

1 The inositol pyrophosphate synthesis pathway in *Trypanosoma*
2 *brucei* is linked to polyphosphate synthesis in acidocalcisomes

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28 **Summary**

29 Inositol pyrophosphates are novel signaling molecules possessing high-energy pyrophosphate bonds
30 and involved in a number of biological functions. Here, we report the correct identification and
31 characterization of the kinases involved in the inositol pyrophosphate biosynthetic pathway in
32 *Trypanosoma brucei*: inositol polyphosphate multikinase (TbIPMK), inositol pentakisphosphate 2-
33 kinase (TbIP5K) and inositol hexakisphosphate kinase (TbIP6K). TbIP5K and TbIP6K were not
34 identifiable by sequence alone and their activities were validated by enzymatic assays with the
35 recombinant proteins or by their complementation of yeast mutants. We also analyzed *T. brucei*
36 extracts for the presence of inositol phosphates using polyacrylamide gel electrophoresis and high
37 performance liquid chromatography. Interestingly, we could detect inositol phosphate (IP), inositol
38 4,5-bisphosphate (IP₂), inositol 1,4,5-trisphosphate (IP₃) and inositol hexakisphosphate (IP₆) in *T.*
39 *brucei* different stages. Bloodstream forms unable to produce inositol pyrophosphates, due to
40 downregulation of *TbIPMK* expression by conditional knockout, have reduced levels of polyphosphate
41 and altered acidocalcisomes. Our study links the inositol pyrophosphate pathway to the synthesis of
42 polyphosphate in acidocalcisomes, and may lead to better understanding of these organisms and
43 provide new targets for drug discovery.

44

45 **Introduction**

46 *Myo*-inositol is an essential precursor for the synthesis of soluble inositol phosphates (IPs) and lipid-
47 bound inositols called phosphoinositides (PIPs) (Irvine *et al.*, 2001). After inositol incorporation into
48 the lipid phosphatidylinositol (PI), the inositol ring is phosphorylated to PIPs such as
49 phosphatidylinositol 4,5-bisphosphate (PIP₂) through the action of a phosphatidylinositol phosphate
50 (PIP) kinase. PIP₂ is cleaved by a phosphoinositide phospholipase C (PI-PLC) (Cocco *et al.*, 2015) to
51 inositol 1,4,5-trisphosphate (IP₃) (Fig. 1) and 1,2-diacylglycerol (DAG), which are important second
52 messengers. While DAG stimulates a protein kinase C (Nishizuka, 1986), IP₃ stimulates an IP₃

53 receptor to release Ca^{2+} from intracellular stores (Berridge, 2009) and can be further metabolized to
54 other soluble IPs by several kinases and phosphatases.

55 The inositol phosphate multikinase (IPMK) has dual 3-kinase/6-kinase activity and catalyzes the
56 conversion of IP_3 into inositol tetrakisphosphate (IP_4) and inositol pentakisphosphate (IP_5). IP_5 is
57 converted into inositol hexakisphosphate (IP_6), the fully phosphorylated *myo*-inositol also known as
58 phytic acid, by the 2-kinase activity of inositol pentakisphosphate kinase (IP5K, or IPPK). Further
59 phosphorylation of IP_6 by the inositol hexakisphosphate kinase (IP_6 kinase or IP6K) results in the
60 production of diphosphoinositol polyphosphates (PP-IPs), also known as inositol pyrophosphates.
61 These are IPs characterized by containing one or more high-energy pyrophosphate moiety. PP-IPs
62 were discovered in the early 1990's, in *Dictyostelium discoideum* (Europe-Finner *et al.*, 1991, Mayr
63 GW, 1992, Stephens *et al.*, 1993), *Entamoeba histolytica* (Martin *et al.*, 1993), and in mammalian cells
64 (Menniti *et al.*, 1993). The best-characterized member of this class is 5-diphosphoinositol
65 pentakisphosphate (5-PP- P_5 or IP_7), which has five of the *myo*-inositol hydroxyls monophosphorylated,
66 while the sixth, at the 5-position, contains a pyrophosphate group (Albert *et al.*, 1997). The IP6K can
67 also metabolize IP_5 to diphosphoinositol tetrakisphosphate (PP- IP_4) (Saiardi *et al.*, 2000, Losito *et al.*,
68 2009). Another isomer of IP_7 , containing a pyrophosphate at the 1-position, can also be formed by a
69 more recently identified enzyme termed diphosphoinositol pentakisphosphate kinase (PP-IP5K),
70 though this enzyme appears to be predominantly associated physiologically with the formation of
71 diphosphoinositol hexakisphosphate (PP- IP_4 or IP_8) (Choi *et al.*, 2007).

72 Among the many roles attributed to PP-IPs are the regulation of telomere length (Saiardi *et al.*,
73 2005, York *et al.*, 2005), DNA repair by homologous recombination (Luo *et al.*, 2002, Jadav *et al.*,
74 2013), response to hyperosmotic stress (Pesesse *et al.*, 2004, Choi *et al.*, 2007), vesicle trafficking
75 (Saiardi *et al.*, 2000, Saiardi *et al.*, 2002), apoptosis (Morrison *et al.*, 2001, Nagata *et al.*, 2005),
76 autophagy (Nagata *et al.*, 2010), binding of pleckstrin homology domains to phospholipids and
77 proteins (Luo *et al.*, 2003, Gokhale *et al.*, 2013), transcription of glycolytic enzymes (Szijgyarto *et al.*,

78 2011), hemostasis (Ghosh *et al.*, 2013), phagocytic and bactericidal activities of neutrophils (Prasad *et*
79 *al.*, 2011), epigenetic modifications to chromatin (Burton *et al.*, 2013) and exocytic insulin secretion
80 (Illies *et al.*, 2007). PP-IPs may signal through allosteric interaction with proteins (i.e. binding to
81 pleckstrin homology (PH) or other domains of proteins) or by phosphotransfer reactions (Saiardi, 2012,
82 Shears, 2015, Wild *et al.*, 2016). The phosphotransfer reaction is non-enzymatic and requires a
83 phospho-serine residue within an acidic region and consists in adding a second phosphate to the
84 phosphor-serine resulting in pyrophosphorylation (Saiardi, 2012).

85 *Trypanosoma brucei*, which belongs to the group of parasites that causes African trypanosomiasis
86 (sleeping sickness), possesses a PI-PLC that is stimulated by very low Ca²⁺ concentrations (King-
87 Keller *et al.*, 2015) and an IP₃ receptor that localizes to the acidocalcisomes instead of the endoplasmic
88 reticulum (Huang *et al.*, 2013). We now found that they also possess orthologs to IPMK, IP5K and
89 IP6K, but do not have recognizable orthologs to PP-IP5K, inositol 1,4,5-trisphosphate 3-kinases
90 (ITPKs) and inositol tetrakisphosphate 3-kinase 1 (ITPK1) (Table S1). The ortholog to IPMK
91 (TbIPMK) was recently reported as essential for the bloodstream forms of the parasites (Cestari *et al.*,
92 2015), suggesting that the soluble inositol phosphate pathway is essential for the parasite. The
93 orthologs to IP5K and IP6K were not recognizable by sequence only and were wrongly annotated as a
94 putative hypothetical protein and as inositol polyphosphate-like protein, respectively. In the present
95 study, we thoroughly characterized the soluble inositol phosphate pathway of *T. brucei*. We cloned,
96 expressed and biochemically characterized the recombinant enzymes from *T. brucei*, complemented
97 yeast mutants to demonstrate their function, analyzed their products, studied the inositol phosphate
98 metabolism of *T. brucei* cells, and revealed the link of this pathway to the synthesis of polyphosphate
99 in acidocalcisomes.

100

101 **Results**

102

103 *Sequence analysis of T. brucei inositol phosphate kinases*

104

105 Gene homology searches followed by validation of their activity (see below) have allowed to identify
106 in the *T. brucei* genome (<http://www.tritrypdb.org/tritrypdb/>) the presumably gene orthologs to the
107 inositol phosphate kinases encoding inositol polyphosphate multikinase (IPMK in mammals, and
108 Arg82p or Ipk2p in yeast) (Tb427tmp.211.3460); the IP₅ kinase (IPPK or IP5K in mammals, and
109 Ipk1p in yeast) (Tb427.04.1050); and the IP₆ kinase (IP6K in mammals, and Kcs1p in yeast)
110 (Tb427.07.4400), (Fig. 1), and named *TbIPMK*, *TbIP5K*, and *TbIP6K*, respectively (Table S1). No
111 orthologs to diphosphoinositol pentakisphosphate kinase (PP-IP5K in mammals, or Vip1 in yeasts)
112 were found, although orthologs to this gene are present in Apicomplexan (Laha *et al.*, 2015) and
113 *Giardia* (EuPathDB). The orthologs to *TbIPMK*, *TbIP5K*, and *TbIP6K* identified in *T. cruzi*
114 (TcCLB.510741.110, TcCLB.506405.90, TcCLB.504213.90) and *Leishmania major* (LmjF.35.3140,
115 LmjF.34.3700, LmjF.14.0340) shared 45%, 36%, 35%, and 29%, 28%, 24% amino acid identity,
116 respectively. Those of *T. brucei* share 15%, 16%, and 15% identity with the human enzymes,
117 respectively. Structural analyses (ELM and TMHMM servers) predicted no transmembrane domains.
118 A signal peptide was predicted for *TbIP5K*, but not for *TbIPMK* or *TbIP6K*. Mature proteins of 342,
119 461, and 756 amino acids with predicted molecular weights of 38.8, 51, and 82.6 kDa, for *TbIPMK*,
120 *TbIP5K*, and *TbIP6K*, respectively, were also predicted. Amino acids 138-147 of *TbIPMK*, and 588-
121 596 of *TbIP6K* contained the conserved sequence PCVLDL(I)KL(M)G demonstrated previously as
122 the putative inositol phosphate binding site that catalyzes the transfer of phosphate from ATP to
123 inositol phosphates (Bertsch *et al.*, 2000). *TbIP5K* possesses the sequence PVLDIELL (amino acids
124 269-276) instead. Both *TbIPMK* and *TbIP6K* have a SASLL or TSSLL domain present in most
125 members of this family of enzymes and required for enzymatic activity (Saiardi *et al.*, 2001b,
126 Nalaskowski *et al.*, 2002).

127 We utilized homologous recombination to add a hemagglutinin (HA) or c-Myc tag to the
128 endogenous loci (Oberholzer *et al.*, 2006) of TbIPMK, TbIP5K and TbIP6K. All three inositol
129 phosphate kinases are expressed in procyclic forms (PCF) of *T. brucei* (Fig. 2A). Although the
130 predicted MW of TbIP6K is 82.6 kDa the enzyme has multiple phosphorylations (Urbaniak *et al.*,
131 2013) and these post-translational modifications (in addition to the HA tag) could result in a higher
132 apparent MW. Interestingly, TbIP5K revealed no expression when using the HA-tag, but a protein
133 with the expected size was detected when using a c-Myc tag (Fig. 2A). In addition, we tagged the three
134 IP kinases in *T. brucei* bloodstream forms (BSF) but no clear bands were detected by western blot
135 analyses although the tagged genes were expressed at the mRNA level (data not shown), suggesting
136 that protein expression is lower in BSF than in PCF.

137

138 *Characterization of the inositol phosphate multikinase (TbIPMK)*

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140 To characterize the enzymatic activity of TbIPMK we expressed it as fusion protein with an *N*-
141 terminal polyhistidine tag, purified and tested its activity *in vitro*. We found that it catalyzes the
142 formation of IP₅ from IP₃ or IP₄, as detected by polyacrylamide gel electrophoresis (Fig. 2B). Inositol-
143 1,4,5-trisphosphate (I(1,4,5)P₃) but not inositol-1,3,4-trisphosphate (I(1,3,4)P₃) could be used as
144 substrate while both inositol-1,3,4,5-tetraphosphate (I(1,3,4,5)P₄) and inositol-1,4,5,6-tetraphosphate
145 (I(1,4,5,6)P₄) could be used for the generation of inositol-1,3,4,5,6-pentakisphosphate (I(1,3,4,5,6)P₅)
146 (Fig. 2B), indicating that TbIPMK has a dual 3-kinase/6-kinase activity. An additional product, which
147 runs closely but not identically to IP₆, was also detected when IP₃, IP₄, or IP₅ was used as substrate
148 (Figs. 2B and 2C). The ability of IPMK to form PP-IP₄, an inositol pyrophosphate containing 6
149 phosphates and thus migrating closely to IP₆, has been demonstrated for the mammalian and yeast
150 ortholog (Saiardi *et al.*, 2001a, Zhang *et al.*, 2001), and we therefore suspected that TbIPMK could
151 have the same activity. A treatment with perchloric acid (PA), which degrades high-energy

152 phosphoanhydride bonds (pyrophosphates) and is inactive against the phosphoester bond of IP₆ (Fig.
153 2C) (Pisani *et al.*, 2014), demonstrated that the highly phosphorylated product of TbIPMK is a
154 pyrophosphate containing species, therefore PP-IP₄. The pH optimum of rTbIPMK was determined.
155 TbIPMK has the maximum activity for IP₃ at the pH range of 6.5-7.0 (Fig. 2D). We also tested the
156 ability of TbIPMK to phosphorylate different isomers of IP₅. Recombinant TbIPMK was able to
157 phosphorylate I(1,2,4,5,6)P₅ and I(1,2,3,4,5)P₅ to IP₆ after short incubation times, but it was not able to
158 use I(2,3,4,5,6)P₅, I(1,3,4,5,6)P₅, I(1,2,3,5,6)P₅, or I(1,2,3,4,6)P₅ as substrate (Fig. 2E). Although
159 I(1,2,4,5,6)P₅ and I(1,2,3,4,5)P₅ would not be physiological substrates, the results again confirms a
160 3/6-kinase activity. Interestingly, TbIPMK could also phosphorylate I(1,4)P₂ to IP₄ (Fig. 2B). The
161 mammalian IPMK has been reported to have PI3-kinase activity that produces PIP₃ from PIP₂
162 (Resnick *et al.*, 2005). However, our *in vitro* activity tests using PIP₂ as substrate revealed no such
163 activity (data not shown) in agreement with the results of a previous report (Cestari *et al.*, 2016).

164 The ability of TbIPMK to act on IP₃ *in vivo* was tested by complementation of a *null* mutant for its
165 ortholog ARG82 (*arg82Δ* in *Saccharomyces cerevisiae*. Fig. 3A shows the HPLC analysis of soluble
166 inositol phosphates isolated from yeast labeled with [³H]inositol. Arg82p phosphorylates IP₃ to
167 produce IP₄ and IP₅, and in its absence there is accumulation of IP₃, instead of the accumulation of IP₆
168 that occurs in wild type yeast (Fig. 3A). The metabolic pathway from IP₃ to IP₆ was restored by
169 complementation with *TbIPMK* (Fig. 3A). These results indicate that TbIPMK function as part of the
170 IP₆ biosynthetic pathway established in yeast (York *et al.*, 1999). We also examined the ability of
171 TbIPMK to rescue the growth defect of *arg82Δ* yeast. Complementation of *arg82Δ* with *TbIPMK*
172 rescued their growth defect (Fig. 3B, and 3C). Therefore, TbIPMK was able to complement yeast
173 deficient in its ortholog Arg82p, providing molecular evidence of its function. The results also suggest
174 that the pathway for IP₅ synthesis is similar to that present in yeast with conversion of I(1,4,5)P₃ into
175 I(1,4,5,6)P₄ and I(1,3,4,5,6)P₅, TbIPMK acting as a 3/6-kinase. This is different from the pathway for

176 synthesis of I(1,3,4,5,6)P₅ present in humans, where the major activity of IP₄ kinase is phosphorylation
177 at the D-5 position (Chang *et al.*, 2002).

178

179 *Characterization of the inositol pentakisphosphate kinase (TbIP5K)*

180

181 Although expression of polyhistidine-tagged TbIP5K was obtained in bacteria and the recombinant
182 protein had the expected molecular mass, we were not able to detect its activity *in vitro*, even in the
183 presence of different isomers of IP₅ (data not shown) suggesting that additional post-translational
184 modifications are needed. In this regard, activity of human IP5K could only be obtained when
185 expressed in insect cells (Verbsky *et al.*, 2002). However, *TbIP5K* was able to complement *null*
186 mutant yeast deficient in its ortholog *IPK1* (*Ipk1Δ*) (Fig. 3D). *Ipk1p* phosphorylates IP₅ to produce IP₆,
187 and in its absence there is accumulation of IP₅, instead of the accumulation of IP₆ that occurs in wild
188 type yeast. The metabolic pathway from IP₅ to IP₆ was restored by complementation with *TbIP5K* (Fig.
189 3D). The presence of a shoulder close to the PP-IP₄ eluting peak in the mutant yeast suggests the
190 existence of two isomeric PP-IP₄ species. We also complemented yeast mutants for both *ipk1Δ* (*IP5K*)
191 and *kcs1Δ* (*IP6K*). These mutants accumulate IP₂, IP₃, IP₄, and IP₅ but no PP-IPs. While
192 complementation with either *TbIPMK* or *TbIP6K* (not shown) alone did not change appreciably the
193 inositol polyphosphate profile, synthesis of IP₆ was restored by complementation with *TbIP5K* alone
194 (Fig. 3E), demonstrating that TbIP5K is the only inositol phosphate kinase identified in *T. brucei*
195 genome that can produce IP₆.

196

197 *Characterization of the inositol hexakisphosphate kinase (TbIP6K)*

198

199 TbIP6K catalyzes the formation of IP₇ from IP₆. *TbIP6K* was also tagged with an HA tag using
200 homologous recombination with the endogenous gene loci (Oberholzer *et al.*, 2006). We detected
201 expression of the enzyme in *T. brucei* procyclic forms (PCF) by western blot analysis (Fig. 2A).
202 Recombinant TbIP6K was found to generate PP-IP₄ from IP₅ and IP₇ from IP₆ (Fig. 4A). Interestingly
203 TbIP6K was not able to generate IP₈ using a 5PP-IP₇ as substrate, which suggests that, as IP6K from
204 yeast and mammals, TbIP6K phosphorylates phosphate position D-5. Therefore, TbIP6K is able to
205 generate two PP-IPs *in vitro*: PP-IP₄, and IP₇. The activity of TbIP6K was tested *in vivo* by
206 complementation of a *null* mutant for its IP6K ortholog (*KCSI*) in *S. cerevisiae*. In the absence of
207 *KCSI* there is no accumulation of IP₇, but the metabolic pathway from IP₆ to IP₇ is restored by
208 complementation with *TbIP6K* (Fig. 4B). Complementation of *Kcs1Δ TbIP6K* also rescued the growth
209 defect of these mutants (Fig. 4C and 4D). The TbIP6K enzymatic activity has optimum pH 6.0-7.0
210 (Fig. 4E).

211

212 *Characterization of inositol phosphates from T. brucei cells*

213

214 Previous attempts to characterize soluble inositol phosphates from *T. brucei* (Moreno *et al.*, 1992) and
215 *T. cruzi* (Docampo *et al.*, 1991) only detected IP, IP₂ and IP₃. We used increased labeling time to 40
216 hours (BSF) and 75 hours (PCF) with [³H]inositol and used an improved protocol for purifying and
217 analyzing inositol phosphates (see Materials and methods). Using these conditions, we were able to
218 detect a small peak of IP₆ in PCF but not in BSF of the parasite (Figs. 5A, and 5B). The inability to
219 detect radiolabeled IP₆ in the BSF might simply reflect the lower number of cells that can be obtained
220 in culture. To improve the detection of IP₆ we used a different approach that does not require
221 metabolic labeling with [³H]inositol. We extracted IPs from large amounts of cells (see Materials and
222 methods) and assayed extracts by 35% polyacrylamide gel electrophoresis (PAGE). A band that runs
223 like the IP₆ standard and that disappears after treatment of the extracts with phytase (Phy) was

224 observed in both PCF and BSF (Figs. 5C, and 5D). Other highly phosphorylated inositol phosphates
225 were not detected. These results confirm that both PCF and BSF TbIPMK and TbIP5K can
226 sequentially synthesize IP₆ in *T. brucei*.

227

228 *Biological relevance of the TbIPMK pathway*

229

230 Yeast lacking Arg82p have no observable inorganic polyphosphate accumulation (Lonetti *et al.*, 2011).
231 As polyphosphate has important roles in trypanosomes, including growth, response to osmotic stress,
232 and maintenance of persistent infections (Lander *et al.*, 2016), we investigated whether deletion of
233 soluble inositol polyphosphates affected the levels of polyphosphate in *T. brucei*. We used the
234 *TbIPMK* conditional knockout BSF cell line previously described (Cestari *et al.*, 2015). Removal of
235 tetracycline to induce the knockdown of *TbIPMK* dramatically reduced its expression more than 100-
236 fold (Fig. 6A). Growth stalled after the first day without tetracycline (Fig. 6B). A resulting progressive
237 reduction in polyphosphate levels was detected (Fig. 6C). Acidocalcisomes are the main cellular
238 storage compartment for polyphosphate in trypanosomes (Lander *et al.*, 2016). However, examination
239 of the cells by super-resolution microscopy with antibodies against the vacuolar proton
240 pyrophosphatase (TbVP1) showed no apparent difference in labeling or distribution of
241 acidocalcisomes between control and *TbIPMK* mutant cells (Figure S1). In previous work we
242 demonstrated that a knockdown of the TbVtc4, which catalyzes the synthesis and translocation of
243 polyphosphate into acidocalcisomes, results in less electron-dense organelles, as examined by electron
244 microscopy (Ulrich *et al.*, 2014). We hypothesized that if the polyphosphate reduction observed (Fig.
245 6C) was primarily within acidocalcisomes, we should observe similar changes in the *TbIPMK* mutant
246 cells. Indeed, electron microscopy of the *TbIPMK* mutants showed a reduction in the number (Fig. 6D),
247 size, and electron density (compare Fig. 6E and 6F) of electron-dense organelles identifiable as

248 acidocalcisomes. This result indicates that acidocalcisome polyphosphate synthesis is disrupted by
249 ablation of the inositol phosphate signaling pathway.

250

251 **Discussion**

252

253 Our work establishes the presence of an inositol pyrophosphate (PP-IPs) synthesis pathway in *T.*
254 *brucei*. We demonstrated that genes encoding proteins with homology to kinases involved in the
255 generation of IP₅ from IP₄ and IP₃ (TbIPMK), of IP₆ from IP₅ (TbIP5K), and of IP₇ from IP₆ (TbIP6K)
256 are present in the *T. brucei* genome (*TbIPMK*, *TbIP5K*, and *TbIP6K*). To demonstrate that these genes
257 encode for functional enzymes we complemented yeast strains deficient in their corresponding
258 orthologs and compared their products with those produced in the wild type strain providing *in vivo*
259 genetic evidence of their function. We did not compare them with the knockout strains overexpressing
260 the endogenous genes because the heterologous gene expression is often hampered by a diverse
261 genetic code usage and by the lack of yeast specific post-translational processing. Thus, the
262 heterologous genes are regularly expressed from a stronger promoter. The overexpressing of the
263 endogenous gene from a stronger promoter might generate, to the contrary, ‘hyper’ phenotype and not
264 a normal WT phenotype and our aim was to demonstrate their function and not to compare their
265 activities to those of the overexpressed endogenous genes. Suppression of this pathway in *T. brucei*
266 BSF resulted in a significant decrease in polyphosphate levels and in morphological alterations of the
267 acidocalcisomes. The results suggest that this pathway is important for polyphosphate synthesis in
268 acidocalcisomes.

269 Examination of the protein sequences of TbIPMK, TbIP5K, and TbIP6K indicated low identity
270 with the mammalian enzymes but conservation of the putative binding site that catalyzes the transfer
271 of phosphate from ATP to IPs, as well as of other domains required for enzymatic activity. The
272 expression of these three kinases is very low in BSF since no clear bands were detected by western

273 blot analyses of endogenous tagged lines, although gene expression is detectable at the mRNA level.
274 Conversely, all three kinases can be easily identified by western blot analysis of PCF. Our results
275 suggest, in agreement with the presence of these enzymes in other unicellular organisms such as *D.*
276 *discoideum* (Europe-Finner *et al.*, 1991, Mayr GW, 1992, Stephens *et al.*, 1993), and *E. histolytica*
277 (Martin *et al.*, 1993), an early emergence of this pathway preceding the origin of multicellularity.

278 The application of polyacrylamide gel electrophoresis (PAGE) and toluidine blue staining (Losito
279 *et al.*, 2009, Pisani *et al.*, 2014) allowed the characterization of the IPs synthesizing kinases of *T.*
280 *brucei* and the identification of the products of each reaction bypassing the need for extraction under
281 the strong acidic conditions required for HPLC analysis that has been shown to degrade some of the
282 most highly phosphorylated species (Losito *et al.*, 2009).

283 Previous work has indicated that TbIPMK is essential for growth (Cestari *et al.*, 2015) and
284 infectivity (Cestari *et al.*, 2016) of *T. brucei* BSF, and partially characterized the recombinant enzyme
285 (Cestari *et al.*, 2016). We confirmed that TbIPMK prefers I(1,4,5)P₃ and I(1,3,4,5)P₄ as substrates
286 (Cestari *et al.*, 2016) and found that it does not phosphorylate I(1,3,4)P₃. We also confirmed that
287 TbIPMK cannot phosphorylate the lipid PIP₂ to PIP₃ (Cestari *et al.*, 2016), as the human enzyme does
288 (Resnick *et al.*, 2005). In addition, we found that the enzyme can use I(1,3,4,5)P₄ and I(1,4,5,6)P₄ for
289 the generation of I(1,3,4,5,6)P₅ indicating that TbIPMK has a dual 3-kinase/6-kinase activity. This is
290 in contrast to the human enzyme, where the major activity of IP₄ kinase is phosphorylation at the D-5
291 position (Chang *et al.*, 2002). Moreover, we demonstrated that TbIPMK is able to generate PP-IP₄ *in*
292 *vitro*, using either I(1,4,5)P₃, I(1,3,4,5)P₄ or I(1,3,4,5,6)P₅, as well as IP₆ from I(1,2,4,5,6)P₅, or
293 (I(1,2,3,4,5)P₅) as substrate, again indicating a 3/6-kinase activity. TbIPMK has a neutral pH optimum
294 for phosphorylation of both IP₃ and IP₄. Previous work (Cestari *et al.*, 2016) described inhibitors of
295 this enzyme that inhibited *T. brucei* BSF growth. However, their IC₅₀s against the enzymes were
296 higher (3.4-5.33 μM) than the EC₅₀s for their growth inhibition (0.51-0.83 μM), suggesting that either
297 the drugs are accumulated or other targets might be involved in the sensitivity of *T. brucei* BSF to

298 those inhibitors. The search for more specific inhibitors is warranted to demonstrate the relevance of
299 this pathway to human disease and drug therapy. Interestingly, a recombinant multi-domain protein
300 from *Plasmodium knowlesi* termed PkIPK1 was shown to have IPMK-like activity and was able to
301 generate I(1,3,4,5)P₄ from I(1,4,5)P₃ and I(1,2,4,5,6)P₅ from either I(1,2,5,6)P₄ or I(1,3,4,6)P₄,
302 showing 3/5-kinase activity (Stritzke *et al.*, 2012).

303 We were not able to detect activity of the recombinant IP5K in the presence of different isomers of
304 IP₅ suggesting that, as proposed for the mammalian enzyme, post-translational modifications are
305 needed for its activity (Verbsky *et al.*, 2002). However, *TbIP5K* was able to complement *null* mutant
306 yeast deficient in its ortholog *IPK1* (*Ipk1Δ*), providing genetic evidence of its function.

307 Recombinant TbIP6K was able to generate PP-IP₄ from IP₅ and IP₇ from IP₆, but was not able to
308 generate IP₈ using a 5-PP-IP₅ as substrate suggesting that, as IP6K from mammalian cells (Draskovic
309 *et al.*, 2008), TbIP6K phosphorylates phosphate at position D-5. Therefore, TbIP6K is able to generate
310 two PP-IPs in vitro: PP-IP₄, and IP₇. Complementation of yeast deficient in its ortholog confirmed the
311 function of this enzyme.

312 *T. brucei* incorporates poorly the radioactive tracer [³H]inositol a feature previously observed in
313 *Dictyostelium discoideum* (Losito *et al.*, 2009). Nevertheless, improved metabolic labeling with
314 [³H]inositol resulted in detection of IP, IP₂, IP₃ and IP₆ by HPLC analysis of PCF extracts. In contrast
315 to the results obtained using similar methods in yeasts (Azevedo *et al.*, 2006), plants (Phillippy *et al.*,
316 2015) or animal cells (Guse *et al.*, 1993), only very low levels of IP₆ were detected and no labeled IP₆
317 was detected by HPLC using BSF extracts. However, IP₆ was clearly detected by PAGE and toluidine
318 blue staining when large numbers of parasites were used. No inositol pyrophosphates were detected
319 since to purify and visualize IPs we removed the abundant inorganic polyphosphate (polyP) by acidic
320 treatment, procedure that would degrade IP₇ to IP₆. However, the absence of IP₇ could be also
321 attributed to the high turnover of these important signaling molecules (Glennon *et al.*, 1993, Burton *et*
322 *al.*, 2009). Some cells accumulate IP₆ and produce IP₇ upon signaling events. For instance,

323 *Cryptococcus neoformans* requires synthesis of IP₇ for successful establishment of infection (Li *et al.*,
324 2016). A recent study demonstrated that IP₇ binds the SPX domain of proteins involved in phosphate
325 homeostasis in plants, yeast and humans with high affinity and specificity and postulated the role of
326 this domain as a polyphosphate sensor domain (Wild *et al.*, 2016, Azevedo *et al.*, 2017). Two proteins
327 in *T. brucei* possess SPX domains, TbVtc4 (Lander *et al.*, 2013), which is involved in polyphosphate
328 synthesis and translocation, and TbPho91 (Huang *et al.*, 2014), a phosphate transporter. Both proteins
329 localize to acidocalcisomes (Huang *et al.*, 2014), the main polyphosphate storage of these cells. Our
330 results, showing lower levels of polyphosphate and altered acidocalcisomes in *TbIPMK* BSF mutants,
331 support the link between PP-IPs and polyphosphate metabolism.

332 In summary, both recombinant enzymes, TbIPMK and TbIP6K, are able to generate inositol
333 pyrophosphates. The essentiality of the first enzyme of this pathway, TbIPMK, for growth and
334 infectivity of *T. brucei* BSF (Cestari *et al.*, 2015, Cestari *et al.*, 2016) suggests that the study of the PP-
335 IPs pathway in trypanosomes could lead to the elucidation of potentially multiple important roles of
336 these compounds, possibly linked to the synthesis of polyphosphate. Differences between mammalian
337 and trypanosome metabolism of these compounds could provide potential targets for drug
338 development.

339

340 **Experimental procedures**

341

342 *Chemicals and reagents*

343

344 Mouse antibodies against HA were from Covance (Hollywood, FL). Inositol, myo-[1,2-³H(N)] (30-80
345 Ci/mmol, ART 0261A) was from American Radiolabeled Chemicals, Inc. Goat anti-mouse antibodies
346 were from LI-COR Biosciences (Lincoln, NE). Laemmli sample buffer was from Bio-Rad
347 Laboratories (Hercules, CA). The bicinchoninic (BCA) protein assay kit was from Pierce (Thermo

348 Fisher Scientific, USA). Titanium dioxide (TiO₂) beads (Titansphere ToO 5 μm) were from GL
349 Sciences (USA). PrimeSTAR HS DNA polymerase was from Clontech Laboratories Inc. (Takara,
350 Mountain View, CA). Vector pET32 Ek/LIC was from Novagen (Merck KGaA, Darmstadt, Germany).
351 Acrylamide mix was from National Diagnostics (Chapel Hill, NC). CelLytic M cell lysis reagent,
352 P8340 protease inhibitor, protease inhibitors, Benzonase Nuclease, antibody against c-Myc, inositol
353 phosphates, and other analytical reagents were from Sigma-Aldrich (St. Louis, MO).

354

355 *Cell cultures*

356

357 *T. brucei* Lister strain 427 BSF and PCF were used. The BSF were cultivated at 37°C in HMI-9
358 medium (Hirumi *et al.*, 1989) supplemented with 10% heat inactivated fetal bovine serum (FBS,
359 Sigma). The PCF were cultivated at 28°C in SDM-79 medium (Cunningham, 1977) supplemented
360 with 10% heat-inactivated FBS and hemin (7.5 μg/ml). To determine the presence of IP₆ by PAGE
361 analysis *T. brucei* BSF were also isolated from infected mice (Balb/c, female, 6-8 weeks old) and rats
362 (Wistar, male retired breeders), as described previously (Cross, 1975). *T. brucei* IPMK conditional
363 knockout cell line was obtained and grown as described previously (Cestari *et al.*, 2015).

364

365 *Yeast strains*

366

367 The yeast strains used in this study are isogenic to DDY1810 (MATa leu2-3,112 trp1-Δ901 ura3-52
368 prb1-1122 pep4-3 prc1-407), except for the *ipk1Δkcs1Δ* strain that is isogenic to BY4741 (MATa
369 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) and was previously described (Saiardi *et al.*, 2002). The DDY1810
370 protease deficient strain is often used to increase the expression of exogenous proteins upon
371 overexpression due to a deletion on the Pep4 protease. The generation of DDY1810 *kcs1Δ* strain was

372 previously described (Onnebo *et al.*, 2009). The *arg82Δ*, *ipk1Δ* yeast strains in the DDY1810 genetic
373 background were generated following standard homologous recombination techniques (Gueldener *et*
374 *al.*, 2002) using oligonucleotides listed in Table S2. Initially diagnostic PCR was performed to confirm
375 the correct integration of the deletion constructs. Subsequently, the soluble inositol polyphosphate
376 profile of these new strains was used to phenotypically validate the correct homologous recombination
377 event.

378

379 *Epitope tagging, cloning expression and biochemical characterization of inositol phosphate kinases*

380

381 We followed a one-step epitope-tagging method (Oberholzer *et al.*, 2006) to produce the C-terminal
382 HA- or cMyc-tagging cassettes for transfection of *T. brucei* PCF (Table S2). Briefly, the tagging
383 cassettes containing selection markers were generated for cell transfection by PCR using pMOTag4H
384 and pMOTag33M as templates with the corresponding PCR primers of the genes (Table S2).
385 Transfection was performed using 2.5×10^7 PCF parasites from log phase. Cells were harvested at
386 1,000 x *g* for 10 min, washed with 10 ml of ice-cold sterile Cytomix buffer (2 mM EGTA, 3 mM
387 MgCl₂, 120 mM KCl, 0.5% glucose, 0.15 mM CaCl₂, 0.1 mg/ml bovine serum albumin, 10 mM
388 K₂HPO₄/KH₂PO₄, 1 mM hypoxanthine, 25 mM Hepes, pH 7.6), centrifuged at 1,000 x *g* for 7 min,
389 suspended in 0.5 ml Cytomix and transferred to an ice-cold 4 mm gap cuvette (Bio-Rad) containing 15
390 μg of PCR amplicon. Cuvettes were incubated 5 min on ice and immediately electroporated twice in
391 Bio-Rad GenePulser Xcell™ Electroporation System at 1.5 kV, 25 μF. Cuvettes were kept on ice for
392 one minute between electroporation pulses. Cell mixture was transferred to SDM-79 medium with
393 15% FBS. After 6 h appropriate antibiotics were added. The sequences of the three kinases *TbIPMK*,
394 *TbIP5K* and *TbIP6K* were amplified from genomic DNA by PCR (Table S2) using PrimeSTAR HS
395 DNA polymerase and cloned into ligation independent expression vector pET32 Ek/LIC, as
396 recommended by the manufacturer. Constructs were cloned into *Escherichia coli* BL21-

397 CodonPlus(DE3) and protein expression was induced with 1 mM isopropyl β -D-1-
398 thiogalactopyranoside (IPTG) in Luria Bertani broth for 3 h. Protein purification was performed using
399 affinity chromatography HIS-Select® Cartridge, according to the manufacturer's instructions. We
400 tested activity of the kinases on commercially available substrates. Enzyme assays were performed at
401 37°C using approximately 50 ng of recombinant protein, 20 mM Hepes buffer, pH 7.0, 0.2-0.5 mM
402 substrate, 6 mM MgCl₂, 100 mM NaCl, 1 mM dithiotreitol (DTT), 0.5 mM ATP, 10 mM
403 phosphocreatine, and 40 U creatine kinase. Enzymatic reactions were stopped with 3 μ l of 100 mM
404 EDTA and kept on ice or frozen until further use. Reaction products were resolved by PAGE using
405 35% acrylamide/bis-acrylamide 19:1 gels in Tris/Borate/EDTA (TBE) buffer as described by (Losito
406 *et al.*, 2009). Gels were stained with toluidine blue (Losito *et al.*, 2009).

407

408 *RNA quantification*

409

410 The *TbIPMK* conditional knockout cell line was grown with or without 1 μ g/ml tetracycline and
411 harvested at room temperature. RNA was extracted with TRI reagent (Sigma) and used as template for
412 cDNA synthesis with SuperScript III RNA Polymerase (ThermoFisher) and oligo-dT as recommended
413 by the manufacturer. We then performed qRT-PCR analysis using specific primers (Table S2) and
414 SYBR Green Supermix (Bio-Rad). Relative *TbIPMK* gene expression relative to actin was calculated
415 using CFX Manager™ Software (Bio-Rad).

416

417 *Western blot analyses*

418

419 Cells were harvested, washed twice in PBS, and lysed with CelLytic M cell lysis reagent containing
420 protease inhibitor cocktail (Sigma P8340) diluted 1:250, 1 mM EDTA, 1 mM phenylmethanesulfonyl
421 fluoride (PMSF), 20 μ M *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane (E64) and 50 U/ml

422 Benzonase Nuclease (Millipore). The protein concentration was determined by using a BCA protein
423 assay kit. The total cell lysates were mixed with 2X Laemmli sample buffer at 1:1 ratio (vol/vol) and
424 directly loaded in 10% SDS-PAGE. The separated proteins were transferred onto nitrocellulose
425 membranes using a Bio-Rad transblot apparatus. The membranes were blocked with 5% (wt/vol)
426 nonfat milk in PBS containing 0.5% Tween-20 (PBS-T) at 4°C overnight. The blots were incubated for
427 1 hour with mouse antibodies against HA (1:1000) or mouse antibodies against c-Myc (1:1000). After
428 five washings with PBS-T the blots were incubated with goat anti-mouse antibodies at a dilution of
429 1:15000 and developed using an Odyssey CLx Infrared Imaging System (LI-COR) according to the
430 manufacturer instructions.

431

432 *Yeast complementation*

433

434 *S. cerevisiae* strains generated from DDY1810 were used: *arg82Δ*, *ipk1Δ*, *kcs1Δ*, *ipk1Δkcs1Δ*.
435 *TbIPMK*, *TbIP5K* and *TbIP6K* were amplified from *T. brucei* Lister 427, cloned into plasmid
436 pADH:GST (pYES-ADH1-GST) (Azevedo *et al.*, 2009). Yeast cells were grown for 48 h in CSM
437 plates. One colony was collected and suspended in 0.2 M lithium acetate with 25% polyethylene
438 glycol solution and 0.1 M DTT. Cells were homogenized in 100 μl of solution with 100 ng of plasmid
439 DNA and 5 μl of salmon sperm (Sigma D76560). Cells were incubated at 42°C for 30 min and
440 immediately plated in CSM -URA plates. Colonies were used for further experiments.

441

442 *Titanium dioxide bead extraction*

443

444 We adapted the method of Wilson *et al.* (Wilson *et al.*, 2015) for cell extraction of inositol
445 polyphosphates. Cells (5×10^9) were harvested and washed twice in washing buffer A with glucose
446 (BAG, 116 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO₄, 50 mM Hepes, ph 7.3, and 5.5 mM glucose).

447 The pellet was then mixed with 1 M perchloric acid, resuspended by sonication (40% amplitude) for
448 10 s and kept at room temperature for 15 min. The sample was centrifuged at 18,000 \times g for 5 min
449 and the supernatant was transferred to a new tube and boiled for 30 min to remove the large amount of
450 polyphosphate present in *T. brucei*. Seven mg of TiO₂ beads were washed with water and 1 M
451 perchloric acid, and added to the sample and left rotating for 30 min. Beads were centrifuged at 3,500
452 \times g and inositol phosphates eluted with 1 M KOH, 10 mM EDTA. The sample was neutralized with
453 perchloric acid and split into two. One half was digested with phytase (0.1 mg/ml) in the same medium
454 at pH 5.0 and 37°C for 1 h. Extracts were resolved by 35% PAGE analysis as described above.

455

456 *HPLC analysis*

457

458 Inositol phosphate analysis was performed according to (Azevedo *et al.*, 2006). Briefly, yeast liquid
459 cultures were diluted to OD₆₀₀ 0.005 in inositol free media supplemented with 5 μ Ci/ml [³H] inositol
460 and grown overnight at 30°C with shaking. Cells were washed twice with water and immediately
461 incubated with ice-cold 1 M perchloric acid and 3 mM EDTA. Glass beads were added and cells lysed
462 by vortexing at 4°C for 2 min, 3 times. Lysates were centrifuged and supernatants neutralized with 1
463 M K₂CO₃ and 3 mM EDTA. Samples were analyzed by strong anion exchange HPLC using SAX
464 4.6125 mm column (Whatman cat. no. 4621-0505). The column was eluted with two slightly different
465 gradients generated by mixing buffer A (1 mM Na₂EDTA) and buffer B [buffer A plus 1.3 M
466 (NH₄)₂HPO₄ (pH 3.8 with H₃PO₄)] as follows: 0–5 min, 0% B; 5–10 min, 0–30% B; 10–60 min, 30–
467 100% B; 60–80 min, 100% B; or as follow: 0–5 min, 0% B; 5–10 min, 0–10% B; 10–85 min, 20–
468 100% B; 85–100 min 100% B. Four mL of Ultima-Flo AP liquid scintillation cocktail (Perkin-Elmer
469 cat. no. 6013599) was added to each fraction, mixed and radioactivity quantified in a scintillation
470 counter.

471

472 *T. brucei* labeling for HPLC analysis

473

474 *T. brucei* PCF ($\sim 3 \times 10^6$ cells) were labeled with 5 $\mu\text{Ci/ml}$ of 1,2- ^3H -inositol in SDM-79 medium
475 (with 10% FBS) and grown for approximately 72 h. *T. brucei* BSF ($\sim 2 \times 10^5$ cells) were labeled with 5
476 $\mu\text{Ci/ml}$ of 1,2- ^3H -inositol in HMI-9 medium (with 10% FBS) and grown for approximately 40 h.
477 Cells were washed with PBS or BAG twice and frozen immediately. Soluble inositol phosphates were
478 extracted and analyzed as described before (Azevedo *et al.*, 2006), with minor modifications. Briefly,
479 cells were suspended in ice-cold perchloric acid and broken by vortexing for 2 min. All steps were
480 performed at 4°C. Lysates were centrifuged for 5 min at 18,000 $\times g$ and supernatants transferred to
481 new tubes, where the pH was neutralized with 1 M K_2CO_3 and 3 mM EDTA. Samples were stored at
482 4°C and resolved by HPLC.

483

484 *Polyphosphate extraction and measurement*

485

486 Short chain polyphosphate was extracted from BSF *T. brucei* and quantified as described previously
487 (Ulrich *et al.*, 2014).

488

489 *Immunofluorescence Assay*

490 *T. brucei* BSF were washed with BAG and fixed with 2% paraformaldehyde in BAG for 1 h at room
491 temperature. Then they were adhered to poly-L-lysine coated coverslips and permeabilized with 0.1%
492 Triton X-100 in PBS for 5 min. Blocking was performed overnight at 4°C in PBS containing 100 mM
493 NH_4Cl , 3% BSA, 1% fish gelatin and 5% goat serum. Cells were then incubated with anti-TbVPI
494 polyclonal Guinea pig antibody (1:100) for 1 h and subsequently with Alexa 488-conjugated goat anti-
495 Guinea pig antibody (1:1000) for 1h. Microscopy images were taken with a 100X oil immersion
496 objective, a high-power solid-state 405 nm laser and EM-CCD camera (Andor iXon) under

497 nonsaturating conditions in a Zeiss ELYRA S1 (SR-SIM) super resolution microscope. Images were
498 acquired and processed with ZEN 2011 software with SIM analysis module.

499

500 *Electron microscopy*

501 Imaging of whole *T. brucei* BSF and determination of morphometric parameters were done as
502 described previously (Ulrich *et al.*, 2014).

503

504 *Statistical analysis*

505

506 All experiments were repeated at least three times (biological replicates) with several technical
507 replicates as indicated in the figure legends, and where indicated results are expressed as means \pm s.d.
508 or s.e.m. of n experiments. Statistical analyses were performed using the Student's t-test. Results are
509 considered significant when $P < 0.05$.

510

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521

522 **Competing interests**

523 The authors declare no competing or financial interests

524

525 **Author contributions**

526 C.C., A.S. and R.D. designed the experiments and analyzed the data. C.C. and A.S. conducted the
527 experiments. R.D. wrote the majority of the manuscript, with specific sections contributed by C.C.,
528 and A.S. R.D. and A.S. supervised the work and contributed to the analysis of experiments.

529

530 **Supporting information**

531 Supplementary information available online at:

532

533 **Abbreviated Summary**

534 The work identifies the enzymes involved in the inositol pyrophosphate synthesis pathway in
535 *Trypanosoma brucei* and establishes a link between this pathway and the synthesis of polyphosphate
536 in acidocalcisomes.

537

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539

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724

725 **FIGURE LEGENDS**

726

727 Fig. 1. Inositol phosphate pathway in *Trypanosoma brucei*. The soluble IP pathway starts with
728 hydrolysis of PIP₂ by TbPI-PLC1, releasing IP₃ that is phosphorylated by TbIPMK to generate IP₄ and
729 IP₅. IP₅ is phosphorylated by TbIP5K to generate IP₆. IP₅ and IP₆ can be further phosphorylated by
730 TbIPMK or TbIP6K to generate inositol pyrophosphates PP-IP₄ and IP₇. Names of the equivalent yeast
731 enzymes are in green.

732

733 Fig. 2. Western blot analyses and enzymatic activity of TbIPMK.

734 A. Western blot analyses of *T. brucei* PCF expressing epitope-tagged TbIPMK, TbIP5K and TbIP6K.
735 *Left panel* are HA tagged cell lines: 1, wild-type; 2, TbIPMK-HA; 3, wild-type; 4, TbIP6K-HA. *Right*
736 *panel* is a c-Myc tagged line: 5, wild-type; 6, TbIP5K-cMyc.

737 B. Kinase reactions performed with recombinant TbIPMK (2 μ g) using the indicated substrates at 250
738 μ M for 1 hour at 37°C. TbIPMK can phosphorylate I(1,4,5)P₃ but not I(1,3,4)P₃ to produce I(1,3,4)P₅
739 and PP-IP₄, and can phosphorylate I(1,3,4,5)P₄, and I(1,4,5,6)P₄ to produce IP₅ and PP-IP₄. It can also
740 phosphorylate I(1,3,4,5,6)P₅ to PP-IP₄. Other *arrows* show bands corresponding to ATP, IP₄, and IP₃.
741 TbIPMK can phosphorylate I(1,4)P₂ to produce IP₄, and I(1,4,5)P₃ to produce IP₅ and PP-IP₄.
742 C. Treatment of the sample with perchloric acid (PA) eliminates the band corresponding to PP-IP₄ but
743 has no effect on IP₆. Other *arrows* indicate bands corresponding to ATP and IP₃.
744 D. Optimum pH for TbIPMK activity is within the physiological range.
745 E. TbIPMK can only phosphorylate positions 3 and 6 of different IP₅ derivatives to generate IP₆. Note
746 the lower synthesis of PP-IP₄ using I(1,3,4,5,6)P₅ as substrate compared to results obtained in (B) and
747 (C). We observed that shorter enzymatic reaction time resulted in less PP-IP₄ synthesis.
748 All results are representative of three or more independent experiments.

749

750 Fig. 3. *TbIPMK*, and *TbIP5K* complementation of yeast mutants.

751 A. HPLC analysis of soluble inositol phosphates of *S. cerevisiae arg82Δ* mutants transformed with an
752 empty vector (*red*) or a vector containing the entire open reading frame of *TbIPMK* (*blue*), and
753 compared to those of wild-type (WT) yeast transformed with empty vector (*black*).

754 B. Growth of the same cells in liquid medium as estimated by measuring optical density at 660 nm.
755 *arg82Δ* mutants had reduced growth, which was restored by expression of *TbIPMK*. Mean \pm s.d. for
756 three independent experiments, each one with 6 duplicates.

757 C. WT, and *arg82Δ* transformed with empty vector or *arg82Δ* transformed with *TbIPMK* (serially
758 diluted 10-fold, 10⁶-10 cells/spot from left to right) were spotted on YPD plates and incubated at 30°C
759 for 2 days.

760 D. HPLC analysis of soluble inositol phosphates of *Scipk1Δ* mutants transformed with an empty vector
761 (*red*) or a vector containing the entire open reading frame of *TbIPMK* (*blue*) and compared with wild
762 type transformed with an empty vector (*black*).

763 E. HPLC analysis of *Scipk1ΔKcs1Δ* complemented with empty vector (*red*) or *TbIP5K* (*green*) shows
764 reconstitution of IP₆ synthesis. In *black*, wild type transformed with empty vector.

765 All results are representative of three or more independent experiments.

766

767 Fig. 4. TbIP6K activity and complementation of yeast mutants.

768 A. Kinase reactions performed with recombinant TbIP6K (2 μg) using the indicated substrates at 150
769 μM for 1 hour at 37°C. TbIP6K can phosphorylate I(1,3,4,5,6)P₅ to PP-IP₄ and IP₆ to produce IP₇
770 (5PP-IP₅) but cannot phosphorylate IP₇ to produce IP₈. Other *arrows* show bands corresponding to
771 ATP, and IP₅.

772 B. HPLC analysis of soluble inositol phosphates of *S. cerevisiae kcs1Δ* mutants transformed with an
773 empty vector (*red*) or a vector containing the entire open reading frame of *TbIP6K* (*blue*).

774 C. Growth of the same cells in liquid medium as estimated by measuring optical density at 660 nm.
775 *kcs1Δ* mutants had reduced growth, which was restored by expression of *TbIP6K*. Mean ± s.d. for
776 three independent experiments, each one with 6 duplicates.

777 D. WT, and *kcs1Δ* transformed with empty vector or *kcs1Δ* transformed with *TbIP6K* (serially diluted
778 10-fold, 10⁶-10 cells/spot from left to right) were spotted on YPD plates and incubated at 30°C for 2
779 days.

780 E. Optimum pH for TbIP6K activity is under acidic conditions. We detected a higher activity at pH 6.0
781 and 6.5.

782 All results are representative of three or more independent experiments.

783

784 Fig. 5. HPLC and PAGE analyses of soluble inositol phosphates from *T. brucei* PCF and BSF.
785 A. PCF showed the presence of IP, IP₂, IP₃ and IP₆.
786 B. BSF showed the presence of IP, and IP₂. Cells were labeled with [³H]inositol as described under
787 *Experimental Procedures*.
788 C-E. PAGE analyses of extracts from PCF (C) or BSF (D) or standard IP₆ (E). Samples in (C) and (D)
789 (5 x 10⁹ cells) were treated with phytase (Phy) (0.1 mg/ml, pH 5.0, at 37°C for 1 hour) to confirm that
790 the bands correspond to IP₆.
791 E. Phytase control activity with IP₆ standard.
792 All results are representative of three or more independent experiments.
793

794 Fig. 6. Phenotypic changes of mutant BSF deficient in *TbIPMK*.
795 A. qRT-PCR analysis of gene expression of *TbIPMK* at time 0 and after 1 and 3 days in the absence of
796 tetracycline as compared to expression of control actin. Values are means ± s.e.m., *n* = 3. *P* < 0.001 at
797 days 1 and 3 without tetracycline. Student's *t* test.
798 B. In vitro growth of BSF in the presence (+*Tet*) or absence (-*Tet*) of 1 μM tetracycline. Values are
799 means ± s.e.m., *n* = 3 (bars are smaller than symbols).
800 C. Quantification of short-chain polyphosphate in control (+*Tet*) and induced (-*Tet*) *TbIPMK*
801 conditional knockout BSF. Values are means ± s.e.m, *n* = 3, **P* < 0.05. Student's *t* test.
802 D. Numeric distribution of acidocalcisomes in BSF. Whole unfixed parasites were observed by
803 transmission electron microscopy and the number of acidocalcisomes per cell in ~100 cells of control
804 (+*Tet*) and conditional *TbIPMK* mutants (-*Tet*) were counted (the results from 3 independent
805 experiments were combined).
806 E, F. Scanning transmission electron microscopy (STEM) images from control (E) or *TbIPMK*
807 conditional mutant BSF showing acidocalcisomes. Bar = 1 μm. *Insets* show acidocalcisomes
808 highlighted in (E) and (F) at higher magnification. Bars = 0.5 μm.

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