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Leishmania dual-specificity tyrosine-regulated kinase 1 (DYRK1) is required for sustaining Leishmania stationary phase phenotype

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1 ***Leishmania* Dual Specificity Tyrosine Regulated Kinase 1**
2 **(DYRK1) is required for sustaining *Leishmania* stationary**
3 **phase phenotype**

4
5 **Running Title: *Lin*DYRK1 is essential for *Leishmania* stationary phase biology**

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32 **Keywords:** *Leishmania*, DYRK, differentiation, cell cycle, growth arrest

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48 **Summary**

49 Although the multiplicative and growth-arrested states play key roles in *Leishmania* development,
50 the regulators of these transitions are largely unknown. In an attempt to gain a better
51 understanding of these processes, we characterised one member of a family of protein kinases
52 with dual specificity, *LinDYRK1*, which acts as a stasis regulator in other organisms. *LinDYRK1*
53 over-expressing parasites displayed a decrease in proliferation and in cell cycle re-entry of
54 arrested cells. Parasites lacking *LinDYRK1* displayed distinct fitness phenotypes in logarithmic
55 and stationary growth phases. In logarithmic growth-phase, *LinDYRK1*^{-/-} parasites proliferated
56 better than control lines, supporting a role of this kinase in stasis, while in stationary growth-
57 phase, *LinDYRK1*^{-/-} parasites had important defects as they rounded up, accumulated vacuoles
58 and lipid bodies and displayed subtle but consistent differences in lipid composition. Moreover,
59 they expressed less metacyclic-enriched transcripts, displayed increased sensitivity to
60 complement lysis and a significant reduction in survival within peritoneal macrophages. The
61 distinct *LinDYRK1*^{-/-} growth phase phenotypes were mirrored by the distinct *LinDYRK1*
62 localisations in logarithmic (flagellar pocket and endosomes) and late stationary phase
63 (mitochondrion). Overall, this work provides first evidence for the role of a DYRK family
64 member in sustaining promastigote stationary phase phenotype and infectivity.

65

66 **Keywords:** *Leishmania*, DYRK, differentiation, cell cycle, growth arrest

67

68 **Introduction**

69 *Leishmania* is a protozoan parasite responsible for a group of diseases, termed leishmaniases, that
70 shows multiple clinical manifestations, ranging from self-healing cutaneous to lethal visceral
71 leishmaniasis if left untreated (Murray *et al.*, 2005). These diseases are endemic in tropical and

72 subtropical countries, causing severe morbidity and mortality and presenting a worldwide
73 incidence of approximately 1 500 000 clinically reported cases (Alvar *et al.*, 2012).

74 *Leishmania* spp have a digenetic life cycle, involving a motile, extracellular promastigote
75 stage that parasitises the alimentary tract of a sand-fly vector, and an immotile amastigote stage
76 that survives and replicates in the phagolysosomes of mononuclear phagocytes (Bates, 1994,
77 Sacks & Perkins, 1984). To complete its life cycle, the parasite has to successfully undergo
78 different developmental transitions induced by environmental stimuli. In the midgut of the sand-
79 fly, sequential development of promastigotes inside the sugar-rich environment initially involves
80 the rapid division of non-infective procyclic promastigotes (Burchmore & Barrett, 2001,
81 McConville & Naderer, 2011). These parasites differentiate into distinct forms, including the cell
82 cycle arrested nectomonads and the dividing leptomonads that will ultimately differentiate to
83 infective, metacyclic promastigotes in the anterior parts of the digestive tract (Gossage *et al.*,
84 2003). During a blood meal, metacyclics will be transmitted to the mammalian host, where they
85 will encounter a marked environmental temperature (26°C to 34-37°C) and pH (7.4 to 5.5) shift,
86 as well as changes in nutrients including a sugar-poor and fatty-acid- and amino-acid-rich
87 environment. Parasites that will successfully adapt to these new environmental changes and
88 subvert the immune system of the host will differentiate and proliferate as amastigotes
89 (Burchmore & Barrett, 2001, McConville & Naderer, 2011).

90 *Leishmania* promastigote differentiation in the sand-fly requires homeostatic control and
91 signalling events that are largely unknown (Sacks & Perkins, 1984, Sacks & Perkins, 1985).
92 Procyclic to metacyclic promastigote transitions in the sand fly can be mirrored in cell culture
93 (Gossage *et al.*, 2003) and are characterised by specific molecular, morphological, biochemical
94 and cell cycle changes (Inbar *et al.*, 2017, Sacks *et al.*, 1985, Sacks & Perkins, 1984, Sacks &
95 Perkins, 1985). These transitions are initiated by environmental changes (Cunningham *et al.*,
96 2001, Serafim *et al.*, 2012), and are communicated by signalling cascades, including
97 phosphosignalling events (Tsigankov *et al.*, 2014, Tsigankov *et al.*, 2013). Despite the importance

98 of phosphosignalling in differentiation, there is little knowledge on the nature of kinases that
99 mediate these transitions. Amongst these, protein kinase A (PKA) activity is known to be
100 required for the promastigote to amastigote transition (Bachmaier *et al.*, 2016), whereas Target of
101 Rapamycin (TOR) 3 is known to be associated with the development of the infectious stage
102 (Madeira da Silva & Beverley, 2010).

103 The dual specificity tyrosine regulated kinases (DYRKs) are members of the CMGC
104 group of kinases (Kannan & Neuwald, 2004) are characterised by their dual specificity,
105 presenting an auto-phosphorylation activity of a critical tyrosine residue of the activation loop and
106 a serine/threonine kinase activity towards substrates (Becker & Joost, 1999). Based on the
107 homology of their kinase domain, these proteins are divided into 3 subfamilies: the
108 homeodomain-interacting protein kinases (HIPKs) known to regulate a wide variety of stress
109 signals (Schmitz *et al.*, 2014), the pre-mRNA processing protein 4 kinases (PRP4s) involved in
110 pre-mRNA processing and transcriptional regulation (Huang *et al.*, 2000) and the DYRKs
111 (Aranda *et al.*, 2011). The DYRK subfamily comprises of the YAKs, class I (DYRK1A and
112 DYRK1B) and class II (DYRK2, DYRK3 and DYRK4) DYRKs (Aranda *et al.*, 2011).

113 The DYRK subfamily members are multifaceted kinases, regulating a great variety of
114 cellular processes including gene expression, proteasomal degradation, or chromatin remodelling.
115 Stasis regulation and differentiation however, is the hallmark of DYRK activity (Aranda *et al.*,
116 2011). The DYRK subfamily shares a conserved structure which includes a DYRK homology
117 (DH) box, an aspartic rich sequence that precedes the kinase domain (Becker & Joost, 1999), a
118 characteristic HCD motif in the catalytic loop, instead of the highly conserved HRD (Kannan &
119 Neuwald, 2004), and a YxY motif with the second tyrosine being auto-phosphorylated and
120 required for DYRK activity (Lochhead *et al.*, 2005). Regulatory sequences including nuclear
121 localisation sequences (NLSs) and a PEST sequence known to act as signal for protein
122 degradation., are present in specific members of the DYRK family (Aranda *et al.*, 2011).

123 Recently, a role in differentiation was shown for a DYRK related kinase of the
124 trypanosomatid parasite *Trypanosoma brucei*. Mony *et al* performed a genome-wide RNAi target
125 screen to identify genes whose down-regulation resulted in a cAMP unresponsive phenotype
126 (Mony *et al.*, 2014). This phenotype was associated with a differentiation defect, whereby
127 parasites failed to differentiate from the "proliferative slender" to the "arrested stumpy" form, the
128 latter stage being responsible for transmission to the tsetse flies (Mony *et al.*, 2014). In addition,
129 another DYRK member from *T. brucei* has been shown to be required for parasite survival in the
130 mouse (Fernandez-Cortes *et al.*, 2017).

131 Interestingly, in *Leishmania* the DYRK family consists of 8 members. DYRK1
132 homologue (*LinJ.15.0180*, *LinDYRK1*) is a single-copy gene which is a *Leishmania* DYRK1
133 homologue having the highest conservation to mammalian DYRK1A and DYRK1B orthologues.
134 Herein, we combined genetic manipulation and phenotypic analyses to generate null mutants and
135 report that *LinDYRK1* is a pro-survival kinase and its deletion is associated with a deregulated
136 stationary phase biology.

137

138 **Results**

139 **Evolutionary relationship and characterisation of *Leishmania* DYRK family** 140 **members**

141 To investigate the identity and evolutionary relationship of *Leishmania* DYRK family proteins,
142 we employed phylogenetic analyses of animal, plant, insect, yeast and protist DYRK protein
143 sequences. Our analysis revealed the presence of 8 *Leishmania* DYRK proteins, in accordance to
144 previous reports (Parsons *et al.*, 2005). From the *Leishmania* DYRK members, five belong to all
145 three DYRK subfamily groups, namely the DYRK (*LinJ.15.0180-LinDYRK1*, *LinJ.14.0890* and
146 *LinJ.33.1930*), the HIPK (*LinJ.19.0360*) and the Prp4 (*Lin.J.36.4460*) (**Fig 1A**). The other three

147 family members (*LinJ.21.2010*, *LinJ.14.1140* and *LinJ.35.1850*) did not cluster with the known
148 DYRK subfamilies (**Fig 1A**).

149 *LinDYRK1* possesses sequences, including the HCD triplet in the catalytic loop and a
150 Tyrosine (HR_Y⁵²³), aligning to the second tyrosine of the DYRK subfamily activation loop
151 sequence YxY that conform to DYRK constraints (**Fig 1B**, **S1 Fig**). In addition, *LinDYRK1* is
152 more similar to mammalian DYRKs than to lower eukaryotic YAKs (**S1 Fig**). For example,
153 *LinDYRK1* is closer in sequence identity to *H. sapiens* (*Hs*) DYRK1A (48.5% identity, query
154 cover 97%, E value: $1 * e^{-63}$) and *HsDYRK1B* (49.5%, query cover: 96%, E value: $4 * e^{-65}$), than to
155 *S. cerevisiae* Yak1p (46.55% identity, query cover 59%, E value: $1 * e^{-35}$).

156 In *Leishmania*, the kinase with the highest similarity to *LinDYRK1* is *LinJ.14.0890* (**Fig.**
157 **1B**). The full protein sequences share an overall 54% sequence identity (54% query cover, E
158 value: $8e^{-74}$), while both proteins have a long N terminal domain of unknown function, unique to
159 *Leishmania*. The two kinases lack a NLS and contain predicted PEST sequences (**Fig 1B**). The
160 DH box, while conserved in *LinDYRK1*, is not present in *LinJ.14.0890*, whereas *LinJ.14.0890*
161 also possesses a conserved tyrosine (PK_Y⁷⁴³), in its predicted activation loop. This kinase
162 however, has insertions in the activation loop between subdomains VII and VIII and between
163 subdomains X and XI (**Fig 1B**), and thus its catalytic activity merits experimental validation.

164 Apart from *LinJ.14.0890*, other *Leishmania* DYRK family members have important
165 modifications of consensus sequences within the kinase domain. For example, *LinJ.21.2010* and
166 *LinJ.14.1140* are characterised by the atypical glycine rich loops DXAXSXXV and
167 GXAXSXXV respectively, that replace the highly conserved GXGXXGXV required for the
168 positioning of ATP (**S2 Fig**). Interestingly, *LinJ.19.0360* contains an atypical DFS cation binding
169 site instead of the highly conserved DF/LG (**S2 Fig**) and thus the catalytic activities of these
170 kinases require confirmation.

171

172 ***LinDYRK1* localisation**

173 To investigate the subcellular localisation of *LinDYRK1*, we expressed a tagged version of
174 *LinDYRK1* with eGFP at its N-terminus (GFP-*LinDYRK1*) from an episome. In logarithmic
175 cultures, *LinDYRK1*-GFP was mainly localised at the anterior side of the cell. Co-localisation
176 with mitotracker was used in an attempt to compare *LinDYRK1* localisation in logarithmic cells
177 with respect to the mitochondrion. Our results showed that GFP-*LinDYRK1* in logarithmic cells,
178 exhibited no co-localisation with mitotracker (**Fig 2A**). Moreover, concanavalin A (ConA)
179 conjugated to Alexa594 (ConA–Alexa594) used to label the surface glycocalyx and the flagellar
180 pocket (Ilgoutz *et al.*, 1999), revealed that GFP-*LinDYRK1* occupied a portion of the flagellar
181 pocket area (**Fig 2B**). GFP-*LinDYRK1* was also found outside the flagellar pocket at the anterior
182 end of the kinetoplast (**Fig 2B**) and between the nucleus and kinetoplast (**Fig 2A** and **Fig 2B**). In
183 order to further investigate the localisation of *LinDYRK1* in logarithmic cells, FM4-64 dye was
184 used as a marker of endosomes (Besteiro *et al.*, 2006). Areas of intense localisation of GFP-
185 *LinDYRK1* were observed, similar to the localisation of FM4-64, in a 3 min pulse chase
186 experiment followed by an incubation time of 20 min incubation at RT (**Fig 2C**). GFP-
187 *LinDYRK1* in late logarithmic/ early stationary phase promastigotes extended more throughout
188 the cell body (**Fig 2A**) but showed a distinct localisation with respect to mitotracker (**Fig 2A**). In
189 late (stationary phase day 7 of cell culture, day 3 of stationary phase) however, we observed a
190 change in GFP-*LinDYRK1* localisation to areas, where mitotracker was present (**Fig 2A**).

191 To ensure that the observed *LinDYRK1* localisation was not an artefact, we expressed
192 from an episome a second fusion protein, tagged with eGFP at its C-terminus (*LinDYRK1*-GFP).
193 *LinDYRK1*-GFP had a similar localisation to GFP- *LinDYRK1* (**S3A** and **S3B Fig**). Despite the
194 fact that episomal expression can sometimes cause excessive expression of the target protein, that
195 might result in potential mislocalisation (Tanz *et al.*, 2013), this does not seem to be the case in
196 our system. The levels of N and C terminal tagged fusion proteins were much lower than GFP,
197 and below the detection limit by western blot analysis in total protein extracts (data not shown).

198 In addition, the fact that similar localisation for both C and N terminal fusion proteins was
199 observed, is a strong indication that they represent the localisation of the endogenous protein.

200

201 ***LinDYRK1* over-expression has a negative effect on promastigote growth**

202 To have a better understanding of the *LinDYRK1* function, we created a *L. infantum* cell line that
203 over-expresses the protein from a *Leishmania* expression plasmid (pXNG4). This plasmid
204 expresses Herpes Virus Thymidine kinase (HSV-TK) and can be subject to negative selection by
205 antiviral drug ganciclovir (GCV), which is phosphorylated by HSV-TK into a toxic product
206 (Murta *et al.*, 2009). Parasites over-expressing *LinDYRK1* {*LinDYRK1*^{+/+}[pXNG-*LinDYRK1*]}
207 had a proliferation defect with respect to control parasites {wild type (*LinDYRK1*^{+/+}), parasites
208 that had lost the episome after treatment with GCV {*LinDYRK1*^{+/+}[pXNG-*LinDYRK1*]} and
209 parasites transfected with plasmid alone {*LinDYRK1*^{+/+}[pXNG]} visible at seeding density of
210 10⁶ mL⁻¹ (**Fig 3A**). In order to measure more precisely the growth defect, a lower seeding density
211 was used (3 *10⁵ mL⁻¹) for *LinDYRK1*^{+/+}[pXNG-*LinDYRK1*] and *LinDYRK1*^{+/+}[pXNG]
212 promastigotes. The logarithmic slopes of the growth curves were compared and our results
213 demonstrated that *LinDYRK1* over-expressing parasites proliferated 33% less well than parasites
214 bearing plasmid alone (**Fig 3A**). Cell cycle progression of *LinDYRK1* over-expressing parasites
215 was also investigated by initially synchronising *LinDYRK1*^{+/+}[pXNG-*LinDYRK1*] and
216 *LinDYRK1*^{+/+}[pXNG] promastigotes at the G₀/G₁-S boundary with an inhibitor of ribonucleotide
217 reductase, hydroxyurea (HU). Subsequently cell cycle re-entry was allowed by removal of HU.
218 Cell cycle was investigated 0 h, 3 h and 6.5 h after HU removal. At 6.5 h post HU removal 51%
219 of *LinDYRK1*^{+/+}[pXNG-*LinDYRK1*] parasites had not entered in S-G₂/M phases in contrast to
220 only 15% of control *LinDYRK1*^{+/+}[pXNG] parasites (**Fig 3B, S1 Table**), and revealed that
221 *LinDYRK1* over-expression resulted in delayed cell cycle re-entry of resting cells.

222

223 ***LinDYRK1* gene deletion is tolerated in logarithmic phase promastigotes**

224 Assuming initially that *LinDYRK1* was essential, we decided to generate *LinDYRK1* facilitated
225 knockouts, a process that overcomes a potentially lethal phenotype (Dacher *et al.*, 2014, Murta *et*
226 *al.*, 2009). The episome [pXNG4] mentioned above, also expresses GFP, which is used as a
227 reporter for monitoring its loss (Dacher *et al.*, 2014, Murta *et al.*, 2009). By sequentially
228 introducing the puromycin N-acetyl-transferase (PAC) and then the hygromycin B (HYG)
229 targeting cassettes, we replaced the two *LinDYRK1* alleles in *L. infantum* promastigotes
230 transfected with [pXNG-*LinDYRK1*] (**S4A Fig**). Following this strategy, we generated two
231 homozygous facilitated mutants from two independent heterozygous clones (**A** and **B**). We
232 confirmed incorporation of the targeting of cassettes into the *LinDYRK1* genomic locus by
233 diagnostic PCR (**S4B Fig**).

234 We then tested if *LinDYRK1* was essential for promastigote survival. To this end, we
235 applied negative selection by replacing the positive selection drug NTC with the negative
236 selection drug GCV and analysed the loss of the episome by measuring the GFP fluorescence by
237 flow cytometry. Analysis of the GFP fluorescence of both clones **A** and **B** for 4 passages in
238 logarithmic phase revealed that the *LinDYRK1* expressing episome could be eliminated after the
239 addition of GCV, in a similar manner to the episome in wild type promastigotes (**Fig. 4A**).
240 Moreover, the mean GFP fluorescence gradually was reduced to similar levels of wild type
241 *LinDYRK1*^{+/+} promastigotes (**Fig 4B**). To be certain that the loss of fluorescence in the pools of
242 parasites correlated with the loss of the [pXNG-*LinDYRK1*] episome, we analysed by western
243 blotting the GFP amounts of individual clones isolated after 15 passages of cell-culture, using
244 alpha-tubulin as a loading control (**Fig 4C**). We did not observe GFP expression in any of the
245 clones tested (**Fig 4C**), revealing that *LinDYRK1*^{-/-} parasites can be viable without the episome,
246 while GFP was detected in the control *LinDYRK1*^{+/+}[pXNG-*LinDYRK1*] pool treated with NTC
247 (**Fig 4C**). To rule out the possibility of an extra copy of *LinDYRK1* anywhere within the genome
248 of *L. infantum*, we performed diagnostic PCR with internal *LinDYRK1* primers (p1 and p2) in

249 selected clones after GCV selection, and failed to amplify a product (**S4C Fig**). Overall, our
250 findings showed that the loss of the [pXNG-*LinDYRK1*] episome could be tolerated in
251 logarithmic cells and that *LinDYRK1* is not essential for viability.

252 The experiment was also repeated without the presence of the episome, in order to
253 ascertain that GCV does not alter the phenotype of these cells. We were able to generate and
254 *LinDYRK1*^{-/-} promastigotes, termed here direct heterozygous *LinDYRK1*^{+/-direct} and direct
255 homozygous *LinDYRK1*^{-/-direct} mutants. Correct incorporation of targeting cassettes and lack of
256 internal *LinDYRK1* copy were validated by PCR (**S4D Fig**).

257

258 ***LinDYRK1* deletion affects stationary growth phase**

259 To further investigate the biological role of *LinDYRK1*, we analysed some phenotypes of
260 *LinDYRK1*^{-/-} parasites, including growth comparisons. In the logarithmic phase clones
261 *LinDYRK1*^{-/-}**B1** and *LinDYRK1*^{-/-}**B2** displayed a higher proliferation rate (40% and 66% for
262 clones **B1** and **B2** respectively) and higher maximal cell density than their parental cell line
263 *LinDYRK1*^{-/-}[pXNG-*LinDYRK1*]**B** (**S5A Fig**). A higher proliferation was also observed for
264 *LinDYRK1*^{-/-}**A1** and *LinDYRK1*^{-/-}**A2** clones with respect to their parental line (data not shown).
265 In addition, growth comparison between a *LinDYRK1A1*^{-/-} add back cell line and *LinDYRK1A1*^{-/-}
266 ^{-/-} promastigotes transfected with plasmid alone {*LinDYRK1*^{-/-}[pXNG]}, revealed that add backs
267 displayed 30% reduced proliferation rate and lower maximal cell density (**S5B Fig**). Moreover,
268 the homozygous *LinDYRK1*^{-/-direct} also proliferated better than the heterozygous *LinDYRK1*^{+/-direct}
269 (**S5C Fig**). These results altogether, allow us to conclude that the lack of *LinDYRK1* has a
270 positive effect on the growth rate of logarithmic cells.

271 Despite the fitness of *LinDYRK1*^{-/-} mutants in logarithmic growth phase, in stationary
272 phase these cells displayed defects including morphological alterations. All experiments in
273 stationary growth phase, were performed the second day of stationary growth phase, unless stated
274 otherwise. Stationary *LinDYRK1*^{-/-} parasites, were less elongated, had greater width and were

275 more rounded than wild type, parental *LinDYRK1*^{-/-}[pXNG-*LinDYRK1*] lines and an add back
276 clone (**Fig 5A**). Similar morphological alterations were observed with direct null mutants (**S6A**
277 **Fig**). Flow cytometry analysis confirmed these findings which showed higher forward scatter
278 (FSC) values for both facilitated *LinDYRK1*^{-/-} (**S6B Fig**) and the *LinDYRK1*^{-/-direct} mutants (**S6C**
279 **Fig**) in comparison to control promastigotes. Moreover, null mutants in stationary phase
280 accumulated a greater percentage of cells with two nuclei (N) and two kinetoplasts (K) (17% and
281 29% of clones *LinDYRK1*^{-/-}**A1** and *LinDYRK1*^{-/-}**A2**, respectively), and a greater percentage of
282 cells with 2N1K (7% and 12.5% for clones *LinDYRK1*^{-/-}**A1** and *LinDYRK1*^{-/-}**A2**, respectively)
283 versus ≤3.3% of 2N2K and ≤1% of 2N1K of control cells (parental line *LinDYRK1*^{-/-}[pXNG-
284 *LinDYRK1*]**A** and *LinDYRK1*^{+/+}) (**S6D Fig**). This could be an indication that these promastigotes
285 are in a stress related G₂/M cell cycle arrest, which in due time results in cell death (Azzopardi *et*
286 *al.*, 2017).

287 Cell death was increased in late stationary phase in facilitated and direct *LinDYRK1*^{-/-}
288 clones (**Fig 5B**, **S7 Fig**, **Table S2A**). For facilitated clones *LinDYRK1*^{-/-}**A1** and *LinDYRK1*^{-/-}**A2**,
289 46.3% and 33.8% respectively had hypodiploid DNA content, versus 4.9% of wild type
290 *LinDYRK1*^{+/+}, 15.2% of *LinDYRK1*^{-/-} [pXNG-*LinDYRK1*]**A** and 9% of *LinDYRK1*^{-/-}**A1**[pXNG-
291 *LinDYRK1*] add back (**Fig 5B**, **Table S2A**). In early stationary phase (day 5 of growth curve, day
292 1 of stationary phase) *LinDYRK1*^{-/-} mutants did not show increase of hypodiploid DNA content
293 (**S7A Fig**), whereas late stationary phase (day 7 of growth curve, day 3 of stationary phase).

294

295 ***LinDYRK1*^{-/-} stationary phase promastigotes exhibit severe surface aberrations, lipid body** 296 **formation and display notable lipid content changes**

297 To further investigate the morphological defects of *LinDYRK1*^{-/-} parasites in stationary
298 phase, we performed scanning (SEM) and transmission (TEM) electron microscopy SEM
299 analysis of facilitated *LinDYRK1*^{-/-} mutants revealed had surface aberrations, with folds on the
300 surface and membrane invaginations (**Fig 6A**). TEM analysis revealed that *LinDYRK1*^{-/-}

301 promastigotes displayed an abnormal subcellular structure. These parasites exhibited intense
302 vacuolisation of the cytoplasm, (**Fig. 6B**{III, IV, V, VI}). Vacuoles often contained membrane
303 blebs, where the kinetoplast membrane dissociated inside a vacuole (**Fig. 6B**{V}). Another
304 prominent ultrastructural feature of *LinDYRK1*^{-/-} parasites was the presence of lipid vacuoles or
305 bodies observed by their low electron density and homogeneous appearance (Charron & Sibley,
306 2002). Lipid bodies were found to be adjacent to vacuoles and often were merging with the cell
307 membrane, leaving an open invagination full of lipids (**Fig. 6B** {III and IV}). In addition some
308 parasites had fragmented DNA (**Fig. 6B** IV), suggesting that they were already in the process of
309 cell death.

310 As lipid droplet formation may be linked to lipid metabolism (Walther & Farese, 2012),
311 we further investigated the lipid composition of *LinDYRK1*^{-/-} promastigotes. To this end, we
312 analysed the total phospholipid content by nano-electrospray mass spectrometry (nESI-MS), and
313 by gas chromatography mass spectrometry (GC-MS) the total fatty acid and sterol content. We
314 analysed two knockout clones *LinDYRK1*^{-/-}**A** and *LinDYRK1*^{-/-}**B** from different parental strains
315 to normalise for colony specific effects, and compared them with wild type parasites
316 (*LinDYRK1*^{+/+}) and the parental clone *LinDYRK1*^{-/-}[pXNG-*LinDYRK1*] **A**. Our results showed
317 that *LinDYRK1* mutants had higher amounts of saturated fatty acids (**Fig. 6C, Table 1**). The most
318 evident difference was a decrease in the percentage of 18:2, and an increase in that of 18:0 (**Table**
319 **1**), resulting in a C18:2/C18:0 fatty acid ratio of ~0.7 and ~0.8 for the clones *LinDYRK1*^{-/-}**A1** and
320 *LinDYRK1*^{-/-}**B1** respectively, versus 1.7 for wild type cells. Furthermore, sterol analysis showed
321 that *LinDYRK1*^{-/-} parasites displayed relatively lower cholesterol levels than the pool of
322 *LinDYRK1*^{+/+} parasites (14-15% mutants versus 27% wild type) and higher ergosta-7,24-dien-3β-
323 ol levels (17.8%-22% mutants versus 11% wild type) (**Table 1**). Finally, our analysis
324 demonstrated that *LinDYRK1* knockout does not significantly affect phospholipid composition
325 (**S8 Fig**).

326

327 ***LinDYRK1*^{-/-} promastigotes exhibit defects in markers of infective promastigotes,**
328 **increased thermosensitivity and reduced survival in host macrophages**

329 To ensure survival within the mammalian host, *Leishmania* promastigotes develop
330 resistance to complement-mediated lysis, as they progress from the logarithmic to stationary
331 growth phase (Franke *et al.*, 1985). In an attempt to relate *LinDYRK1*^{-/-} stationary phase defects
332 with parasite infectivity, the ability of null mutants to endure complement lysis in logarithmic and
333 stationary phase was assessed. *LinDYRK1*^{-/-} promastigotes were more susceptible to complement
334 lysis (70% logarithmic and 30% stationary phase) than *LinDYRK1*^{+/+} (46.7% logarithmic and
335 17.8% stationary phase) and *LinDYRK1*^{-/-}[pXNG-*LinDYRK1*] (53% logarithmic and 16.5 %
336 stationary phase) promastigotes (**Fig 7A**).

337 The number of infective metacyclics and the expression of metacyclic enriched
338 transcripts were also measured for stationary phase *LinDYRK1*^{-/-} mutants and control cells. For
339 measuring metacyclics, the peanut agglutinin (PNA) assay was performed, which relies on the
340 ability of the PNA lectin to bind and agglutinate non-infective procyclics but not infective
341 metacyclic promastigotes (Sacks *et al.*, 1985). The assay revealed that that the percentage of
342 unagglutinated *LinDYRK1*^{-/-} parasites was 2.8 times less than wild type *LinDYRK1*^{+/+} and 3.6
343 times less than *LinDYRK1*^{-/-}[pXNG-*LinDYRK1*] (**Fig 7B**). In addition, *LinDYRK1*^{-/-} parasites
344 expressed less well transcripts known to be enriched in metacyclic promastigotes. More
345 specifically levels of prohibitin {*LinJ.16.1710* (Almeida *et al.*, 2004)}, meta1 {*LinJ.17.0990*,
346 (Nourbakhsh *et al.*, 1996)} and HASPB {*LinJ23.1220*} (Sadlova *et al.*, 2010)} transcripts
347 normalised with ribosomal protein S29 transcript (*LinJ.28.2360*) were compared. The ratios of
348 normalised transcripts were significantly reduced with respect to wild type *LinDYRK1*^{-/-} A1 (7.14
349 fold less for prohibitin, 5 fold less for meta1 and 4.17 fold less for HASPB) and to *LinDYRK1*^{-/-}
350 [pXNG-*LinDYRK1*]A (2.57 fold less for prohibitin, 2.6 fold less for meta1 and 1.46 less for

351 HASPB) (**Fig. 7C**). These results suggest that stationary phase defects are not only morphological
352 in *LinDYRK1*^{-/-} but also biochemical, and are consistent with defects in metacyclogenesis.

353 In addition to metacyclogenesis, other factors may influence stage differentiation as the
354 ability to survive a 26→37°C upshift in temperature, which mimics the sand-fly to mammalian
355 host temperature transition. As DYRK related kinases are known to regulate thermotolerance
356 (Hartley *et al.*, 1994), we hypothesised that *LinDYRK1*^{-/-} promastigotes were less thermotolerant.
357 We subjected parasites to a 16 h 26→37°C heat-shock and compared the percentage of
358 hypodiploid cells in facilitated (**Fig 7D, S2C Table**) and direct mutants (**S2D Table, S6 Fig**) with
359 the corresponding control cells. Our analyses showed that all *LinDYRK1* knockouts were more
360 sensitive to heat-shock than control cell lines (**Fig 7D, S7 Fig, S2C&D Table**). More specifically
361 28% and 29% of *LinDYRK1*^{-/-} clones **A1** and **A2** had hypodiploid DNA content, versus 17.1% of
362 the parental strain, 16.12% of the *LinDYRK1*^{-/-}[pXNG-*LinDYRK1*] add back and 12.5% of the
363 wild type *LinDYRK1*^{+/+} promastigotes (**Fig 7D, S2C&D Table**). Comparison of direct mutants
364 also confirmed that lack of *LinDYRK1* gave rise to a more thermosensitive phenotype (**S9 Fig,**
365 **S2C&D Table**), reinforcing that heat shock sensitivity is specifically related to loss of
366 *LinDYRK1* expression.

367 We then performed an *in vitro* infection assay and compared the percentage of infection
368 and survival of *LinDYRK1*^{-/-}, *LinDYRK1*^{-/-}[pXNG-*LinDYRK1*] and *LinDYRK1*^{+/+} stationary
369 phase parasites in murine peritoneal macrophages. The infection rate of *LinDYRK1*^{-/-} parasites (%
370 of infected macrophages and number of parasites per macrophage) was dramatically reduced by
371 10.5-fold (2.7 less % infected macrophages and 3.9 times less parasites per macrophage) 72 h
372 post infection, compared the beginning of infection (**Fig 7E**). The controls, *LinDYRK1*^{+/+} and
373 *LinDYRK1*^{-/-}[pXNG-*LinDYRK1*], presented similar rates or moderate reduction of infection rates
374 72 h post infection compared to the beginning of infection (**Fig 7E**). In addition, we attempted to
375 measure the *in vivo* infectivity of these parasites. To this end, efforts were made to recover freshly
376 isolated wild type and transgenic parasites cultivated in parallel, from livers of BALB/c mice.

377 During these attempts, it was possible to recover only *LinDYRK1*^{-/-} [pXNG-*LinDYRK1*]
378 parasites in one of the three mice used per group. Parasite burden of animals infected was
379 measured two weeks post-infection by qPCR using liver DNA to estimate the amount of the
380 *Leishmania* specific arginine permease gene AAP3 (Tellevik *et al.*, 2014) and corresponded to
381 1530 parasites per 8700 hepatocyte nuclei. This result is suggestive of the reduced ability of
382 *LinDYRK1*^{-/-} promastigotes to survive in mice, as the ability of parasites to survive in mice is
383 compromised even in wild type background parasites due to the continuous cultivations and
384 manipulations.

385

386 **Discussion**

387 In this work, we determined the evolutionary relationship of the *Leishmania* DYRK
388 family members. A clustering of *Leishmania* DYRK family members both within (for 5 out of 8),
389 and outside (for 3 out of 8) of the known subfamilies (DYRKs, HIPKs, Prp4s), suggests that the
390 DYRK taxonomy in its current form, requires revisiting and the inclusion of a more
391 representative repertoire of eukaryotic organisms. Differences in highly conserved sequences
392 (glycine rich loop, cation binding site) of the kinase catalytic domain for specific *Leishmania*
393 DYRK family members indicate that these are either pseudokinases or kinases with atypical
394 features mirroring the unique biology of the parasite. *LinDYRK1* is one of the genes that shares
395 all the features of an active kinase, and recombinant *LinDYRK1* has been shown to be an active
396 kinase (Hombach-Barrigah *et al.*, 2019).

397 *LinDYRK1* had a distinct localisation in logarithmic, stationary and late stationary
398 growth phases. *LinDYRK1* localisation in proliferative and early stationary phases, in the
399 flagellar pocket area, a structure known to carry out endo- and exo-cytosis (Stierhof *et al.*, 1994)
400 and endosomes involved in endocytic cycle (Besteiro *et al.*, 2006) and mitochondrial localisation
401 in late stationary phase, show that *LinDYRK1* signaling is growth stage dependent. The

402 endosomal localisation data is reminiscent of a known role for mammalian DYRK1A in
403 endocytosis and vesicle recycling (Murakami *et al.*, 2009, Murakami *et al.*, 2012).

404 In this study, it was shown that *LinDYRK1* knockouts were viable and that in the
405 logarithmic growth phase had a higher proliferation rate than control cell lines. At the same time,
406 over-expression of *LinDYRK1* in *Leishmania* resulted in decreased proliferation and delayed cell
407 cycle re-entry after synchronisation by HU. These results could be in line with the known role of
408 DYRKs in various cell types, in regulating the decision between cell cycle re-entry and cell cycle
409 exit (Soppa & Becker, 2015) in mitogen-poor environments (Becker, 2018, Garrett & Broach,
410 1989). In *Leishmania* however, further enquiry is needed to address if there is a direct role for
411 *LinDYRK1* in cell cycle control and proliferation, including the identification of partners and
412 substrates.

413 One of the most prominent phenotypes observed in this study was the deregulated
414 phenotype of stationary phase *LinDYRK1*^{-/-} mutants. Stationary phase phenotypes in unicellular
415 organisms are known to have activated the internal systems of protection against stress and are
416 able to survive better under adverse or changing environmental settings (Pletnev *et al.*, 2015). In
417 *Leishmania* too, stationary phase promastigotes have increased ability to survive in the
418 intracellular environment of the mammalian host (Wozencraft & Blackwell, 1987) and this ability
419 is associated with metacyclogenesis (da Silva & Sacks, 1987). In our system *LinDYRK1*^{-/-}
420 stationary phase promastigotes displayed several defects. Knockout parasites were more round
421 and exhibited an intense vacuolisation of the cytoplasm. The formation of vacuoles in wild type
422 cells could be the result of an ongoing autophagic process, a survival process (Smirlis *et al.*,
423 2010) that also occurs during the procyclic to metacyclic promastigote stage differentiation
424 (Williams *et al.*, 2006). Prolonged, autophagy results in cell death (Smirlis *et al.*, 2010), and
425 could explain the cell death observed in long range stationary phase promastigotes.

426 The accumulation of lipid bodies and the switch in the ratio of saturated (18:0) and
427 polyunsaturated (18:2) fatty acids in stationary phase *LinDYRK1*^{-/-} promastigotes, may be related

428 to the formation of lipid bodies, as this switch of identical lipids in mammalian cells corroborates
429 to the formation of large lipid droplets (Arisawa *et al.*, 2016). Most importantly, large lipid
430 droplets are induced by stress (Petan *et al.*, 2018), while changes in relative ratios of saturated
431 fatty acids are anticipated in normal procyclic to metacyclic stage differentiation (Silva *et al.*,
432 2011). Hence our results suggest abnormal differentiation processes and/or stress response. Sterol
433 metabolism is also known to be affected differentially in *Leishmania* logarithmic and stationary
434 phase cultures (Jacobs *et al.*, 1982). The decrease observed for the relative amount of cholesterol
435 in *LinDYRK1*^{-/-} parasites, is suggestive of a disruption in uptake. This is less explainable for the
436 parent cell line *LinDYRK1*^{-/-}[pXNG-*LinDYRK1*] but even in these cells some vacuolisation is
437 visible. Conversely, the alteration in sterol composition may be entirely due to upregulation of the
438 synthesis of some of endogenous ergostane-based sterols, clearly visible in the putative terminal
439 molecule of the pathway, ergosta-7,24-dien-3 β -ol (Yao & Wilson, 2016).

440 In addition, defects in metacyclogenesis, infectivity and thermotolerance of *LinDYRK1*^{-/-}
441 parasites mirror the importance of the kinase in the completion of the *Leishmania* life-cycle. The
442 enrichment of the *S. cerevisiae* YAK1 in differentiated quiescent yeast stationary-phase cultures
443 (Aragon *et al.*, 2008) and its thermoprotective function in yeast (Hartley *et al.*, 1994), may reveal
444 a central role of DYRK kinases in stationary phase biology and thermoprotection.

445 Overall, our findings suggest that *LinDYRK1* signalling is needed to normal promastigote
446 development. This finding is one step further for gaining insight into the contribution of protein
447 phosphorylation in the growth-stress response balance, and it opens new areas of research and
448 future challenges, including search of DYRK1 substrates the role *LinDYRK1* in the amastigote
449 stage.

450

451 **Experimental Procedures**

452 **Multiple sequence alignment**

453 To search DYRK kinases in *Leishmania*, BLAST analysis was performed with blastp algorithm
454 using protein sequences of known DYRK genes from *H. sapiens*, *D. melanogaster*, *S. cerevisiae*
455 as queries. Sequences from *L. infantum*, *T. cruzi* and *T. brucei* were retrieved from the NCBI
456 (<http://www.ncbi.nlm.nih.gov/protein/>) and TriTrypDB databases (<http://tritrypdb.org/tritrypdb/>).
457 Protein alignment was performed using CLUSTALW 2.0.12 using the built-in programs
458 (Bioedit). Unrooted phylogenetic tree defined by amino acid sequence similarities was generated
459 using Interactive Tree of Life (<http://itol.embl.de/>). Sequence accession numbers are shown in **S3**
460 **Table**.

461

462 **Plasmids**

463 For the generation of [pXNG-*LinDYRK1*] construct, the open reading frame (ORF) of
464 *LinDYRK1* (*LinJ.15.0180*) was amplified by polymerase chain reaction (PCR) from *L. infantum*
465 (L4, MHOM/GR/78/L4) genomic DNA and cloned into the *Leishmania* expression plasmid
466 [pXNG4]. To this end, the p1/p2 primer pair (**S4 Table**) were used and *LinDYRK1* ORF was
467 cloned into the BglII (New England Biolabs) site of [pXNG4] plasmid.

468 *LinDYRK1* targeting constructs, encoding for HYG and PAC resistance markers to
469 antibiotics hygromycin B and puromycin respectively, flanked by the ~ 900 bp 5' and 3' UTR of
470 the *LinDYRK1* gene, were generated for replacing the genomic *LinDYRK1* ORF. To this end, the
471 900 bp 5'-UTR and 3'-UTR of *LinDYRK1* were PCR-amplified using primer pairs p3/p4 and
472 p5/p6, respectively (**S4 Table**), and cloned by TA cloning in the appropriate orientation into
473 [pGEM-T] and [pGEM-T Easy] vectors (Promega) respectively, resulting in pGEM-5'UTR and
474 [pGEM-T Easy-^{3'}UTR] plasmids. The *LinDYRK1* 3'UTR was released using NotI restriction
475 enzyme (New England Biolabs) and cloned into the NotI site of pGEM-5'UTR in the appropriate
476 orientation, to generate plasmid [pGEM-5'UTR-3'UTR]. HYG and PAC coding sequences were
477 PCR-amplified from plasmids [pX63-Hyg] and [pXG-PAC], using primer pairs p7/p8 and p9/p10

478 and cloned into the SpeI/BamHI restriction sites of [pGEM-5'UTR-3'UTR]. Primers p3 and p6
479 also contained an internal PmeI (S4 Table) restriction site, which was used for the generation of
480 linear constructs prior to electroporation.

481 To generate a GFP-*LinDYRK1* fusion construct, the *LinDYRK1* insert was sub-cloned
482 from [pXNG-*LinDYRK1*] in the BglII site of [pXG-GFP⁺²] plasmid. To generate the
483 [*LinDYRK1*-GFP] fusion construct, the *LinDYRK1* insert encoding the ORF was amplified by
484 PCR from genomic *L. infantum* (L4, MHOM/GR/78/L4) DNA with the use of the p1/p21 primer
485 pair (S4 Table). The PCR product was digested with BglII and BamHI and inserted in the
486 appropriate orientation in the BamHI site of [PXG-PAC] *Leishmania* expression plasmid to
487 generate plasmid [*LinDYRK1*-carboxyterminal]. The eGFP was PCR amplified from
488 [pEGFP(N3)] (Clontech®) using the p22/ p23 primers pair (S4 Table), digested with BamHI and
489 BglII. The product was cloned in the appropriate orientation in the BamHI site of plasmid
490 [*LinDYRK1*-carboxyterminal], to generate plasmid [pXG-PAC-*LinDYRK1*-GFP]. All constructs
491 were confirmed by sequencing.

492

493 **Cell culture and transfection**

494 *L. infantum* L4 strain (L4, MHOM/GR/78/L4) promastigotes were cultivated at 26° C in M199
495 medium supplemented with 10% foetal bovine serum, 100 µM adenine, 10 µg mL⁻¹ hemin, 40
496 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic and acid) (pH 7.4), and 50 units of
497 Penicillin-Streptomycin (Gibco Laboratories) (Kapler *et al.*, 1990) and *L. amazonensis* parasites
498 (MHOM/BR88/BA-125, Leila strain) were cultured in Schneider's insect medium
499 (Sigma Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (Gibco Laboratories,
500 Gaithersburg, MD).

501 Parasite transfection was performed in logarithmic phase promastigotes that were washed
502 in electroporation buffer (21 mM HEPES pH 7.5, 137 mM NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄
503 and 6 mM glucose) and transfected by electroporation with 20 µg of plasmids DNA ([pXNG-

504 *LinDYRK1*] , pXG-GFP-*LinDYRK1*] or [pXG-PAC-*LinDYRK1*-GFP]), according to previously
505 established protocols (Beverley & Clayton, 1993). Parasites transfected with [pXNG-*LinDYRK1*]
506 and [pXGGFP²-*LinDYRK1*] constructs were selected in fully supplemented culture medium in
507 the presence of 150 µg mL⁻¹ NTC, 50 µg mL⁻¹ G418 and 30 µg mL⁻¹ puromycin, respectively.

508 For the generation of *LinDYRK1* facilitated mutants, we followed the methodology
509 described (Dacher *et al.*, 2014). More specifically, *LinDYRK1*^{+/+}[pXNG-*LinDYRK1*] *L. infantum*
510 promastigotes were first transfected with 3 µg of PmeI linearised and dephosphorylated PAC
511 targeting cassette. Parasites were plated 12 h post transfection in solid medium (fully
512 supplemented M199 containing 1% (w/v) noble agar, 60 µg mL⁻¹ puromycin and 100 µg mL⁻¹
513 NTC. Two independent clones were selected to check for correct PAC cassette genomic
514 integration. Integration was validated by PCR using genomic DNA of the two colonies as
515 template and primers p14/p11 (**S4 Table**). After the confirmation of the cassette replacement into
516 the *DYRK1* genomic locus, two independent *LinDYRK1*^{+/+}[pXNG-*LinDYRK1*] heterozygous
517 clones **A** and **B**, were transfected with 3µg of PmeI linearised and dephosphorylated HYG
518 targeting cassette and selected in solid medium (see above), which contained 60 µg mL⁻¹
519 puromycin, 60 µg mL⁻¹ hygromycin B and 100 µg mL⁻¹ NTC. Representative homozygous clones
520 derived from both **A** and **B** heterozygous clones were selected and analysed for the correct
521 integration of the HYG replacement cassette and the absence of genomic *LinDYRK1* ORF with
522 the primer pairs p14/p12 and p14/p13, respectively (**S4 Table**). For generating “direct
523 knockouts”, the same procedure was performed in wild type cells that had not been previously
524 transfected with the construct [pXNG-*LinDYRK1*].

525 To negatively select against [pXNG-*LinDYRK1*], the NTC antibiotic was replaced by 50
526 µg mL⁻¹ of GCV in *LinDYRK1*^{-/-}[pXNG-*LinDYRK1*] and *LinDYRK1*^{+/+}[pXNG-*LinDYRK1*]
527 parasites, used as control, as previously described (Dacher *et al.*, 2014). For passive loss of the
528 [pXNG-*LinDYRK1*] episome, the NTC antibiotic was omitted from the culture medium. For
529 selecting single colonies parasites were plated on M199 agar plates (Dacher *et al.*, 2014).

530 Promastigote growth was assessed microscopically by counting fixed cells daily in a
531 malassez cell-counting chamber or by flow cytometry. All growth curve experiments were
532 performed at least three times.

533

534 **SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting**

535 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli
536 (Laemmli, 1970). For immunoblotting, proteins were transferred on a nitrocellulose filter
537 (Hybond C, Amersham Biosciences) and immunoblotting was performed as previously described
538 (Papageorgiou & Soteriadou, 2002). For the analysis of GFP expression, we performed
539 immunoblotting using monoclonal mouse antibodies anti-GFP (Miltenyi) and mouse anti- α
540 tubulin (Sigma), both at 1:2000 dilution. A mouse horseradish peroxidase-conjugated antibody
541 (Pierce) was finally added, and the image acquired by ECL® Plus (enhanced chemiluminescence)
542 (GE Healthcare), according to the manufacturer's instructions. After the development of the first
543 signal corresponding to GFP, the blots were stripped and reprobated with anti-tubulin antibody.

544

545 **Flow cytometry**

546 For measuring the GFP fluorescence of live parasites, parasites were incubated with 50 $\mu\text{g mL}^{-1}$
547 propidium iodide (PI). GFP was analysed in the FL1 channel or 525/40 BP filter and PI in FL2 or
548 610/20 BP of FACS Calibur (Becton Dickinson) and Cytotflex 5 (Beckman Coulter), respectively.
549 For cell cycle analysis, logarithmic parasites were synchronised in the G₁/S boundary with 2.5
550 mM of HU (Sigma Aldrich) for 12 h, as previously reported. The DNA content of promastigotes
551 was analysed by PI staining of RNase A treated and ethanol fixed parasites, as previously
552 described (Smirlis *et al.*, 2006, Hombach-Barrigah *et al.*, 2019). More specifically, cells were
553 fixed by addition of ice-cold ethanol and parasitic DNA content was measured by flow cytometry
554 following the incubation with 50 $\mu\text{g mL}^{-1}$ PI and 100 $\mu\text{g mL}^{-1}$ RNase A (Invitrogen) in PBS in

555 the FL2 or 610/20BP channels. For all experiments 10,000 events were collected and analysed.
556 Flow Jo (Miltenyi), MODFIT (Becton-Dickinson) and CELLQuest™ (Becton-Dickinson)
557 software were used to perform the analyses. All flow cytometry experiments were performed at
558 least three times and results presented are from one representative experiment.

559

560 **Fluorescence microscopy**

561 For immunofluorescence analysis, *L. infantum* promastigotes were incubated with 4% (w/v)
562 paraformaldehyde (PF) for 30 min at room temperature (RT). Cells were left to adhere in poly L-
563 lysine coated slides for 20 min. Parasites were blocked with 1% (w/v) BSA in PBS (Sigma
564 Aldrich) and permeabilised with 0.1% (v/v) triton X-100 (Sigma Aldrich) for 30 min and
565 subsequently stained with 1:200 diluted in PBS/ 0,1% (w/v) BSA, mouse anti-alpha tubulin
566 antibody (Sigma, 051M4771) followed by 1 $\mu\text{g mL}^{-1}$ anti-mouse IgG antibody conjugated with
567 Alexa Fluor® 594. Prior to mounting parasites were stained with 2 μM Hoechst 33342 for 15
568 min. Slides were mounted in Vectashield (Vector Laboratories).

569 Analysis of the localisation of *LinDYRK1* fusions with eGFP, was performed with
570 staining of live cells with ConA-Alexa594 (Molecular Probes), MitoTracker® Orange
571 CMTMRos (Thermo Fisher Scientific) and FM®4-64FX (Molecular Probes), aided with DNA
572 co-staining with 16 μM Hoechst 33342 (Molecular Probes) according to the manufacturer's
573 instructions. In brief, promastigotes were incubated with 5 $\mu\text{g mL}^{-1}$ of ConA and 20 $\mu\text{g mL}^{-1}$
574 FM4-64 for 3 min on ice followed by incubation at RT at various time points , and with 100 nM
575 mitotracker for 30 min at RT. ConA stained promastigotes were subjected to live imaging while
576 MitoTracker Orange and FM4-64 stained cells were fixed with 4% (w/v) PF and mounted, as
577 previously reported.

578 Images were collected using the ApoTome inverted (ZEISS) and the confocal
579 microscopes FluoView 1000 (Olympus; Tokyo, Japan) and TCS SP8P (Leica Microsystems

580 GmbH, Germany). For analysing the morphology of the parasites, images were collected and
581 analysed in Image J software.

582

583 **Transmission and scanning electron microscopy**

584 Parasites in stationary growth phase were fixed in a solution of 2.5% (v/v) glutaraldehyde, 2 %
585 (v/v) formaldehyde and 2.5 mM CaCl₂ in 0.1 M sodium cacodylate buffer pH 7.2, followed by
586 post-fixation in 1% osmium tetroxide and 0.8 % potassium ferrocyanide in the same buffer and
587 acetone gradient dehydration. Finally, the material was embedded in Poly/Bed resin; ultrathin
588 sections were stained with uranyl acetate and lead citrate and observed under a JEM 1320
589 transmission electron microscope (JEOL). For scanning electron microscopy, parasites were fixed
590 as abovementioned and washed in 0.1 M cacodylate buffer. Cells were left to adhere in poly L-
591 lysine coated slides for 20 min, and post-fixed with a solution of osmium tetroxide containing
592 0.8–1% of potassium ferrocyanide for 30 min and dehydrated with increasing concentrations of
593 ethanol (30%, 50%, 70%, 90% and 100% (v/v). Samples were subjected to the critical point,
594 metalised with gold and analysed in a JEOL JSM-6390LV scanning electron microscope.

595

596 **Lectin-mediated agglutination and complement lysis**

597 Agglutination assays using PNA (100 µg µL⁻¹; Sigma) were performed as previously described
598 (Sacks *et al.*, 1985). Stationary phase (day 2 stationary phase) were washed 2 times in PBS and
599 incubated at a density of 3*10⁸ mL⁻¹ with PNA in PBS at RT for 30 min and the suspension was
600 centrifuged at 150 x g for 5 min.

601 Complement lysis of promastigotes was performed according Späth *et al.*, 2003 (Späth *et*
602 *al.*, 2003). Promastigotes (10⁶), were washed and incubated for 30 minutes in 10% (v/v) human
603 serum, that previously separated by centrifugation at 6,000 x g from clotted donor blood, and 50

604 $\mu\text{g mL}^{-1}$ propidium iodide (Sigma). The percentage of fluorescence (lysis) was determined by
605 flow cytometry.

606

607 **cDNA synthesis and real-time quantitative reverse transcription-PCR**

608 First strand cDNA was synthesised from 1 μg total RNA using the (Protoscript M-MuLV Taq
609 RT-PCR kit, New England Biolabs) with oligo(dT)18 primers following the manufacturer's
610 instructions. The resulting cDNA was diluted 5 times (10 ng mL^{-1}) with nuclease-free water
611 (NEB). 50 ng of cDNA template were used in a final volume of 20 μL 1X SYBR Green (Kappa
612 Biosystems) based quantitative PCR reactions on a SaCycler-96 RUO cycler (Sacace
613 Biotechnologies, Italy). The protocol used for the qPCR was: 10 s at 94°C , 40 cycles comprising
614 of 30 s at 94°C , 20 s at 52°C , 30 s at 72°C , and a final step of 10 min at 4°C . Primer pairs p15-
615 p16, p17-p18 and p19-p20 were used to amplify meta 1 transcript (*LinJ.17.0990*), HASPB
616 (*LinJ.23.1220*) and prohibitin (*LinJ.16.1710*) respectively (**S4 Table**). The sequences of primer
617 pairs for ribosomal protein S29 transcript (*LinJ.28.2360*) have been previously described
618 (Alexandratos *et al.*, 2013). Gene expression levels were calculated using the comparative C_t
619 method as previously described (Alexandratos *et al.*, 2013), from duplicate or triplicate PCRs of
620 cDNAs synthesised from RNAs recovered from parasites in three different experiments.

621

622 **Infection of mice and determination of parasite burden with quantitative real-time** 623 **polymerase chain reaction (qPCR)**

624 Male BALB/c mice (6–8 weeks old) used in the present study were obtained from the breeding
625 unit of the Hellenic Pasteur Institute (HPI; Athens, Greece) and reared in institutional facilities
626 under specific pathogen-free environmental conditions at an ambient temperature of 25°C . Mice
627 were provided with sterile food and water ad libitum. Stationary-phase promastigotes were
628 harvested by centrifugation at $800\times g$ for 10 min at 4°C . The pellet was washed by PBS and

629 resuspended at a concentration of 4×10^8 cells mL⁻¹. A volume of 100 μ L of this preparation was
630 injected intravenously in the lateral tail vein of each mouse. Two weeks post infection, mice were
631 sacrificed by CO₂ asphyxiation and 2 mg of liver tissues were frozen for subsequent analysis.
632 DNA was extracted from about 2 mg of liver tissue from infected mice using NucleoSpin® DNA
633 Rapid Lyse (Macherey-Nagel) according to the manufacturer's instructions. Parasite burden was
634 determined using TaqMan-based qPCR assay as previously described (Margaroni *et al.*, 2017,
635 Tellevik *et al.*, 2014). Briefly, the assay was performed in the presence of 15 pmol of forward (5'-
636 7GGCGGCGGTATTATCTCGAT-3') and 5 pmol of reverse (5'-
637 ACCACGAGGTAGATGACAGACA-3') primers (VBC Biotech, Vienna, Austria) (targeting a
638 74- bp region of the gene encoding the arginine transporter AAP3 gene) and 25 pmol of TET-
639 labeled TaqMan® probe (TET-5'-ATGTCGGGCATCATC-3'-BHQ; VBC Biotech) and DNA
640 template (50 ng and 100 ng) in a TaqMan master mix (2 \times) (Kapa Biosystems, Wilmington, MA,
641 USA). Each qPCR test was run in triplicate on a SaCycler-96 RUO cycler (Sacace
642 Biotechnologies, Como, Italy). The cycling conditions were 95°C for 10 min, followed by 40
643 cycles at 95°C, 15 s and 62°C, 60 s. The standard curve method for absolute quantification of
644 parasite number was used. Quantification was performed using standard curves prepared from
645 DNA extracted from ten-fold serial dilution of *L. infantum* parasites (range 1– 1×10^5).

646

647 **Macrophage infection**

648 Peritoneal macrophages exudate from 6- to 8-week-old female BALB/c mice were collected after
649 4 days injection of 3% (v/v) thioglycollate (Sigma Aldrich), as described previously (Gomes *et*
650 *al.*, 2003). Macrophages were obtained by flushing the peritoneal cavity once with 10 mL ice-
651 cold saline and centrifuged at 300 \times g for 10 min. Cells were resuspended in complete RPMI
652 medium, seeded at 2×10^5 /well in 24 well plates containing 13mm-rounded glass slides, and
653 incubated at 37°C, 5% CO₂ overnight. Stationary phase *L. infantum* parasites were plated at a
654 parasite:host cell ratio of 10:1 and incubated for 24 h (time zero) at 37° C 5% CO₂. Infected

655 cultures were washed extensively with PBS and incubated at the same conditions during 24, 48
656 and 72 h. Cultures were then fixed with 4% (w/v) paraformaldehyde. DNA was marked with 3
657 μM DAPI (Sigma Aldrich). Plates were incubated at RT for 10 min followed by wash with PBS.
658 *In vitro* macrophage infections were performed in triplicates in at least three independent
659 experiments. One hundred macrophages were counted per replicate.

660

661 **Lipid extraction and analysis**

662 Total lipids were extracted from *L. infantum* by the Bligh and Dyer method (Bligh & Dyer, 1959).
663 Briefly, cells in stationary phase were collected by centrifugation ($800\times g$, 10 min), washed with
664 PBS, resuspended in 100 μL PBS and transferred to a glass tube. Following the addition of 375
665 μL of 1:2 (v/v) CHCl_3 : MeOH the samples were vortexed thoroughly before further vigorous
666 agitation for 1 hour at 4°C . Biphasic mixtures were generated by the addition of 125 μL of CHCl_3
667 with vortexing and then 125 μL of H_2O again with vortexing followed by centrifugation at
668 $1000\times g$ at RT for 5 min. The lower chloroform-rich phase was transferred to a new glass vial,
669 dried under nitrogen and stored at 4°C . The lipid extracts were dissolved in 50 μL
670 chloroform:methanol (1:2), 50 μL of acetonitrile:isopropanol:water (6:7:2) and 10 mM
671 ammonium acetate and analysed with a Sciex 4000 QTrap, a triple quadrupole mass spectrometer
672 equipped with an AdvionTriVersaNanoMate® source. The nanoelectrospray interface allowed
673 the delivery of samples by direct infusion ($\sim 125 \text{ nL min}^{-1}$) with lipids analysed in both positive
674 and negative ion modes using a capillary voltage of 1.25 kV with each spectrum encompassing at
675 least 50 repetitive scans. Tandem mass spectra (MS/MS) were obtained for precursors of
676 phosphatidylinositol (PI)/inositol-phosphoceramide (IPC) species in negative ion mode, with
677 parent-ion scanning of m/z 241 (collision energy of 70V); and phosphatidylcholine (PC)/
678 sphingomyelin (SM) species in positive mode, with parent-ion scanning of m/z 184 (collision
679 energy of 50V). Assignment of phospholipid species is based upon a combination of survey,
680 daughter and precursor scans, as well as previous assignments (Fernandez-Prada *et al.*, 2016,

681 Richmond *et al.*, 2010) and use of the LIPID MAPS: Nature Lipidomics Gateway
682 (www.lipidmaps.org).

683

684 **Identification and characterisation of fatty acids**

685 Total fatty acids were isolated from the *L. infantum* lipid extracts and converted to fatty acid
686 methyl esters (FAME) for GC-MS characterisation following the method of Ichihara and
687 Fukubayashi (2010) (Ichihara & Fukubayashi, 2010). This trans-methylation reaction forms
688 methyl esters of all fatty acids in the sample, regardless of the lipid they were attached to. Briefly,
689 lipid samples were resuspended in 300 µL of 8% (w/v) solution of HCl in methanol water (85:15
690 v/v) and 200 µL of toluene and 1.5 mL of methanol were added prior to incubation overnight at
691 45°C. Samples were dried down under nitrogen and 500 µL hexane and 500 µL distilled water
692 were added to create a biphasic mixture, of which the FAME containing upper hexane layer is
693 extracted and dried again under nitrogen. FAME samples were dissolved in 20 µL
694 dichloromethane and analysed by injection of 1 µL into a Agilent Technologies GC-MS (GC-
695 6890N, MS detector-5973) using a ZB-5 column (30 M x 25 mm x 25mm, Phenomenex) with a
696 temperature program of 50°C for 10 min followed by a rising gradient to 220°C at 5°C /min and
697 hold at 220°C for a further 15 min. Mass spectra were continuously acquired in the range of 50-
698 500 amu and peak identification was performed by comparison of the retention times and
699 fragmentation patterns with a bacterial FAME standard mixture that contains a cyclopropyl C19Δ
700 FAME, in addition to common fatty acids (SIGMA Supelco 47080-U).

701

702 **Identification and characterisation of sterols**

703 *L. infantum* lipid extracts were resuspended in 100 µL dichloromethane with 1 µL analysed by
704 gas chromatography-mass spectrometry (GC-MS) on an Agilent Technologies mass selective
705 detector (GC-6890N, MS detector-5973) with a ZB-50 column (15 M x 32 mm ID x 0.5 mm

706 thickness, (Phenomenex), injector at 270°C with a temperature program of 100°C for 1 min
707 followed by a rising gradient to 200°C at 8°C /min and hold at 200°C for a further 2 min,
708 followed by a second gradient at 3°C /min up to 300°C for a further 15 min. Electron ionisation
709 (EI) was at 70 eV energy, with an emission current of 50 µA and an ion source of 230°C. Mass
710 spectra were acquired from 50-550 amu. Identification was carried out by comparison of the
711 retention times and fragmentation patterns with sterols in the NIST/EPA/NIH Mass Spectral
712 Library and those reported in previous publications (Cauchetier *et al.*, 2002, Fernandez-Prada *et*
713 *al.*, 2016, Yao & Wilson, 2016). To examine changes in composition, the area under each sterol
714 peak was used for relative quantification.

715

716 **Ethics statement**

717 All animal protocols were conducted in strict accordance with the National Law 56/2013, which
718 adheres to the European Directive 2010/63/EU for animal experiments, and complied with the
719 Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines. The study was carried
720 out according to the approved by the Ministry of Rural Development and Food license no. 433-
721 01-2018. The human blood donor sampled was an author of this project. The Bioethics
722 Committee of Hellenic Pasteur Institute was aware of experiments that required human blood in
723 this project, and a waiver was received prior to the initiation of experiments (no.3156).

724

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736

737 **Conflict of Interest**

738 None of the authors of the above manuscript has declared any conflict of interest.

739

740 **Author contributions**

741 VPCR, MD, SYAM, GFS, MBPS and DS have made major contributions to the conception or
742 design of the study, VPCR,MD, SYAM, FK, AE, GFS, MBP and DS to the acquisition, analysis,
743 or interpretation of the data; VPCR GFS, SYA, MBPS and DS to the writing of the manuscript.

744

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949 **Tables**

950 **Table 1: Fatty acid and sterol content of *LinDYRK1*^{-/-} promastigotes and control wild type**
 951 ***LinDYRK1*^{+/+} and parental *LinDYRK1*^{-/-}[pXNG-*LinDYRK1*] cell lines**

| | | Mean mol % ±SD | | | |
|-------------|-------|--------------------------------|---|--------------------------------------|--------------------------------------|
| | | <i>LinDYRK1</i> ^{+/+} | <i>LinDYRK1</i> ^{-/-} [pXNG- <i>LinDYRK1</i>]A | <i>LinDYRK1</i> ^{-/-} A1 | <i>LinDYRK1</i> ^{-/-} B1 |
| Fatty acids | 14:00 | 1.70± 0.5 | 0.51 ± 0.09 | 0.63± 0.07 | 0.47± 0.32 |
| | 16:01 | 0.79± 0.56 | 0.42 ± 0.29 | 0.63 ± 0.19 | 1.10± 0.18 |
| | 16:00 | 8.23 ± 1.70 | 9.37 ± 4.25 | 11.71± 1.67 | 9.33± 0.94 |
| | 18:02 | 24.50± 1.70 | 19.24 ± 5.58 | 14.98 ± 6.36* | 15.73± 0.52* |

| | | | | | |
|--|---|-------------|--------------|---------------|---------------|
| | 18:01 | 41.57± 2.37 | 44.12 ± 3.31 | 41.87± 0.75 | 42.66± 0.29 |
| | 18:00 | 14.14± 0.73 | 17.19 ± 3.70 | 22.05± 3.01** | 19.91± 1.68** |
| | C19Δ | 5.46± 0.55 | 4.24 ±1.12 | 4.75± 1.12 | 7.12± 0.51 |
| | 20:02 | 2.64± 0.19 | 2.62± 0.00 | 2.09 ± 0.13 | 2.30± 0.06 |
| | 20:00 | 0.98± 0.21 | 2.29 ±1.68 | 1.29 ± 0.20 | 1.40± 0.09 |
| | SFA | 25.0± 3.14 | 29.4± 9.72 | 35.7± 4.81 | 31.1± 0.52 |
| | MUFA | 47.8± 1.26 | 48.8± 4.12 | 47.2± 1.67 | 50.9± 0.98 |
| | PUFA | 27.1± 1.88 | 21.9± 5.57 | 17.1± 6.48 | 18.0± 0.46 |
| Sterol (retention time min ⁻¹) | Cholesterol (35.21) | 27.66± 2.98 | 15.27± 0.01 | 14.49± 2.23 | 14.97± 4.66 |
| | Zymosterol (33.59) | 4.14± 0.75 | 7.47± 4.97 | 12.48± 1.28 | 4.51± 1.45 |
| | Cholesta-5,7,24-trien-3β-ol (30.21) | 8.57± 0.08 | 6.50± 1.46 | 7.55± 0.2 | 5.79± 2.37 |
| | Ergosterol (35.21) | 30.23± 0.54 | 29.92± 2.08 | 31.44± 3.82 | 30.83± 2.05 |
| | Ergosta-7,24-dien-3β-ol (35.76) | 11.71±0 | 18.66± 3.97 | 17.84± 0.28 | 22.18± 1.12 |
| | 14α-methylergosta-8,24(28)-dien-3β-ol (36.02) | 4.53± 0.31 | 4.57± 2.22 | 5.38± 1.59 | 3.33± 0.09 |
| | 14α-methylergosta-5,7,24(28)-trien-3β-ol | 6.42± 0.20 | 3.46± 0.79 | 3.80± 1.36 | 3.02± 0.21 |
| | Lanosterol (36.47) | 0.00± 0.00 | 4.99± 7.05 | 0.00± 0.00 | 8.93± 0.21 |
| | 4,14α-dimethylergosta-8,24(28)-dien-3β-ol (36.75) | 2.23±0.70 | 3.42± 1.97 | 3.11± 2.94 | 1.67± 2.36 |
| | Stigmasta-7,24(28)-dien-3β-ol (37.85) | 4.51± 2.20 | 5.74± 1.71 | 3.91± 0.96 | 4.77± 1.56 |

952 **Table 1 legend:** Fatty acid and sterol composition was determined by GC analysis as described
953 in Materials and Methods section. Data, expressed as mole percent, are means ±SD of 2
954 independent experiments. SFA = Saturated fatty acids, MUFA = monounsaturated fatty acids,
955 PUFA = polyunsaturated fatty acids. **p*< 0.01, two-tail, paired student's *t* test between the
956 replicates of mutants and the corresponding control values (*LinDYRK1*^{+/+} and *LinDYRK1*^{-/-}
957 [pXNG-*LinDYRK1*]; ** *p*=0.057, two-tail, paired Student's *t* test between the replicates of mutants
958 and the corresponding control values (*LinDYRK1*^{+/+} and *LinDYRK1*^{-/-}[pXNG-*LinDYRK1*]).

959

960 Figure legends

961 **Fig 1. DYRK family in *Leishmania* and sequence comparison of two related *Leishmania***
962 **DYRKs: *LinDYRK1* (*LinJ.15.0180*) and *LinJ.14.0890***

963 (A) Representation of unrooted phylogenetic tree of DYRK kinases using as input full protein
964 sequences. DYRK families and sub-families are indicated. The leishmanial DYRKs are
965 highlighted according to DYRK subfamilies colour. *LinDYRK1* (*Lin*.15.0180) is in bold red.
966 Accession numbers are shown in **S3 Table**. (B) Sequence comparison of two related *Leishmania*
967 DYRKs: *LinDYRK1* (*LinJ*.15.0180) and *LinJ*.14.0890. Conserved identical residues are shaded
968 in black. Different protein sequences or motifs are shown. PEST sequence is shown in violet
969 [predicted by epestfind (<http://emboss.bioinformatics.nl/cgi-bin/emboss/epestfind>)] and
970 DYRK homology box (DH box) in green. Secondary structure predictions (α helix blue, β sheet
971 orange) of the kinase domains corresponding to the DYRK1A kinase domain ([http://](http://www.rcb.org/pdb/home.home.do)
972 www.rcb.org/pdb/home.home.do). Lines indicate kinase subdomains (I-XI) according to Hanks
973 and Hunter, 1995 (Hanks & Hunter, 1995) and functional features including the ATP anchor,
974 phosphate anchor, catalytic loop, cation binding site, activation loop, P+1 loop and CMGC insert
975 (red line). Grey represents important insertions in the sequence of *LinJ*.14.0890. Red arrow
976 represents the activation loop tyrosine (Y).

977

978 **Fig 2. Localisation of GFP-*LinDYRK1* in *Leishmania* promastigotes** (A) Fluorescence
979 confocal microscopy showing the localisation of the GFP-*LinDYRK1* hybrid protein (GFP-
980 *LinDYRK1*, green) in logarithmic (scale bar = 10 μ m), early stationary (scale bar = 10 μ m) and
981 late stationary promastigotes (scale bar upper row= 10 μ m; scale bar lower row= 5 μ m).
982 Promastigotes were stained with mitotracker (mitotracker, red) to visualise the mitochondrion and
983 Hoechst 33342 (Hoechst, blue) to visualise the nucleus (N) and kinetoplast (K). Bright field and
984 images with merged channels are also shown. (B) Live imaging showing the localisation of GFP-
985 *LinDYRK1* (green) *L. amazonensis* logarithmic promastigotes, in cells treated for 3 min with
986 ConA-Alexa594 (ConA, red) and Hoechst (blue) to visualise the nucleus (N) and kinetoplast (K).
987 Scale bar= 5 μ m. (C) Fluorescence confocal microscopy showing the localisation of the GFP-
988 *LinDYRK1* fusion protein (GFP-*LinDYRK1*, green) in logarithmic cells stained with FM®4-

989 64FX (FM4-64, Red) (pulse chase experiment) for 20 min at 4°C to visualise the endosomal
990 compartment and Hoechst 33342 (Hoechst, blue) to visualise the nucleus (N) and kinetoplast (K).
991 Bright field and images with merged channels are also shown Scale bar=10 µm, Insets: 2-fold
992 magnification

993

994 **Fig 3. Growth-curve and cell cycle analysis of *LinDYRK1* over-expressing promastigotes**

995 (A) Growth-curve of *LinDYRK1* over-expressing parasites. Left panel: 10^6 ml⁻¹ starting
996 inoculum. The growth of wild type promastigotes (*LinDYRK1*^{+/+}), of promastigotes transfected
997 with episome only (*LinDYRK1*^{+/+}[pXNG]), of promastigotes transfected with pXNG-*LinDYRK1*
998 (*LinDYRK1*^{+/+}[pXNG-*LinDYRK1*]) and of promastigotes that had lost the episome after 15
999 passages of negative selection in GCV (*LinDYRK1*^{+/+}[pXNG-*LinDYRK1*]GCV), seeded at 10^6
1000 mL⁻¹ was monitored daily by cell count. Bars indicate the mean ± standard deviation (SD) of five
1001 independent experiments. Right panel: 3×10^5 mL⁻¹ starting inoculum. The growth of
1002 promastigotes transfected with episome only (*LinDYRK1*^{+/+}[pXNG]), of promastigotes
1003 transfected with pXNG-*LinDYRK1* (*LinDYRK1*^{+/+}[pXNG-*LinDYRK1*]). Bars indicate the mean
1004 ± standard deviation (SD) of three independent experiments. (B) Cell cycle analysis of
1005 *LinDYRK1* transgenic parasites showing overlaid histogram plots of *LinDYRK1*^{+/+}[pXNG-
1006 *LinDYRK1*] (light blue) and *LinDYRK1*^{+/+}[pXNG] (pink) promastigotes. Cell cycle analysis was
1007 performed in promastigotes synchronised in the G₁/S cell boundary with 2.5 mM HU for 12 h or
1008 in promastigotes after 3 h and 6.5 h post-HU release. Diploid (2N) and tetraploid (4N) DNA
1009 content is indicated. This experiment is representative of three experiments performed.

1010

1011 **Fig 4. Time-course analysis of *LinDYRK1*^{-/-}[pXNG-*LinDYRK1*] by negative GCV selection**

1012 (A) Green fluorescent histograms (FL-1) of *LinDYRK1*^{-/-}[pXNG-*LinDYRK1*] clones (A and B)
1013 during 4 cell culture passages with 50 µg mL⁻¹ GCV (GCVp1- GCVp4) are used to quantify GFP
1014 levels and hence the presence of [pXNG-*LinDYRK1*]. GCV treated clones were compared to

1015 clones cultivated with $150 \mu\text{g mL}^{-1}$ of the positive selection drug, NTC (NTC). *LinDYRK1*^{+/+}
1016 [pXNG-*LinDYRK1*] parasites were used as control, and wild type cells were used to determine
1017 the background fluorescence level. The perpendicular line separates GFP positive fluorescence
1018 from the background fluorescence level. (B) Histograms that show the percentage of retained
1019 GFP fluorescence for *LinDYRK1*^{-/-}[pXNG-*LinDYRK1*] clones A and B, of 4 passages of
1020 selection with $50 \mu\text{g mL}^{-1}$ GCV (GCVp1- GCVp4) with respect to the GFP fluorescence of the
1021 same cells treated with $150 \mu\text{g mL}^{-1}$ NTC (NTC). *LinDYRK1*^{+/+} [pXNG-*LinDYRK1*] parasites
1022 were used as control. This experiment was performed three times and error bars represent the
1023 standard deviation of three different experiments. (C) Western blot analysis after 15 passages of
1024 selection with $50 \mu\text{g mL}^{-1}$ GCV. Twenty μg of total protein was extracted from late logarithmic
1025 phase promastigotes, was analysed by immunoblotting with anti-GFP antibody (GFP, and anti-
1026 alpha tubulin antibody (alpha tubulin) for normalisation. Left: Total protein extracts were
1027 analysed from late logarithmic *LinDYRK1*^{+/+}[pXNG-*LinDYRK1*] pool maintained in NTC, or
1028 after 15 passages of negative selection with GCV or of passive selection (no drug). Right: Total
1029 protein extracts from individual clones derived from pools of *LinDYRK1*^{-/-}[pXNG-*LinDYRK1*]
1030 cells (clone B) after 15 passages of negative selection (GCV) and of the same pool cultivated in
1031 the presence of NTC as a positive control. Here we only show the western blots derived from
1032 heterozygous clone **B**, but similar results were observed for clone **A**.

1033

1034 **Fig 5. *LinDYRK1*^{-/-} display aberrant morphology and increased cell-death in stationary**
1035 **growth-phase and during heat-shock**

1036 (A) Morphology of *LinDYRK1*^{-/-} promastigotes in stationary phase. Upper panel and lower left:
1037 Morphometric measurements showing roundness, body length and body width of *LinDYRK1*^{-/-} **A1**
1038 and **A2** clones, *LinDYRK1*^{-/-}[pXNG-*LinDYRK1*]A parental clone, *LinDYRK1*^{-/-} add back (**A1**
1039 clone) and *LinDYRK1*^{+/+} wild type cells. Bars represent mean \pm SEM. * Statistically significant
1040 ($p < 0.05$). Lower right: Immunofluorescence analysis of stationary phase *LinDYRK1*^{-/-}

1041 promastigotes and add back showing the morphology of alpha tubulin and DNA stained cells.
1042 Fluorescence microscopy shows nuclear and kinetoplast DNA, after labelling with Hoechst 33342
1043 (Hoechst, blue), and alpha tubulin (α tubulin, red) after labelling with anti-alpha tubulin antibody
1044 and secondary anti-mouse Alexa Fluor® 594 (alpha tubulin, red). Scale bar= 1.5 μ m. (B) DNA
1045 content determination of *LinDYRK1*^{-/-} promastigotes in logarithmic and late stationary (day 3)
1046 growth phase by flow cytometry. Flow cytometry profiles showing the DNA content of control
1047 wild type parasites *LinDYRK1*^{+/+}, parental cell line *LinDYRK1*^{-/-}[pXNG-*LinDYRK1*]A and two
1048 independent knockout mutants *LinDYRK1*^{-/-}A1 and *LinDYRK1*^{-/-}A2 and the *LinDYRK1*^{-/-}A1 add
1049 back. Diploid (2N) and tetraploid (4N) DNA content is indicated. Arrows indicate great increase
1050 of hypodiploid DNA content. This is a representative experiment of three.

1051

1052 **Fig 6. Ultrastructural alterations and fatty acid composition in *LinDYRK1* null mutants**

1053 (A) Scanning electron microscopy (SEM) of *LinDYRK1* promastigotes in stationary phase. SEM
1054 showing the rounded promastigote cell shape and membrane alterations of stationary phase
1055 *LinDYRK1*^{-/-} null mutants and comparison with wild type *LinDYRK1*^{+/+} and parental cell line
1056 *LinDYRK1*^{-/-}[pXNG-*LinDYRK1*] in the same growth phase. Arrows indicate sites of surface
1057 irregularities and membrane invaginations of null mutants. Scale bars are indicated on each image
1058 (B) Transmission electron microscopy (TEM) of *LinDYRK1* promastigotes in stationary phase.
1059 TEM analysis of (I) wild type *LinDYRK1*^{+/+}, (II) *LinDYRK1*^{-/-}[pXNG-*LinDYRK1*] and (III-VI)
1060 *LinDYRK1*^{-/-} stationary phase promastigotes. Figure shows increased accumulation of lipid (Ld)
1061 bodies in null mutant parasites. Black arrow in V indicates kinetoplast membrane inside a vacuole
1062 and white arrows (III, IV) show open membrane invaginations filled with lipids (IV, V). Scale
1063 bars are indicated on each image. N, nuclei; K, kinetoplast; Fp, flagellar pocket; V, vacuole. (C)
1064 Fatty acid composition of *L. infantum* promastigotes in stationary phase. Composition of total
1065 fatty acids isolated from *LinDYRK1*^{+/+}, *LinDYRK1*^{+/+}[pXNG-*LinDYRK1*] and *LinDYRK1*^{-/-}
1066 mutants. Total fatty acids from two independent cultures of *LinDYRK1*^{+/+}, *LinDYRK1*^{+/+}[pXNG-

1067 *LinDYRK1*]**A** and two independent clones derived from different parental mutants *LinDYRK1*^{-/-}
1068 **A** and *LinDYRK1*^{-/-}**B** were analysed with gas chromatography mass spectrometry (GC-MS). Bars
1069 show the mean values percentages of saturated (0 double bonds), monounsaturated (1 double
1070 bond) and polyunsaturated fatty acids (≥ 2 double bonds).

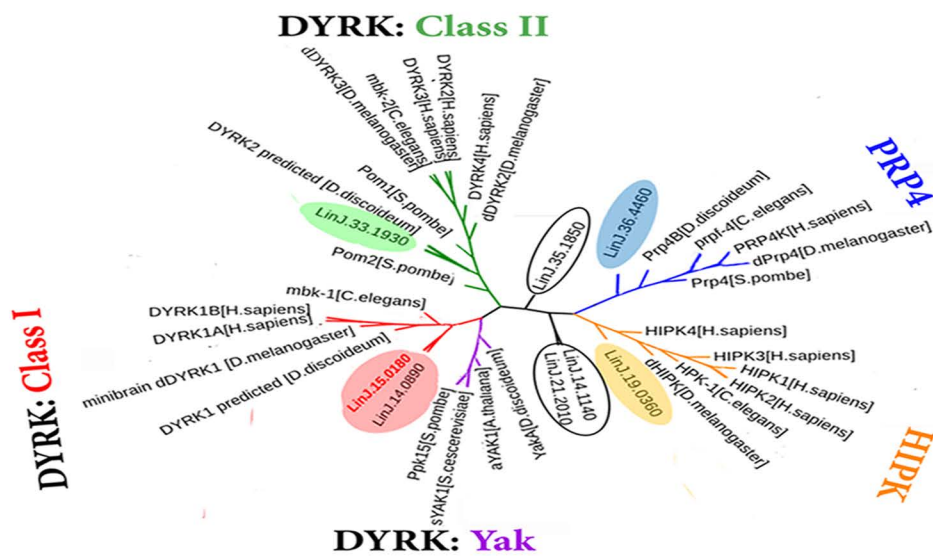
1071

1072 **Fig 7. Reduction of virulent traits, compromised survival in mouse peritoneal macrophages**
1073 **and increased heat shock sensitivity of *LinDYRK1*^{-/-} promastigotes**

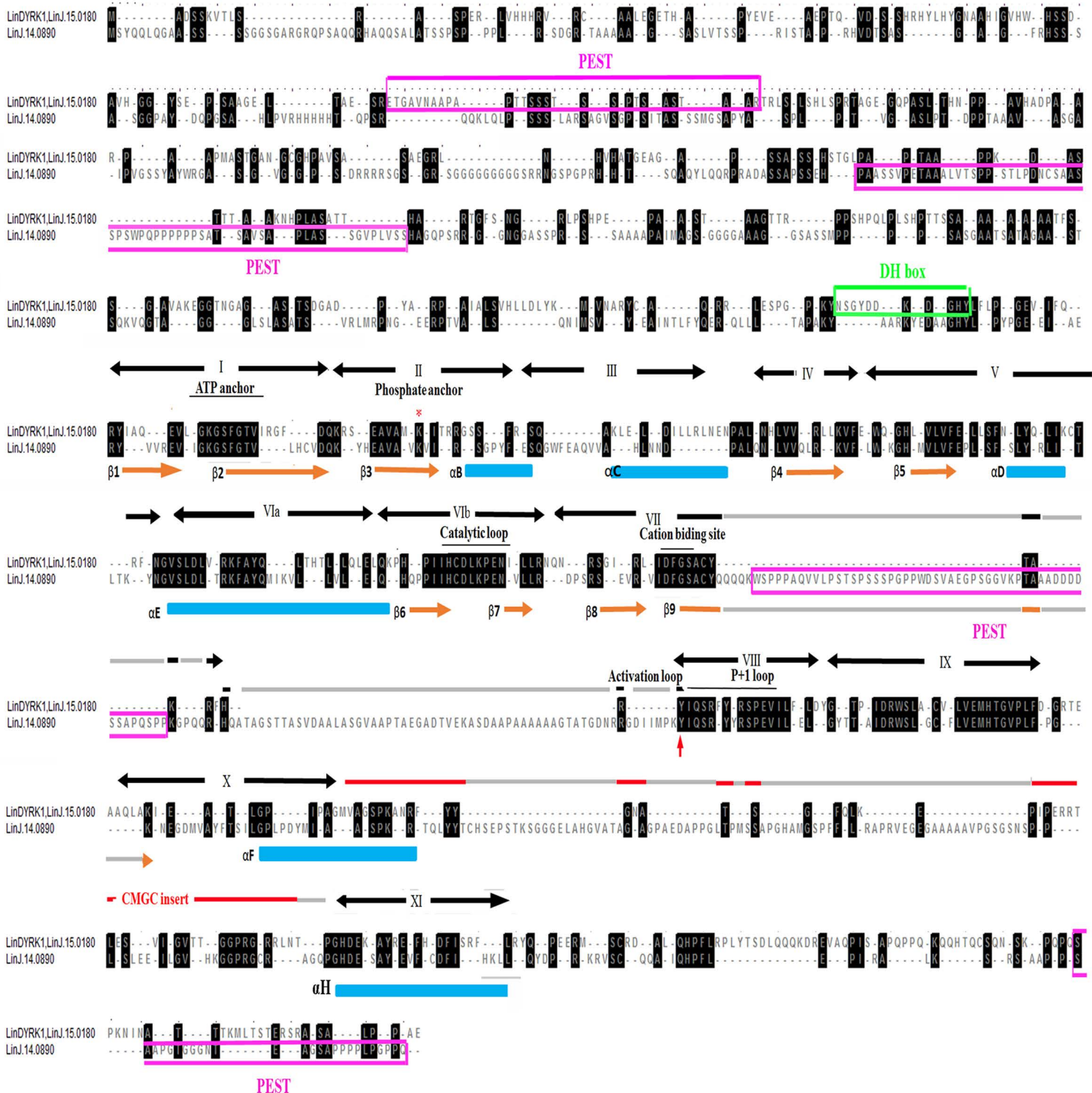
1074 (A) Column charts displaying the percentage of lysed (propidium iodide positive, PI+)
1075 *LinDYRK1*^{+/+}, *LinDYRK1*^{-/-}[pXNG-*LinDYRK1*] and *LinDYRK1*^{-/-} logarithmic (Log) and
1076 stationary (Stat) growth phase promastigotes by serum complement. Bars show the mean \pm
1077 standard error of the mean (SEM) of at least three experiments performed. Statistically significant
1078 * ($p < 0.05$) (Student's *t* paired test). (B) Column charts displaying the percentage of live
1079 (propidium iodide negative, PI-) unagglutinated *LinDYRK1*^{+/+}, *LinDYRK1*^{-/-}[pXNG-*LinDYRK1*]
1080 and *LinDYRK1*^{-/-} promastigotes after PNA agglutination assay. Bars show the mean \pm standard
1081 deviation (SD) of at least three experiments performed. Statistically significant * ($p < 0.01$), **
1082 ($p < 0.05$) (Student's *t* paired test). (C) Expression of prohibitin (*LinJ.16.1710*), meta 1
1083 (*LinJ.17.0990*) and HASPB (*LinJ23.1220*) transcripts Meta-1 transcripts, relative to ribosomal
1084 protein S29 transcript (*LinJ.28.2360*) in stationary phase *LinDYRK1*^{+/+}, *LinDYRK1*^{-/-}[pXNG-
1085 *LinDYRK1*] and *LinDYRK1*^{-/-} promastigotes by qPCR assessment. Expression levels are the
1086 mean of 3 independent experiments (3 independent cDNAs per cell line). Statistically significant
1087 * ($p < 0.05$). (D) Cell cycle analysis of *LinDYRK1*^{-/-} promastigotes under heat-shock.
1088 *LinDYRK1*^{+/+}, *LinDYRK1*^{-/-}[pXNG-*LinDYRK1*], *LinDYRK1*^{-/-}A1 and *LinDYRK1*^{-/-}A2 and
1089 *LinDYRK1*^{-/-} add back (A1 clone) promastigotes in logarithmic growth phase cultivated at 26°C
1090 and 37°C for 16 h. Diploid (2N) and tetraploid (4N) DNA content is indicated. Results are
1091 representative of 3 experiments. Arrows show the increased percentage of hypodiploid cells. (E)
1092 Reduced intracellular survival in peritoneal macrophages of *LinDYRK1*^{-/-} parasites in comparison

1093 to survival of *LinDYRK1*^{-/-}[pXNG-*LinDYRK1*] and *LinDYRK1*^{+/+} promastigotes. The numbers
1094 of parasites per cell, as well as the percentage of infection at 0 h and 72 h post-infection were
1095 calculated based on the number of parasite and host nuclei. Bars show the mean ± SEM of three
1096 experiments performed.

A

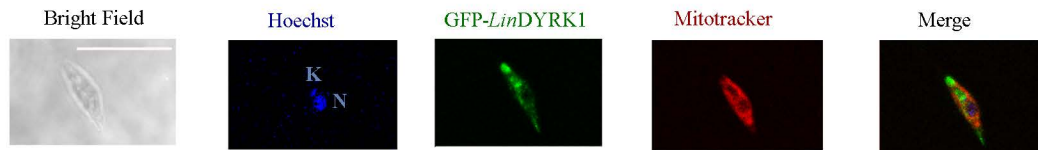


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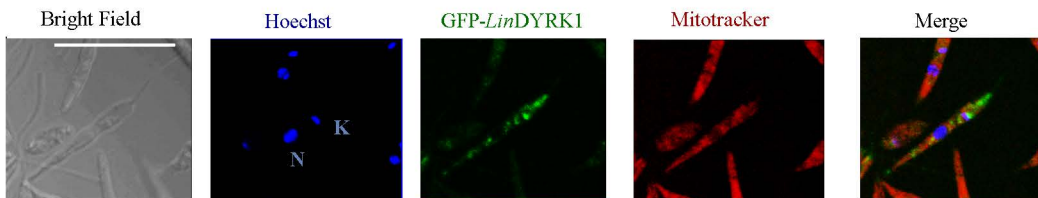


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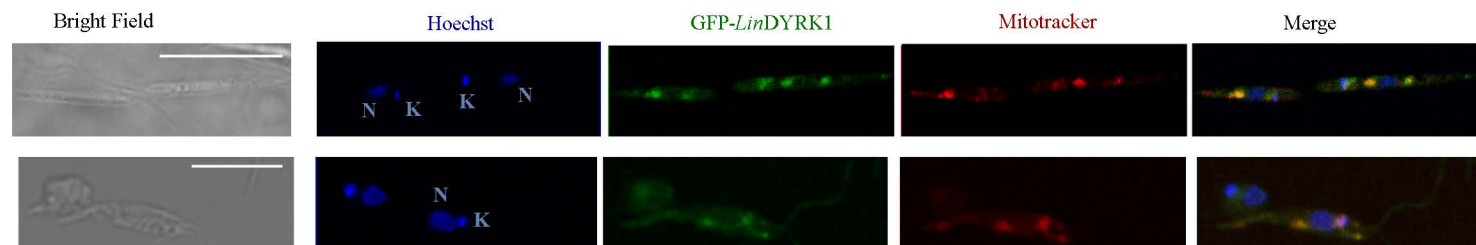
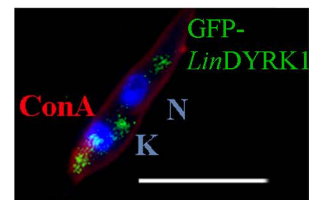
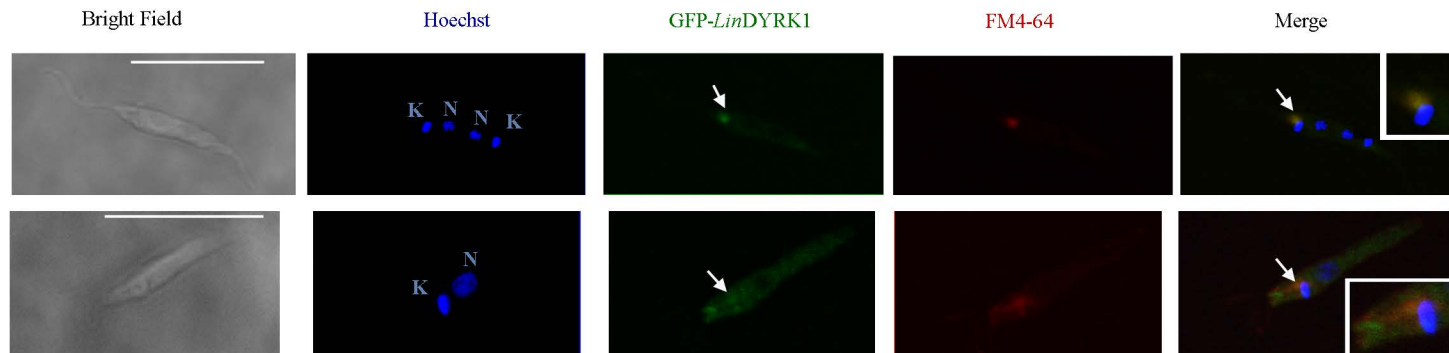
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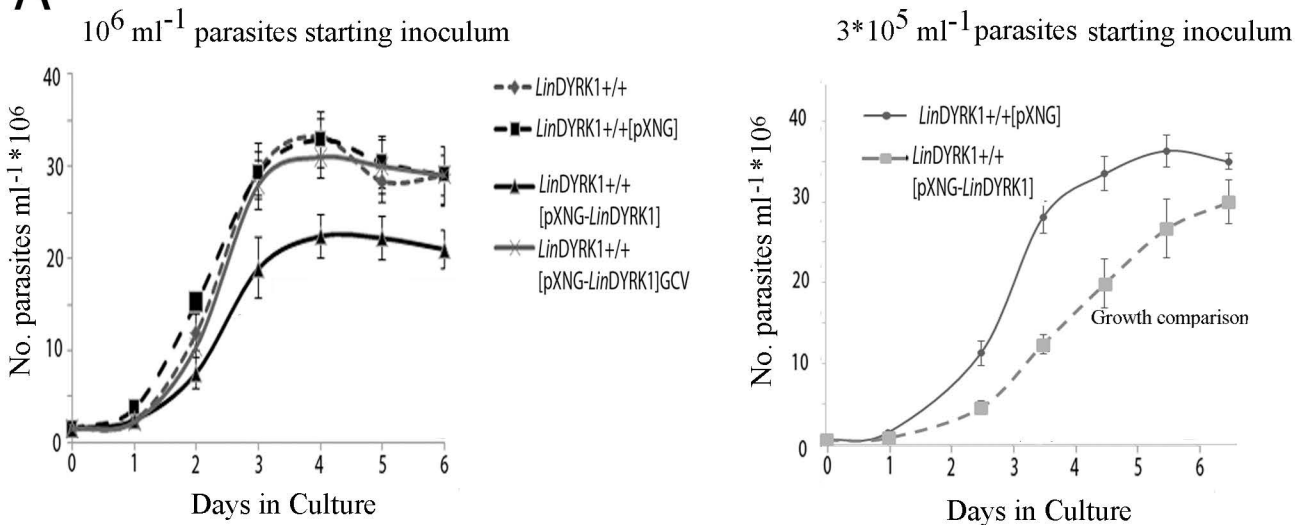
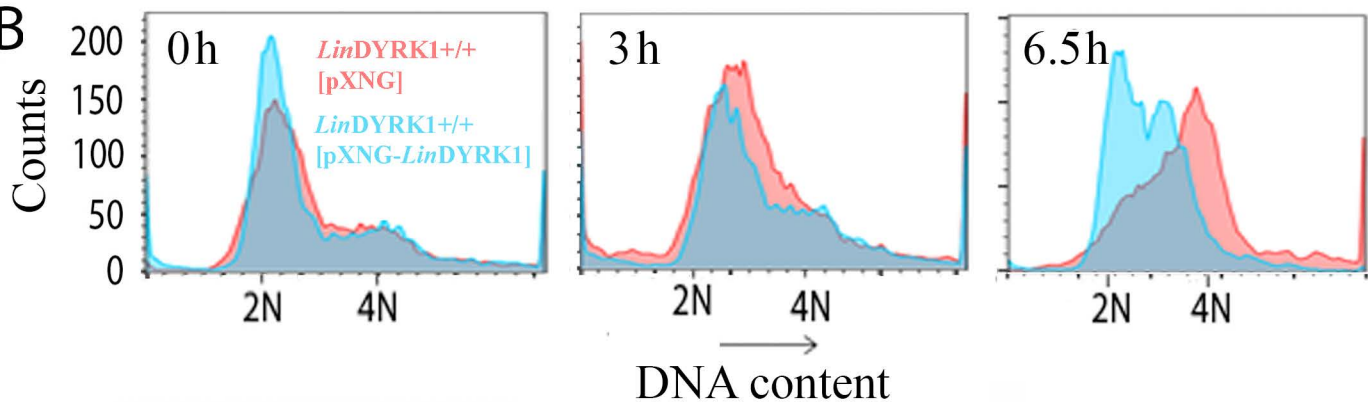


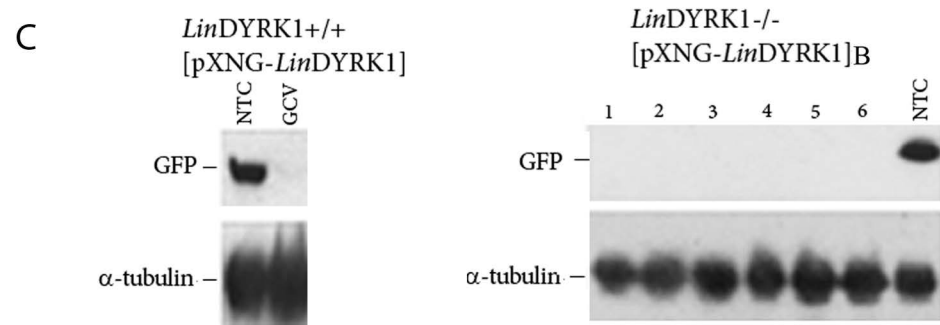
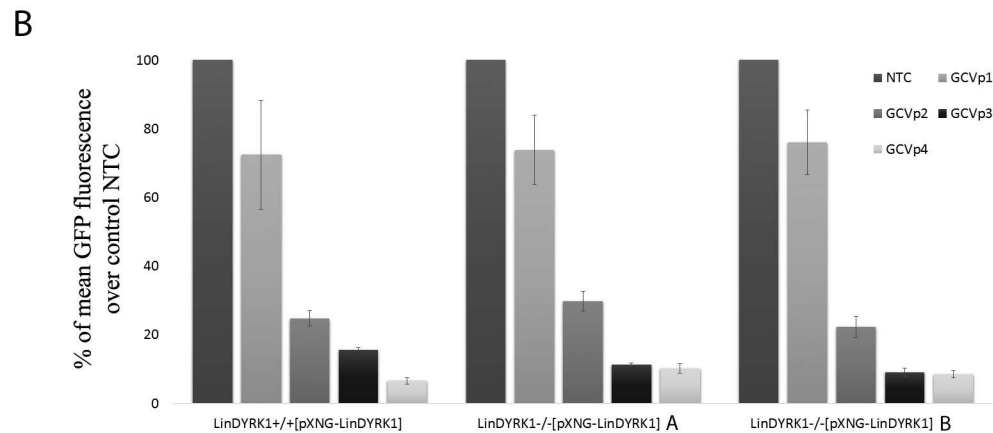
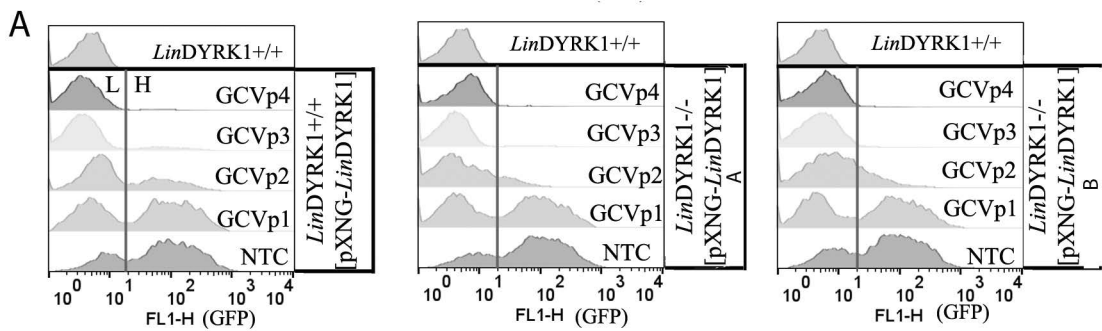
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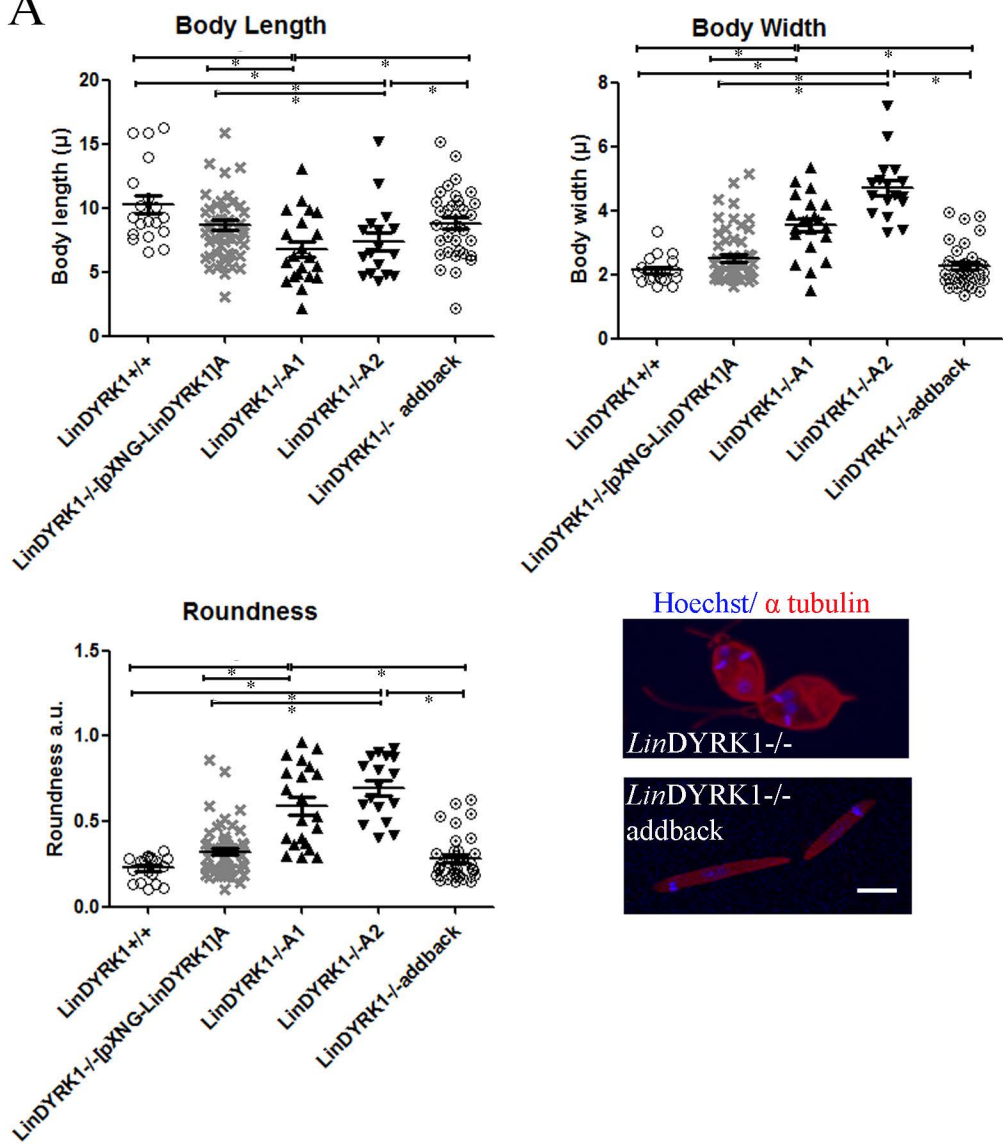
late stationary

**B****C**

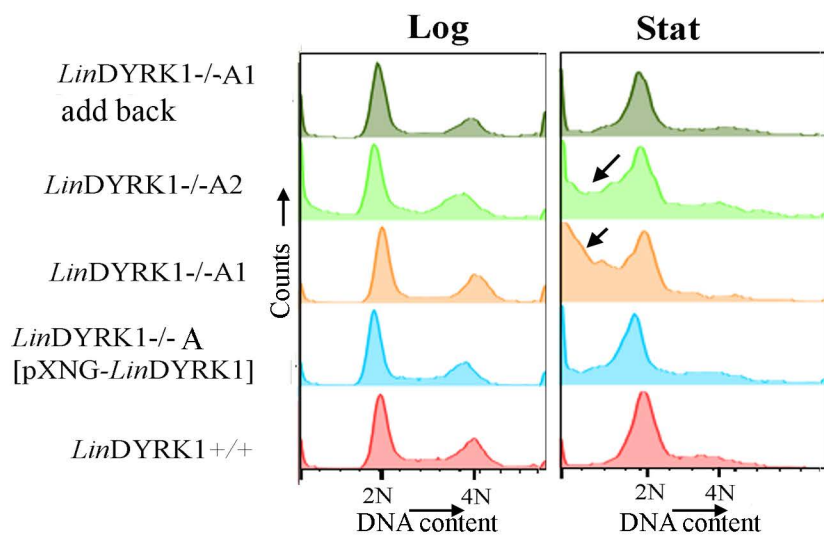
A**B**

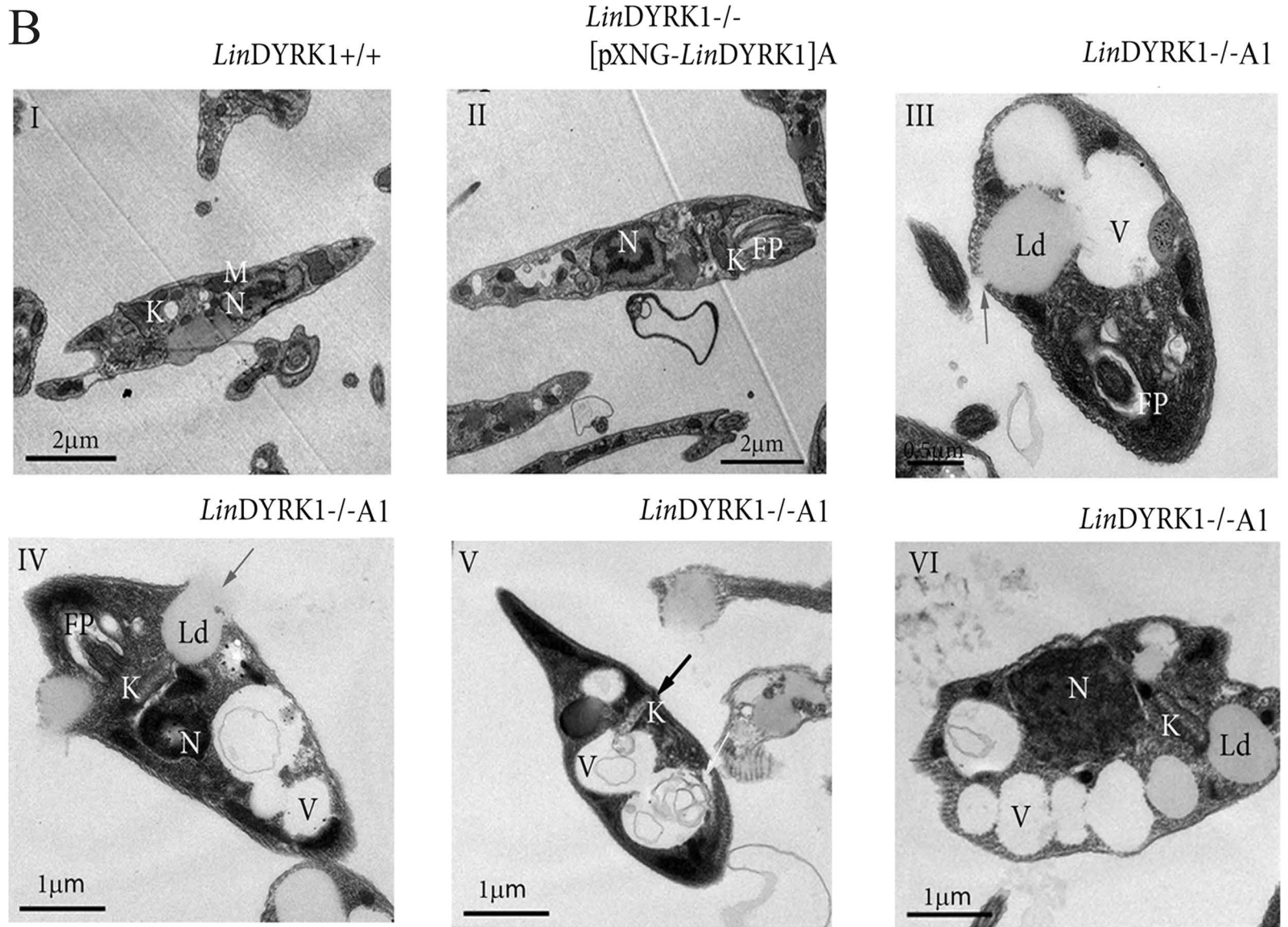
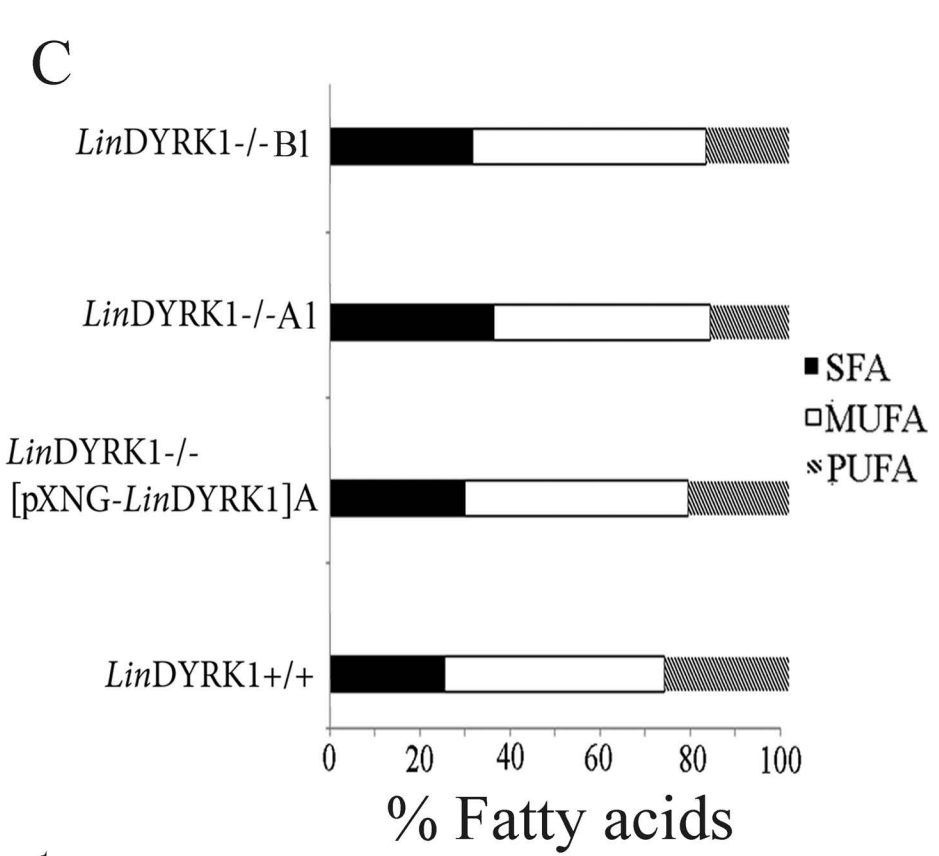
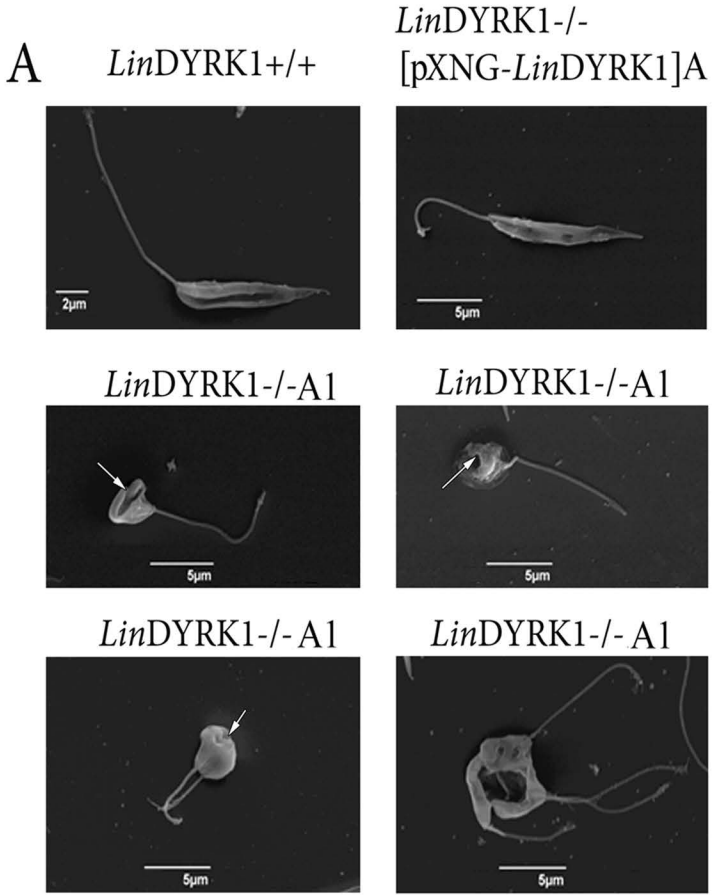


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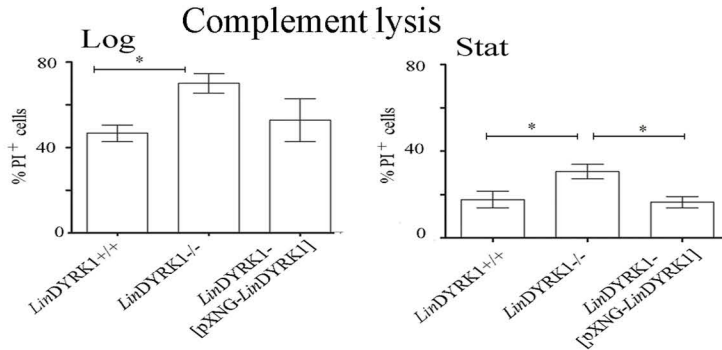


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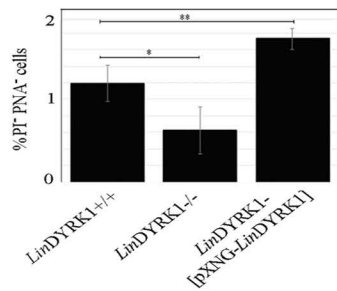




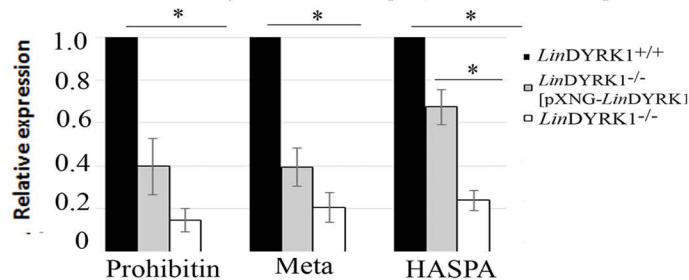
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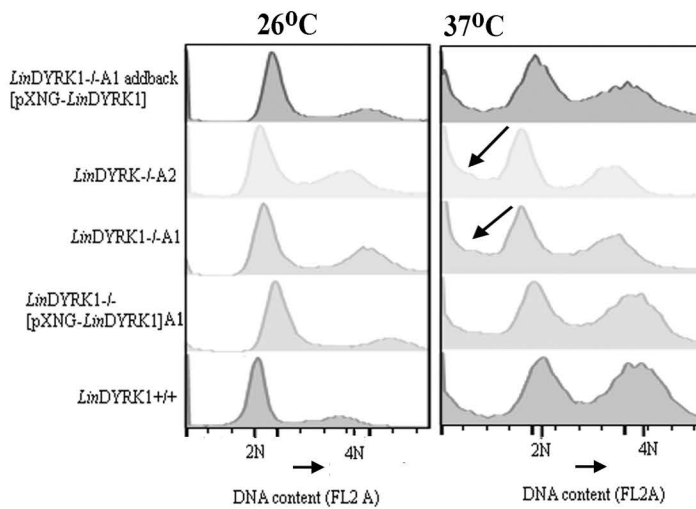
B Peanut agglutinin assay



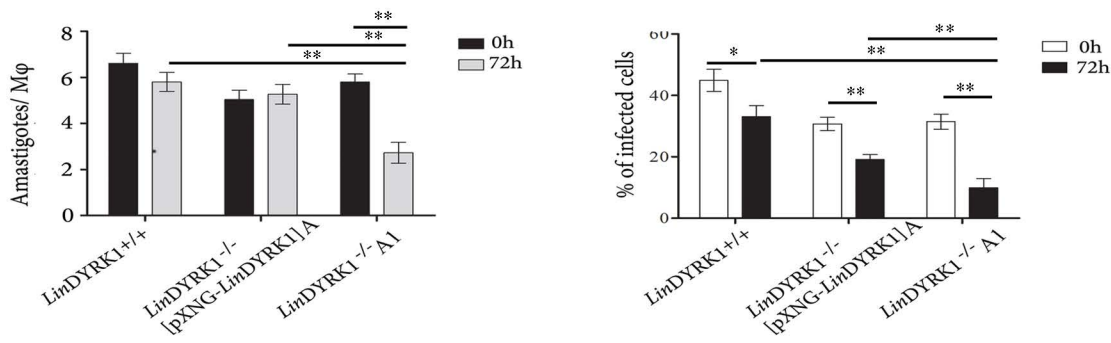
C Normalised target transcript expression relative to s39 ribosomal protein transcript



D



E



1 ***Leishmania* dual-specificity tyrosine-regulated kinase 1**
2 **(DYRK1) is required for sustaining *Leishmania* stationary**
3 **phase phenotype**

4
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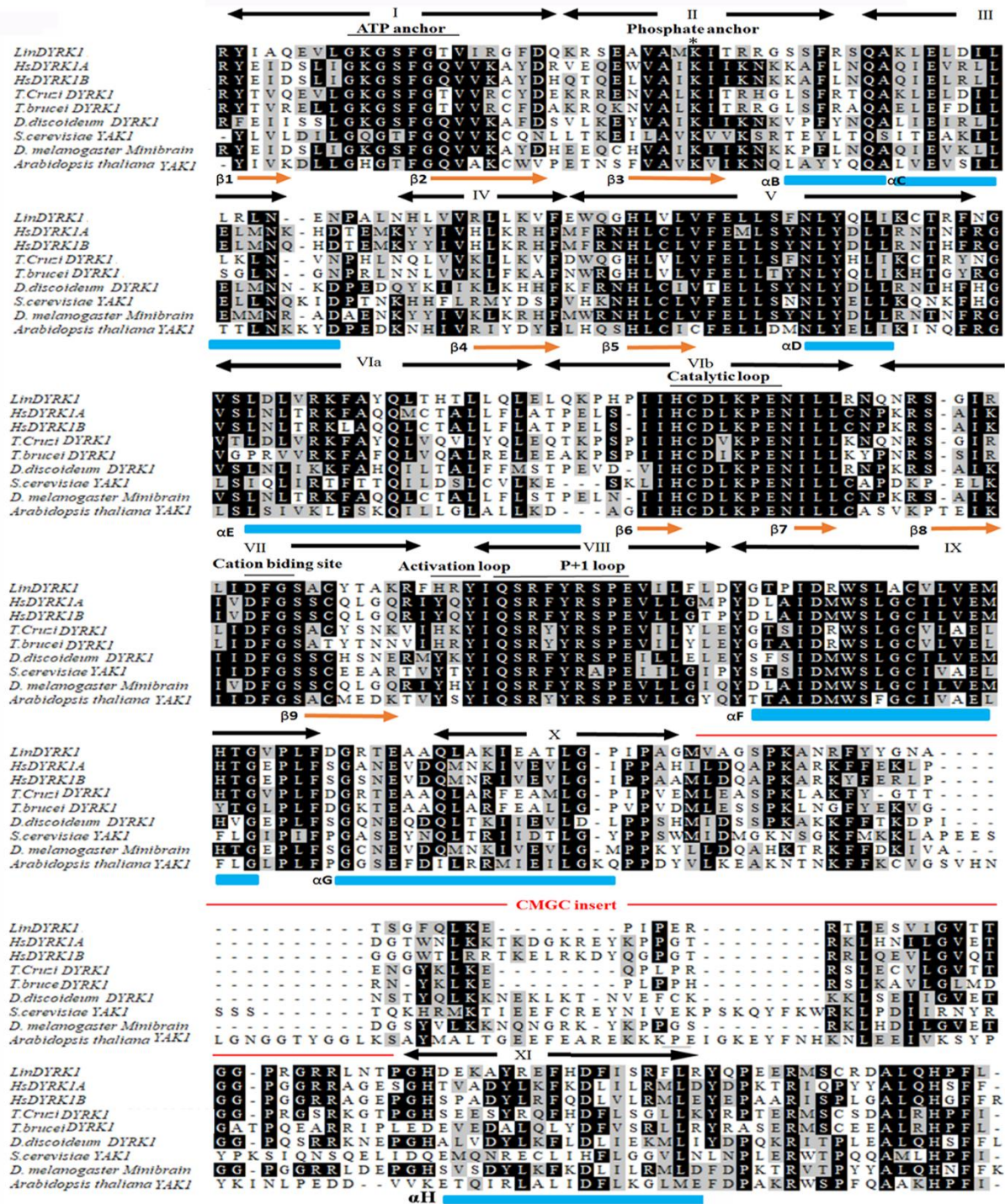
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24 S1 Fig. Kinase domain alignment of DYRK1 and related kinases from different organisms.

25 Alignment of the kinase domains of DYRK1 kinases and Yaks syntenic trypanosomatid

26 DYRK1s [*T. cruzi* DYRK1 (A0A422PWG1), *T. brucei* DYRK1 (AAZ11280)], of the *H. sapiens*

27 (Hs) DYRK1A (Q13627) and HsDYRK1B (Q9Y463), of *S. cerevisiae* YAK1 (P14680), *D.*
28 *discoideum* DYRK1 (Q76NV1) and of *D. melanogaster* Minibrain (P49657). Identical aminoacids
29 are shaded in black and similar in grey. Secondary structure predictions (α helix blue, β sheet
30 orange) of the kinase domains correspond to the DYRK1A kinase domain
31 (<http://www.rcb.org/pdb/home.home.do>). Lines indicate kinase subdomains (I-XI), according to
32 Hanks and Hunter (Hanks & Hunter, 1995) and functional features including the ATP anchor,
33 phosphate anchor, catalytic loop, cation binding site, activation loop, P+1 loop and CMGC insert
34 (red line).

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52 **S2 Fig**



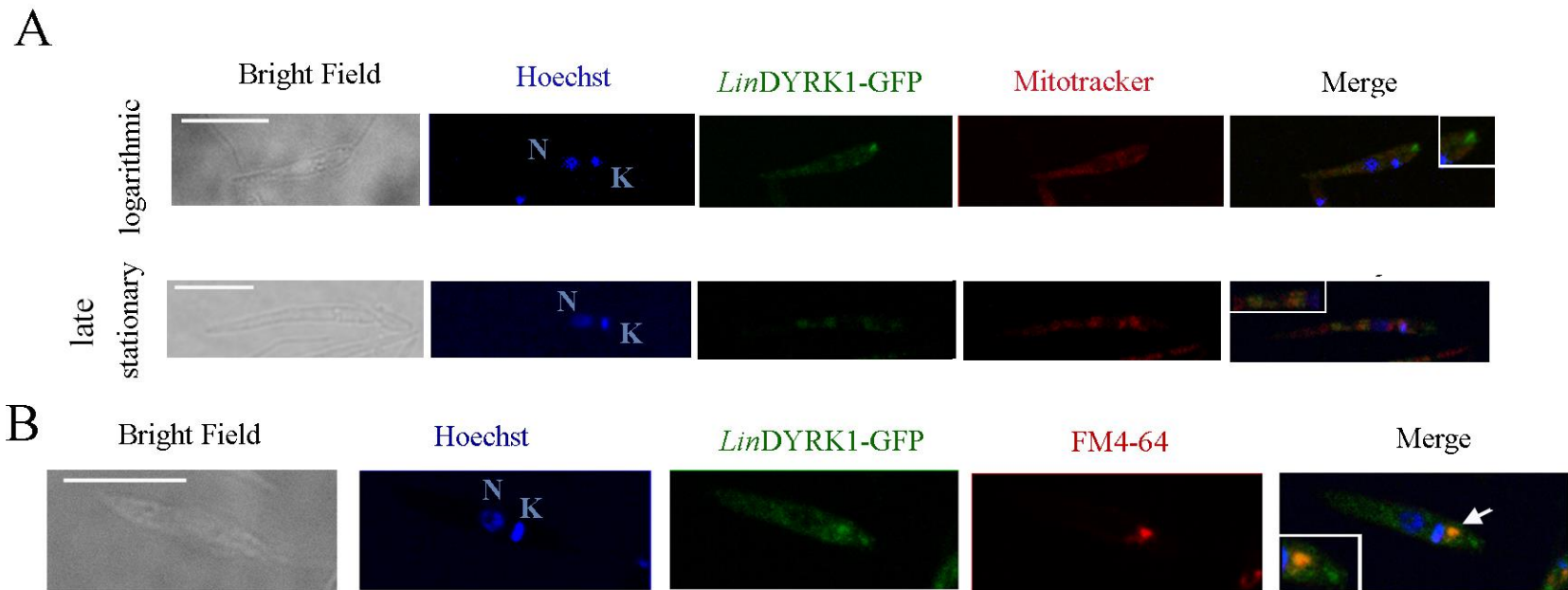
53

54 **S2 Fig. Multiple sequence alignment.** Protein sequence alignment of the kinase domain of the
 55 kinase domain of the eight *L. infantum* DYRKs using CLUSTALW as described in “Materials
 56 and Methods”. Identical amino acids are shaded in black and similar in grey. Secondary structure

57 predictions (α helix blue, β sheet orange) of the kinase domains correspond to the DYRK1A
58 kinase domain (<http://www.rcb.org/pdb/home.home.do>). Lines indicate kinase subdomains (I-XI)
59 according to Hanks and Hunter (Hanks & Hunter, 1995) and functional features including the
60 ATP anchor, phosphate anchor, catalytic loop, cation binding site, activation loop, P+1 loop and
61 CMGC insert (red line).

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82 Fig. S3



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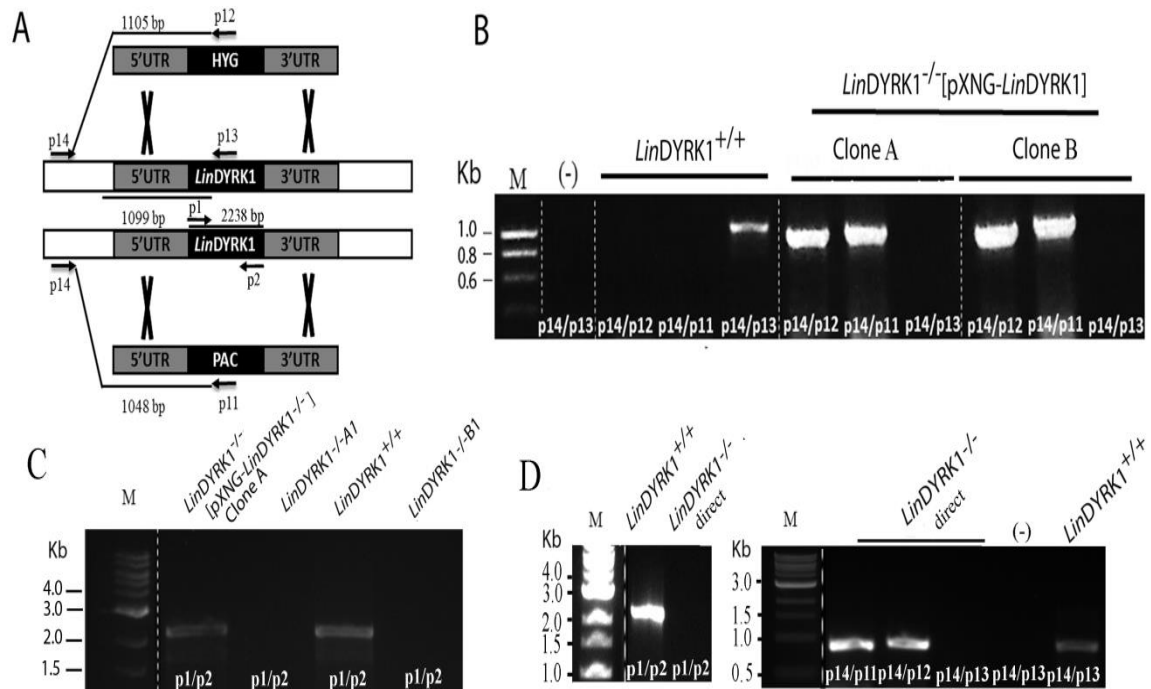
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85 **S3 Fig. Localisation of *LinDYRK1-GFP*.** Fluorescence confocal microscopy showing the localisation of the *LinDYRK1-GFP* hybrid protein
86 (*LinDYRK1-GFP*, green) in logarithmic (scale bar = 5 μ m), and late stationary promastigotes (scale bar= 5 μ m). Promastigotes were stained with
87 mitotracker (mitotracker, red) to visualise the mitochondrion and Hoechst 33342 (Hoechst, blue) to visualise the nucleus (N) and kinetoplast (K).
88 Bright field and images with merged channels are also shown. (C) Fluorescence confocal microscopy showing the localisation of the GFP-
89 *LinDYRK1* hybrid protein (*LinDYRK1-GFP*, green) in logarithmic cells stained with FM®4-64FX (FM4-64, Red) (pulse chase experiment) for 20

90 min at 4°C to visualise the endosomal compartment and Hoechst 33342 (Hoechst, blue) to visualise the nucleus (N) and kinetoplast (K). Bright
91 field and images with merged channels are also shown Scale bar= 5 μm, Insets: 1.5 fold magnificati

Supporting information

92 **S4 Fig**



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94 **S4 Fig. Generation of *LinDYRK1* facilitated null mutants.** (A) Schematic representation

95 showing the genomic structure of *LinDYRK1* and the gene replacement strategy. The two alleles

96 of *LinDYRK1* were replaced by HYG and PAC resistance cassettes yielding homozygous

97 facilitated null mutants in *LinDYRK1*^{-/-}[pXNG-*LinDYRK1*] (facilitated) or in wild type

98 *LinDYRK1*^{+/+} cells. The primers sets used for PCR amplifications and the predicted sizes of the

99 PCR products in base pairs (bp) are indicated. (B) Diagnostic PCR to evaluate the establishment

100 of facilitated knockouts generating independent clones *LinDYRK1*^{-/-}[pXNG-*LinDYRK1*]**A** and **B**.

101 PCR analysis from wild type *LinDYRK1*^{+/+} genomic DNA is used as well as a negative control a

102 PCR reaction with no DNA (-). The DNA marker with the band sizes indicated is also presented

103 (M). The size of the expected amplicons is indicated in Panel A. (C) Diagnostic PCR to evaluate

104 the presence of an endogenous *LinDYRK1* copy with the use of internal primers p1/p2, after the

105 loss of the pXNG-*LinDYRK1* episome in *LinDYRK1*^{-/-}**A1** and *LinDYRK1*^{-/-}**B1** mutants. As a

106 control wild type promastigotes (*LinDYRK1*^{+/+}) and *LinDYRK1*^{-/-}[pXNG-*LinDYRK1*] were also

Supporting information

107 included. The DNA marker with the band sizes indicated is also presented (M). (D) Diagnostic
108 PCR to evaluate to evaluate the establishment of conventional knockout clone (*LinDYRK1*^{-/-}
109 direct). Right panel: Primer pairs p11/p14 and p12/p14 were used to validate the placement of the
110 PAC and HYG cassettes respectively in *LinDYRK1*^{-/-direct} mutants. The p13/p14 primer set was
111 also used to validate the presence of an extra genomic copy of *LinDYRK1* within the *DYRK1*
112 genomic locus. As a positive control PCR analysis is performed with primer sets p13/14 and as
113 negative control PCR reaction with no DNA (-). The DNA marker with the band sizes indicated is
114 also presented (M). Left Panel: Diagnostic PCR to validate the absence of any genomic
115 *LinDYRK1* copy outside the *LinDYRK1* locus in *LinDYRK1*^{-/-direct} mutants. Primer set p1/p2 was
116 used to establish the absence of endogenous copy of *LinDYRK1* in *LinDYRK1*^{-/-direct} mutants
117 (*LinDYRK1*^{-/-direct}). As a positive control analysis of wild type (*LinDYRK1*^{+/+}) genomic DNA
118 was performed. (-). The DNA marker with the band sizes indicated is also presented (M).

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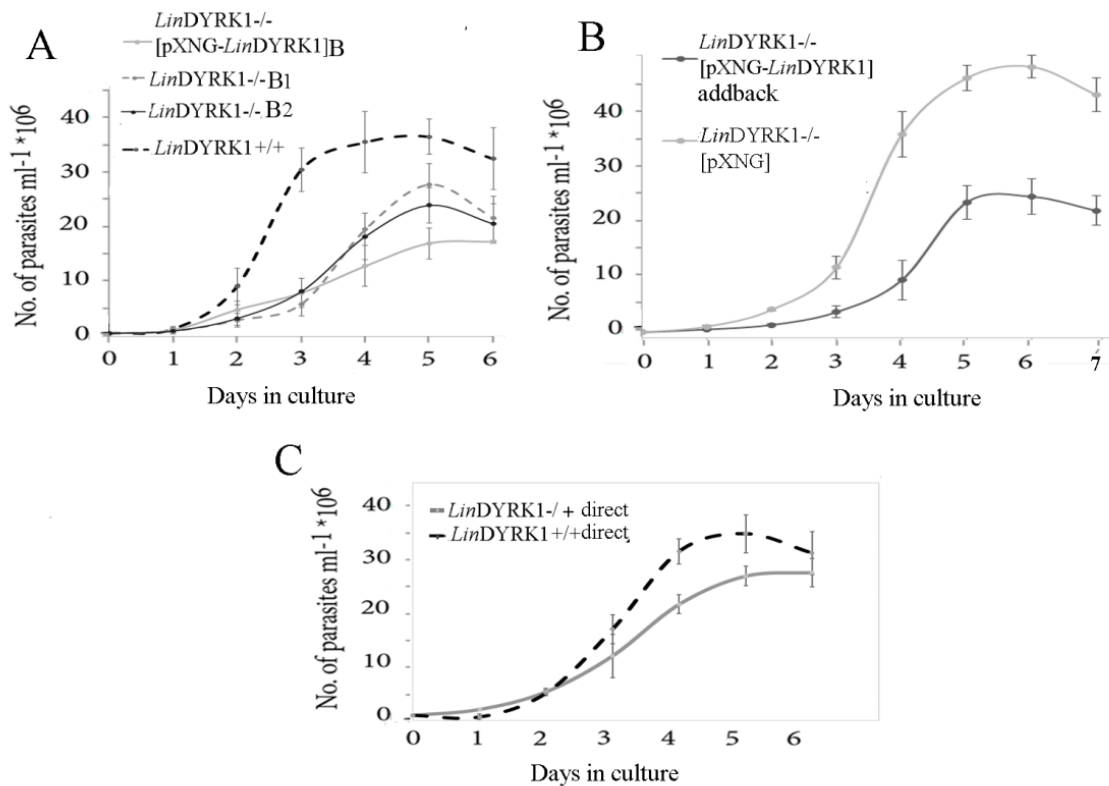
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Supporting information

132 S5 Fig

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135 S5 Fig. *LinDYRK1*^{-/-} growth curves (A) Growth curves of *LinDYRK1*^{-/-}B1 and *LinDYRK1*^{-/-}B2

136 with respect to the parental clone *LinDYRK1*^{-/-}[pXNG-*LinDYRK1*]B1 and to wild type cells.

137 Growth of parasites was monitored daily by cell count followed for six days. Error bars show

138 standard deviation of 5 different experiments. (B) Growth curves of *LinDYRK1*^{-/-}[pXNG] (clone

139 A1) and *LinDYRK1*^{-/-}[pXNG-*LinDYRK1*] addbacks (clone A1). Growth of parasites was

140 monitored daily by cell count followed for seven days. Error bars show standard deviation of 4

141 different experiments. (C) Growth curve of conventional mutant *LinDYRK1*^{-/-} (*LinDYRK1*^{-/-direct})

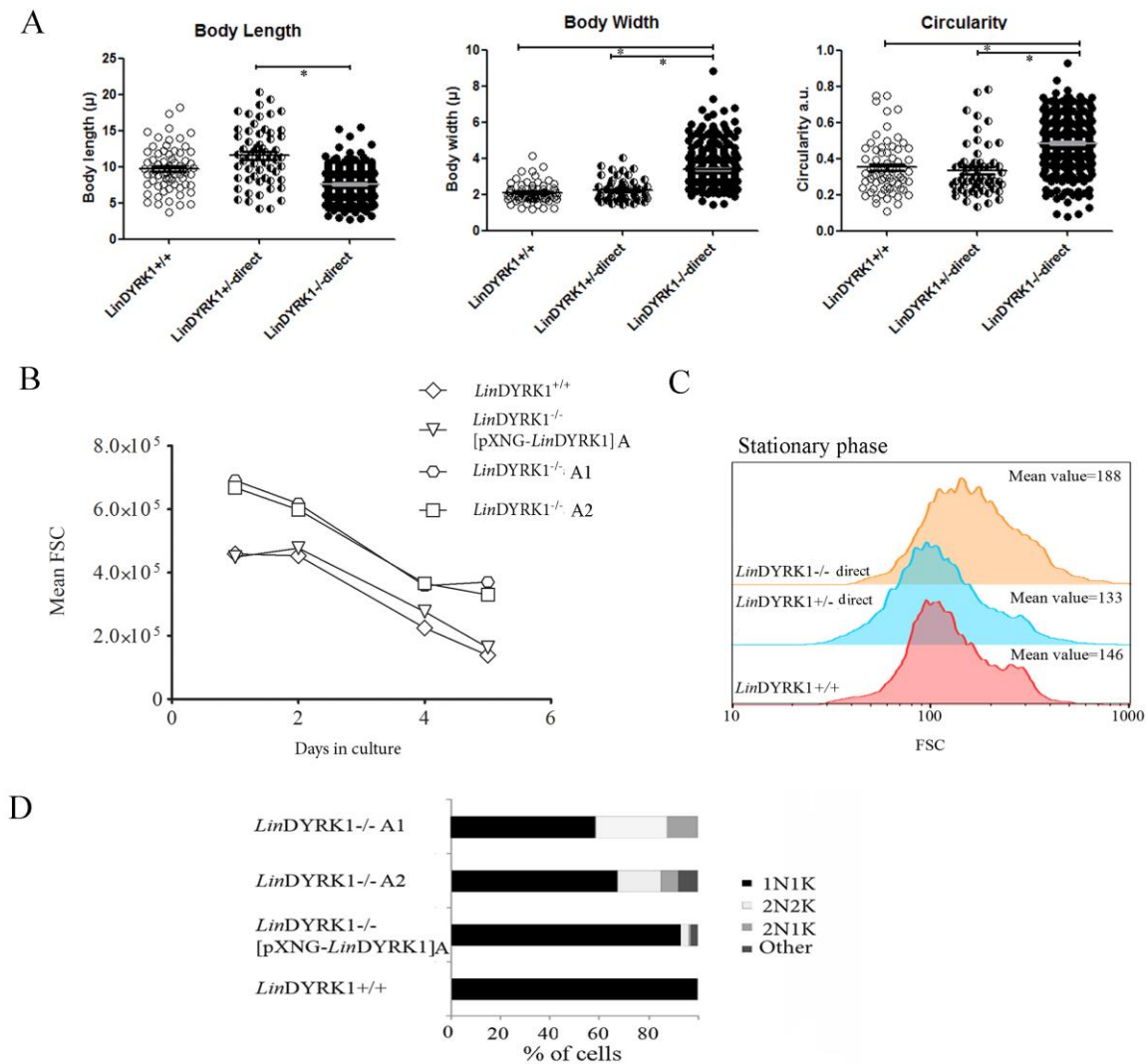
142 with respect to its heterozygous parental clone *LinDYRK1*^{+/+} (*LinDYRK1*^{+/+direct}), and wild type

143 promastigotes (*LinDYRK1*^{+/+}). Error bars show standard deviation of 4 different experiments.

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Supporting information

145 S6 Fig



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147 **S6 Fig. Morphology and DNA content of *LinDYRK1*^{-/-} stationary parasites (A)**

148 Morphometric measurements showing roundness, body length and body width of *LinDYRK1*^{-/-}

149 direct, *LinDYRK1*^{+/-direct} and *LinDYRK1*^{+/+} wild type cells. Bars represent mean \pm error. *

150 Statistically significant ($p < 0.05$). (B) The forward scatter (FSC) was analysed as an estimation of

151 cell size/morphology by flow cytometry in *LinDYRK1*^{-/-}A1, *LinDYRK1*^{-/-}A2 and their

152 comparison to wild type *LinDYRK1*^{+/+} and parental clone *LinDYRK1*^{-/-}[pXNG-*LinDYRK*]A. The

Supporting information

153 figure represents the mean FSC of 10,000 events per each cell line. (C) FSC histogram plots of
154 *LinDYRK1*^{-/-direct} (orange), *LinDYRK1*^{+/-direct} (blue) and wild type *LinDYRK1*^{+/+} (red)
155 promastigotes in mid stationary phase. Mean value is shown for 10,000 events. (D) Nuclear and
156 kinetoplast DNA content analysis of mid stationary promastigotes (25-40 *10⁶ mL⁻¹). Nuclei and
157 kinetoplasts of at least 50 parasites per clone were counted.

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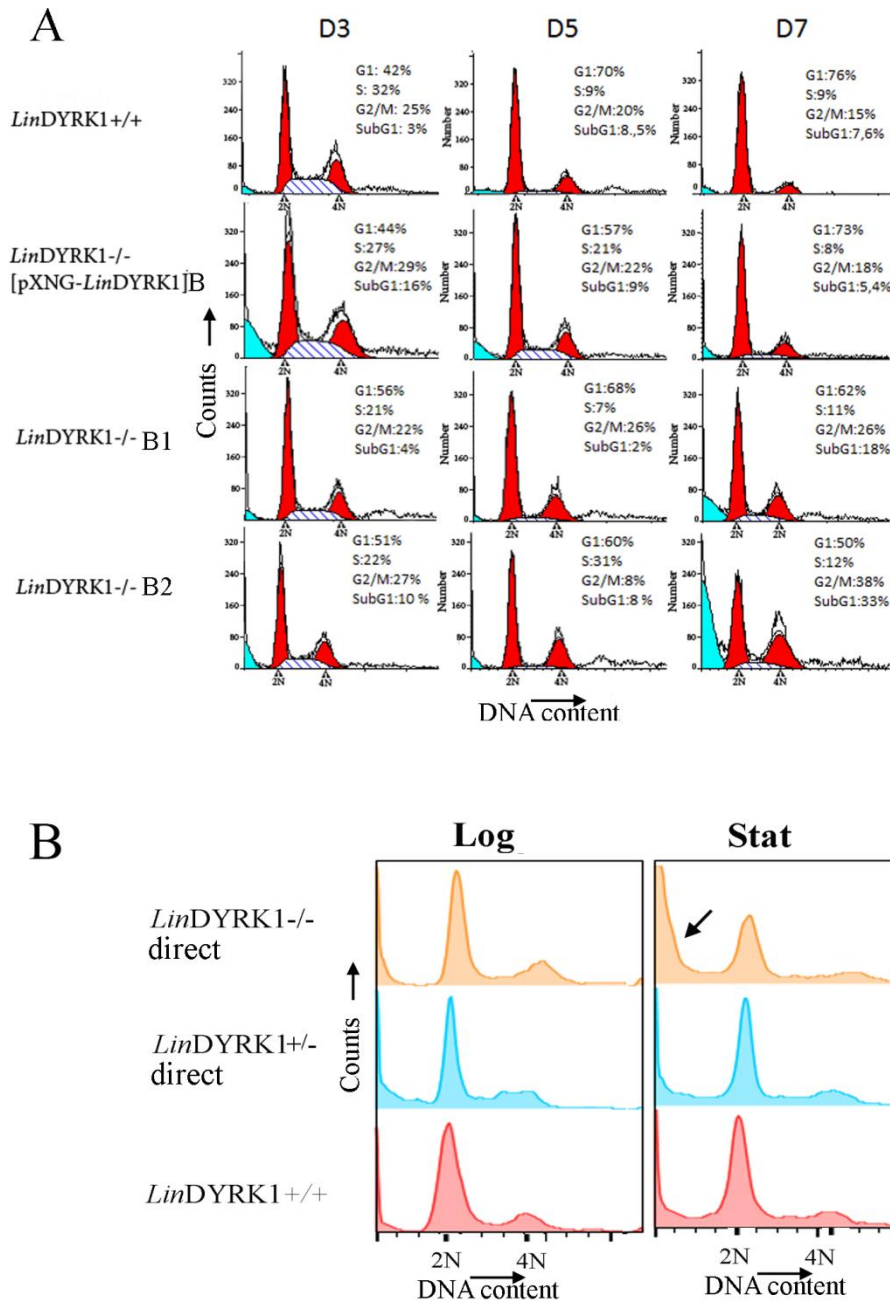
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Supporting information

178 S7 Fig



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180 **S7 Fig. Cell cycle analysis of *LinDYRK1*^{-/-} mutants at different days of cell culture.** (A) Cell
 181 cycle analysis of wild type *LinDYRK1*^{+/+}, parental *LinDYRK1*^{-/-} [pXNG-*LinDYRK1*]B pool and
 182 two *LinDYRK1*^{-/-} B clones (*LinDYRK1*^{-/-}B1 and *LinDYRK1*^{-/-}B2). Cell cycle analysis was
 183 performed on day 3 (logarithmic), day 5 (mid stationary phase), and day 7 (late stationary phase

Supporting information

184 promastigotes). Diploid (2N) and tetraploid (4N) DNA content is indicated. Cell cycle
185 distribution is presented on the right hand side of each image. This analysis is representative of
186 three independent culture experiments. (B) Cell cycle analysis of wild type *LinDYRK1*^{-/-direct},
187 parental heterozygous and wild type cells in logarithmic (Log) and late stationary phase (Stat)
188 Diploid (2N) and tetraploid (4N) DNA content is indicated. Cell cycle distribution is presented on
189 the right hand side of each image. Arrow represents a great increase in hypodiploid DNA content.
190 This analysis is representative of three independent culture experiments.

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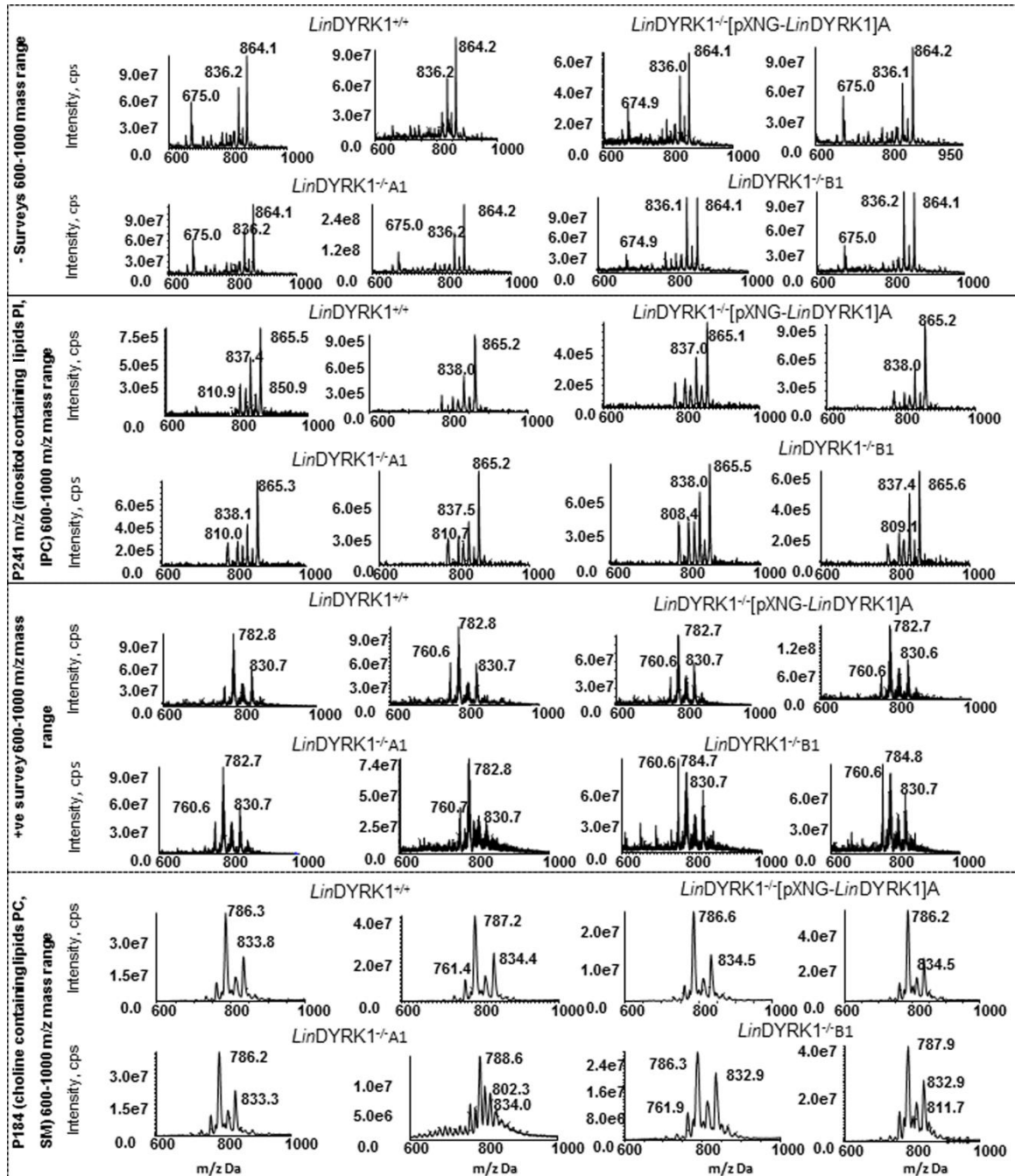
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205 **S8 Fig**

Supporting information



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207 **S8 Fig. Phospholipid analysis** Lipids isolated from *LinDYRK1^{+/+}*, *LinDYRK1^{-/-}[pXNG-*
 208 *LinDYRK1]B*, *LinDYRK1^{-/-}B* and *LinDYRK1^{-/-}B* in stationary phase were analysed in both
 209 positive and negative ion modes. Tandem mass spectra (MS/MS) were obtained for
 210 phosphatidylinositol (PI)/inositol-phosphoceramide (IPC) species in negative ion mode (parent-

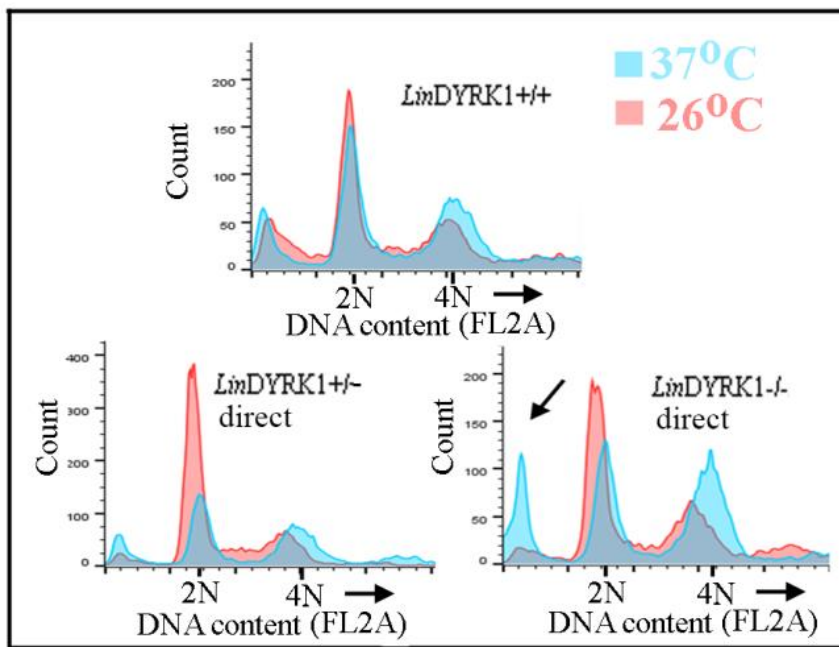
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211 ion scanning of m/z 241); and phosphatidylcholine (PC)/sphingomyelin (SM) species in positive
212 mode (parent-ion scanning of m/z 184).

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215 **S9 Fig**



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218 **S9 Fig. Cell cycle analysis of *LinDYRK1*^{-/-direct} promastigotes under heat-shock.** Overlaid

219 histograms plots showing the DNA content (FL2A) of logarithmic *LinDYRK1*^{+/+}, *LinDYRK1*^{+/-}

220 ^{direct}, *LinDYRK1*^{-/-direct} cultivated at 26°C (pink) and 37°C (blue) for 16 h. Diploid (2N) and

221 tetraploid (4N) DNA content is indicated. Results are representative of 3 experiments. Black

222 arrow show the increased percentage of hypodiploid cells.

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Supporting information

S1 Table: Cell cycle distribution of *LinDYRK1*^{+/+}[pXNG-*LinDYRK*] and *LinDYRK1*^{+/+}[pXNG] cells after HU withdrawal.

| | Cell cycle distribution after synchronisation | | | | | | | | |
|---|---|----|-------------------|-----|----|-------------------|-------|----|-------------------|
| | 0 h | | | 3 h | | | 6.5 h | | |
| | G1 | S | G ₂ /M | G1 | S | G ₂ /M | G1 | S | G ₂ /M |
| <i>LinDYRK1</i> ^{+/+} [pXNG] | 71 | 11 | 17 | 61 | 21 | 18 | 15 | 20 | 65 |
| <i>LinDYRK1</i> ^{+/+} [pXNG- <i>LinDYRK1</i>] | 76 | 9 | 15 | 70 | 15 | 15 | 51 | 39 | 10 |

Percentage of cell cycle phases *LinDYRK1*^{+/+}[pXNG] and *LinDYRK1*^{+/+}[pXNG-*LinDYRK1*] 0 h, 3 h and 6.5 h post hydroxyurea release. This is one representative of three experiments.

S2 Table: Cell cycle distribution of *LinDYRK1*^{-/-} mutants and control cells parasites in logarithmic, stationary phase and in heat-shock

| A. | | CELL CYCLE ANALYSIS OF FACILITATED <i>LinDYRK1</i> ^{-/-} MUTANTS AND <i>LinDYRK1</i> ^{-/-} ADDBACK COMPARISON IN LOGARITHMIC AND STATIONARY CELL CULTURE GROWTH PHASE | | | | |
|-----------------|----------|---|--|-----------------------------------|-----------------------------------|--|
| | | <i>LinDYRK1</i> ^{+/+} | <i>LinDYRK1</i> ^{-/-} [pXNG- <i>LinDYRK1</i>]A | <i>LinDYRK1</i> ^{-/-} A1 | <i>LinDYRK1</i> ^{-/-} A2 | <i>LinDYRK1</i> ^{-/-} ADDBACK |
| Log (% phases) | SubG1 | 2 | 3.1 | 2.2 | 11 | 1.8 |
| | G1 | 49.9 | 54 | 52.3 | 56.8 | 60.7 |
| | S | 13.1 | 12.6 | 9.7 | 12 | 8.7 |
| | G2/M | 34.7 | 29.6 | 35.6 | 29.9 | 28.6 |
| Stat (% phases) | SubG0/G1 | 4.9 | 15.2 | 46.3 | 33.8 | 9.4 |
| | G1 | 70.9 | 59.5 | 43.6 | 42.6 | 64.8 |
| | S | 7.1 | 6.8 | 4.3 | 5.8 | 5.5 |
| | G2/M | 17.7 | 18.1 | 5.6 | 17.5 | 20.1 |

| B. | | CELL CYCLE ANALYSIS OF <i>LinDYRK1</i> ^{-/-} direct MUTANTS IN LATE LOGARITHMIC AND STATIONARY CELL CULTURE GROWTH PHASE | | |
|----------------|-----------|---|-------------------------------------|--------------------------------------|
| | | <i>LinDYRK1</i> ^{+/+} | <i>LinDYRK1</i> ^{+/direct} | <i>LinDYRK1</i> ^{-/-direct} |
| Log (% phases) | SubG0 /G1 | 4.4 | 15.9 | 9.9 |
| | G1 | 68.9 | 52.6 | 59.6 |
| | S | 10 | 13.9 | 12.3 |
| | G2/M | 16.5 | 17.4 | 18 |

Supporting information

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|-----------------------|----------|------|------|------|
| Stat (% phases) | SubG0/G1 | 11.5 | 16.9 | 49.2 |
| | G1 | 55.4 | 56 | 34.5 |
| | S | 14.6 | 8.5 | 5.5 |
| | G2/M | 18.2 | 18.5 | 5.7 |

| C. | | CELL CYCLE ANALYSIS OF FACILITATED <i>LinDYRK1</i> ^{-/-} MUTANTS AND <i>LinDYRK1</i> ^{-/-} ADBACK COMPARISON AT 26 °C AND 37°C | | | | |
|-------------------------|----------|--|---|-----------------------------------|-----------------------------------|--|
| | | <i>LinDYRK1</i> ^{+/+} | <i>LinDYRK1</i> ^{-/-} [pXNG- <i>LinDYRK1</i>] A | <i>LinDYRK1</i> ^{-/-} A1 | <i>LinDYRK1</i> ^{-/-} A2 | <i>LinDYRK1</i> ^{-/-} A1 [pXNG- <i>LinDYRK1</i>] addback |
| T=26°C (% phases) | SubG0/G1 | 6.7 | 4.6 | 1.45 | 4.8 | 3.5 |
| | G1 | 63.4 | 61.7 | 53.8 | 47.2 | 63.6 |
| | S | 9.8 | 12.4 | 11.5 | 14.7 | 8.5 |
| | G2/M | 19.9 | 21.1 | 33.21 | 33.1 | 24.2 |
| T=37°C (% phases) | SubG1 | 12.5 | 17.1 | 29.03 | 28.0 | 16.12 |
| | G1 | 35.9 | 33.7 | 29.03 | 38.5 | 38.0 |
| | S | 11.3 | 12.11 | 10.7 | 7.8 | 11.6 |
| | G2/M | 40.2 | 6.9 | 31.1 | 25.4 | 34.1 |

| D. | | CELL CYCLE ANALYSIS OF <i>LinDYRK1</i> ^{-/-} direct MUTANTS AT 26 °C AND 37°C | | |
|-------------------------|-------|--|---------------------------------------|---------------------------------------|
| | | <i>LinDYRK1</i> ^{+/+} | <i>LinDYRK1</i> ^{+/+} direct | <i>LinDYRK1</i> ^{-/-} direct |
| T=26°C (% phases) | SubG1 | 22 | 5.5 | 7.5 |
| | G1 | 42.8 | 57.7 | 53 |
| | S | 10.5 | 11.6 | 6.8 |
| | G2/M | 25.7 | 18.2 | 32 |
| T=37°C (% phases) | SubG1 | 15.8 | 15.6 | 24.5 |
| | G1 | 41.5 | 40.5 | 29 |
| | S | 6.5 | 5.8 | 4.5 |
| | G2/M | 39 | 38 | 42 |

Percentages of cell cycle phases in logarithmic, stationary growth phase and during a 16 h 26→37 °C heat-shock are shown.

S3 Table: List of DYRK sequences used for the phylogenetic analysis

| Protein name | Species | Accession number |
|--------------|----------------------------------|------------------------|
| Yak1p | <i>Saccharomyces cerevisiae</i> | NP_012394; GI:6322320 |
| Pom1p | <i>Schizosaccharomyce spombe</i> | NP_592974; GI:19113886 |

Supporting information

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|--------------------------------|----------------------------------|-------------------------------|
| Ppk15p | <i>Schizosaccharomyces pombe</i> | NP_593830; GI:19114742 |
| Ppk5p | <i>Schizosaccharomyces pombe</i> | NP_593081; GI:63054495 |
| Prp4p | <i>Schizosaccharomyces pombe</i> | NP_588261; GI:19075761 |
| Dyrk1 (predicted) | <i>Dictyostelium discoideum</i> | XP_642598; GI:66817490 |
| Dyrk2 (predicted) | <i>Dictyostelium discoideum</i> | XP_628965; GI:66800079 |
| Prp4B | <i>Dictyostelium discoideum</i> | XP_641608; GI:66814997 |
| YakA | <i>Dictyostelium discoideum</i> | XP_638920; GI:66810395 |
| HPK-1 | <i>Caenorhabditis elegans</i> | NP_741762; GI:25146827 |
| MBK-2 | <i>Caenorhabditis elegans</i> | NP_502492; GI:71988995 |
| MBK-1 | <i>Caenorhabditis elegans</i> | NP_510460; GI:25152628 |
| PRPF4 | <i>Caenorhabditis elegans</i> | NP_492008; GI:17506739 |
| dDyrk2 (Smi35A) | <i>Drosophila melanogaster</i> | NP_523564; GI:17737415 |
| dDyrk3 (CG40478) | <i>Drosophila melanogaster</i> | NP_001033810; GI:85724756 |
| dHipk (CG17090) | <i>Drosophila melanogaster</i> | NP_728531; GI:24654780 |
| Minibrain (dDyrk1) | <i>Drosophila melanogaster</i> | NP_728104; GI:24642876 |
| dPrp4 (CG7028) | <i>Drosophila melanogaster</i> | NP_612010; GI:19922978 |
| PRP4K | <i>Homo sapiens</i> | NP_003904; GI:89276756 |
| HIPK1 | <i>Homo sapiens</i> | NP_938009; GI:38201640 |
| HIPK2 | <i>Homo sapiens</i> | NP_073577; GI:164420685 |
| HIPK3 | <i>Homo sapiens</i> | NP_005725; GI:114796624 |
| HIPK4 | <i>Homo sapiens</i> | NP_653286; GI:31542768 |
| DYRK1A | <i>Homo sapiens</i> | NP_001387; GI:18765758 |
| DYRK1B (Mirk) | <i>Homo sapiens</i> | NP_004705; GI:4758222 |
| DYRK2 | <i>Homo sapiens</i> | NP_006473; GI:153281169 |
| DYRK3 (REDK) | <i>Homo sapiens</i> | NP_003573; GI:51702240 |
| DYRK4 | <i>Homo sapiens</i> | NP_003836; GI:28827774 |
| AtYak1 | <i>Arabidopsis thaliana</i> | NP_198447; GI:42568145 |
| LinJ.14.0890 | <i>Leishmania infantum</i> | XP_001464268.1; GI: 146081498 |
| LinJ.14.1140 | <i>Leishmania infantum</i> | XP_001464290.1; GI: 146081591 |
| LinJ.15.0180 (LinDYRK1) | <i>Leishmania infantum</i> | XP_001464356.1; GI: 146081806 |
| LinJ.19.0360 | <i>Leishmania infantum</i> | XP_001464999.1; GI: 146084408 |
| LinJ.21.2010 | <i>Leishmania infantum</i> | XP_001465461.2; GI: 339898159 |
| LinJ.33.1930 | <i>Leishmania infantum</i> | XP_001468297.1; GI: 146098030 |
| LinJ.35.1850 | <i>Leishmania infantum</i> | XP_001469005.1; GI: 146101011 |
| LinJ.36.4460 | <i>Leishmania infantum</i> | XP_001470018.2; GI: 339899360 |

Table shows Fasta (NP) or Uniprot (XP) accession numbers of proteins whose sequences were used for the phylogenetic analysis

S4 Table: List of primers used in PCR reactions.

| Ref. | Primer name | Primer sequence | Added restriction sites | Use |
|-----------|-------------------------------|--|-------------------------|---|
| p1 | pXNG- <i>LinDYRK1</i> for | 5'CTCTAGATCTGGAGCATGGCTGACTCTTCG AAGGTG ^{3'} | BglII | <i>LinDYRK1</i> internal primers and amplification for cloning into [pXNG4]. Forward primer also used with p21 to amplify insert for generating construct [<i>LinDYRK1</i> -carboxyterminal] |
| p2 | pXNG- <i>LinDYRK1</i> _rev | 5'CTCTAGATCTCTATTTCGGCTGGAGGAAGAG CA ^{3'} | BglII | |

Supporting information

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|-----|---------------------------------------|---|--|--|
| p3 | <i>LinDYRK1</i> 5'UTR_for | 5' <u>GTTTAAACC</u> CACTTCCCTCCCTCTGAAC ^{3'} | PmeI | 5'UTR amplification of for cloning into [pGEM-T] |
| p4 | <i>LinDYRK1</i> 5' 5'UTR_rev | 5' <u>ACTAGTTGCTGATCTCCCCC</u> CTTCGC ^{3'} | SpeI | |
| p5 | <i>LinDYRK</i> 3'UTR_for | 5' <u>GGATCC</u> CAGATGGGAGGCAGGCGGGGACTA ^{3'} | BamHI | 3'UTR amplification for cloning into [pGEM-T easy]. |
| p6 | pXNG- <i>LinDYRK1</i> 3'UTR_for | 5' <u>GTTTAAACC</u> CTTGAAGGAGAGGCTCCAC ^{3'} | PmeI | |
| p7 | hph_for | 5'TCATCCGGGTCCGAGC <u>ACTAGT</u> GATGAA ^{3'} | SpeI intern | Amplification of <i>hph</i> gene from [pX63-Hyg] for construction of targeting cassette |
| p8 | hph_rev | 5'GACAGGATCCTCTATTCCCTTGGCCCTCGGA ^{3'} | BamHI | |
| p9 | PAC_for | 5'AAGG <u>ACTAGT</u> CCTTCCATGACCGAGTACAA G ^{3'} | SpeI | Amplification of <i>hph</i> gene from [pXG-PAC] for construction of targeting cassette |
| p10 | PAC_rev | 5'GTTT <u>GATC</u> CTAGGCACCGGGCTTGCGGGT ^{3'} | BamHI | |
| p11 | PAC_integratio n_rev | 5'CGTGGGCTTGTACTCGGTCATG ^{3'} | * | Validation of PAC cassette integration |
| p12 | HYG_integratio n_rev | 5'CATCAGGTCGGAGACGCTGTC ^{3'} | * | Validation of HYG cassette integration |
| p13 | internal_DYRK _rev | 5'CTCCACCTCATACGGCGCATG ^{3'} | * | Cassette integration/ genomic <i>LinDYRK1</i> copy |
| p14 | Genomic DYRK | 5'GTGTGTGGGTTGTTCGTCGAGG ^{3'} | * | Presence of genomic <i>LinDYRK1</i> copy |
| p15 | Meta1_for | 5'ATGGAGATGAAAAAACTTGCTTGG ^{3'} | * | Amplification of Meta-1 transcript |
| p16 | Meta1_rev | 5'TTGATGATGTGTGTTTACTGACTGC ^{3'} | * | |
| p17 | HASPB_for | 5'ATGGGAACTTCTTGTACAAAG ^{3'} | * | Amplification of HASPB transcript |
| p18 | HASPB_rev | 5'TCCGTGATTGGCCTCAGT ^{3'} | * | |
| p19 | Prohibitin_for | 5'CTTCTGCTCATTCTCCATCAG ^{3'} | * | Amplification of Prohibitin transcript |
| p20 | Prohibitin_rev | 5'CTTAGCGTGACAGCTGC ^{3'} | * | |
| p21 | RDYRK1Cterm GFP | 5'CACAGGATCCTTCGGCTGGAGGAAGAGCA ^{3'} | BamHI | Reverse primer used to amplify <i>LinDYRK1</i> insert with primer p1 for generating construct [<i>LinDYRK1</i> -carboxyterminal] |
| p22 | FGFPN3 | 5'GTGGGAGGTCTATATAAGCAGAGC ^{3'} | * (BamHI site internal inside the amplified product) | Amplification of eGFP from [pEGFP(N3)] for cloning into [<i>LinDYRK1</i> -carboxyterminal], to generate construct [pXG-PAC- <i>LinDYRK1</i> -GFP] |
| p23 | RGFPN3 | 5'CTCTCAGATCTTACTTGTACAGCTCGTCCATG ^{3'} | BglII | |

Table shows the names of the primers and describes the purpose of their use during the experimental procedures