1 Title
2 **Differential requirements of cyclase associated protein (CAP) for actin turnover during**
3 **the lytic cycle of *Toxoplasma gondii***
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17
18 Abstract
19 *Toxoplasma gondii* contains a limited subset of actin binding proteins. Here we show that
20 ablation of the putative actin regulator cyclase-associated protein (TgCAP) leads to significant
21 defects in some but not all actin dependent processes, including a defect in cell-cell
22 communication, but surprisingly not synchronicity of division. Two CAP isoforms originate from
23 alternative translational start sites and are beneficial for parasite fitness while a single isoform
24 is sufficient for virulence in mice. Examination of the mutant parasites by 3D electron
25 microscopy reveals that loss of CAP results in a defect to form a normal residual body, but all
26 parasites remain connected within the vacuole. This dissociates synchronicity of division and
27 parasite rosetting and reveals that establishment and maintenance of the residual body may
28 be more complex than previously thought. These results highlight the different spatial
29 requirements for actin turnover in *Toxoplasma*, controlled by a reduced subset of actin binding
30 proteins.
31
32 Introduction
33 *Toxoplasma gondii* is an obligate intracellular parasite, belonging to the Apicomplexa phylum.
34 The Apicomplexa, which also include *Plasmodium* and *Cryptosporidium* species, pose a
35 significant global public health burden. *Toxoplasma*, specifically, is one of the most prevalent
36 human pathogens, chronically infecting ~30% of the world's population (Swapna & Parkinson,
37 2017). While most infections are asymptomatic, in congenitally infected and
38 immunocompromised patients, disease outcomes are often severe and potentially fatal
39 (Halonen & Weiss, 2013). During acute infection of the host, the asexual tachyzoite stage of
40 *Toxoplasma gondii* undergoes cycles of active invasion, replication and egress from host cells.
41 This lytic cycle leads to rapid proliferation and dissemination of the parasite throughout the
42 host (Black & Boothroyd, 2000). To facilitate these processes, *Toxoplasma* utilises a unique
43 form of locomotion, called gliding motility, which relies on actin and an unconventional myosin
44 motor (Frénal, Dubremetz, et al., 2017). This motor allows the parasite to actively invade host
45 cells, where it forms a protective parasitophorous vacuole (PV). PV structural integrity and
46 stability is sustained through the parasite's release of dense granule proteins from secretory
47 vesicles (Heaslip et al., 2016). Additionally, several dense granule proteins are transported
48 into the host cell where they co-opt or interfere with host cell functions (Hakimi et al., 2017).
49 Within the PV, *Toxoplasma* begins a unique form of cell division called endodyogeny (Sheffield
50 & Melton, 1968). Here, two daughter cells are synchronously assembled within the mother cell
51 before daughter cell budding (Delbac et al., 2001). This initiates at the apical pole of the mother
52 cell and once complete, the daughter cells bud from the mother cell but remain attached at
53 their basal pole to the residual body: a membranous compartment containing maternal
54 remnants of organelles and cytoskeletal elements (Muñiz-Hernández et al., 2011). During cell
55 division, organelles such as mitochondria and the apicoplast, a unique and essential organelle

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56 which contributes to isoprenoid synthesis (Vaishnava & Striepen, 2006), also divide and are
57 distributed between the two daughter parasites. Following multiple rounds of cell division,
58 parasites organise into a rosette-like pattern around the residual body. Formation of the
59 characteristic rosette pattern requires actin, several myosins, and other actin-binding proteins
60 (Frénal, Jacot, et al., 2017; Haase et al., 2015; Jacot et al., 2013; Periz et al., 2017; Tosetti et
61 al., 2019). It has been hypothesised that the inter-parasite connections via the residual body
62 are not only important for parasite organisation, but also play a key role in cell-cell
63 communication. Such communication was measured by the transfer of reporter proteins
64 between parasites and is believed to ensure the synchronous division of parasites within a
65 vacuole (Frénal, Jacot, et al., 2017). The parasites continue to replicate until host cell lysis and
66 parasite egress. Following egress, parasites migrate to and invade new host cells and the lytic
67 cycle repeats, leading to tissue destruction (Black & Boothroyd, 2000). Actin plays an essential
68 role in the parasite's lytic cycle through function of the actin cytoskeleton and actomyosin motor
69 complex. Despite this crucial role in apicomplexan biology, there has been difficulty visualising
70 actin filaments in apicomplexan species (Bannister & Mitchell, 1995; Sahoo et al., 2006; Shaw
71 & Tilney, 1999) and it has been suggested that as much as 98% of parasite actin is monomeric
72 (G-actin) and not incorporated into filaments (F-actin) (Dobrowolski et al., 1997). This, along
73 with structural differences found in actin of Apicomplexa (Pospich et al., 2017), led to the
74 hypothesis that *Toxoplasma* F-actin has reduced stability, forming abnormally short filaments
75 that are rapidly recycled to maintain essential cellular function (Pospich et al., 2017; Skillman
76 et al., 2011). However, recent development of the actin-chromobody has allowed for the
77 visualisation of long F-actin structures both in the parasite and extensive networks within the
78 PV (Periz et al., 2017). Taken together, along with biochemical evidence, apicomplexan actin
79 appears to be different to actin from other organisms (Frénal, Dubremetz, et al., 2017).
80
81 Actin turnover is regulated by actin binding proteins, of which *Toxoplasma* possesses a
82 reduced repertoire, including ADF/cofilin, profilin, coronin and cyclase-associated protein
83 (CAP) (Baum et al., 2006). Functional studies have shown ADF/cofilin and profilin to be
84 essential for *Toxoplasma* progression through the lytic cycle, while coronin depletion had a
85 modest impact on parasite invasion and egress (Mehta & Sibley, 2011; Plattner et al., 2008;
86 Salamun et al., 2014). Apart from its localisation, the function of CAP in *Toxoplasma* has not
87 been investigated. In the majority of eukaryotes, CAP is a highly conserved multidomain
88 protein that regulates actin filament dynamics via two distinct mechanisms (Ono, 2013). CAP
89 can bind and sequester G-actin using its CAP and X-linked retinitis pigmentosa 2 protein
90 (CARP) domain and can regulate actin filament disassembly by promoting ADF/cofilin
91 mediated severing using its helical folded domain (HFD). Through regulation of actin dynamics,
92 by interacting with actin and other actin binding proteins, it has been shown that mouse CAP1
93 plays important roles in cell morphology, migration and endocytosis (Bertling et al., 2004).
94 However, species belonging to the Apicomplexa phylum possess a truncated form of CAP,
95 retaining only the conserved C-terminal G-actin-binding CARP domain (Hliscs et al., 2010).
96 This conserved β-sheet domain has been shown to interact directly with monomeric actin,
97 providing either sequestration or nucleotide exchange of G-actin in a concentration-dependent
98 manner (Hliscs et al., 2010; Makkonen et al., 2013; Mattila et al., 2004). As such, apicomplexan
99 CAP is hypothesised to regulate actin turnover solely through interaction with monomeric actin.
100 Biochemical analysis of *Cryptosporidium parvum* CAP identified the formation of a dimer and
101 G-actin sequestering activity, while *Plasmodium falciparum* CAP was shown to facilitate
102 nucleotide exchange, loading ADP-actin monomers with ATP (Hliscs et al., 2010; Makkonen
103 et al., 2013). A *Plasmodium berghei* CAP KO demonstrated that while PbCAP is dispensable
104 for asexual blood stages *in vivo*, there is a complete defect in oocyst development in the insect
105 vector which was overcome through complementation with *C. parvum* CAP (Hliscs et al.,
106 2010). A PbCAP overexpression study revealed no defect in ookinete motility or oocyst
107 development, however sporozoites displayed impaired gliding motility, invasion and salivary
108 gland colonisation (Sato et al., 2016). Taken together, these results suggest that apicomplexan
109 CAP may function as a dimer with the ability to interact with G-actin monomers to sequester
110 them and/or facilitate their nucleotide exchange. While CAP has yet to be functionally

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111 characterised in *Toxoplasma*, Lorestani *et al* reported that CAP localises to the apex of
112 intracellular parasites, a hub for events leading to egress and motility (Graindorge et al., 2016;
113 Lorestani et al., 2012; Tosetti et al., 2019). Intriguingly, following host cell lysis, relocalisation
114 of CAP to the parasite cytosol was observed (Lorestani et al., 2012). Furthermore, we have
115 previously identified differential phosphorylation of TgCAP in parasites with a delayed egress
116 phenotype (Treeck et al., 2014). The correlation between TgCAP redistribution and
117 phosphorylation, following host cell lysis, hints at a potential role for TgCAP in actin regulation
118 during rapid egress. Taken together, these results prompted us to characterise the role of
119 TgCAP in *Toxoplasma* biology.
120
121 Here we show that TgCAP is produced in two distinctly localised isoforms through alternative
122 translation initiation: a membrane bound isoform, localised to the apical tip (longCAP) and a
123 cytosolically dispersed isoform (shortCAP). Conditional knockout of TgCAP, using a second
124 generation DiCre strain, identified an important function of TgCAP in some, but not all, actin-
125 dependent processes. Invasion, egress, motility, correct daughter cell orientation and dense
126 granule trafficking were all perturbed in CAP depleted parasites, while apicoplast inheritance
127 was not. This suggests different spatial requirements for CAP in actin turnover within the cell.
128 Furthermore, the characteristic rosette organisation of parasites in the vacuole was completely
129 lost, but synchronicity of division was unaffected. Strikingly, while we observe rapid protein
130 transfer only between two adjacent cells in a vacuole, all parasites remain connected through
131 a decentralised residual body, potentially explaining why synchrony of division is unaffected.
132 In the mouse *in vivo* infection model, CAP-depleted type I RH parasites display normal lethality,
133 while CAP depletion renders type II Pru parasites avirulent, with markedly reduced cyst
134 formation. Furthermore, the cytoplasmic isoform of TgCAP was sufficient for the infection of
135 mice and the formation of latent stages in the brain, indicating that the apically localised CAP
136 isoform provides only a small fitness benefit under the conditions tested here.
137
138 Results
139 **TgCAP contains two translational start sites which results in the production of two**
140 **differentially localised protein isoforms.**
141 TgCAP was previously shown to localise to the apex of intracellular parasites and rapidly
142 redistribute to the cytoplasm of extracellular parasites following host cell lysis (Lorestani et al.,
143 2012). This suggested that TgCAP localisation may be influenced by post-translational
144 modifications that enable CAP to regulate actin dynamics at different locations in the cell.
145 TgCAP, and CAP from *Neospora* and *Hammondia* species contain a unique predicted N-
146 terminal extension that is not present in other Apicomplexa, such as *Plasmodium* (Fig 1A). The
147 extension contains two predicted palmitoylation sites and CAP was identified in an analysis of
148 palmitoylated proteins in *Toxoplasma gondii* (Foe et al., 2015). Furthermore, two
149 phosphorylation sites in the N-terminus of TgCAP are substantially phosphorylated upon
150 ionophore-induced egress (Treeck et al., 2014). These phosphorylation events are dependent
151 on the calcium-dependent kinase 3 (TgCDPK3) (Treeck et al., 2014), which has been shown
152 to be important in mediating rapid exit from the host cell and is localised to the plasma
153 membrane (Black et al., 2000; Garrison et al., 2012; Lourido et al., 2012; McCoy et al., 2012).
154 Collectively, these observations allow for the possibility that re-localisation of CAP is important
155 for egress and is mediated by dynamic post-translational modifications. To visualise TgCAP
156 we expressed it as a GFP fusion. TgCAP-GFP localises to the apex of the parasite, as
157 previously shown, but also to the cytoplasm of intracellular tachyzoites (Fig. 1B). We
158 additionally demonstrated this dual localisation of TgCAP by C-terminally tagging the
159 endogenous TgCAP locus with a HA epitope tag (Supplementary Fig. 1). To rule out any mis-
160 localisation hof the protein as a result of tagging, we expressed recombinant TgCAP, spanning
161 residues 37 to 203, and generated antibodies against TgCAP which confirmed the dual
162 localisation (Fig 1C). Western blot analysis of parasite lysates revealed the presence of two
163 bands close to the expected size of TgCAP, which are expressed at a constant level, relative
164 to the *Toxoplasma* loading control, across the first 24 hours following host cell invasion (Fig.
165 1D). The dual localisation of CAP could be due to the expression of two isoforms from a single

gene, through use of alternate translational start sites, as previously observed for protein kinase G (Brown et al., 2017). Indeed, sequence comparison of TgCAP to PfCAP reveals a second in-frame methionine at position 37 in *Toxoplasma* that aligns with the PfCAP start methionine (Fig. 1A). Two additional methionine residues, M71 and M161, are present in the CAP primary sequence. However, these are unlikely used as translational start sites as their products would lead to a truncated and potentially inactive CARP domain.

Translational start sites in eukaryotic mRNAs are preceded by a translation initiation sequence (Kozak, 1987a, 1987b). Consensus translation initiation sequences have been determined for many different organisms and are known as the Kozak sequence (Nakagawa et al., 2008). In *Toxoplasma*, the Kozak sequence was elucidated by identifying nucleotides commonly found in highly abundant proteins, such as SAG1 (Seeber, 1997). This Kozak sequence contains an adenine at the -3 position, relative to the start ATG, and was identified as the most important factor in ribosomal recognition of the start ATG (Seeber, 1997). Absence of an adenine can result in ribosome "leaky scanning" and translation from an internal ATG. We therefore analysed the translation initiation sequence around CAP's first (M1) and second (M37) putative translational start sites. The M1 translation initiation sequence conforms less with the *Toxoplasma* Kozak sequence than the sequence preceding M37; as the former is lacking an adenine at the -3 position (Fig. 1E). This suggested that alternative translation could lead to the generation of two CAP isoforms: longCAP, which is translated from the first start ATG, and shortCAP, which is translated from the second start ATG. To test this, we generated parasite strains that expressed either the WT sequence or variants where either the first (M1), or the second methionine (M37) of TgCAP was mutated to leucine, precluding their use as translational start sites (Fig. 1F). We used the endogenous promotor (i.e. 969 bp upstream of the first start ATG) and introduced the C-terminal HA-tagged TgCAP variants into the *UPRT* locus of the RH DiCreΔ*ku80*Δ*hxgprt* parasite strain. To determine whether longCAP and shortCAP show differential localisation, as predicted by the presence of putative palmitoylation sites in longCAP, we analysed their subcellular localisation using the HA-tag. While WT CAP parasites showed the expected dual localisation, mutants expressing shortCAP showed exclusively cytoplasmic staining while those expressing longCAP showed predominantly apical staining, with some further signal throughout the parasite (Fig. 1G). As longCAP contains two putative palmitoylation sites not present in the shortCAP sequence, we next evaluated whether palmitoylation was important for the apical localisation of the long CAP isoform. We mutated the two cysteines in the N-terminus to alanine residues (CAP_C6,8A). CAP_C6,8A appeared cytosolic with no detectable accumulation of CAP at the apical end of the parasites (Fig. 1G). Western blot analysis of the HA-tagged TgCAP variants confirmed that WT CAP is identified as two distinct protein bands, which correlate with the predicted size for longCAP and shortCAP (23.9 and 20 kDa). Parasites expressing shortCAP displayed only the lower molecular weight band, while longCAP-expressing parasites only showed the higher molecular weight band. We observed that the protein levels of longCAP appear reduced compared to its isoform in parasites expressing WT CAP, whereas the shortCAP isoform shows an increase (Fig. 1H). As expected, despite CAP_C6,8A not being detected at the apical end of the parasite, both isoforms were detected by Western blot at levels comparable to parasites expressing WT CAP. Collectively, these results show that in *Toxoplasma*, and potentially closely related coccidian parasites of *Hammondia* and *Neospora*, CAP is produced as two differentially localised isoforms using alternative translational start sites and the apical localisation of the long isoform is likely palmitoylation dependent.

Generation of a more stable RH DiCre Δ*ku80* cell line
While attempting to generate a conditional knock out (cKO) of CAP using the DiCre strategy, we observed a frequent loss of one of the DiCre subunits, resulting in dysfunctional floxed CAP parasite strains that lacked the ability to excise CAP. This is possibly because both DiCre subunits are driven by identical 5' and 3' UTRs, allowing for potential recombination in the Δ*ku80* parental line, which possesses an increased efficiency of homologous recombination (Fox et al., 2009; Huynh & Carruthers, 2009). To prevent loss of DiCre subunits, we generated

221 a new DiCre construct, DiCre_T2A, that expresses the two DiCre subunits from a single
222 promotor using T2A skip peptides (Kim et al., 2011). To further minimise the potential for loss
223 of DiCre, we placed a chloramphenicol acetyltransferase (CAT) selectable marker between
224 the two subunits (Fig. 2A). This would lead to the production of the two separate Cre subunits
225 and the CAT selectable marker. We inserted this construct, into the modified *KU80* locus of
226 the RH Δ*ku80Δhxgprt* strain (Huynh & Carruthers, 2009) using CRISPR/Cas9. To test whether
227 expression of the DiCre subunits in the resulting line, RH DiCre_T2A Δ*ku80Δhxgprt*, is stable
228 over time, we integrated the loxP-KillerRed-loxP-YFP reporter construct used in Andenmatten
229 *et al*., into the *UPRT* locus (Andenmatten et al., 2013). As expected, non-treated parasites
230 express KillerRed which, upon RAP treatment, is excised and leads to expression of YFP (Fig.
231 2B). As extracellular stress can lead to increased loss of DiCre activity in the original DiCre
232 line (M. Meissner, personal communication, 02.2019), we subjected the new DiCre parasite
233 line (RH DiCre_T2A Δ*ku80Δhxgprt*) to frequent extracellular stress over the course of 65 days.
234 On average, parasites were passaged every 2.3 days, leaving parasites extracellular for ~32
235 hours in the presence or absence of continuous chloramphenicol selection. We also
236 simultaneously passaged the original DiCre line (Andenmatten et al., 2013) under standard,
237 non-stressing, culturing conditions. The new DiCre_T2A line excision efficiency varied
238 between 98 and 99% for replicates on day 1 and was maintained throughout the experiment
239 with a maximal loss of 3% of excision efficiency, irrespective of the presence or absence of
240 chloramphenicol selection, by day 65 (Fig. 2C). In contrast, the original DiCre line, cultured
241 under standard non-stress conditions, lost 42% of excision capacity by day 65 (Supplementary
242 Fig. 2), although this was only done as a single replicate. This shows that the second
243 generation DiCre line, RH DiCre_T2A Δ*ku80Δhxgprt*, retains high excision capacity over long
244 periods of time, even when exposed to extracellular stress.
245
246 **TgCAP is important but not essential for *in vitro* growth and deletion can be largely**
247 **restored by the short cytoplasmic isoform, but only partially by the membrane bound**
248 **isoform.**
249 To investigate CAP function, we generated a conditional knock out (cKO) of *CAP* using the
250 DiCre strategy. Here, *CAP* with a C-terminal HA tag is flanked by two loxP sites, that recombine
251 upon dimerisation of two split-Cre subunits, a process mediated by the small molecule
252 rapamycin (RAP) (Andenmatten et al., 2013). To create the *CAP* conditional knockout line, we
253 integrated a floxed, recodonised and HA-tagged CAP cDNA sequence into the endogenous
254 locus of the RH DiCreΔ*ku80Δhxgprt* line (Fig. 3A). Correct integration into the locus was
255 confirmed by PCR (Fig. 3B). However, due to the DiCre issues detailed above, we were not
256 able to successfully induce DiCre-mediated excision of the *CAP* gene. As the DiCre_T2A
257 strategy demonstrated consistently high excision rates over time, during our testing with a
258 reporter construct, we integrated the DiCre_T2A construct into the *KU80* locus of the non-
259 excising floxed *CAP* parasite strain. This generated the parasite line RH DiCre_T2A
260 DiCreΔ*ku80Δhxgprt_LoxPCAP-HA*, called LoxPCAP hereafter. As expected, LoxPCAP
261 displayed dual localisation by IFA (Fig. 3C). RAP treatment resulted in a complete loss of CAP
262 (ΔCAP), as shown by IFA (Fig. 3C) and Western blot (Fig. 3D).
263
264 To assess the requirements of CAP and its isoforms for various *Toxoplasma* functions, we
265 complemented LoxPCAP parasites with either wildtype, the short or the long CAP isoform by
266 integration of HA-tagged variants into the *UPRT* locus to generate merodiploid lines (named
267 LoxPCAP^{CAP}, LoxPCAP^{shortCAP} and LoxPCAP^{longCAP}, respectively). We then excised the
268 endogenous CAP copy by RAP treatment. Clones were subsequently obtained by limiting
269 dilution and excision verified by PCR (Supplementary Fig. 3).
270
271 This resulted in parasite strains that express only the WT complemented form (ΔCAP^{CAP}), the
272 short complemented form (ΔCAP^{shortCAP}) or the long complemented form (ΔCAP^{longCAP}). We
273 confirmed the differential localisation and translation of these isoforms by IFA (Fig. 3E) and
274 Western blot (Fig. 3F). No differences in the protein levels of the two CAP isoforms could be

275 observed in the CAP complemented strain (ΔCAP^{CAP}) over the first 24 hours following host cell
276 invasion, relative to the loading control (Supplementary Fig. 4).
277
278 To reliably quantify the contribution of CAP, and its two isoforms independently, to the lytic
279 cycle, we performed a competition assay in which growth of ΔCAP^{CAP} was compared to ΔCAP,
280 ΔCAP^{shortCAP} and ΔCAP^{longCAP}. To do so, we integrated an mCherry expressing cassette into
281 the $\Delta ku80$ locus, replacing the DiCre_T2A cassette in each of these lines. Note, the DiCre_T2A
282 cassette was no longer required because CAP had already been excised in these parasites.
283 After 15 days in growth competition with ΔCAP^{CAP}, ΔCAP parasites were largely depleted
284 (>97.3%) from the population, while ΔCAP^{shortCAP} showed a reduction of only 4%. In contrast,
285 ΔCAP^{longCAP} showed an intermediate level of depletion (33.3%). These phenotypes were
286 exaggerated after 30 days in culture; ΔCAP parasites were largely depleted (>99.9%),
287 ΔCAP^{shortCAP} showed a reduction of 12.4%, and ΔCAP^{longCAP} growth was reduced by 43.1%
288 relative to ΔCAP^{CAP} (Fig. 4A). Collectively, these findings demonstrate that CAP plays an
289 important but non-essential role in cell culture and that its function can be largely restored by
290 the short CAP isoform but only partially by the long isoform.
291
292 **TgCAP contributes to motility, invasion, egress and dense granule trafficking**
293 While the competition assays highlight the importance of CAP during the *in vitro* lytic cycle,
294 they do not clarify which step of the cycle is affected. To test whether the growth differences
295 between the lines were merely a result of differences in parasite replication rates, we counted
296 the parasites per vacuole for each parasite line. No significant differences were observed
297 (Supplementary Fig. 5). As CAP is a predicted actin regulator, we next focused our phenotypic
298 analysis on *Toxoplasma* processes for which actin is known to be important, such as motility,
299 egress, invasion and dense granule trafficking (Heaslip et al., 2016; Periz et al., 2017; Whitelaw
300 et al., 2017).
301
302 In the absence of CAP (ΔCAP) significantly fewer parasites are able to initiate gliding motility
303 in 3D motility assays (Leung et al., 2014) compared to the RH strain (Fig. 4B). In the motile
304 population, trajectory displacement, trajectory length and maximum achieved speeds were all
305 significantly reduced, although mean speed was not significantly different (Fig. 4B). To
306 determine which isoform(s) contribute to parasite 3D motility, the motility parameters of
307 ΔCAP^{CAP}, ΔCAP^{shortCAP} and ΔCAP^{longCAP} were compared. Both ΔCAP^{shortCAP} and ΔCAP^{longCAP}
308 parasites showed similar levels of motility initiation, track displacement, track lengths and
309 speed compared to ΔCAP^{CAP} (Supplementary Fig. 6). These data indicate that CAP plays a
310 role in initiation of motility and in controlling speed and track length once motile.
311 Complementation with single isoforms shows that initiation of motility can be rescued by either
312 CAP isoform and, once motile, either can maintain speed and track length.
313
314 As invasion and egress of host cells rely on active motility, we next compared invasion
315 efficiency of ΔCAP and the different complementation lines. ΔCAP showed a significant
316 reduction of invasion capacity (50.2% reduction compared to WT complemented lines), which
317 was restored by both the short and long isoforms (Fig. 4C). We also performed egress assays
318 in the presence of BIPPO, which results in a strong calcium response in *Toxoplasma* parasites
319 causing synchronised and rapid egress from host cells (Howard et al., 2015). ΔCAP parasites
320 showed a substantial delay in egress from host cells at 30 s after induction (73.1% less egress
321 in ΔCAP compared to ΔCAP^{CAP}), while at 2 minutes the majority of parasites have egressed
322 (10.6% less egress in ΔCAP compared to ΔCAP^{CAP}) (Fig. 4D). This defect was fully restored
323 in ΔCAP^{shortCAP} parasites, while ΔCAP^{longCAP} showed slightly lower levels of egress after 30 s of
324 treatment but by 60 s were indistinguishable from WT or short CAP complemented lines. We
325 also observed live egress events in which ΔCAP, following host cell egress, showed decreased
326 movement away from the host cell when compared to ΔCAP^{CAP}, ΔCAP^{shortCAP} and ΔCAP^{longCAP}
327 (Videos 1-4).
328

6

329 A recent study revealed that directed dense granule transport is dependent on filamentous
330 actin (Heaslip et al., 2016). Therefore, dense granule trafficking was assessed in ΔCAP and
331 the three complemented lines. We measured the run frequency (# of directed
332 runs/parasite/minute), run length and velocity of directed motions. (Fig. 4E). Both the mean
333 run length and the mean velocity were significantly increased in ΔCAP (34.8% and 52.9%,
334 respectively), but not in either of the complements. However, the frequency of directed runs
335 was reduced in ΔCAP (14.8%), but did not reach significance. These results indicate that both
336 CAP isoforms play a supporting role in dense granule trafficking and that, upon CAP deletion,
337 dense granules are trafficked further distances at higher speed. Collectively these data show
338 that CAP plays a role in the actin-dependent processes described above and that either
339 isoform is able to fulfil these CAP functions.
340
341 **ΔCAP parasites display a defect in efficient cell-cell communication**
342 Actin, formin 3 and myosin I and J have been shown to be important for both parasite rosette
343 organisation and cell-cell communication, as assessed by measuring the rapid transfer of
344 reporter proteins between individual parasites in a vacuole (Frénal, Jacot, et al., 2017; Periz
345 et al., 2017; Tosetti et al., 2019). Upon CAP deletion, we observed a complete loss of the
346 characteristic rosette formation normally seen in *Toxoplasma*. This aberrant phenotype was
347 fully rescued upon complementation with either WT CAP or the short CAP isoform (Fig. 5A).
348 ΔCAP^{longCAP} displayed partial restoration with 44.9% of parasites forming phenotypically normal
349 rosettes while the remainder were disorganised within the vacuole. This was also shown by
350 scanning electron images of infected human fibroblasts in which the host cell and the vacuole
351 membrane was removed ("unroofed") as previously described (Magno et al., 2005)
352 (Supplementary Fig. 7).
353
354 It was previously shown that mutant parasite lines which lost rosetting capacity also lost the
355 **r**apid **t**ransfer **o**f **r**eporter **p**roteins (called RTORP hereafter) between them. This has been
356 established by photobleaching of individual parasites in a vacuole, which express a fluorescent
357 reporter protein. Under normal conditions, all parasites in a vacuole contribute to the
358 fluorescence recovery of the photobleached parasite (Frénal, Jacot, et al., 2017; Periz et al.,
359 2017; Foe et al., 2018; Tosetti et al., 2019). Such transfer of reporter proteins has previously
360 been used as a readout for cell-cell communication and, by extension, parasite connectivity
361 (Frénal, Jacot, et al., 2017; Periz et al., 2017). To examine whether loss of cell-cell
362 communication also accompanied the disrupted rosetting observed in ΔCAP parasites, we
363 performed fluorescence recovery after photobleaching (FRAP) experiments on ΔCAP and its
364 complementation lines. We chose vacuoles that contained 8 parasites/ vacuole as at this stage
365 parasites organise in rosettes and individual cells can be easily monitored. We bleached one
366 parasite in the vacuole and recorded both the recovery of fluorescence in the bleached parasite
367 and the fluorescence levels of all other parasites in the vacuole. As expected, the WT-
368 complemented ΔCAP^{CAP} photobleached parasites display rapid recovery of fluorescence to
369 which, in most cases, all parasites in the vacuole appear to contribute (Fig. 5Bi, 5C and Video
370 5). Conversely, ΔCAP photobleached parasites were supported in their rapid fluorescence
371 recovery predominantly by just one other parasite, usually the parasite closest to the bleached
372 cell, resulting in slow recovery (Fig. 5Bii, 5C and Video 6). In some cases, no recovery was
373 observed in ΔCAP parasites.
374
375 Loss of rapid protein transfer between parasites could be explained by a structural disruption
376 of inter-parasite connections provided by the residual body. However, in addition to the residual
377 body, an intravacuolar network (IVN) of tubule-like structures is present in the parasitophorous
378 vacuole. Because of its tubular structure, it could also be involved in cell-cell communication.
379 To determine whether the observed defect in the RTORP was dependent on the presence of
380 the IVN, we performed a FRAP assay in RH Δku80Δgra2 parasites (Rommereim et al., 2016)
381 where the IVN fails to form but parasites still organise in rosettes. The results show that
382 RTORP between parasites was not negatively affected (Fig. 5Biii and Video 7). The IVN,
383 therefore, is unlikely involved in cell-cell communication.

7

384
385   To test if CAP deletion leads to a defect in cell-cell communication directly, or whether this is
386 a direct consequence of the inability to form rosettes, we tested whether the RTORP varied
387 between rosetting (r) and non-rosetting parasites (nr) of the $\Delta CAP^{longCAP}$ line. These
388 experiments revealed that if $\Delta CAP^{longCAP}$ parasites rosette, they show normal RTORP between
389 all cells in the vacuole, while non-rosetting parasites display the same defect as the ΔCAP line,
390 with fluorescence recovery from only one other parasite in the vacuole (Fig. 5C). Interestingly,
391 $\Delta gra2$ vacuoles showed a slight increase in the frequency of photobleach recovery from
392 multiple parasites, but we have not further investigated this phenomenon here. The
393 connectivity of parasites was also assessed based on the percentage of recovery after
394 photobleaching. As expected, ΔCAP and non-rosetting $\Delta CAP^{longCAP}$ photobleached parasites
395 recovered significantly less fluorescence than their rosetting counterparts (Fig. 5D).
396
397   Next, we sequentially photobleached individual ΔCAP parasites in a vacuole and identified
398 which parasites appear to be physically connected based on the transfer of mCherry (Fig. 5E).
399 This revealed that only parasite pairs in close proximity are rapidly communicating. Collectively
400 the results show that CAP deletion leads to a loss of both rosetting and the rapid transfer of
401 reporter proteins between more than two parasites. Furthermore, RTORP appears to be
402 dependent on parasite rosette organisation, but not on presence of the IVN.
403
404 **ΔCAP parasites have a defect in daughter cell orientation but not in synchronised**
405 **division or apicoplast inheritance**
406 Previously, cell-cell communication has been hypothesised to control synchronous division
407 between parasites within the same vacuole (Frénal, Jacot, et al., 2017). To determine whether
408 ΔCAP parasites display phenotypes previously observed for mutants with defective cell-cell
409 communication, we used IMC3 antibodies to visualise synchronicity of forming daughter cells
410 (Fig. 6A). Surprisingly, ΔCAP vacuoles exhibited synchronous division with no significant
411 difference to the WT complemented line (Fig. 6B). IMC3 staining was also used to assess
412 daughter cell orientation. While in WT CAP complemented parasites 94.6% of daughter cells
413 grew in the same orientation, a significant defect was observed in ΔCAP and $\Delta CAP^{longCAP}$
414 strains; only 42.9% (ΔCAP) and 67.7% ($\Delta CAP^{longCAP}$) of daughter cells orientated in the same
415 direction. $\Delta CAP^{shortCAP}$ parasites were able to largely overcome this defect, with 89.5% of
416 daughter cells growing in the same orientation (Fig. 6C). Such improper orientation of daughter
417 cells following budding raises the possibility of improper organelle segregation too. To test this,
418 we looked at apicoplast segregation, another actin-dependent process (Andenmatten et al.,
419 2013; Jacot et al., 2013). Using streptavidin as a marker for the apicoplast, we identified no
420 significant differences in apicoplast inheritance rates between ΔCAP^{CAP} and ΔCAP strains
421 (Supplementary Fig. 8). These data show that despite a loss of rosette organisation and
422 RTORP between parasites in a ΔCAP vacuole, as well as disordered daughter cell orientation,
423 parasite division remains synchronous. Furthermore, while CAP is supporting many actin-
424 dependent processes in *Toxoplasma*, it appears dispensable for apicoplast division.
425
426 **CAP deletion leads to the formation of a decentralised residual body in which all**
427 **parasites remain connected, despite loss of RTORP**
428 The observed synchronicity of division in the ΔCAP strain, despite loss of RTORP, was
429 unexpected. It suggested that synchronicity is either independent of the residual body, or that
430 ΔCAP parasites actually maintain connections that allow flow of proteins or metabolites to
431 synchronise divisions. To investigate this, we established a connectivity map of parasites in a
432 vacuole using correlative light and electron microscopy (CLEM). This allowed us to first analyse
433 connectivity of parasites based on FRAP analysis, and then reconstruct a 3D electron
434 microscopy image of the parasites and their connections by focused ion beam scanning
435 electron microscopy (FIB SEM).
436
437 In the ΔCAP^{CAP} strain, as expected for a WT complemented line, all parasites in the vacuole
438 contribute to recovery of a photobleached parasite (Fig. 7A) and a normal residual body is

439 formed, connecting all parasites in the vacuole (Fig. 7B and Video 8), with one tubular
440 extension extending away from the residual body with no apparent connections at the distal
441 end. In contrast, for the ΔCAP strain, fluorescence recovery of the photobleached parasite was
442 predominantly observed from those cells in close proximity (Fig. 7C). To investigate how these
443 parasites are connected, we analysed FIB SEM images obtained from the photobleached
444 vacuole in Fig. 7C. This revealed membrane bound tubular connections of approximately 300
445 nm thickness between parasites, despite the aberrant transfer of mCherry and loss of rosetting
446 (Fig. 7Di and ii). Following the lumen of the connections across 3 dimensions demonstrates
447 that all parasites in the vacuole are connected by these tubular connections, likely representing
448 a decentralised residual body that forms as a result of an inability to keep the posterior ends
449 of the parasites in close proximity (Fig.7D.iii, Supplementary Fig. 9 and Video 9). Correlation
450 of the FRAP data with the FIB SEM images showed that rapid transfer of mCherry was always
451 between parasites in close proximity at their basal ends.
452
453 While ΔCAP parasites appear to be connected via a decentralised residual body, it could be
454 that transfer of material through these connections is limited by physical barriers, such as the
455 mitochondria which have previously been observed in the residual body (Frénal, Jacot, et al.,
456 2017). Close examination of the residual body connections showed only one such connection
457 contained two mitochondria and a constriction of the decentralised residual body lumen to ~50
458 nm, while all other connections appeared free of large physical barriers, indicating that this is
459 unlikely an explanation for the lack of RTORP between parasites. However, we observed a
460 complex network of tubules and sheet-like structures, likely representing the endoplasmic
461 reticulum, in the connections (Fig. 7E) (Puhka et al., 2012; Schroeder et al., 2019; Tomavo et
462 al., 2013; West et al., 2011). One of these tubular structures, tracked in three dimensions, was
463 shown to enter multiple parasites via the basal pole, suggesting a possible continuum between
464 them, which was also observed in then WT CAP complemented parasites. This has not been
465 further examined here but could contribute to exchange of material between parasites.
466
467 Collectively, while CAP is important for rosette organisation of parasites, it is not essential for
468 forming and sustaining a residual body with connectivity to all parasites in the vacuole.
469 Furthermore, it shows that the RTORP is not an indicator of parasite connectivity.
470
471 **Deletion of CAP results in completely avirulent type II parasites, but not in the type I RH**
472 **strain.**
473 The short CAP isoform complements most phenotypes in cell culture while the long CAP
474 isoform, in most cases, only shows a partial rescue. This raises the question about the
475 evolutionary roles of the two different isoforms. To better discriminate the functions of the short
476 and longCAP isoforms, we wanted to examine their respective roles in natural infections, where
477 parasites encounter a number of additional stresses, including shear stress and the immune
478 system. Accordingly, we addressed the essentiality of CAP and its isoforms in mouse
479 infections. We hypothesised that if both isoforms are essential for parasite survival in a natural
480 host, loss of either of the two isoforms would manifest in a fitness cost. First, we injected male
481 C57BL/6 mice with ~25 ΔCAP or ΔCAP^{CAP} tachyzoites of the virulent RH strain, and monitored
482 them over the course of 10 days. In both instances mice began to succumb to infection after 9
483 days, indicating that, despite the motility and rosetting phenotypes, CAP depletion in a type I
484 RH background still results in high virulence in mice (Fig. 8A). As RH parasites have frequently
485 been associated with hypervirulence and do not form cysts *in vivo*, we next generated ΔCAP
486 parasites and complemented versions in the type II Pru strain. In stark contrast to the RH line,
487 upon injection of ~50,000 tachyzoites, Pru ΔCAP parasites showed no virulence in mice while
488 the Pru ΔCAP^{CAP}, Pru ΔCAP^{shortCAP}, and Pru ΔCAP^{longCAP} complemented parasites led to a lethal
489 infection, with most mice succumbing to the parasites 8-10 days post-infection (Fig. 8B). These
490 data show that expression of either individual isoform is sufficient to cause a lethal infection.
491 Next, to look at formation of tissue cysts, which leads to a chronic infection, we injected a lower
492 dose of ~5,000 tachyzoites of the Pru lines into mice. The majority of mice survived until day
493 32 post-infection, although 2 ΔCAP^{CAP}-infected mice and 1 ΔCAP^{shortCAP}-infected mouse died

9

494 before reaching this end-point. At day 32, the mice were sacrificed, brain samples were
495 collected and serum tested for anti-*Toxoplasma* antibodies, confirming that all mice were
496 successfully infected with *Toxoplasma* (Fig. 8C). Both the Pru ΔCAP and Pru ΔCAP^{longCAP}
497 infections demonstrated significantly lower cyst loads compared to Pru ΔCAP^{CAP} and Pru
498 ΔCAP^{shortCAP} infected mice (Fig. 8D). These results show that CAP plays an essential role in
499 the virulence of the type II Pru parasite strain, but not the type I RH strain. Moreover, while the
500 short CAP isoform is able to fulfil all functions of TgCAP in cell culture and in the mouse model,
501 the long isoform, despite its ability to complement most phenotypes to at least ~50% of WT
502 complement levels, has a significant defect in establishing a chronic infection at the infectious
503 dose used here.
504
505 Discussion
506 *Toxoplasma* actin is important for a range of cellular processes, from organelle segregation
507 and cell-cell communication, to gliding motility: a crucial factor in parasite dissemination
508 (Andenmatten et al., 2013; Egarter et al., 2014; Periz et al., 2017). Despite the key role of actin
509 in parasite biology, our understanding of actin dynamics and its regulation remains incomplete.
510 CAP is a ubiquitous protein with a conserved role in regulating actin dynamics. In this study,
511 we established that *Toxoplasma* CAP is expressed by an alternative translation initiation site,
512 giving rise to two independent isoforms: shortCAP and longCAP. Through sequence alignment
513 we identified that the alternate translation initiation site is conserved only within *Toxoplasma,*
514 *Neospora and Hammondia*. These are all members of the Toxoplasmatinae, a subfamily of the
515 Apicomplexa phylum, suggesting that while CAP is present in all apicomplexa, the long isoform
516 is specific to *Toxoplasma* and its closest relatives. Here we investigated the role of both CAP
517 isoforms in the *Toxoplasma* lytic cycle. Through *in vitro* competition assays, we show that
518 complementation with shortCAP is enough to overcome the majority (87.6%), but not all, of the
519 growth defect associated with CAP depletion. This suggests that longCAP is performing a
520 specific function important for parasite fitness, which shortCAP is unable to compensate for.
521 Furthermore, longCAP complementation restored 56.9% of the growth defect, arguing for a
522 degree of functional overlap between the two isoforms. Our experiments have not uncovered
523 a phenotype that only longCAP can rescue, which would have revealed a unique function. Its
524 apical localisation, however, makes it temping to speculate a function in actin regulation during
525 invasion, which is a phenotype it can fully rescue. It could be that higher actin turnover is
526 required at the apex and the concentration of longCAP here is fulfilling this need by influencing
527 G-actin levels. Indeed, it has previously been hypothesised in other organisms that increased
528 local concentration of CAP results in the sequestration of actin monomers (Ono, 2013).
529 However, providing evidence for this hypothesis is limited by the fact that the shortCAP isoform
530 can fully rescue all phenotypes in cell culture under the conditions tested. Given that cell culture
531 assays do not fully represent the environment *Toxoplasma* normally encounters, we aimed to
532 tease apart the functions of the two isoforms in mice. Surprisingly, even here the short isoform
533 appears able to compensate for the lack of longCAP. This suggests that even under conditions
534 encountered in the natural host, the longCAP isoform plays only a minor role, although dosage
535 effects or routes of infection may well be confounding factors when assessing virulence of the
536 different strains. Competition experiments between the mutants *in vivo* may help to tease apart
537 the independent importance of the isoforms in the future. However, from the *in vitro* competition
538 assays it can be predicted that parasites expressing two isoforms fare better than those
539 expressing just one isoform. We cannot rule out that the differences in proteins levels of
540 longCAP and shortCAP in the single isoform producing lines are affecting the phenotypic
541 observations. However, both isoforms are able to rescue most phenotypes indicating that the
542 protein levels do not appear to substantially affect CAP function.
543
544 Despite being dispensable for type I RH parasite virulence, CAP is essential for type II Pru
545 parasite virulence in mice, and complementation with either shortCAP or longCAP restored
546 lethality. At lower, non-lethal doses, longCAP complementation led to markedly reduced cyst
547 formation in the brain. The underlying basis for this has not been explored here and it could be
548 that it is the reduced fitness of the strain, rather than stage conversion phenotype, that causes

10

549 a reduction of parasites reaching the brain. Therefore, the observed virulence of the longCAP
550 complemented strain is possibly due to the high dosage used, allowing the parasite to
551 proliferate at high enough rates to overwhelm the immune system. Nevertheless, the basis for
552 reduced fitness of ∆CAP and ∆CAP^{longCAP} parasites in the murine infection model is likely
553 multifactorial and we hypothesise that other actin-dependent processes that we have not
554 assayed here could also contribute to the reduced virulence in mice.

556 The most pronounced phenotype of CAP deletion is loss of rosetting: the highly symmetrical
557 physical distribution of parasites within the vacuole. Previous studies on actin, myosin I, myosin
558 J, ADF and formin 3 have suggested that the actomyosin motor is important for rosetting
559 (Frénal, Dubremetz, et al., 2017; Haase et al., 2015; Periz et al., 2017; Tosetti et al., 2019).
560 Our ∆CAP data further supports this hypothesis. Interestingly, complementing ∆CAP parasites
561 with longCAP restored rosetting in just under half of all vacuoles, with the remainder appearing
562 as equally disordered as the ∆CAP vacuoles. Despite this mixed population, we did not
563 observe vacuoles with a combination of organised and disorganised parasites, suggesting
564 rosetting is a binary outcome; either all parasites in a vacuole are connected by a central
565 residual body, or not. This may suggest that the ability to produce highly organised rosettes is
566 established during the first round of cell division when the residual body forms. This mixed
567 population of rosetting and non-rosetting parasites, in an isogenic strain, gave us a unique
568 opportunity to determine whether rosetting is important for efficient intravacuolar cell-cell
569 communication. Using longCAP complemented parasites, we show that in rosetting vacuoles
570 there is efficient RTORP with all parasites able to transfer material to the bleached parasite.
571 Within the same population, non-rosetting vacuoles displayed severe defects in the RTORP
572 with parasites seemingly only communicating in pairs. However, despite apparently only
573 communicating in pairs, all parasites in the vacuole remained synchronised in their stage of
574 replication. This is different to other studies which suggested that defects in RTORP between
575 parasites leads to asynchrony in division (Frénal, Jacot, et al., 2017; Periz et al., 2017). Our
576 results show that neither RTORP between daughter cells nor the formation of a rosette are
577 predictors for synchronicity of division.

579 The synchronous division in ∆CAP parasites could be explained by our FIB SEM results.
580 Despite their disorganised appearance and loss of rapid cell-cell communication, ∆CAP
581 parasites are still connected by a decentralised residual body. This connection, although not
582 facilitating rapid transfer of proteins between parasites that are further apart, could allow for
583 slow or minimal transfer of proteins which is sufficient to synchronise divisions. An alternative
584 hypothesis could be that metabolites, not proteins, are required to synchronise divisions, and
585 their diffusion through the decentralised residual body is quicker. It is also a possibility that the
586 seemingly continuous ER in the residual body, observed here connecting most if not all
587 parasites, could contribute to synchronicity of division. Whatever the basis for synchronicity is,
588 rosetting and rapid cell-cell communication are not essential and their analysis cannot be
589 reliably used to predict whether connections between parasites exist. Recently, Foe *et al*
590 reported that upon deletion of *ASH4*, a serine hydrolase, parasites were only connected in
591 pairs but retained synchronicity of division (Foe et al., 2018), similar to our results. An
592 explanation could be that ASH4 mutants display a phenotype similar to the CAP phenotype
593 described here, where, despite the loss of RTORP, parasites remain connected by a
594 decentralised residual body, allowing synchronicity of division.

596 In summary, our results strongly support an actin regulatory role for CAP in *Toxoplasma*.
597 Interestingly, actin dependent processes were affected to differing extents in ∆CAP parasites,
598 such as rosetting being completely lost while apicoplast inheritance was unaffected. This
599 surely reflects the different spatial requirements for actin turnover within a cell. It is likely that
600 the local concentration of actin, actin binding proteins such as the formins and the different
601 myosins facilitate this. The results obtained here also leave open a few questions that are
602 interesting to study in the future. How does actin help to initiate and maintain the centralised
603 residual body? How are ER connections between parasites in a decentralised residual body

604 maintained and what is the function of this connection? What is the functional role of longCAP
605 with its high concentration at the apex of the parasite? Why are some actin dependent
606 processes completely reliant on CAP while others are not? Each of these questions will require
607 careful analysis and the cell lines described here will likely provide useful tools to investigate
608 these in the future.
609
610 Materials and methods
611 **Plasmid construction**
612 All primers used in this study are listed in Supplementary File 1. All synthetic DNA used in this
613 study is listed in Supplementary File 2.
614
615 To generate the CAP-GFP fusion plasmid, pUPRT_CAP_GFP, the *Toxoplasma CAP* gene
616 (TGME49_310030) 5'UTR was amplified from genomic DNA using primer pair P1/P2 and
617 Gibson assembled (Gibson et al., 2010) with a synthetic *CAP* cDNA-*XmaI*-eGFP sequence
618 (GeneArt strings, Life Technologies, Massachusetts, United States) into *BamHI* and *PacI*
619 digested UPRT targeting vector pUPRT-HA (Reese et al., 2011).
620
621 To generate the CAP cKO plasmid, pG140_CAP_cKO_LoxP111, the *CAP* 5'UTR with a LoxP
622 site inserted 111 bp upstream of the *CAP* start codon, and a recodonised *CAP* cDNA-HA
623 sequence, were synthesised (GeneArt strings, Life Technologies). These DNA fragments were
624 Gibson cloned (Gibson et al., 2010) into the parental vector p5RT70loxPKillerRedloxPYFP-HX
625 (Andenmatten et al., 2013) which had been digested using *ApaI* and *PacI*, creating an
626 intermediate plasmid. Next, the *CAP* 3'UTR was amplified from genomic DNA using primer
627 pair P3/P4 while mCherry flanked by *GRA* gene UTRs was amplified from pTKO2c (Caffaro et
628 al., 2013) using primers P5/P6. These PCR products were Gibson cloned (Gibson et al., 2010)
629 into the SacI digested intermediate plasmid to create pG140_CAP_cKO_LoxP111*.*
630
631 To generate pUPRT_CAP, the *CAP* 5'UTR was amplified from genomic DNA using primer pair
632 P1/P2. This DNA fragment and a synthetic *CAP* cDNA-*BamHI*-HA sequence (GeneArt strings,
633 Life Technologies), were Gibson cloned (Gibson et al., 2010) into *BamHI* and *PacI* digested
634 UPRT targeting vector pUPRT-HA (Reese et al., 2011).
635
636 To generate pUPRT_CAP_C6,8A, the *CAP* 5'UTR was amplified from genomic DNA using
637 primer pair P1/P2 and, alongside a synthetic *CAP* cDNA-HA sequence with C6,8A mutations
638 (GeneArt strings, Life Technologies), was Gibson cloned (Gibson et al., 2010) into *BamHI* and
639 *PacI* digested UPRT targeting vector pUPRT-HA (Reese et al., 2011).
640
641 To generate pUPRT_CAP_M1L, pUPRT_CAP was amplified with primer pair P7/P8 to
642 introduce the M1L point mutation.
643
644 To generate pUPRT_CAP_M37L, pUPRT_CAP was amplified with primer pair P9/P10 to
645 introduce the M37L point mutation.
646
647 To generate pG140_DiCre, the plasmid containing DiCre_T2A, two synthetic DNA fragments
648 were produced containing *Ku80* homology region-alpha-tubulin promoter-FRB-Cre60-T2A-
649 chloramphenicol resistance cassette-T2A-FKBP-Cre59-*SAG1* 3'UTR-*Ku80* homology region
650 (gBlock gene fragments, Integrated DNA Technologies, Iowa, United States). These DNA
651 fragments were Gibson cloned (Gibson et al., 2010) (into *ApaI* and *SacI* digested parental
652 vector, p5RT70loxPKillerRedloxPYFP-HX (Andenmatten et al., 2013)
653
654 To generate plasmids expressing CAS9 and a single guide RNA (sgRNA), we used plasmid
655 pSAG1::CAS9-U6::sgUPRT as a backbone (Addgene plasmid #54467) (Shen et al., 2014).
656 The plasmid was amplified with primer P11 and another containing a sgRNA to replace the
657 UPRT-targeting sgRNA. Guide RNA sequences were selected using the Eukaryotic Pathogen
658 CRISPR gRNA Design Tool (Peng & Tarleton, 2015). All sgRNA-expressing plasmids used in

659　this study were generated by this strategy. The sgRNA-containing primers are listed in
660　Supplementary File 1.
661
662　For creation of plasmids with multiple sgRNAs, first, two separate vectors, each with a sgRNA,
663　were generated as described above using the parental vector pSAG1::CAS9-U6::sgUPRT.
664　Then, primers P12/P13 were used to amplify one of the sgRNA regions which was Gibson
665　cloned (Gibson et al., 2010) into the other *KpnI* and *XhoI* digested plasmid, creating a multiple
666　sgRNA plasmid.
667

Culturing of parasites and host cells

669　*T. gondii* tachyzoites were cultured in human foreskin fibroblasts (HFF) and in Dulbecco's
670　modified Eagle's medium (DMEM) with GlutaMAX (Invitrogen, California, United States)
671　supplemented with 10% fetal bovine serum and maintained at 37°C with 5% CO_2.
672

Generation of parasite lines

674　To generate the conditional *CAP* knockout strain (RH DiCre$\Delta ku80\Delta hxgprt\_$LoxCAP-HA,
675　referred to here as LoxPCAP), first, the linearised plasmid pG140_CAP_cKO_LoxP111,
676　carrying the *HXGPRT* cassette, was transfected into the RH DiCre$\Delta ku80\Delta hxgprt$ strain
677　(Andenmatten et al., 2013). Resistant parasites were cloned. Next, the DiCre conditional
678　knockout function of the strain was restored. The linearised pG140_DiCre plasmid, carrying
679　the chloramphenicol resistance cassette and homology with the *Ku80* UTRs, was transfected
680　into the strain. To increase *Ku80*-specific insertion efficiency, a plasmid with multiple *Ku80*-
681　targeting sgRNAs was generated as described above using primer pairs P11/P14 and
682　P11/P15. Resistant parasites were cloned. Integration into the *CAP* endogenous locus was
683　confirmed using primer pairs P16/P17 and P18/P19. Replacement of *CAP* gDNA was
684　confirmed using primers P20/P21. Excision of the floxed *CAP* sequence was confirmed with
685　primer pairs P16/P20.
686

687　To complement the LoxPCAP strain with CAP-expressing constructs, the linearised plasmid
688　pUPRT_CAP, pUPRT_CAP_M1L or pUPRT_CAP_M37L was transfected alongside
689　pSAG1::CAS9-U6::sgUPRT. 5-fluorodeoxyuridine (FUDR) resistant parasites were cloned.
690

691　*CAP* was subsequently excised from the above strains by addition of 50 nM rapamycin in
692　DMSO for 4 hr at 37°C with 5% CO2, before washout, and excised parasites were cloned.
693　Next, to aid with experimentation, an mCherry fluorescent construct was integrated into the
694　*Ku80* locus, replacing the present DiCre_T2A construct. mCherry flanked by *GRA* UTRs was
695　amplified from pG140_CAP_cKO_LoxP111 using primer pair P22/P23 which also carries 30
696　bp homology with the *Ku80* locus. To increase *Ku80*-specific insertion efficiency, a plasmid
697　with multiple *Ku80*-targeting sgRNAs was generated as described above using primer pairs
698　P11/P24 and P11/P25 and co-transfected with the PCR product. A population of parasites
699　expressing mCherry were sorted by flow cytometry using a BD Influx cell sorter (BD
700　Biosciences). Parasites were subsequently cloned, generating the strains ΔCAP, ΔCAP^{CAP},
701　ΔCAP^{shortCAP} and ΔCAP^{longCAP}.
702

703　The Pru $\Delta ku80\Delta cap$ strain was generated by amplifying the *HXGPRT* resistance cassette from
704　the pG140_CAP_cKO_LoxP111 plasmid using primer pair P26/P27. To direct insertion of the
705　PCR product to the *CAP* locus, a plasmid with multiple *CAP*-targeting sgRNAs was generated
706　as described above using primer pairs P11/P28 and P11/P29 and co-transfected with the PCR
707　product. Resistant parasites were cloned. To complement the Pru $\Delta ku80\Delta cap$ strain with CAP-
708　expressing constructs, the linearised plasmid pUPRT_CAP, pUPRT_CAP_M1L or
709　pUPRT_CAP_M37L was transfected alongside pSAG1::CAS9-U6::sgUPRT. FUDR resistant
710　parasites were cloned.
711

712　To generate fluorescent $\Delta gra2$ parasites for FRAP experimentation, mCherry flanked by *GRA*
713　UTRs was amplified from pG140_CAP_cKO_LoxP111 using primer pair P30/P31 with *UPRT*

714 locus overhangs. This PCR product was co-transfected with pSAG1::CAS9-U6::sgUPRT into
715 the RH Δ*ku80* Δ*gra2* strain (Rommereim et al., 2016). An FUDR resistant population was
716 obtained.

718 The RH DiCre_T2A Δ*ku80*Δ*hxgprt* line was generated by integrating the DiCre construct into
719 the *Ku80* locus in RH Δ*ku80*Δ*hxgprt* parasites (Huynh & Carruthers, 2009). The DiCre
720 construct was amplified from pG140_DiCre using primer pair P32/P33 which also carries 30
721 bp homology with the *Ku80* locus. To increase *Ku80*-specific insertion efficiency, a plasmid
722 with a *Ku80*-targeting sgRNA was generated as described above using primer pairs P34/P11
723 and co-transfected with the PCR product. Resistant parasites were cloned.

725 To assess the efficiency of rapamycin-dependent excision of the DiCre strains, the Killer Red
726 gene-swap construct was amplified from p5RT70loxPKillerRedloxPYFP-HX (Andenmatten et
727 al., 2013) using primer pair P35/P36. This PCR product was co-transfected with
728 pSAG1::CAS9-U6::sgUPRT for targeted insertion into the *UPRT* locus. FUDR resistant
729 parasites were cloned.

731 To generate a CAP C-terminal endogenous HA-tagged line, a synthetic DNA repair template
732 was produced containing *CAP* gDNA (a section of which is recodonised), a HA tag and the
733 *CAP* 3'UTR (gBlock gene fragments, Integrated DNA Technologies). The DNA repair template
734 was amplified using primer pair P37/P38. To direct insertion of the PCR product to the *CAP*
735 locus, a plasmid with a single *CAP*-targeting sgRNA was generated as described above using
736 the primer pairs P39/P11 and was co-transfected with the PCR product into RH Δ*ku80*
737 parasites. A population of parasites expressing the co-transfected Cas9-GFP-containing
738 plasmid were sorted by flow cytometry using a BD Influx cell sorter (BD Biosciences). ~70% of
739 these parasites expressed the HA peptide by IFA.

740 **Parasite transfection and selection**
742 To generate stable transformants, $0.5 – 1 \times 10^7$ freshly lysed parasites were transfected with
743 either 25 μg of linearised template DNA, 5 μg of linearised template DNA and 20 μg of a
744 corresponding gRNA-specific CRISPR/CAS9 plasmid or template DNA produced from 1
745 ethanol precipitated PCR and 20 μg of a corresponding gRNA-specific CRISPR/CAS9 plasmid.
746 Selection on the basis of 5-fluorodeoxyuridine (20 μM), mycophenolic acid (25 μg ml^{-1}),
747 xanthine (50 μg ml^{-1}) or chloramphenicol (21 μM) was performed according to the selection
748 cassette used.

750 **Preparation of parasite genomic DNA**
751 Genomic DNA was extracted from *T. gondii* tachyzoites to use as a PCR template by pelleting
752 parasites and resuspending in PBS. DNA extraction was then performed using the Qiagen
753 QIAamp DNA blood mini kit as per the manufacturer's protocol.

755 **IFA**
756 Parasites were seeded onto HFFs grown on coverslips. 16 – 24 h after seeding, the coverslips
757 were fixed in 3% formaldehyde for 15 min at room temperature then permeabilised in 0.2%
758 Triton X-100/PBS for 3-10 min and blocked in 3% BSA/PBS for 1 h. Staining was performed
759 using appropriate primary antibodies and goat Alexa Fluor 488-, Alexa Fluor 594- and Alexa
760 Fluor 647-conjugated secondary antibodies (1:2000) alongside DAPI (5 μg/ml). Coverslips
761 were mounted on glass slides with SlowFade gold antifade mountant (Life Technologies).
762 Antibody concentrations used were: rat anti-HA high affinity (Roche, Basel, Switzerland)
763 (1:1000), rabbit anti-TgCAP (1:2000), mouse anti-GFP (Roche) (1:1000).

765 Widefield images were generated with a Ti-E Nikon microscope using a 63x or 100x objective
766 (Tokyo, Japan). Images were processed with Nikon Elements software. Confocal images were
767 taken using a Zeiss LSM-780 inverted confocal laser scanning microscope with a 63x
768 objective. Images were processed with Zeiss Zen Black software (Oberkochen, Germany).

14

769
770 **Western blot**
771 Western blot samples were obtained by scraping and lysing intracellular parasites in 200 µl 1x
772 Laemmli buffer (2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.002% bromophenol blue
773 and 125 mM Tris HCl, pH 6.8). Samples were subjected to SDS-PAGE under reducing
774 conditions before being transferred to a nitrocellulose membrane. Immunoblotting was
775 performed with appropriate primary antibodies in 0.1% Tween 20, 3% skimmed milk/PBS.
776 Bound secondary fluorochrome-conjugated antibodies were visualised using the Odyssey
777 Infrared Imaging System (LI-COR Biosciences, Nebraska, United States).
778
779 Antibody concentrations used were: rat anti-HA high affinity (Roche) (1:1000), rabbit anti-
780 TgCAP (1:2000), mouse anti-*Toxoplasma* [TP3] (Abcam, Cambridge, United Kingdom)
781 (1:1000). Goat anti-mouse IRDye 800CW (LI-COR) (1:20000), Donkey anti-rabbit IRDye
782 680LT (LI-COR) (1:20000), Goat anti-rat IRDye 680LT (LI-COR) (1:20000).
783
784 **Generation of TgCAP antibody**
785 To generate the shortCAP expression plasmid, pET-28_CAP_A38toC203, a *Toxoplasma*
786 shortCAP (M37toC203) recodonised sequence was synthesised (gBlock gene fragments,
787 Integrated DNA Technologies) and Gibson cloned (Gibson et al., 2010) into *BamHI* and *NdeI*
788 digested pET-28a(+) plasmid (Merck, Darmstadt, Germany). This allowed for expression of an
789 N-terminal 6xHis tagged shortCAP recombinant protein in *Escherichia coli* BL21 cells under
790 the control of T7 *lac* promoter. Short CAP was expressed and His tag purified using Ni-NTA
791 affinity purification under native conditions using the standard manufacturer's protocol (Qiagen,
792 Hilden, Germany). The shortCAP recombinant protein was used to immunize female New
793 Zealand white rabbits (Covalab, Cambridge, United Kingdom) for generation of polyclonal
794 antibodies.
795
796 **Flow cytometry analysis of DiCre excision**
797 Parasites were added to a HFF monolayer and allowed to invade for 1 h. Then, cre
798 recombinase-mediated recombination was induced by addition of 50 nM rapamycin in DMSO
799 for 4 hr before washout. 22 h after infection, parasites were lysed, pelleted and washed twice
800 in PBS. Parasites were resuspended in 0.5 ml 3% formaldehyde and fixed for 10 min. The
801 suspension was centrifuged and the pellet washed in PBS before resuspension in PBS. To
802 remove debris, samples were passed through a 30 µm pre-separation filter (Miltenyi Biotec,
803 Bergisch Gladbach, Germany). 20,000 events were recorded using a BD LSR II flow cytometer
804 (BD Biosciences California, United States). Killer Red was excited by the 561 nm laser and
805 detected by a 600 long pass filter and either a 582/15, 610/20 or 620/40 band pass filter. YFP
806 was excited by the 488 nm laser and detected a 505 long pass filter and either a 525/50 or
807 530/30 band pass filter. For quantification, and elimination of debris, total number of Killer Red+
808 parasites was considered 100%. This was performed at day 0, 35 and 65 of the experiment.
809 For each condition, three biological replicates were analysed. At least 10000 Killer Red+
810 events were counted for each individual time point.
811
812 <u>**Phenotypic characterisations**</u>
813 **Competition Assay**
814 mCherry-expressing ΔCAP, ΔCAP^{CAP}, ΔCAP^{shortCAP} or ΔCAP^{longCAP} parasites were mixed with
815 non-fluorescent ΔCAP^{CAP} parasites at an average ratio of 60/40. At day 0, 15 and 30 in culture,
816 the ratio was determined by flow cytometry for two biological replicates. Parasites were lysed,
817 pelleted and washed twice in PBS. Parasites were resuspended in 0.5 ml 3% formaldehyde
818 and fixed for 5 min. The suspension was centrifuged and the pellet resuspended in 5 µg/ml
819 DAPI/PBS for 10 min. The pellet was washed and resuspended in PBS. To remove debris,
820 samples were passed through a 30 µm pre-separation filter (Miltenyi Biotec). Events were
821 recorded using a BD LSR II flow cytometer (BD Biosciences). DAPI was excited by the 355
822 nm laser and detected by a 450/50 band pass filter. mCherry was excited by the 561 nm laser
823 and detected by a 600 long pass filter and a 610/20 band pass filter. To eliminate debris from

15

824 the analysis, events were gated on DAPI fluorescence. The ratio of control parasites
825 (DAPI+/mCherry-) to individual CAP complements (DAPI+/mCherry+) was calculated and
826 normalised to the day 0 ratio. The data represent two (day 15) and three (day 30) independent
827 experiments. At least 1500 DAPI+ events were obtained for each individual time point. The
828 results were statistically tested with a two-way ANOVA test plus a multiple comparison Sidak's
829 test individually comparing day 15 or day 30 means to their respective day 0 mean, in
830 GraphPad Prism® 7. The data presented are as mean $\pm$ s.d.
831
832 **Intracellular growth and rosetting assay**
833 Parasites were harvested from a T-25 and added to a coverslip coated with a HFF monolayer.
834 After 20 h the coverslips were fixed with 3% formaldehyde for 15 min at room temperature.
835 Coverslips were mounted and mCherry expression used to identify parasites. For each
836 replicate, 4 random fields were imaged with a 40x objective. Counts were performed in a
837 blinded manner in duplicate for two independent experiments. The number of parasites per
838 vacuole was determined by counting at least 265 vacuoles per strain. The number of vacuoles
839 that rosette was determined by looking at 8-pac vacuoles, at least 90 vacuoles per strain were
840 counted. The results were statistically tested with a one-way ANOVA test plus a multiple
841 comparison Dunnett's test comparing all means to the ΔCAP^{CAP} mean in GraphPad Prism® 7.
842 The data presented are as mean $\pm$ s.d.
843
844 **Invasion assay**
845 Red/green invasion assays were performed. mCherry-expressing parasites were lysed in an
846 invasion non-permissive buffer, Endo buffer (44.7 mM K_2SO_4, 10 mM $MgSO_4$, 106 mM
847 sucrose, 5 mM glucose, 20 mM Tris–H_2SO_4, 3.5 mg/ml BSA, pH 8.2). 250 µl of $8x10^5$
848 parasites/ml in Endo buffer were added to each well of a 24-well flat-bottom plate [Falcon],
849 which contains a coverslip with a confluent HFF monolayer. The plates were spun at 129 x g
850 for 1 min at 37°C to deposit parasites onto the monolayer. The Endo buffer was gently removed
851 and replaced with invasion permissive medium (1% FBS/DMEM). These parasites were
852 allowed to invade for 1 min at 37°C after which the monolayer was gently washed twice with
853 PBS and fixed with 3% formaldehyde for 15 min at room temperature. Extracellular parasites
854 were stained with mouse anti-*Toxoplasma* antigen B1247M (Abcam) 1:1000 and goat anti-
855 mouse Alexa Fluor 488, following the IFA protocol. For each replicate, 3 random fields were
856 imaged with a 40x objective. Three independent experiments were performed in duplicate. The
857 number of intracellular (mCherry+/488-) and extracellular (mCherry+/488+) parasites was
858 determined by counting, in a blinded fashion, at least 758 parasites per strain. The results were
859 statistically tested with a one-way ANOVA test plus a multiple comparison Dunnett's test
860 comparing all means to the ΔCAP^{CAP} mean in GraphPad Prism® 7. The data presented are as
861 mean $\pm$ s.d.
862
863 **Egress assay**
864 Parasites were added to a HFF monolayer, in a ibidi µ-plate 96 well, and grown for 30 h. The
865 wells were washed twice with PBS and the media was exchanged for 80 µl Ringers solution
866 (155 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 3 mM NaH_2PO_4, 10 mM HEPES, 10
867 mM glucose). To artificially induce egress, 40 µl of Ringer's solution containing 150 µM BIPPO
868 (50 µM final conc) was added to each well. At specified time points the cells were fixed by
869 adding 26 µl 16% formaldehyde (3% final conc) for 15 min. Cells were washed in PBS and
870 stained with DAPI (5 µg/ml). Automated image acquisition of 25 fields per well was performed
871 on a Cellomics Array Scan VTI HCS reader (Thermo Scientific, Massachusetts, United States)
872 using a 20× objective. Image analysis was performed using the Compartmental Analysis
873 BioApplication on HCS Studio (Thermo Scientific). Egress levels were determined in triplicate
874 for three independent assays. At least 11987 vacuoles per strain were counted at t = 0 s.
875 Subsequent time point vacuole counts were normalised to t=0 to determine how many
876 vacuoles had egressed. The results were statistically tested with a two-way ANOVA test plus

877 a multiple comparison Dunnett's test comparing all means to the ΔCAP^{CAP} mean, at each time
878 point separately, in GraphPad Prism® 7. The data presented are as mean ± s.d.
879
880 **Live egress**
881 Parasites were added to a HFF monolayer, in a ibidi μ-plate 96 well, and grown for 30 h. The
882 wells were washed twice with PBS and the media was exchanged for 80 μl Ringers solution
883 (155 mM NaCl, 3 mM KCl, 2 mM CaCl$_2$, 1 mM MgCl$_2$, 3 mM NaH$_2$PO$_4$, 10 mM HEPES, 10
884 mM glucose). The plate was then transferred to a Ti-E Nikon microscope with a 37°C
885 environmental chamber. To artificially induce egress, 40 μl of Ringer's solution containing 150
886 μM BIPPO (50 μM final conc) was added to each well after imaging had commenced. Images
887 were captured every 1.8 s.
888
889 **Apicoplast segregation assay**
890 Parasites were added to a HFF monolayer and grown for 20 h before fixation with ice cold
891 methanol for 2 min at room temperature. IFAs were performed using a streptavidin-Alexa Fluor
892 594 conjugate (Invitrogen) as a marker for the apicoplast. Correct apicoplast segregation was
893 determined in duplicate for three independent assays. At least 228 vacuoles were counted per
894 strain. Counts were performed in a blinded manner. The results were statistically tested with
895 an unpaired t test in GraphPad Prism® 7. The data presented are as mean ± s.d.
896
897 **Synchronicity of division and daughter cell orientation assay**
898 Parasites were added to a HFF monolayer and grown for 20 h before fixation with 3%
899 formaldehyde for 15 min at room temperature. IFAs were performed using rat anti-IMC3
900 antibodies. To determine the synchronicity of cell division within the vacuoles, anti-IMC3
901 staining was used to evaluate the stage of daughter cell development. Vacuoles were scored
902 as synchronous if all daughter cells were at the same stage of development. Vacuoles were
903 counted blind in triplicate for three independent experiments. At least 275 vacuoles were
904 counted per strain. The results were statistically tested with an unpaired t test in GraphPad
905 Prism® 7. The data presented are as mean ± s.d. Daughter cell orientation was quantified in
906 triplicate for three independent experiments. At least 312 mother cells were counted blind, per
907 strain. The results were statistically tested with a one-way ANOVA test plus a multiple
908 comparison Dunnett's test comparing all means to the ΔCAP^{CAP} mean in GraphPad Prism® 7.
909 The data presented are as mean ± s.d.
910
911 **3D motility assay**
912 Motility assays were performed as previously described (Leung et al., 2014), with minor
913 modifications. Parasites were syringe-released from a HFF monolayer (one heavily infected
914 T75 flask per strain) by passing through a 27 gauge needle and filtering through a 3 μm
915 Nuclepore filter. Parasites were then centrifuged (1,000 x g for 2 min) and resuspended in 40
916 μl motility media supplemented with 0.3 mg/mL Hoescht 33342. Matrigel was thawed on ice to
917 prevent polymerization and combined with parasites and motility media in a ratio of 3:1:3
918 respectively. Pitta chambers were perfused with 10 μL of this suspension and incubated at
919 27°C for 7 minutes on a thermoplate. Chambers were incubated in the heated (35°C +/- 1°C)
920 microscope enclosure for 3 min prior to imaging. Parasite nuclei were imaged, using a 20x
921 objective, capturing 61 – 63 stacks of 41 z-slices 1 μm apart. To ensure conditions remained
922 constant between parasite lines, samples used for capture were alternated. Datasets were
923 exported to Imaris x64 v9.2.1. Using the ImarisTrack module parasites were tracked in a region
924 of interest from which 1 μm has been cropped from the x and y edges to eliminate edge
925 artefacts. Parasites were identified, after background subtraction, using spot detection with
926 estimated size of 4 μm (xy) and 8 μm (z). Spots were filtered to exclude all that had duration
927 of less than 3 seconds to minimise tracking artifacts. An autoregressive motion tracking
928 algorithm was applied with a maximum distance of 6 μm and a maximum gap size of 3.
929 Datasets were manually inspected to ensure appropriate tracking and to remove artifacts and
930 trajectories tracked from multiple identified spots. Percent moving was calculated as the

931   number of trajectories (>2 µm displacement) / number of objects in frame 3 (>3 s duration).
932   Trajectory parameters were extracted directly from Imaris software. Data shown are derived
933   from 4 independent biological replicates, each consisting of a minimum of two technical
934   replicates. For all analysis, means and standard deviation were calculated for four independent
935   biological replicates before statistical analysis using an unpaired t-test in GraphPad Prism ®7.
936   For the three strain experiment, $\Delta CAP^{shortCAP}$ and $\Delta CAP^{longCAP}$ were each compared to
937   ΔCAP^{CAP}. All technical replicates are presented in the figures, together with their mean ± s.d.
938
939   **Dense granule trafficking assay**
940   For each condition 1×10^7 parasites were transiently transfected with 30 µg of pTub SAG1ΔGPI-
941   mCherry plasmid (Heaslip et al., 2016) and immediately added to a confluent HFF monolayer
942   in a Mattek® 35 mm dish, coverslip 1.5. 12-15 h after infection the monolayers were washed
943   3 times with pre-warmed Gibco® Fluorobrite™ DMEM supplemented with 4% Fetal Bovine
944   Serum. The coverslips were immediately used for imaging. The acquisitions were made with
945   an Olympus IX71 coupled to a DELTAVISION™ Elite imaging system in a 37°C environmental
946   chamber. The acquisition for each condition were made sequentially from the washing step to
947   the acquisition with a random order to avoid any artifactual data. The acquisition analysis was
948   made with Fiji and the MTrackJ plugin. The data represent three independent experiments. At
949   least 43 parasites and 273 direct runs were counted per strain. The results were statistically
950   tested with a one-way ANOVA test plus a multiple comparison Dunnett's test comparing all
951   means to the ΔCAP^{CAP} mean in GraphPad Prism® 7. The data presented are as mean ± s.d.
952
953   **FRAP**
954   Parasites were inoculated on a confluent layer of HFFs 20 h before experiments were
955   performed using a Zeiss LSM-780 inverted confocal laser scanning microscope at 37°C.
956   Acquisition and processing were performed with the Zeiss Zen Black software. Images were
957   taken for 2 min (one image per second). Three pre-bleach images were recorded before the
958   region of interest was photobleached ten times with a 561 nm laser at 100% power.
959   Fluorescence intensity is presented as a percentage relative to the same area pre-bleach.
960   These normalised intensity values were also used for the calculations below. To calculate
961   percentage of fluorescence recovery, the final reading (116 s post-bleach) was subtracted from
962   the reading immediately post-bleach, t = 3 s, to give percentage recovery after 116 s. To
963   calculate percentage loss of fluorescence, the reading immediately post-bleach, t = 3 s, was
964   subtracted from the final reading (116 s post-bleach) to give percentage loss after 116 s. These
965   percentages were used to generate the heat map. At least 11 vacuoles were counted per strain
966   across at least two independent experiments. The results were statistically tested with a one-
967   way ANOVA test plus a multiple comparison Dunnett's test comparing all means to the
968   ΔCAP^{CAP} mean in GraphPad Prism® 7. The data presented are as mean ± s.d. To assess
969   recovery type, at least 11 vacuoles were counted per strain across at least two independent
970   experiments. The results were statistically tested with a Chi-square in GraphPad Prism® 7.
971
972   **Tape unroofing SEM**
973   Parasites were inoculated on a confluent HFF monolayer 24 h before fixation in EM fixative
974   (2.5% gluteraldehyde, 4% formaldehyde in 0.1 M phosphate buffer) for 30 min. Cells were
975   washed in 0.1 M phosphate buffer (PB) and stored in 1% formaldehyde in PB at 4°C. Cells
976   were then washed in PB at room temperature, then washed in ddH$_2$O at RT. The cells were
977   dehydrated stepwise from 70% to 100% ethanol before critical point drying from acetone in a
978   CPD300 (Leica Microsystems, Vienna, Austria). After drying, the coverslips were mounted on
979   stubs, and the HFF cells were unroofed by placing Scotch tape on the coverslips and gently
980   peeling it off, exposing the host cytoplasm and the parasitophorous vacuoles. The cells were
981   coated with 7 nm platinum in a Q150R Sputter Coater (Quorum Tech, East Sussex, UK)
982   before viewing in a Phenom ProX SEM (Thermo Scientific) at 10 kV, 1024 x 1024 pixel
983   frame, on 'high' quality.

18

984

985 **FIB SEM**

986 Immediately following FRAP experimentation, as described above, parasites were fixed in 4%
987 formaldehyde for 15 min at 37°C before washing in 0.1 M PB. Cells were then fixed in EM
988 fixative (2.5% gluteraldehyde, 4% formaldehyde in 0.1 M PB) for 30 min at room temperature.
989 Cells were washed in 0.1 M PB and stored in 1% formaldehyde in 0.1 M PB. After fixation,
990 samples were transferred to a Pelco BioWave Pro+ microwave (Ted Pella) for processing
991 using a protocol adapted from the NCMIR protocol (Deerinck et al., 2010). See Supplementary
992 File 3 for full BioWave program details. The SteadyTemp plate was set to 21°C unless
993 otherwise indicated. Each step was performed in the microwave, except for the buffer
994 and ddH$_2$O wash steps, which consisted of two washes on the bench and two washes in the
995 microwave (250 W for 40 s). The cells were washed (as above) in 0.1 M PB, stained with 2%
996 osmium tetroxide and 1.5% potassium ferricyanide (v/v) for 14 min under vacuum (with/without
997 100 W power at 2 min intervals), and then washed in ddH$_2$O (as above). Next, the cells were
998 incubated in 1% thiocarbohydrazide in ddH$_2$O (w/v) for 14 min (vacuum, 100 W on/off at 2
999 min intervals) with SteadyTemp plate set to 40°C, followed by ddH$_2$O washes (as above), and
1000 then a further stain with 2% osmium tetroxide in ddH$_2$O (w/v) for 14 min (vacuum, 100 W on/off
1001 at 2 min intervals), followed by ddH$_2$O washes (as above). The cells were then incubated
1002 in 1% aqueous uranyl acetate (vacuum, 100 W on/off at 2 min intervals, SteadyTemp 40°C),
1003 and then washed in ddH$_2$O (as above, except with SteadyTemp at 40°C). Walton's lead
1004 aspartate was then applied (vacuum, 100 W on/off at 2 min intervals, SteadyTemp 50°C), and
1005 the cells were washed (as above) and dehydrated in a graded ethanol series (70%, 90%, and
1006 100%, twice each), at 250 W for 40 s without vacuum. Exchange into Durcupan ACM® resin
1007 (Sigma-Aldrich, Missouri, United States) was performed in 50% resin in ethanol, at 250 W for
1008 3 min, with vacuum cycling (on/off at 30 sec intervals), and then pure Durcupan was infiltrated
1009 in four microwave steps with the same settings, before embedding at 60°C for 48 h.

1010

1011 Focused ion beam scanning electron microscopy (FIB SEM) data was collected using a
1012 Crossbeam 540 FIB SEM with Atlas 5 for 3-dimensional tomography acquisition (Zeiss).
1013 Segments of the cell monolayer containing the cells of interest were trimmed, polished with a
1014 diamond knife (removing uneven resin at the base of the monolayer to provide a flat surface
1015 for tracking marks), mounted on a standard 12.7 mm SEM stub using conductive epoxy (ITW
1016 Chemtronics), and coated with a 5 nm layer of platinum.

1017

1018 The specific cells of interest were relocated by imaging through the platinum coating at an
1019 accelerating voltage of 20 kV and correlating to previously acquired fluorescence microscopy
1020 images. After preparation for milling and tracking, images were acquired at 5 nm isotropic
1021 resolution throughout each region of interest, using a 10 µs dwell time. During acquisition the
1022 SEM was operated at an accelerating voltage of 1.5 kV with 1 nA current. The EsB detector
1023 was used with a grid voltage of 1,200 V. Ion beam milling was performed at an accelerating
1024 voltage of 30 kV and current of 700 pA.

1025

1026 After cropping to the specific region of interest comprising the entire extent of the PV (ΔCAP^{CAP};
1027 3080 x 634 x 2509 pixels; 15.4 x 3.17 x 12.545 µm; ΔCAP; 4065 x 1136 x 4490 pixels; 20.325
1028 x 5.68 x 22.45 µm) and aligning the dataset (gradient align; Atlas 5), the images were
1029 processed to supress noise and slightly enhance sharpness (gaussian blur 0.75 radius,
1030 followed by unsharp mask radius 1, strength 0.6; Fiji) prior to reorienting and reslicing in the
1031 YZ plane (assigned as the XY plane for segmentation), and scaling to 10 nm isotropic
1032 resolution for segmentation and display.

1033

1034 Selected structures were segmented manually from the FIB SEM datasets and 3D
1035 reconstructions were made using the 3dmod program of IMOD (Kremer et al., 1996). The
1036 normal and decentralised residual body structures were manually skeletonised by tracing the
1037 approximate central axis of the structure from the posterior pore of each tachyzoite using open
1038 contours. Thus, the green skeleton follows the lumen of the connections between parasites.

1039 The minimum number of points were placed that would still ensure the contour remained at
1040 the central axis of the volume. Where the structure branched, points linking contours from
1041 multiple extensions were placed at the approximate centre of the branch point volume. The
1042 contours were then rendered as a 50 or 100 nm tube to aid visualisation (tube diameter chosen
1043 depending on view). This model was then inspected in the X, Y, and Z-planes and corrections
1044 made to ensure the skeleton followed the approximate centre through the volume. Since not
1045 all RB-like structure extensions ended at the posterior pole of a tachyzoite, the posterior poles
1046 were highlighted by segmenting them with open contours with points at every 40 nm in Z,
1047 following the edge of the cytosol (the ribosome-containing electron lucent space where it meets
1048 the intermediate electron density surrounding the inner membrane complex), and meshing the
1049 contour as a 50 nm tube. A selected region of the putative ER lipid bilayer outer leaflet was
1050 segmented with closed contours drawn every 5-20 nm in Z (smaller Z intervals where needed
1051 to capture fenestrations/complexity); from an approximately square region around the inner
1052 face of two of the basal pores up to an arbitrary point along the decentralised residual body
1053 structure. Contour gaps were placed at the edge of this region. Two tachyzoites were also
1054 highlighted by partial coarse segmentation; closed contours drawn every 250 nm in Z through
1055 the main body of the cell (segmentation of the complex top and bottom of the tachyzoites was
1056 omitted for clarity).
1057

1058 **Animals**
1059 C57BL/6 (wild type) mice were bred and housed under pathogen-free conditions in the
1060 biological research facility at the Francis Crick Institute in accordance with the Home Office UK
1061 Animals (Scientific Procedures) Act 1986. All work was approved by the UK Home Office
1062 (project license PDE274B7D), the Francis Crick Institute Ethical Review Panel, and conforms
1063 to European Union directive 2010/63/EU. All mice used in this study were male and between
1064 7- to 9-week old.
1065

1066 Mice were infected with *T. gondii* tachyzoites by intraperitoneal injection (i.p.) with either 25, 5
1067 x 10^3 parasites (cyst formation) or 5 x 10^4 parasites (survival) in 200 µl medium on day 0. Mice
1068 were monitored and weighed regularly for the duration of the experiments.
1069 For serum samples, mice were euthanized and blood collected into blood serum collection
1070 tubes (SAI, Infusion technologies) by puncturing the jugular vein. Blood was allowed to clot at
1071 room temperature for 30 min, before tubes were centrifuged at 1500 x g for 10 min. Serum
1072 was collected and stored at -20°C until analysis.
1073

1074 ***Toxoplasma* serum antibody ELISA**
1075 *Toxoplasma* soluble antigens were extracted as previously described (Silva et al., 2007). In
1076 short, parasites were syringe-lysed, washed once with PBS, and adjusted to 1 x 10^8
1077 tachyzoites/ml with PBS containing protease inhibitors (cOmplete mini, Roche). Parasites
1078 were lysed by five freeze-thaw cycles (liquid nitrogen/37°C), followed by ultrasound sonication
1079 on ice (five 60 Hz cycle for 1 min each). Samples were centrifuged at 10,000 x g for 30 min at
1080 4 °C before supernatants were collected and protein content was determined using the BCA
1081 Protein Assay Kit (Pierce, Thermo Fisher Scientific) following the manufacturer's instructions.
1082

1083 To detect *Toxoplasma* antibodies in murine serum samples, 96-well plates (flat bottom, high-
1084 binding) were coated overnight with 2 µg/ml *Toxoplasma* soluble antigens at 4°C. Plates were
1085 washed with PBS/0.05% Tween-20 (v/v) (PBS-T) before blocked with 1% BSA (w/v) in PBS
1086 for 2 h at room temperature. Bound antigens were incubated with murine sera diluted 1/10 in
1087 1% BSA/PBS for 2 h at room temperature, washed three times with PBS-T and bound
1088 antibodies detected by incubation for 2 h at room temperature with anti-mouse
1089 Immunoglobulins (HRP conjugate, Darko) diluted 1/1000 in 1% BSA/PBS. Finally, plates were
1090 washed three times with PBS-T and developed by adding TMB substrate solution (Thermo
1091 Fisher Scientific). The TMB reaction was stopped by adding 2 N sulphuric acid and the
1092 absorbance measured (OD_{450} minus OD_{540} wave length correction) using the VersaMax^{TM}
1093 Microplate Reader with SoftMax® Pro Software.

20

Mouse brain collection and preparation for cyst counting

To determine the number of cysts in the brain of infected animals, mice were euthanized and the brain extracted from the skull. The brain was homogenised in 1 ml PBS and stained with Rhodamine-conjugated *Dolichos biflorus* agglutinin (1/1000; Vector Laboratories) for 1 h at room temperature. Fluorescently labelled cysts were counted using a Ti-E Nikon microscope.

Statistical analysis

Statistical tests used are stated in individual sections above. *P*-values significance thresholds were set at: **** P <0.0001, *** P <0.001, ** P <0.01 and * P <0.05. All significant results are labelled with a line and asterisk(s) in the graphs.

Competing interests

The authors have declared that no competing interests exist.

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1385 **Figure legends**
1386
1387 **Figure 1: Alternative translational start sites lead to the generation of two different CAP**
1388 **isoforms.**
1389 **(A)** Sequence alignment of the first 57 amino acid residues of TgCAP with that of other
1390 Apicomplexa. Green shading indicates cysteines which are putative palmitoylation sites, yellow
1391 shading indicates methionines which are putative alternative translational start sites. **(B)**
1392 Subcellular localisation of a CAP-GFP fusion. Scale bar, 2 μm. **(C)** Subcellular localisation of
1393 CAP by immunofluorescence assay (IFA) using rabbit anti-TgCAP antibodies. Scale bar, 2 μm.
1394 **(D)** Western blot of CAP expression levels over the first 24 hours following host cell invasion
1395 using anti-TgCAP antibodies. Anti-*Toxoplasma* antibodies were used as a loading control. **(E)**
1396 Alignment of the *Toxoplasma* consensus translation initiation (Kozak) sequence (Seeber,
1397 1997) with the translation initiation sequences of *CAP*'s first (M1) and second (M37) putative
1398 translational start sites. Green shading indicates bases that correspond to the Kozak
1399 sequence. **(F)** Schematic of TgCAP with annotations for the two putative CAP isoforms and
1400 the mutation experiments performed to test their expression: Mutation of M1 to leucine (L) to
1401 produce shortCAP, and mutation of M37 to L producing longCAP. Green diamonds indicate
1402 putative palmitoylation sites. **(G)** IFA and **(H)** western blot of ectopic HA-tagged *Tg*CAP
1403 isoforms and cysteine mutants (C6 and C8). Inclusion of a HA-tag makes the protein run more
1404 slowly than the untagged protein. Anti-*Toxoplasma* antibodies were used as a loading control
1405 in (H). Scale bar in (G), 5 μm.
1406
1407 **Figure 2: A second generation RH Δ*ku80* DiCre_T2A parasite strain stably expresses**
1408 **DiCre.**
1409 **(A)** Schematic of the DiCre_T2A expression construct. The chloramphenicol resistance
1410 cassette (CAT) is flanked by T2A skip peptides. The two Cre subunits (FRB_Cre2 and
1411 FKBP_Cre1) are located on either side of the T2A::CAT::T2A cassette. The fusion protein is
1412 driven by the alpha-tubulin promotor with a *SAG1* 3' UTR. The red hexagon indicates the
1413 position of the stop-codon. **(B)** Flow cytometry analysis to determine excision efficiency of the
1414 RH DiCre_T2A Δ*ku80* line following 65 days of frequent extracellular stress. Excision is
1415 determined by a shift from Killer Red[(+)] to Killer Red[(+)] and YFP[(+)] expression. Parasites were
1416 analysed 22 h after induction with 50 nM rapamycin (RAP) for 4 h. Due to analysing 22 h after
1417 induction of excision, parasites still have residual KillerRed signal. **(C)** Table summarizing the
1418 excision efficiency of RAP treated RH DiCre_T2A Δ*ku80* parasites over time in the presence
1419 or absence of chloramphenicol selection. "Day" refers to the number of days in cell culture
1420 while "rep" corresponds to biological replicates.
1421
1422 **Figure 3: Generation of CAP conditional knockout and complementation strains**
1423 **(A)** Schematic of the CAP conditional knock out strategy using double homologous integration.
1424 The position of the 5' LoxP site in the CAP promotor is indicated as well as the predicted sizes
1425 of the PCR amplicons. **(B)** Agarose gel showing the expected PCR products for correct
1426 integration at the endogenous locus. **(C)** IFA 46 hr after treatment with DMSO or 50 nM
1427 rapamycin for 4 h. Scale bar, 5 μm. **(D)** Western blot showing absence of CAP protein in the
1428 cloned ΔCAP parasites using anti-TgCAP antibodies. Anti-*Toxoplasma* antibodies were used
1429 as a loading control. **(E)** Subcellular localisation and **(F)** Western blot of ectopic HA-tagged
1430 *Tg*CAP isoforms, in the ΔCAP background. Inclusion of a HA-tag makes the protein run more
1431 slowly than the untagged protein. IFA images have been individually contrast adjusted to aid
1432 in visualising protein localisation. Scale bar in (E), 5 μm. Anti-*Toxoplasma* antibodies were
1433 used as a loading control in (F).
1434
1435 **Figure 4: CAP plays an important but not essential role during the lytic cycle in cell**
1436 **culture**

1437 **(A)** Overview of the flow cytometry competition assay (left). Competition assays of mCherry-
1438 expressing ΔCAP and CAP complementation lines with non-fluorescent WT-complemented
1439 parasites (right). The ratio of mCherry$^{(+)}$ to mCherry$^{(-)}$ parasites was analysed by flow cytometry
1440 at day 0, 15 and 30. Data are represented as mean ± s.d. (D0 and D30, n=6. D15, n=4). Two-
1441 way ANOVA followed by a multiple comparison Sidak's test was used to compare means
1442 between time points. **(B)** 3D Matrigel-based motility assays performed in the absence of
1443 inducers of motility. Results are expressed as mean ± s.d. (n=4). Each data point corresponds
1444 to a single technical replicate from one of four independent biological replicates, on which
1445 significance was assessed using an unpaired t-test. **(C)** Invasion assay comparing ΔCAP to
1446 the complemented lines. Data are represented as mean ± s.d. (n=3). One-way ANOVA
1447 followed by Dunnett's test was used to compare means to the ΔCAP^{CAP} mean **(D)** Egress
1448 assay. Graph shows number of egressed vacuoles in response to BIPPO over time. Data are
1449 represented as mean ± s.d. (n=3). Two-way ANOVA followed by Dunnett's test was used to
1450 compare means to the ΔCAP^{CAP} mean. Stated significance is in comparison to ΔCAP^{CAP}. **(E)**
1451 Dense granule trafficking assay. ΔCAP and CAP complemented lines were transiently
1452 transfected with SAG1ΔGPI-mCherry that allows visualisation of dense granules. The length
1453 and speed of directed runs were recorded using fluorescence microscopy and analysed using
1454 ImageJ. Data are represented as mean ± s.d. (n=3). One-way ANOVA followed by Dunnett's
1455 test was used to compare means to the ΔCAP^{CAP} mean.
1456
1457 **Figure 5: CAP is important for rosetting and rapid cell-cell communication**
1458 **(A)** Representative fluorescence images of mCherry expressing ΔCAP parasites and
1459 complemented isoforms (left). Quantification of rosetting vs. non-rosetting parasites (right).
1460 Data are represented as mean ± s.d. (n=2). One-way ANOVA followed by Dunnett's test was
1461 used to compare means to the ΔCAP^{CAP} mean. **(B-E)** FRAP experiments to measure transfer
1462 of mCherry between individual parasites in a vacuole. **(B)** Heatmaps showing the percentage
1463 change in fluorescence of individual parasites in **(i)** CAP complemented, **(ii)** ΔCAP and **(iii)**
1464 Δ*gra2* parasite lines. A recovery plot and image for representative vacuoles are included.
1465 Regions of interest are numbered; the bleached parasite is "1". Numbers are allocated based
1466 on proximity to the bleached parasite. The yellow lightning bolt indicates which parasite was
1467 photobleached. Data are representative of two independent experiments. **(C, D)** Graphs
1468 quantifying the type (C) and amount (D) of recovery for all parasite lines. Data are represented
1469 as mean ± s.d. (ΔCAP and ΔCAP^{CAP} n=4, all other lines, n=2). Statistical significance was
1470 assessed by either Chi-square test (D) or one-way ANOVA followed by Dunnett's test to
1471 compare means to the ΔCAP^{CAP} mean (C). **(E)** FRAP analysis of ΔCAP parasites. The images
1472 and graphs represent sequential photobleaching and recovery measurements of individual
1473 parasites within the vacuole. The yellow lightning bolt indicates which parasite was
1474 photobleached. The white arrow identifies the parasite from which the majority of recovery was
1475 observed. All scale bars, 5 μm.
1476
1477 **Figure 6: CAP is important for daughter cell orientation, but not synchronous division**
1478 **(A)** Parasites stained with anti-IMC 3 antibodies to visualise daughter cell orientation and
1479 division. Scale bar, 5 μm. **(B)** Quantification of synchronicity of division in parasite vacuoles
1480 using daughter cell staining from (A) reveals no defect in the synchronicity of ΔCAP parasites.
1481 Data are represented as mean ± s.d. (n=3). Significance was assessed using an unpaired two-
1482 tailed t-test. **(C)** Quantification of daughter cell orientation in parasite vacuoles reveals a
1483 significant defect in the daughter cell orientation of ΔCAP parasites. Data are represented as
1484 mean ± s.d. (n=3). One-way ANOVA followed by Dunnett's test was used to compare means
1485 to the ΔCAP^{CAP} mean.
1486
1487 **Figure 7: CAP KO parasites are still connected by a decentralised residual body**
1488 **(A)** FRAP analysis of ΔCAP^{CAP} parasites. The images and graphs represent sequential
1489 photobleaching and recovery measurements of individual parasites within the vacuole. **(B) (i)**

1490 FIB SEM of the vacuole from (A) with a 3D model highlighting the residual body (green skeleton
1491 representing how the approximate centre can be traced through to the basal poles) and
1492 parasite openings at the basal pole (orange ring). Note that the illustrated connections display
1493 the distance between the parasite's posterior ends, and do not represent tubular connections
1494 between them. Numbering of parasites is consistent with (A). **(ii)** Zoomed image of the residual
1495 body from (i), **(iii)** zoomed image of the residual body from (i) overlaid with the 3D model. **(C)**
1496 FRAP analysis of ∆CAP parasites. The images and graphs represent sequential
1497 photobleaching and recovery measurements of individual parasites within the vacuole. **(D) (i)**
1498 FIB SEM of the vacuole from (A), numbering of parasites is consistent with (C). **(ii)** Zoomed
1499 image of the boxed region from (i). Green arrows indicate the residual body (left panel). A 3D
1500 model highlights the residual body (green skeleton drawn through the lumen of the connections
1501 between parasites), parasite openings at the basal pole (orange ring) (right panel)**. (iii)** A 3D
1502 model of selected features in the vacuole from (C), highlighting the residual body (green),
1503 parasite openings at the basal pole (orange ring) and coarse segmentation of two of the
1504 parasites (yellow) (left panel). This model is shown with an orthoslice from the FIB SEM volume
1505 (right panel). **(E)** Zoomed images of the boxed region from (Di). Blue arrows indicate a putative
1506 ER structure (left). A 3D model highlights this putative ER (blue) and parasite openings at the
1507 basal pole (orange ring) (right)**. (i)** View facing a Z orthoslice. **(ii)** View facing an X orthoslice.
1508 The yellow lightning bolt indicates which parasite was photobleached and the white arrow
1509 identifies the parasite from which recovery was observed (A,C). Scale bar, 5 µm (A, C) or 1
1510 µm (B, D, E).
1511
1512 **Figure 8: CAP is essential for virulence in type II but not type I parasites.**
1513 **(A)** Survival rates of C57BL/6 mice infected with 25 RH ∆CAP^{CAP} or RH ∆CAP parasites. **(B)**
1514 Survival rates of C57BL/6 mice infected with 50,000 Pru ∆CAP parasites or with
1515 complementing CAP variants. **(C)** ELISA testing sera reactivity of naïve or *Toxoplasma*
1516 infected mice using *Toxoplasma* antigen. **D)** Cyst burden in the brain of C57BL/6 mice 32 days
1517 post-infection with 5,000 Pru ∆CAP parasites or with complementing CAP variants. For all
1518 experiments, five animals were infected for each strain.
1519
1520 **Supplementary Figure 1. Endogenous tagging of CAP**
1521 **(A)** Schematic of CAP endogenous tagging strategy. Arrowhead indicates CRISPR/CAS9 cut
1522 site. **(B)** IFA of the resulting HA-tagged line. Scale bar, 2 µm.
1523
1524 **Supplementary Figure 2. Excision efficiency testing of RH DiCre ∆*ku80*∆*hxgprt***
1525 Flow cytometry analysis to determine excision efficiency of the original RH DiCre
1526 ∆*ku80*∆*hxgprt* line following 65 days of standard, non-stressing, culture conditions. Excision is
1527 determined by a shift from Killer Red$^{(+)}$ to Killer Red$^{(+)}$ and YFP$^{(+)}$ expression. Parasites were
1528 analysed 22 h after induction with 50 nM rapamycin (RAP) for 4 h. Due to analysing 22 h after
1529 induction of excision, parasites still have residual KillerRed signal.
1530
1531 **Supplementary Figure 3. Excision testing of the CAP conditional knockout lines**
1532 **(A)** Schematic of the *CAP* locus in the LoxPCAP conditional knockout lines. Rapamycin
1533 induced excision of CAP is detected by PCR3. **(B)** Diagnostic PCR of the *CAP* locus in the **(i)**
1534 LoxPCAP lines 5 d after treatment with either DMSO (D) or rapamycin (R). **(ii)** Excised clones
1535 were subsequently obtained from these rapamycin treated populations.
1536
1537 **Supplementary Figure 4. CAP-HA expression in the ∆CAP^{CAP} line**
1538 Western blot on CAP expression levels in the ∆CAP^{CAP} strain over the first 24 hours following
1539 host cell invasion using anti-TgCAP antibodies. Anti-*Toxoplasma* antibodies were used as a
1540 loading control.
1541
1542 **Supplementary Figure 5. Replication analysis of ∆CAP and the complemented lines.**

1543 Data are represented as mean ± s.d. (*n*=2). One-way ANOVA followed by Dunnett's test was
1544 used to compare means to the ΔCAP^{CAP} mean. "Other" refers to vacuoles with a non-power of
1545 2 number of parasites.
1546
1547 **Supplementary Figure 6. 3D Matrigel-based motility assays performed in the absence**
1548 **of inducers of motility.**
1549 Results are expressed as mean ± s.d. (*n*=4). Each data point corresponds to a single technical
1550 replicate from one of four independent biological replicates, on which significance was
1551 assessed using an unpaired *t*-test.
1552
1553 **Supplementary Figure 7. Scanning electron micrographs of intracellular ΔCAP and**
1554 **complemented lines**
1555 Vacuole organisation is disrupted in ΔCAP and ΔCAP^{longCAP} parasite strains.
1556
1557 **Supplementary Figure 8. Apicoplast segregation analysis**
1558 Apicoplast segregation was not affected in the ΔCAP line. Representative IFA images (left)
1559 and quantification of apicoplast segregation (right). Data are represented as mean ± s.d. (*n*=3).
1560 Significance was assessed using an unpaired two-tailed *t*-test. Scale bar, 5 μm.
1561
1562 **Supplementary Figure 9. Close up FIB SEM images of parasite connections in the ΔCAP**
1563 **vacuole from (Fig. 7C-E)**
1564 The 3D model highlights the residual body (green skeleton representing how the approximate
1565 centre can be traced through to the basal poles), parasite openings at the basal pole (orange
1566 ring) and coarse segmentation of two of the parasites (yellow). Scale bars are not shown since
1567 these are oblique views – see Fig. 7 and Methods for scale information.
1568
1569 **Video 1. Live egress imaging of a ΔCAP^{CAP} vacuole**
1570 Egress was induced by addition of 50 μM BIPPO at 0 s. Image taken every 1.8 s. The video is
1571 played at 6.6 fps and the time is indicated in seconds. Scale bar, 10 μm.
1572
1573 **Video 2. Live egress imaging of a ΔCAP vacuole**
1574 Egress was induced by addition of 50 μM BIPPO at 0 s. Image taken every 1.8 s. The video is
1575 played at 6.6 fps and the time is indicated in seconds. Scale bar, 10 μm.
1576
1577 **Video 3. Live egress imaging of a ΔCAP^{shortCAP} vacuole**
1578 Egress was induced by addition of 50 μM BIPPO at 0 s. Image taken every 1.8 s. The video is
1579 played at 6.6 fps and the time is indicated in seconds. Scale bar, 10 μm.
1580
1581 **Video 4. Live egress imaging of a ΔCAP^{longCAP} vacuole**
1582 Egress was induced by addition of 50 μM BIPPO at 0 s. Image taken every 1.8 s. The video is
1583 played at 6.6 fps and the time is indicated in seconds. Scale bar, 10 μm.
1584
1585 **Video 5. FRAP on a ΔCAP^{CAP} vacuole**
1586 A single parasite was photobleached at 3 s. Images taken every 1 s. The video is played at 2
1587 fps and the time is indicated in seconds. Scale bar, 5 μm.
1588
1589 **Video 6. FRAP on a ΔCAP vacuole**
1590 A single parasite was photobleached at 3 s. Images taken every 1 s. The video is played at 2
1591 fps and the time is indicated in seconds. Scale bar, 5 μm.
1592
1593 **Video 7. FRAP on a ΔGra2 vacuole**
1594 A single parasite was photobleached at 3 s. Images taken every 1 s. The video is played at 2
1595 fps and the time is indicated in seconds. Scale bar, 5 μm.
1596

1597 **Video 8. FIB SEM of the ∆CAP^{CAP} vacuole from Fig. 7A-B with 3D model overlay**
1598 All FIB SEM slices of the ∆CAP^{CAP} vacuole Fig. 7A-B. The 3D model highlights the residual
1599 body (green skeleton representing how the approximate centre can be traced through to the
1600 basal poles, along with a structure that extends from that centre but has no connection) and
1601 parasite openings at the basal pole (orange ring). The volume of the FIB SEM dataset shown
1602 is indicated in the Materials and methods.
1603
1604 **Video 9. FIB SEM of the ∆CAP vacuole from Fig. 7C-E with 3D model overlays**
1605 All FIB SEM slices of the ∆CAP vacuole Fig. 7C-E. The 3D model highlights the residual body
1606 (green skeleton drawn through the approximate central axis of the lumen of the connections
1607 between parasites), parasite openings at the basal pole (orange ring), coarse segmentation of
1608 the central part of two of the parasites (yellow), and part of the putative ER (blue, note that only
1609 a selected region was segmented; from the inner face of two of the basal pores through part
1610 of the decentralised residual body). The volume of the FIB SEM dataset shown is indicated in
1611 the Materials and methods.
1612
1613 **Supplementary File 1. Primers listed in the Materials and methods**
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1615 **Supplementary File 2. Synthetic DNA listed in the Materials and methods**
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1617 **Supplementary File 3. BioWave program details for FIB SEM**
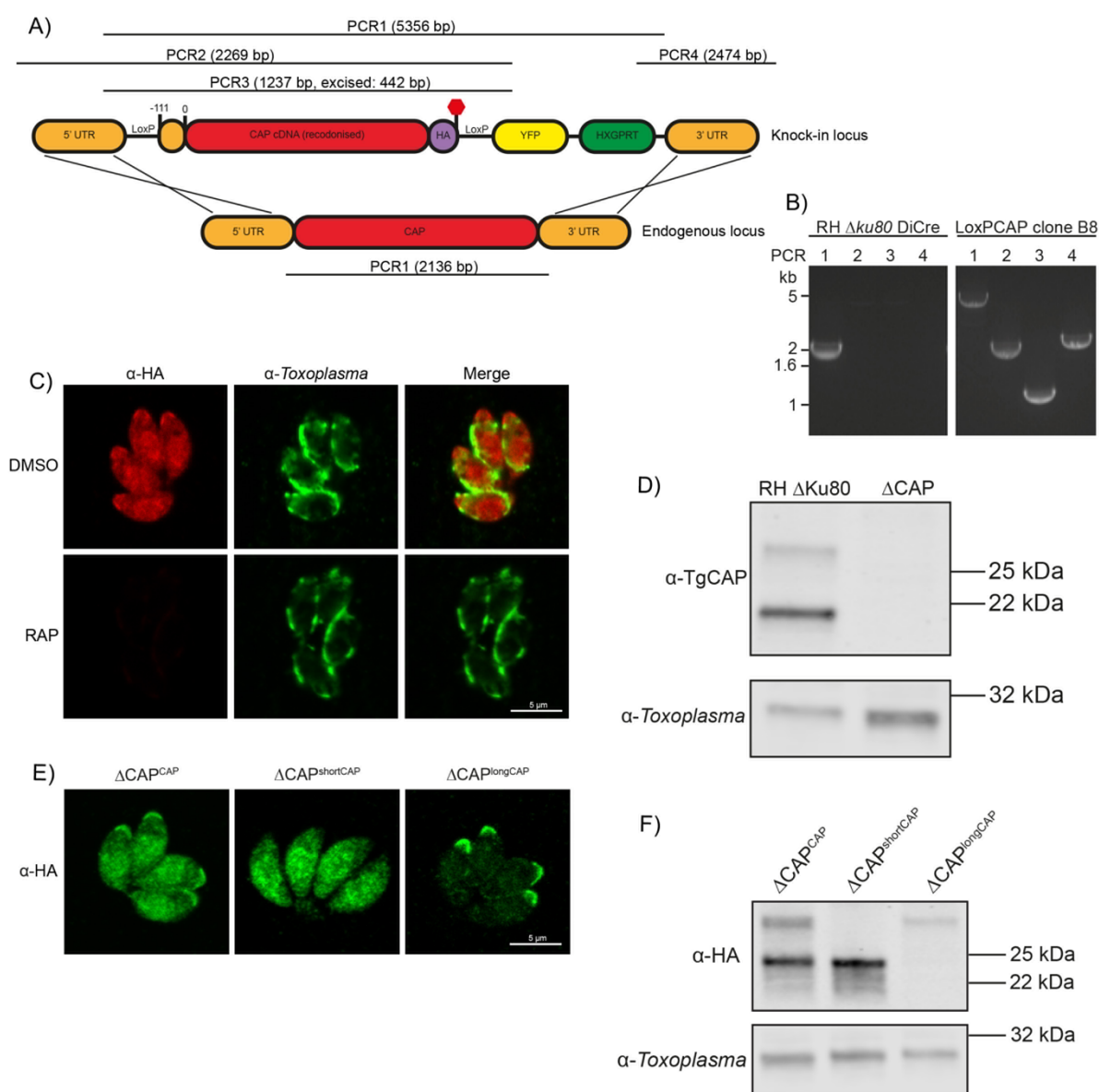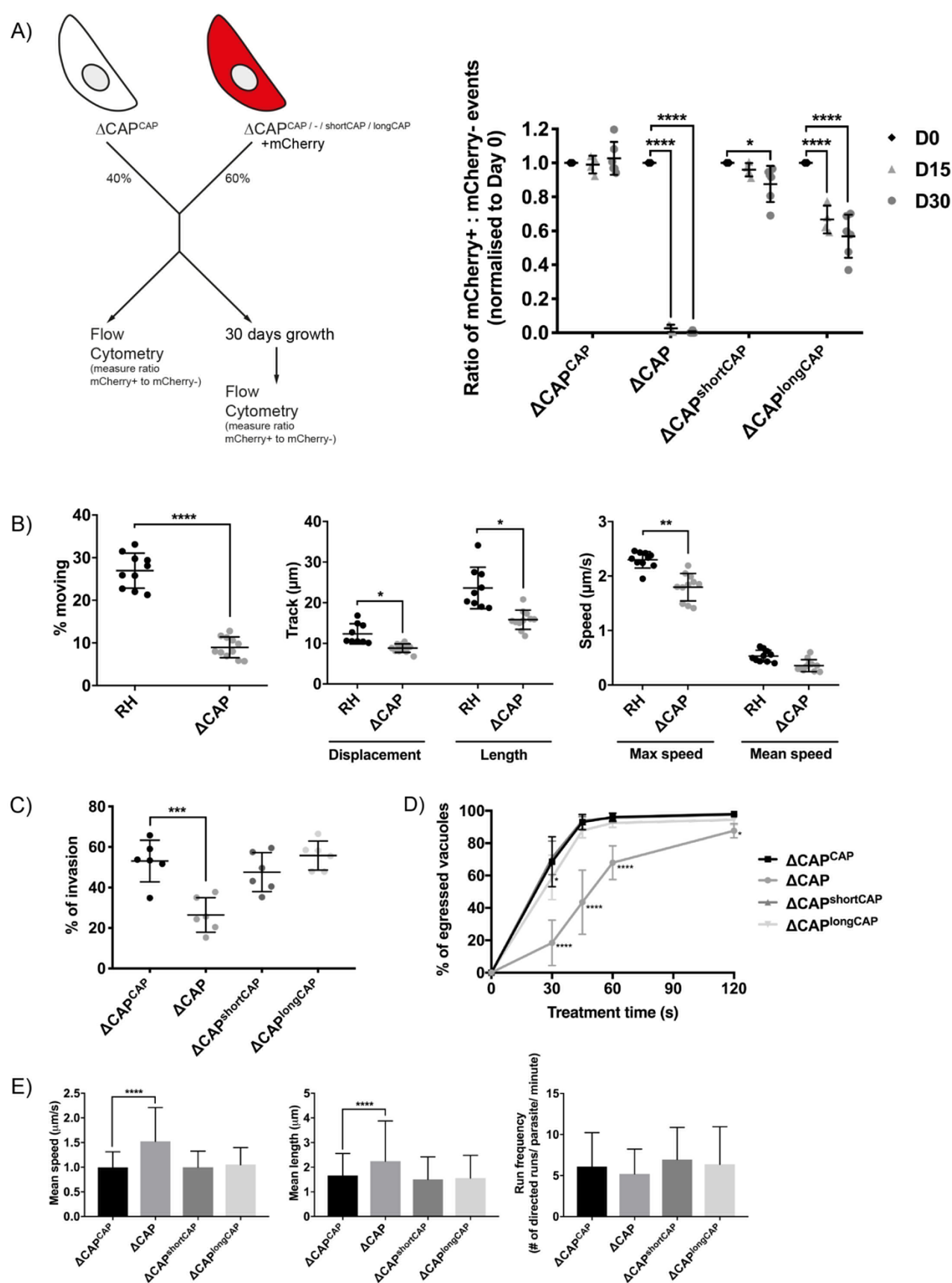
1618 **Figure 1**
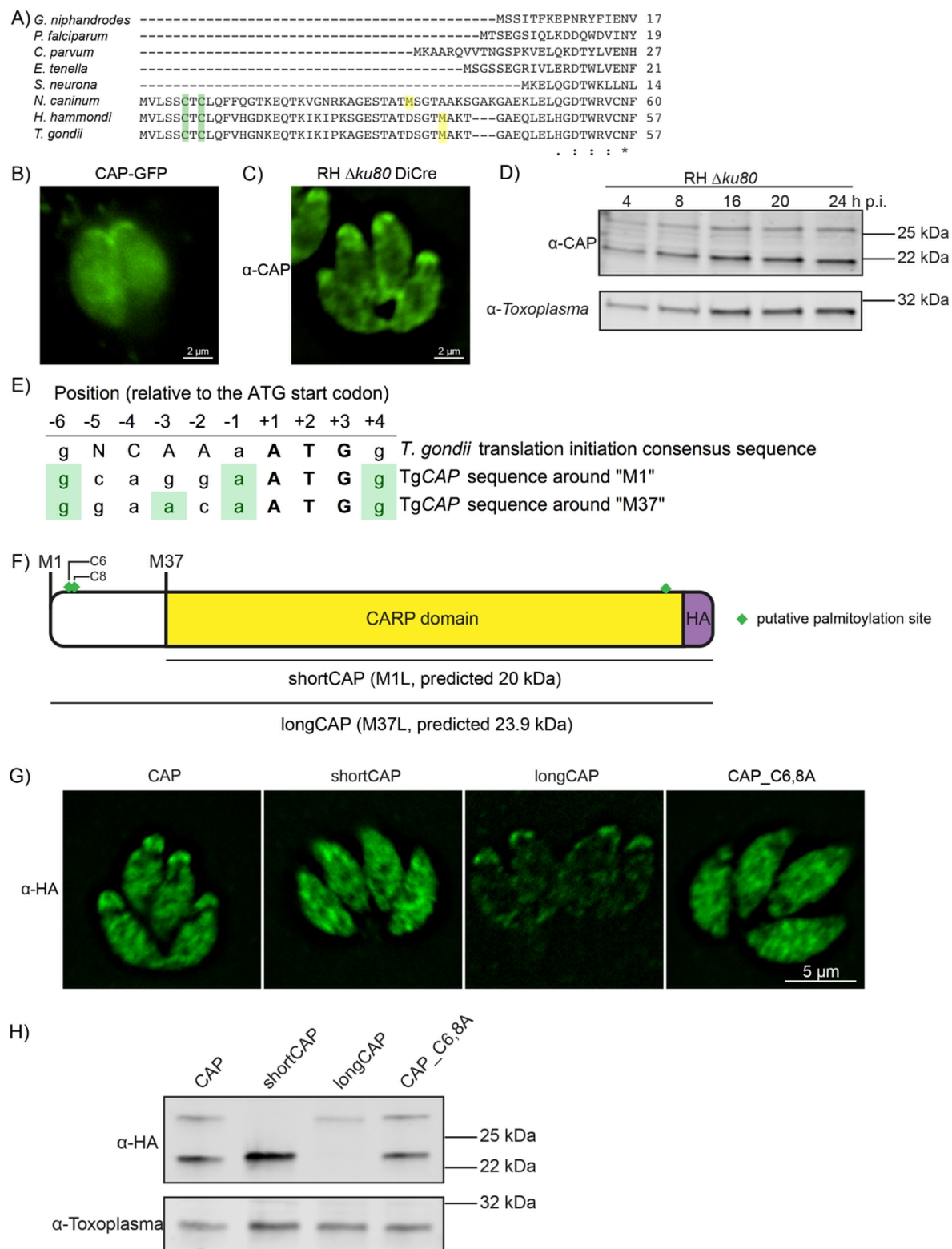
1625 **Figure 2**

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Figure 3

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1671   **Figure 4**

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1674 **Figure 5**

1675   **Figure 6**
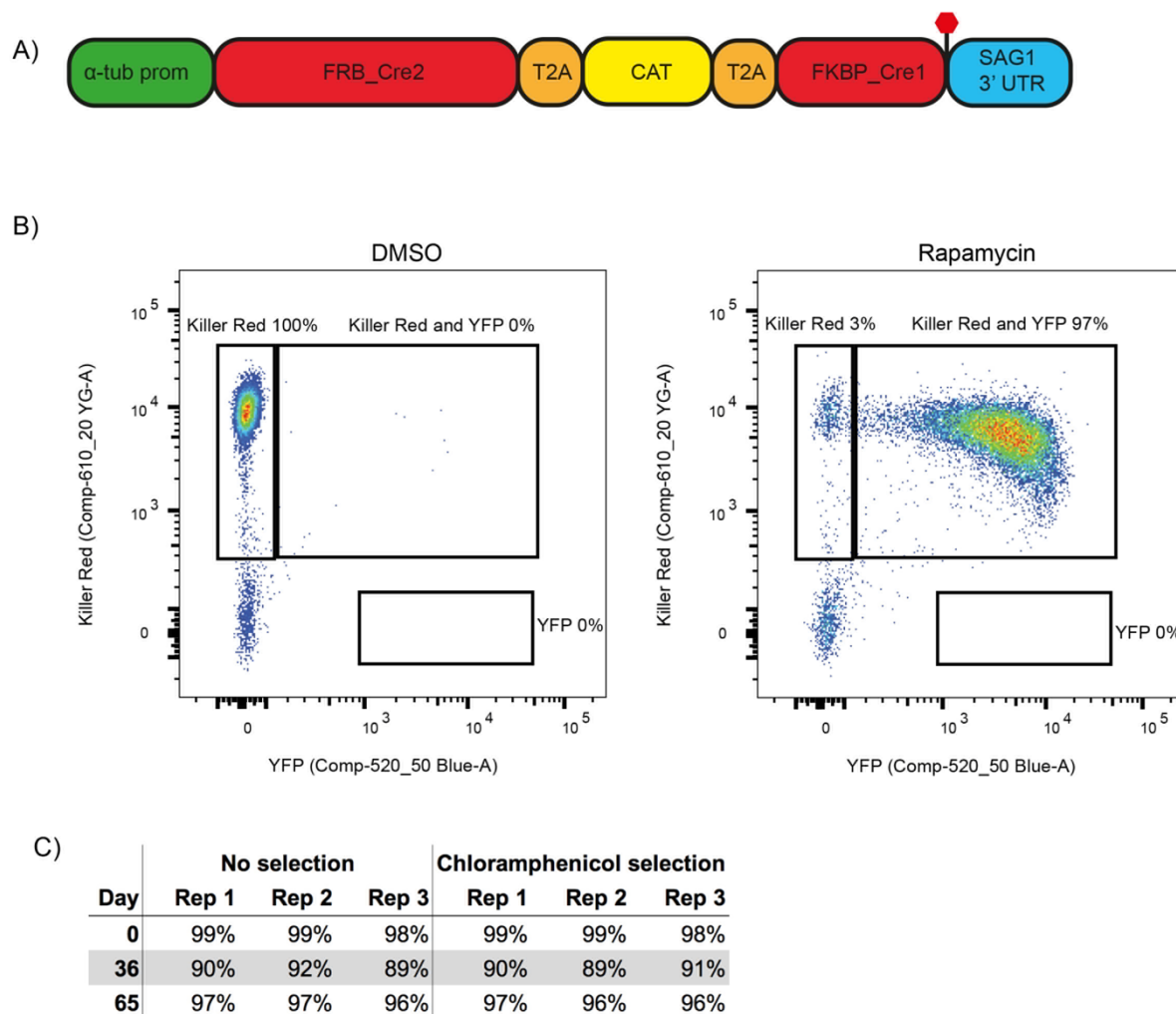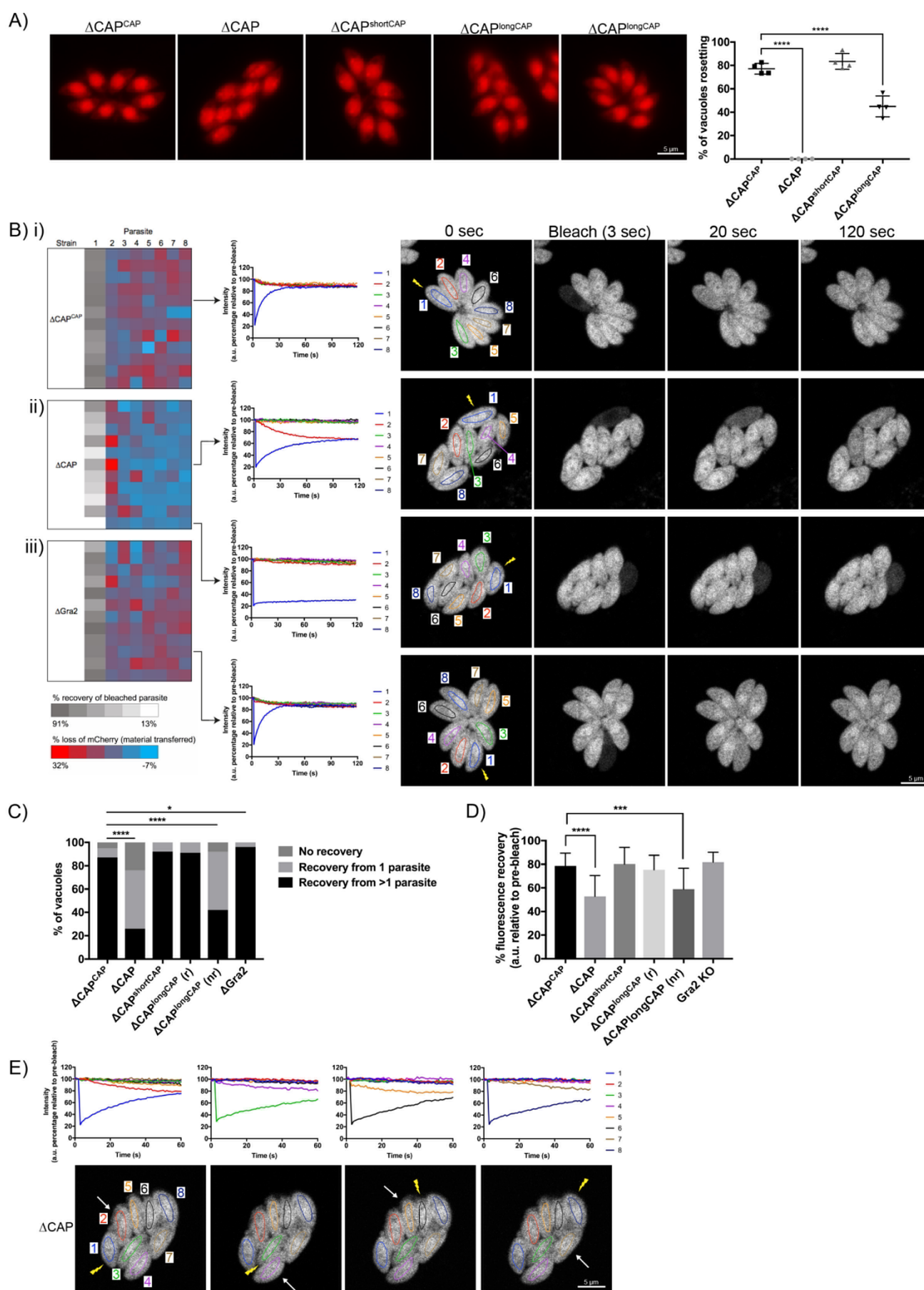
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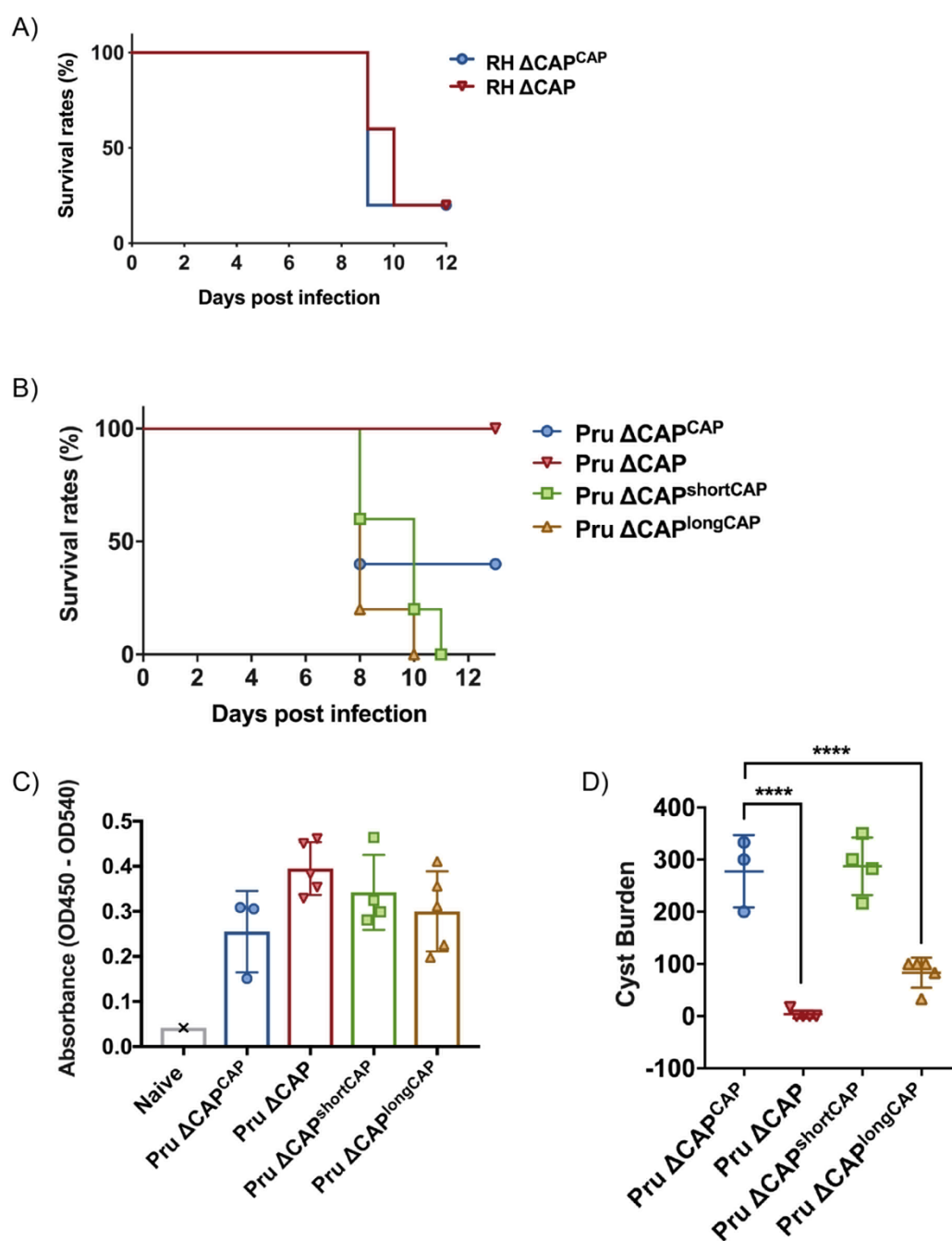1694
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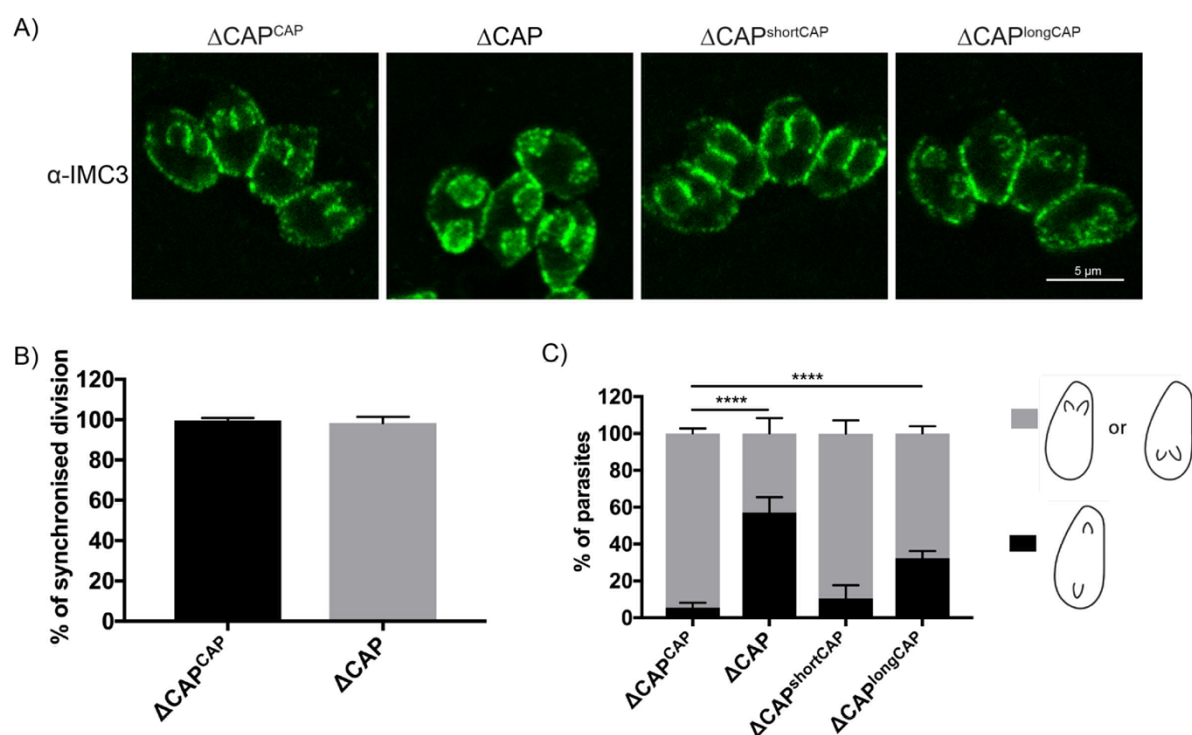
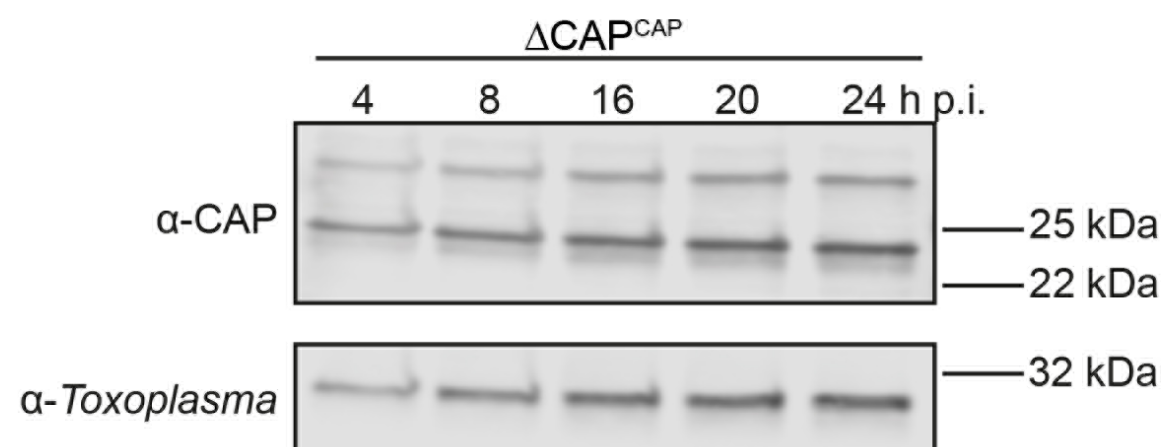1707   **Figure 7**
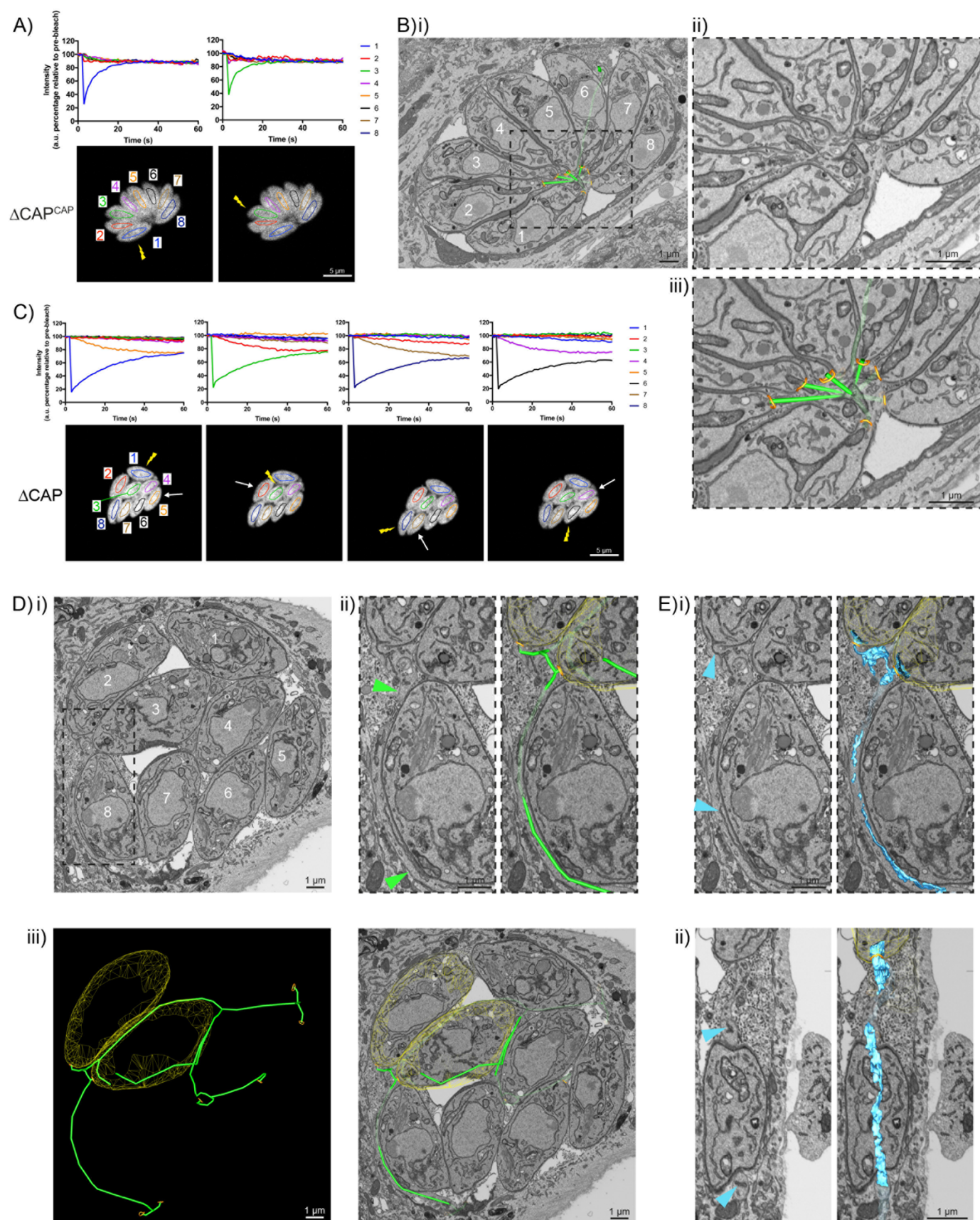
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Figure 8

Supplementary Figure 1

A)

CAP | HA | 3' UTR

5' UTR | CAP | 3' UTR

CRISPR cut site

5' UTR | CAP | HA | 3' UTR

B)

CAP-HA

2 µm

1807 **Supplementary Figure 2**

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Supplementary Figure 3

1885 **Supplementary Figure 4**
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1924 **Supplementary Figure 5**

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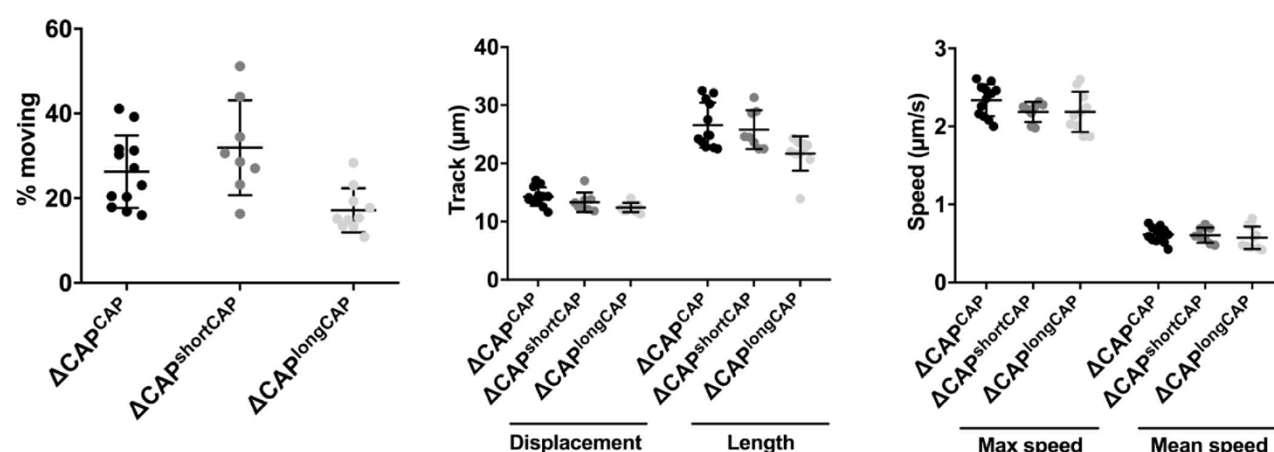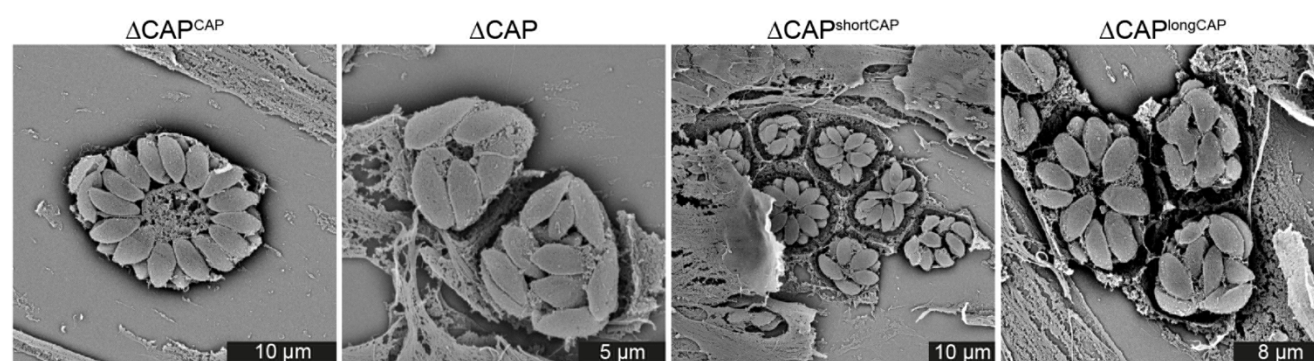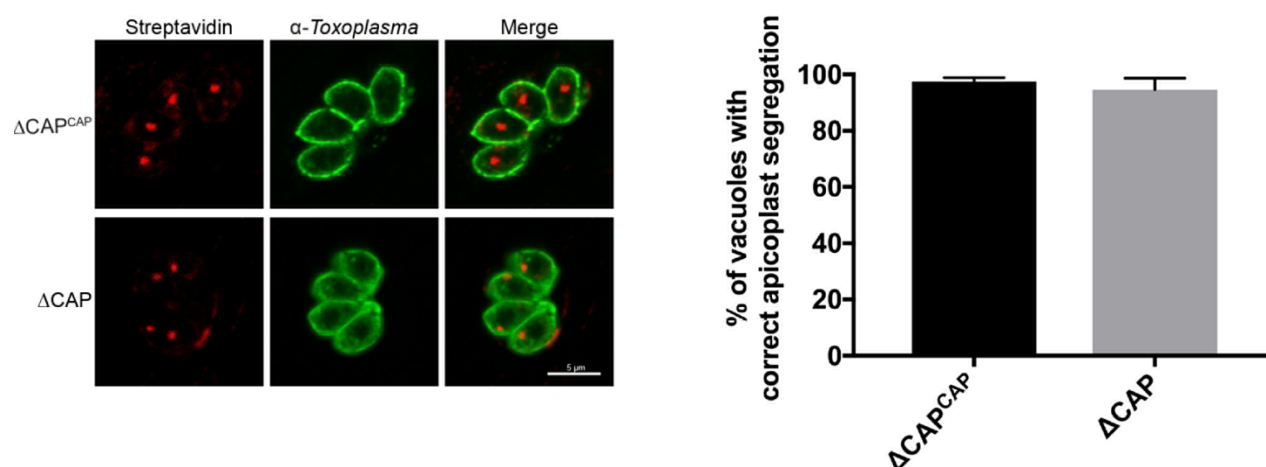**Supplementary Figure 6**

2002 **Supplementary Figure 7**

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2045 **Supplementary Figure 8**
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2086   **Supplementary Figure 9**
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