

1 **A positive feedback loop mediates crosstalk between calcium, cyclic nucleotide**
2 **and lipid signalling in *Toxoplasma gondii*.**

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18 **Abstract**

19 Fundamental processes of obligate intracellular parasites, such as *Toxoplasma gondii*
20 and *Plasmodium falciparum*, are controlled by a set of plant-like calcium dependent
21 kinases (CDPKs), the conserved cAMP- and cGMP-dependent protein kinases (PKA and
22 PKG), secondary messengers and lipid signalling. While some major components of the
23 signalling networks have been identified, how these are connected remains largely
24 unknown. Here, we compare the phospho-signalling networks during *Toxoplasma* egress
25 from its host cell by artificially raising cGMP or calcium levels to activate PKG or CDPKs,
26 respectively. We show that both these inducers trigger near identical signalling pathways
27 and provide evidence for a positive feedback loop involving CDPK3. We measure
28 phospho- and lipid signalling in parasites treated with the Ca²⁺ ionophore A23187 in a
29 sub-minute timecourse and show CDPK3-dependent regulation of diacylglycerol levels

30 and increased phosphorylation of four phosphodiesterases (PDEs), suggesting their
31 function in the feedback loop. Disruption of CDPK3 leads to elevated cAMP levels and
32 inhibition of PKA signalling rescues the egress defect of Δ CDPK3 parasites treated with
33 A23187. Biochemical analysis of the four PDEs identifies PDE2 as the only cAMP-specific
34 PDE among these candidates, while the other PDEs are cGMP specific, two of which are
35 inhibited by the predicted PDE inhibitor BIPPO. Conditional deletion of the four PDEs
36 supports an important, but non-essential role for PDE1 and PDE2 in growth, with PDE2
37 controlling A23187-mediated egress. In summary we uncover a positive feedback loop
38 that enhances signalling during egress and links several signalling pathways together.

39 Introduction

40 The Apicomplexa are obligate intracellular parasites that pose a considerable challenge
41 to human and animal health. The most prevalent member of this phylum, *Toxoplasma*
42 *gondii*, infects virtually all warm-blooded animals, including an estimated 30% of humans
43 worldwide (Pappas, Roussos and Falagas, 2009). To ensure its survival in the host,
44 *Toxoplasma*, like all apicomplexan parasites, must actively invade host cells to initiate
45 replication inside a parasitophorous vacuole. Following several rounds of division, or in
46 response to adverse environmental changes, tachyzoites are triggered to egress from
47 host cells, allowing for subsequent cycles of reinvasion and growth.

48 At any stage during the replicative cycle, *T. gondii* may be triggered to egress from
49 infected cells in response to deleterious environmental changes. Both extrinsic and
50 intrinsic stimuli play a role in this process. Extrinsic signals include low potassium (K^+),
51 low pH, and serum albumin (Moudy, Manning and Beckers, 2001; Roiko, Svezhova and
52 Carruthers, 2014; Brown, Lourido and Sibley, 2016), while the accumulation of
53 phosphatidic acid (PA) produced in the parasitophorous vacuole serves as an intrinsic
54 signal to induce natural egress, although this occurs in a more gradual manner after
55 several cycles of endodyogeny (Bisio *et al.*, 2019).

56 Irrespective of the egress trigger, it is clear that secondary messengers play a key role in
57 driving the process forward once initiated. Indeed, calcium (Ca^{2+}) (Carruthers and Sibley,
58 1999), purine cyclic nucleotides (cGMP and cAMP) (Wiersma *et al.*, 2004; Ono *et al.*,
59 2008), and phosphatidic acid (PA) (Bullen *et al.*, 2016) have all been implicated (a model
60 is shown in Supp Fig. 1). Upon initiation of egress, migration, or invasion, cytosolic Ca^{2+}
61 levels rise substantially (Lourido and Moreno, 2015). It has been hypothesised that

62 inositol triphosphate (IP₃), generated by phosphoinositide phospholipase C (PI-PLC)-
63 mediated cleavage of phosphatidylinositol 4,5-bisphosphate (PIP₂), opens an (as yet
64 unidentified) IP₃-sensitive channel to release Ca²⁺ from organelles that otherwise
65 sequester Ca²⁺ during immotile replicative stages (Lovett *et al.*, 2002; Garcia *et al.*, 2017).
66 Once released, Ca²⁺ activates a range of effectors, including a group of 'plant-like' Ca²⁺-
67 dependent protein kinases (CDPKs) (Billker, Lourido and Sibley, 2009) and proteins
68 involved in vesicle exocytosis (Farrell *et al.*, 2012). PI-PLC-mediated cleavage of PIP₂
69 also leads to the production of diacylglycerol (DAG), which can be interconverted to PA
70 by the apicomplexan-specific DAG-kinase 1 (DGK1). In conjunction with Ca²⁺, PA is
71 believed to play an indispensable role in microneme secretion by interacting with the PA
72 receptor, acylated pleckstrin-homology (PH) domain-containing protein (APH), to
73 facilitate microneme exocytosis (Bullen *et al.*, 2016).

74 The apicomplexan cGMP-dependent protein kinase (PKG) has been identified as a key
75 regulator of the above-mentioned Ca²⁺ signalling cascade by facilitating the production of
76 IP₃ precursors (Brochet *et al.*, 2014; Katris *et al.*, 2020). Moreover, several studies have
77 suggested that PKG, beyond its regulation of the phosphoinositide pathway, may also
78 exert further control over egress by targeting as yet unidentified substrates required for
79 microneme secretion (Brown, Lourido and Sibley, 2016; Brown, Long and Sibley, 2017)
80 In *T. gondii* tachyzoites, the cAMP-dependent protein kinase catalytic subunit 1 (PKAc1),
81 meanwhile, has been proposed to act as a negative regulator of PKG signalling by
82 inhibiting egress induced by parasite-dependent acidification (Jia *et al.*, 2017; Uboldi *et al.*,
83 2018). PKAc1 has been suggested to indirectly regulate PKG by phosphorylating and
84 possibly activating a putative cGMP phosphodiesterase (PDE) which would result in the
85 degradation of cGMP and a subsequent down-regulation of PKG activity (Jia *et al.*, 2017).
86 It is important to note that the cAMP signalling pathway plays no role in regulating *P.*
87 *falciparum* egress during the blood stages of infection, and instead appears to be
88 important for mediating invasion (Leykauf *et al.*, 2010; Flueck *et al.*, 2019; Patel *et al.*,
89 2019).

90 Although many experimental observations place PKG (Brown, Long and Sibley, 2017; Jia
91 *et al.*, 2017) and phosphoinositide (Bullen *et al.*, 2016) signalling upstream of cytosolic
92 Ca²⁺ flux (and by extension the activation of CDPKs) a further level of interaction between
93 cGMP signalling and CDPK3 has become apparent (Lourido, Tang and David Sibley,
94 2012). CDPKs, comprised of a serine/threonine kinase domain fused to a calmodulin-like

95 domain, belong to a superfamily of kinases that feature prominently in the Ca²⁺ signalling
96 pathways of plants and some ciliates. Although *Toxoplasma* has numerous CDPK
97 encoding genes (Long, Wang and Sibley, 2016), CDPK1 and CDPK3 have been most
98 extensively studied. CDPK1 has been implicated in microneme exocytosis and the
99 subsequent initiation of gliding motility (Lourido, Tang and David Sibley, 2012), while
100 CDPK3 has been shown to be important for rapid Ca²⁺ ionophore-induced egress, where
101 the addition of the calcium ionophore A23187 or BIPPO leads to concerted parasite exit
102 from the host cell in seconds (Black and Boothroyd, 2000; Garrison *et al.*, 2012; Lourido,
103 Tang and David Sibley, 2012; McCoy *et al.*, 2012). Intriguingly, while a marked delay in
104 egress is evident when CDPK3 depleted/inhibited parasites are treated with Ca²⁺
105 ionophore, this phenotype is partially rescued when tachyzoites are induced to egress
106 with PDE inhibitors such as zaprinast and BIPPO (Lourido, Tang and David Sibley, 2012;
107 Howard *et al.*, 2015). The Ca²⁺ ionophore A23187 forms lipid-soluble complexes with
108 divalent cations and is thought to induce a discharge of Ca²⁺ from cytosolic stores. Both
109 zaprinast and BIPPO, known inducers of *Toxoplasma* egress (Lourido, Tang and David
110 Sibley, 2012; Howard *et al.*, 2015) are thought to induce an elevation in cytosolic cGMP
111 levels by inhibiting cGMP hydrolysing PDEs, activating PKG activity via elevated cGMP
112 levels. Accordingly, these findings suggest a compensatory role for PKG signalling in the
113 absence of CDPK3.

114 While this compensatory mechanism has not been examined in any great detail, it is
115 possible that PKG and CDPK3 substrate specificity may overlap. Multiple kinases
116 converging on shared targets can provide multiple layers of regulation to a single
117 pathway, and this is a known feature of nucleotide-activated kinases, including PKG
118 (Pearce, Komander and Alessi, 2010). Alternatively, it is plausible that BIPPO's
119 compensatory effects are explained by a more direct link between CDPK3 and PKG
120 activity; if CDPK3 were to play a feedback-mediated role in the positive regulation of PKG
121 signalling, pharmacological activation of PKG (e.g. by BIPPO/zaprinast treatment) would
122 also diminish the requirement for CDPK3 during egress. Interestingly, this is reminiscent
123 of the *P. falciparum* CDPK5, where the egress block of CDPK5-deficient parasites can be
124 rescued by hyperactivation of PKG (Absalon *et al.*, 2018).

125 While the above literature forms a common understanding that CDPKs, PKG, PKA, lipid
126 and second messenger signalling are important across lifecycle stages in *Toxoplasma*

127 and *Plasmodium* species, how they are spatially and temporally regulated, how they
128 intersect and how specific signalling outcomes are achieved is not well described.

129 Here, we report on the phospho-, lipid and cyclic nucleotide signalling networks activated
130 during the pharmacological induction of *Toxoplasma* tachyzoite egress using either
131 A23187 or BIPPO. Collectively, our data highlights the presence of a feedback loop
132 between A23187-regulated Ca^{2+} release and cyclic nucleotide as well as
133 phosphoinositide signalling. This mechanism appears to be regulated, at least in part, by
134 CDPK3 and the cAMP-specific phosphodiesterase PDE2.

135

136 **Results**

137 **Generation of Calcium Reporter Lines to align BIPPO and A23187 signalling** 138 **pathways.**

139 To investigate how the cGMP and calcium signalling pathways converge and differ, we
140 compared their phosphorylation dynamics using two activators of these pathways:
141 BIPPO, a PDE inhibitor, and the calcium ionophore A23187.

142 The signalling kinetics following Ca^{2+} ionophore and BIPPO treatment vary, so we first
143 determined a timepoint at which both pathways should be comparable. Common to both
144 treatments is a raise in intracellular calcium levels before egress. We therefore chose
145 peak intracellular calcium levels as a reference point to facilitate a direct comparison
146 between BIPPO- and A23187-treated parasites. To this end, we generated a stable
147 calcium sensor line that co-expresses, through use of a T2A ribosomal skip peptide, an
148 internal GFP control and the genetically encoded ruby Ca^{2+} biosensor jRCaMP1b (Alves
149 *et al.*, 2021) from a single promoter (Fig. 1A-B). The expression of the biosensor did not
150 have any discernible effects on Ca^{2+} ionophore (A23187) or BIPPO induced egress rates
151 (Supp Fig. 2) therefore all subsequent experiments were performed with this line
152 (henceforth referred to as WT). While some variability of jRCaMP1b fluorescence was
153 observed between vacuoles at a per-well level upon stimulation, ratiometric quantitation
154 of jRCaMP1b fluorescence upon BIPPO or A23187 treatment of cytochalasin D-
155 immobilised parasites illustrated distinct Ca^{2+} response curves; BIPPO treatment led to a
156 rapid increase in Ca^{2+} levels, (Fig. 1Ci), while the cytosolic Ca^{2+} rise detected upon
157 A23187 treatment appeared more gradual (Fig. 1Cii). To facilitate optimal alignment, and

158 to account for the rapid kinetics of these signalling pathways, treatment timings of 15s
159 (BIPPO) and 50s (A23187) were selected for subsequent phosphoproteomics
160 experiments.

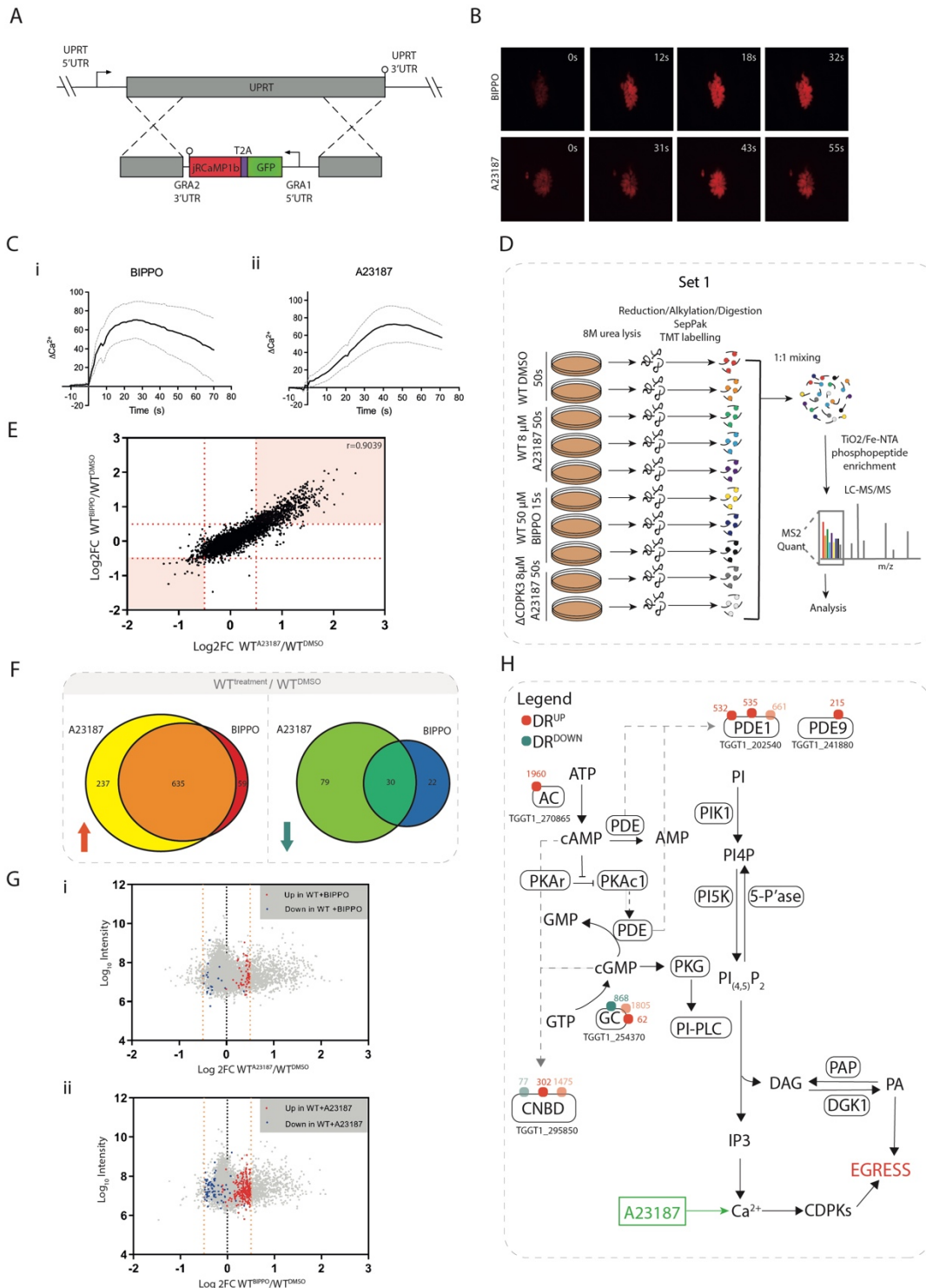
161 **A23187- and BIPPO-treated Wildtype Parasites Exhibit Highly Correlative**
162 **Phosphoproteomic Responses at temporally aligned calcium flux.**

163 Having identified the optimal BIPPO and A23187 treatment timings to achieve maximal
164 calcium release, we wanted to identify and compare phosphorylation events that take
165 place during BIPPO- and A23187-induced signalling cascades at these timepoints. We
166 used multiplexed tandem-mass-tags (TMT) and LC-MS/MS to quantify the
167 phosphoproteome of intracellular WT tachyzoites treated with vehicle (DMSO), 50 μ M
168 BIPPO (15s) or 8 μ M A23187 (50s) at 37°C. DMSO-treated parasite samples were
169 generated in 2 biological replicates, while BIPPO- and A23187-treated samples were
170 generated in 3 biological replicates (Fig. 1D). At these timepoints parasites remained
171 intracellular. Samples were lysed, digested, and labelled with different TMT tags. Labelled
172 samples were pooled and subjected to TiO₂/Fe-NTA phosphopeptide enrichment prior to
173 LC-MS/MS analysis. Of note, this experiment (Set 1), also contains two biological
174 replicates of an A23187-treated calcium-dependent kinase 3 deletion (Δ CDPK3) parasite
175 line. This allowed us to identify Δ CDPK3 dependency of signalling events during A23187
176 and BIPPO induced egress and is explained further below.

177 We quantified changes in phosphorylation states by calculating the log₂-transformed
178 intensity ratios (log₂FC) of A23187- or BIPPO-stimulated WT parasites versus DMSO-
179 treated WT parasites (DataS1). In total we quantified 7,811 phosphorylation sites across
180 these conditions.

181 Differentially regulated (DR) phosphorylation sites were selected if the log₂FC exceeded
182 3x the median absolute deviation (MAD), rounded to the nearest tenth. This was
183 log₂FC>0.5 for up-regulated sites and log₂FC<-0.5 for down-regulated sites; (Supp Fig.
184 3A) and applied across all datasets.

185 The rapid signalling progression upon treatment with BIPPO and A23187 inevitably
186 results in variability in phosphosite intensities between replicates, where despite our best
187 efforts, signalling may be stopped with several seconds difference between experiments.
188 As such variability results in poor p-values in classical t-tests and, by extension, an under-



189 **Figure 1. Comparative phosphoproteomics of BIPPO vs A23187-induced GFP-T2A-jRCaMP1b (WT)**
 190 **parasites at peak cytosolic calcium levels (A)** Generation of the calcium sensor line GFP-T2A-
 191 jRCaMP1b by integration into the UPRT locus. **(B)** Video microscopy of intracellular GFP-T2A-jRCaMP1b
 192 parasites (red channel), following addition of 50 μM BIPPO or 8 μM A23187 at 0s. **(C)** Ratiometric tracking
 193 of mean Ca^{2+} response (jRCaMP1b/GFP normalized to 0), following addition of (i) 50 μM BIPPO or (ii) 8 μM

194 A23187. Grey dotted lines represent \pm SEM. Red dotted line indicates the timepoint selected for subsequent
195 phosphoproteomic experiments. Data was collected from ≥ 10 vacuoles (in separate wells) over ≥ 6 days.
196 **(D)** Schematic summary of the TMT-10-plex experiment (set 1) and workflow used to quantify the
197 phosphoproteomes of intracellular WT tachyzoites treated with 50 μ M BIPPO (15s) or 8 μ M A23187 (50s).
198 Δ CDPK3 parasites treated with 8 μ M A23187 (50s) were included to facilitate later analyses. **(E)** Correlation
199 of phosphosite regulation (\log_2 FC) in BIPPO (y axis) and A23187 (x axis)-treated WT parasites. Each data
200 point corresponds to a single phosphosite. Red dotted lines represent 3xMAD outlier thresholds used to
201 determine differential site regulation. Phosphosites that fall within the pink shaded regions are differentially
202 regulated upon treatment with both BIPPO and A23187. **(F)** Overlap between phosphosites that are
203 differentially regulated in GFP-T2A-jRCaMP1b (WT) tachyzoites following treatment with 8 μ M A23187
204 (50s) or 50 μ M BIPPO (15s). Red arrow represents up-regulated sites (\log_2 FC >0.5), blue arrow represents
205 down-regulated sites (\log_2 FC <-0.5). Only sites confidently detected in both treatment conditions were
206 included for analysis. **(G)** Differential phosphosite regulation (\log_2 FC) vs \log_{10} total reporter intensity
207 following treatment of WT tachyzoites with **(i)** 8 μ M A23187 (50s) or **(ii)** 50 μ M BIPPO (15s). **(H)** Each data
208 point corresponds to a single phosphosite. Only sites confidently detected in both BIPPO and A23187
209 samples are shown. In (i) Coloured data points highlight sites that were differentially up- or down-regulated
210 (red and blue, respectively) upon BIPPO, but not A23187 treatment, while in (ii) coloured data points
211 highlight sites that were differentially up- or down-regulated (red and blue, respectively) upon A23187, but
212 not BIPPO treatment. Orange dotted lines represent 3xMAD outlier thresholds used to determine differential
213 site regulation (\log_2 FC >0.5 for up-regulated sites and \log_2 FC <-0.5 for down-regulated sites). **(H)**
214 Differentially A23187-regulated phosphosites detected on targets implicated in the regulation of cyclic
215 nucleotides. Red and blue dots represent sites that are differentially up- or down-regulated, respectively.
216 Numbers refer to site position within protein. Dots with reduced opacity represent sites with a localization
217 score difference <10 .

218

219 reporting of true treatment-regulated sites, we did not subject DR sites to further p-value-
220 based thresholding. However, the reporter intensities associated with DR sites correlated
221 well across replicates ($r>0.89$, Supp Fig. 3B). This suggests that despite some of the
222 aforementioned replicate variability, the overall trends across replicates were consistent,
223 and these scores could therefore be confidently averaged to provide values that are
224 representative of a site's phosphorylation state at the timepoint of interest.

225 Comparison of the \log_2 FCs observed in BIPPO- and A23187-treated samples shows
226 strong correlation between the phosphorylation responses of these conditions ($r=0.9039$)
227 (Fig. 1E), suggesting that the signalling pathways at these selected timepoints align
228 sufficiently well to directly compare them.

229 **Phosphoproteomic Analysis Cannot Confidently Distinguish BIPPO- from A23187-**
230 **Induced Signalling.**

231 To investigate the signalling events that are shared between or are unique to BIPPO and
232 A23187 treatment, we identified DR sites for each treatment condition. We then identified
233 DR sites that were successfully quantified in both treatments, which allowed us to
234 examine their behaviour under both conditions. In total we identified 746 BIPPO and 981
235 A23187 DR sites. A large overlap was detected between treatments for both up- and
236 down-regulated phosphosites (DR^{UP} and DR^{DOWN}, respectively); ~91% of phosphosites
237 up-regulated following BIPPO treatment showed similar regulation upon A23187 addition
238 and ~58% of BIPPO down-regulated sites behaved similarly following A23187 treatment
239 (Fig. 1F).

240 We also observed some dissimilar regulation between conditions; 59 phosphorylation
241 sites were found to be up-regulated following BIPPO treatment only, while 237 sites were
242 phosphorylated exclusively following A23187 treatment. Of the DR^{DOWN} phosphorylation
243 sites, 22 were found to be unique to BIPPO treatment, while 79 were unique to A23187
244 treatment. These treatment-specific sites may originate from distinct signalling pathways,
245 activated by each of the compounds. To discern whether these disparate site behaviours
246 are truly treatment-specific effects, or whether they are the result of imperfect alignment
247 of the treatment timings, we visualised phosphorylation site log₂FCs following A23187
248 treatment, and highlighted phosphorylation sites that were only DR following BIPPO
249 treatment (Fig. 1Gi). Similarly, we also visualised phosphorylation site log₂FCs following
250 BIPPO treatment, and highlighted phosphorylation sites that were only DR following
251 A23187 treatment (Fig. 1Gii). In both instances, most sites approached the DR thresholds
252 for up- or down-regulation. While this does not preclude the possibility that some of the
253 BIPPO- and A23187-specific DR^{UP/DOWN} sites are regulated in a drug-exclusive manner,
254 it is likely that the majority of these sites would pass the DR threshold within seconds,
255 and that minor changes in treatment timing can make the difference between surpassing
256 the DR threshold or not.

257 Collectively, these findings demonstrate that at temporally aligned calcium release within
258 the parasite, it is nearly impossible to detect clear signalling features that confidently
259 distinguish the BIPPO-activated signalling pathway from the signalling cascade activated
260 upon cytosolic Ca²⁺ elevation by A23187 treatment.

261 **A23187 Treatment Leads to Differential Regulation of Targets Implicated in the PKG**
262 **Signalling Pathway.**

263 A substantial overlap between BIPPO-regulated and A23187-regulated sites was
264 expected given the increase of cytosolic Ca²⁺ in both treatment conditions. However, the
265 inability to confidently distinguish BIPPO from A23187 signalling was unexpected, as
266 these agents are believed to initiate egress by activating distinct, albeit interconnected,
267 signalling responses (Lourido, Tang and David Sibley, 2012). Previous studies have
268 placed PKG activation upstream of Ca²⁺ release (Brochet *et al.*, 2014; Stewart *et al.*,
269 2017) and it was therefore to our surprise that within the A23187 and BIPPO response
270 overlap, DR phosphorylation sites were detected on proteins implicated in the catalysis
271 and hydrolysis of the cyclic nucleotides (cNMPs) cGMP and cAMP, key molecules
272 involved in PKG activation. Subsequent examination of all A23187-regulated
273 phosphorylation sites (including those for which we lacked quantifications in BIPPO
274 samples) identified differential phosphorylation on proteins including, but not limited to,
275 enzymes important for cNMP signalling: PDEs (TGGT1_202540 (PDE1) and
276 TGGT1_241880 (PDE9)), an adenylate cyclase (TGGT1_270865), a guanylyl cyclase
277 (TGGT1_254370), and a cyclic nucleotide (cNMP) binding domain (CNBD) containing
278 protein (TGGT1_295850) (Fig. 1H).

279 The differential phosphorylation of several proteins in the upstream pathway of cNMP
280 production/regulation hints at a putative Ca²⁺-mediated feedback loop that regulates
281 cGMP and/or cAMP signalling. The existence of such a feedback mechanism could
282 account for our inability to confidently discern PKG-specific signalling upon BIPPO
283 treatment, as such signalling would be activated upon treatment with both BIPPO and
284 A23187.

285 **Deletion of CDPK3 leads to signalling perturbations in both A23187 and BIPPO**
286 **treatment conditions.**

287 Following our analyses of BIPPO- and A23187- induced egress, we set out to explore the
288 role of CDPK3 in these signaling pathways. The observation that BIPPO/zaprinast-
289 mediated activation of PKG partially compensates for a loss of CDPK3 has led to the
290 hypothesis that, given the function of both kinases in egress, the kinases' substrate
291 specificities may overlap (Lourido, Tang and David Sibley, 2012). In such a scenario,

292 BIPPO treatment would facilitate PKG-mediated phosphorylation of CDPK3 targets, thus
293 overcoming the egress delay otherwise seen in A23187-treated parasites.

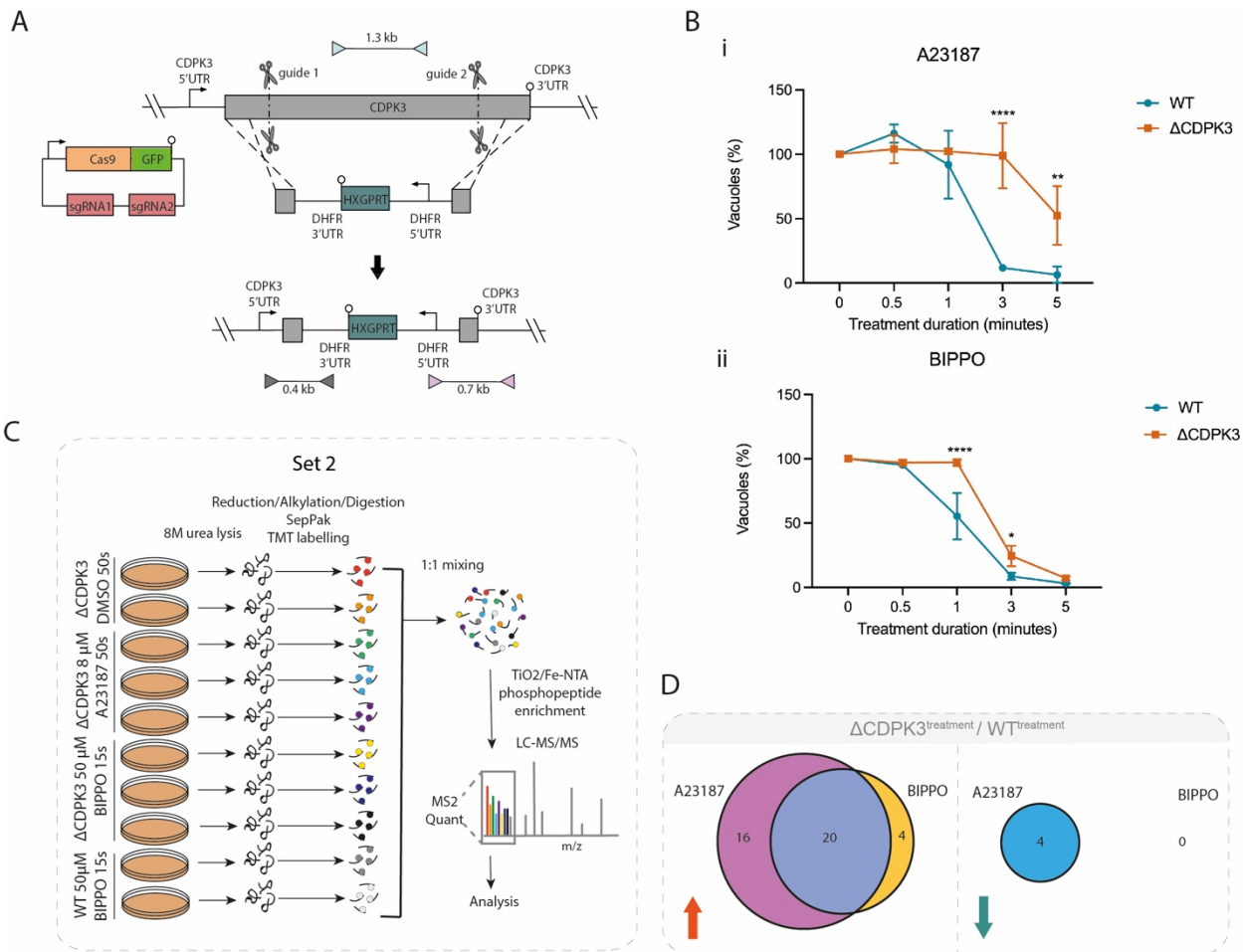
294 To identify phosphorylation sites that might fit such criteria, we wanted to identify
295 phosphorylation sites that are CDPK3-dependent upon A23187s treatment, but CDPK3-
296 independent upon BIPPO treatment. To do this, we generated a Δ CDPK3 parasite line by
297 replacing the endogenous CDPK3 locus in the RH GFP_T2A_jRCaMP1b line with a
298 HXGPRT expression cassette (henceforth known as Δ CDPK3; Fig. 2A) and confirmed
299 deletion of CDPK3 by PCR (Supp Fig. 4). We performed an egress assay to validate the
300 known A23187-induced egress delay reported for Δ CDPK3 parasites (Black and
301 Boothroyd, 2000; Garrison *et al.*, 2012; Lourido, Tang and David Sibley, 2012; McCoy *et*
302 *al.*, 2012). As expected, we found that A23187-induced egress was substantially inhibited
303 in this line (Fig. 2Bi), while a less severe egress delay was observed in BIPPO-treated
304 Δ CDPK3 parasites (Fig. 2Bii). This recapitulates previous findings (Lourido, Tang and
305 David Sibley, 2012) that activation of PKG partially compensates for a loss of CDPK3.

306 We next quantified phosphorylation events in Δ CDPK3 parasites treated with DMSO, 50
307 μ M BIPPO (15s) or 8 μ M A23187 (50s) at 37°C (Fig. 2C) in biological replicates (2x
308 DMSO, 3x A23187, 3x BIPPO). In this experiment (set 2), we included 2 biological
309 replicates of BIPPO-treated WT parasites. In conjunction with the ionophore-treated
310 Δ CDPK3 parasites included in set 1 (Fig. 1D), this allowed us to identify CDPK3-
311 dependent phosphorylation sites during BIPPO- and A23187-induced egress (Data S2).
312 We first identified DR phosphorylation sites across all datasets for which we had
313 quantifications and tested for CDPK3 dependency. Of the 498 phosphosites detected, 44
314 sites (~8.5%) were CDPK3-dependent (Fig. 2D, Data S2). 40 sites were classed as DR^{UP};
315 16 were exclusive to A23187 treatment, 20 were identified upon both A23187 and BIPPO
316 treatment, and 4 were detected upon BIPPO treatment only. By contrast, only 4 sites were
317 classed as DR^{DOWN}, all in a seemingly A23187-exclusive manner.

318 The 16 phosphorylation sites that show CDPK3 dependency exclusively upon A23187
319 treatment constituted putative candidates for PKG/CDPK3 substrate overlap. We
320 reasoned that, if a DR^{UP} site was found to be CDPK3-dependent upon A23187 treatment
321 only, this phosphorylation site should be recovered in Δ CDPK3 parasites following BIPPO
322 treatment. Only 3 of these phosphorylation sites showed this behaviour and were located
323 on two hypothetical proteins (TGGT1_243460, TGGT1_232120) and a DnaJ domain-

324 containing protein (TGGT1_203380) (Data S2). While these findings do not completely
 325 rule out the ‘substrate overlap’ theory to account for BIPPO’s compensatory effects, the
 326 putative overlap is extremely small, and none of these proteins contain predicted domains
 327 that would explain the rescue of CDPK3 mutants by BIPPO in induced egress.

328 Collectively, our current findings provide more evidence for a Ca^{2+} -regulated feedback
 329 loop model than for PKG/CDPK3 substrate overlap.



330 **Figure 2. Comparative phosphoproteomics of BIPPO vs A23187-induced GFP-T2A-jRCaMP1b**
 331 **Δ CDPK3 parasites at peak cytosolic calcium levels (A)** Generation of the GFP-T2A-jRCaMP1b
 332 Δ CDPK3 line using CRISPR/Cas9 to increase site-directed integration. Scissors represent Cas9 cleavage
 333 sites and lollipops depict stop codons. Coloured triangles represent primer pairs used to detect WT, 5'
 334 integration and 3' integration loci (light blue, grey and pink respectively). PCR results using these primer
 335 pairs are shown in Supp Fig. 4 **(B)** Egress assay of GFP-T2A-jRCaMP1b (WT) and GFP-T2A-jRCaMP1b
 336 Δ CDPK3 (Δ CDPK3) parasites following treatment with **(i)** 8 μ M A23187 or **(ii)** 50 μ M BIPPO. Graph shows
 337 the remaining % of un-egressed vacuoles (relative to untreated) following A23187/BIPPO treatment. Data
 338 are represented as mean \pm s.d. (n=3). Two-way ANOVA with Holm-Sidak *post hoc* comparison. ****, $P \leq$

339 0.0001 **(C)** Schematic summary of the TMT-10-plex experiment (set 2) and workflow used to quantify the
340 phosphoproteomes of intracellular Δ CDPK3 tachyzoites treated with 50 μ M BIPPO (15s), 8 μ M A23187
341 (50s), and WT parasites treated with 50 μ M BIPPO (15s). **(D)** Overlap between differentially regulated
342 phosphosites that display CDPK3 dependency following treatment with 50 μ M BIPPO (15s) or 8 μ M A23187
343 (50s) (data derived from set 1 and 2). Red and blue arrows represent up- and down-regulated sites,
344 respectively. Only phosphosites found to be differentially regulated upon treatment with both A23187 and
345 BIPPO were included for analysis.

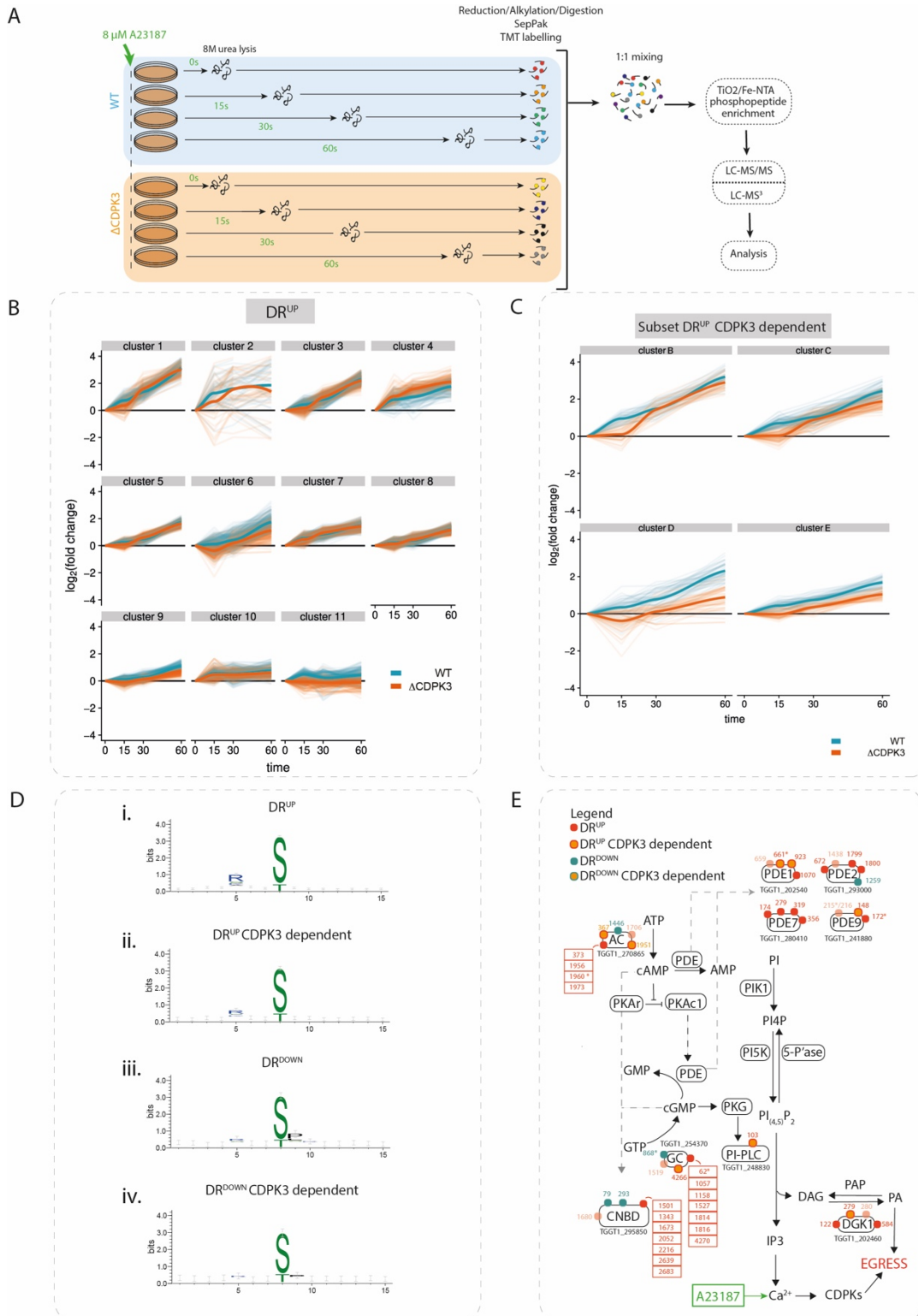
346

347 **A sub-minute timecourse of CDPK3 dependent and independent signalling** 348 **progression in ionophore-induced egress.**

349 The experiments delineated above, exploring the phosphosignalling networks in WT and
350 Δ CDPK3 parasites triggered upon treatment with A23187 or BIPPO, examined only a
351 single treatment timepoint. These experiments therefore offer only a limited 'signalling
352 snapshot' that precludes any insights into the dynamics of signalling progression over
353 time. To further investigate the putative activation of cNMP-induced signalling pathways
354 in WT and Δ CDPK3 mutants upon A23187 treatment we performed a sub-minute
355 phosphosignalling timecourse. We treated intracellular WT and Δ CDPK3 tachyzoites with
356 8 μ M A23187 for 15, 30 or 60 seconds at 37°C (Fig. 3A), during which the parasites
357 remained intracellular. As before, samples were subjected to TMT-based quantitative
358 analysis of phosphoproteomic changes. Fold changes were calculated relative to a 0s
359 (DMSO) control. In total we quantified 11,021 phosphorylation sites (Data S3).

360 DR thresholds were set at 3x MAD of the log₂FC across each WT timepoint (15s, 30s
361 and 60s). Phosphorylation sites were considered differentially regulated if at any given
362 timepoint their log₂FC surpassed these thresholds. CDPK3 dependency was determined
363 for each phosphorylation site by calculating the log₂ ratios of A23187-treated WT and
364 Δ CDPK3 parasites for each timepoint. The resulting ratios were used to calculate the
365 MAD at each timepoint, and the most stringent score was used to set 3X MAD outlier
366 thresholds. A DR site was considered CDPK3-dependent if, at any given timepoint, it
367 simultaneously passed the DR and CDPK3 dependency thresholds.

368 We identified 2,408 phosphorylation sites (DR^{UP}) upon A23187 treatment in WT
369 parasites, which were also quantified in Δ CDPK3 parasites (Data S3). To examine
370 whether this dataset recapitulates our previous findings, we compared the DR^{UP} sites



371 **Figure 3. A23187 treatment results in partially CDPK3-dependent phosphorylation of targets**
 372 **implicated in PKG signalling (A) Schematic of A23187-treatment timecourse experimental design. (B)**
 373 **Gaussian mixture-model-based clustering of all DR^{UP} sites in the A23187-treatment time courses. Log₂FC**

374 values from both WT and Δ CDPK3 samples were combined to cluster on six dimensions (WT 15s, 30s and
375 60s and Δ CDPK3 15s, 30s and 60s). Thin lines represent the time course traces of individual
376 phosphorylation sites. Thick lines represent Loess regression fits of all traces. **(C)** Gaussian mixture-model-
377 based clustering of a subset of DR^{UP} CDPK3-dependent sites in the A23187-treatment time courses.
378 Log₂FC values from both WT and Δ CDPK3 samples were combined to cluster on six dimensions (WT 15s,
379 30s and 60s and Δ CDPK3 15s, 30s and 60s). Thin lines represent the time course traces of individual
380 phosphorylation sites. Thick lines represent Loess regression fits of all traces. Four clusters (B-E) best
381 illustrating the transient phosphorylation delay in Δ CDPK3 parasites are shown. **(D)** Results of
382 phosphorylation motif enrichment analysis using rmotifx 1.0. Results are shown for the analysis of **(i)** DR^{UP}
383 **(ii)** DR^{UP} CDPK3-dependent **(iii)**DR^{DOWN} and **(iv)** DR^{DOWN} CDPK3-dependent phosphorylation sites. **(E)**
384 Differentially A23187-regulated phosphosites detected on targets implicated in the regulation of PKG
385 signalling. Red and blue dots represent sites that are differentially up- or down-regulated, respectively. Dots
386 with an orange centre indicate CDPK3-dependent sites. Numbers refer to site position within protein. Dots
387 with reduced opacity represent sites with a localisation score difference < 10. Asterisks highlight sites that
388 were previously detected in this study's A23187/BIPPO phosphoproteome experiment (see Fig. 1D & Fig.
389 2C).

390

391 identified at the 60s timepoint in this experiment, with those identified after 50s A23187
392 treatment from the preceding experiments (Fig. 2C). Of the 572 DR phosphorylation sites
393 identified 50s after A23187 treatment in our initial experiment, 503 sites (~88%) also
394 passed the threshold for differential (up)regulation in the timecourse at the 60s post-
395 treatment timepoint. Reassuringly, we observed many proteins previously identified as
396 being phosphorylated in a CDPK3-dependent manner (Treeck *et al.*, 2014; Wallbank *et*
397 *al.*, 2019), including the CRAL/TRIO domain containing protein (TGGT1_254390), a
398 putative P-type ATPase4 (TGGT1_278660), CDPK2A (TGGT1_206590) and the tyrosine
399 transporter ApiAT5-3 (TGGT1_257530).

400 To get an overview of the progression of signalling cascades in DMSO- and A23187-
401 treated parasites, we performed a clustering analysis, as previously described (Invergo
402 *et al.*, 2017), of DR phosphorylation sites identified in WT and, separately, of DR sites
403 found to be CDPK3-dependent in our timecourse experiments (see Table S1 for all
404 clusters). We obtained 11 clusters showing distinct up-regulation dynamics (Fig. 3B) and
405 10 clusters showing down-regulation dynamics in WT parasites (Supp Fig. 5A). Analysis
406 of CDPK3-dependent DR sites, meanwhile, yielded 10 up-regulated clusters and 6 down-
407 regulated clusters Fig. 3C), (Supp Fig. 5B-C).

408 In the up-regulated clusters, we identified a preponderance for phosphorylation motifs
409 with arginine in the -3 position (Fig. 3Di), a consensus sequence that has previously been
410 shown to be preferentially phosphorylated by CDPK1 (Lourido *et al.*, 2013) and possibly
411 CDPK3 (Treeck *et al.*, 2014). Reassuringly, this consensus motif was also observed
412 among DR^{UP} CDPK3-dependent sites (Fig. 3Dii). Down-regulated phosphorylated sites,
413 meanwhile, show a clear enrichment for proline in the +1 position (Fig. 3Diii & Fig. 3Div).
414 This indicates that while CDPK activity (and/or activity of kinases with a similar substrate
415 preference) is being induced by calcium-signalling, a distinct set of one or more kinases
416 with this phosphorylation motif preference is being inactivated concurrently. Alternatively,
417 this could be mediated by the activation of a specific phosphatase. We observed that
418 several of the less phosphorylated proteins in the timecourse are secreted into the
419 parasitophorous vacuole (PV), which physically separates the parasite from the host cell
420 cytoplasm. Several proteins that are secreted into the PV have been shown to play a role
421 in mediating egress. This includes GRA41, which has been shown to be important for
422 A23187-induced egress (LaFavers *et al.*, 2017). It is therefore possible that the secreted
423 proteins identified in our timecourse may be implicated in wider signalling events
424 occurring in the PV that are required for egress.

425 Several functionally related proteins were phosphorylated with similar dynamics, as
426 revealed by Gene Ontology (GO) term enrichment (see Tables S2-5 for DR^{UP}, DR^{DOWN},
427 CDPK3-dependent DR^{UP} and CDPK3-dependent DR^{DOWN} GO term enrichments,
428 respectively). Most notably, two up-regulation clusters (clusters 1 and 2) were enriched
429 in terms related to signal transduction (GO:0007165, GO:0007154, GO:0023052) and
430 hydrolase activity (GO:0016787, GO:0042578, GO:0016462, GO:0016817, GO:0016818,
431 GO:0017111), respectively (Fig. 3B). These enrichments were, in part, driven by
432 phosphorylation of PDEs and cyclases involved in cyclic nucleotide signalling. Thus, not
433 only are the enzymes potentially upstream of PKG being phosphorylated upon exposure
434 to ionophore, but also the dynamics of phosphorylation are similar between them.

435 We also found significant enrichment of membrane proteins in CDPK3-dependent
436 clusters (GO:0044425, GO:0016021, GO:0031224, GO:0016020). These are predicted
437 to play roles in nutrient transport and ion-exchange, including the sodium-hydrogen
438 exchangers NHE1 and NHE3 (which have previously been linked to egress) (Arrizabalaga
439 *et al.*, 2004; Francia *et al.*, 2005), and the tyrosine transporter ApiAT-5-3 (a known target
440 of CDPK3 phosphorylation)(Treeck *et al.*, 2014; Wallbank *et al.*, 2019).

441 Further GO enrichment analysis of up-regulated clusters revealed other potential
442 downstream targets of ionophore-induced signalling, including transcription (e.g.
443 GO:0006355, GO:1903506, GO:2001141) by AP2-family transcription factors,
444 magnesium chelatase activity (GO:0016851) by DNA replication licensing factors, and
445 regulation of a GTPase-mediated process (GO:00423087, GO:0051336). Intriguingly, we
446 found that phosphorylation of several targets associated with these GO terms also
447 showed CDPK3 dependency, including AP2VIII-2 (TGGT1_233120), AP2XI-2
448 (TGGT1_310900), MCM7 (TGGT1_237220), and a predicted GTPase, Ras-related
449 Rab11 (TGGT1_289680). The function of these proteins in the CDPK3 signalling cascade
450 is less clear but may point towards biological processes related to DNA replication and
451 transcription.

452 In addition to its likely involvement in the numerous signalling processes mentioned
453 above, CDPK3 also directly or indirectly regulates the activities of other signalling
454 proteins. For example, CDPK3-dependent down-regulation was detected at a site within
455 the protein kinase domain of the cell-cycle-associated protein kinase GSK
456 (TGGT1_265330 S214). Similarly, CDPK3-dependent DR^{UP} sites were found within the
457 PI3Ka domain of PI3/4-kinase (TGGT1_215700) and the EF-hand domains of centrin 2
458 (TGGT1_250340) and calmodulin (TGGT1_305050).

459 It is important to note that visualisation of the CDPK3-dependent DR^{UP} clusters revealed
460 that for many sites, the effect of CDPK3 deletion was temporary, such that there was an
461 initial delay in phosphorylation, but by 60s the sites reached only slightly lower log₂FC
462 values than in WT (Fig. 3C). This may point to the redundant or compensatory activity of
463 a protein kinase other than PKG, which could in part account for the fact that egress still
464 occurs in CDPK3-depleted parasites, albeit at a delayed pace. Such a delay was not
465 clearly detectable in the CDPK3-dependent DR^{DOWN} clusters.

466 The GO terms “signal transduction” and “hydrolase activity” mentioned above contained
467 numerous phosphorylation sites on proteins potentially involved in cNMP signalling,
468 including the PDEs TGGT1_202540 (PDE1) and TGGT1_241880 (PDE9), the adenylate
469 cyclase TGGT1_270865, the guanylyl cyclase TGGT1_254370, and the CNBD
470 containing protein TGGT1_295850 (Fig. 3E; Data S3). In addition to these previously
471 detected targets, we also observed increased phosphorylation of PDEs TGGT1_280410
472 (PDE7) and TGGT1_293000 (PDE2) as well as components of phosphoinositide

473 signalling including the PI-PLC TGGT1_248830 and the DAG kinase 1 (DGK1)
474 TGGT1_202460. Many phosphorylation sites on these enzymes are found to be CDPK3-
475 dependent (Fig. 3E). PI-PLC is required for the production of IP₃ which triggers calcium
476 release and has been implicated as a key downstream mediator of PKG activity (Brochet
477 *et al.*, 2014), while the DGK1 has been shown to play an important role in the conversion
478 of intracellular DAG to PA and, by extension, the activation of microneme secretion and
479 subsequent egress (Bullen *et al.*, 2016). Collectively, these findings further substantiate
480 our hypothesis that the A23187-mediated release of Ca²⁺ activates a CDPK3-dependent
481 feedback loop that regulates the PKG signalling pathway.

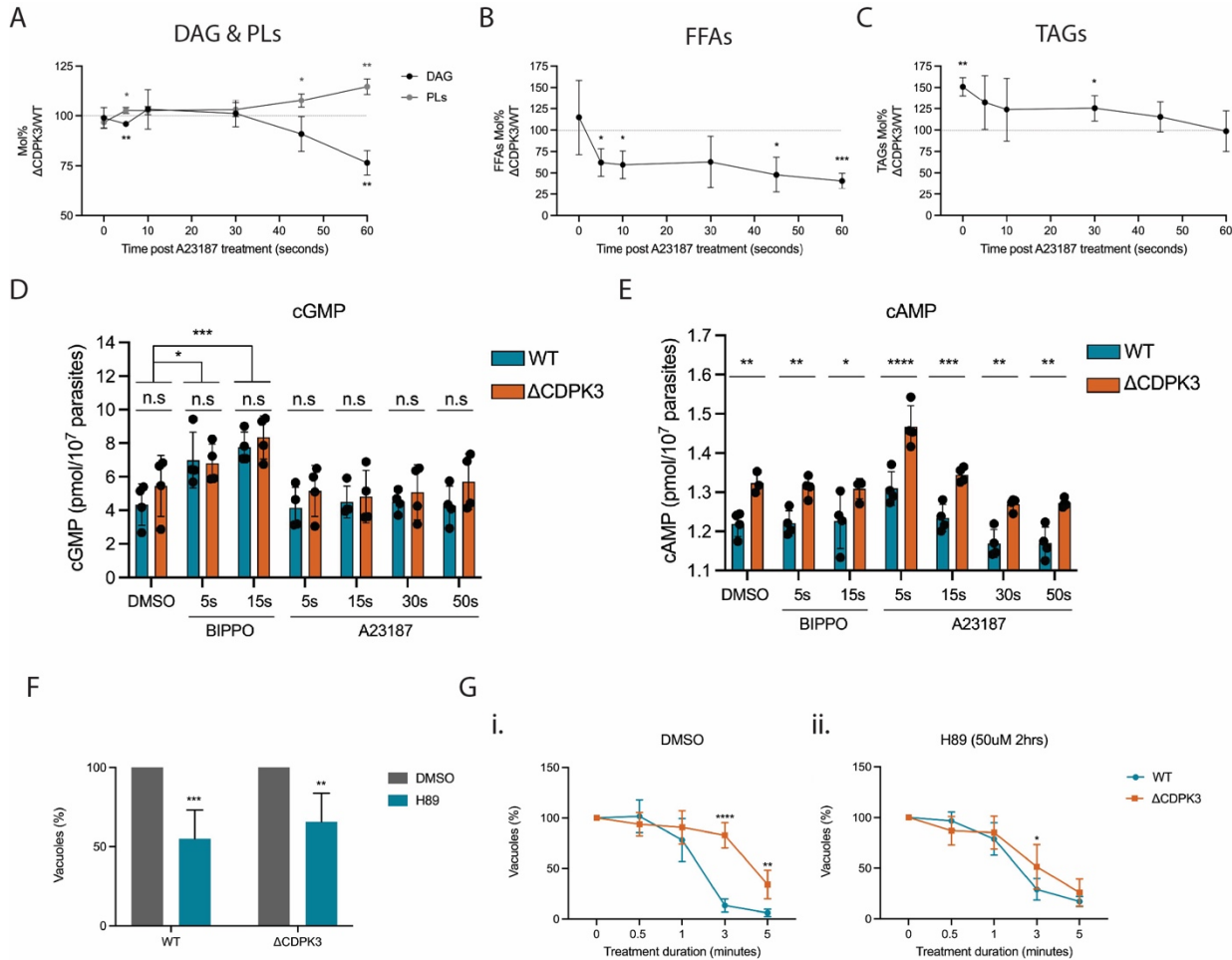
482 **Deletion of CDPK3 leads to disruptions in cAMP levels and lipid signalling** 483 **following A23187 treatment.**

484 While it is uncertain whether the above-mentioned phosphorylation events modulate the
485 function of the proteins that they are found on, the putative CDPK3-dependent regulation
486 of cNMP signalling is intriguing, as altered hydrolysis of either cAMP or cGMP has defined
487 regulatory consequences for PKG activation. Similarly, both PI-PLC and the DAG kinase
488 are understood to be key players in the PKG signalling pathway leading to egress
489 (Brochet *et al.*, 2014; Bullen *et al.*, 2016). We therefore set out to determine whether
490 deletion of CDPK3 would lead to disruptions in these signalling pathways.

491 To test whether levels of DAG and global lipid production are dysregulated following
492 disruption of CDPK3, we performed kinetics experiments in which we analysed DAG and
493 phospholipid levels in WT and Δ CDPK3 parasites before and after stimulus with the
494 calcium ionophore A23187, in a similar manner to the previous phosphoproteomic
495 timecourse. Extracellular WT and Δ CDPK3 parasites were shifted to 37°C for 60 seconds
496 to acclimatise, and then stimulated by addition of media containing A23187 similar to
497 established methods (Katris *et al.*, 2020). The parasites were incubated for 5, 10, 30, 45
498 or 60 seconds before quenching to stop the signal chain, followed by lipid analysis. A 0s
499 (DMSO) control was also included. After 60 seconds of A23187 stimulus, WT parasites
500 produced slightly more DAG than Δ CDPK3 parasites, however there was no difference
501 with the DMSO control (Fig. 4A, Supp Fig. 6A). Accordingly, WT parasites began to show
502 less phospholipids than Δ CDPK3 parasites after 45 seconds of stimulus (Fig. 4A, Supp
503 Fig. 6B), consistent with a lack of turnover of phospholipids to produce DAG in the
504 Δ CDPK3 knockout parasites. While DAG-related proteins were the primary lipid related

505 proteins affected in Δ CDPK3 based on our timecourse phosphoproteome, we also
506 identified other proteins involved in palmitoylation and triacylglycerol synthesis that were
507 differentially regulated, so we further investigated other lipids including Free Fatty Acids
508 (FFAs) and triacylglycerols (TAGs). We observed a trend towards an increase in the
509 levels of free fatty acids (FFAs) in WT parasites following A23187 stimulus (Fig. 4B, Supp
510 Fig. 6C), which remained unchanged in Δ CDPK3 parasites. This was accompanied by a
511 concomitant change in triacylglycerols (TAGs) whereby prior to stimulus, Δ CDPK3
512 parasites had more TAGs than WT parasites, but after A23187 stimulus, WT tachyzoites
513 produced more TAGs over time so that levels became similar between both parasite lines
514 (Fig. 4C, Supp Fig. 6D). This shows that following A23187 treatment, Δ CDPK3 parasites
515 have altered FFA and TAG abundance necessary for lipid recycling and storage,
516 consistent with a speculated role for CDPK3 in metabolic regulation (Treeck *et al.*, 2014).
517 A full lipidomic analysis of individual phospholipid species including
518 lysophosphatidylinositol (LPI), lysophosphatidylcholine (LPC) phosphatidylinositol (PI),
519 phosphatidylserine (PS), phosphatidylthreonine (PT) phosphatidic acid (PA),
520 phosphatidylethanolamine (PE), FFAs, sphingomyelin (SM) and phosphatidylcholine
521 (PC) found no significant difference in any phospholipids between WT and Δ CDPK3
522 parasites under normal cell culture conditions (Supp Fig. 6E), showing that defects in lipid
523 signalling in Δ CDPK3 parasites can only be seen following A23187 calcium stimulus.

524 Having identified several cNMP-related proteins that were differentially phosphorylated in
525 Δ CDPK3 parasites in our phosphoproteome timecourse, we next wanted to determine
526 whether there are any perturbations to cNMP signalling upon deletion of CDPK3 by
527 measuring the changes in intracellular levels of cAMP and cGMP. To do this WT and
528 Δ CDPK3 extracellular parasites that were syringe lysed in BSA free Endo buffer (Endo *et al.*,
529 1987) were treated with vehicle (DMSO), 50 μ M BIPPO for 5 or 15 seconds or 8 μ M
530 A23187 for 5, 15, 30 or 60 seconds at 37°C. We found that basal levels of cGMP were
531 identical in WT and Δ CDPK3 parasites and treatment with the PDE inhibitor BIPPO
532 resulted in elevated cGMP levels in both parasite lines compared to baseline (Fig. 4D).
533 In contrast, and somewhat surprisingly, we found no changes in cGMP levels following
534 A23187 treatment over the course of 60 seconds. It is important to note that our
535 measurements of cGMP levels following treatment with A23187 differ from results shown
536 in Stewart *et al.* (2017). One key difference is that we kept tachyzoites in Endo buffer, a
537 potassium-rich buffer which mimics the intracellular environment. Stewart *et al.* used



538 **Figure 4. Disruption of CDPK3 leads to perturbations in lipid and cAMP signalling following A23187**
 539 **treatment** Pulse experiment of WT and Δ CDPK3 parasites treated with DMSO or 8 μ M A23187 for 0, 5,
 540 10, 30, 45 or 60 seconds analysing levels of **(A)** DAG and PLs **(B)** FFAs and **(C)** TAGs, with data expressed
 541 as a ratio of Δ CDPK3/WT levels. Data are represented as mean \pm s.d. (n=4). Significance was assessed
 542 using a one same t and Wilcoxon test. **, $P \leq 0.01$; *, $P \leq 0.05$. **(D)** Comparison of intracellular cGMP and
 543 **(E)** cAMP levels in WT and Δ CDPK3 tachyzoites following treatment with DMSO for 60 seconds; BIPPO
 544 for 5 or 15 seconds; or A23187 for 5, 15, 30 or 60 seconds. All samples were lysed in 0.1 M HCl to inactivate
 545 all PDEs, and extracts were analyzed by using commercial ELISA-based cGMP and cAMP detection
 546 assays. Data are represented as mean \pm s.d. (n=4). Two-way ANOVA with Sidak multiple comparisons.
 547 ****, $P \leq 0.0001$; ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$; n.s, not significant. **(F)** Quantification of natural
 548 egress of WT and Δ CDPK3 parasites following treatment with DMSO or 50 μ M H89 (2 hrs). Graph shows
 549 the remaining % of un-egressed vacuoles (relative to untreated). Data are represented as mean \pm s.d.
 550 (n=5). Significance was assessed using an unpaired two-tailed t-test. ***, $P \leq 0.001$; **, $P \leq 0.01$ **(G)** Egress
 551 assay of **(i)** DMSO- and **(ii)** H89-pre-treated WT and Δ CDPK3 parasites following treatment with 8 μ M
 552 A23187. Data are represented as mean \pm s.d. (n=5). Two-way ANOVA with Holm-Sidak *post hoc*
 553 comparison. ****, $P \leq 0.0001$; **, $P \leq 0.01$.

554 extracellular parasites in low potassium buffer, which may have profound effects on
555 downstream signalling pathways, given that those parasites have received signals that
556 they are outside of the host cell. Indeed, McCoy et al have shown that the A23187-
557 mediated egress defect of Δ CDPK3 parasites can be rescued if saponin permeabilised
558 infected host cells are incubated in extracellular buffer, but not when incubated in
559 intracellular buffer (McCoy *et al.*, 2012), suggesting that there are key signalling
560 differences between intra and extracellular parasites.

561 Basal cAMP levels, by contrast, were 8.3% higher in knockout parasites compared to WT.
562 Following treatment with A23187, we found that cAMP levels initially rose in both WT and
563 knockout parasites at 5 seconds post treatment, with a gradual decrease to below basal
564 levels at 60 seconds post treatment (Fig. 4E). We observed no immediate change in
565 cAMP levels following BIPPO treatment, suggesting that BIPPO does not inhibit cAMP-
566 specific PDEs in *T. gondii*. These findings point towards perturbations in cAMP signalling
567 in Δ CDPK3 parasites which have elevated basal levels that further increase upon A23187
568 treatment.

569 Since basal cAMP levels are elevated in Δ CDPK3 parasites compared to WT, we
570 reasoned that inhibition of cAMP signalling would overcome the A23187-mediated egress
571 delay observed in knockout parasites. To test this, we treated both WT and Δ CDPK3
572 parasites with the ATP-competitive PKAc inhibitor H89 (50 μ M). After 2 hours of
573 treatment, there was a significant amount of premature egress in both WT and Δ CDPK3
574 parasites (~45% & ~34%, respectively; Fig. 4F) consistent with previous reports that have
575 shown that the downregulation of cAMP signalling by genetic disruption of PKAc1
576 stimulates premature egress in *Toxoplasma* (Jia *et al.*, 2017; Uboldi *et al.*, 2018).
577 Intriguingly, when we investigated A23187-induced egress rates of the remaining
578 intracellular H89 pre-treated parasites, we found that the egress delay normally observed
579 in Δ CDPK3 parasites was largely rescued with H89 pre-treatment (Fig. 4Gi-ii). This finding
580 suggests that pharmacological inhibition of cAMP signalling is sufficient to partially
581 compensate for the deletion of CDPK3.

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585 **Cell biological and biochemical characterisation of PDE1, 2, 7 and 9.**

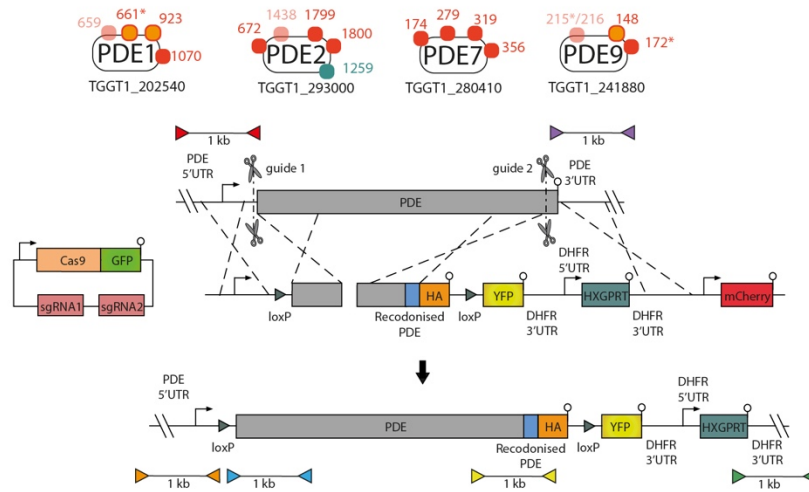
586 The preponderance of A23187-induced phosphorylation on several PDEs suggests that
587 they may play an important role in the cAMP- and cGMP-mediated signalling cascades
588 that lead to egress assuming that phosphorylation may directly, or indirectly control their
589 activity. Specifically, we predicted a cAMP-specific PDE would play an important role
590 given the rescue of the egress defect observed in Δ CDPK3 parasites by the PKA inhibitor
591 H89. As the specificity for the majority of *Toxoplasma* PDEs has not been experimentally
592 validated, we generated HA-tagged conditional knockouts (cKOs) of the 4 PDEs identified
593 as being phosphorylated following A23187 treatment in order to characterise them and
594 identify which are capable of hydrolysing cAMP (Fig. 5A). For each line, integration of
595 both repair templates was validated by PCR (Supp Fig. 7). Western blot analysis
596 confirmed that they migrate at their predicted sizes (Fig. 5B), and we found that each PDE
597 occupies a distinct cellular localisation (Fig. 5C), in agreement with a previous report (Vo
598 *et al.*, 2020; Moss *et al.*, 2021). To identify the substrate specificity of the PDEs, we
599 immunoprecipitated each via the HA-tag from parasite lysates and measured their
600 hydrolytic activity *in vitro*. We included the *P. falciparum* PDE β that was previously shown
601 to be dual-specific as a positive control (Flueck *et al.*, 2019). PDEs 1, 7 and 9 were able
602 to hydrolyse cGMP, while PDE2 is specific for cAMP (Fig. 5D). Only PfPDE β displayed
603 dual-hydrolytic activity in our hands.

604 To further confirm the hydrolytic specificity of the PDEs, we treated each of the samples
605 with BIPPO. While the specificity of BIPPO has not been experimentally validated in
606 *Toxoplasma*, cGMP-specific PDE1 and 7 were significantly inhibited by BIPPO, while the
607 cAMP-specific PDE2 was refractory to BIPPO inhibition (Fig. 5E). Interestingly, PDE9, a
608 cGMP-specific PDE appears less sensitive to BIPPO treatment. This is in agreement with
609 a previous study (Vo *et al.*, 2020), although in our hands PDE9 is cGMP-specific and not
610 dual-specific. Collectively these data show that BIPPO is a cGMP-specific inhibitor in
611 *Toxoplasma* and lends further support that PDE2 is a cAMP-specific PDE. Interestingly,
612 we found that both the cAMP- and cGMP-hydrolysis activities of PfPDE β are inhibited
613 with BIPPO. It will be interesting in the future to evaluate the structural differences
614 between the PDEs and the inhibitory potential of BIPPO.

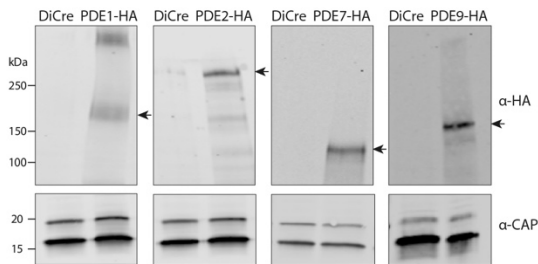
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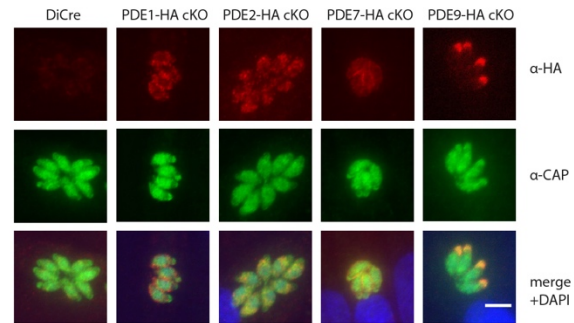
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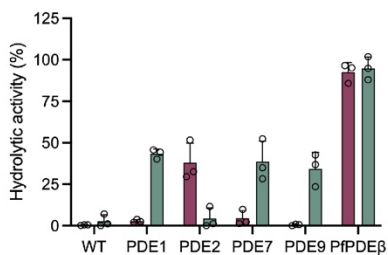
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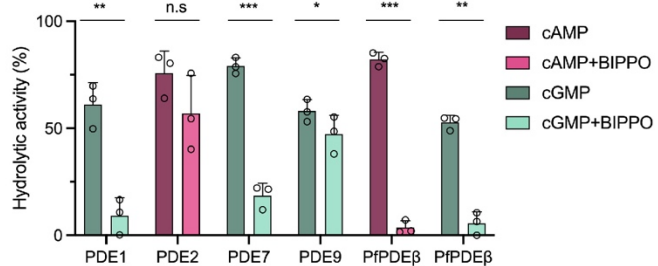
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D



E



617 **Figure 5. Candidate PDEs occupy distinct cellular localisations and are differentially inhibited by**
 618 **BIPPO (A)** Schematic representation of the PDEs identified in the timecourse phosphoproteome (see Fig.
 619 2E) and the strategy use to generate the conditional PDE knockout lines. CRISPR/Cas9 was used to
 620 generate two cuts in the gene and two separate repair templates were provided to integrate one loxP site
 621 (green triangle) upstream of the PDE gene, and another repair template to tag the PDE with a C-terminal
 622 HA epitope tag (orange) and introduce a second loxP site, a YFP sequence and the HXGPRT cassette.
 623 Scissors represent Cas9 cleavage sites and lollipops depict stop codons. Coloured triangles represent
 624 primer pairs used to detect WT, 5' integration and 3' integration loci for 5' loxP integration (red, orange and
 625 blue respectively) and 3' tagging (purple, yellow and green respectively). PCR results using these primer

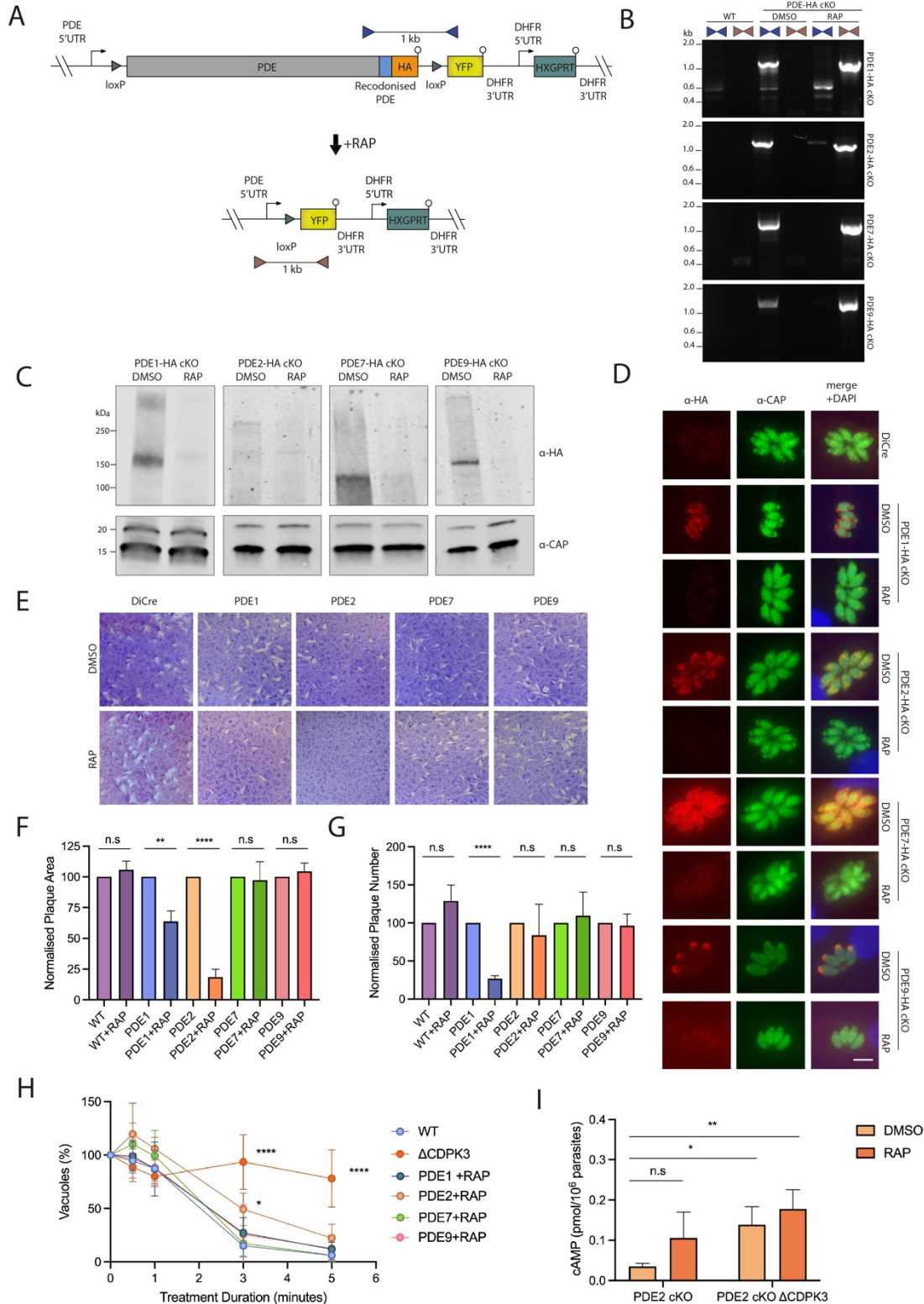
626 pairs are shown in Supp Fig. 7. **(B)** Western blot analysis of parental DiCre and HA-tagged PDE1, PDE2,
627 PDE7 and PDE9 cKO parasites probed with α -HA antibodies showing migration of the PDEs at their
628 expected molecular weights as depicted by arrows. A non-specific band >250 kDa is observed in the PDE1-
629 HA cKO line. Blots were probed with α -CAP antibodies as a loading control. **(C)** Immunofluorescence
630 analysis of DiCre and HA-tagged PDE1, PDE2, PDE7 and PDE9 cKO lines probing with α -HA (red) and α -
631 CAP (green) antibodies. Scale bar, 5 μ m. **(D)** Hydrolytic activity of immunoprecipitated HA-tagged PDE1,
632 PDE2, PDE7, PDE9 and the PfPDE β using either cAMP or cGMP as a substrate. Lysates from the WT
633 parental line were also included as a control. Data are represented as mean \pm s.d. (n=3). **(E)** Hydrolytic
634 activity of immunoprecipitated HA-tagged PDE1, PDE2, PDE7, PDE9 and PfPDE β after incubating with
635 DMSO (vehicle) or 25 μ M BIPPO. cAMP was used as a substrate for PDE2 and PDE β , while cGMP was
636 used as a substrate for PDE1, PDE7, PDE9 and PDE β . Data are represented as mean \pm s.d. (n=3).
637 Significance was assessed using a paired t-test. ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$; n.s. not significant.

638

639 **Functional assessment of the candidate PDEs reveals that PDEs 1 & 2 are**
640 **important but not essential during the lytic cycle while PDEs 7 & 9 are dispensable.**

641 We next wanted to establish which of the aforementioned PDEs were essential for lytic
642 growth. Addition of rapamycin (RAP) to the HA-tagged PDE cKO lines leads to excision
643 of the PDE gene of interest in the respective cKO lines (Fig. 6A). Despite near complete
644 excision in all lines as observed by PCR 24 hours post RAP treatment (Fig. 6B), it was
645 only until 3 days post-treatment that we saw complete protein depletion below detectable
646 levels (Fig. 6C-D). Therefore, all subsequent experiments were conducted with parasites
647 3 days post RAP treatment. To assess the impact of PDE disruption on parasite viability
648 and growth, we performed plaque assays using DMSO- and RAP-treated PDE cKO
649 parasites and measured the size and number of plaques after 5 days (Fig. 6E-G). All
650 knockout lines were able to form plaques, with deletion of PDE7 and PDE9 resulting in
651 no significant changes in plaque size or number. However, PDE1 and PDE2 formed much
652 smaller plaques, with a 37% and 81% reduction in plaque sizes respectively. Despite a
653 marked reduction in plaque size for PDE2 knockout parasites, there was no significant
654 change in the number of plaques compared to the DMSO-treated line. Deletion of PDE1
655 on the other hand resulted in a 4-fold reduction in the number of plaques formed. Overall,
656 these results suggest that both PDE1 & PDE2 are important but not essential for lytic
657 growth and that PDE1 may be important for egress and/or invasion due to the reduced
658 number of plaques formed following its disruption. Reassuringly, our observed

659



660 **Figure 6. PDE1 and PDE2 are important for lytic growth, with Δ PDE2 parasites displaying an A23187-**
 661 **mediated egress defect similar to Δ CDPK3 parasites (A) Schematic representation of PDE2 rapamycin**
 662 **mediated deletion of PDE cKO lines. Addition of rapamycin leads to excision of the entire gene, placing**

663 YFP under the control of the PDE promoter. Coloured triangles represent primer pairs used to detect
664 unexcised (blue) and excised (brown) loci. **(B)** PCR analysis of DMSO- and RAP-treated PDE cKO parasite
665 lines showing near complete excision for all lines. **(C)** Western blot analysis of PDE cKO lines showing near
666 complete loss of the PDEs at the protein level following treatment with RAP **(E)** Representative images of
667 plaque assays performed on DMSO- and RAP-treated WT and PDE cKO lines after a period of 5 days.
668 Assays were performed in biological triplicates. **(F)** Measurement of plaque area shown in Fig. 6E. Data are
669 represented as mean \pm s.d. (n=3). Significance was assessed using multiple t-tests. ****, $P \leq 0.0001$; **, P
670 ≤ 0.01 ; n.s, not significant. **(G)** Quantification of plaque numbers shown in Fig. 6E. Data are represented
671 as mean \pm s.d. (n=3). Significance was assessed using multiple t-tests. ****, $P \leq 0.0001$; n.s, not significant.
672 **(H)** Egress assay of GFP-T2A-jRCaMP1b expressing WT, Δ CDPK3 and RAP-treated PDE cKO parasites
673 following treatment with 8 μ M A23187. Data are represented as mean \pm s.d. (n=3). Two-way ANOVA. ****,
674 $P \leq 0.0001$; *, $P \leq 0.05$. **(I)** Quantification of intracellular cAMP levels of PDE2 cKO and PDE2 cKO Δ CDPK3
675 parasites treated with either DMSO or RAP. Data are represented as mean \pm s.d. (n=4). Two-way ANOVA
676 with Turkey's multiple comparisons. **, $P \leq 0.01$; *, $P \leq 0.05$.

677

678 phenotypes for PDE 1, 2, 7 and 9 KOs are in agreement with a recent study which
679 knocked down all predicted *Toxoplasma* PDEs (Moss *et al.*, 2021).

680

681 We next wanted to determine whether disruption of any of the PDEs would lead to an
682 A23187-mediated egress delay, similar to Δ CDPK3 parasites. We reasoned that if one of
683 the PDEs is involved in the A23187-mediated feedback loop, then disruption of this PDE
684 would mimic, at least partially, the A23187-mediated egress delay observed in Δ CDPK3
685 parasites. Using a medium-throughput plate-based egress assay we found that only
686 deletion of PDE2 showed a modest egress defect (Fig. 6H, Supp Fig. 8). This suggests
687 an important, but non-essential role for PDE2 in cAMP signalling. Since the egress defect
688 observed upon deletion of PDE2 did not reach the severity of the egress defect observed
689 in Δ CDPK3 parasites, it is likely that other PDEs, and/or cyclases are involved in the
690 dysregulation of cAMP levels found in our study, some of which may have been missed
691 in our phosphoproteome or may not be regulated by phosphorylation. To test this, we
692 generated a Δ CDPK3 KO in the PDE2 cKO line using a similar approach to the one used
693 in Fig. 2A, however we substituted the HXGPRT cassette with a DHFR-TS selection
694 cassette. Upon deletion of PDE2, we observed a further increase of cAMP levels
695 compared to either single gene deletion (Fig. 6I) suggesting that CDPK3 is regulating
696 another unknown cAMP-specific PDE, or an adenylyl cyclase.

697 Discussion

698 In this study we have aimed to unravel the complexity of the signalling pathways that
699 govern the control of host cell egress of *Toxoplasma* from its host cell. Several signalling
700 components conserved in higher eukaryotes have previously been identified, and their
701 connectivity, to some extent described. However, the published data is not currently
702 supported by a model that fits most experimental results. A deeper understanding of how
703 the signalling pathways are interconnected is essential for our understanding of the
704 regulation of host cell egress and of the integration of environmental or endogenous
705 signals in general. This is important since we do not fully understand how *Toxoplasma*
706 parasites sense and react to their environment. Furthermore, the plethora of calcium-
707 dependent kinases and phosphodiesterases imply a highly complex and sensitive
708 interplay of signalling pathways to finetune cellular responses to inputs. Our results are
709 likely of importance beyond *Toxoplasma gondii* research: the plant-like calcium-
710 dependent kinases (CDPKs) are conserved in *Plasmodium* species where it has been
711 shown that PDE inhibitors can overcome disruption of CDPKs (Absalon *et al.*, 2018).

712 Our data quite clearly support previous data on the importance of cNMP, Ca²⁺ and lipid
713 signalling on parasite exit from the host cell. They furthermore add an important new layer
714 of information: CDPK3, and potentially other kinases that depend on CDPK3 function, are
715 part of a feedback loop that enables rapid signalling. This feedback loop does not control
716 release of Ca²⁺ from internal stores, since McCoy *et al* (2012) have shown that disruption
717 of CDPK3 does not lead to a delay in Ca²⁺ release. It also does not appear to act through
718 elevation of cGMP levels as we show both that treatment with ionophore does not lead to
719 a measurable increase in cGMP levels and that cGMP levels are not dependent on
720 CDPK3.

721 More likely, and in keeping with our cAMP measurements, CDPK3 directly or indirectly
722 downregulates levels of cAMP. This, in turn, alters activity of the cAMP-dependent protein
723 kinase, PKAc. It is interesting to note that Jia and colleagues found a clear dependency
724 on PKG for parasites to egress upon PKAc depletion, but they were equally unable to
725 reliably ascertain cGMP accumulation in intracellular parasites (Jia *et al.*, 2017). While it
726 is possible that our collective inability to observe elevated cGMP levels is explained by
727 the sensitivity limits of the assay employed, it is similarly possible that cAMP-mediated
728 signalling is exerting its effects on the PKG signalling pathway in a cGMP-independent

729 manner. While no such mechanism has been described, it is possible that
730 phosphorylation of PKG may lead to changes in its affinity for cGMP or it may regulate
731 the activity of the kinase itself. Further work is needed to clarify the role of cGMP levels
732 in these conditions. We also identified dysregulation of DAG and phospholipid signalling
733 in Δ CDPK3 parasites following A23187 treatment, which could be contributing to the
734 delayed egress phenotype observed in the KO parasites. Having identified CDPK3-
735 dependent phosphorylation sites on both DGK1 and PI-PLC in our timecourse
736 phosphoproteome, it is possible that these perturbations are being directly mediated by
737 CDPK3. Alternatively, and as outlined above, any changes in cGMP levels or PKG activity
738 in Δ CDPK3 parasites could also lead to the dysregulation of phospholipid signalling we
739 observed.

740 We identify PDE2 as one contributor of cAMP control, however, through double gene
741 deletions of PDE2 and CDPK3, we show that other cAMP signalling components likely
742 contribute to a further cAMP imbalance. This could be either via as yet unidentified PDEs
743 with cAMP specificity or, more likely, an adenylate cyclase. In support of the latter, we
744 identified several CDPK3-dependent phosphorylation sites on an AC β following A23187
745 treatment. We also found that deletion of PDE2 alone leads to a modest egress
746 phenotype that does not reach Δ CDPK3 levels. This is not surprising: our data, and
747 previous studies have identified many CDPK3-dependent targets (Treeck *et al.*, 2014;
748 Gaji *et al.*, 2015; McCoy *et al.*, 2017; Wallbank *et al.*, 2019), and the CDPK3-mediated
749 phenotype is likely caused by a combination of the phosphorylation events identified here.
750 However, we did not detect any CDPK3-dependent phosphosites on PDE2, so a direct
751 link between CDPK3 and PDE2 is currently missing. However, it is possible that CDPK3-
752 dependent phosphorylation sites on PDE2 were not detected in our mass-spectrometry
753 experiments for technical reasons, or that PDE2 is indirectly regulated by CDPK3. It has,
754 for instance, been reported that CDPK3 promotes egress by phosphorylating the egress
755 suppressor SCE1 (McCoy *et al.*, 2017). Deletion of SCE1 in Δ CDPK3 parasites largely
756 rescues several CDPK3-dependent phosphosites, suggesting that another SCE1-
757 suppressed kinase is able to partially compensate for loss of CDPK3, and likely amplifies
758 regular CDPK3-mediated egress under normal conditions.

759 The research community is also continuously identifying novel components involved in
760 signalling which, once identified, could shed light on how different pathways are
761 interconnected. For example, a recent report has identified SPARK, a novel kinase that

762 appears to mediate Ca²⁺ release in a PKG-dependent manner and can be largely
763 bypassed via treatment with A23187 (Smith *et al.*, 2021). While A23187 treatment
764 appears to restore absolute levels of Ca²⁺ release in SPARK depleted parasites, the rate
765 of both calcium release and egress remains partially delayed. These findings suggest that
766 PKG-regulated SPARK still contributes, to some degree, to A23187-mediated egress.
767 This observation is in keeping with our proposition that A23187 signalling feeds back into
768 the PKG signalling pathway.

769 While our study provides strong evidence for a CDPK3-mediated feedback loop to control
770 rapid egress and nearly overlapping signalling pathways at peak calcium flux, we cannot
771 draw conclusions about the signalling events at the onset of calcium release.
772 Nevertheless, our timecourse data identifies rapid CDPK3-dependent differences on
773 proteins involved in cNTD signalling as early as 15 seconds post-induction, suggesting
774 that it plays a role at the very onset of the signalling cascades, well before calcium release
775 peaks. We are still far from identifying all players in the signalling cascades that lead to
776 egress from the host cell. Arrayed CRISPR screens targeting of the *Toxoplasma* kinome
777 in Δ CDPK3 parasites (Young *et al.*, 2019; Smith *et al.*, 2021) will likely shed further light
778 on these signalling pathways.

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790 **Materials and Methods**

791 **Parasite culture and transfection**

792 *T. gondii* tachyzoite RH strains lacking KU80 ($\Delta ku80$) and HXGPRT ($\Delta hxgprrt$) (Fox *et al.*,
793 2009; Huynh and Carruthers, 2009) were cultured in a confluent monolayer of human
794 foreskin fibroblasts (HFFs) maintained in Dulbecco's Modified Eagle medium
795 GlutaMAX™ (DMEM+ GlutaMAX™, Gibco) supplemented with 10% foetal bovine serum
796 (FBS), at 37°C and 5% CO₂.

797

798 **Plasmid and parasite strain generation**

799 Primers used throughout this study are listed in Table S6. The calcium sensor construct
800 was generated as recently described (Alves *et al.*, 2021). The construct was linearised
801 using NaeI and transfected into RH $\Delta ku80\Delta hxgprrt$ parasites as described previously
802 (Soldati and Boothroyd, 1993) to generate the GFP-T2A-jRCaMP1b calcium sensor line.
803 Transgenic parasites were subjected to 5'-fluo-2'-deoxyuridine (FUDR) selection (5 μ M)
804 24 hrs after transfection. To generate the GFP-T2A-jRCaMP1b Δ CDPK3 line, the
805 *HXGPRT* cassette (flanked by 5' and 3' DHFR UTR sequences) was PCR amplified from
806 *pGRA-HA_HXGPRT* (Coppens *et al.*, 2006) using primers 1/2 (introducing 40bp CDPK3
807 homology regions to the amplified fragment) and co-transfected into RH $\Delta ku80\Delta hxgprrt$
808 with pSag1::Cas9-U6::dbl-sgCDPK3. The pSag1::Cas9-U6::dbl-sgCDPK3 vector was
809 generated by inverse PCR amplification of the pSag1::Cas9-U6 (Behnke *et al.*, 2014)
810 vector using primer pairs 3/4 and 3/5 to generate intermediate constructs pSag1::Cas9-
811 U6::sg1CDPK3 (comprising sgRNA1) and pSag1::Cas9-U6::sg2CDPK3 (comprising
812 sgRNA2) respectively. Following circularization of both intermediate constructs using
813 KLD reaction buffer (NEB), a region comprising sgRNA1 was PCR amplified with primers
814 6 and 7 from pSag1::Cas9-U6::sg1CDPK3 and Gibson assembled into Kpn1/XhoI
815 linearised pSag1::Cas9-U6:: sg2CDPK3 to generate the double sgRNA plasmid
816 pSag1::Cas9-U6::dbl-sgCDPK3. Recombinant parasites were selected 24 hrs post
817 transfection by addition of mycophenolic acid (MPA; 25 μ g/mL) and xanthine (XAN; 50
818 μ g/mL) to culture medium. Integration of the HXGPRT cassette at the CDPK3 locus was
819 confirmed using primer pairs 8/9 and 10/11 to confirm 5' and 3' integration respectively.
820 Absence of the endogenous CDPK3 locus was confirmed using primers 12/13.

821

822 To generate the PDE1, PDE2, PDE7 and PDE9 HA-tagged conditional knockout lines,
823 two separate repair templates were generated for each gene; one which would integrate
824 a loxP site 100 bp upstream of the start codon, and one that would introduce a C-terminal
825 HA epitope tag along with a second loxP site and an HXGPRT cassette downstream of
826 the gene.

827 To generate the pUC19_PDE1_5'loxP repair construct, a 1 kb 5' homology region and a
828 1 kb 3' homology region were PCR amplified from genomic DNA using primers 14/15 and
829 16/17 respectively, with the primers designed to introduce a loxP site between the 5' and
830 3' homology regions. The fragments were then Gibson cloned into the BamHI and EcoRI
831 sites of the pUC19 vector. To generate the pG140_PDE1-HA_3'loxP_HXGPRT plasmid,
832 a 1 kb 5' homology region was amplified from genomic DNA using primers 18/19 and the
833 HA tag was amplified from an unpublished in-house plasmid using primers 20/21. These
834 fragments were Gibson cloned into the HindIII & PacI sites of the pG140 plasmid to
835 generate an intermediate plasmid. A 1 kb 3' homology region was PCR amplified from
836 genomic DNA using primers 22/23, while an mCherry coding sequence flanked by Gra
837 gene UTRs was amplified from pTKO2C (Caffaro *et al.*, 2013) using primers 24/25. These
838 fragments were subsequently Gibson cloned into the SacI sites of the intermediate
839 plasmid to generate pG140_PDE1-HA_3'loxP_HXGPRT.

840 The pSag1::Cas9-U6::dbl-sgPDE1 vector was generated by inverse PCR amplification of
841 the pSag1::Cas9-U6 (Behnke *et al.*, 2014) vector using primer pairs 3/26 and 3/27 to
842 generate intermediate constructs pSag1::Cas9-U6::sg1PDE1 (comprising sgRNA1) and
843 pSag1::Cas9-U6::sg2PDE1 (comprising sgRNA2) respectively. Following circularization
844 of both intermediate constructs using KLD reaction buffer (NEB), a region comprising
845 sgRNA1 was PCR amplified with primers 6 and 7 from pSag1::Cas9-U6::sg1PDE1 and
846 Gibson assembled into KpnI/XhoI linearised pSag1::Cas9-U6:: sg2PDE1 to generate the
847 double sgRNA plasmid pSag1::Cas9-U6::dbl-sgPDE1.

848 After linearising pUC19_PDE1_5'loxP with HindIII & EcoRI and pG140_PDE1-
849 HA_3'loxP_HXGPRT with HindIII & SapI, the two repair templates were co-transfected
850 with pSag1::Cas9-U6::dbl-sgPDE1 into the RH DiCre $\Delta ku80\Delta hxgprt$ line (Hunt *et al.*,
851 2019). Recombinant parasites were selected 24 hrs post transfection by addition of
852 mycophenolic acid (MPA; 25 μ g/mL) and xanthine (XAN; 50 μ g/mL) to culture medium.

853 The same cloning strategy was used for all other PDE cKO lines with the primer pairs
854 used in each step listed in table S1.

855 To generate the PDE2-HA-cKO Δ CDPK3 line, the *DHFR-TS* cassette (flanked by GRA1 5'
856 and GRA2 3' UTR sequences) was PCR amplified from an unpublished in-house plasmid
857 using primers 28/29 (introducing 40bp CDPK3 homology regions to the amplified
858 fragment) and co-transfected into RH $\Delta ku80\Delta hxxgprt$ with pSag1::Cas9-U6::dbl-
859 sgCDPK3. Recombinant parasites were selected 24 hrs post transfection by addition of
860 pyrimethamine (1 μ M) to culture medium.

861 **Egress assay**

862 Fresh tachyzoites were harvested and seeded onto confluent HFF monolayers in black
863 96-well imaging μ -plates (Ibidi) at an MOI of 0.5. After 28 hours of growth, egress assays
864 were performed in triplicate at 37 °C in Ringers buffer (155 mM NaCl, 3 mM KCl, 2 mM
865 CaCl₂, 1 mM 556 MgCl₂, 3 mM NaH₂PO₄, 10 mM HEPES, 10 mM glucose). The
866 parasites were incubated with 8 μ M Ca²⁺ ionophore A23187 (BioVision) or 50 μ M BIPPO
867 (generated in-house) for variable timings. Wells were subsequently fixed by adding 16%
868 FA to a final concentration of 3% for 15 mins. Wells were washed with PBS and stained
869 with 5 μ g/ml DAPI. Automated image acquisition of 25 fields per well was performed on
870 a Cellomics 561 Array Scan VTI HCS reader (Thermo Scientific) using a 20 \times objective.
871 Image analysis was performed using the Compartmental Analysis BioApplication on HCS
872 Studio (Thermo Scientific).

873

874 **Live imaging of calcium sensor line**

875 Fresh tachyzoites were harvested and seeded (at an MOI of 0.5) onto confluent HFF
876 cells grown on IBIDI tissue culture treated 8 well chamber slides and allowed to grow for
877 28 hrs in DMEM + 10%FBS. Prior to imaging, wells were washed once with PBS, and
878 supplemented with 100 μ l Ringer's media. Wells were treated for 5 mins at 37°C with 100
879 μ l 2 μ g/ml Cytochalasin D in Ringer's buffer (final concentration 1 μ g/ml) to prevent
880 egress. Imaging was performed on the Nikon Eclipse Ti-U inverted fluorescent
881 microscope, 60x/1.4 NA Oil immersion objective, in environmental chamber (OKOLAB)
882 with temperature maintained at 37°C. Image capture was managed by Nikon NIS-

883 Elements software with acquisition 1/s for 70s. At 15s following image acquisition, 100 μ l
884 of A23187 (24 μ M) or BIPPO (150 μ M) in Ringer's buffer was added by pipette (to final
885 concentrations of 8 μ M and 50 μ M respectively). ≥ 10 vacuoles across ≥ 10 wells were
886 imaged across ≥ 7 days for each condition. Image analysis was performed using Nikon
887 NIS-Elements analysis software. jRCaMP1b and GFP signals at 0s were set to 0 (zero)
888 and 1 respectively. jRCaMP1b/GFP was used as a readout for ΔCa^{2+} .

889

890 **Phosphoproteome analysis**

891 *Lysis and protein digestion*

892 Parasites were seeded onto HFF monolayers in 15cm culture dishes at an MOI of 5. 24
893 hours post-inoculation, plates were washed once with PBS and treated with 50 μ M BIPPO
894 (15s) or 8 μ M A23187 (variable timings depending on experiment) in Ringer's buffer.
895 Following the appropriate treatment duration, treatments were rapidly removed and plates
896 placed on a supercooled salt water ice bath to inhibit further signalling. Lysis was
897 performed by scraping cells in ice cold 8 M urea, 75 mM NaCl, 50 mM Tris, pH 8.2,
898 supplemented with protease (complete mini, Roche) and phosphatase (PhosSTOP,
899 Roche) inhibitors. Lysis was followed by sonication to reduce sample viscosity (30% duty
900 cycle, 3 x 30 seconds bursts, on ice). Protein concentration was measured using a BCA
901 protein assay kit (Pierce). Lysates (1mg each) were subsequently reduced with 5 mM
902 DTT for 30 minutes at 56 $^{\circ}$ C and alkylated in the dark with 14 mM iodoacetamide for 30
903 minutes at RT. Following iodoacetamide quenching with 5 mM DTT for 15 minutes in the
904 dark, lysates were diluted with 50 mM ammonium bicarbonate to < 4 M urea, and digested
905 with LysC (Promega) for 2-3 hours at 37 $^{\circ}$ C. Lysates were further diluted with 50 mM
906 ammonium bicarbonate to < 2 M urea and digested with trypsin (Promega) overnight at
907 37 $^{\circ}$ C. After digestion, samples were acidified with trifluoroacetic acid (TFA) (Thermo
908 Fisher Scientific) to a final concentration of 1% (v/v). All insoluble material was removed
909 by centrifugation and the supernatant was desalted on Sep-Pak C₁₈ cartridges (Waters).

910

911 *TMT labelling*

912 Samples were dissolved at 1 mg/ml in 50 mM Na-Hepes, pH 8.5 and 30% acetonitrile
913 (v/v) and labelled with respective TMT reagents (Thermo Fisher Scientific, 2.4 mg
914 reagent/1 mg sample) for 1 hour at RT. Labelling was then quenched with 0.3%
915 hydroxylamine for 15 minutes at RT and samples acidified (pH~2) with formic acid. After
916 verification of labelling efficiency via mass spectrometry, the lysates were mixed in a 1:1
917 ratio, vacuum dried and desalted on Sep-Pak C₁₈ cartridges.

918

919 *Phosphopeptide enrichment*

920 Desalted and vacuum dried samples were solubilised in 1 ml of loading buffer (80%
921 acetonitrile, 5% TFA, 1 M glycolic acid) and mixed with 5 mg of TiO₂ beads (Titansphere,
922 5 µm GL Sciences Japan). Samples were incubated for 10 minutes with agitation,
923 followed by a 1 minute 2000 × g spin to pellet the beads. The supernatant was removed
924 and used for a second round of enrichment as explained below. Beads were washed with
925 150 µl loading buffer followed by two additional washes, the first with 150 µl 80%
926 acetonitrile, 1% TFA and the second with 150 µl 10% acetonitrile, 0.2% TFA. After each
927 wash, beads were pelleted by centrifugation (1 minute at 2000 × g) and the supernatant
928 discarded. Beads were dried in a vacuum centrifuge for 30 minutes followed by two elution
929 steps at high pH. For the first elution step, beads were mixed with 100 µl of 1% ammonium
930 hydroxide (v/v) and for the second elution step with 100 µl of 5% ammonium hydroxide
931 (v/v). Each time beads were incubated for 10 minutes with agitation and pelleted at 2000
932 × g for 1 minute. The two elutions were removed following each spin, and subsequently
933 pooled together before undergoing vacuum drying. The supernatant from the TiO₂
934 enrichment was desalted on Sep-Pak C₁₈ and the High Select Fe-NTA phosphopeptide
935 enrichment kit (Thermo Fisher Scientific) was used according to manufacturer's
936 instructions for a second round of enrichment.

937

938 *Sample fractionation and desalting*

939 Combined TiO₂ and Fe-NTA phosphopeptide eluates were fractionated using the Pierce
940 High pH Reversed-Phase kit (Thermo Fisher Scientific) according to manufacturer's
941 instructions. Resulting fractions were taken to dryness by vacuum centrifugation and
942 further desalted on a stage tip using Empore C18 discs (3M). Briefly, each stage tip was

943 packed with one C18 disc, conditioned with 100 µl of 100% methanol, followed by 200 µl
944 of 1% TFA. The sample was loaded in 100 µl of 1% TFA, washed 3 times with 200 µl of
945 1% TFA and eluted with 50 µl of 50% acetonitrile, 5% TFA. The desalted peptides were
946 vacuum dried in preparation for LC-MS/MS analysis.

947

948 *LC-MS/MS*

949 Samples were resuspended in 0.1% TFA and loaded on a 50 cm Easy Spray PepMap
950 column (75 µm inner diameter, 2 µm particle size, Thermo Fisher Scientific) equipped
951 with an integrated electrospray emitter. Reverse phase chromatography was performed
952 using the RSLC nano U3000 (Thermo Fisher Scientific) with a binary buffer system
953 (solvent A: 0.1% formic acid, 5% DMSO; solvent B: 80% acetonitrile, 0.1% formic acid,
954 5% DMSO) at a flow rate of 250 nl/minute. The samples were run on a linear gradient of
955 5-60% B in 150 minutes with a total run time of 180 minutes including column conditioning.
956 The nanoLC was coupled to an Orbitrap Fusion Lumos mass spectrometer using an
957 EasySpray nano source (Thermo Fisher Scientific). The Orbitrap Fusion Lumos was
958 operated in data-dependent mode using two acquisition methods. For the MS2 method,
959 HCD MS/MS scans (R=50,000) were acquired after an MS1 survey scan (R=120, 000)
960 using MS1 target of 4E5 ions, and MS2 target of 2E5 ions. The number of precursor ions
961 selected for fragmentation was determined by the “Top Speed” acquisition algorithm with
962 a cycle time of 3 seconds, and a dynamic exclusion of 60 seconds. The maximum ion
963 injection time utilised for MS2 scans was 86 ms and the HCD collision energy was set at
964 38. For the MS3 method, CID MS/MS scans (R=30,000) were acquired after an MS1
965 survey scan with parameters as above. The MS2 ion target was set at 5E4 with multistage
966 activation of the neutral loss (H3PO4) enabled. The maximum ion injection time utilised
967 for MS2 scans was 80 ms and the CID collision energy was set at 35. HCD MS3 scan
968 (R=60,000) was performed with synchronous precursor selection enabled to include up
969 to 5 MS2 fragment ions. The ion target was 1E5, maximum ion injection time was 105 ms
970 and the HCD collision energy was set at 65. Acquired raw data files were processed with
971 MaxQuant (Cox and Mann, 2008; Cox *et al.*, 2011) (version 1.5.2.8) and peptides were
972 identified from the MS/MS spectra searched against *Toxoplasma gondii* (combined TG1,
973 ME48 and VEG proteomes, ToxoDB) and *Homo sapiens* (UniProt, 2018) proteomes
974 using Andromeda (CITE Cox et al. 2011) search engine. TMT based experiments in

975 MaxQuant were performed using the ‘reporter ion MS2 or MS3’ built-in quantification
976 algorithm with reporter mass tolerance set to 0.003 Da. Cysteine carbamidomethylation
977 was selected as a fixed modification. Methionine oxidation, acetylation of protein N-
978 terminus, deamidation (NQ) and phosphorylation (S, T, Y) were selected as variable
979 modifications. The enzyme specificity was set to trypsin with a maximum of 2 missed
980 cleavages. The precursor mass tolerance was set to 20 ppm for the first search (used for
981 mass re-calibration) and to 4.5 ppm for the main search. The datasets were filtered on
982 posterior error probability to achieve a 1% false discovery rate on protein, peptide and
983 site level. ‘Match between runs’ option was enabled for fractionated samples (time
984 window 0.7 min) and “Unique and razor peptides” mode was selected to allow
985 identification and quantification of proteins in groups (razor peptides are uniquely
986 assigned to protein groups and not to individual proteins). All mass spectrometry
987 acquisition files and MaxQuant processing files have been deposited to the
988 ProteomeXchange Consortium via the PRIDE (Perez-Riverol *et al.*, 2019) partner
989 repository (currently awaiting dataset identifier).

990

991 **Phosphoproteome data processing**

992 *A23187/BIPPO analysis (set1 and set2)*

993 The data were analyzed using Perseus (Tyanova *et al.*, 2016) (version 1.5.0.9) and
994 Microsoft Office Excel 2016. Briefly, the data were filtered to remove common
995 contaminants, IDs originating from reverse decoy sequences and sites originating from
996 the host (human) proteome. Individual TMT reporter intensities (MS2-based acquisition)
997 and total intensity were log₂ and log₁₀ transformed, respectively. Log₂ reporter
998 intensities for each sample were subsequently normalised (centered) by subtracting the
999 median log₂ reporter intensity value calculated for all non-phosphorylated peptides
1000 detected in the same sample. Data were then filtered by 1 valid value to retain only the
1001 quantified phosphosites and log₂ fold changes in reporter intensity between conditions
1002 were calculated. Differentially regulated (DR) phosphorylation sites were identified by
1003 calculating the median absolute deviation (MAD) for the log₂FC in each comparative
1004 dataset. The largest of these was used to set an outlier threshold of 3x MAD (rounded to
1005 the nearest tenth; log₂FC>0.5 for up-regulated sites and log₂FC<-0.5 for down-regulated
1006 sites) and applied across all datasets.

1007

1008 *A23187 timecourse analysis*

1009 The data were analyzed using Perseus (Tyanova *et al.*, 2016) (version 1.5.0.9) and
1010 Microsoft Office Excel 2016. TMT reporter intensities obtained via MS2 and MS3-based
1011 acquisition were filtered to remove common contaminants, IDs originating from reverse
1012 decoy sequences and sites originating from the host (human) proteome. MS2/MS3
1013 reporter intensities and the total intensity were then log2 and log10 transformed,
1014 respectively. Log2 reporter intensities for each sample were subsequently normalised
1015 (centered) by subtracting the median log2 reporter intensity value calculated for all non-
1016 phosphorylated peptides detected in the same sample. Data were then filtered by 1 valid
1017 value to retain only the quantified phosphosites. Finally, log2 fold changes were
1018 calculated relative to a 0s (DMSO) control, separately for the MS2 and MS3 data, to obtain
1019 per site response to ionophore treatment. For downstream analysis responses obtained
1020 by the MS2 and MS3 based quantification were averaged.

1021 DR thresholds were determined in a timepoint-specific manner by calculating the log2FC
1022 MADs scores across each WT timepoint (15s, 30s and 60s), and setting 3x MAD outlier
1023 thresholds for each (rounded to the nearest tenth: 15s log2FC<-0.5 for DR^{DOWN} and 15s
1024 log2FC>0.5 for DR^{UP}; 30s log2FC<-0.6 for DR^{DOWN} and 30s log2FC>0.6 for DR^{UP}; 60s
1025 log2FC<-0.9 for DR^{DOWN} and 60s log2FC>0.9 for DR^{UP}). Phosphorylation sites were
1026 considered to be differentially regulated if at any given timepoint their log2FC surpassed
1027 these thresholds.

1028 CDPK3 dependency, was determined for each phosphorylation site by calculating the
1029 log2 ratios of A23187-treated WT and Δ CDPK3 parasites (Δ CDPK3^{A23187}/WT^{A23187}) for
1030 each timepoint. The resulting ratios were used to calculate the MAD at each timepoint,
1031 and the most stringent score was used to set 3X MAD outlier thresholds (rounded to the
1032 nearest tenth: log2FC<-0.6 for CDPK3 dependency in DR^{UP} sites and log2FC>0.6 for
1033 CDPK3 dependency in DR^{DOWN} sites). A DR site was considered to be CDPK3-dependent
1034 if, at any given timepoint, it simultaneously passed the appropriate DR and CDPK3
1035 dependency thresholds.

1036

1037

1038 *Clustering*

1039 Phosphosite log₂FC values from the timecourse experiment were clustered using a
1040 Gaussian finite mixture model-based method (Scrucca *et al.*, 2016) log₂FC values from
1041 both the WT and Δ CDPK3 samples were combined, thus the clustering was performed
1042 on six dimensions: WT 15s, 30s and 60s and Δ CDPK3 15s, 30s and 60s. The method
1043 was restricted to spherical models with equal or unequal volumes (models “EII” and “VII”)
1044 and models with up to 11 clusters were tested. The clustering method was applied
1045 separately to the sites designated as up-regulated, down-regulated, and CDPK3-
1046 dependent.

1047 *Gene Ontology Enrichment*

1048 Each cluster was tested for an enrichment in Gene Ontology annotations using goatools
1049 version 0.8.12 (Klopfenstein *et al.*, 2018). The ontology was downloaded from
1050 <https://geneontology.org> on 2019 April 17. All tests were performed using Fisher’s Exact
1051 Test and p-values were adjusted for false discovery rate.

1052 *Motif Analysis*

1053 The sequence surrounding each DR timecourse phosphosite, +/-7 residues, was
1054 subjected to a motif analysis using rmotifx 1.0 (motif enrichment; Wagih, Reimand, &
1055 Bader, 2015) and WebLogo 3.7.1 (Crooks *et al.*, 2004). The analysis was performed for
1056 each cluster as well as for the combined sets of phosphosites designated as up-regulated,
1057 down-regulated and CDPK3-dependent.

1058

1059 **Measurement of cyclic nucleotide levels in extracellular parasites**

1060 Parasites were seeded onto HFF monolayers in T175 flasks. After 24-30 hours, flasks
1061 were washed once with PBS, then scraped and syringe lysed in endo buffer (44.7 mM
1062 K₂SO₄, 10 mM MgSO₄, 106 mM sucrose, 5 mM glucose, 20 mM Tris–H₂SO₄, pH 8.2)/
1063 After counting, the parasites were aliquoted into eppendorfs and treated with 50 μ M
1064 BIPPO, 8 μ M A23187 or the equivalent volume of DMSO for the variable timings while
1065 maintained at 37°C. The samples were then lysed by adding two volumes of 0.1 M HCl
1066 and left on ice for 10 minutes with intermittent vortexing. The levels of cAMP and cGMP
1067 levels in the samples was determined using the enzyme-linked immunosorbent assay

1068 (ELISA)-based high-sensitivity direct cAMP and cGMP colorimetric assay kits (Enzo Life
1069 Sciences). Samples and standards were acetylated in order to improve sensitivity. All
1070 samples and standards were set up in duplicate. Absorbance was measured at 405nm
1071 using a FLUOstar Omega plate reader. The detection ranges were 0.078 to 20 pmol/ml
1072 and 0.08 to 50 pmol/ml for the cAMP and cGMP assays, respectively.

1073

1074 **DAG & Global Lipid Metabolomics**

1075 For the DAG and lipid kinetics experiments, parasites were grown for 3 days in DMEM
1076 containing 10% FBS and syringe released by passing through a 23-gauge needle. Lysed
1077 parasites were filtered through a 10 µm polycarbonate filter, counted and then pelleted
1078 (1,800 rpm, 10 min). After washing pellets with DMEM containing 10 mM HEPES,
1079 parasites were aliquoted to achieve 1×10^8 cells per tube and maintained at room
1080 temperature. Parasites were shifted to 37°C and allowed to equilibrate for 60 seconds
1081 before addition of pre-warmed DMEM containing either DMSO or 8 µM A23187. Parasites
1082 were allowed to incubate for the desired time and quenched immediately on dry
1083 ice/ethanol for 5 seconds then left on ice. Cells were pelleted (8,000 rpm, 2 min) and
1084 washed with 3x with 1 ml of ice-cold PBS. Cells were pelleted, supernatant aspirated and
1085 pellets stored at -80°C until required.

1086

1087 Alternatively for global lipidomics, scraped tachyzoite cultures without stimulus were
1088 rapidly quenched in a dry ice ethanol bath and placed on ice. Tachyzoites were then
1089 needle passed and filtered on ice and pelleted (1800 rpm, 10 minutes) at 4°C. Cells were
1090 washed twice with ice cold 1x PBS and then pellets were stored at -80 until required.

1091

1092 **Lipid analysis**

1093 Total lipids and internal standards were extracted using chloroform:methanol, 1:2 (v/v)
1094 and chloroform:methanol, 2:1 (v/v) in the presence of 0.1 M HCl with periodic sonication.
1095 The organic phase was dried under N₂ gas and dissolved in 1-butanol. For DAG, total
1096 lipid was then separated by 1D-HPTLC using hexane : diethyl-ether : formic Acid, 40:10:1.
1097 For global phospholipid analysis including PA, total lipid was spiked with 1 µg

1098 PA(C17:0/C17:0) (Avanti Polar lipids) and then separated by 2D-HPTLC using
1099 chloroform/methanol/28% NH₄OH, 60:35:8 (v/v) as the 1st dimension solvent system and
1100 chloroform/acetone/methanol/acetic acid/water, 50:20:10:13:5 (v/v) as the 2nd dimension
1101 solvent system (Amiar *et al.*, 2016). All lipid spots including PA were visualised with
1102 primulin and scraped. Lipids and additional standards were then prepared for GC-MS
1103 analysis in hexane (Agilent 5977A- 7890B) after derivatisation by methanolysis using 0.5
1104 M HCl in methanol incubated at 85°C for 3.5 hrs. Fatty acid methyl esters were identified
1105 by their mass spectrum and retention time compared to authentic standards. Individual
1106 lipid classes were normalised according to internal standards.

1107

1108 **Parasite protein extraction, SDS-PAGE, and immunoblotting**

1109 Intracellular parasites were scraped and syringe released HFFs by passing through a 23-
1110 gauge needle. Extracellular parasites were pelleted (8,000 rpm, 10 min) then lysed in an
1111 NP40 buffer (150mM NaCl, 0.5mM EDTA, 1% NP-40, 10mM Tris [pH 7.5]) supplemented
1112 with cOmplete EDTA-free protease inhibitor (Roche). Samples were incubated on ice for
1113 10 min, then centrifuged at 12,000xg for 10 min at 4°C and supernatants collected.
1114 Following the addition of SDS sample buffer, the samples were electrophoresed on 4-
1115 20% Mini-Protean TGX stain-free precast gels (Bio-Rad) then transferred onto
1116 nitrocellulose mem- branes using a semidry Trans-Blot Turbo transfer system (Bio-Rad).
1117 The membranes were blocked using 10% skimmed milk in PBS containing 0.1% Tween
1118 20 (PBST) and then incubated with rat anti-HA high affinity (1:1,000; Roche) and rabbit
1119 anti-T. gondii CAP (1:2,000; Hunt *et al.*, 2019) for 1 hour, followed by donkey anti-rabbit
1120 IRDye 680LT (1:20,000; LI-COR) and goat anti-rat IRDye 800CW (1:20,000; LI-COR) for
1121 1 hour. After several washed with PBS, the remaining bound near-infrared conjugated
1122 secondary antibodies were visualised using the Odyssey Infrared Imaging System (LI-
1123 COR Biosciences, Nebraska, United States).

1124

1125 **Immunofluorescence microscopy**

1126 Parasites were seeded onto HFFs grown in chambered coverslip slides (Ibidi). After 18-
1127 24 hours, the chambers were washed with PBS, then fixed in 3% formaldehyde in PBS
1128 for 15 minutes. The cells were then permeabilised using 0.1% Triton X-100 in PBS for 10

1129 minutes, then blocked in 3% BSA in PBS for 1 hour. The samples were then incubated
1130 rat anti-HA high affinity (1:1,000; Roche) and rabbit anti-T. gondii CAP (1:2,000; Hunt et
1131 al., 2019) for 1 hour followed by goat anti-rabbit Alexa Flour 594 (1:2,000; Life
1132 Technologies) and donkey anti-rat Alexa Flour 488 (1:2,000; Life Technologies)
1133 secondary antibodies along with 5 µg/ml DAPI for 1 hour. Images were obtained using a
1134 Nikon Eclipse Ti-U inverted fluorescent microscope using a 100x objective and processed
1135 using ImageJ software.

1136

1137 **PDE Pulldown**

1138 HFF monolayers infected with WT or HA-tagged PDE1, PDE2, PDE7 or PDE9 lines were
1139 scraped, syringe-released and counted. A total of 1×10^7 cells per condition were pelleted
1140 (8,000 rpm, 10 min) then lysed in 10 µl of NP-40 buffer (150mM NaCl, 0.5mM EDTA, 1%
1141 NP-40, 10mM Tris [pH 7.5]) supplemented with cOmplete EDTA-free protease inhibitor
1142 (Roche) for 10 minutes on ice. Samples were centrifuged at 12,000 xg for 10 min at 4°C,
1143 the supernatants collected and the volume adjusted to 100 µl with IP buffer (150mM NaCl,
1144 0.5mM EDTA, 10mM Tris [pH 7.5] and cOmplete EDTA-free protease inhibitor) to give a
1145 final detergent concentration of 0.1%. To pull down HA-tagged PDEs, Pierce anti-HA
1146 conjugated magnetic beads (5 µl per condition, Thermo Fisher) were washed 3x with IP
1147 buffer to equilibrate the beads, after which the diluted lysate samples was added to the
1148 beads and incubated for 2 hours at 4°C on a rotating wheel. After incubation, the
1149 supernatant was discarded and the beads washed 3x with IP buffer.

1150

1151 **PDE Assay**

1152 The hydrolytic activity of immunoprecipitated PDEs bound to anti-HA magnetic beads was
1153 measured using the PDE-Glo assay (Promega), a bioluminescence-based assay which
1154 quantifies the amount of cAMP or cGMP hydrolysed by a given PDE. Briefly, the PDE-
1155 bound magnetic beads were resuspended in assay buffer and incubated with either 1 µM
1156 cAMP or 10 µM cGMP for 1 hour at room temperature and the reaction terminate by the
1157 addition of termination buffer. Detection buffer was added and incubated for 20 minutes,
1158 followed by the addition of Kinase-Glo detection solution which was incubated for a further
1159 10 minutes. The supernatants were then transferred to a white 96-well plate and

1160 luminescence measured using a FLUOstar Omega plate reader. To test the inhibitory
1161 effects of BIPPO on the activity of the PDEs, 25 μ M BIPPO or the equivalent volume of
1162 DMSO was added to the reaction buffer and left to incubated for 10 minutes before
1163 addition of cyclic nucleotide.

1164

1165 **Plaque Assays**

1166 Intracellular parasites were treated with 50 nM RAP or the equivalent volume of DMSO
1167 for 4 hours, after which the media was replaced, and the parasites left to grow for 3 days
1168 to ensure efficient turnover of the PDEs in the RAP-treated samples. DMSO- and RAP-
1169 treated parasites were harvested by syringe lysis, counted and 250 parasites seeded on
1170 confluent HFFs grown in T25 flasks. Plaques were allowed to form for 5 days, after which
1171 cells were fixed in 100% ice cold methanol for 2 minutes and then stained with 0.1%
1172 crystal violet for 10 minutes to visualise the plaques. Images of the plaques were acquired
1173 with a 4x objective using an Olympus CKX53 microscope fitted with an Olympus DP74
1174 camera. Plaque areas were determined using ImageJ software.

1175

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1417 **List of supplementary documents associated with the manuscript**

1418

1419 **Data S1** – Phosphopeptide quantifications and calculated logFCs from A23187/BIPPO
1420 treated WT/ Δ CDPK3 parasites. Tabs include data subsets that were subjected to
1421 thresholding for differential regulation in A23187 and/or BIPPO treatment conditions.
1422 Includes data from TMT sets 1 and 2.

1423

1424 **Data S2** – Phosphopeptide quantifications and calculated logFCs for peptides that were
1425 (i) differentially regulated following A23187/BIPPO treatment and (ii) were detected
1426 during CDPK3 dependency analysis. Tabs include data subsets that were subjected to
1427 thresholding for differential regulation and CDPK3 dependency. Includes data from TMT
1428 sets 1 and 2.

1429

1430 **Data S3** – Phosphopeptide quantifications and calculated logFCs from WT/ Δ CDPK3
1431 parasites subjected to A23187 treatment timecourse. Tabs include data subsets
1432 subjected to thresholding for differential regulation and CDPK3 dependency.

1433

1434 **Table S1** – List of proteins (identified in A23187 timecourse) assigned to clusters
1435 identified in the Gaussian mixture-model-based clustering analysis. The clusters are
1436 listed across four different tabs based on whether they are differentially up- or down-
1437 regulated and whether this shows CDPK3-dependency.

1438

1439 **Table S2** – Gene ontology analysis results of proteins found to be differentially up-
1440 regulated in A23187 timecourse.

1441

1442 **Table S3** – Gene ontology analysis results of proteins found to be differentially down-
1443 regulated in A23187 timecourse.

1444

1445 **Table S4** – Gene ontology analysis results of proteins found to be differentially up-
1446 regulated and CDPK3 dependent in A23187 timecourse.

1447

1448 **Table S5** – Gene ontology analysis results of proteins found to be differentially down-
1449 regulated and CDPK3 dependent in A23187 timecourse.

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1451 **Table S6** – List of primers used in this study