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1           **Conditional gene deletion with DiCre demonstrates an essential role for CRK3 in**  
2                           *Leishmania mexicana* cell cycle regulation

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7  
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14 Running Head: Conditional gene deletion in *Leishmania*

15  
16 Key words: genome engineering, DiCre recombinase, cyclin-dependent kinase

17  
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19  
20  
21 **Abstract**

22 *Leishmania mexicana* has a large family of cyclin-dependent kinases (CDKs) that reflect the  
23 complex interplay between cell cycle and life cycle progression. Evidence from previous  
24 studies indicated that Cdc2 related kinase 3 (CRK3) in complex with the cyclin CYC6 is a  
25 functional homologue of the major cell cycle regulator CDK1, yet definitive genetic evidence  
26 for an essential role in parasite proliferation is lacking. To address this, we have implemented  
27 an inducible gene deletion system based on a dimerised Cre recombinase (diCre) to target  
28 CRK3 and elucidate its role in the cell cycle of *L. mexicana*. Induction of diCre activity in  
29 promastigotes with rapamycin resulted in efficient deletion of floxed *CRK3*, resulting in  
30 G2/M growth arrest. Co-expression of a *CRK3* transgene during rapamycin-induced deletion  
31 of *CRK3* resulted in complementation of growth, whereas expression of an active site  
32 *CRK3*<sup>T178E</sup> mutant did not, showing that protein kinase activity is crucial for CRK3 function.  
33 Inducible deletion of *CRK3* in stationary phase promastigotes resulted in attenuated growth in  
34 mice, thereby confirming CRK3 as a useful therapeutic target and diCre as a valuable new  
35 tool for analysing essential genes in *Leishmania*.

36  
37 **Introduction**

38

39 The leishmaniasis, diseases caused by protozoan parasites of the genus *Leishmania*, have  
40 diverse clinical manifestations dependent on the species and host immune response.  
41 Leishmaniasis is a substantial public health issue, causing an estimated 40,000 deaths  
42 annually and approximately 0.2 to 0.4 and 0.7 to 1.2 million visceral and cutaneous  
43 manifestations of the disease respectively (Alvar *et al.*, 2012). Existing drug therapies are  
44 problematic due to high treatment costs, toxicity and undesirable administration routes,  
45 making the development of novel and effective drug therapies to expand the current  
46 repertoire crucial. Phenotypic strategies to identify drug targets in the mammalian infective  
47 amastigote life cycle stage are of particular importance for drug discovery programs.

48

49 As unicellular organisms, *Leishmania* depend on stringent control of cellular division to  
50 propagate and maintain infection. Protein kinases elicit pronounced effects on the *Leishmania*  
51 cell cycle by regulation of cell signalling pathways, and a number of protein kinases have  
52 been identified that are essential for promastigote viability (Wang *et al.*, 2005; Dacher *et al.*,  
53 2014). The cyclin-dependent kinases (CDK) are of particular interest due to their pivotal  
54 roles as cell cycle regulators. The use of CDK inhibitors in cancer therapy (Cicenas and  
55 Valius, 2011; Knapp and Sundström, 2014) and the relative expansion of this protein family  
56 in *Leishmania* relative to other unicellular organisms distinguishes them as suitable drug  
57 targets. In particular, the CDK related kinase CRK3 has been demonstrated as being  
58 important for regulation of the *L. mexicana* promastigote cell cycle by existing genetic  
59 manipulation techniques and cell cycle arrest following treatment with CDK inhibitors (Grant  
60 *et al.* 1998; Hassan *et al.* 2001; Grant *et al.* 2004). Recombinant protein kinase activity assays  
61 (Gomes *et al.*, 2010) and yeast recovery mutants (Wang *et al.*, 1998) have provided further  
62 validation of CRK3 as a drug target, leading to the identification and synthesis of a number of  
63 CRK3 inhibitors (Grant *et al.*, 2004; Cleghorn *et al.*, 2011; Walker *et al.*, 2011; Goyal *et al.*,  
64 2014; Řezníčková *et al.*, 2015). Regulation of CRK3 expression in *L. mexicana* is desirable to  
65 further assess its function in both procyclic promastigote and amastigote life cycle stages,  
66 however, no system exists for conditional deletion of essential genes. Recent application of  
67 plasmid shuffle methodology has addressed this issue by enabling the generation of partial  
68 *null* mutants to further study essentiality and important residues within coding sequences  
69 (Morales *et al.*, 2010; Dacher *et al.*, 2014), however the gene is not deleted and this prevents  
70 phenotyping of a *null* mutant.

71

72 To address this limitation, we have implemented a rapamycin-inducible gene deletion system  
73 using a dimerised Cre recombinase (diCre) (Jullien *et al.*, 2003; Collins *et al.*, 2013;  
74 Andenmatten *et al.*, 2013) to target *CRK3* and elucidate its role in the cell cycle of *L.*  
75 *mexicana*. *L. mexicana* is generally diploid (Rogers *et al.*, 2011) and both *CRK3* alleles were  
76 replaced with a ‘floxed’ *CRK3* open reading frame and the diCre coding sequence through  
77 promastigote transfection and homologous recombination. This system was used to  
78 conditionally delete *CRK3* during promastigote growth and so prove that *CRK3* mediates the  
79 transition through G2/M. Induced loss of *CRK3* was complemented by expression of a *CRK3*  
80 transgene but not by expression of an inactive site (T178E) *CRK3* mutant, showing that  
81 protein kinase activity is crucial for *CRK3* function. Significantly, conditional deletion of  
82 *CRK3* in stationary phase promastigotes and subsequent attenuation during murine infection  
83 demonstrates that *CRK3* activity is essential for establishing infection. This system represents  
84 a new method to directly assess whether a gene is essential to parasite viability and provides  
85 novel insight into the function of essential genes in *Leishmania*.

86

## 87 **Results**

88

89 *DiCre activity is tightly regulated in L. mexicana promastigotes and amastigotes*

90 To test the activity of diCre in *L. mexicana* promastigotes, a reporter cell line was generated  
91 by integration of a loxP-flanked *GFP* into the ribosomal locus: [*SSU GFP<sup>Flox</sup>*]. This cell line  
92 was transfected with a diCre construct containing the two dimerizable Cre recombinase  
93 subunits with the homologous flanks of *crk3* to generate the heterozygous line  
94 ( $\Delta crk3::DICRE/CRK3$  [*SSU GFP<sup>Flox</sup>*]). Integration of the diCre construct at the *CRK3* locus  
95 was confirmed by PCR analysis (Fig. S1A). No effect on the growth of *SSU GFP<sup>Flox</sup>* or  
96  $\Delta crk3::DICRE/CRK3$  [*SSU GFP<sup>Flox</sup>*] was observed in the presence of the dimerization ligand,  
97 rapamycin, up to the highest dose of 250 nM (Fig. S1B). *GFP* excision following incubation  
98 with increasing concentrations of rapamycin was investigated by PCR using specific primers  
99 flanking *GFP*. A single 1.45 kb PCR product, the floxed *GFP* fragment, was detected in the  
100 absence of rapamycin, whilst a 0.69 kb PCR product, representing the excised locus, was  
101 detected following rapamycin treatment only (Fig. 1A), indicating tight regulation of diCre  
102 activity.  $\Delta crk3::DICRE/CRK3$  [*SSU GFP<sup>Flox</sup>*] and [*SSU GFP<sup>Flox</sup>*] promastigotes grown for 5  
103 days in the presence or absence of increasing concentrations of rapamycin were analysed by  
104 flow cytometry to measure levels of *GFP* expression (Fig. 1B). Treatment of

105  $\Delta crk3::DICRE/CRK3$  [*SSU GFP<sup>Flox</sup>*] promastigotes with greater than 5 nM rapamycin  
106 resulted in substantial loss of GFP expression compared with the untreated controls, whilst  
107 GFP expression in [*SSU GFP<sup>Flox</sup>*] was the same following growth in all concentrations of  
108 rapamycin. GFP loss in  $\Delta crk3::DICRE/CRK3$  [*SSU GFP<sup>Flox</sup>*] promastigotes grown in the  
109 presence or absence of 100 nM rapamycin for 5 days was further assessed by Western  
110 blotting of total protein extracts using anti-GFP antibody (Fig. 1C). Rapamycin treated  
111 promastigotes had considerably reduced GFP compared with the untreated controls, thereby  
112 demonstrating that gene loss results in reduced protein expression. These data also  
113 demonstrate that expression of *diCre* from the *CRK3* locus is sufficient to efficiently excise  
114 the *GFP* transgene at rapamycin concentrations above 5 nM, and that no background diCre  
115 activity can be detected in the absence of ligand. 100 nM rapamycin was chosen as the  
116 optimum concentration to induce diCre activity in promastigotes whilst having no effect on *in*  
117 *vitro* cell growth.

118 To test diCre functionality in amastigotes, infectious promastigotes of the experimental line  
119  $\Delta crk3::DICRE/CRK3$  [*SSU GFP<sup>Flox</sup>*] were inoculated into BALB/c footpads and amastigotes  
120 purified from the resulting lesion. *Ex vivo* amastigotes retained high levels of green  
121 fluorescence and were incubated with rapamycin for 24 hrs in Schneider's medium prior to  
122 infection of bone-marrow derived macrophages. Efficient excision of *GFP<sup>Flox</sup>* was detected  
123 by PCR amplification of a 0.69 kb fragment representative of *GFP* loss in all rapamycin  
124 treated samples (Fig. 1D) and *GFP<sup>-</sup>* (non-fluorescent) amastigotes were observed by  
125 comparing images obtained through fluorescence live cell imaging (Fig. S1C). Residual  
126 *GFP<sup>+</sup>* amastigotes were still visible by microscopy (Fig. S1C) and could be detected by flow  
127 cytometry (Fig. S1D); this was possibly due to the slow replication rate of amastigotes  
128 leading to a low rate of GFP turnover. These data demonstrate inducible diCre activity in  
129 amastigotes.

### 130 *Inducible deletion of CRK3 in L. mexicana promastigotes*

131 The functional and efficient levels of diCre-mediated excision of *GFP* underpinned the  
132 development of a system for conditional deletion of essential genes. Gateway recombineering  
133 was used to flank appropriate diCre and loxP expression constructs with gene-specific,  
134 homologous flanks (Fig. S2). Plasmids were generated by this method to replace the two  
135 alleles of *CRK3*, an essential gene in *L. mexicana* (Hassan *et al.*, 2001) (Fig S3A). The first  
136 allele of *CRK3* was replaced with *DICRE* ( $\Delta crk3::DICRE/CRK3$ ) and the second allele of

137 *CRK3* was subsequently replaced with a floxed C-terminal GFP-tagged *CRK3* version  
138 ( $\Delta crk3::DICRE/\Delta crk3::CRK3^{Fllox}$ ; Figs. 2A and S3B). In addition, an *mCherry* red fluorescent  
139 protein coding sequence was incorporated downstream from the floxed *CRK3-GFP* to  
140 facilitate flow cytometry and microscopy analysis. Transfection resulted in multiple clones  
141 with the expected genetic modifications, as confirmed by PCR analysis (Fig. S3B).

142

143 The growth of promastigotes from two  $\Delta crk3::DICRE/\Delta crk3::CRK3^{Fllox}$  clones were assessed  
144 following diCre-mediated excision induced with 100 nM rapamycin (Fig. 2B). Cells were  
145 counted over the course of 5 days, revealing a pronounced growth defect and reduction in cell  
146 number in rapamycin treated cells compared with uninduced controls. PCR analysis of  
147 promastigotes grown in the presence or absence of 100 nM rapamycin for 24 h and 48 h  
148 confirmed efficient loss of the *CRK3* gene (Fig. 2C) by the amplification of a single 1.36 kb  
149 DNA fragment for both rapamycin treated clones. The retention of the 3.4 kb amplicon  
150 containing the *CRK3* gene in both untreated clones is evidence that no background diCre  
151 activity can be detected in the absence of rapamycin. To test for loss of the CRK3-GFP  
152 protein, total protein extracts of clone 2 promastigotes grown for 96 h in the presence or  
153 absence of 100 nM rapamycin were analysed by Western blot analysis with anti-GFP  
154 antibody (Fig. 2D) Very low levels of protein were detected in the treated promastigotes  
155 compared to the untreated cells, confirming that the conditional gene loss leads to reduced  
156 protein levels. Treatment with 100 nM rapamycin did not result in any noticeable effect on *L.*  
157 *mexicana* promastigote growth (Fig. S1B), however the pronounced growth arrest arising  
158 from loss of the essential gene could possibly result in cellular stress that synergises with  
159 rapamycin. These data show that this is a viable genetic manipulation strategy and that loss of  
160 *CRK3* resulted in growth arrest and reduced cell numbers, both phenotypes consistent with  
161 loss of an essential gene.

162

163 *Cell cycle analysis of CRK3-deficient promastigotes.*

164

165 Previous attempts to impair *CRK3* function in *Leishmania* by treatment with protein kinase  
166 inhibitors may have resulted in off-target effects (Grant *et al.*, 2004; Reichwald *et al.*, 2008;  
167 Cleghorn *et al.*, 2011; Jorda *et al.*, 2011; Efstathiou *et al.*, 2014; Řezníčková *et al.*, 2015).  
168 Here the utilisation of diCre mediated gene deletion enabled the effect of *CRK3* depletion on  
169 the cell cycle to be investigated. Firstly, microscopic analysis of the cells at 96 h post-  
170 induction showed an accumulation of large, aberrant cells with altered organelle homeostasis

171 as evidenced by the presence of cells with multiple flagella (Fig. 3A). DAPI labelling of such  
172 multi-flagellated cells to visualise cellular DNA revealed the presence of enlarged nuclei  
173 indicative of an arrest in mitosis. Interestingly, cells were also observed that lacked a nucleus  
174 but retained the kinetoplast ('zoids'), a cell cycle defect observed previously by the treatment  
175 of promastigotes with CDK inhibitors (Grant *et al.*, 2004). Secondly, flow cytometry was  
176 performed to determine the overall DNA content of  $\Delta crk3::DICRE/\Delta crk3::CRK3^{Flox}$   
177 promastigotes grown in the presence or absence of 100 nM rapamycin for 72 and 96 h (Fig.  
178 3B). This analysis showed that conditional deletion of *CRK3* resulted in the accumulation of  
179 cells with 4C DNA content, associated with cell cycle arrest at G2/M, whilst an increasing  
180 population of cells with DNA content <1C indicates the accumulation of zoids. Finally, to  
181 assess the rate of cell death occurring in *CRK3*-deficient cells a viability assay was performed  
182 on promastigotes after growth in the presence or absence of 100 nM rapamycin for 72 h  
183 (Figs. 3C & S4). After 72 h the proportion of propidium iodide positive cells (PI<sup>+</sup>) was  
184 around 40% indicating a high level of cell death, which likely resulted from the accumulation  
185 of anucleated zoids at this time point. Flow cytometry analysis of cell size (using forward  
186 scatter) was in agreement with the microscopy analysis and showed that *CRK3* deficient cells  
187 were substantially larger than cells retaining the gene (Fig. S4). Taken together, these data  
188 provide evidence that *CRK3* plays an essential role in regulating mitosis in replicating  
189 promastigotes.

190

### 191 *Active CRK3 is required for cell cycle progression in promastigotes*

192 We demonstrated that diCre could be used to efficiently delete a floxed copy of *CRK3*, so we  
193 exploited the efficiency of this system to further study gene function through  
194 complementation. Such a system was established by expressing a histidine-tagged *CRK3*  
195 (*CRK3<sup>his</sup>*) (Hassan *et al.*, 2001) transgene in  $\Delta crk3::DICRE/\Delta crk3::CRK3^{Flox}$  promastigotes.  
196 No significant difference in growth was noted in the presence or absence of rapamycin over a  
197 5 day period (Fig. 4A). Efficient excision of floxed *CRK3* in the induced culture was  
198 confirmed by PCR amplification of the diagnostic 1.36 kb fragment by 24 h post-treatment  
199 with 100 nM rapamycin (Fig. 4B). The proliferation of promastigotes, despite loss of floxed  
200 *CRK3*, indicates *CRK3* transgene complementation in the induced  $\Delta crk3$  cell line. Previous  
201 studies have shown that recombinant *L. mexicana* *CRK3*<sup>T178E</sup> protein lacks H1 kinase activity  
202 (Gomes *et al.*, 2010) and an *L. major* *CRK3*<sup>T178E</sup> mutant fails to complement a *cdc2-33(ts)*  
203 yeast mutant (Wang *et al.*, 1998). To test whether active *CRK3* is required for cell growth,

204 we exploited this complementation approach by generation of the cell line  
205  $\Delta crk3::DICRE/\Delta crk3::CRK3^{Flox}$  [*SSU CRK3<sup>T178E</sup>*] expressing a T-loop residue mutated  
206 version of *CRK3* from the ribosomal locus. Growth curves indicate that expression of the  
207 *CRK3<sup>T178E</sup>* transgene failed to complement the loss of *CRK3<sup>Flox</sup>* following induction with  
208 rapamycin (Fig. 4A, B) thereby demonstrating that *CRK3<sup>T178E</sup>* cannot rescue loss of active  
209 *CRK3*. The overall growth rate of both complementation mutants was reduced relative to the  
210 parental line (Table 1) and may explain the growth arrest at 72 h following excision of *CRK3*  
211 in  $\Delta crk3::DICRE/\Delta crk3::CRK3^{Flox}$  [*SSU CRK3<sup>T178E</sup>*] compared with a more rapid onset of  
212 growth arrest in the parental line (Fig. 2B). These data show that active *CRK3* is required for  
213 parasite growth. The *CRK3* deficient cells were analysed by flow cytometry and fluorescence  
214 microscopy showing that  $\Delta crk3::DICRE/\Delta crk3::CRK3^{Flox}$  [*SSU CRK3<sup>T178E</sup>*] cells were  
215 blocked in G2/M (Fig. 4C) and were multi-nucleate and aberrant (Fig. 4D). These data are in  
216 agreement with the phenotype observed following excision of *CRK3* in wild-type cells (Fig.  
217 3A & B), thereby indicating the importance of the T-loop in regulating *CRK3* activity. Based  
218 on these results, we conclude that transgene complementation can be used to confirm the  
219 specificity of conditional deletion of essential genes and also to probe the function of genes  
220 following mutagenesis.

221

### 222 *CRK3 is essential for in vivo infection of murine hosts*

223 The lack of a conditional system to regulate expression of essential genes is a major obstacle  
224 for *in vivo* studies of essentiality, with such studies having crucial applications for drug target  
225 validation. To address this we tested if *CRK3* activity is essential for survival of the parasite  
226 over the course of *in vivo* infection. Monitoring infection by detection of the light signal  
227 emitted from bioluminescent *Leishmania* using an *in vivo* imaging system (IVIS) is an  
228 established, longitudinal and non-invasive method to correlate signal with pathogen load  
229 (Lang *et al.*, 2005; Lecoecur *et al.*, 2007; Talmi-Frank *et al.*, 2012; Vasquez *et al.*, 2015). To  
230 assess the outcome of *CRK3* loss on the proliferation of *L. mexicana in vivo*, bioluminescent  
231 lines were generated by transfection of *L. mexicana* wild-type and  
232  $\Delta crk3::DICRE/\Delta crk3::CRK3^{Flox}$  promastigotes with a ribosomal integration construct  
233 encoding red-shifted firefly luciferase, Ppy RE9H (Branchini *et al.*, 2010; McLatchie *et al.*,  
234 2013). Both lines were bioluminescent as determined by luciferase expression assays on  
235 logarithmic stage promastigotes. The resulting  $\Delta crk3::DICRE/\Delta crk3::CRK3^{Flox}$  [*SSU RE9H*]  
236 cell line produced 5 fold higher bioluminescence compared with the wild-type [*SSU RE9H*]



237 control (Fig. S4). Footpad bioluminescence detected with an *in vivo* imaging system (IVIS)  
238 correlated well with parasite burden in mice infected with *L. mexicana* expressing Ppy RE9H  
239 (Fig. 5A;  $y = 4.8 + 0.43x$ ,  $R^2 = 0.743$  and  $p < 0.0001$ ). The slope of the linear regression line  
240 (0.43) revealed smaller increases in bioluminescence with increasing parasite burden. This  
241 may be related to tissue absorbance of light *in vivo* or limited substrate availability with  
242 increasing numbers of amastigotes within the lesion. Nevertheless, these data show that  
243 parasite burdens can be predicted from bioluminescence and that IVIS could be used for the  
244 non-invasive monitoring of parasite growth in mice over 10 weeks of infection. Following  
245 treatment of  $\Delta crk3::DICRE/\Delta crk3::CRK3^{Flox}$  stationary phase promastigotes with rapamycin  
246 for 24 h the amplification of a 1.36 kb fragment (Fig. 5B) indicated that the majority of  
247 parasites had successfully excised floxed *CRK3*. The presence of small amounts of a 3.4 kb  
248 amplicon corresponding to the intact floxed *CRK3* gene, however, also suggested that some  
249 parasites had retained the gene. These stationary phase  $\Delta crk3::DICRE/\Delta crk3::CRK3^{Flox}$  [*SSU*  
250 *RE9H*] promastigotes either rapamycin treated (+ Rap) or not treated (- Rap) were then  
251 inoculated into the footpads of BALB/c mice. The *in vivo* bioluminescence in footpads of  
252 mice infected with the rapamycin-treated  $\Delta crk3::DICRE/\Delta crk3::CRK3^{Flox}$  [*SSU RE9H*] was  
253 significantly reduced compared to the uninduced control by 5 weeks post-infection ( $p$   
254  $< 0.001$ ) and this continued up to 9 weeks post-infection ( $p < 0.005$ ) (Fig. 5C, D). From 5 to 9  
255 weeks the bioluminescence from footpads infected with rapamycin-treated parasites  
256 increased 100-fold and was likely due to the proliferation of parasites that had not responded  
257 to rapamycin treatment and persisted in the lesion. To investigate this possibility, viable  
258 amastigotes were purified from the lesions of four mice at 10 weeks post-infection and  
259 analysed for the presence of *CRK3*<sup>Flox</sup> by PCR after a single round of *in vitro* culture (Fig.  
260 5E). A 3.4 kb PCR product containing *CRK3* was amplified from all samples, indicating the  
261 persistence of parasites that had escaped diCRE mediated excision of *CRK3*.

262

263 The ability of *CRK3* deficient promastigotes to establish infection was further assessed by  
264 measuring footpad sizes at weekly intervals (Fig. 5F). The footpad sizes of mice infected with  
265 either untreated or rapamycin-treated  $\Delta crk3::DICRE/\Delta crk3::CRK3^{Flox}$  [*SSU RE9H*] parasites  
266 were similarly low until about 4 weeks post-infection. Subsequently, footpads containing  
267 untreated parasites increased steadily over the course of infection, whilst those infected with  
268 rapamycin-treated  $\Delta crk3::DICRE/\Delta crk3::CRK3^{Flox}$  [*SSU RE9H*] remained low until 9 weeks  
269 post infection. Comparison of the bioluminescence and lesion sizes suggest that there is a  
270 delay in lesion development despite parasite proliferation and that the lesions only increase

271 significantly when parasite load reaches a certain level (equating to bioluminescence  $\approx 10^7$   
272 photons/sec); in the case of the untreated parasites this occurred from about 5 weeks while for  
273 rapamycin-treated parasites this level of parasite burden had still not been reached by 9  
274 weeks. Altogether these data show that loss of active CRK3 impairs the establishment of  
275 infection *in vivo*, and that a later resurgence of parasites likely results from a small population  
276 of cells which previously escaped *CRK3* conditional deletion.

277

## 278 **Discussion**

279

280 We have developed an inducible system for the genetic manipulation of essential genes in  
281 *Leishmania*. Inducible diCre was used to demonstrate the requirement for CRK3 activity in  
282 the regulation of mitosis. A distinct growth defect was observed 48 h after induced deletion  
283 of *CRK3* (Fig. 2) resulting in cells arrested in G2/M, as well as an accumulation of zoids and  
284 eventually a population of enlarged, multi-flagellated cells (Fig. 3). This phenotype was  
285 rescued by expression of a *CRK3* transgene from the ribosomal locus, confirming that loss of  
286 CRK3 caused mitotic arrest (Fig. 4). Arrest in G2/M and the accumulation of zoids have  
287 previously been reported following incubation of *L. mexicana* promastigotes with the CRK3  
288 inhibitors flavopiridol (Hassan *et al.*, 2001) and indirubin (Grant *et al.* 2004), showing  
289 correlation between genetic and chemical downregulation of CRK3 activity. In *Trypanosoma*  
290 *brucei* RNAi knockdown of the syntenic orthologue of *CRK3* in the procyclic form also  
291 results in G2/M arrest and zoid formation (Tu and Wang, 2004), with the accumulation of  
292 such aberrant cells explained by the lack of a checkpoint controlling exit from mitosis and  
293 entry in cytokinesis (Ploubidou *et al.*, 1999; Hammarton *et al.*, 2003). Inducible deletion of  
294 *CRK3* indicates that this checkpoint is also absent in *L. mexicana* promastigotes, resulting in  
295 impairment of mitotic progression, followed by re-initiation of G1 in the absence of  
296 cytokinesis. It appears that these abnormal cells can eventually undergo cytokinesis; however  
297 the daughter cell lacks a nucleus and is often multi-flagellated (see bi-flagellated zoid in Fig  
298 3A), whilst the high levels of cell death occurring 72 h after gene loss show that such progeny  
299 are not viable.

300

301 CRK3 is active at different stages in the cell cycle by forming complexes with cyclin partners  
302 such as CYC6 and CYCA, therefore CRK3 deletion could impact the cell cycle at multiple  
303 stages. RNAi of the CYC6 in *T. brucei* procyclic forms results in growth arrest within 48 h of  
304 induction and the accumulation of zoids and cells in G2/M (Hammarton *et al.*, 2003). A

305 similar phenotype was found in this study with *CRK3* inducible deletion, suggesting that the  
306 *CRK3:CYC6* complex is involved in regulation of mitosis (Walker *et al.*, 2011). Less is  
307 known about the activity of *CRK3:CYCA*. Protein expression assays of *L. donovani* *CYC1*  
308 (the functional orthologue of *CYCA*) demonstrates an increased abundance during S-phase  
309 (Banerjee *et al.*, 2006) coupled with histone phosphorylation by an active *CRK3:CYC1*  
310 complex (Maity *et al.*, 2011), which is suggestive of S-phase kinase activity. Active,  
311 recombinant *L. mexicana* *CRK3:CYCA* has also been engineered, with phosphorylation of  
312 the T-loop residue T178 by the CDK activating kinase (CAK) Civ-1 increasing activity  
313 (Gomes *et al.*, 2010). The T178 residue is essential for *CRK3* activity as T178E mutagenesis  
314 inhibits functional rescue in *S. pombe* (Wang 1998) and ablates kinase activity in  
315 recombinant *CRK3<sup>T178E</sup>:CYCA* (Gomes *et al.*, 2010). The necessity of T178 was tested  
316 directly in this study, with excision of floxed *CRK3* in the  $\Delta crk3::DICRE/\Delta crk3::CRK3^{Flox}$   
317 [*SSU CRK3<sup>T178E</sup>*] line leading to cell cycle arrest in G2/M and zoid formation. The growth  
318 rate of this line and  $\Delta crk3::DICRE/\Delta crk3::CRK3^{Flox}$  [*SSU CRK3*] were reduced when  
319 compared to  $\Delta crk3::DICRE/\Delta crk3::CRK3^{Flox}$  (Table 1), indicative of generally reduced  
320 growth rate when expressing a transgene. Episomal complementation with *CRK3* did not  
321 result in an observable growth defect (Hassan *et al.*, 2001), but this may result from the  
322 modulation of the number of episomal copies, as has been observed previously following  
323 complementation of the essential *MCA* gene (Ambit *et al.*, 2008). Integration into the 18s  
324 rRNA locus results in consistently high levels of expression (Misslitz *et al.*, 2000) leading to  
325 non-physiological levels of *CRK3* and subsequent *CRK3:CYC6* activity at potentially  
326 inappropriate stages of the life cycle.

327

328 The reduced growth rate of promastigotes overexpressing *CRK3<sup>T178E</sup>* is likely due to a partial  
329 dominant negative effect, whereby inactive *CRK3<sup>T178E</sup>* binds endogenous *CYC6* leading to  
330 impaired protein kinase activity even in the presence of active *CRK3*. This reduced growth  
331 rate may explain both the cell cycle arrest at 72 h in the [*SSU CRK3<sup>T178E</sup>*] complemented line  
332 (Fig. 4A) compared to arrest at 48 hours in  $\Delta crk3::DICRE/\Delta crk3::CRK3^{Flox}$  (Fig. 2B) and  
333 additionally the lower proportion of zoids when analysed by flow cytometry (Fig. 4C). The  
334 accumulation in G2/M suggests that mutation ablates *CRK3:CYC6* activity, rather than  
335 *CRK3:CYCA*, where an increase of cells in G1/S might be anticipated. Both induced and  
336 uninduced  $\Delta crk3::DICRE/\Delta crk3::CRK3^{Flox}$  [*SSU CRK3<sup>T178E</sup>*] have dramatically reduced  
337 flagellum length and are immotile (Fig. 4D).. The reduced size of the flagellum and a growth  
338 defect are similar phenotypes to those observed in cell lines deficient in *ATG5*, a key

339 component of the autophagic pathway (Williams *et al.*, 2012). This is likely a result of their  
340 impaired ability to salvage material through the autophagic pathway, imparting selection on  
341 the parasites to reduce energy through flagellum regression. The partial dominant negative  
342 effect of CRK3<sup>T178E</sup> may also result in metabolic stress in these cells leading to the phenotype  
343 observed. The importance of T178 as an active site residue for regulating progression through  
344 G2/M implicates upstream modifiers of this residue as essential regulators of the *L. mexicana*  
345 cell cycle. In mammalian cells CDK7 acts as a CAK to regulate CDK1 by phosphorylation at  
346 this T-loop residue, yet no CAK homologues have been identified in the *Leishmania* genome  
347 (Gomes *et al.*, 2010). The identification of potential post-transcriptional modifiers of the  
348 CRK3 T-loop residue that act in an analogous fashion to CDK7 would therefore yield  
349 promising targets for drug discovery. The phenotype of the induced cell line shows the  
350 importance of the T-loop residue for CRK3 activity and mitotic function within the cell,  
351 endorsing this complementation assay as a rational approach for active site investigation.

352

353 The assessment of gene essentiality for amastigote viability is an important approach in the  
354 context of drug target validation as this life cycle stage is the pathologically significant form.  
355 The recent utilisation of plasmid shuffle has facilitated the study of *Leishmania* genes  
356 involved in life cycle differentiation and essentiality both in amastigote and promastigote  
357 forms by the generation of partial *null* mutants (Morales *et al.*, 2010; Dacher *et al.*, 2014).  
358 Retention of an episomal gene in a *null* mutant cell line after murine infection is a useful  
359 approach to assess that gene as necessary to amastigote survival *in vivo* (Wiese, 1998).  
360 Despite such elegant utilisation of reverse genetic methods to probe gene function, no method  
361 exists for the generation of conditional *null* mutants during *in vivo* infection. Our study does  
362 not address this lack directly due to the sensitivity of amastigotes to rapamycin, however as  
363 diCre activity remains high in stationary-phase promastigotes *CRK3* was efficiently excised  
364 (Fig. 5B) to probe the subsequent infectivity of CRK3-deficient promastigotes. By tracking  
365 the progression of infection with reporter parasites expressing the highly sensitive red-shifted  
366 luciferase (Branchini *et al.*, 2010; McLatchie *et al.*, 2013) and by footpad size measurement,  
367 we demonstrate that the *CRK3*-deficient *L. mexicana* are unable to proliferate in their  
368 mammalian host (Figs. 5C, 5D and 5F). Importantly, the wild-type line expressing luciferase  
369 grows normally in mice following rapamycin treatment, which indicates that lack of growth  
370 of the *CRK3*-deficient mutant is not a result of the drug treatment. The average light  
371 intensities emitted from footpads infected with the wild-type [*SSU RE9H*] line and those from  
372 footpads infected with the  $\Delta crk3::DICRE/\Delta crk3::CRK3^{Fllox}$  [*SSU RE9H*] line retaining floxed

373 *CRK3* are at similar levels throughout infection, yet mean footpad size is larger in wild-type  
374 [*SSU RE9H*] infected mice after 3 weeks post infection; such disagreement may be a result of  
375 the 5 fold lower signal intensity of the wild-type [*SSU RE9H*] compared with the  
376 experimental line (Fig. S5) and therefore an overall higher burden of the wild-type line is  
377 likely masked by a reduced bioluminescent signal intensity.

378

379 Interestingly, parasite burden as measured by total flux remains consistently above the  
380 background intensity (dashed line, Fig. 5D) in those footpads infected with the *CRK3*-  
381 deficient line, suggestive of the survival of a low number of bioluminescent parasites. The  
382 outgrowth of these parasites was observed through an increased bioluminescence signal at 9  
383 weeks post infection compared with 5 weeks (Fig. 5C and 5D). Purification and PCR analysis  
384 of these parasites shows they retained the floxed *CRK3* (Fig. 5E) and that the persistence of  
385 signal and subsequent increase are a result of incomplete excision of floxed *CRK3* during the  
386 24 h incubation with rapamycin. These data further demonstrate the essentiality of *CRK3*  
387 activity for establishing infection.

388

389 This is the first time an essential gene in promastigotes has been studied *in vivo* by  
390 conditional deletion, representing a useful tool to probe gene function. We are validating the  
391 feasibility of conditional gene deletion *ex vivo* and *in vivo* using rapamycin and non-immuno-  
392 inhibitory rapamycin analogues ('rapalogs'), with such work being useful for the future of  
393 drug target validation. DiCre activity has been demonstrated *in vivo* (Jullien *et al.*, 2007),  
394 however rapamycin treatment may be a limitation due to influence on the host immune  
395 response and on amastigote proliferation. Our attempts to study the effect of *CRK3*<sup>Flox</sup>  
396 deletion in lesion-derived amastigotes grown in axenic culture medium was problematic due  
397 to reduced proliferation of both experimental and wild-type *L. mexicana* at the relatively low  
398 dose of 50 nM rapamycin, therefore the use of rapalogs would be a rational approach for  
399 induction of diCre activity if they have reduced binding affinity for *Leishmania* TORs  
400 (Madeira da Silva and Beverley, 2010). A second generation diCre is currently in  
401 development and may present an alternative method for inducible gene deletion *in vivo*. In  
402 diCre2, each subunit is fused to mutant FKBP domains that are dimerised by the rapalog  
403 AP20187, which is amenable to *in vivo* use (Collins *et al.*, 2013). Such a system could be  
404 applied for use in *Leishmania* and would complement our existing floxed gene replacement  
405 approach.

406

407 In conclusion we have developed a highly efficient inducible gene deletion system that when  
408 used with transgene complementation allows for the first time the function of essential  
409 *Leishmania* genes to be elucidated. We have applied this approach to show that CRK3 is  
410 required for promastigote progression through mitosis, with gene deletion mutants showing a  
411 G2/M arrest and an accumulation of zoids, indicative of a lack of a cell cycle checkpoint in  
412 cytokinesis. Inducible deletion of CRK3 in stationary phase promastigotes attenuates  
413 infection in a murine host, providing further genetic validation of CRK3 as a potential drug  
414 target (Grant et al. 1998; Hassan et al. 2001; Grant et al. 2004; Gomes et al. 2010; Walker et  
415 al. 2011). Our diCre method provides a powerful tool for analysing genes essential for  
416 promastigote proliferation and to the study of the differentiation of promastigotes to  
417 amastigotes.

418

## 419 **Figure legends**

420

421 **Fig. 1.** Validation of inducible diCre in *L. mexicana*: conditional deletion of *GFP* in

422 promastigotes and amastigotes.

423 A. Gene excision analysed by PCR amplification. Schematic (lower) shows the *SSU GFP<sup>Flox</sup>*  
424 locus and the recombination event expected after treatment with rapamycin (Rap). (upper)  
425 PCR amplification with oligonucleotides 4287 and 4288 from experimental  
426 ( $\Delta crk3::DICRE/CRK3$  [*SSU GFP<sup>Flox</sup>*]) and control [*SSU GFP<sup>Flox</sup>*] promastigotes at 5 days  
427 post-treatment with different concentrations of rapamycin.

428 B. Flow cytometry assessment of GFP intensity of experimental and control promastigotes  
429 incubated in the presence or absence of rapamycin for 5 days.

430 C. Western blotting analysis with anti-GFP and anti-EF1 $\alpha$  loading control antibodies of  
431 protein extracted from experimental promastigotes grown for 5 days in the presence or  
432 absence of 100 nM rapamycin.

433 D. PCR analysis of *GFP<sup>Flox</sup>* loss (as described in A) in amastigotes after 24 h rapamycin  
434 treatment (0 – 1000 nM), followed by 120 h infection in bone-marrow derived macrophages.

435 Lane 2 contains a 1 kb+ DNA ladder.

436

437 **Fig. 2.** Generation of a *CRK3* conditional deletion cell line.

438 A. Schematic showing the replacement of endogenous *CRK3* to generate

439  $\Delta crk3::DICRE/\Delta crk3::CRK3^{Flox}$ . One allele contains a *loxP* flanked *CRK3-GFP* coding

440 sequence with mCherry red-fluorescent protein cassette (*RFP*) and puromycin drug selectable  
441 marker (*PAC*). The other allele contains genes encoding both diCre subunits (*CRE59*,  
442 *CRE60*) each linked with rapamycin binding domains (not shown: *FKBP12* and *FRB*  
443 respectively) and a blasticidin resistance cassette (*BSD*). Each construct was flanked with 500  
444 bp arms of homology (light grey) by Gateway recombination to facilitate integration at the  
445 *CRK3* locus. All coding sequences are flanked by regulatory elements (dark grey). *L.*  
446 *mexicana* parasites were transfected sequentially with the *diCre* construct and floxed *CRK3*  
447 to confer resistance to blasticidin and puromycin antibiotics respectively.  
448 B. Clones 2 and 8 promastigotes were seeded at a density of  $5 \times 10^5$  cells ml<sup>-1</sup> and grown in  
449 the presence or absence (+/-) of 100 nM rapamycin for 5 days. Cell density was determined  
450 by counting at 24 h intervals and mean  $\pm$  SD of triplicate values was plotted.  
451 C. (lower) A schematic representation of the floxed *CRK3* locus after excision. PCR  
452 amplification shows the primers binding upstream of the 5' *CRK3* homologous flank and  
453 within the *PAC* cassette. (upper) PCR amplification of clones 2 and 8 at 24 h and 48 h +/-  
454 100 nM rapamycin treatment was conducted and the resulting amplicons resolved on an  
455 agarose gel.  
456 D. Western blotting analysis with anti-GFP and anti-EF1 $\alpha$  loading control antibodies of  
457 protein extracted from experimental clone 2 promastigotes grown for 4 days in the presence  
458 or absence of 100 nM rapamycin.

459

460 **Fig. 3.** Analysis of *CRK3* deficient promastigotes.

461 A. Representative images of cells grown in the absence (top) or presence (bottom two rows)  
462 of 100 nM rapamycin for 96 h. Promastigotes (clone 2) were stained with DAPI to observe  
463 nuclear and kinetoplast content alongside mCherry expression by fluorescence microscopy.  
464 Scale bar represents 5 $\mu$ m.  
465 B. (upper) DNA content analysis of clone 2 promastigotes at 72 and 96 h post treatment.  
466 Cells were fixed with methanol and stained with propidium iodide for flow cytometry  
467 analysis of 100,000 cells to examine nuclear content. Arrows indicate the positions of cells in  
468 G<sub>1</sub> phase (2C), in G<sub>2</sub>/M (4C) and low DNA content associated with increased incidence of  
469 <1C zoids. (lower) Graphical representation of the DNA content of each population based on  
470 the flow cytometry plots.  
471 C. The viability of cells grown in the absence (-) or presence (+) of 100 nM rapamycin for 72  
472 h. Promastigotes (clone 2) were incubated with 5  $\mu$ g ml<sup>-1</sup> propidium iodide (PI) for 15 min

473 and analysed by flow cytometry. A heat lysed (HL) control in which half the sample was  
474 lysed by incubation at 70°C for 3 min was included to enable an appropriate live / dead gate  
475 to be drawn. Numbers represent the percentage of cells assessed as PI positive (PI+) based on  
476 the HL control. Data shown are the means of 3 technical replicates, data are representative of  
477 2 independent experiments.

478

479 **Fig. 4.** *CRK3* wild type and active site mutant complementation assays.

480 A. Wild type complemented ( $\Delta crk3::DICRE/\Delta crk3::CRK3^{Flox}$  [*SSU CRK3*], left graph) and  
481 mutant complemented ( $\Delta crk3::DICRE/\Delta crk3::CRK3^{Flox}$  [*SSU CRK3<sup>T178E</sup>*], right graph) cell  
482 lines were seeded as promastigotes at  $1 \times 10^5$  cells ml<sup>-1</sup> and grown +/- 100 nM rapamycin for  
483 5 days. Cell density was determined by counting at 24 h intervals and the mean  $\pm$  SD of  
484 triplicate values was plotted.

485 B. The resulting amplicons generated by PCR amplification of each cell line at 24 and 48 h  
486 after growth +/- 100 nM rapamycin.

487 C. (left) DNA content analysis of  $\Delta crk3::DICRE/\Delta crk3::CRK3^{Flox}$  [*SSU CRK3<sup>T178E</sup>*]  
488 promastigotes after methanol fixation and staining with propidium iodide for flow cytometry  
489 analysis (100,000 cells) to examine nuclear content. Arrows indicate the positions of cells in  
490 G<sub>1</sub> phase (2C), in G<sub>2</sub> (4C) and low DNA content associated with increased incidence of <2C  
491 zoids. (right) Graphical representation of the DNA content of each population based on the  
492 flow cytometry analysis.

493 D. Representative images of  $\Delta crk3::DICRE/\Delta crk3::CRK3^{Flox}$  [*SSU CRK3<sup>T178E</sup>*] promastigotes  
494 grown in the absence (top) or presence (bottom two rows) of 100 nM rapamycin for 96 h.  
495 Parasites were stained with DAPI to detect nuclear and kinetoplast DNA by fluorescence  
496 microscopy. Scale bar represents 5 $\mu$ m.

497

498 **Fig. 5.** *CRK3* conditional deletion in stationary phase promastigotes and *in vivo* infection.

499 A. Correlation between *in vivo* bioluminescence (total flux in photons per second) and  
500 parasite burdens from the same infected footpads. BALB/c mice were infected with *L.*  
501 *mexicana* WT or Ppy RE9H-expressing stationary phase promastigotes and imaged weekly  
502 using an *in vivo* imaging system (IVIS). At 2, 4, 6 and 8 weeks post-infection mice were  
503 sacrificed after imaging and parasite burdens in infected footpads determined using limiting  
504 dilution assays. Each point shows the total flux and parasite burden from the footpad in one



505 mouse (n = 3-4 mice per time point). Linear regression line and R<sup>2</sup> was calculated from the  
506 log transformed data.

507 B. PCR amplification of the floxed *CRK3* locus of  $\Delta crk3::DICRE/\Delta crk3::CRK3^{Fllox}$  [*SSU*  
508 *RE9H*] stationary phase promastigotes after incubation in the presence (+) or absence (-) of 1  
509  $\mu$ M rapamycin for 24 h.

510 C. Control (-) or 24 h rapamycin-treated (+) stationary phase promastigotes were inoculated  
511 into the footpads of BALB/c mice. The total flux (photons/sec) emitted from the infected  
512 footpad region of interest (ROI) was quantified weekly.

513 D. The total flux measured from infected footpads was plotted over 9 weeks of infection.  
514 Data shown represent the mean flux and SD from groups of four mice. The dotted line  
515 indicates the average background flux emitted from uninfected footpads measured 1 week  
516 post infection (n=12). A significant difference in the mean total flux emitted between the  
517 footpads of mice infected with untreated and rapamycin-induced parasites was observed at 5  
518 and 9 weeks post infection (2-way ANOVA, \*\*\*P=<0.001; \*\*P=<0.005).

519 E. PCR amplification of the floxed *CRK3* locus of  $\Delta crk3::DICRE/\Delta crk3::CRK3^{Fllox}$  [*SSU*  
520 *RE9H*] + Rap after purification of amastigotes from the footpads of 10-week infected mice.  
521 Cells were propagated *in vitro* to obtain sufficient genomic DNA for PCR analysis.

522 F. Footpad sizes were recorded by weekly caliper measurement. Data shown represent the  
523 mean footpad size and SD from groups of four mice (Unpaired *t*-test \*P=<0.05).

524

525

526 **Table 1.** Comparisons of the growth rates of conditional *CRK3* deletion lines measured  
527 during logarithmic growth.

528

## 529 **Experimental Procedures**

### 530 *Ethics statement*

531 Animal studies were carried out under UK Home Office regulations (Project licence PPL  
532 60/4442).

### 533 *Parasite culture and transfection*

534 *Leishmania mexicana mexicana* (MNYC/BZ/62/M379) promastigotes were cultured at 25°C  
535 in HOMEM supplemented with 10% heat inactivated foetal calf serum (HI-FCS) and 1%

536 penicillin/streptomycin (PEN/STREP). Amastigotes were cultured in Schneider's Insect  
537 Medium supplemented with 20% HI-FCS, 1% PEN/STREP and 15 $\mu\text{g mL}^{-1}$  Hemin at pH5.5.  
538 Mid-log phase *L. mexicana* promastigotes were transfected with 10 $\mu\text{g}$  of digested DNA by  
539 electroporation using the Nucleofector system with the Human T-Cell kit (Lonza) as  
540 described previously (Castanys-Muñoz *et al.*, 2012). Transgenic cell lines were grown in the  
541 presence of appropriate antibiotics at the following concentrations: G418 50  $\mu\text{g mL}^{-1}$ ,  
542 blasticidin 10  $\mu\text{g mL}^{-1}$  and puromycin 10  $\mu\text{g mL}^{-1}$  (InvivoGen).

#### 543 *Construct design and development*

544 A full list and descriptions of all primers (Table S1) and plasmids (Table S2) used in this  
545 study are available. To produce a diCre expression vector, the diCre coding sequences Cre59-  
546 FKBP12 and Cre60-FRB were each flanked by actin and  $\beta$ -tubulin sequences in array with  
547 blasticidin resistance cassette flanked by *DHFR-TS* regulatory elements. The sequence was  
548 synthesised and sub-cloned into the pDONR221 vector (GenScript). The backbone of the  
549 loxP vector containing the loxP sites flanking a multiple cloning site and other restriction  
550 enzyme regions flanked by regulatory elements was synthesised (GenScript). The *PAC*,  
551 *mCherry* and *CRK3-GFP* cassettes were inserted by enzymatic restriction digest mediated  
552 ligation, and subsequently sub-cloned into pDONR221. Addition of *CRK3* homology  
553 flanking homology was performed by MultiSite Gateway 3-fragment vector construction  
554 (Invitrogen) as per manufacturers' guidelines. Briefly, flanks were amplified by PCR by  
555 Phusion polymerase (New England BioLabs) using oligonucleotides conferring *attB*  
556 recombination sites to the amplicons. Subsequent BP reactions inserted the flanks into  
557 appropriate pDONR vectors containing *attL* sites for site-specific recombination. An LR  
558 reaction resulted in the flanking of diCre and loxP vectors into a pDEST vector for  
559 transfection. Finally, complementation plasmids were generated by inserting the *CRK3*,  
560 *CRK3*<sup>T178E</sup> and *RE9H* genes (Branchini *et al.*, 2010; McLatchie *et al.*, 2013) into a modified  
561 version of pGL631 (Misslitz *et al.*, 2000) containing a G418r cassette for SSU integration  
562 construct by *XhoI* & *NotI* restriction enzyme digestion and ligation.

#### 563 *Induction of diCre mediated gene deletion*

564 All experiments were conducted using cells in the early to mid log stage of exponential  
565 growth (between 1-5 x 10<sup>6</sup> cells mL<sup>-1</sup>) with the exception of the stationary phase inducible

566 gene deletion. Between 1nM to 1µM rapamycin (Abcam) was administered by inoculation  
567 into the cell culture medium from a 100 µM working stock.

#### 568 *Conditional gene deletion analysis*

569 Taq polymerase (NEB) was used to PCR amplify the regions surrounding *GFP<sup>Flox</sup>* and  
570 *CRK3<sup>Flox</sup>* using primers shown in Table S1 and a  $T_A$  calculated using an online  $T_m$  calculator  
571 (New England BioLabs) and 30 cycles for amplification.

#### 572 *Western Blot Analysis*

573 For western blotting analysis, either  $1 \times 10^7$  cells were loaded per lane or equal  
574 concentrations of protein extract as quantified by Bradford assay of a 10% NuPAGE Bis-Tris  
575 gel (Invitrogen) in MOPS running buffer and transferred onto Hybond-C nitrocellulose  
576 membranes (GE Healthcare). Primary antibodies against GFP were used to detect GFP and  
577 CRK3-GFP expression at 1:1000 whilst anti-EF1α was used as a loading control at 1:5000.  
578 Membranes were washed three times in TBST, incubating for 10 min each time, before  
579 incubation with horse radish peroxidase (HRP)-conjugated secondary rabbit and mouse  
580 antibodies at 1:5000 dilution for 1 h at room temperature. After washing three times in TBST,  
581 the membrane was treated with an ECL (enhanced chemiluminescence) kit (SuperSignal  
582 West Pico Chemoluminescent Substrate, Pierce) according to manufacturer's instructions and  
583 then exposed on Kodak photographic film.

#### 584 *Infection of mice*

585 BALB/c mice were purchased from Charles River (MA., USA) and infected in the right  
586 footpad with  $2 \times 10^6$  stationary-phase *L. mexicana* promastigotes in 1 x PBS. Lesion size was  
587 monitored weekly and  $\Delta crk3::DICRE/CRK3$  [*SSU GFP<sup>Flox</sup>*] amastigotes were purified before  
588 the lesions reached a thickness of 5mm.

#### 589 *Purification of lesion derived amastigotes*

590 Lesion derived  $\Delta crk3::DICRE/CRK3$  [*SSU GFP<sup>Flox</sup>*] amastigotes were purified by  
591 homogenising the extracted lesion in 1xPBS and passing the solution through a 20 µm cell  
592 strainer. Amastigotes were pelleted by centrifugation at 2,000 g for 10 mins, followed by re-  
593 suspension in culture medium. To prevent cells from clumping together and ensure accurate  
594 cell counting, amastigote cultures were first centrifuged at 2,000 g for 10 mins and the

595 supernatant removed to leave the pellet in 500uL volume. The pellet was re-suspended in this  
596 volume by gentle syringing through a blunt 16G needle and the single cell suspension added  
597 back to the culture medium. Cell counting was performed by mixing the homogenised culture  
598 1:1 with Trypan blue and cell counting with a Haemocytometer (Neubauer).

#### 599 *Macrophage differentiation and amastigote infection*

600 Non-differentiated monocytes were extracted from the femurs and tibia of BALB/c mice by  
601 dissection to remove the bones. RPMI 1640 medium was used to wash the bone marrow out  
602 of the intact bones by syringing with a 25G needle. Extracted cells were quantified by  
603 dilution in Trypan blue (1:1) and counting with a haemocytometer. Monocytes were seeded at  
604  $5 \times 10^5$  cells  $\text{ml}^{-1}$  in M $\Phi$  Medium (DMEM + L-Glut + 20%FCS + 1% P/S + 30% L-Cell M) in  
605 8 ml volumes in Petri dishes and incubated at 37°C with 5% CO<sub>2</sub> for 3 days to induce  
606 differentiation to monocyte-derived macrophage. After this period the medium was replaced  
607 and by day 5 the cells were removed from the dishes using a cell scraper with ice- cold RPMI  
608 1640. Bone marrow derived macrophage were adhered at a concentration of  $5 \times 10^5$  cells  $\text{ml}^{-1}$   
609 overnight in DMEM medium with 10 % HIFCS at 37°C in 5% CO<sub>2</sub> onto 8-chamber tissue  
610 culture slides (LAB-TEK) for microscopic analysis or 12 well plates for DNA extraction and  
611 flow cytometry analysis. Macrophages were then infected at a ratio of 5 parasites per  
612 macrophage with lesion-derived  $\Delta crk3::DICRE/CRK3$  [*SSU GFP<sup>Flox</sup>*] amastigotes, which had  
613 been previously grown in axenic medium in the presence or absence of rapamycin for 24 h.  
614 Wells were washed at 24 h post infection to remove extracellular parasites and media  
615 replenished with DMEM/10% HIFCS. Cells were removed from the plates for DNA  
616 extraction and flow cytometry analysis by gentle scraping in ice cold RPMI at the 120 h end  
617 time point.

#### 618 *Fluorescence microscopy analysis*

619 For imaging,  $2 \times 10^6$  parasites were washed in 1 x PBS, re-suspended in Fluoromount-G  
620 (SouthernBiotech) DAPI infused mounting medium and mounted on glass slides for analysis.  
621 Parasite morphology was observed by DIC and mCherry fluorescent imaging, and DNA  
622 content observed by DAPI fluorescent imaging using a Delta Vision core (Image Solutions)  
623 inverted microscope equipped with mCherry and DAPI filter sets. Images were processed  
624 using Photoshop CS (Adobe) image software. GFP expression of intracellular amastigotes  
625 was assessed by fluorescent microscopy. Cells were imaged between 24 and 120 h after

626 infection in the DeltaVision Core environmental chamber at 37°C and 5 % CO<sub>2</sub> upon  
627 incubation in 1 x PBS infused with DAPI.

#### 628 *DNA content and GFP expression analysis by flow cytometry*

629 Parasites were prepared for DNA content analysis as described previously (Paul Hassan *et*  
630 *al.*, 2001) with the exceptions that a MacsQuant flow cytometer was used to analyse 100,000  
631 cells per sample. Cell distribution was modelled using FlowJo software (Tree Star). For  
632 determining GFP expression of promastigotes and amastigotes by flow cytometry analysis,  
633 live cells were washed twice in 1xPBS and passed through a nitex mesh prior to acquisition.

#### 634 *Viability assay*

635 Log-phase promastigotes were seeded at 5 x 10<sup>5</sup> cells ml<sup>-1</sup> and grown in the presence or  
636 absence of 100 nM rapamycin. At 72 h post treatment 1 x 10<sup>7</sup> cells were washed once with 1  
637 x PBS and incubated with 5ug ml<sup>-1</sup> propidium iodide (PI) for 15 minutes at room temperature  
638 in the dark. A heat lysed (HL) control in which half the sample was lysed by incubation at  
639 70°C for 3 min was included to enable an appropriate live / dead gate to be drawn. Cells were  
640 washed with 1 x PBS and used to acquire 100,000 events per group by flow cytometry using  
641 a MacsQuant flow cytometer.

#### 642 *In vivo imaging*

643 For imaging, mice were anaesthetised with 4.0% isofluorane/1.5 L O<sub>2</sub> per minute and  
644 inoculated by subcutaneous injection with 200µl D-luciferin (15 mg ml<sup>-1</sup> in Mg/Ca-free  
645 Dulbecco's modified PBS). Light emission was recorded 10 minutes after inoculation using  
646 an IVIS Spectrum bioluminescence imaging system (PerkinElmer). Imaging was performed  
647 with an open emission filter, for 30-60 second exposures, large binning, and 1 f/stop, and  
648 captured with a charge-coupled device (CCD) camera. The absolute unit of photon emission  
649 was given as radiance (photons /second/cm<sup>2</sup> /steradian). Images were analysed using Living  
650 Image Software (PerkinElmer) and regions of interest (ROI) of equal size were selected over  
651 the infected footpads to quantify the amount of photon emission as total photon flux in  
652 photons per second (photons/sec).

#### 653 *Statistical analysis*

654 Statistical analysis was performed using GraphPad Prism 5. The analysis of significance of

655 the data was performed by 2-way ANOVA when comparing data from induced (+Rap) and  
656 uninduced (-Rap)  $\Delta crk3::DICRE/\Delta crk3::CRK3^{Fllox}$  [*SSU RE9H*] infections and by paired t-test  
657 when comparing footpad sizes.

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## 664 **Conflict of Interest**

665 The authors declare no conflict of interest.

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Figure S1

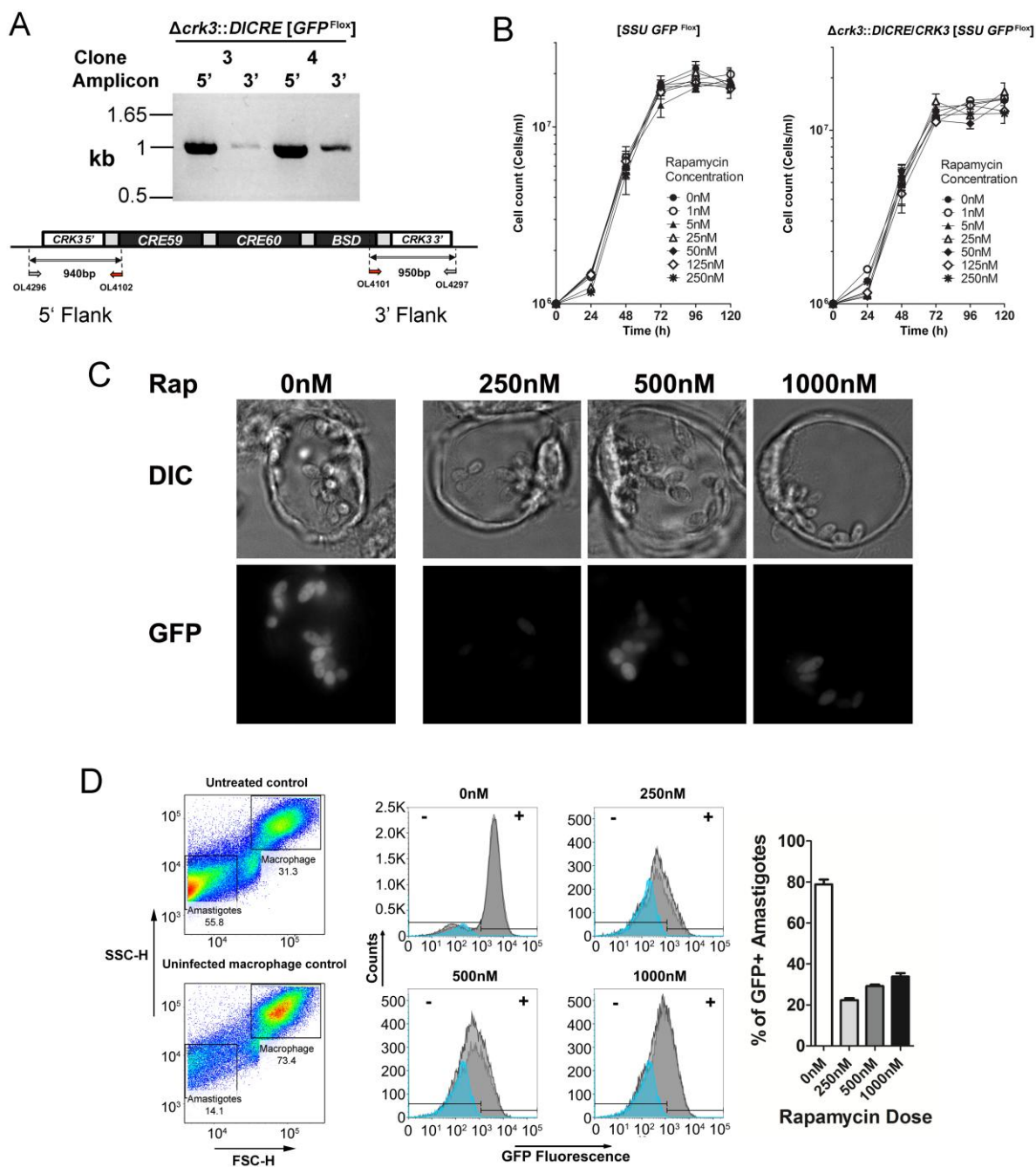


Fig. S1. A. Replacement of a single copy of *CRK3* by diCre construct integration into the  $[SSU GFP^{Flox}]$  cell line was confirmed by PCR amplification of genomic DNA extracted from two clones (3 and 4). Oligonucleotides (OL) that bind outside the integration site (grey arrows) and within the diCre coding sequence (red arrows) were used to amplify 940 bp and 950 bp amplicons. Clone 3 was designated as the experimental line  $\Delta crk3::DICRE/CRK3 [SSU GFP^{Flox}]$ .

B. Experimental  $\Delta crk3::DICRE/CRK3$  [*SSU GFP<sup>Flox</sup>*] or control [*SSU GFP<sup>Flox</sup>*] *L. mexicana* promastigotes were seeded at  $1 \times 10^6$  cells  $\text{ml}^{-1}$  and incubated in the presence or absence of between 1 to 250 nM rapamycin. Cell density was determined at 24 hour intervals by cell counting (N=1-3 technical replicates, error SEM).

C. Representative DIC (upper) and GFP (lower) images from live cell imaging of amastigotes-infected macrophages at 5 days post-infection. GFP expression from live amastigotes was imaged using a Delta Vision core fluorescent microscope.

D. GFP intensity loss in amastigotes extracted at day 5 post *in vitro* macrophage infection; (left) amastigotes were gated from large, granular macrophage by forward scatter (FSC) for size and side-scatter (SSC) for granularity. (middle) Histograms of amastigote GFP intensity were generated from amastigote gates with retention of GFP expression at  $>10^3$  fluorescence intensity based on rapamycin untreated controls. Blue plots represent the amastigote gate plotted from a macrophage only control group to represent background cellular 'debris' as a result of macrophage lysis following sample preparation (left).  $>20,000$  amastigote events were analysed per treatment group based on two biological replicates shown as dark and light grey plots. (right) Retention of GFP signal as a % of amastigote gate displayed as bar graphs for each treatment group (Data represent means  $\pm$  SEM).

Figure S2

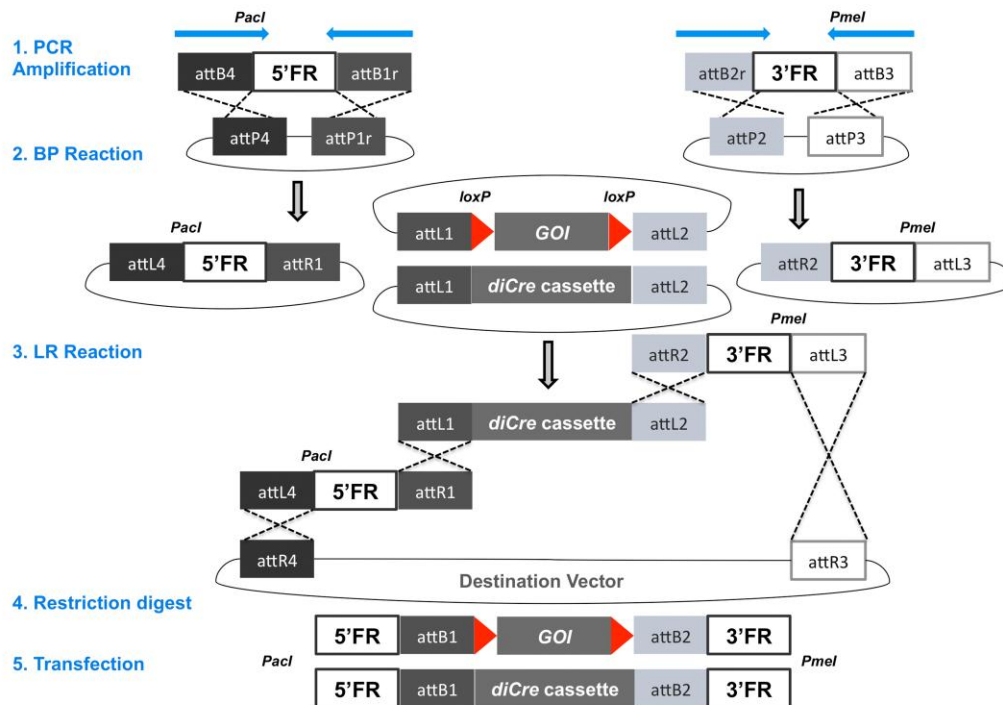


Fig. S2. Pipeline of Gateway-mediated addition of target gene homologous flanks to diCre and loxP vectors. (1) Primers (blue arrows) containing appropriate att sites and 5' *PacI* or 3' *PmeI* unique restriction sites amplify a 0.5-1 kb region up- and downstream of the gene. (2) BP clonase catalyses the insertion of these flanks into their appropriate vectors. (3) The resulting 5', 3' and diCre or loxP vectors are recombined into a pDEST vector by LR clonase. (4) The final vector is linearised by *PacI* and *PmeI* digest for (5) transfection into *L. mexicana*. This method enables flanking of both the floxed gene of interest (GOI) expression cassette and diCre expression cassette.

Figure S3

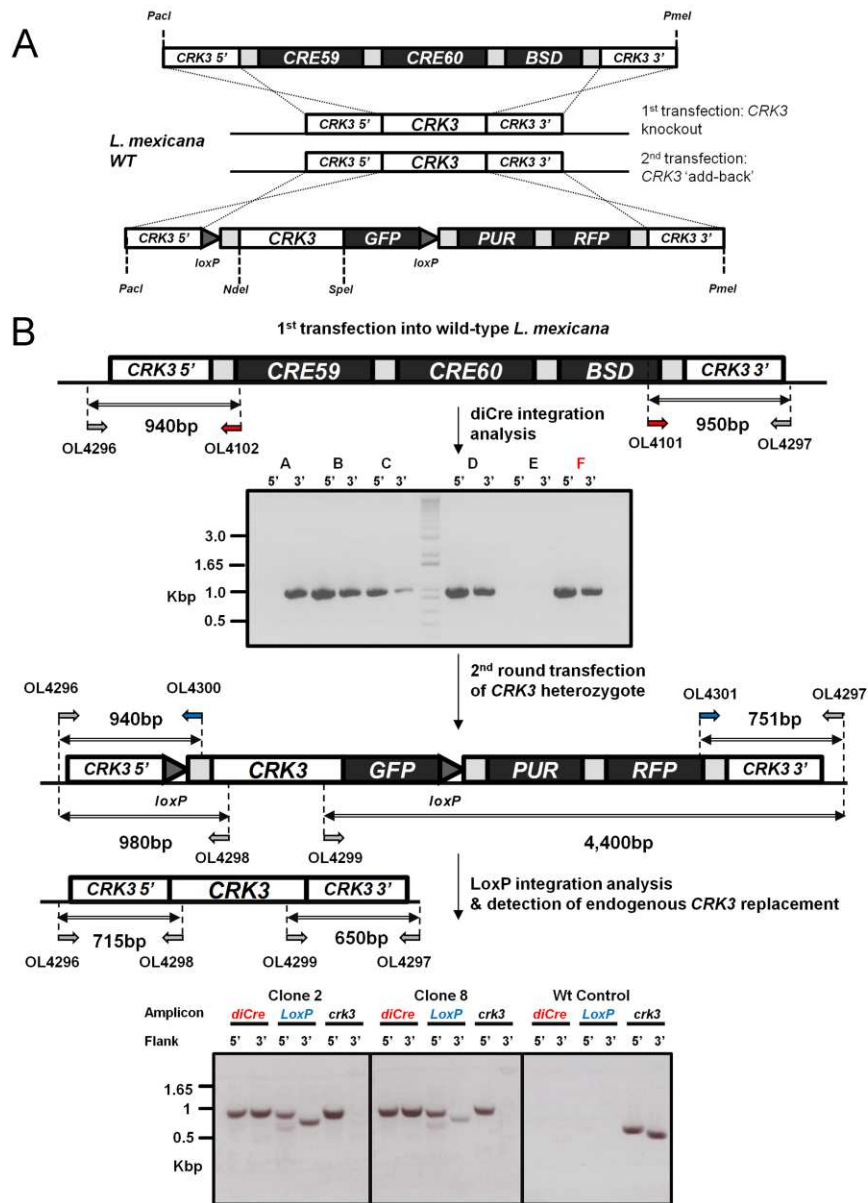


Fig. S3. A. Schematic representing the diCre and floxed *CRK3* replacement strategy. Homologous recombination was facilitated by Gateway flanking of both diCre and loxP vectors with ~500 bp of *crk3* 5' and 3' homologous regions to replace both alleles. B. Transfection of wild-type *L. mexicana* with the diCre construct: integration was confirmed by PCR amplification of genomic DNA extracted from six clones with oligonucleotides (OL) binding outside the integration site (grey arrows) and within the diCre coding sequence (blue arrows) to amplify 940 and 950 bp amplicons. A single blasticidin (BSD) resistant clone F with *diCre* integrated at the *crk3* locus was subsequently transfected with the loxP construct to replace the remaining endogenous *crk3* allele with a floxed *CRK3* fused to a 3' *GFP* tag, thereby generating a diCre-mediated conditional deletion line:  $\Delta crk3::DICRE/$

$\Delta crk3::CRK3^{Flox}$ . PCR amplification of genomic DNA extracted from two blasticidin/ puromycin (PUR) double resistant clones (2 and 8) with oligonucleotides binding outside the integration site (grey arrows), within the *crk3* coding sequence (grey arrows), within the loxP vector (blue arrows) and diCre sequences (red arrows).

Figure S4

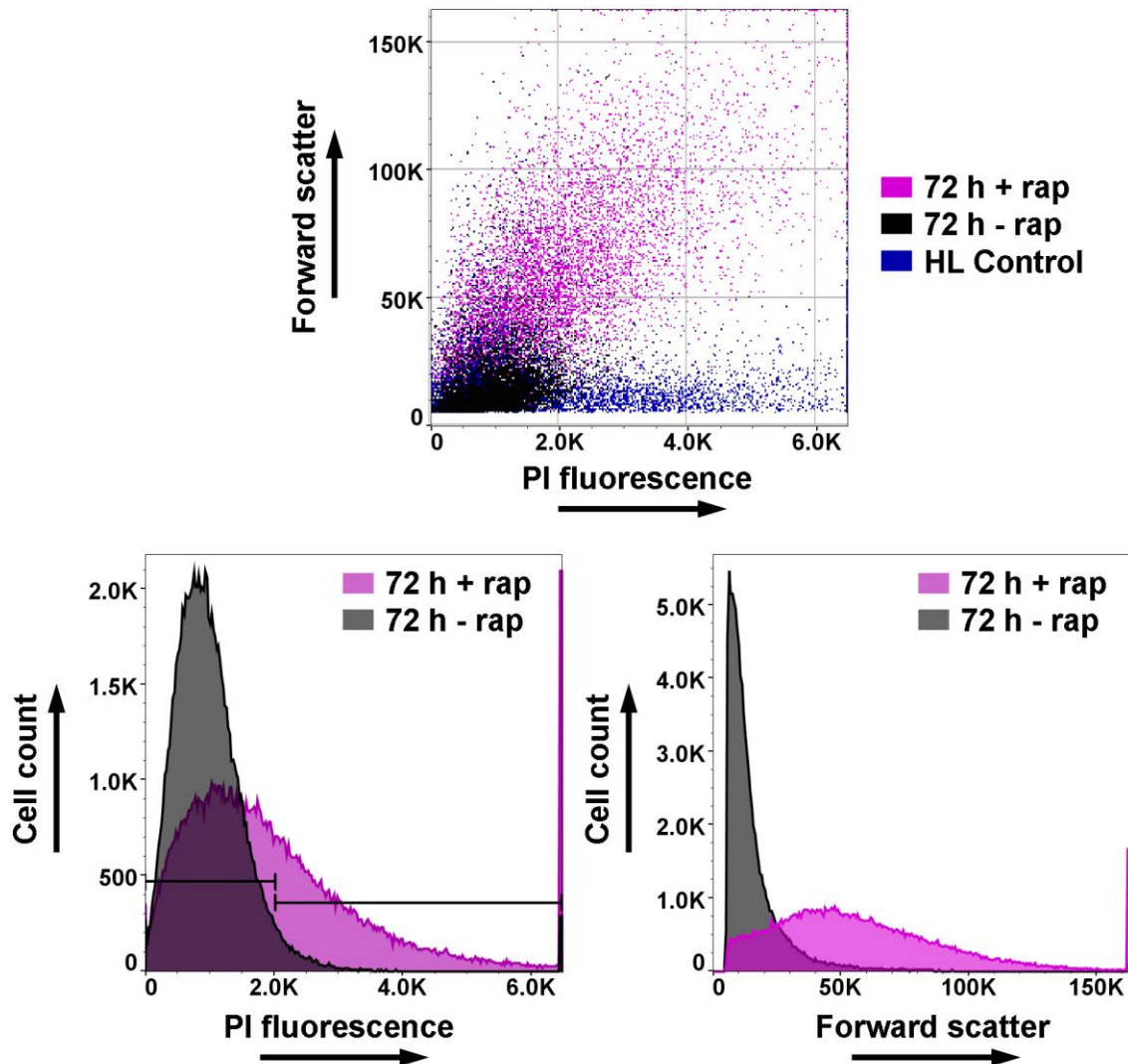


Figure S4. Viability assay of  $\Delta crk3::DICRE/\Delta crk3::CRK3^{Flx}$  promastigotes. Cells were grown in the presence or absence of 100 nM rapamycin for 72 h. Live cells were incubated with  $5\mu\text{g ml}^{-1}$  propidium iodide (PI) for 15 minutes and uptake measured by flow cytometry alongside a heat lysed (HL) control in which half the cells were lysed by incubation at  $70^{\circ}\text{C}$  for 3 min prior to flow cytometry analysis. Top panel shows cell size as measured by forward scatter in the y-axis and cell lysis by increasing PI fluorescence along the x-axis. Bottom left panel shows the gating strategy whereby cells are defined as + or – in PI uptake based on the HL control. Bottom right panel is an analysis of promastigote cell size following incubation in the presence or absence of rapamycin. Results are representative of 2 independent experiments.

Figure S5

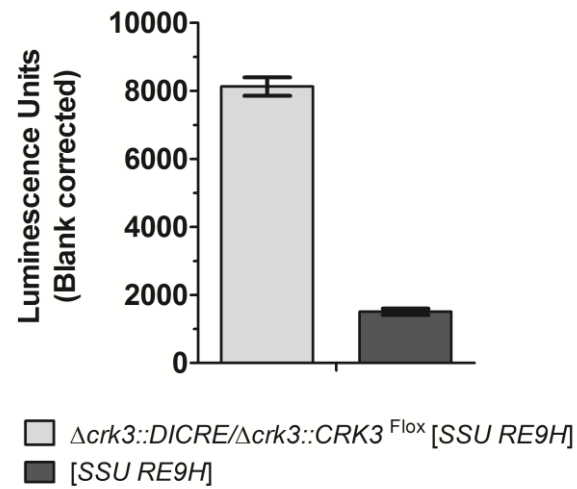


Figure S5. *In vitro* bioluminescence expression assay of experimental and control promastigotes. Promastigotes were assayed during logarithmic growth and luminescence expression data was acquired 30 minutes post luciferin treatment. Error bars represent the SEM of two technical replicates per clone.



# Table S1

Oligo No.		Description	Sequence
<b>Gateway cloning of CRK3 homologous flanks</b>			
OL4249	F	Amplification of a 5' <i>CRK3</i>	GGGGACAACCTTTGTATAGAAAAGTTGCCCTTAATTA AAAAGGTAGAGGATGCCGTTTT
OL4250	R	homologous flank with attB4/P1r	GGGGACTGCTTTTTGTACAAACTTGCTTGAATGTTGCAGGGAGAAA
OL4251	F	Amplification of a 3' <i>CRK3</i>	GGGGACAGCTTCTTGTACAAAAGTGGGGAGTGGAAAAGGCATGACTGAA
OL4252	R	homologous flank with attB2r/B3	GGGGACAACCTTTGTATAATAAAAGTTGCCGTTTAAACTTTCCTCCCAGCACGCACAC
<b>Generation of <i>CRK3</i> loxP expression and complementation vectors</b>			
OL4065	F	Amplification of puromycin resistance cassette from pGL631	GATCCTGCAGCGCGTGGATGTCGCGCAG
OL4066	R		GATCGCTAGCCTAGGCACCGGGCTTGCG
OL4293	F	Amplification of SAS-HASPB- <i>mCherry</i> from pGL1893 to integrate at reporter site	GATCCTCGAGAATTGCCCGCTTCCAT
OL4294	R		GATCGCGGCCGCGGGATCCTCAATGATGA
OL4316	F	Amplification of GFP from pGL1773 for integration as N-terminal tag	GATCCATATGATGGTGAGCAAGGGCGAG
OL4317	R		GATCGGTACCCCTGTACAGCTCGTCCAT
OL4318	F	Amplification of 6xHA integration as N-terminal tag	GATCCATATGTACCCTTACGATGTGCCT
OL4319	R		GATCGGTACCTGCGTAATCGGGCACATC
OL4320	F	Amplification of GFP from pGL1773 for integration as C-terminal tag	GATCACTAGTATGGTGAGCAAGGGCGAG
OL4321	R		GATCTCTAGATCACTTGTACAGCTCGTCCAT
OL4541	F	Amplification of SAS-HASPB- <i>mCherry</i> for insertion via <i>HindIII</i> : enables the replacement of HASPB- <i>mCherry</i> by <i>XhoI</i> and <i>NotI</i>	GATCAAGTTAATGCCCGCTTCCATTCG
OL4542	R		GATCGCGGCCGCGGGATCCTCAATGATGATGAT
OL4067	F	Amplification of the <i>CRK3</i> CDS for insertion into the loxP MCS: no Stop codon amplified due to C-terminal <i>GFP</i> fusion	GATCCATATGTCTTCGTTTGGCCGTGTG
OL4103	R		GATCATCGATCCAACGAAGGTCGCTGAA
OL4388	F	Amplification of the <i>CRK3</i> CDS for insertion into the loxP MCS: Stop codon amplified due to N-terminal <i>GFP</i> fusion	GATCACTAGTCTTCGTTTGGCCGTGTGACC
OL4389	R		GATCTCTAGACTACCAACGAAGGTCGCTGAA
OL4591	F	Amplification of <i>CRK3-his</i> for insertion into pGL2277 to generate an 18S RNA integration vector for complementation of the floxed <i>CRK3</i> inducible deletion line	CTCGAGATGTCTTCGTTTGGCCGT
OL4592	R		GCGGCCGCTAATGATGATGATGATGCCAACG AAGGTCGCTGAA
OL4601	F	Mutagenesis primers for T178 mutation to a glutamic acid residue to create <i>CRK3</i> <sup>T178E</sup>	GCACACCTACGAGCAGGAGTGG
OL4602	R		ATGGGCACCTTGAACGCAC
<b>Primers for analysis of vector integration and floxed gene loss by PCR amplification</b>			
OL4101	F	Internal forward (BLA) and reverse (FKBP12) primers to detect diCre integration into the genome	CTGGTTATGTGTGGGAGG
OL4102	R		GATGGTTTCCACCTGCAC
OL4287	F	Upstream and downstream primers to amplify the floxed <i>GFP</i> fragment to detect gene loss by diCre induction	GCTCGCGTGTGTTGAGCC
OL4288	R		CATTCGTGGGCTCCAGCT
OL4296	F	Primers binding out-with the <i>CRK3</i> integration site	GATCGTGGGAAGGGGAAG
OL4297	R		GGAAGTCCAAGTAGCGCG
OL4298	R	Primers binding the <i>CRK3</i> gene	GGTCACACGGCCAAACGA
OL4299	F		GCCAAGGAGGCCCTACAG
OL4300	R	Primers binding the loxP vector at the 5' splice acceptor site (SAS) and 3' poly-adenylation site (PAS)	GGTGGACGGCTCAACACA
OL4301	F		GTGTGCTGTGCGTTCAGC
OL4781	F	Upstream and downstream primers for amplification of a floxed <i>CRK3-GFP</i> fragment to detect gene loss	AACTGGCAGCAGCGATTTGGCAGGGG
OL4782	R		GCACCGTGGGCTTGTACTCGGTCATG
OL4748	F	Primers to check for integration of <i>RE9H</i> construct (pGL2398) into the ribosomal locus	TCGTGAGACGCCAGCGAATG
OL4750	R		ACCGACGCCACATCGAGGTG

Table S1. A list of the oligonucleotides used in this study.

## Table S2

pGL No.	Gene ID	Gene Name	Backbone	Description
2313	N/A	<i>diCre</i>	pDONR221	DiCre expression cassette entry vector
2314	N/A	<i>loxP-C-6xHA</i>	pDONR221	LoxP (empty) expression cassette: c-terminal 6xHA tag
2315	N/A	<i>loxP-C-GFP</i>	pDONR221	LoxP (empty) expression cassette: c-terminal GFP tag
2316	N/A	<i>loxP-N-GFP</i>	pDONR221	LoxP (empty) expression cassette: n-terminal GFP tag
2375	LmxM.36.0550	<i>CRK3</i>	pGL631	WT <i>CRK3</i> ribosomal SSU integration vector
2376	LmxM.36.0550	<i>CRK3</i> <sup>T178E</sup>	pGL631	Mutated <i>CRK3</i> <sup>T178E</sup> ribosomal SSU integration vector
2398	N/A	<i>RE9H</i>	pGL631	Red-shifted luciferase bioluminescent protein in G418r pRib
2445	LmxM.36.0550	5' <i>CRK3</i> flank	pDONR P41-Pr	5' Flank (500bp) ready for Gateway recombination
2446	LmxM.36.0550	3' <i>CRK3</i> flank	pDONR P2r-P3	3' Flank (500bp) ready for Gateway recombination
2455	N/A	<i>diCre</i>	pDEST R4-R3	DiCre cassette flanked with <i>CRK3</i> homologous arms
2456	LmxM.36.0550	<i>CRK3</i>	pDEST R4-R3	<i>CRK3-GFP</i> <sup>lox</sup> cassette flanked with <i>CRK3</i> homology
2461	N/A	<i>GFP</i> <sup>lox</sup>	pGL631	Floxed <i>GFP</i> in pRib: for functional analysis of diCre

Table S2. A list of the plasmids generated in this study.