respectively. To analyze parasite development in the mosquito,

779 rapamycin was administered to infected mice 24 hours prior to transmission to mosquitoes, as

780 described [19]. Midguts were dissected out at day 14 post infection. The haemolymph was

781 collected by flushing the haemocoel with complete DMEM, day 14 to 16 post infection. Salivary

782 gland sporozoites were collected between 21–28 days post feeding from infected mosquitoes,

783 by hand dissection and homogenization of isolated salivary glands in complete DMEM. Live

784 samples (infected mosquito midguts or salivary glands, sporozoites) were mounted in PBS

785 and visualized live using a Zeiss Axio Observer.Z1 fluorescence microscope equipped with a

786 LD Plan-Neofluar 403/0.6 Corr Ph2 M27 objective. The exposure time was set according to

787 the positive control and maintained for both untreated and rapamycin-treated parasites, in

788 order to allow comparisons. All images were processed with ImageJ for adjustment of contrast.

789

790 ***In vitro* sporozoite assays**

791 HepG2 cells were seeded at a density of 30,000 cells/well in a 96-well plate for flow cytometry

792 analysis or 100,000 cells/well in 8 well μ-slide (IBIDI) for immunofluorescence assays, 24 hours

793 prior to infection with sporozoites. On the day of the infection, the culture medium in the wells

794 was discarded and fresh complete DMEM was added along with 10,000 sporozoites, followed

795 by incubation for 3 hours at 37°C. After 3 hours, the wells were washed twice with complete

796 DMEM and then incubated for another 24-48 hours at 37°C and 5% CO₂. For quantification of

797 EEF numbers, the cells were trypsinized after two washes with PBS, followed by addition of

798 complete DMEM and one round of centrifugation at 4°C. After discarding the supernatant, the
799 cells were either directly re-suspended in complete DMEM for flow cytometry, or fixed with 2%
800 PFA for 10 minutes, subsequently washed once with PBS and then re-suspended in PBS for
801 FACS acquisition. For quantification of traversal events, fluorescein-conjugated dextran
802 (0.5mg/ml, Life Technologies) was added to the wells along with sporozoites followed by an
803 incubation at 37°C for 3 hours. After 3 hours, the cells were washed twice with PBS, trypsinized
804 and resuspended in complete DMEM for analysis by flow cytometry.

805

806 **RON4 immunoprecipitation and mass spectrometry**

807 Freshly dissected RON4-mCherry sporozoites were lysed on ice for 30 min in a lysis buffer
808 containing 0.5% w/v NP40 and protease inhibitors. After centrifugation (15,000 × g, 15 min,
809 4°C), supernatants were incubated with protein G-conjugated sepharose for preclearing
810 overnight. Precleared lysates were subjected to mCherry immunoprecipitation using RFP-Trap
811 beads (Chromotek) for 2h at 4°C, according to the manufacturer's protocol. PbGFP parasites
812 with untagged RON4 were used as a control. After washes, proteins on beads were eluted in
813 2X Laemmli and denatured (95°C, 5min). After centrifugation, supernatants were collected for
814 further analysis. Samples were subjected to a short SDS-PAGE migration, and gel pieces were
815 processed for protein trypsin digestion by the DigestProMSi robot (Intavis), as described [10].
816 Peptide were separated on an Aurora UHPLC column from IonOpticks (25 cm x 75 µm, C18),
817 using a 30 min gradient from 3 to 32% ACN with 0.1% formic acid, and analyzed on a timsTOF
818 PRO mass spectrometer (Bruker). Mascot generic files were processed with X!Tandem
819 pipeline (version 0.2.36) using the PlasmoDB_PB_39_PbergheiANKA database, as described
820 [10]. The mass spectrometry proteomics data have been deposited to the ProteomeXchange
821 Consortium via the PRIDE [48] partner repository with the dataset identifier PXD031463.

822

823 **Immunofluorescence assays**

824 Blood-stage schizonts were fixed with 4% PFA and 0.0075% glutaraldehyde for 30 mins at
825 37°C with constant shaking. The samples were then quenched/permeabilized with 125mM

826 glycine /0.1% Triton X-100 for 15 minutes, blocked with PBS/3% BSA, then incubated with Rat
827 anti-AMA1 antibodies (1:250, clone 28G2, MRA-897A, Bei Resources) followed by Alexa Fluor
828 goat anti-rat 405 antibodies (1:1000, Life Technologies). The samples were mounted in PBS
829 and immediately visualized under a fluorescence microscope. Sporozoites were resuspended
830 in PBS, added on top of poly-L-lysine coated coverslips and allowed to air dry. The sporozoites
831 were then fixed with 4% PFA for 30 mins, followed by quenching with 0.1M glycine for 30 mins
832 and two washes with PBS. In the next step, the sporozoites were permeabilized with 1% Triton-
833 X100 for 5 mins, washed twice with PBS, then blocked with PBS 3%BSA for 1hr at RT and
834 incubated with anti-AMA1 antibody (1:250) diluted in blocking solution. Following 3 washes
835 with PBS, the sporozoites were incubated with the secondary antibody (anti-Rat Alexa Fluor
836 647) diluted in blocking solution. Following 3 washes with PBS, the coverslips were mounted
837 onto a drop of prolong diamond anti-fade mounting solution (Life Technologies), sealed with
838 nail polish and imaged using a fluorescence microscope. Infected HepG2 cell cultures were
839 washed twice with PBS, then fixed with 4% PFA for 20 minutes, followed by two washings with
840 PBS and incubation with goat anti-UIS4 primary antibody (1:500, Sicgen), followed by donkey
841 anti-goat Alexa Fluor 594 secondary antibody (1:1000, Life Technologies). For fluorescence
842 imaging of entire glands, freshly dissected salivary glands were fixed in 4% PFA for 30 minutes
843 and permeabilized in acetone for 90 seconds, as described [40]. Samples were incubated with
844 Phalloidin-iFluor 647 (Abcam) and Hoechst 77742 (Life Technologies) overnight at 4°C,
845 washed and mounted in PBS before imaging. Acquisitions were made on a Zeiss Axio
846 Observer Z1 fluorescence microscope using the Zen software (Zeiss). Images were processed
847 with ImageJ for adjustment of contrast.

848

849 **Serial block face-scanning electron microscopy**

850 For Serial Block Face-Scanning Electron Microscopy (SBF-SEM), salivary glands were
851 isolated from infected mosquitoes at day 15 or 21 post-feeding, and fixed in 0.1 M cacodylate
852 buffer containing 3% PFA and 1% glutaraldehyde during 1 hour at room temperature. Intact
853 salivary glands were then prepared for SBF-SEM (NCMIR protocol) [49] as follows: samples

854 were post-fixed for 1 hour in a reduced osmium solution containing 1% osmium tetroxide, 1.5%
855 potassium ferrocyanide in PBS, followed by incubation with a 1% thiocarbohydrazide in water
856 for 20 minutes. Subsequently, samples were stained with 2% OsO₄ in water for 30 minutes,
857 followed by 1% aqueous uranyl acetate at 4 °C overnight. Samples were then subjected to en
858 bloc Walton's lead aspartate staining [50], and placed in a 60 °C oven for 30 minutes. Samples
859 were then dehydrated in graded concentrations of ethanol for 10 minutes in each step. The
860 samples were infiltrated with 30% agar low viscosity resin (Agar Scientific Ltd, UK) in ethanol,
861 for 1 hour, 50% resin for 2 hours and 100% resin overnight. The resin was then changed and
862 the samples were further incubated during 3 hours, prior to inclusion by flat embedding
863 between two slides of Aclar® and polymerization for 18 hours at 60 °C. The polymerized blocks
864 were mounted onto aluminum stubs for SBF-SEM imaging (FEI Microtome 8 mm SEM Stub,
865 Agar Scientific), with two-part conduction silver epoxy kit (EMS, 12642-14). For imaging,
866 samples on aluminum stubs were trimmed using an ultramicrotome and inserted into a
867 TeneoVS SEM (ThermoFisher Scientific). Acquisitions were performed with a beam energy of
868 2 kV, 400 pA current, in LowVac mode at 40 Pa, a dwell time of 1 µs per pixel at 10 nm pixel
869 size. Sections of 50 nm were serially cut between images. Data acquired by SBF-SEM were
870 processed using Fiji and Amira (ThermoFisher Scientific). Data alignment and manual
871 segmentation were performed using Amira.

872

873 **Quantification and statistical analysis**

874 *In vitro* experiments were performed with a minimum of three technical replicates per
875 experiment. Statistical significance was assessed by two-way ANOVA, one-way ANOVA
876 followed by Tukey's multiple comparisons, Fisher's exact or ratio paired t tests, as indicated in
877 the figure legends. All statistical tests were computed with GraphPad Prism 5 (GraphPad
878 Software). The quantitative data used to generate the figures and the statistical analysis are
879 presented in **Table S3**.

880

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896

897

898

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1070

1071 **Supporting information**

1072 **Supplemental tables**

1073

1074 **S1 Table.** Mass spectrometry analysis of co-IP from RON4-mCherry sporozoites.

1075 **S2 Table.** List of oligonucleotides used in the study.

1076 **S3 Table.** Quantitative data and statistical analysis.

1077

1078

1079 **Supplemental figures**

1080

1081 **S1 Fig. Generation of *ama1*Δutr parasites using the DiCre system**

1082 **A.** Strategy to generate *ama1*Δutr parasites. The wild-type locus of *P. berghei ama1* in the
1083 PbDiCre parasite line was targeted with a *ama1*Δutr replacement plasmid containing 2 Lox
1084 sites and 5' and 3' homologous sequences inserted on each side of a GFP-2A-hDHFR
1085 cassette. Upon double crossover recombination, the LoxN sites are inserted upstream of the
1086 3' UTR and downstream of the GFP-2A-hDHFR cassette, respectively. Activation of the DiCre
1087 recombinase with rapamycin results in excision of the 3' UTR together with the GFP-2A-
1088 hDHFR cassette. Genotyping primers and expected PCR fragments are indicated by arrows
1089 and lines, respectively. **B.** Genotyping of parental PbDiCre and *ama1*Δutr transfected parasites
1090 after pyrimethamine selection (pyr) and after rapamycin treatment (rapa) of the final population.
1091 Parasite genomic DNA was analyzed by PCR using primer combinations specific for the
1092 unmodified locus (WT), the 5' integration, 3' integration or excision events. **C.** Flow cytometry
1093 analysis of PbDiCre (parental) and *ama1*Δutr blood stage parasites after pyrimethamine
1094 selection (pyr) or rapamycin exposure (rapa). NI, non-infected red blood cells.

1095

1096 **S2 Fig. Generation of *ama1*Con parasites using the DiCre system**

1097 **A.** Strategy to generate *ama1*Con parasites. The construct is similar to the *ama1*Δutr construct,
1098 except that the first LoxN site is located downstream of the 3' UTR. Upon rapamycin-induced
1099 excision, the *ama1* locus remains intact. **B.** Genotyping of parental PbDiCre and *ama1*Con
1100 transfected parasites after pyrimethamine selection (pyr) and after rapamycin treatment (rapa)
1101 of the final population. Parasite genomic DNA was analyzed by PCR using primer
1102 combinations specific for the unmodified locus (WT), the 5' integration, 3' integration or
1103 excision events. **C.** Flow cytometry analysis of PbDiCre (parental) and *ama1*Con blood stage
1104 parasites after pyrimethamine selection (pyr) or rapamycin exposure (rapa). NI, non-infected
1105 red blood cells.

1106

1107 **S3 Fig. Imaging of *ama1*Con and *ama1*Δutr mosquito stages**

1108 **A.** Fluorescence microscopy images of midguts from mosquitoes infected with untreated (UT)
1109 or rapamycin-treated (rapa) *ama1*Con and *ama1*Δutr parasites. Scale bar = 200 μm. **B.**
1110 Fluorescence microscopy images of salivary glands from mosquitoes infected with untreated
1111 (UT) or rapamycin-treated (rapa) *ama1*Con and *ama1*Δutr parasites. Scale bar = 200 μm.

1112

1113 **S4 Fig. Generation of *ama1*cKO parasites using the DiCre system**

1114 **A.** Strategy to generate *ama1*cKO parasites. The *ama1* locus in rapamycin-treated (excised)
1115 *ama1*Con parasites was targeted with a *ama1*cKO replacement plasmid containing a single
1116 LoxN site and 5' and 3' homologous sequences inserted on each side of a GFP-2A-hDHFR
1117 cassette. Upon double crossover recombination, a second LoxN site is inserted upstream of
1118 the GFP-2A-hDHFR cassette and *ama1* gene. Activation of the DiCre recombinase with
1119 rapamycin results in excision of the entire *ama1* gene together with the GFP-2A-hDHFR
1120 cassette. Genotyping primers and expected PCR fragments are indicated by arrows and lines,
1121 respectively. **B.** Genotyping of PbDiCre, rapamycin-treated (excised) *ama1*Con (parental) and
1122 *ama1*cKO parasites after selection with pyrimethamine (pyr). Parasite genomic DNA was
1123 analyzed by PCR using primer combinations specific for the unmodified locus (WT), the 5'
1124 integration and 3' integration events. **C.** Genotyping of *ama1*cKO blood stage parasites

1125 collected 2 or 6 days after rapamycin exposure or left untreated (UT). Parasite genomic DNA
1126 was analyzed by PCR using primer combinations specific for the non-excised (NE, 5'
1127 integration combination) or excised (E) locus.

1128

1129 **S5 Fig. Imaging of *ama1*cKO mosquito stages**

1130 **A.** Fluorescence microscopy of midguts from mosquitoes infected with untreated (UT) or
1131 rapamycin-treated (rapa) *ama1*cKO parasites. Scale bar = 200 μ m. **B.** Fluorescence
1132 microscopy of salivary glands isolated from mosquitoes infected with untreated (UT) or
1133 rapamycin-treated (rapa) *ama1*cKO parasites. Scale bar = 200 μ m.

1134

1135 **S6 Fig. Analysis of mosquito pericardial structures**

1136 **A.** Imaging of the abdomen of a mosquito infected with rapamycin treated *ama1*cKO parasites,
1137 after removal of the midgut, showing mCherry-labelled pericardial structures. **B.** Quantification
1138 of mosquitoes with mCherry-labelled pericardial cells at D21 post-infection with untreated (UT)
1139 or rapamycin-treated (rapa) *ama1*Con and *ama1*cKO parasites. Ns, non-significant (Two-tailed
1140 ratio paired t test).

1141

1142 **S7 Fig. Generation of *ron2*cKO parasites using the DiCre system**

1143 **A-B.** Two-step strategy to generate *ron2*cKO parasites. In the first step (**A**), the *ron2* locus in
1144 PbDiCre parasites was targeted with a *ron2*-P1 replacement plasmid containing 5' and 3'
1145 homologous sequences and two LoxN sites flanking a GFP-2A- hDHFR cassette. Upon double
1146 crossover recombination, the two LoxN sites are inserted upstream of *ron2*. Activation of the
1147 DiCre recombinase with rapamycin results in excision of the GFP-2A-hDHFR cassette, leaving
1148 a single LoxN site upstream of the gene in excised *ron2*-P1 parasites. In the second step (**B**),
1149 the *ron2* locus in rapamycin-treated (excised) *ron2*-P1 parasites was targeted with a *ron2*-P2
1150 replacement plasmid containing 5' and 3' homologous sequences flanking a GFP-2A- hDHFR
1151 cassette and a single LoxN site. Upon double crossover recombination, the LoxN site is
1152 inserted downstream of *ron2* and the GFP-2A- hDHFR cassette. Activation of the DiCre
1153 recombinase with rapamycin results in excision of the entire *ron2* gene together with the GFP-
1154 2A-hDHFR cassette. Genotyping primers and expected PCR fragments are indicated by
1155 arrows and lines, respectively. **C.** Genotyping of PbDiCre and *ron2*cKO parasites. Parasite
1156 genomic DNA was analyzed by PCR using primer combinations specific for the unmodified
1157 locus (WT), the 5' and 3' integration events.

1158

1159 **S8 Fig. Generation of *ron4*cKO parasites using the DiCre system**

1160 **A-B.** Two-step strategy to generate *ron4*cKO parasites. In the first step (**A**), the *ron4* locus in
1161 PbDiCre parasites was targeted with a *ron2*-P1 replacement plasmid containing 5' and 3'
1162 homologous sequences and two LoxN sites flanking a GFP-2A- hDHFR cassette. Upon double
1163 crossover recombination, the two LoxN sites are inserted upstream of *ron4*. Activation of the
1164 DiCre recombinase with rapamycin results in excision of the GFP-2A-hDHFR cassette, leaving
1165 a single LoxN site upstream of the gene in excised *ron4*-P1 parasites. In the second step (**B**),
1166 the *ron4* locus in rapamycin-treated (excised) *ron4*-P1 parasites was targeted with a *ron4*-P2
1167 replacement plasmid containing 5' and 3' homologous sequences flanking a GFP-2A- hDHFR
1168 cassette and a single LoxN site. Upon double crossover recombination, the LoxN site is
1169 inserted downstream of *ron4* and the GFP-2A- hDHFR cassette. Activation of the DiCre
1170 recombinase with rapamycin results in excision of the entire *ron4* gene together with the GFP-
1171 2A-hDHFR cassette. Genotyping primers and expected PCR fragments are indicated by
1172 arrows and lines, respectively. **C.** Genotyping of PbDiCre and *ron4*cKO parasites. Parasite
1173 genomic DNA was analyzed by PCR using primer combinations specific for the unmodified
1174 locus (WT), the 5' and 3' integration events.

1175

1176 **S9 Fig. Imaging of *ron2*cKO and *ron4*cKO mosquito stages**

1177 **A-B.** Fluorescence microscopy of midguts from mosquitoes infected with untreated (UT) or
1178 rapamycin-treated (rapa) *ron2*cKO (**A**) or *ron4*cKO (**B**) parasites. Scale bar = 200 μ m.

1179

1180 **S10 Fig. Analysis of mosquito pericardial structures**

1181 Quantification of mosquitoes with mCherry-labelled pericardial cells at D21 post-infection with
1182 untreated (UT) or rapamycin-treated (rapa) *ron2cKO* or *ron4cKO* parasites. Ns, non-significant
1183 (Two-tailed ratio paired t test).

1184
1185 **S11 Fig. Serial block face-scanning electron microscopy (SBF-SEM) of infected**
1186 **mosquito salivary glands**

1187 **A-B.** Representative sections of salivary glands from mosquitoes infected with WT (**A**) or
1188 rapamycin-treated *ama1cKO* (**B**) parasites (left panels). Scale bars, 5 μm . WT and AMA1-
1189 deficient sporozoites were observed inside the acinar cells (AC, asterisks) and in the secretory
1190 cavities (SC, arrows). The volume segmentation images (right panels) show the secretory
1191 cavities (yellow) and sporozoites (blue), and correspond to Movie 1 and Movie 2, respectively,
1192 for WT and *ama1cKO* parasites.

1193
1194 **S12 Fig. SBF-SEM analysis of sporozoite distribution inside salivary glands**

1195 **A-B.** SBF-SEM sections from Movie 3, showing WT sporozoites inside salivary gland acinar
1196 cells. The first section (**A**) shows a sporozoite partly surrounded by host cell membranes
1197 (arrow), highlighted in red in the right panel, and a second one seemingly contained inside a
1198 vacuole (asterisk), highlighted in yellow in the right panel. The second section (**B**) shows the
1199 same parasites in a different plane, revealing that the second sporozoite is in fact not enclosed
1200 in a vacuole but instead is interacting with invaginated host cell membranes (asterisk),
1201 highlighted in yellow in the right panel, while the first parasite now seems surrounded by a
1202 membrane (arrow), giving the false impression of being enclosed in a vacuole (highlighted in
1203 red in the right panel). Scale bars, 2 μm . **C.** SBF-SEM section showing an intracellular
1204 rapamycin-treated *ama1cKO* sporozoite surrounded by a cellular membrane (arrow). Scale
1205 bar, 2 μm . AC, acinar cell; SC, secretory cavity. **D-E.** SBF-SEM sections showing WT (**D**) and
1206 rapamycin-treated *ama1cKO* (**E**) sporozoites present inside secretory cavities (SC) and
1207 surrounded by cellular membranes (arrows). Scale bars, 1 μm .

1208
1209 **S13 Fig. SBF-SEM imaging of sporozoite invasion into mosquito salivary glands**

1210 **A-H.** SBF-SEM images of an invading untreated *ama1cKO* sporozoite. Panels A-C show three
1211 XY sections of the invading parasite. The sporozoite is located underneath the basal lamina
1212 (BL), and enters the cell surrounded by a vacuole (white arrowhead). The entry site is marked
1213 by a black arrow. Scale bar, 1 μm . Panel D shows a virtual XZ section, illustrating that the
1214 sporozoite is penetrating tangentially into the acinar cell. The entry aperture is marked by a
1215 black arrowhead. Panels E-H show a volume segmentation of the parasite (in purple) invading
1216 the mosquito cell (in yellow). The entry site is marked by a black arrowhead. In G and H, only
1217 the cell surface is shown, revealing the imprinting of the extracellular portion of the sporozoite
1218 (black arrow). In H, the circular entry site is shown at higher magnification. **I-K.** SBF-SEM
1219 images of another invading untreated *ama1cKO* sporozoite. In I, a XY section cuts the invading
1220 parasite twice (black arrows), with the extracellular portion being positioned between the cell
1221 surface and the basal lamina (BL). Two virtual YZ sections are shown in J and K, illustrating
1222 that the sporozoite is penetrating tangentially into the acinar cell. The entry aperture is marked
1223 by a black arrowhead. A full rhoptry is visible in J and an empty one can be seen in K (arrows).

1224
1225 **S14 Fig. SBF-SEM imaging of sporozoite rhoptries**

1226 **A-B.** SBF-SEM sections of the apical end of an intracellular untreated (wt) *ama1cKO*
1227 sporozoite. In A, two full rhoptries are visible, indicated by white arrows. In B, a different section
1228 of the same parasite reveals an empty rhoptry (black arrow). **C.** SBF-SEM section of an
1229 intracellular rapamycin-treated *ron2cKO* sporozoite, showing two full rhoptries (white arrows)
1230 and one empty one (black arrow). Scale bars, 1 μm .

1231
1232
1233 **S15 Fig. SBF-SEM imaging of RON2-deficient sporozoite invasion into mosquito**
1234 **salivary glands**

1235 **A-F.** SBF-SEM images of two invading rapamycin-treated *ron2cKO* sporozoites. In A, the first
1236 sporozoite (labelled #1) is cut once, while the second one (#2) is cut twice. The entry sites are

1237 indicated by black arrows, and the vacuoles by white arrowheads. Scale bars, 1 μm . Panels B
1238 and C show volume segmentation images of the invading parasites (red and purple,
1239 respectively). The cell is colored in yellow. Panel D shows a virtual XZ section, showing the
1240 vacuole (white arrowhead), a full rhoptry (black arrow) and an empty vesicle (white arrow). **G-**
1241 **J.** SBF-SEM images of another rapamycin-treated *ron2cKO* sporozoites. In G, the entry site is
1242 indicated by a black arrow, and the vacuole by a white arrowhead. Panels H-J show volume
1243 segmentation images of the invading parasite (purple). The cell is colored in yellow. The entry
1244 site is shown at higher magnification in I and J, with or without displaying the sporozoite.
1245

1246 **S16 Fig. SBF-SEM imaging of AMA1- and RON2-deficient sporozoites inside salivary**
1247 **gland cells**

1248 **A-B.** SBF-SEM sections of intracellular rapamycin-treated *ama1cKO* (A) and *ron2cKO* (B)
1249 sporozoites. Both parasites display a strong bending, with the hinge indicated by an arrow.
1250 Scale bars, 2 μm .
1251

1252 **S17 Fig. Cellular alterations in heavily infected mosquito salivary glands**

1253 **A.** SBF-SEM section showing an alteration of the cellular interface with the secretory cavity at
1254 the point of entry of multiple WT sporozoites (asterisk). Intraluminal leakage of cytoplasmic
1255 material is indicated with an arrow. Scale bar, 5 μm . **B.** Fluorescence microscopy images of
1256 salivary gland distal lobes infected with rapamycin-treated *ama1Con* or untreated *ron2cKO*
1257 parasites. Samples were stained with Phalloidin-iFluor 647 (magenta) and Hoechst 77742
1258 (Blue). The right panels show mCherry (red), GFP (green) and Hoechst (blue) merge images.
1259 In both cases, the heavy parasite load is associated with internal alterations of the phalloidin
1260 staining, but the basal border of the lobes is preserved. Scale bars, 50 μm .
1261

1262 **S18 Fig. Infection by AMA1- and RON2-deficient parasites is associated with a loss of**
1263 **integrity of the mosquito salivary gland epithelium**

1264 Representative fluorescence microscopy images of salivary gland lobes infected with
1265 untreated (UT) or rapamycin-treated (+Rapa) *ama1cKO* or *ron2cKO* parasites, day 16 post-
1266 infection. Samples were stained with Phalloidin-iFluor 647 (magenta) and Hoechst 77742
1267 (Blue). The right panels show mCherry (red), GFP (green) and Hoechst (blue) merge images.
1268 Zones of retraction of the acinar epithelial cells are visible in the lobes infected with AMA1-
1269 and RON2-deficient sporozoites (arrows). Scale bars, 50 μm .
1270

1271
1272 **Supplemental movies**

1273
1274 **Movie 1.** 3D segmentation of a mosquito salivary gland infected with WT (PbGFP) sporozoites,
1275 day 21 post-feeding. Parasites appear in blue and secretory cavities in yellow. This movie
1276 corresponds to Fig S11A.
1277

1278 **Movie 2.** 3D segmentation of a mosquito salivary gland infected with rapamycin-treated
1279 *ama1cKO* sporozoites, day 21 post-feeding. Parasites appear in blue and secretory cavities in
1280 yellow. This movie corresponds to Fig S11B.
1281

1282 **Movie 3.** SBF-SEM sections of a mosquito salivary gland infected with WT parasites, day 21
1283 post-feeding. This movie corresponds to Fig S12A-B.
1284

1285 **Movie 4.** 3D segmentation of an untreated *ama1cKO* sporozoite invading a salivary gland cell,
1286 day 15 post-feeding. The invading parasite is colored in purple and the acinar cell in yellow.
1287 This movie corresponds to Fig 5A-F.
1288

1289 **Movie 5.** 3D segmentation of the same invading untreated *ama1cKO* sporozoite as in Movie
1290 4, highlighting the apical organelles. The parasite appears in pink, full rhoptries in blue and
1291 empty vesicles in green. This movie corresponds to Fig 5A-C.
1292

1293 **Movie 6.** 3D segmentation of a rapamycin-treated *ron2cKO* sporozoite invading a salivary
1294 gland cell, day 15 post-feeding. The invading parasite is colored in purple and the acinar cell
1295 in yellow. This movie corresponds to Fig 5G-K.
1296

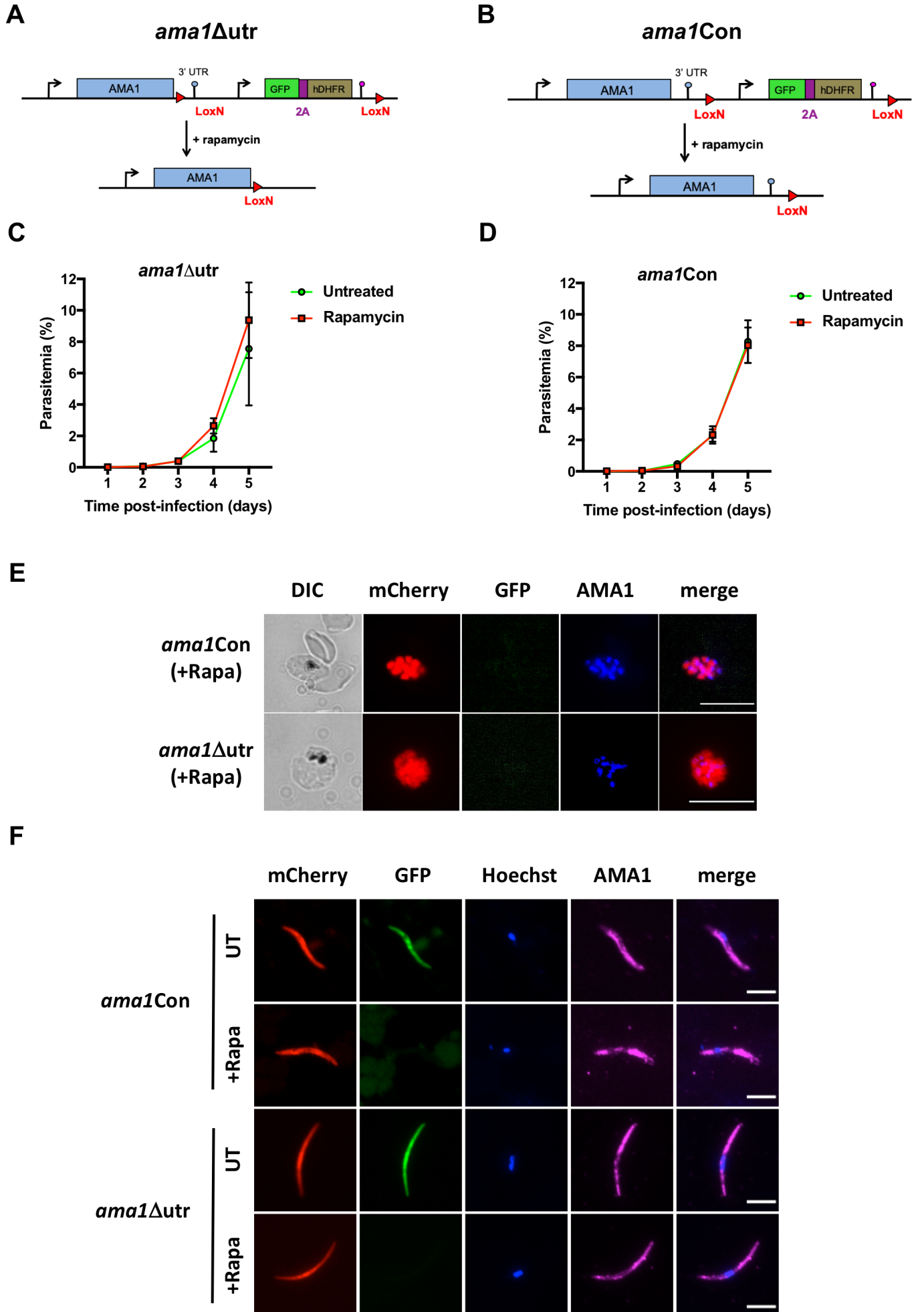
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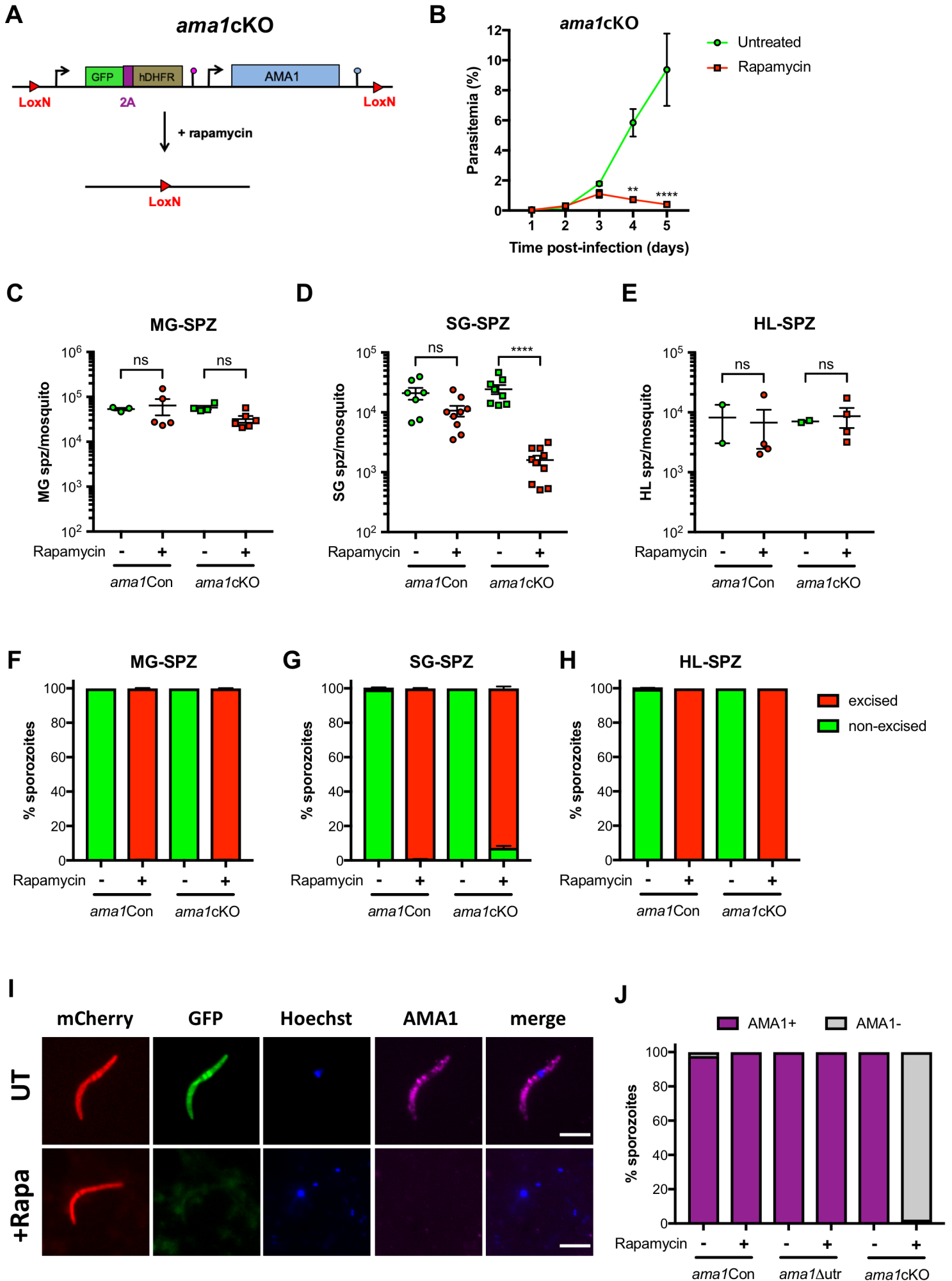
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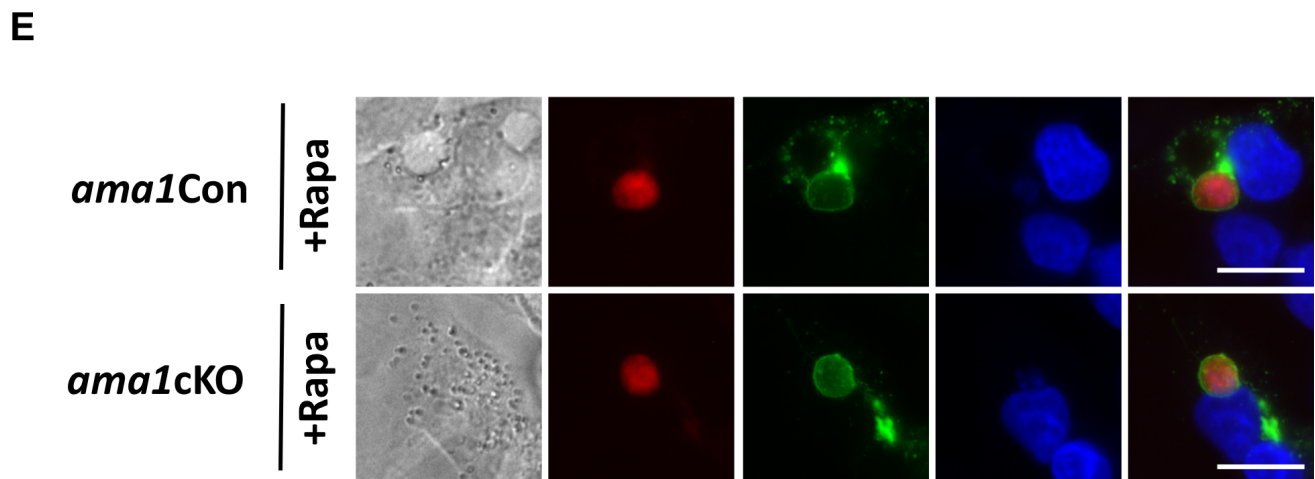
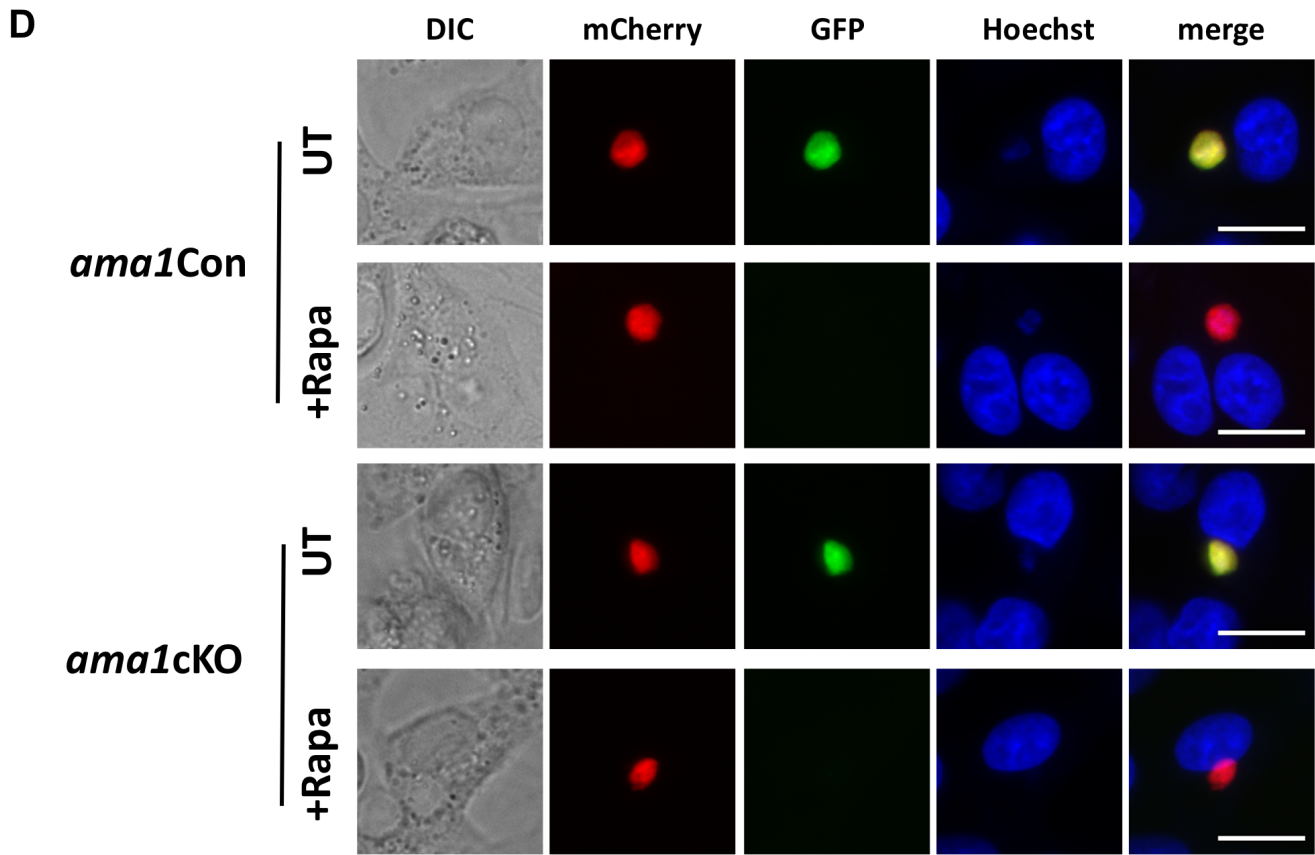
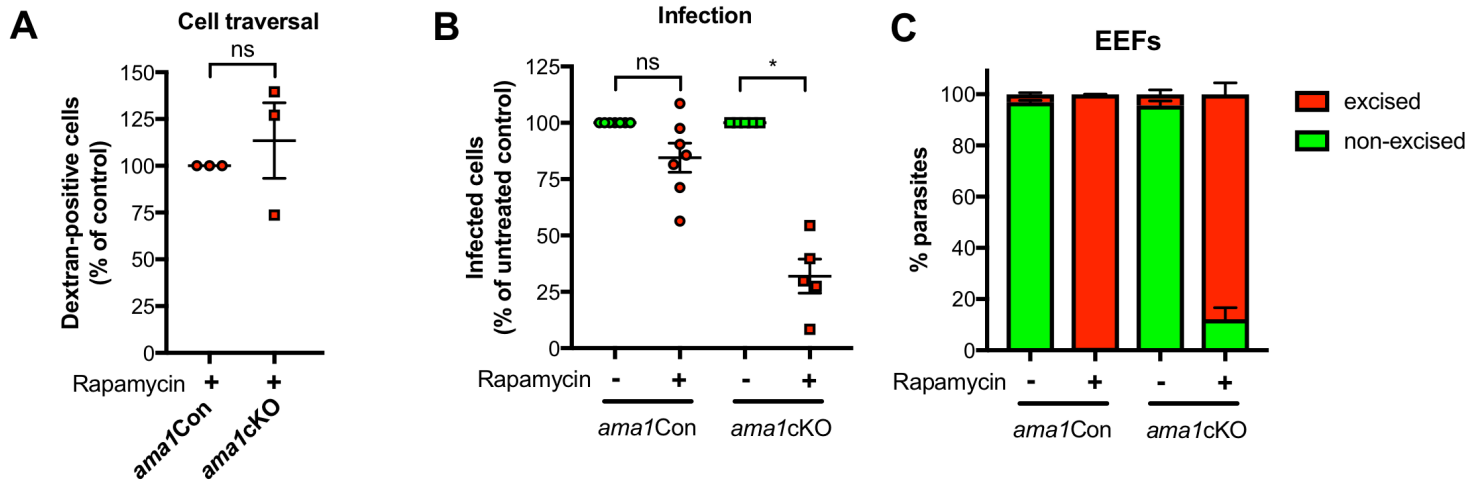
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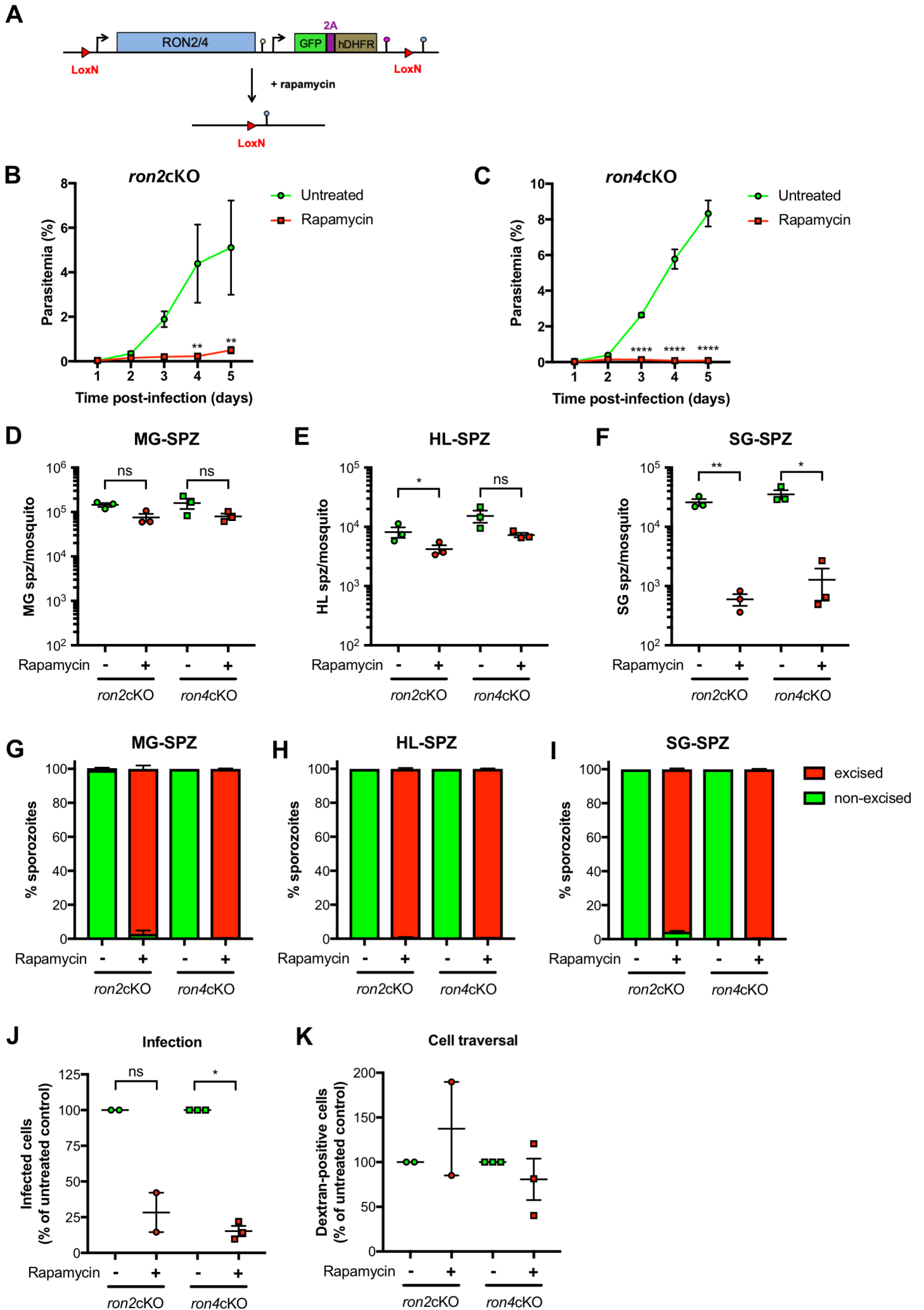
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Fig5

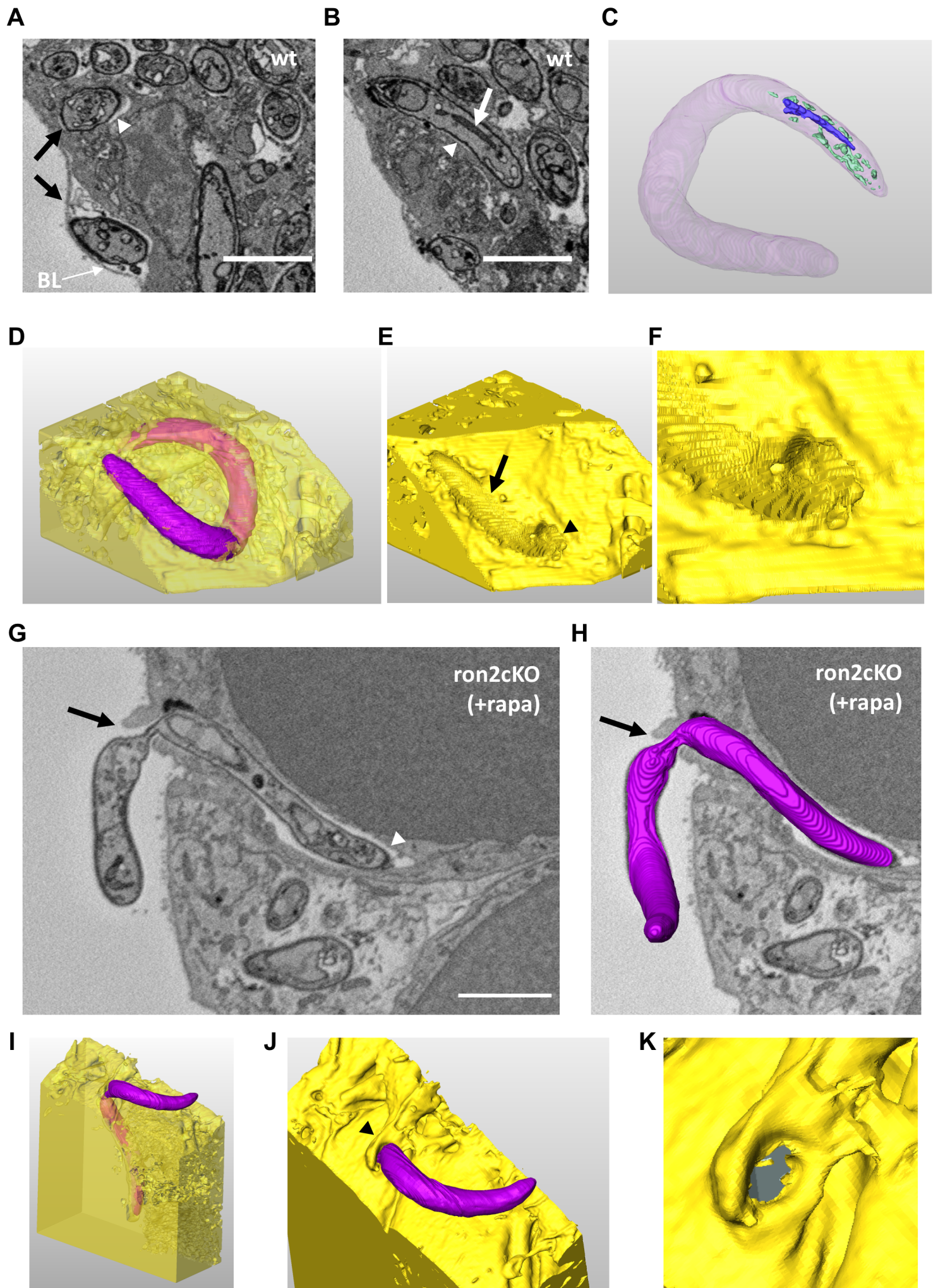


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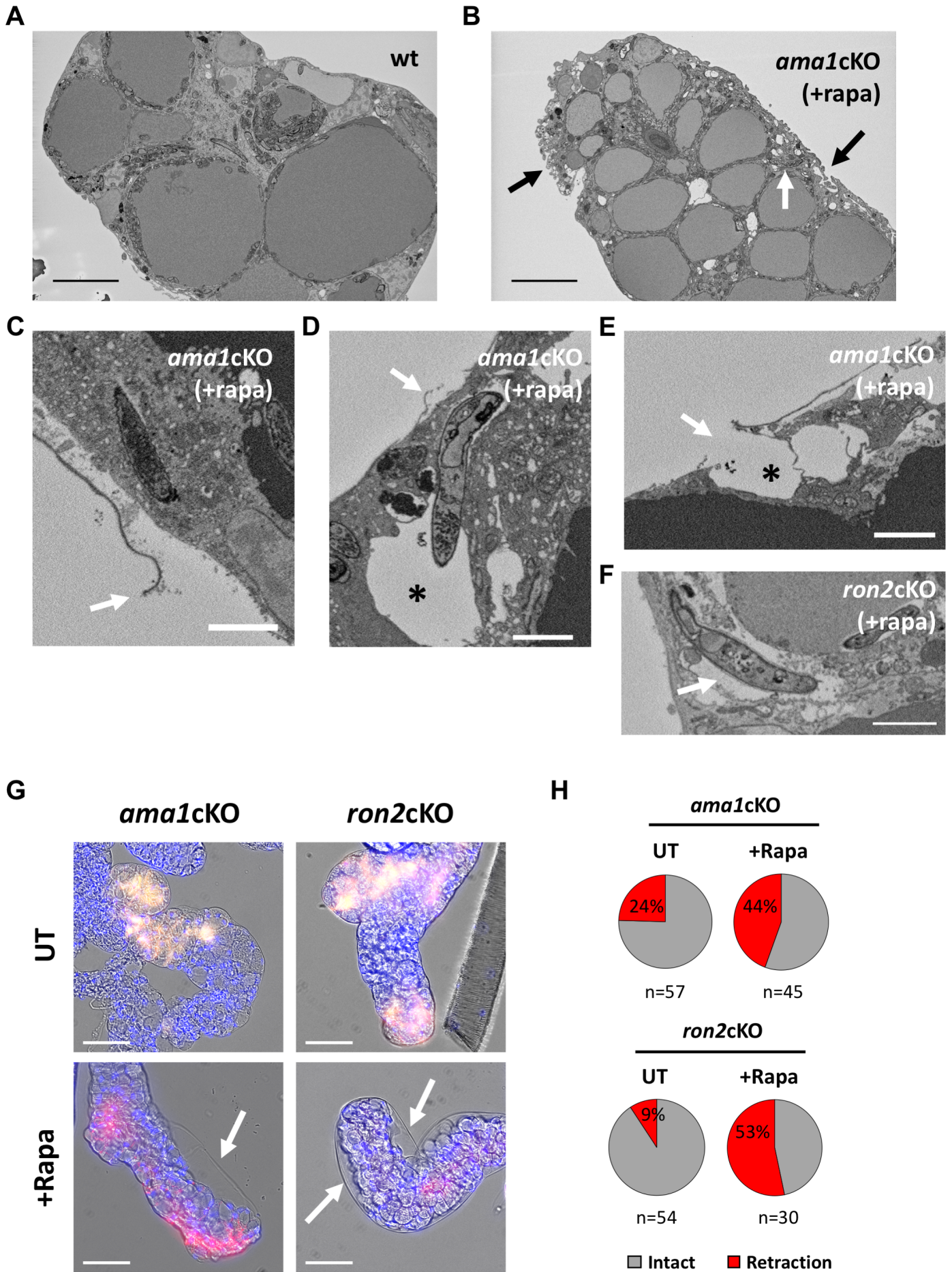
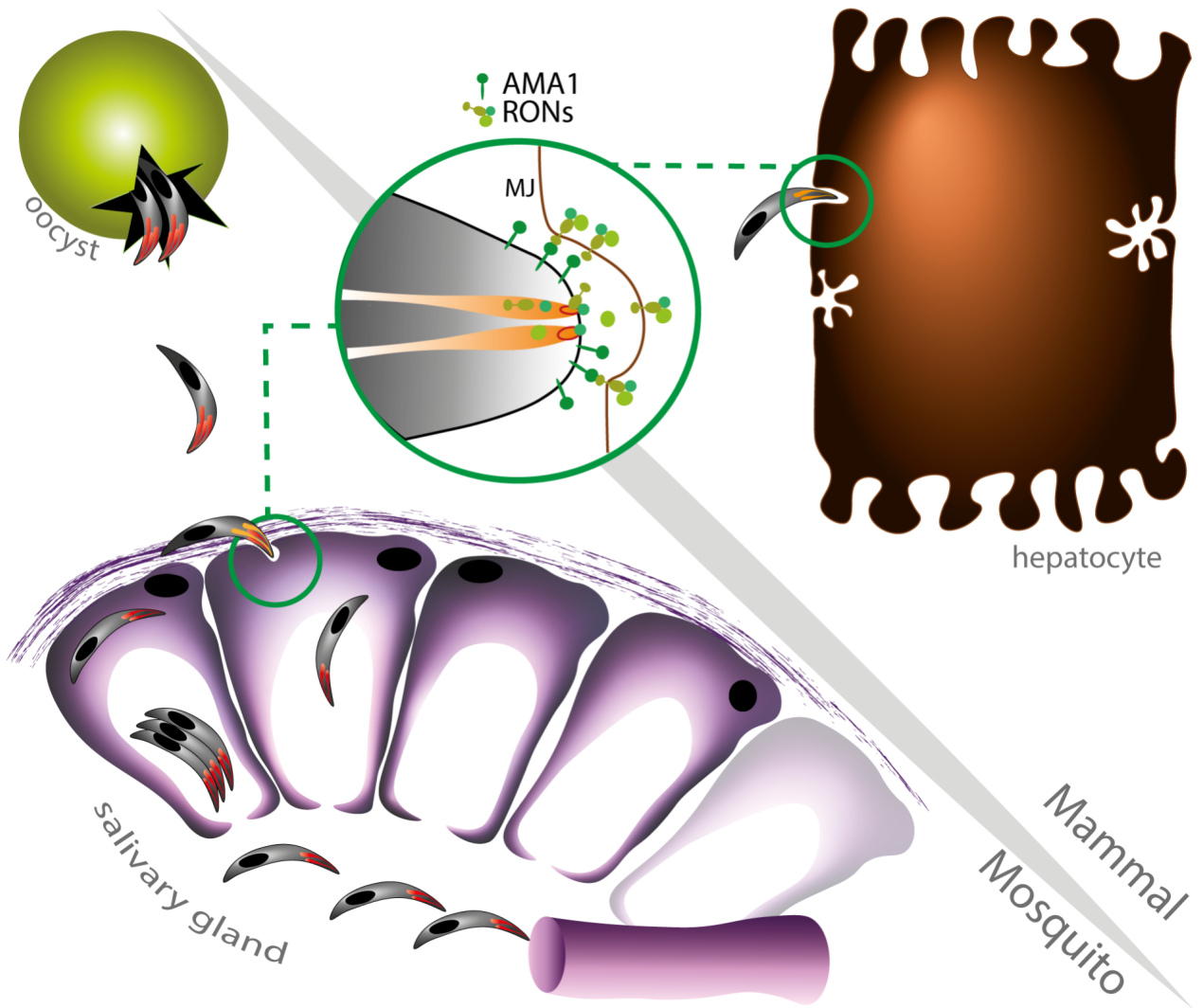
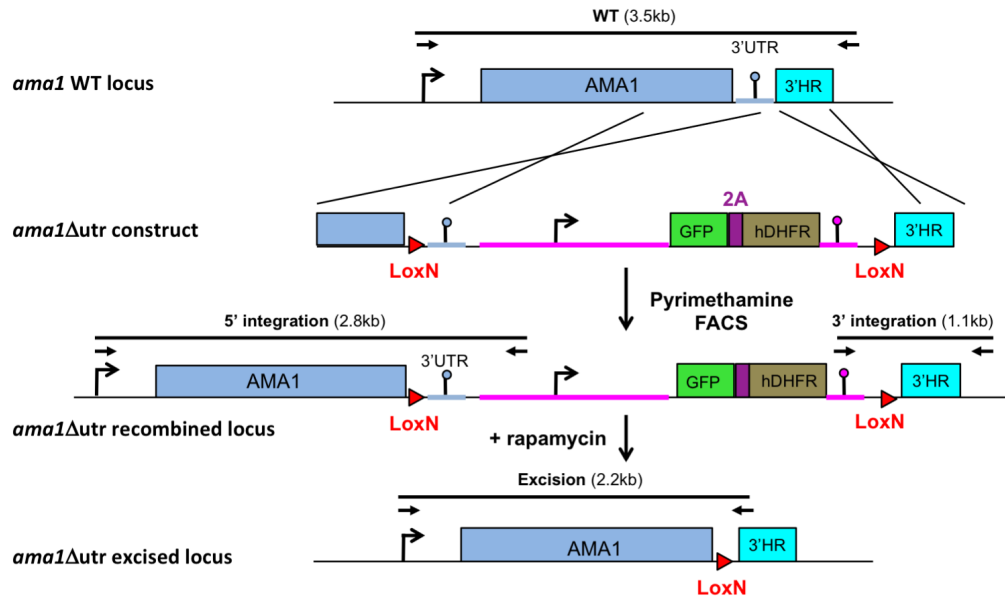


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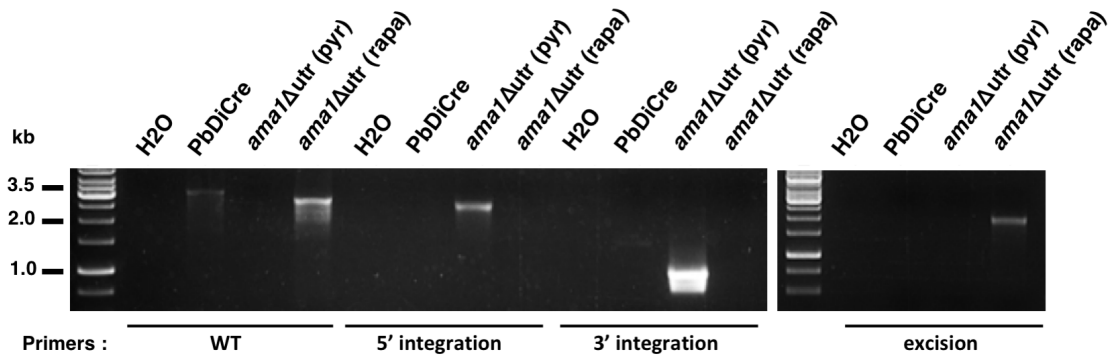


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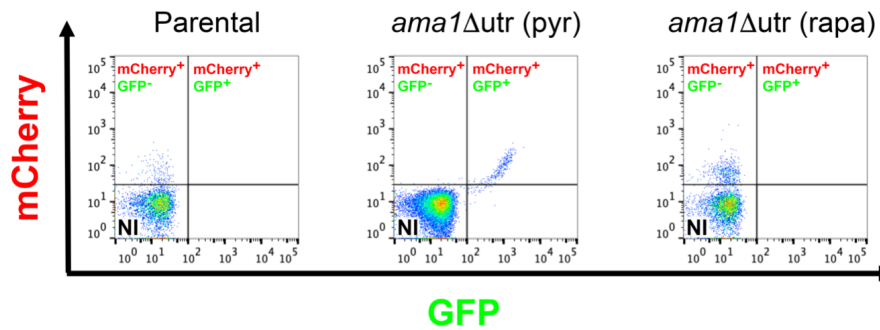
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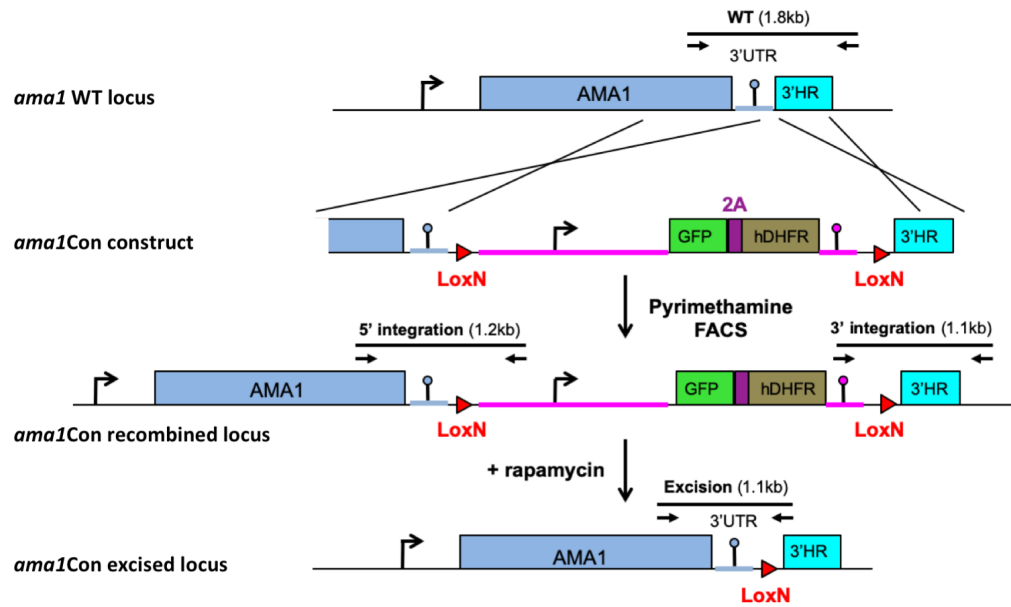


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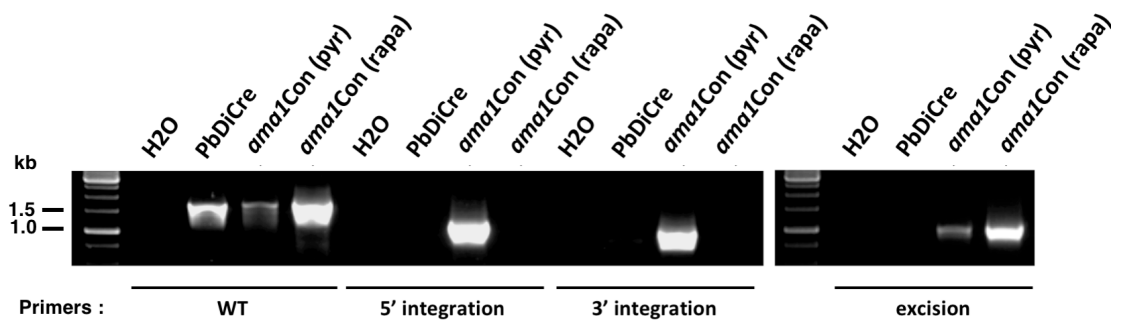


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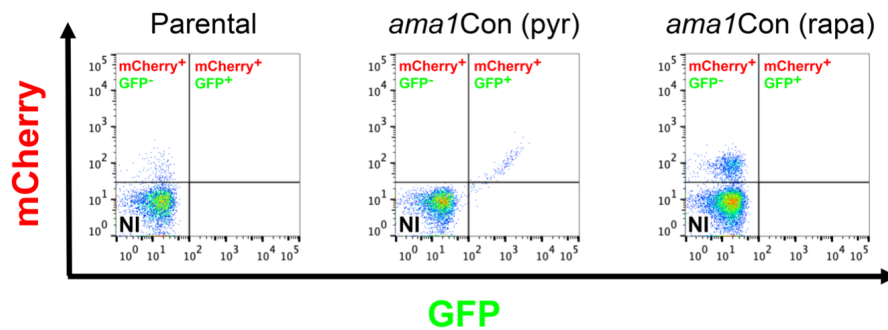
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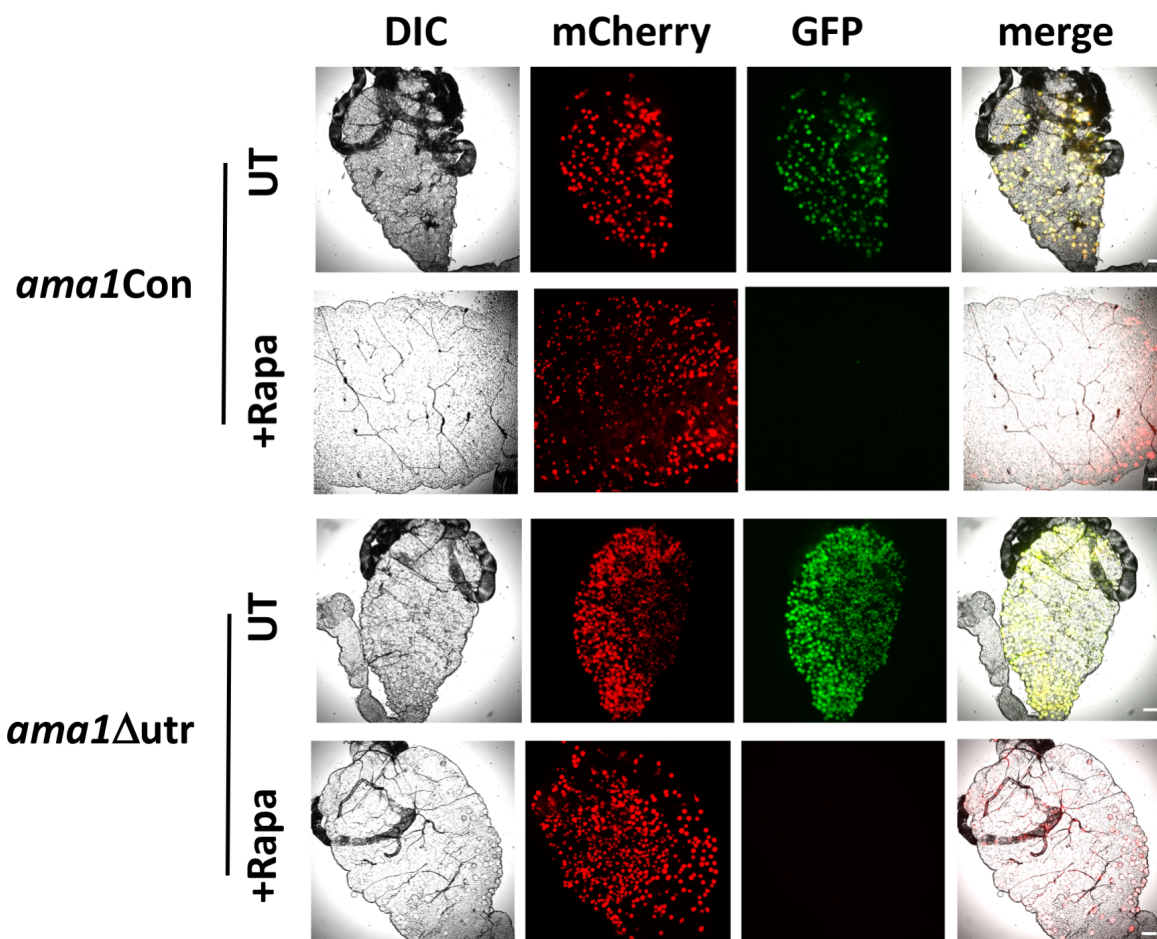


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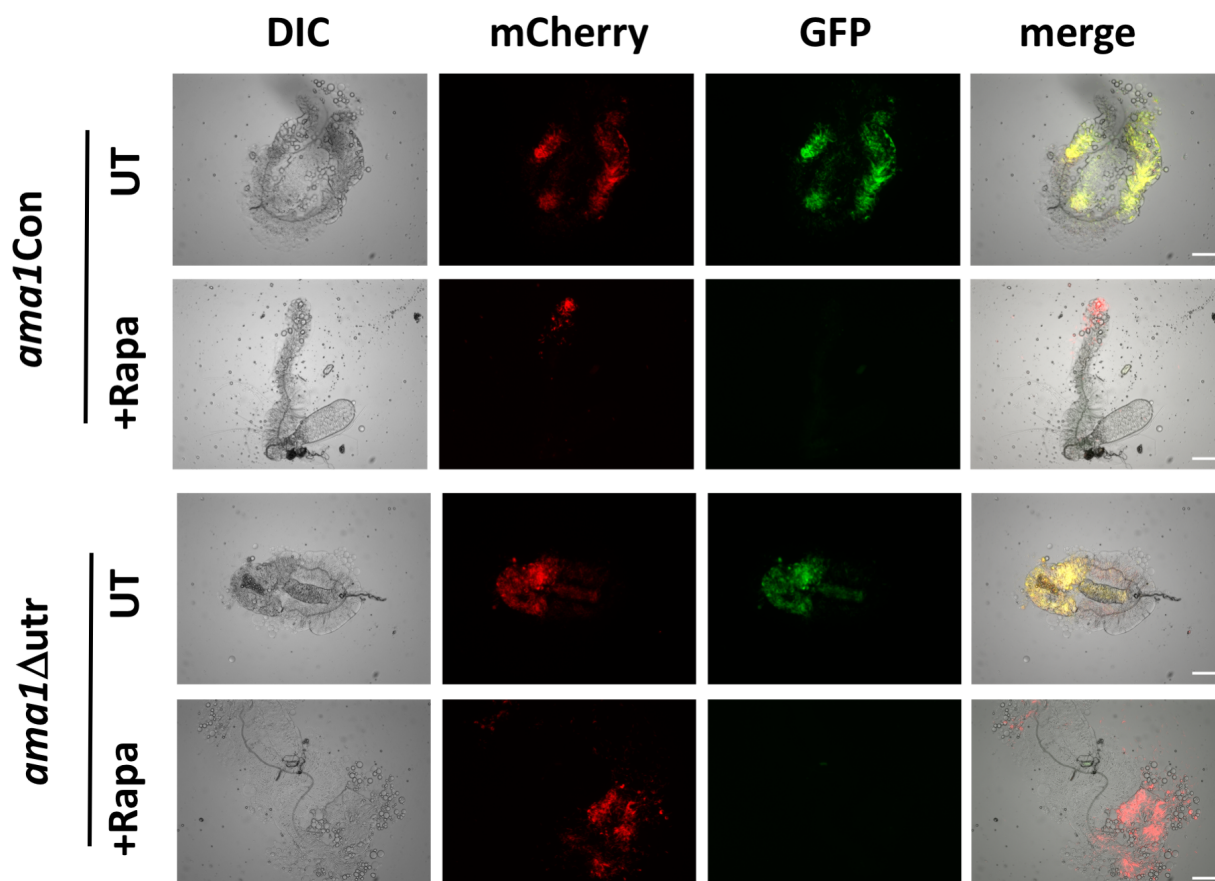


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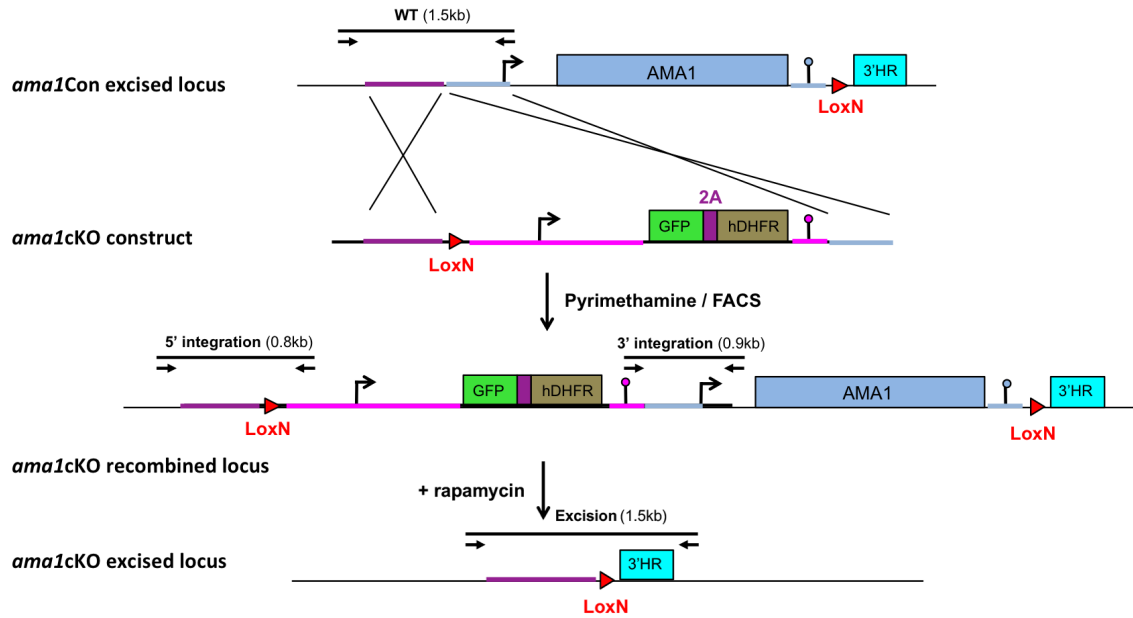


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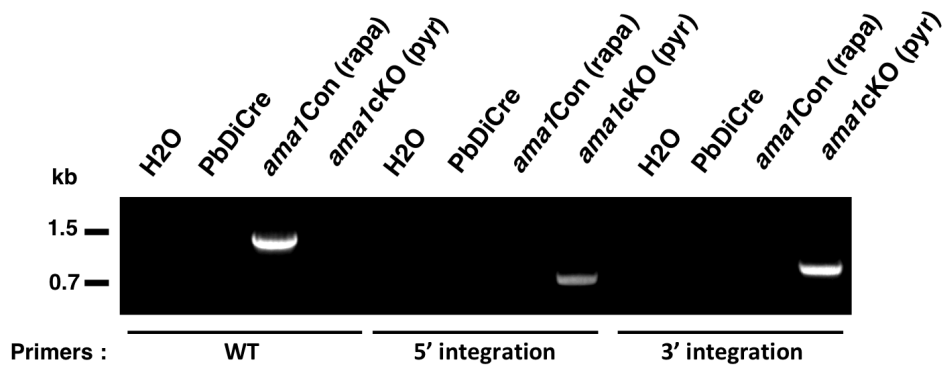


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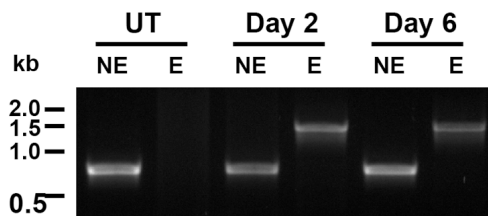
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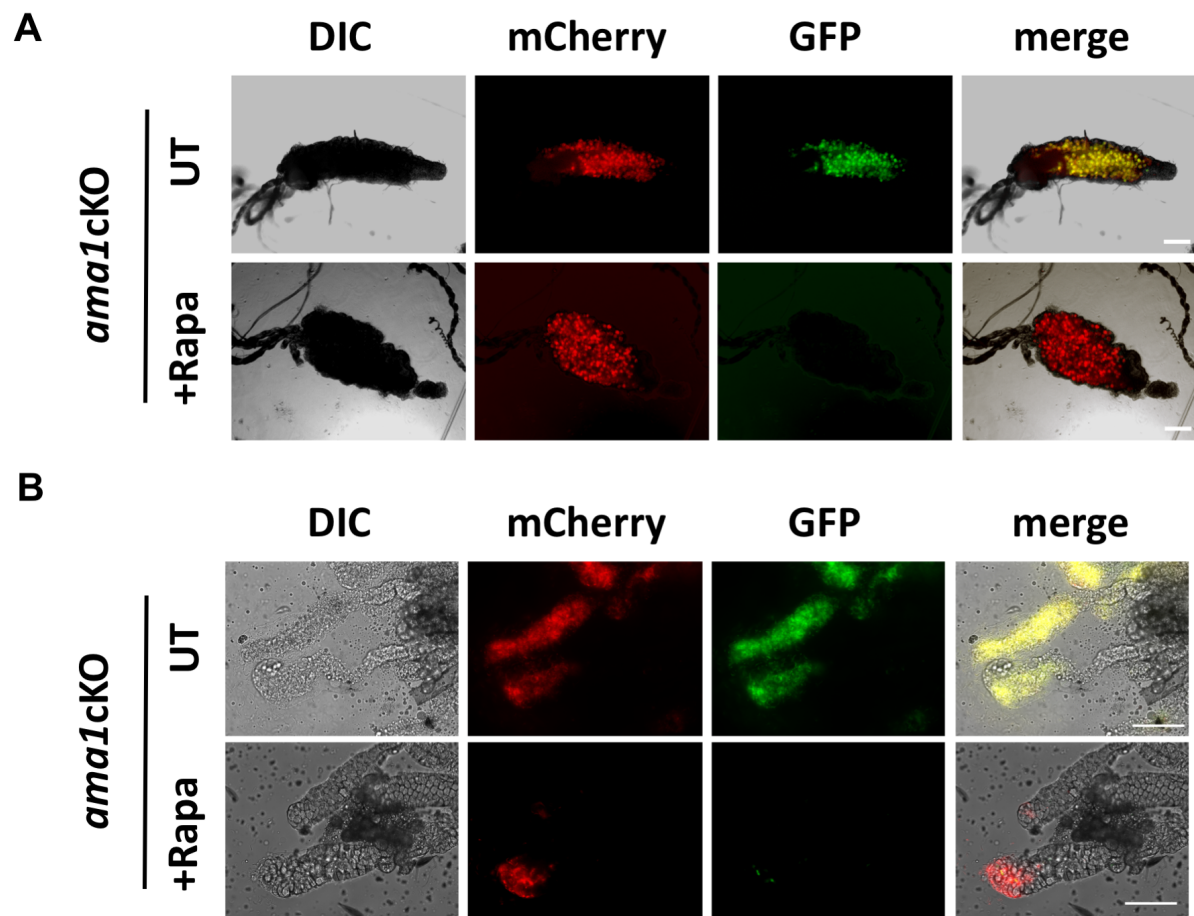
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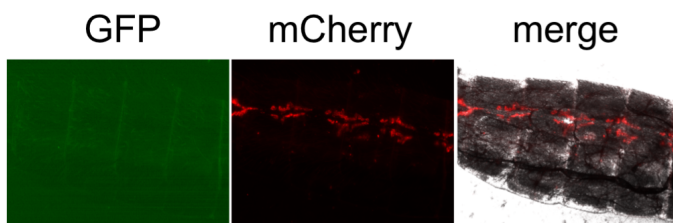


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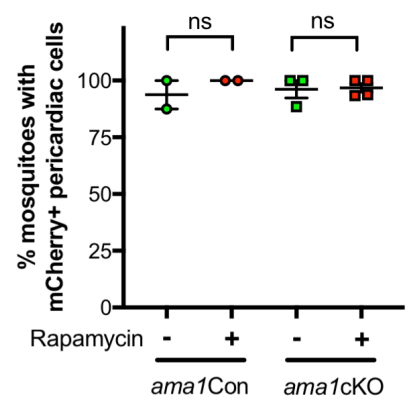


FigS6

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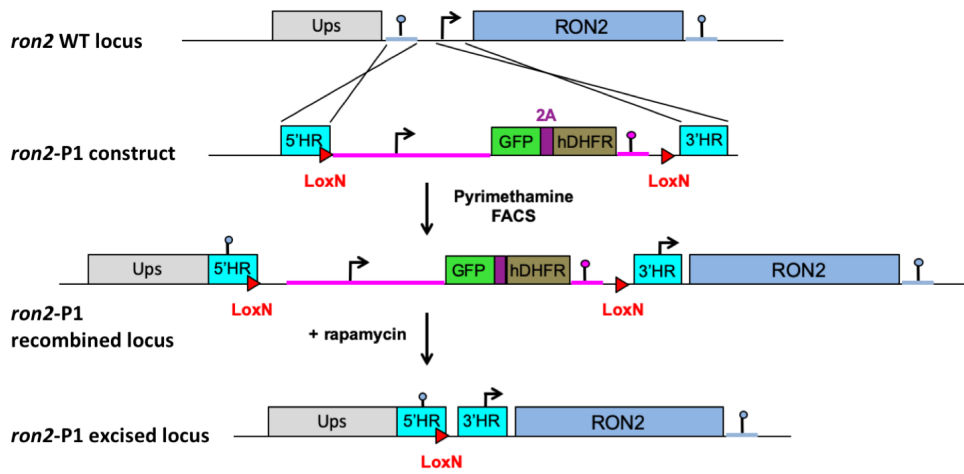


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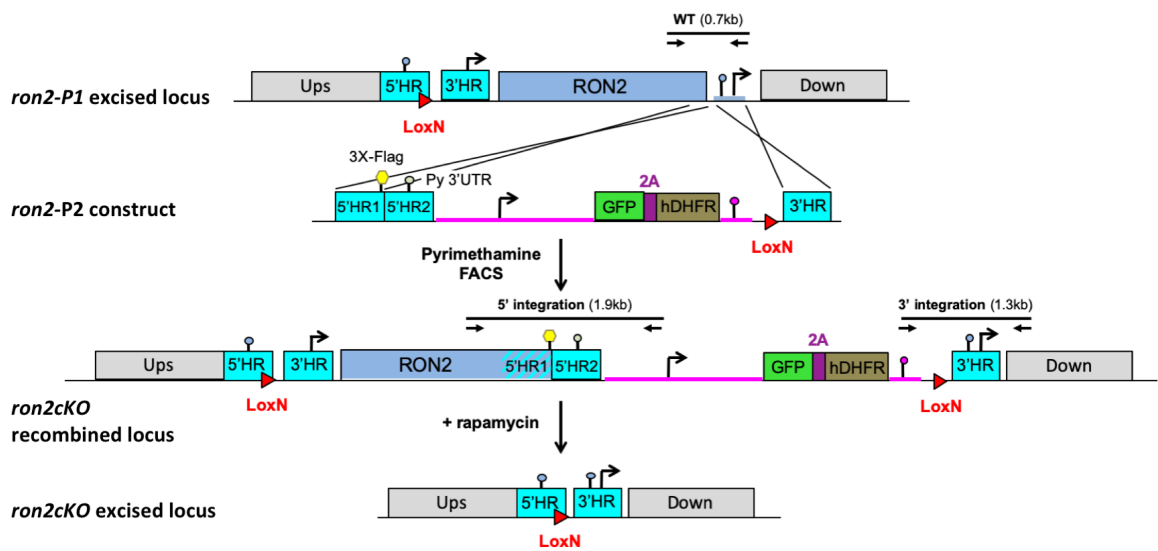


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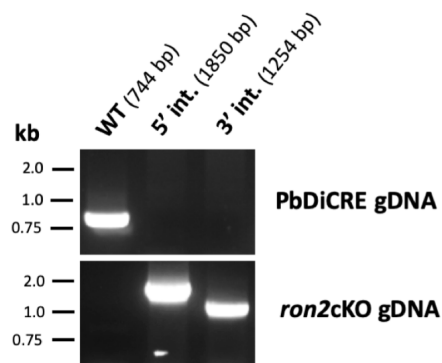
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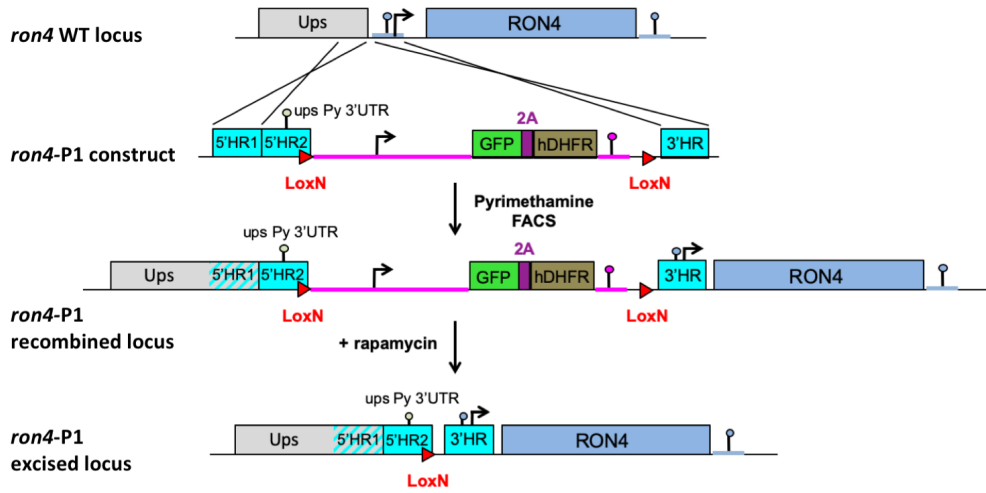


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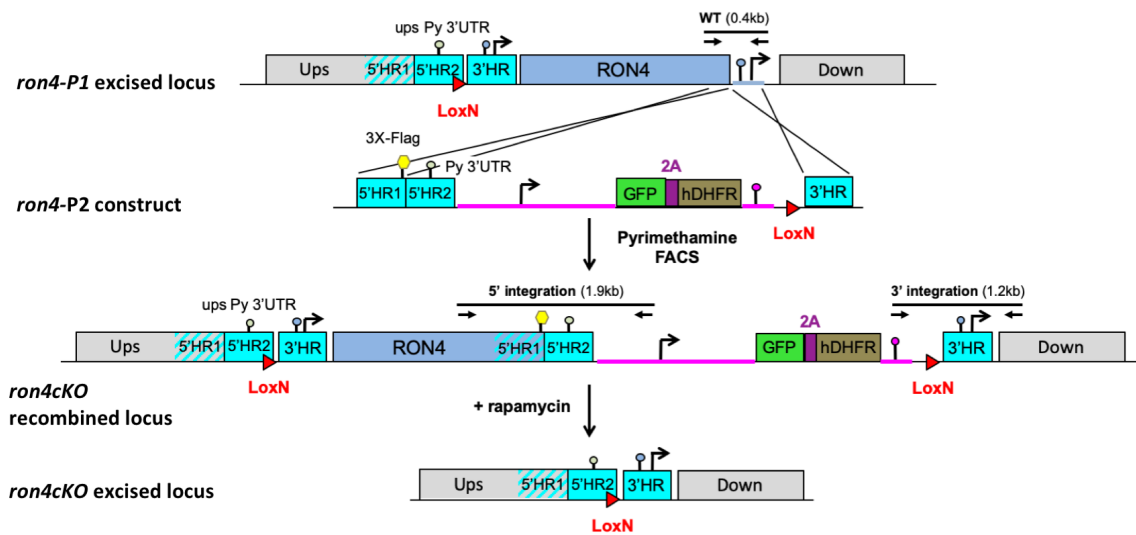


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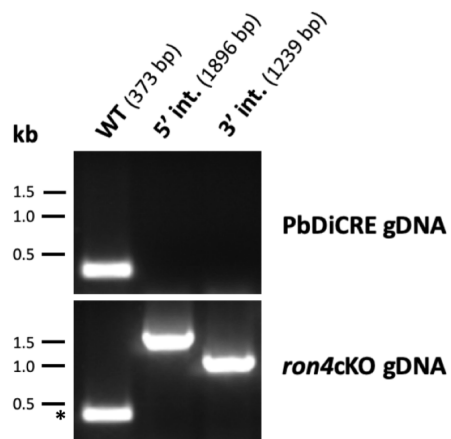
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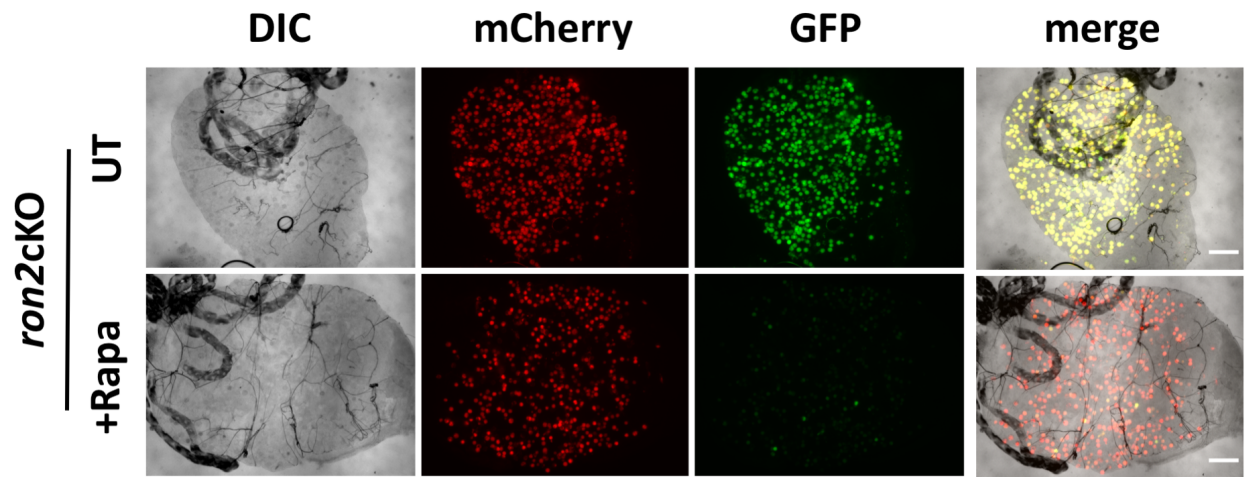


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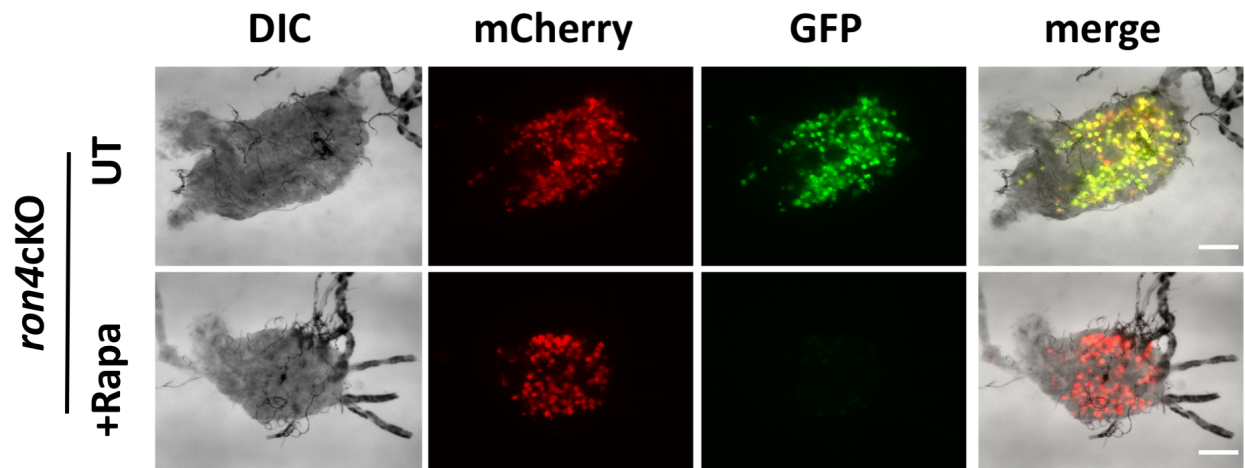


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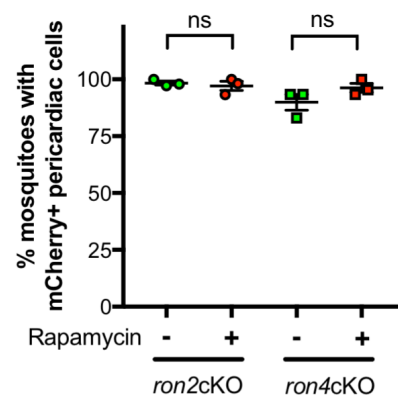
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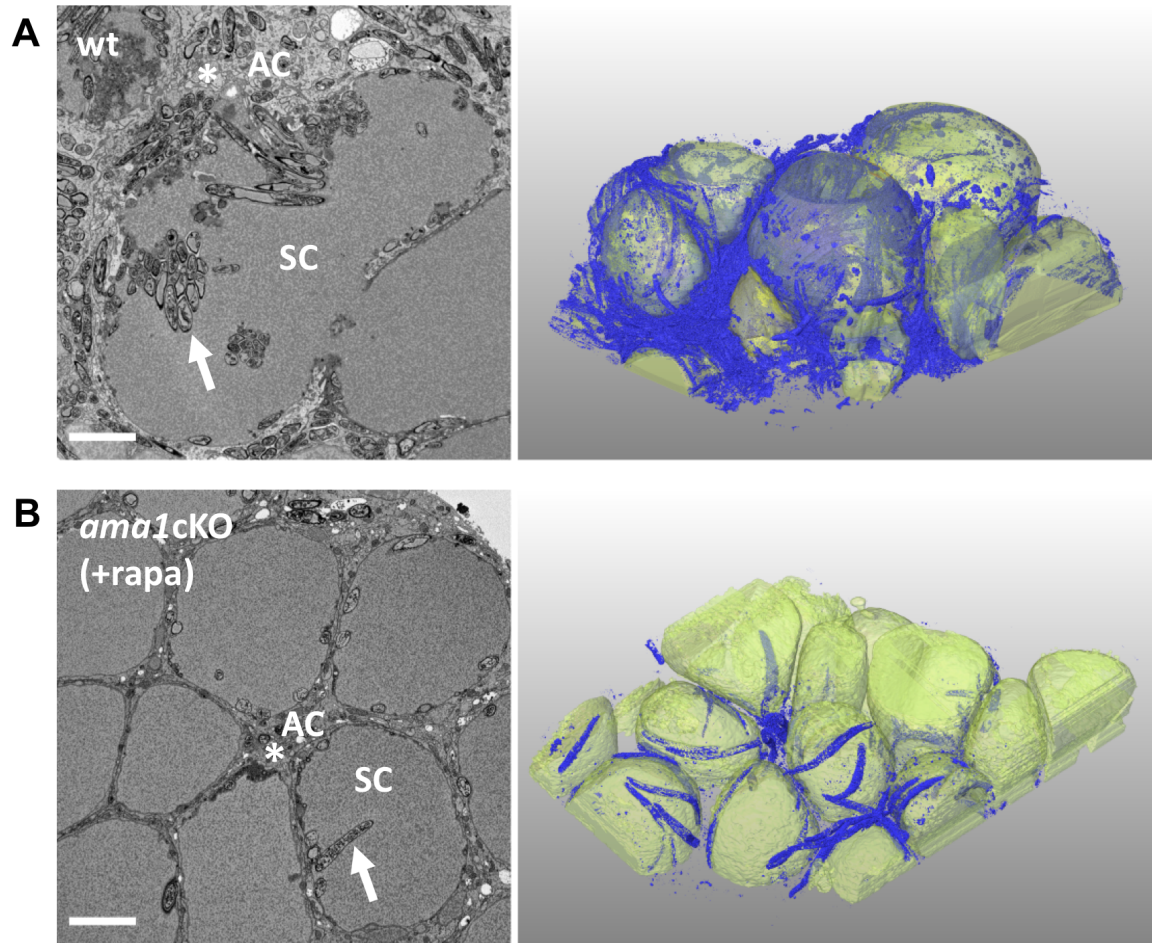
B



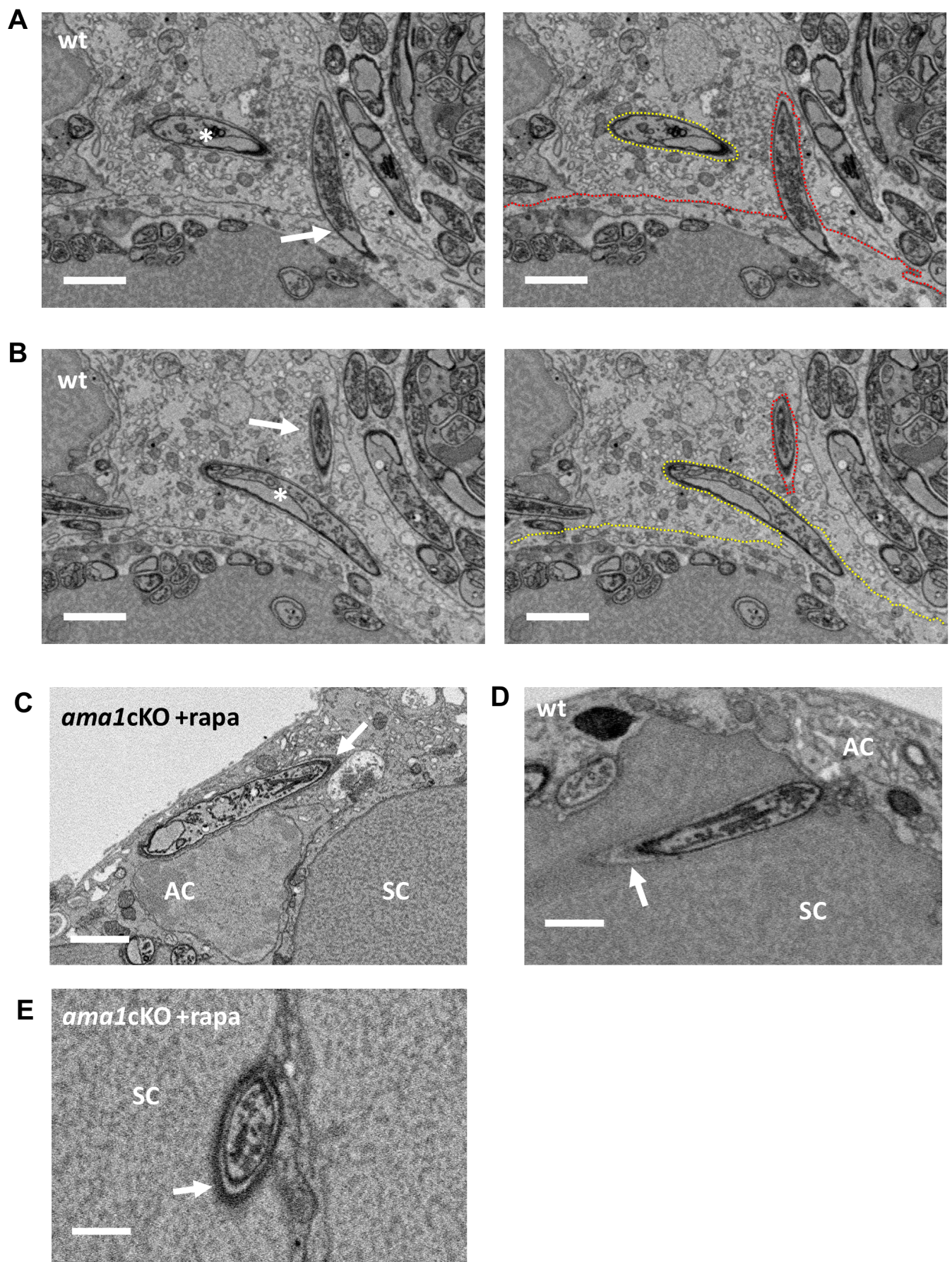
FigS10



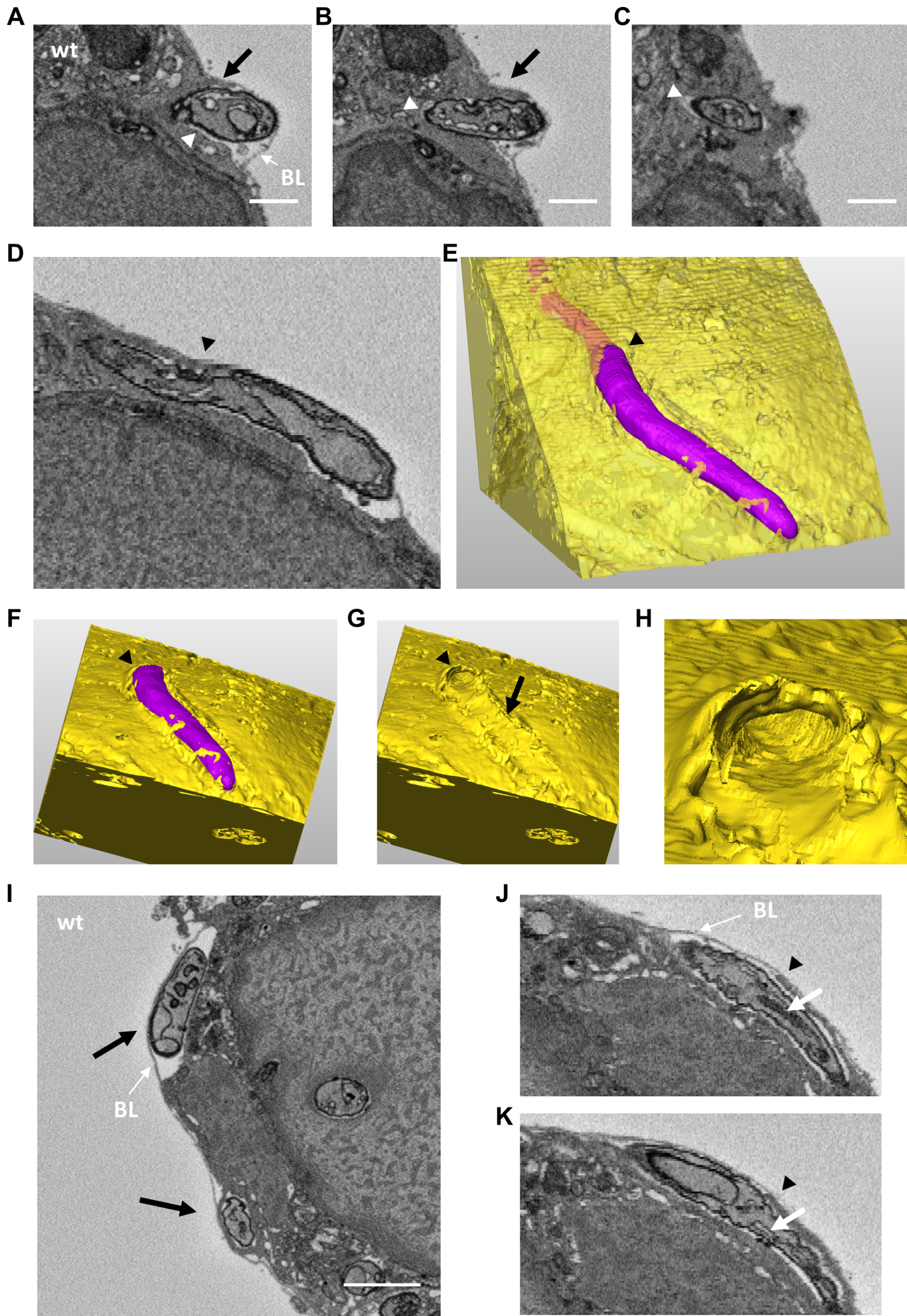
FigS11



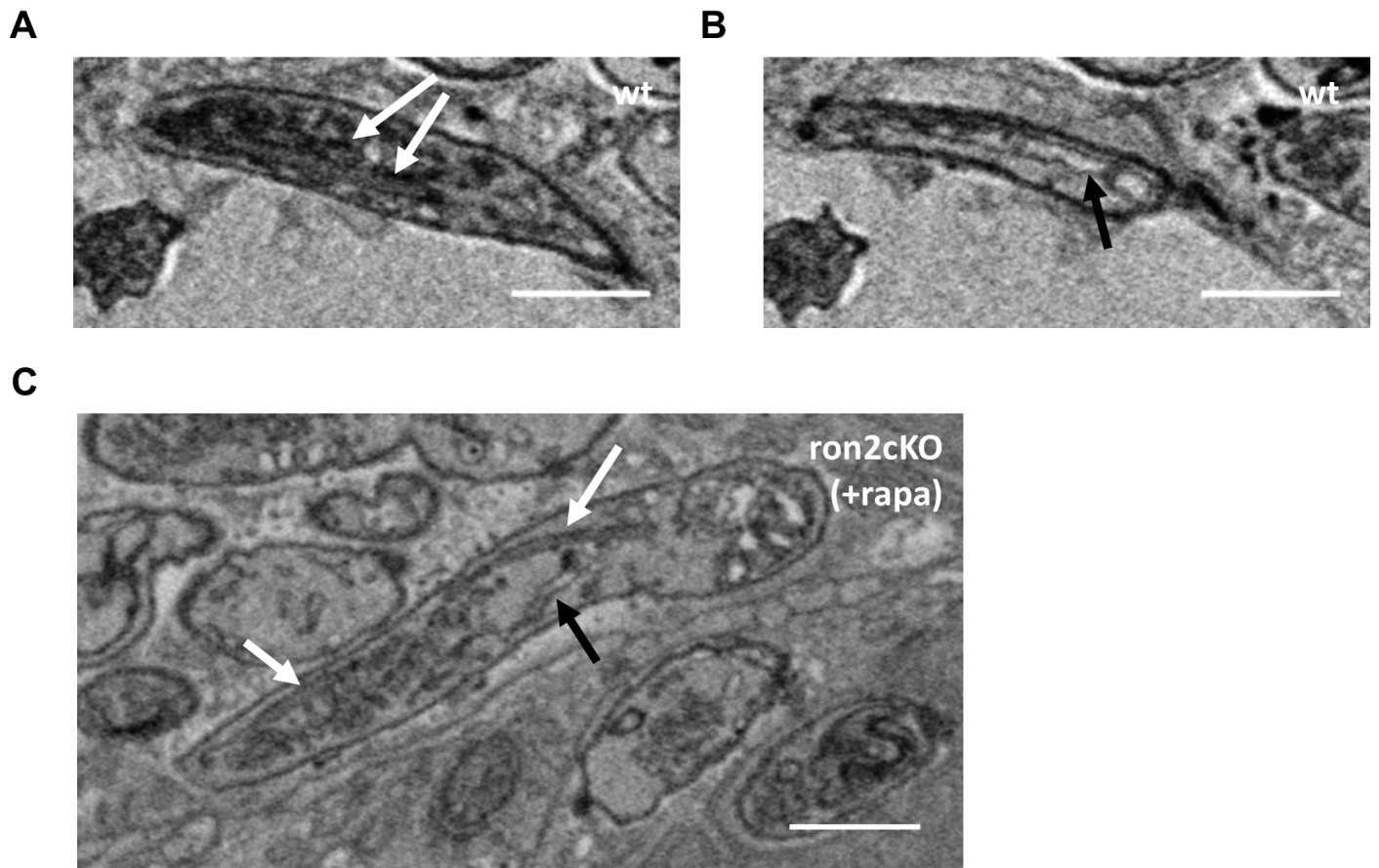
FigS12



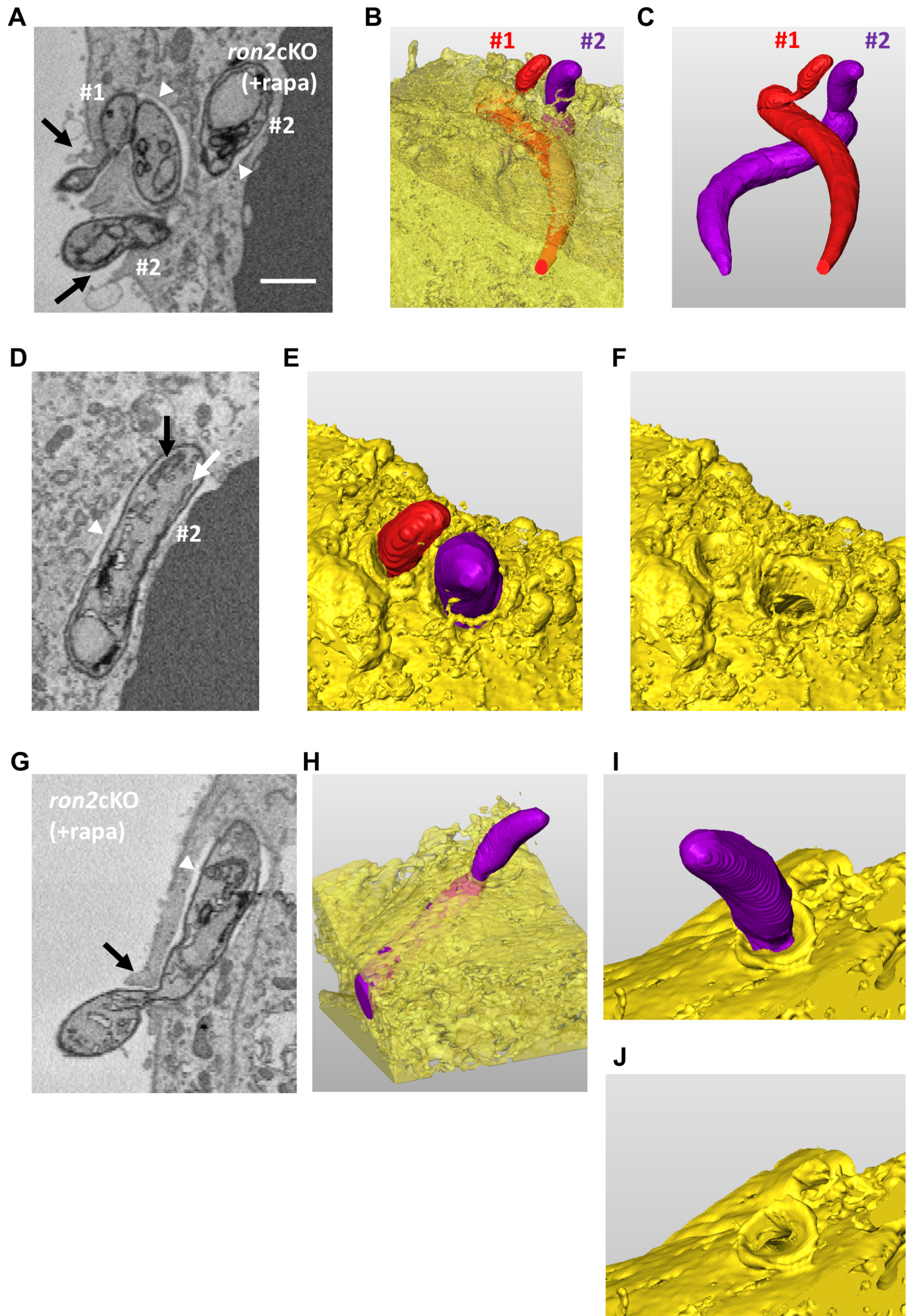
FigS13



FigS14

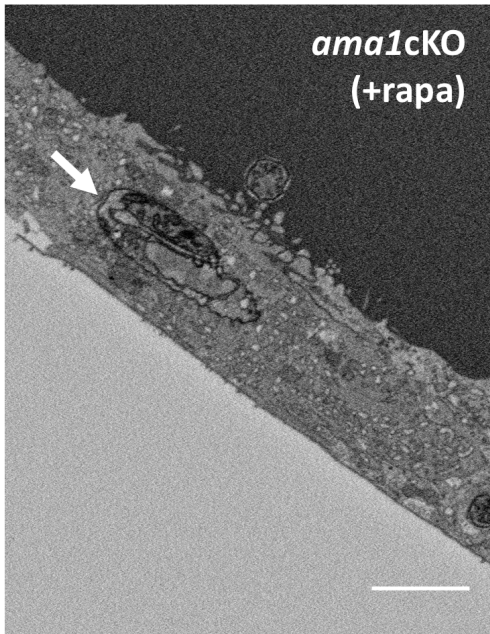


FigS15

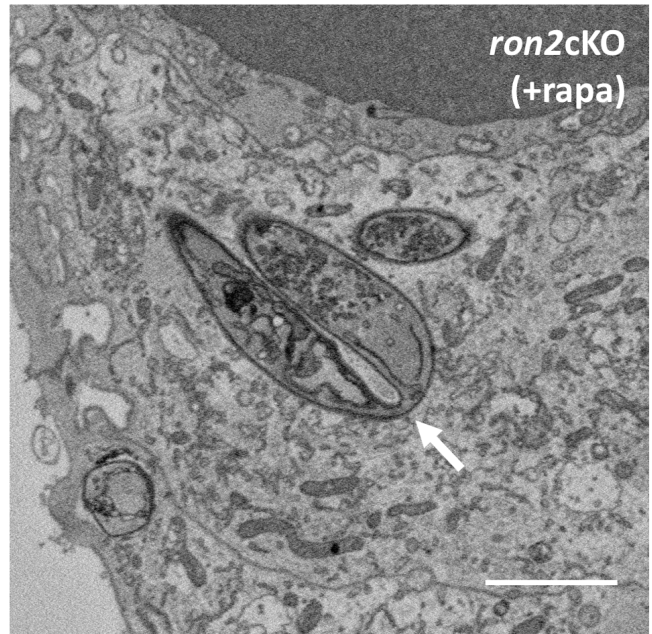


FigS16

A

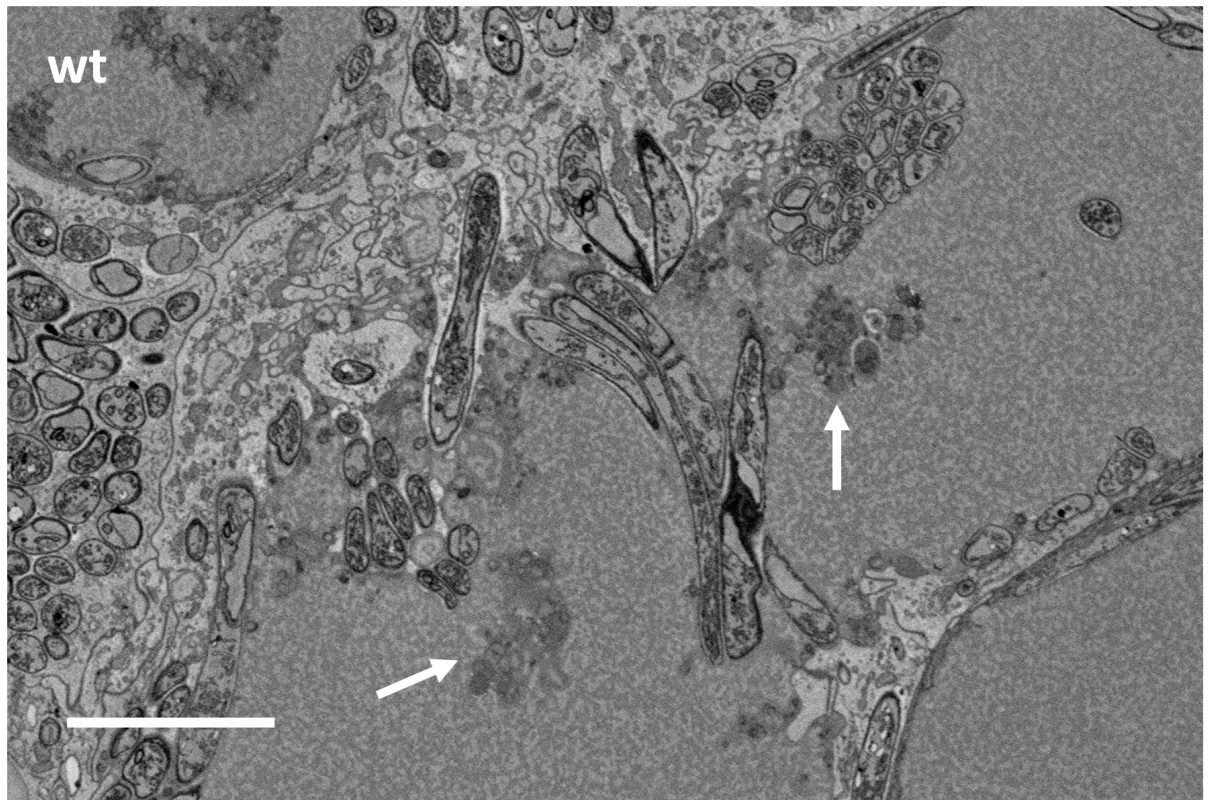


B

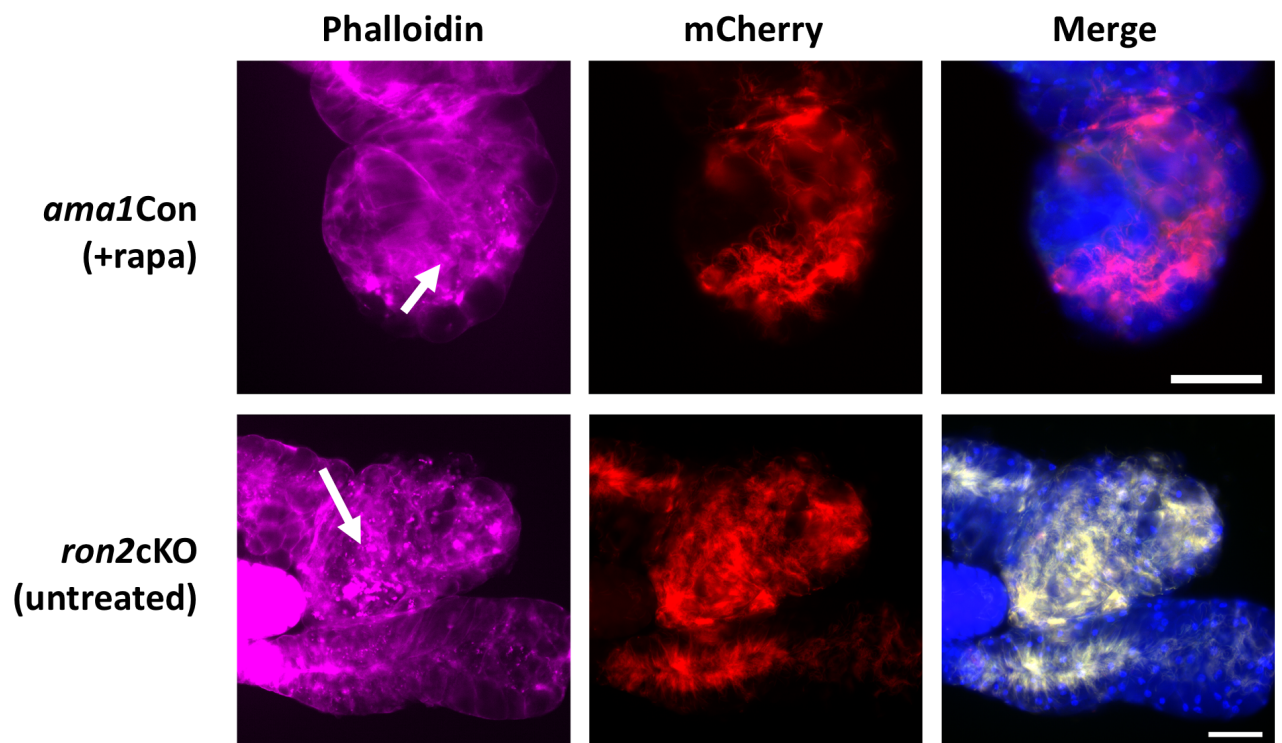


FigS17

A



B



FigS18

