

1 **Bromodomain factor 5 is an essential transcriptional regulator of the *Leishmania* genome.**

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21 transcription.

22
23 **Abstract**

24 *Leishmania* are unicellular parasites that cause human and animal disease. Alongside other
25 organisms in kinetoplastida, they have evolved an unusual genome architecture that requires
26 all RNA polymerase II transcribed genes to be expressed constitutively, with transcriptional
27 start regions denoted by histone variants and histone lysine acetylation. However, the way
28 these chromatin marks are interpreted by the cell is not understood. Seven predicted
29 bromodomain factors (BDF1-7), the reader modules for acetyl-lysine, were identified across
30 *Leishmania* genomes. Using *L. mexicana* as a model, Cas9-driven gene deletions indicate that
31 BDF1-5 are essential for promastigote survival, whilst DiCre inducible gene deletion of the
32 dual bromodomain factor *BDF5* identified it to be essential for both promastigotes and
33 amastigotes. ChIP-seq assessment of BDF5s genomic distribution revealed it as highly
34 enriched at transcriptional start sites. Using an optimised proximity proteomic and
35 phosphoproteomic technique, XL-BioID, we defined the BDF5-proximal environment to be
36 enriched for other bromodomain factors, histone acetyltransferase 2, and proteins essential
37 for transcriptional activity and RNA processing. Inducible deletion of BDF5, led to a disruption
38 of pol II transcriptional activity and global defects in gene expression. Our results indicate the
39 requirement of *Leishmania* to interpret histone acetylation marks for normal levels of gene
40 expression and thus cellular viability.

42 **Introduction**

43

44 Gene transcription in eukaryotic cells is a complex process with multiple levels of regulation¹.
45 Post-translational modifications (PTMs) of histones in nucleosomes can be used to encode an
46 extra layer of information into chromatin, to modify transcriptional activity leading to
47 differential gene expression. Histone modification by lysine acetylation is one predominant
48 modification, it is interpreted by 'reader' proteins called bromodomains. In eukaryotic
49 pathogens such as *Leishmania*, an organism with an unusual genome arrangement, the
50 impact of histone lysine acetylation on transcriptional regulation is not well understood.

51

52 Bromodomains are small protein domains consisting of 100-110 amino acid residues folded
53 into a bundle of 4 helices connected by two loops which form a hydrophobic pocket that can
54 bind to acetyl-lysine modified peptides. Conserved tyrosine and asparagine residues serve as
55 acetyl-lysine recognition elements along with a network of water molecules in the pocket²⁻⁴.
56 Bromodomains are classically found to recognise acetyl-lysine residues of histone tails,
57 allowing bromodomain-containing proteins to act as interpreters of the histone acetylation
58 code. Proteins containing bromodomains are often called bromodomain factors (BDFs). Other
59 accessory domains in the BDF or its binding partners can then conduct other functions such
60 as applying additional PTMs or chromatin remodelling. BDFs can regulate processes at specific
61 regions of genomes such as promoters or enhancers, influencing differential gene expression,
62 leading to cellular proliferation or differentiation. Inhibitors of these interactions have been
63 intensely explored to identify pharmacological interventions for diseases driven by
64 dysregulated BDF-driven processes³⁻⁶.

65

66 Bromodomain proteins are poorly studied in kinetoplastid species, such as *Leishmania*, the
67 causative agents of multiple human and animal diseases. Visceral leishmaniasis infects 50,
68 000 – 90,000 people per year and the cutaneous forms of the disease, including those caused
69 by *L. mexicana*, afflict up to 1 million people per year⁷. Kinetoplastids are deeply branched
70 eukaryotes and their gene expression is radically different to the human host⁸. Genes are
71 arranged into unidirectional polycistronic transcription units (PTU) of hundreds of non-
72 functionally linked genes, and expression is driven from vaguely defined transcriptional start
73 sites (TSS) that are often thousands of bases long⁹. The PTUs can run on either the plus or
74 minus strand from transcriptional start sites at divergent strand switch regions¹⁰. Where PTUs
75 then meet, a convergent SSR occurs, these are transcriptional termination sites (TTS). During
76 transcription of protein-coding genes, RNA polymerase II (pol II) generates polycistronic pre-
77 mRNAs that are processed by co-transcriptional cleavage, polyadenylation and trans-splicing
78 events to produce mature mRNAs. Expression levels of individual genes are then typically
79 regulated by the 3' UTR, which is targeted by RNA binding proteins for stabilisation,
80 sequestration or degradation¹¹. In *Leishmania*, some highly expressed genes are found in
81 tandem arrays or on supernumerary chromosomes to increase gene dosage¹². *Leishmania*
82 also exhibit high levels of mosaic aneuploidy in cell populations as an adaptive survival

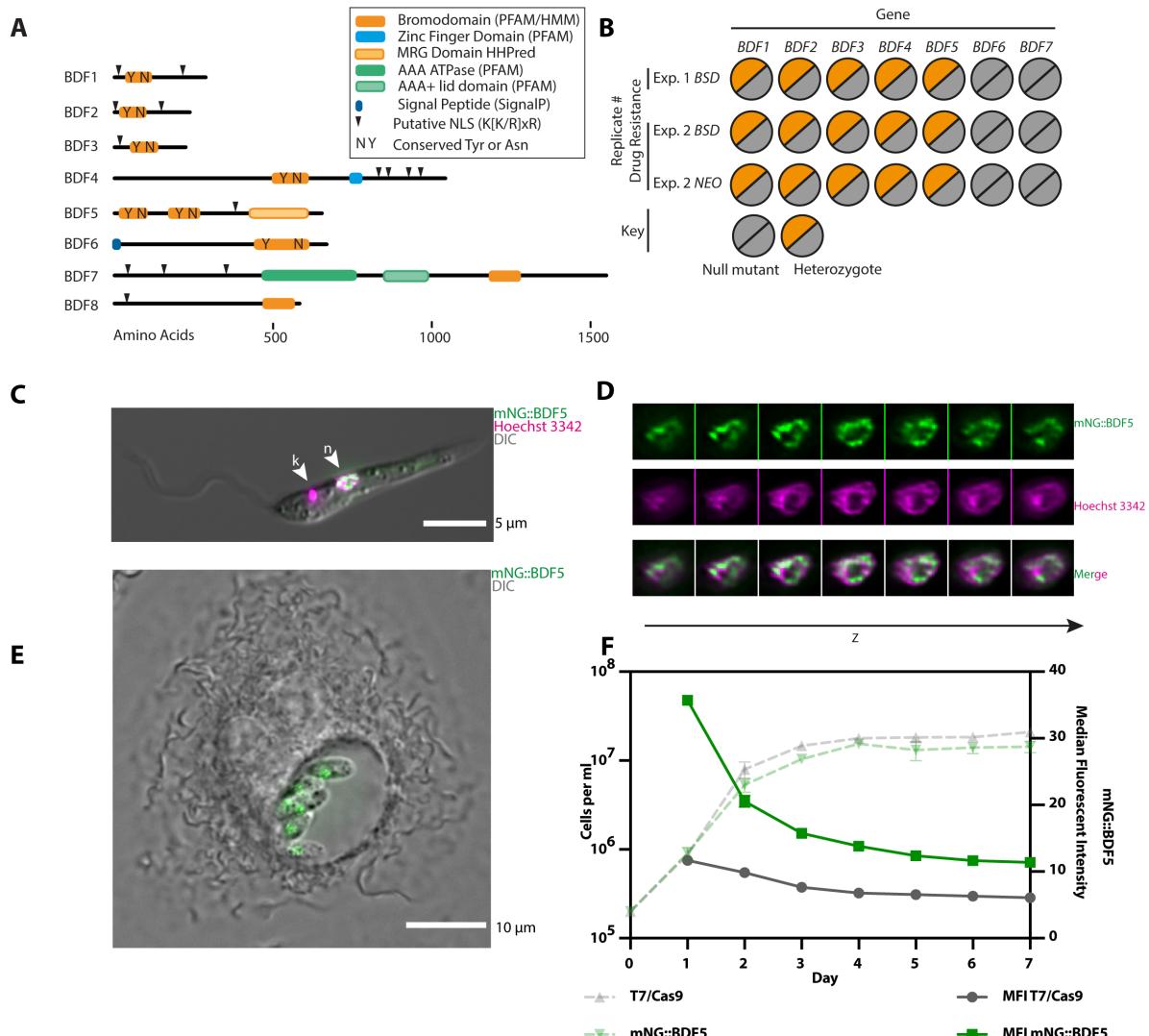
83 strategy allowing plastic variation in gene dose¹³. Consequently, transcription of protein-
84 coding genes by polymerase II was thought to be constitutive with no sequence-defined,
85 promoter-specific regulation¹⁴. However, it appears that cellular demarcation of
86 transcriptionally start sites might be mediated through histone acetylation to provide a
87 platform of accessible chromatin. This suggestion comes from the identification of H3
88 acetylation at TSSs in *L. major*¹⁴. Histone acetyltransferases have been identified as essential
89 for *L. donovani* survival and linked to specific histone modifications^{15–18}.
90

91 *Leishmania* is particularly poorly understood in terms of its transcriptional regulation by
92 acetylation; however, because of the high level of synteny and conservation of their genomic
93 features to *Trypanosoma*, some of their core epigenetic processes may also be conserved.
94 TSSs in *Trypanosoma brucei* are enriched for histone variants and acetylation marks¹⁹, the
95 number of different histone PTMs in *T. brucei* has been shown to range into the hundreds
96^{20,21}. Recently, characterisation of chromatin-associated proteins in *T. brucei* led to the
97 identification of specific networks of bromodomain factors and other proteins at TSS and TTS,
98 with characteristic ChIP-seq profiles suggesting a specific ordering and unexpected
99 complexity of processes at these sites²². It has been reported that after genetic or chemical
100 targeting of TbBDF2 and TbBDF3 in bloodstream stage *T. brucei*, cells undergo a process
101 consistent with aberrant differentiation to the insect stage forms, identifying a potential role
102 in the lifecycle of the parasite²³. Several BDFs have been shown to be essential in multiple life
103 stages of *T. brucei* by using a genome-wide RNAi screen²⁴. TbBDF5 has been individually
104 targeted by RNAi in bloodstream forms and found to be essential for cellular survival²⁵. In
105 *Trypanosoma cruzi* bromodomain factors TcBDF1 and TcBDF3 have been implicated in cellular
106 differentiation, intriguingly TcBDF1 has been reported to localise to glycosomes and TcBDF3
107 has been reported to interact with acetylated tubulin in the flagellum^{26–28}. TcBDF2 has been
108 reported to bind acetylated histone TcH4K10_{ac} and accumulates in cells treated with UV
109 radiation²⁹. Until now, none of these orthologs have been characterised in *Leishmania*.
110

111 In this work, we validate five bromodomains factors as essential in *Leishmania* and
112 characterise the biology co-ordinated by the essential bromodomain factor, BDF5. By
113 applying inducible gene deletion, we were able to establish the requirement for BDF5 in both
114 cell culture and a mammalian host. We applied multiple -omics tools to characterise the
115 function of BDF5, in particular using ChIP-seq to define the genomic distribution of BDF5,
116 proximity proteomics to determine the processes occurring in BDF5-enriched loci and RNA-
117 seq to explore the role of BDF5 in gene expression. Integrating the findings of these
118 approaches we defined BDF5 as an essential factor required for pol II transcriptional activity
119 in *Leishmania*.
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121

122 **Results**

123 *Bromodomain factors in Leishmania*



124

125 **Figure 1: Overview of putative *Leishmania* bromodomain factors.** A. Schematic showing
126 protein domain architecture of *Leishmania* BDFs. B. Overview of Cas9 gene deletion attempts
127 of *BDF1-7* in *L. mexicana* T7/Cas9 promastigotes. Two independent transfections were carried
128 out using either *BSD* or *NEO* as a drug selectable marker individually or in combination. C. Live-
129 cell fluorescent microscopy of *L. mexicana* promastigote expressing mNG::BDF5. Nucleus is
130 denoted by arrowhead labelled n, the kinetoplastid DNA is indicated by arrowhead labelled k.
131 D. Channel separated Z-slices of the nucleus from the cell in (C). E. Live-cell fluorescent
132 microscopy of intra-macrophage *L. mexicana* amastigotes expressing mNG::BDF5
133 endogenously-tagged protein. F. Expression levels of mNG::BDF5 during promastigote growth,
134 determined by mNG signal in individual cells by flow-cytometry. Dashed points denote mean
135 cell density, error bars \pm standard deviation, solid points denote median fluorescence intensity,
136 N=3, 20, 000 events per sample.

137

138 Although bromodomain factors BDF1-5 were readily identifiable in *Leishmania* genomes³⁰,
139 further PFAM and HMM searching identified another two potential bromodomain-containing
140 proteins BDF6 and BDF7 (Fig. 1A, Table 1) (Tallant, C, et al. In Press doi;.

141 10.1021/acsinfecdis.1c00387). BDF1-5 have identifiable tyrosine and asparagine residues in
142 positions consistent with the conserved residues important for peptide binding in canonical
143 bromodomains. BDF1-3 are small proteins <500 residues and contain a single bromodomain
144 and no other identifiable domains. BDF4 is a larger protein with a centrally located
145 bromodomain followed by a predicted CW-type zinc finger. BDF5 is the only *Leishmania* BDF
146 that has tandem bromodomains in the N-terminal half of the protein. We refer to these as
147 BD5.1 and BD5.2 and both are located in the N-terminal half of the protein. A more sensitive
148 HHpred³¹ analysis suggested remote structural homology to an MRG domain-like region
149 (MORF4 (mortality factor on chromosome 4) related gene) in the C-terminal half of the BDF5
150 protein. MRG domains can bind transcriptional regulators and chromatin remodelling
151 factors³²⁻³⁴. BDF6 and BDF7 contain the most divergent bromodomains. BDF6 includes an
152 insert in the bromodomain region and BDF7 lacks the conserved tyrosine and asparagine
153 residues, suggesting that they may be divergent, noncanonical bromodomains or pseudo-
154 bromodomains³⁵. BDF6 has a C-terminal bromodomain and is predicted to have an N-terminal
155 signal peptide when analysed using SignalP4.1. BDF7 is the largest of the BDFs and contains a
156 bromodomain in the C-terminal region of the protein preceded by a predicted ATPase and
157 AAA domain. The bromodomain does not appear to have the conserved tyrosine and
158 asparagine residues that are important for acetyl-lysine binding. However, by HHMER
159 analysis and alignment, it appears that BDF7 might be a homologue of the ATAD2 factor found
160 in many other eukaryotes³⁶⁻³⁸. All of the predicted BDFs, apart from BDF6, contain
161 K[K/R]x[K/R] motifs that can act as a monopartite nuclear localisation signal³⁹. The BDFs of
162 *Leishmania* have orthologs in other parasitic and free-living kinetoplastid organisms, except
163 for BDF1 in *Bodo saltans*^{40,41}. BDF8 was identified by HHpred analysis of a hypothetical gene
164 identified using BDF5-proximity proteomics (this study), it may represent another pseudo-
165 bromodomain.

166
167 To assess the essentiality of the seven BDFs in *Leishmania* promastigotes we used Cas9-
168 targeted gene deletion. sgRNAs and repair templates were generated to target the CDS of
169 each gene and replace it completely (**Fig. S1A**)⁴². Two independent experiments were
170 performed, using either blasticidin (*BSD1* or *BSD* and neomycin (*NEO*) drug resistance repair
171 cassettes, leading to three semi-independent selections. Consistently, BDF6 and BDF7 null
172 mutants could be isolated (**Fig. 1B**, **Fig. S1B**, **S1C**, **S1D**). For BDF1-5 only heterozygote mutants
173 were ever isolated, indicating that although the Cas9 system was functioning, a copy of the
174 gene was required for promastigote survival and thus null mutants could not be generated.

175
176 LmxBDF5, while distinct from BET bromodomains, shares a characteristic tandem
177 bromodomain arrangement reminiscent of human BRD2 and BRD4, or the yeast RSC4
178 protein⁴. These proteins have all been identified to play roles in regulating transcription, so
179 due to this interesting feature of BDF5 we decided to investigate it in greater detail. BDF5
180 homologs are identifiable in all the kinetoplastid genomes available in TriTrypDB. The level of
181 amino acid conservation across the first bromodomain (BD5.1) is higher than the second

182 (BD5.2), but in all cases the conserved tyrosine and asparagine residues are retained in both
183 bromodomains (**Fig. S2A**), these correspond to Y40, N90, Y201 and N247 in LmxBDF5. Both
184 bromodomains have x-ray crystal structures available in the PDB (PDB ID: 5TCM, 5TCK),
185 confirming the bromodomain structural fold and positioning of conserved residues (**Fig. S2B**).
186 BDF5 was endogenously tagged using a Cas9 targeted approach to append a 3xMyc epitope
187 and the green fluorescent protein mNeonGreen to the N-terminus⁴² to generate *mNG::BDF5*.
188 This modification preserves the 3' UTR, which is necessary for regulating endogenous mRNA
189 levels in *Leishmania* allowing for native expression levels and dynamics through growth and
190 lifecycle stages. Live-cell widefield deconvolution microscopy of promastigotes identified that
191 mNG::BDF5 localised to the nucleus (**Fig. 1C**). The distribution of mNG::BDF5 within the
192 nucleus was heterogeneous, with foci found around the periphery of the nucleus and
193 excluded from the nucleolus (**Fig. 1D**). The expression of mNG::BDF5 persisted in amastigotes
194 where it was visualised in a structure consistent with the nucleus of intramacrophage
195 amastigotes (**Fig. 1E**). *BDF5* mRNA was also previously detected to be constitutively expressed
196 in both lifecycle stages⁴³. A seven-day time course experiment was performed where
197 mNG::BDF5 levels in individual cells were measured by flow cytometry to determine the
198 levels of BDF5 during promastigote growth (**Fig. 1F**). mNG::BDF5 levels were highest in rapidly
199 proliferating cells during the first few days of growth and declined as the cells approached
200 stationary phase. By day 7, mNG::BDF5 levels were reduced by >60% compared to day 2.
201 mNG::BDF5 signal was not completely reduced to the levels of the parental control strain,
202 suggesting a low level of BDF5 expression was retained.

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204 **Table 1: Gene IDs for *L. mexicana* BDFs and orthologues in selected trypanosomatids.** PDB
205 identifiers are provided for available structures, shaded boxes represent likely pseudo-
206 bromodomains.

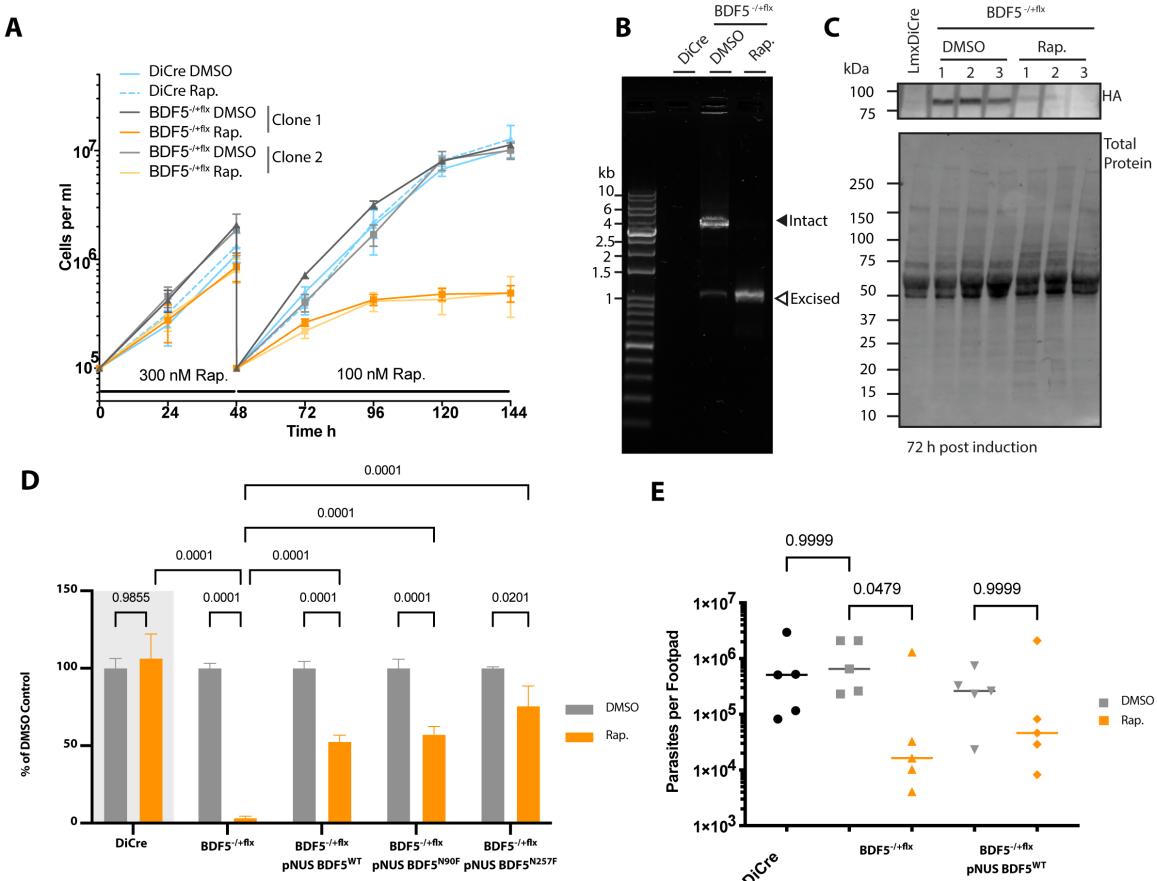
Name	<i>L. mexicana</i> gene	<i>L. donovani</i> gene	PDB ID	<i>T. brucei</i> orthologue	PDB ID	<i>T. cruzi</i> orthologue	PDB ID
BDF1	LmxM.36.6880	LdBPK_367210.1		Tb927.10.8150	5KO4	TcCLB.506247.80	
BDF2	LmxM.36.2980	LdBPK_363130.1	5C4Q	Tb927.10.7420	4PKL, 5CZG, 2N9G	TcCLB.507769.30	6NP7, 6NIM
BDF3	LmxM.36.3360	LdBPK_363520.1	5FEA	Tb927.11.10070	5C8G	TcCLB.509747.110	
BDF4	LmxM.14.0360	LdBPK_140360.1		Tb927.7.4380		TcCLB.508857.150	
BDF5	LmxM.09.1260	LdBPK_091320.1	5TCM, 5TCK	Tb927.11.13400	5K29, 6NEZ	TcCLB.510359.130	6NEY
BDF6	LmxM.12.0430	LdBPK_120390.1		Tb927.1.3400		TcCLB.510889.330	
BDF7	LmxM.11.0910	LdBPK_110910.1		Tb927.11.6350		TcCLB.506297.110	
BDF8	LmxM.33.2300	LdBPK_342070.1		Tb927.4.2340		TcCLB.506559.310	

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210 *BDF5 is essential in promastigotes and for murine infection*



211
212 **Figure 2: Characterisation of inducible knockout of BDF5 using DiCre.** **A.** Growth curve of
213 promastigotes treated with the inducing agent, rapamycin (Rap.), or the vehicle, DMSO. Points
214 and error bars denote mean values \pm standard deviation, n=3. **B.** PCR and agarose gel analysis
215 of *BDF5::6xHA^{flx}* gene excision at the 72 h timepoint in (A). Solid arrowhead denotes the intact
216 *BDF5::6xHA^{flx}* gene and open arrowhead denotes the excised locus after rapamycin addition.
217 The DiCre lane indicates the lack of PCR product in the parental strain. **C.** Western blot showing
218 levels of *BDF5::6xHA* protein at the 72 h timepoint. **D.** Results of clonogenic survival assay
219 comparing BDF5 depleted cells with cell lines carrying episomal complementation of *BDF5* or
220 mutated *BDF5* alleles. Bars denote the mean of the percentage clonal survival where each
221 experiment was normalised to its own DMSO control. Error bars indicate standard deviation,
222 values above are p values from 2-way ANOVA with multiple comparisons by Tukey's test, n=3.
223 Lines denote comparisons performed by two-way ANOVA with associated p-values shown
224 above **E.** Parasite burdens from infected mouse footpads determined by limiting dilution,
225 individual points for each mouse with median values indicated by lines. Comparisons of
226 Kruskal-Wallace test with Dunn's correction indicated with associated p-values written above,
227 n=5.

228
229 To gain a higher quality validation of *BDF5* essentiality⁴⁴ and to investigate the phenotypes
230 resulting from loss of *BDF5* in promastigotes, an inducible knockout strain was generated
231 using the DiCre system^{45,46} (**Fig. S2A, S2B**). An *L. mexicana* strain expressing dimerisable, split
232 Cre recombinase was modified to carry a single, 6xHA epitope-tagged allele of *BDF5* flanked
233 by loxP sites giving *L.mx::DiCre Δbdf5::BDF5::6xHA^{flx}/BDF5*. The second copy of *BDF5* was

234 then deleted using a *HYG* resistance cassette giving the strain
235 *L.mx::DiCreΔbdf5::HYG/Δbdf5::BDF5::6xHA*^{flx}, referred to as *BDF5::6xHA*^{-/+flx}. In the absence
236 of rapamycin, this strain grew normally as per the parental DiCre strain. However, following
237 the addition of rapamycin, there was a marked reduction in the parasite growth (**Fig. 2A**).
238 Rapamycin was added to cultures at 300 nM for 48 h at which point the cultures were diluted
239 to 1 x 10⁵ cells per ml. Rapamycin was then added at 100 nM to suppress escape mutants
240 and the growth phenotype observed. At the 144 h time point, the rapamycin-treated flasks
241 contained ~98% fewer cells than the controls. Rapamycin did not affect the parental DiCre
242 strain, indicating that the effect was specific to the floxed strain where *BDF5* could be deleted.
243 This phenotype was reproducible and observed in an independent, clonal cell line (**Fig. 2A**).
244 PCR analysis of these populations at 72 h after rapamycin addition revealed that the
245 *BDF5::6xHA*^{flx} allele had been excised (**Fig. 2B**). Some leaky excision of the *BDF5::6xHA*^{flx} allele
246 was detectable in the untreated control samples. The levels of *BDF5::6xHA* protein at 72 h
247 were assessed by western blot, revealing a 90% reduction in the rapamycin-treated sample
248 compared to the control samples (**Fig. 2C**). Total protein Stain-Free technology was used to
249 provide loading controls, due to the potential for *BDF5* deletion to impact on transcription of
250 housekeeping genes. To demonstrate that the deletion of *BDF5* was essential for cellular
251 survival a clonogenic assay was applied to characterise the cells resulting from *BDF5* excision
252 (**Fig. 2D**). A 98% reduction in survival of the *BDF5::6xHA*^{-/+flx} strain was observed when cloned
253 in the presence of 100 nM rapamycin, moreover, those cells that survived retained the
254 *BDF5::6xHA*^{flx} allele.

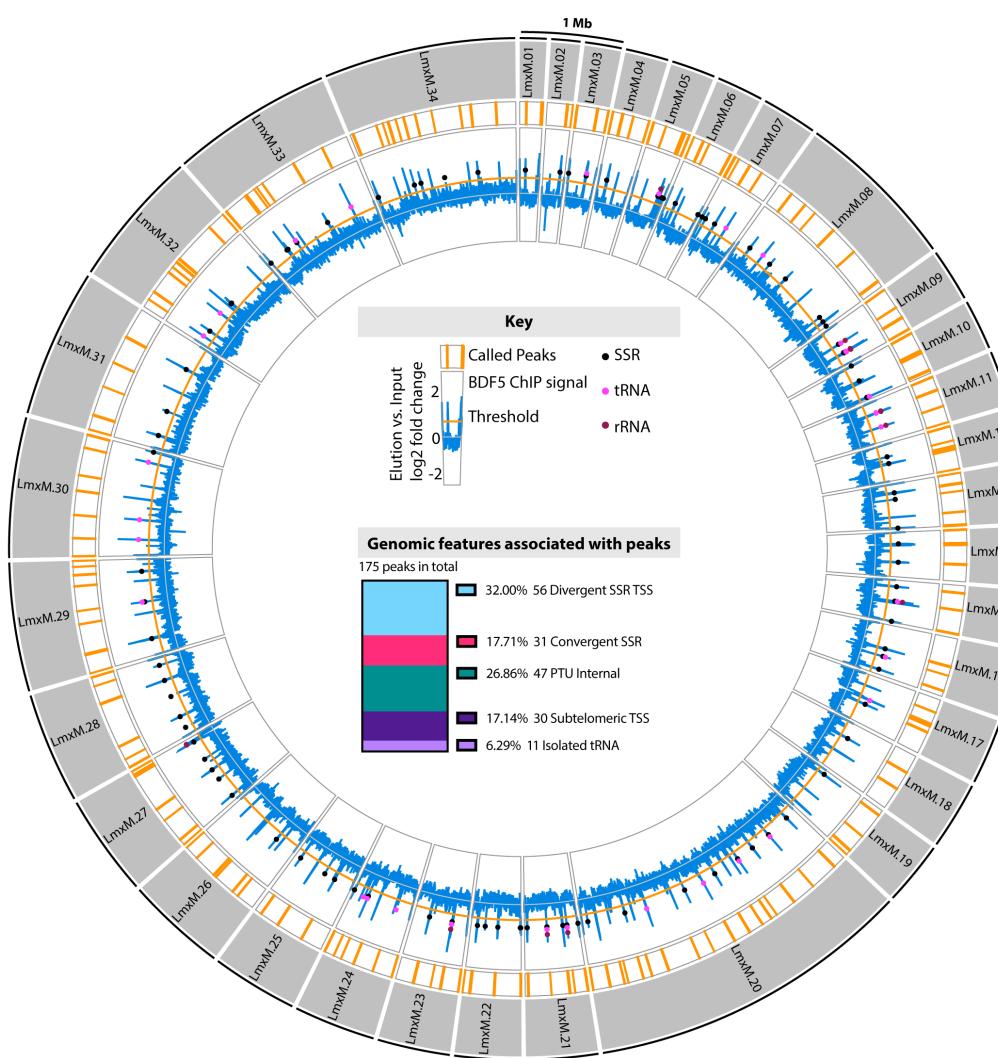
255
256 To ensure the phenotype was specific to *BDF5* deletion and not due to off-target effects, an
257 allele of *BDF5::GFP* was re-introduced to the *BDF5::6xHA*^{-/+flx} strain using the pNUS episome⁴⁷
258 (**Fig. S4**) Clonal survival experiments were performed in media lacking drug selection for the
259 episome allowing for its loss if it confers no selective advantage. Clonal survival of the *BDF5*-
260 complementation strain was ~50% after rapamycin addition, this is 25-fold higher than the
261 non-complemented, induced samples. While not 100% complementation it reflects the
262 potential for parasites to lose the episome (**Fig. 2D**), demonstrating the requirement for *BDF5*
263 for cellular survival. This experimental approach also allowed us to explore the essentiality of
264 the individual bromodomains by making point mutations at the conserved asparagine
265 residues in each bromodomain, N90 and N257 in BD5.1 and BD5.2 respectively (**Fig. S4**). These
266 were mutated to phenylalanine in anticipation that the bulky sidechain would displace any
267 binding peptide from the hydrophobic pocket^{2,48,49}. Clonal survival was restored to similar
268 levels as those observed for the *pNUS BDF5* complementation strain by the *BDF5^{N90F}* and
269 *BDF5^{N257F}* mutants (**Fig. 2D**), indicating that either these mutations are not disruptive, or that
270 any disruption due to mutation of a single *BDF5* BD is tolerated by the cell. Three attempts
271 were made to generate double mutations in N90F/N257F but no viable populations of cells
272 were isolated, suggesting the *BDF5^{N90F/N257F}* is not tolerated by the cells. In light of this, we
273 used a DiCre inducible system⁵⁰ to flip-on expression of an extra *BDF5^{N90F/N257F}::GFP* mutant
274 allele to look for dominant-negative phenotypes (**Fig. S5A, Fig. S5B**). Promastigote cultures

275 induced to express BDF5^{N90F/N257F}::GFP exhibited a significant growth defect (**Fig. S5C, S5D**),
276 whereas those induced to express the BDF5::GFP protein did not exhibit this phenotype.
277 These experiments demonstrate that individually both bromodomains are redundant, but
278 that together they are required for the essential function of BDF5.

279
280 The ability to use the DiCre strains to validate target genes in *Leishmania* amastigotes is
281 restricted due to the toxicity of rapamycin to amastigotes and its immunomodulatory effect
282 in mammals⁴⁵. Therefore, mid-log promastigote cultures of *Lmx::DiCre*, *BDF5::6xHA*^{-/+flx} or
283 *BDF5::6xHA*^{-/+flx} ::*pNUS BDF5::GFP* were treated with 500 nM rapamycin or DMSO for 72 hours
284 allowing them to induce deletion of BDF5 but still allow infectious, metacyclic promastigotes
285 to accumulate in culture. Excision of the BDF5 gene was verified by PCR (**Fig. S6A**) and the
286 stationary cultures were used to infect BALB/c mice by a subcutaneous route into the rear
287 footpad. No apparent differences were observed in the size of the resulting footpad lesions
288 over the 8-week infection period (**Fig. S6B**), however, there was a 50-fold reduction in the
289 parasite burden of the footpads when infected with *BDF5::6xHA*^{-/+flx} rapamycin treated cells
290 compared to the *BDF5::6xHA*^{-/+flx} DMSO treated cells or the parental strain (**Fig. 2E**). The
291 presence of the *pNUS BDF5::GFP* episome restored parasite burden in the rapamycin-treated
292 strain to a level not significantly different to that observed in its uninduced control (**Fig. 2E**).
293 The median parasite burdens of the *BDF5::6xHA*^{-/+flx} strain rapamycin-treated strain in the
294 popliteal lymph nodes was ~10-fold lower than the control strain, but this difference was not
295 statistically significant (**Fig. S6C**). DNA extracted from footpads and lymph nodes, including
296 both host and amastigote DNA, was subjected to PCR analysis which detected non-excised
297 *BDF5::6xHA*^{-/+flx} consistent with BDF5 being essential for amastigote survival as well as
298 promastigote survival (**Fig. S6D**). Clonal promastigote lines lacking the *BDF5::6xHA*^{-/+flx} allele
299 could only be derived from the populations containing an addback copy of BDF5 (**Fig. S6E**).
300 We conclude that BDF5 is essential for successful infection of the mammalian host and is likely
301 to be essential for amastigote survival too.

302

303 *ChIP-seq reveals BDF5 genomic distribution*



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305

306 **Figure 3: Genome-wide distribution of BDF5 determined by ChIP-seq analysis.** Outer circles:
307 Circos plot representing the 32 MB *L. mexicana* genome. The 34 chromosomes are depicted
308 by grey segments. Regions enriched >0.5 log₂fold for BDF5 indicated by the orange bars, the
309 enrichment of BDF5::6xHA in the elution over the input chromatin is indicated in the blue line
310 on a log₂ fold scale, values are the mean derived from 3 ChIP replicates. Genomic features
311 such as strand-switch regions, tRNA genes, and rRNA genes are indicated by coloured circles
312 on this blue line. Inner pane: Key and stacked bar chart showing the genomic features
313 associated with the peaks.

314

315 Due to the importance of BDF5 for the survival of *Leishmania* parasites and the demonstration
316 that it is a nuclear protein, we sought to identify where it might be found in the context of
317 genomic architecture. The BDF5::6xHA protein expressed by the *BDF5::6xHA*^{-/+flx} strain was
318 analysed by chromatin immunoprecipitation sequencing (ChIP-Seq). We identified 175
319 regions where BDF5 was determined to be enriched on the genome; these peaks were
320 distributed across all the 34 chromosomes and could be correlated with specific genomic
321 features (Fig. 3, Fig. S7A, S7B). Of the total peaks, 56 (32%) were associated with TSSs in

322 divergent strand switch regions (dSSRs). A further 30 (17%) were in subtelomeric regions likely
323 to be transcriptional start sites based on the orientation of the polycistronic transcription
324 unit. Forty-seven peaks (27%) were identified in internal regions of polycistronic transcription
325 units (PTUs) and a further 11 peaks overlapped with isolated tRNA genes (6%). Intriguingly,
326 31 peaks (18%) were found at convergent strand switch regions, which are likely
327 transcriptional termination sites. The size of the regions determined to be enriched for BDF5
328 varied, with the mean of peaks found at dSSRs encompassing ~10 kb (**Fig. S7C**). The shape of
329 BDF5 peaks over divergent strand switch regions tended to be broad and even, without
330 exhibiting the “twin-peaks” pattern seen for histone H3 acetylation in *L. major*¹⁴ (**Fig. S7A**,
331 **S7D**). Peaks at both divergent and convergent SSRs tended to be symmetrical although they
332 were narrower and weaker at convergent SSRs (cSSRs) (**Fig. S7D**). Peaks found in PTUs were
333 asymmetric, rising steeply to a peak with a shallow decay in the direction of the PTU
334 transcription. The PTU peak enrichment levels were equivalent to those at dSSRs (**Fig. S7E**).
335 The finding that BDF5 predominantly localises to divergent SSRs and other TSSs suggests it
336 plays a role in polymerase II transcription. However, as a number of termination sites and
337 other classes of small RNA genes were also enriched for BDF5 this indicates it could also play
338 a generalised role in a range of transcriptional processes. Therefore, we sought to analyse the
339 protein complexes associated with BDF5 to give insight into its potential function.

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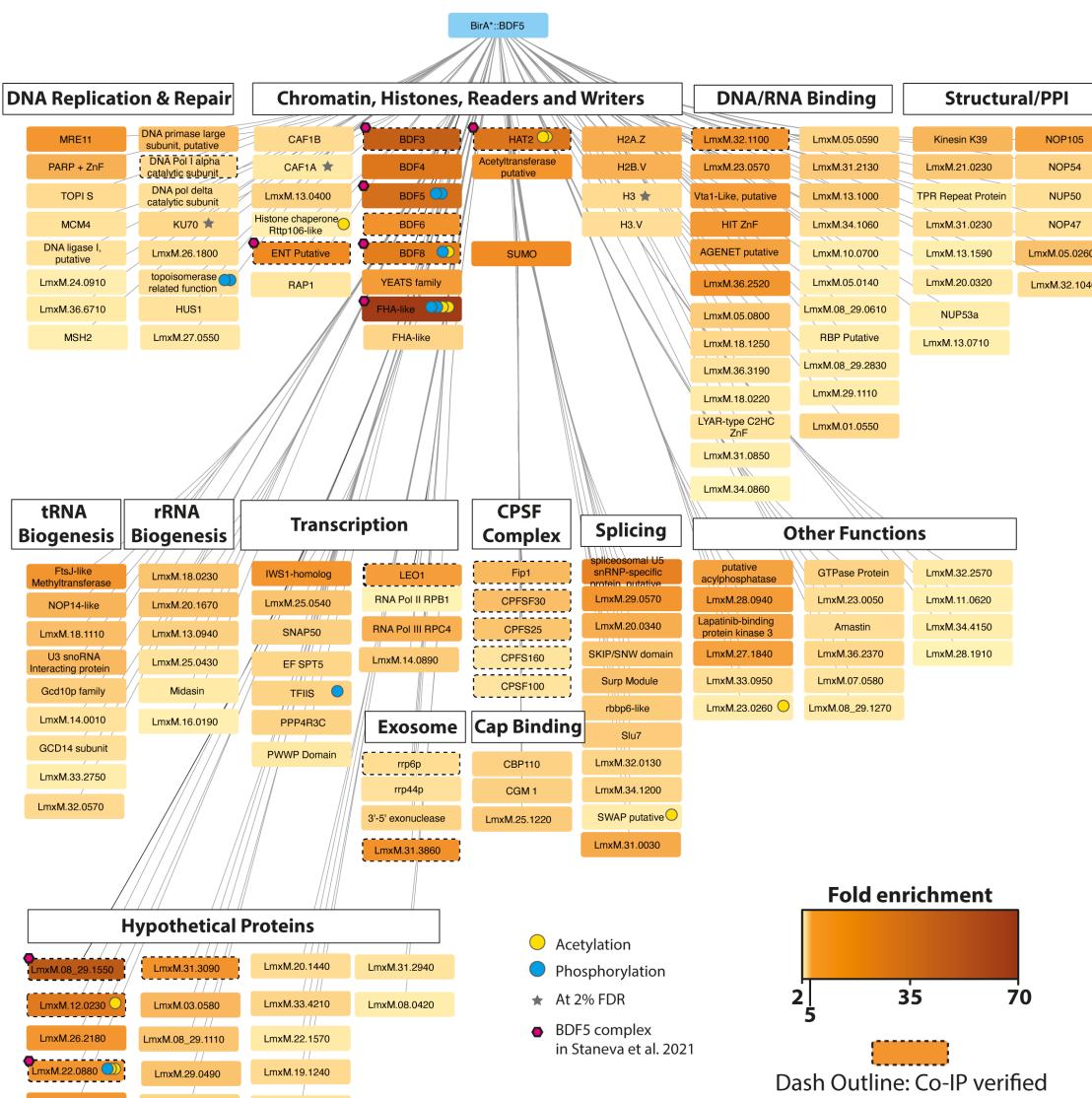
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355 *XL-BioID identifies the BDF5 proximal proteome*



356

357 **Figure 4: The BDF5-proximal proteome determined by XL-BioID.** Network indicates proteins
358 determined to be spatially enriched in proximity to BirA*::BDF5 following biotin labelling and
359 DSP cross-linking. Fold enrichment values are encoded in the colour intensity of the protein
360 boxes; these were calculated from label free protein intensities against a control expressing
361 KKT19::BirA*, from 3 replicate experiments. A dashed outline to a box indicates that BDF5 co-
362 purified with this protein in a reciprocal co-immunoprecipitation experiment. If a post
363 translational modification (PTM) was detected for a protein, this is indicated using a coloured
364 circle. Proteins are grouped by functional annotations or previously published data of
365 complexes in *Leishmania* or *Trypanosoma*. Proteins represented are those identified at 1%
366 false-discovery rate (FDR), those marked with a grey star denote those identified at 2% FDR for
367 select proteins. CPSF stands for cleavage and polyadenylation specificity complex, PPI stands
368 for protein-protein interactions. Magenta hexagons indicate members of the BDF5, BDF3,
369 HAT2 complex reported in *T. brucei*.

370

371 To identify the functional properties of the environment proximal to BDF we applied an in-
372 situ proximity labelling technique, cross-linking BioID (XL-BioID)⁵¹. The promiscuous biotin
373 ligase BirA*, which generates a locally reactive (~10 nm) biotinoyl-5'-AMP⁵², was fused to the

374 N-terminus of BDF5 by endogenous tagging. The resultant parasites were incubated with 150
375 μ M biotin for 18 h to permit labelling of proteins in proximity to BirA*::BDF5. The parasites
376 were then treated with a limited amount of dithiobis(succinimidyl propionate) (DSP) chemical
377 cross-linker, to increase the capture of proximal proteins which enriched with streptavidin,
378 trypsin digested and processed for LC-MS/MS analysis. Importantly, a control cell line was
379 treated in the same way to provide a control dataset of spatially segregated, nuclear proteins.
380 The nuclear-localised protein kinase KKT19⁵³ was chosen as it is expressed at similar levels to
381 BDF5 and localised to a distinct structure, the inner-kinetochore⁵⁴. This provided a way to
382 subtract common background proteins labelled during the synthesis and trafficking of BDF5
383 to the nucleus as well as endogenously biotinylated cellular proteins. Following SAINTq
384 interaction scoring, 156 proteins were determined to be enriched at 1% FDR (**Fig. 4, Table**
385 **S2**). A subset of these proteins was selected for endogenous tagging with 3xHA::mCherry in
386 mNG::BDF5 (which also contains a 3xmyc epitope) expressing strain to allow reciprocal co-
387 immunoprecipitation and verification of the XL-BioID dataset (**Fig. S8, Table S1**). This also
388 served to confirm there was no co-localisation of BDF5 and KKT19, and thus it was an
389 appropriate control protein.

390
391 The BDF5 proximal proteins were assessed for potential function (**Fig. 4**) and assembled into
392 a loose network. We identified a core set of 11 highly enriched proteins (>10-fold), including
393 the bait protein BDF5. Also identified were BDF3, BDF4 and Histone Acetyltransferase 2
394 (HAT2), along with several hypothetical proteins LmxM.35.2500, LmxM.08_29.1550,
395 LmxM.12.0230, LmxM.33.2300, LmxM.24.0530. A component of the spliceosome
396 LmxM.23.0650 was also identified, as was SUMO, which is likely conjugated to proteins in the
397 interactome (SUMO is a PTM common in the nucleus^{55,56}). BDF5 was enriched 35-fold
398 compared to the control samples, BDF3 was enriched 41-fold and BDF4 26-fold. HAT2 was
399 enriched 27-fold, strongly suggesting that these proteins are in very close proximity and that
400 they may even form a stable complex. BDF6 (8-fold) and YEATS, a non-bromodomain acetyl-
401 lysine reader, (4.8-fold) were also identified, consistent with the chromatin environment
402 surrounding BDF5 being important sites of regulation through acetylation. The hypothetical
403 proteins of the interactome were assessed by Phyre2 and HHPRED for remote structural
404 homology to known domains that might indicate their function. LmxM.35.2500 was enriched
405 >65-fold compared to the control samples. HHpred analysis detected remote homology to
406 forkhead-associated domains, suggesting it may play a role in the recognition of
407 phosphorylation sites. LmxM.33.2300 was enriched 22-fold, HHPRED searching detected
408 remote structural homology related to bromodomains in the C-terminal region. However, it
409 appears to lack the conserved asparagine and tyrosine residues. LmxM.33.2300 may
410 therefore represent a degenerate bromodomain, therefore we propose to name it BDF8.
411 LmxM.24.0530, which was enriched almost 14-fold, is predicted to contain an EMSY N-
412 Terminal Domain (ENT). EMSY is a protein implicated in DNA repair, transcription and human
413 tumorigenesis^{57,58}. LmxM.24.1230 was identified as 7-fold enriched, and domain searching
414 identified a putative acetyltransferase in the N-terminal region as well as PHD-Zinc Finger

415 domain. Seventeen other enriched hypothetical proteins remained that lacked structural
416 homology to known protein domains.

417

418 Many proteins identified in the proximal proteome at lower enrichment levels play roles in
419 processes associated with active transcription, broadly separated into RNA transcription and
420 processing, including pre-mRNA cleavage, polyadenylation, splicing, cap-binding and quality
421 control (nuclear exosome), indicating that these processes are occurring in proximity to BDF5.
422 Components of RNA polymerase complexes were identified, including RPC4 associated with
423 RNA polymerase III, RPB1, the largest subunit of RNA polymerase II and the RNA polymerase-
424 associated protein LEO1. LEO1 is a component of the PAF1 complex, which plays numerous
425 roles in transcriptional regulation. The basal transcription factors SNAP50, TFIIS-like protein
426 (LmxM.32.2810), and a hypothetical protein (LmxM.22.0500) with remote homology to TFIIS
427 helical bundle, ISW1 transcriptional elongator (LmxM.22.0500) were identified. Five
428 components of the cleavage and polyadenylation specificity complex (CPFS) were identified
429 and validated by reciprocal co-IP, as this process occurs co-transcriptionally it must be near
430 polymerase complexes. Additionally, proteins associated with the splicing machinery of
431 *Leishmania* were identified, including LmxM.23.065, a component of the spliceosome. Cap
432 binding proteins and members of the nuclear exosome were also identified, all indicative of
433 the mRNA processing and quality control events that occur alongside transcription of the pre-
434 mRNA. These hits are consistent with the ChIP-seq dataset and show BDF5 is located in sites
435 of polymerase II transcriptional activity.

436

437 The ChIP-seq distribution of BDF5 identified it to be enriched not only at TSS regions but also
438 at rRNA and tRNA genes and some polymerase II termination sites (cSSRs). It was interesting
439 to discover proteins in the XL-BioID that are involved in the maturation of both tRNAs and
440 rRNAs, placing BDF5 in proximity to the transcription and maturation of different classes of
441 RNAs. Base J is associated with termination sites in *Leishmania*⁵⁹, and the base J-associated
442 glucosyltransferase JBP1 (LmxM.36.2370) was found to be 3-fold enriched over the control,
443 potentially indicating that BDF5 may occasionally be found at sites linked to transcriptional
444 termination. Interestingly, many factors associated with the detection and repair of DNA
445 damage were also found in the proximal proteome, together with factors associated with
446 DNA replication. It is known that DNA damage can occur in transcriptionally active regions
447 due to the formation of RNA-DNA hybrids called R-loops⁶⁰. Some of the origins of DNA
448 replication in *Leishmania* coincide with transcriptional start sites, suggesting we can detect
449 this association in the XL-BioID data⁶¹.

450

451 Because the samples were trypsin digested, it was difficult to obtain much information on
452 histone tails. Nevertheless, we were able to detect some peptides from the core of histones
453 and histone variants as significantly enriched in proximity to BDF5. Peptides were detected
454 for H2A.Z, H2B.V, H3 and H3.V. In *T. brucei*, the H2A.Z and H2B.V variants have been localised
455 to divergent SSRs¹⁹, where H2A.Z plays a role in the correct positioning of transcription

456 initiation. H2A.Z and H2B.V are also essential for *Leishmania*⁶². H3.V localises to convergent
457 SSRs in *T. brucei* and is not essential for *Leishmania*, nor does it play a role in transcriptional
458 termination in this organism⁶². Mining the XL-BioID data further we were able to detect a
459 number of acetylated peptides. Acetylation sites were detected on HAT2, BDF8, FHA-like
460 protein (LmxM.35.2500), a putative Rttp106-like histone chaperone and several hypothetical
461 proteins (**Table S2**). However, we were again unable to detect any acetylated peptides
462 derived from histones.

463

464 The capacity of XL-BioID to enrich large amounts of proximal material allows it to be combined
465 with other methods, such as phosphoproteomics⁵¹. We engineered a cell line to carry
466 BDF5::miniTurboID for faster labelling kinetics and higher temporal resolution, allowing us to
467 explore BDF5-proximal phosphorylation events across the cell cycle of hydroxyurea
468 synchronised cultures. Following synchronisation release, 30 minute biotinylation timepoints
469 were carried out 0, 4 and 8 h corresponding to G1/S, S and G2/M phase respectively..
470 Samples were processed using the XL-BioID workflow, then proximal phosphopeptides were
471 enriched using Ti-IMAC resin prior to LC-MS/MS analysis. The resulting dataset was compared
472 to a reference phosphopeptide dataset derived from the kinetochore protein KKT3⁵¹. Two
473 BDF5-proximal phosphopeptides were identified in early-S phase which then rose to 19 and
474 13 as the cells progressed through the S and G2/M phases respectively (**Fig. S9, Table S3**). Of
475 these, 14 unambiguous phosphosites were detected in total for proteins in proximity to BDF5,
476 including several for BDF5 itself, pS135, pS133, pS317 and pS330. S135 and S133 are located
477 between the two bromodomains, while S317 and S330 are located after the second
478 bromodomain. LmxM.35.2500, which was highly enriched in the original XL-BioID and
479 identified to contain a putative FHA domain, was itself found phosphorylated at S202, S208
480 and S545. LmxM.33.2300 (BDF8) was found to contain an ambiguous phosphosite at one of
481 six sites in the region of S50-S61 (**Fig. S9**). Despite detecting multiple phosphosites, only a
482 single protein kinase was identified in proximity to BDF5, LmxM.25.1520 (LBPK3), an orphan
483 kinase with unknown function that has been reported to bind lapatinib⁶³.

484

485 The combined ChIP-Seq and XL-BioID data defined where BDF5 localises on the genome, and
486 the protein landscape around it. This points towards a role for BDF5 in promoting
487 transcriptional activity and provides a starting point to develop assays to characterise the
488 phenotypes of BDF5-induced null strains.

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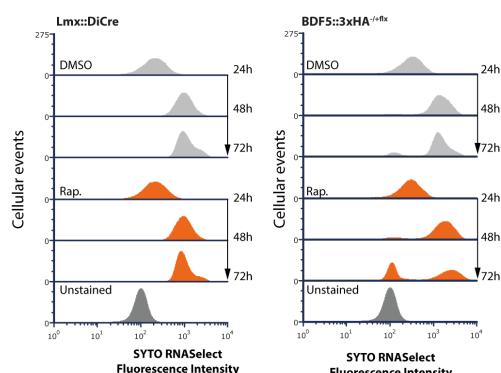
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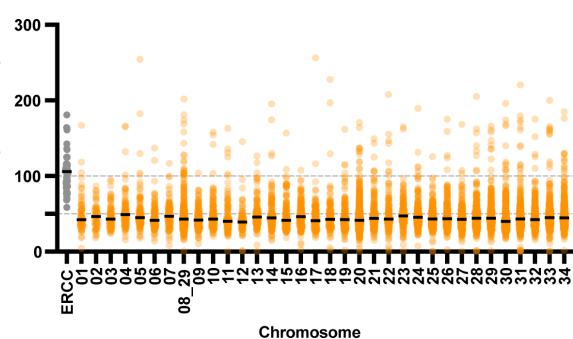
493 *BDF5 depletion results in generalised transcriptional defect.*

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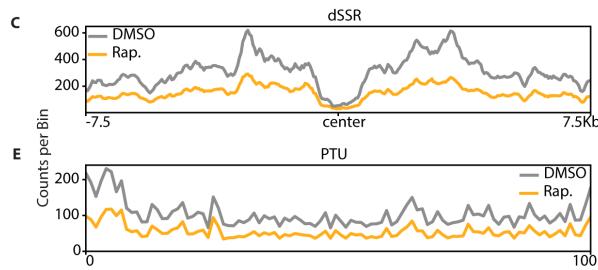
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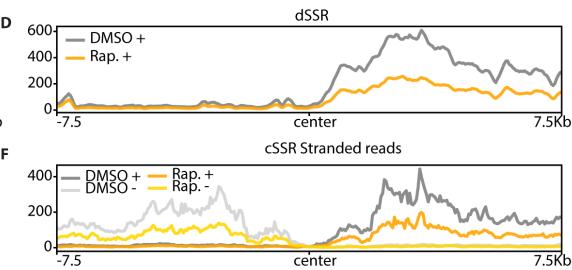
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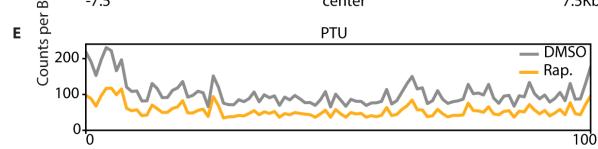
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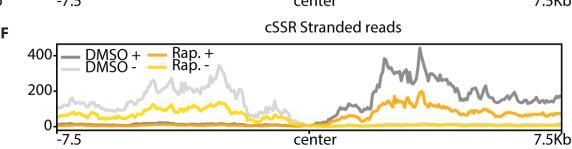
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495

496 **Figure 6: Effect of BDF5 depletion on RNA levels and gene expression.** A. Flow cytometry of
497 cells stained with SYTO RNASelect Stain to measure total RNA levels in *Lmx DiCre* strains or the
498 *BDF5^{+/flx}* strain treated with rapamycin or DMSO over a 72 h time course. 20,000 events
499 measured per condition. B. Dot plot of total RNA-seq reads per protein-coding gene scaled to
500 ERCC spike-in controls, then as a percentage of the DMSO control sample, separated per
501 chromosome, conducted at a 96 h timepoint. Black lines denote the median. N=3 C. Metaplot
502 divergent SSR (n=60) for DMSO treated or rapamycin-treated *BDF5^{+/flx}*. D. Metaplot of reads
503 mapping to the + strand, normalised to ERCC control at divergent SSRs (n=60) of DMSO treated
504 or rapamycin-treated *BDF5^{+/flx}* cultures. E. Metaplot of + stranded RNA-seq reads normalised
505 to ERCC spike-in controls for PTUs (n=120), on a scale of 0-100%. F. Metaplot of reads mapping
506 to the + and – strands, normalised to ERCC control at convergent SSRs (n=40) of DMSO treated
507 or rapamycin-treated *BDF5^{+/flx}* cultures. Metaplot data is from 1 representative of the three
508 replicate RNA-seq datasets.

509

510

511 As most of the BDF5 enriched regions of the genome corresponded to transcriptional start
512 sites, and the proximal proteome contained factors associated with the transcription and
513 maturation of various classes of RNA we sought to assess the effect of BDF5 depletion on
514 cellular RNA levels. Promastigote cultures were stained for total RNA content using SYTO
515 RNASelect fluorescent stain at 24, 48 and 72 h timepoints and measured by flow cytometry
516 (**Fig. 6A**). For *Lmx::DiCre* strain, the addition of rapamycin caused no changes in the levels of
517 total RNA staining. SYTO RNASelect staining increased as cells progressed through log phases
518 of growth at 48-72 h time points. However, once BDF5 was deleted from the *BDF5::6xHA^{+/flx}*

519 cell line by the addition of rapamycin, there was a pronounced increase in the number of
520 cellular events containing very low levels of RNA staining, such that the profile at 72 h overlaps
521 with that for with unstained control cells. This result suggested that total levels of
522 transcription were reduced upon BDF5 deletion from cells. We investigated this in more detail
523 by using total, stranded RNAseq that included External RNA Controls Consortium (ERCC)
524 Spike-in controls²⁰. Cultures of *BDF5::6xHA-/+flx* treated with Rapamycin or DMSO were
525 harvested, then RNA extraction buffer spiked with the 92 synthetic ERCC RNAs was used to
526 lyse the parasites for RNA purification. Following sequencing and read mapping these RNAs
527 were then used to provide a normalisation channel. Overall, a >50% reduction in the median
528 read depth was observed across protein-coding genes on all chromosomes (Fig. 6B). When
529 normalised read depths were compared using metaplots of divergent SSRs, this ~50%
530 reduction in transcriptional levels was reflected (Fig. 6C). However, no positional effects were
531 observed on transcriptional start sites (Fig. 6D). The 50% reduction in read depth was
532 reflected across PTUs (Fig. 6E) and at convergent SSRs (Fig. 6F). Strand-specific read depth at
533 cSSRs did not indicate any increase in transcriptional readthrough in BDF5-induced null cells
534 (Fig. 6F), suggesting the BDF5 located at these termination sites is not playing a role in
535 transcriptional termination. Overall, these results indicate BDF5 is important for global pol II-
536 dependent gene transcription.

537

538 Transcriptionally active regions of kinetoplastid genomes often accumulate DNA damage
539 which occurs due to the formation of DNA-RNA hybrids (R-loops)^{60,64}. As we detected proteins
540 involved in co-ordinating DNA repair in the BDF5 proximal proteome, and that this appears
541 to be a broader feature of BDF protein networks^{65,66}, we examined if there was a link between
542 BDF5 and the DNA damage response in *Leishmania*. BDF5-induced-null promastigotes cease
543 growing quickly, whereas parasites deficient for genome-stability factors often die slowly⁵⁰,
544 suggesting maintaining genome integrity is not the primary role of BDF5. Indeed, after using
545 western blotting to detect γ H2A phosphorylation⁶⁷, a sensitive marker for the cellular
546 response to DNA damage, we could not detect any increase in γ H2A signal in BDF5-depleted
547 cells, nor was there any detectable difference in the γ H2A response of these cells to a non-
548 specific DNA damaging agent, phleomycin (Fig. S10). This indicates that there is no direct or
549 secondary role for BDF5 in DNA damage response. Despite enrichment in the BDF5 proximal
550 proteome for mRNA splicing factors, we did not find evidence to support trans- or cis-splicing
551 defects in BDF5 induced-null mutants using a qualitative RT-PCR assay. This assay was capable
552 of detecting splicing defects caused by inhibition of an analog-sensitised CRK9 by the bulky
553 kinase inhibitor 1NM-PP1 (Fig. S11, S12)⁶⁸.

554

555 **Discussion**

556 Kinetoplastid parasites have evolved a genomic architecture that requires them to conduct
557 most gene transcription constitutively, in an apparently simplified manner and deal with
558 consequences of this using post-transcriptional regulation and specialised solutions to genes
559 requiring a “high-dose”⁸. Pol II transcriptional start sites may simply be maintained as open

560 chromatin. However, recent evidence has indicated these regions are actively regulated,
561 particularly through histone acetylation. How the cell interprets these marks is not completely
562 understood. Bromodomains are clearly critical components of this process in *Leishmania*; we
563 were unable to generate null mutants in five of the seven bromodomain encoding genes, also
564 implying there is no redundancy in their individual functions. Although failure to generate a
565 null is the most basic standard of genetic evidence for essential genes⁴⁴, we were able to
566 generate high-quality, genetic target validation for BDF5, using inducible DiCre both in the
567 promastigote stage and during murine infections. BDF5 expression was confirmed in both
568 stages and expression levels were correlated with cellular growth rate in promastigote stages.
569 Combined with the rapid cytostatic phenotype occurring upon BDF5 inducible deletion,
570 followed by cell death, this identifies BDF5 as a regulator of cell growth and survival. This
571 finding demonstrates that the interpretation of histone acetylation is important for cellular
572 survival (Fig. 7), although for *Leishmania* the specific histone PTMs found at TSSs are not
573 currently defined.

574

575 When functionally characterising BDF5, our starting hypothesis was that BDF5 would localise
576 to polymerase II transcriptional start sites, so it was surprising to find BDF5-enriched peaks
577 associating with many other sites such as rRNA genes, tRNA genes and convergent strand
578 switch regions. This suggests BDF5 plays a multipurpose or generalist role in recruiting or
579 regulating chromatin to promote transcription by multiple polymerase complexes. This was
580 further emphasised by the proximity proteomics dataset, which revealed BDF5 to be close to
581 proteins involved in different processes linked to transcriptionally active chromatin, in
582 particular RNA maturation factors, DNA repair factors and polymerase associated complexes.
583 Our phenotypic analysis appeared to rule out roles for BDF5 in influencing the DNA damage
584 response and cis- and trans-splicing of mRNA but did demonstrate that it was required for
585 normal transcription of polymerase II PTUs. Due to the rRNA depletion method used and low
586 coverage over tRNA genes, we could not assess if pol I or pol III transcripts levels were
587 reduced. This could be determined using qPCR in future studies. Spike-in controlled total RNA
588 seq was previously used to study the influence of HAT1 and HAT2 on transcription in *T. brucei*
589²⁰. It is striking that BDF5 knockout in *L. mexicana* phenocopies HAT1 knockdown in *T. brucei*,
590 both resulting in an overall reduction in transcription levels. In *T. brucei*, HAT1 is required for
591 acetylation of H2A.Z and H2B.V. Depletion of HAT1, and thus H2A.Z levels, leads to a 10-fold
592 decrease in the amount of chromatin-bound pol II, resulting in 50% reduced transcriptional
593 activity. Intriguingly, pol II levels at TSSs were not affected by this; the authors suggested
594 H2A.Z acetylation is required for optimal transcription of bound pol II. As BDF5 knockout
595 phenocopies HAT1 depletion and results in lower pol II activity, it might therefore be involved
596 in reading or applying acetylation of H2A.Z. Surprisingly, although we find HAT2 proximal to
597 BDF5, HAT1 was neither enriched nor detected in our XL-BioID dataset. This suggests that
598 there is a distinct spatial separation between BDF5, HAT2 and HAT1 in *Leishmania* (assuming
599 there is no technical reason HAT1 cannot be labelled by XL-BioID). BDF5 also did not co-
600 precipitate very strongly with HAT2 (Fig. S8), suggesting any interaction between them might

601 be transient or indirect. We did not observe changes in the positioning of transcription
602 initiation, suggesting the HAT1/BDF5 phenotype over-rides any effect on HAT2 dysfunction if
603 this is indeed a BDF5 complex member. Purified *L. donovani* HAT2 has been shown to
604 acetylate H4K10 and it appears to be essential as only heterozygotes can be generated using
605 traditional knockout strategies^{15,18,69}. *L. donovani* HAT2^{+/+} heterozygotes grow slowly and
606 display a cell-cycle defect. No effect was determined on transcriptional initiation positioning
607 at TSSs but an S-phase cell cycle-dependent reduction on *CYC4* and *CYC9* genes was
608 reported¹⁸. Future work could investigate the requirement of BDF5 for cell cycle dependent
609 gene transcription in *Leishmania*, which is an interesting observation given the lack of obvious
610 gene specific promoters.

611
612 A recent immunoprecipitation dataset of *T. brucei* chromatin factors²² defined a complex
613 consisting of BDF5, BDF3, HAT2 and orthologs of the hypothetical genes LmxM.24.0530,
614 LmxM.22.0880, LmxM.35.2500, LmxM.33.2300, LmxM.08_29.1550 that were highly enriched
615 in our proximity proteome (**Fig. 4**). We propose that these proteins represent a Conserved
616 Kinetoplastid Regulator of Transcription (CRKT) Complex, that is recruited to acetylated
617 histones at TSSs (**Fig. 7**). The association of BDF5 at transcriptional termination regions, albeit
618 in lower amounts, could indicate that BDF5 is included in a mobile complex that can progress
619 along chromatin and accumulates at start and termination sites. One complex could be the
620 PAF1 complex, a multifunctional complex associated with pol II initiation, elongation, pausing
621 and termination⁷⁰. However, PAF1-complex is poorly characterised in kinetoplastids and the
622 PAF1 protein itself lacks identifiable orthologs in these organisms. *T. brucei* BDF5 has been
623 suggested as a potential component of a transcription initiation complex due to its dual
624 bromodomains and the interaction with proteins containing homology to TFIID TAF1 (which
625 also contains 2 bromodomains as well as protein kinase and acetyltransferase function).

626
627 Our findings further illustrate the strength of proteomic approaches to studying chromatin
628 regulation in kinetoplastids where the large TSSs allow plentiful material to be derived²⁰.
629 Combined with XL-BioID this allowed for the enrichment of PTMs to be determined for many
630 of the complex members. The phosphorylation of the region connecting the two BDs might
631 represent a site of PTM-dependent regulation of BDF5. Regulation of BDF function by
632 phosphorylation has been reported, for example, BRD4 is phosphorylated in mitosis and
633 hyperphosphorylated BRD4 is associated with greater transcriptional activity during
634 oncogenesis^{71,72}. Adaptation of the XL-BioID workflow to include a chemical derivatisation
635 step (e.g. stable isotope acylation) could allow it to be used to detect histone tail peptides in
636 proximity to BDF5 or other BDFs, potentially identifying their native binding partners and
637 providing locus-specific views of histone PTMs.

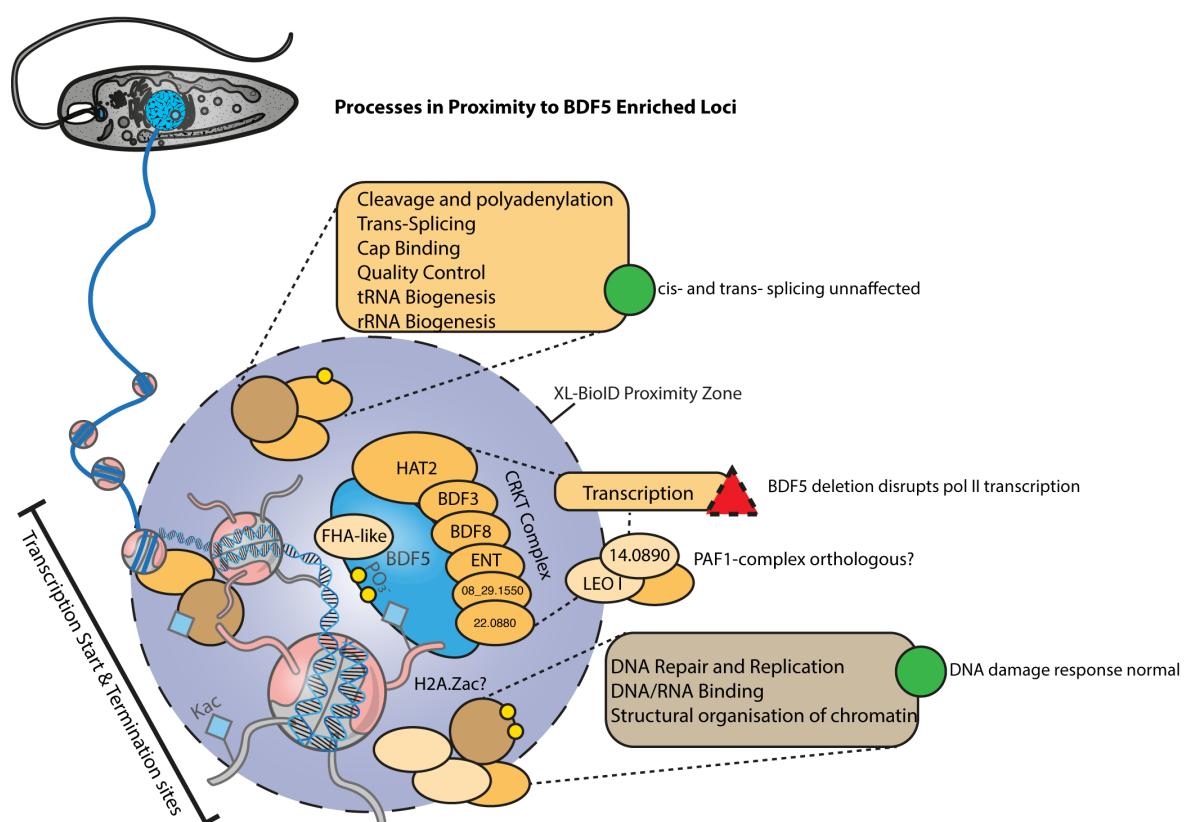
638
639 As bromodomains are chemically tractable targets it may be possible to develop *Leishmania*
640 specific inhibitors that target BDF5. Such a compound would be of high value to the
641 investigation of BDF-dependent transcriptional regulation in kinetoplastids, allowing for

642 precise temporal disruption of BDF5 and the processes that it coordinates. It should be noted
643 that parasites expressing BDF5 with singly mutated bromodomains were viable, requiring
644 both to be disrupted to observe a reduction in parasite growth. Potential BDF5 inhibitors
645 would likely require a bi-specific molecule, a PROTAC (proteolysis targeting chimera) molecule
646 (not yet realised in kinetoplastids), or a mono-specific inhibitor that can perturb the complex
647 enough to be fatal for the cell.

648

649 In summary, our findings identify the importance of the linkage between histone acetylation
650 and transcriptional regulation by bromodomain factors in a eukaryote that is divergent from
651 opisthokonts such as the humans host. Because of their unusual features, kinetoplastids can
652 provide ideal organisms to investigate the evolution of chromatin regulation by acetylation.

653



654

655 **Figure 7: Cartoon of the BDF5-defined chromatin landscape.** BDF5 localised to chromatin with
656 the CRKT complex members depicted as interacting directly and influencing transcription.
657 Juxtaposition of complex members is for illustration only. Not to scale.

658

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668 (former GSK employee) for his critical input to develop the collaboration between University
669 of York and GSK.

670

671

672 **Author Contributions**

673 NGJ, FC, AJW and JCM conceived the project. JCM, AJW, RG, JM and FC supervised the
674 project. NGJ and JCM designed the experiments. NGJ, VG, GM and JBTC performed the
675 experiments. NGJ, VG, GM and KN analysed experimental data. NGJ wrote the manuscript
676 and all other authors revised it. NGJ, RP, IR, FC, AJW and JCM acquired funding.

677

678 **Data Availability**

679 Mass spectrometry data sets and proteomic identifications are available to download from
680 MassIVE (MSV000087750), [doi:10.25345/C5G543] and ProteomeXchange (PXD027080).
681 ChIP-Seq and RNA-Seq reads are available as FASTQ files at the European Nucleotide Archive
682 under the accession code PRJEB46800.

683

684 **Supplemental Files**

685

686 Table S1: Excel spreadsheet, List of Oligonucleotides, plasmids, cell lines and antibodies
687 used in this study.

688

689 Table S2: Excel spreadsheet, SAINTq Analysis of BDF5 XL-BioID data to identify proximal
690 proteins, also contains list of remote homology identified in hypothetical proteins by
691 HHPRED analysis.

692

693 Table S3: Excel spreadsheet, BDF5 proximal phosphosites through the cell cycle as
694 determined by limma analysis of phosphoproteomic XL-BioID samples.

695

696 **Methods**

697

698 *Molecular Biology*

699 Computational sequence analysis, design of vectors, primers and PCR fragments was
700 performed using CLC Main Workbench (Qiagen). Oligonucleotides were synthesised by
701 Eurofins Genomics. High-fidelity PCRs were conducted using Q5 DNA polymerase (NEB)
702 according to manufacturer's instructions. Low-fidelity screening PCRs were conducted using
703 Ultra Mix Red (PCR Biosystems) according to manufacturer's instructions. Vectors for DiCre
704 strain generation were generated as previously described⁴⁵ using Gateway Assembly (Thermo
705 Fisher). PCR amplicons were resolved in 1% agarose (Melford) TBE gels containing 1x SYBRsafe
706 and visualised on a Chemidoc MP (BioRad). A full list of oligonucleotides and vectors are
707 presented in **Table S1**. Sanger sequencing to verify plasmids etc. was conducted by Eurofins
708 Genomics.

709

710 Protein samples of cells were generated by taking 2.5×10^7 log phase promastigotes, lysing in
711 40 μ l LDS (lithium dodecyl sulfate) sample buffer supplemented to 250 mM DTT and heated
712 to 60 °C for 10 minutes, after cooling, 1 μ l of Basemuncher (Abcam) was added and the sample
713 incubated at 37 °C to degrade DNA and RNA. Samples were separated in TGX Stain-Free SDS-
714 PAGE Gels (BioRad) and the total protein labelled and visualised using a propriety trihalo
715 compound activated by UV light in a BioRad ChemiDoc MP. Western blotting was performed
716 using an iBlot II (Invitrogen) and the associated PVDF cassettes, using program P0.
717 Membranes were blocked with 5% milk protein in 1x Tris Buffered Saline Tween-20 0.05%.
718 Primary and secondary antibodies are listed in **Table S1** and were detected using appropriate
719 fluorescent channels of chemiluminescent channels of a Chemidoc MP (BioRad), using Clarity
720 Max Western ECL Substrate (BioRad).

721

722 *Parasites*

723 *Leishmania mexicana* (MNYC/BZ/62/M379) derived strains were grown at 25°C in HOMEM
724 (Gibco) supplemented with 10% (v/v) heat-inactivated foetal calf serum (HIFCS) (Gibco) and
725 1% (v/v) Penicillin/Streptomycin solution (Sigma-Aldrich). Where required parasites were
726 grown with selective antibiotics at the following concentrations: G418 (Neomycin) at 50 μ g ml⁻¹;
727 Hygromycin at 50 μ g ml⁻¹; Blasticidin S at 10 μ g ml⁻¹; Puromycin at 30 μ g ml⁻¹ (antibiotics
728 from InvivoGen).

729

730 *CRISPR/Cas9*

731 Initial screening for bromodomain gene essentiality was performed with a modification of the
732 approach developed by the Gluenz lab⁴². Per gene a single sgRNA was designed with EuPaGDT
733⁷³ to target the interior of the coding DNA sequence. Oligonucleotides are defined in **Table**
734 **S1**. Thirty residue homology flanks were identified adjacent to the CDS and appended to
735 oligonucleotides designed to amplify drug resistance markers from blasticidin and neomycin
736 drug resistance plasmids pGL2208 and pGL2663 respectively. After amplification of the sgRNA

737 and resistance marker the PCR mixes were pooled and precipitated using standard ethanol
738 precipitation, resuspended in sterile water and added to a transfection mix with 1×10^7 mid-
739 log promastigotes. The cell line used was *L. mexicana* T7/Cas9::HYG::SAT⁴². Transfection was
740 performed with an Amaxa Nucleofector 4D using program FI-115 and the Unstimulated
741 Human T-Cell Kit. The mix was resuspended in 10 ml HOMEM 20% FCS and immediately split
742 in two 5 ml aliquots. Following 6-18 h of recovery time the parasites were plated at 1:5, 1:50
743 and 1:500 dilutions in media containing the selective drug blasticidin or G418. Endogenous
744 tagging was performed using the pPLOT 3xMYC::mNG BSD donor vector to install N-terminal
745 tags to BDF5, preserving the 3' UTR for native mRNA regulation (Oligonucleotides defined in
746 **Table S1**).

747

748 *DiCre*

749 *DiCre* strains for BDF5 were generated as previously described⁴⁶. Briefly the BDF5 CDS and
750 flanking regions were assembled into floxing or knockout plasmids using Gateway cloning,
751 BDF5 was cloned into pGL2314 to fuse a 6xHA C-terminal tag and flank with loxP sites
752 (**Oligonucleotides defined in Table S1**). The BDF5::6xHA^{flx} was first integrated into parasites
753 with clones being assessed for correct integration, correct genome copy number and
754 inducibility of the excision of BDF5^{flx} gene prior to the second round of transfections to delete
755 the remaining wild-type allele. The same quality controls were performed when selecting final
756 clones with the genotype *Leishmania mexicana* *DiCre::Puro* Δ *bdf5::HYG*
757 ::*BDF5::6xHA::flx::BSD*.

758

759 Inducible deletion of BDF5^{flx} in *DiCre* cell lines was initiated by the addition of 300 nM
760 rapamycin (Abcam) to promastigotes cultures at 2×10^5 cells ml⁻¹. Cells were grown for 48 h
761 then passaged into new media at a concentration of 2×10^5 cells ml⁻¹; induction was
762 maintained by the addition of 100 nM of rapamycin to suppress escape mutants.

763

764 *Clonogenic assays*

765 For clonogenic assays, mid-log cells were counted and then diluted to 1 cell per 800 μ l and
766 plated out into 200 μ l volumes in 3 x 96-well plates to yield approximately 100 clones. Cells
767 were plated in media \pm 100 nM rapamycin and incubated at 25°C for 3 w before counting of
768 viable colonies by both visual screening and microscopic analysis

769

770

771 *Addback strains*

772 To generate episomal addbacks the BDF5 CDS was amplified from *L. mexicana* genomic DNA
773 and cloned into the pNUS C-Ter GFP NEO (pGL1132) using HiFi Assembly (NEB) to generate a
774 complementation vector. This was used as a base for site-directed mutagenesis using the Q5
775 Mutagenesis product (NEB) to generate mutations in the conserved asparagine residues N90
776 (OL9577 and OL10352), N257 (OL9579 and OL10353) to phenylalanine in BDF5 BD5.1 and

777 BD5.2 (**Table S1**). Log-phase promastigotes were transfected with 2-5 µg plasmid DNA as
778 previously described and maintained as population under G418 selection.

779

780 *Inducible overexpression*

781 An adaptation of a published method was performed whereby BDF5::GFP alleles generated
782 for episomal addback were amplified using PCR primers OL11307 and OL11308, where the
783 oligonucleotide included a directional loxP site as well as a homology region for HiFi Assembly
784 into pRIB Neo (pGL1132). The vector backbone was linearised with PstI/PmeI double digest,
785 separated by agarose gel electrophoresis and purified using QiaEx II gel extraction resin
786 (Qiagen). Log-phase promastigotes were transfected with 1-5 µg and cloned by limiting
787 dilution. Clones were induced to express BDF5::GFP or BDF5^{NN>FF}::GFP

788

789 *Mouse infections*

790 All experiments were conducted according to the Animals (Scientific Procedures) Act of 1986,
791 United Kingdom, and had approval from the University of York Animal Welfare and Ethical
792 Review Body (AWERB) committee. All animal studies were ethically reviewed and carried out
793 in accordance with Animals (Scientific Procedures) Act 1986 and the GSK Policy on the Care,
794 Welfare and Treatment of Animals. Mid-log parasites were treated with 500 nM rapamycin
795 and allowed to progress to stationary phase. These cultures were used to infect BALB/C mice
796 at a dose of 2 x 10⁵ parasites per footpad. Infections were allowed to progress for 8 w, at
797 which point mice were euthanised and footpads and popliteal lymph-nodes were dissected
798 for mechanical disruption and determination of parasite burden by limiting dilution, as
799 previously described⁷⁴.

800

801 *Live-Cell Microscopy*

802 To image mNeonGreen::BDF5 10⁶ mid-log cells were incubated with 1 µg ml⁻¹ Hoechst 3342
803 for 20 mins at 25°C to stain DNA, harvested by centrifugation at 1200 x g for 10 mins and
804 washed twice with PBS. Cell pellets were resuspended in 40 µl CyGel (BioStatus) and 10 µl
805 settled onto SuperFrost+ Slides (Thermo) then cover slip applied. Cells were imaged using a
806 Zeiss AxioObserver Inverted Microscope equipped with Colibri 7 narrow-band LED system and
807 white LED for epifluorescent and white light imaging. Cells were imaged using the x63 or x100
808 oil immersion DIC II Plan APOCHROMAT objectives. Hoechst signal was imaged using the 385
809 nm LED and filter set 49, mNeonGreen with the 469 nm LED and filter set 38. Z-stacks were
810 obtained using the Zen Blue software to control the system and exported as .CZI files to be
811 processed in ImageJ using the Microvolution blind deconvolution module. Wavelength
812 parameters were set for Hoechst (497 nm) and mNeonGreen (517 nm) emission and
813 refractive index parameters were defined for Cygel (1.37). Blind deconvolution was iterated
814 100 times using the scalar setting. Maximum intensity projections were then exported as
815 were individual Z-planes for subpanels in TIFF format. Amastigotes in murine bone marrow
816 derived macrophages were grown on glass bottomed 35 mm dishes (Thermo Scientific) and
817 imaged in FluoroBrite DMEM (Gibco) using a heated plate holder to maintain the samples at

818 35°C. In this instance due to the short imaging duration CO₂ supplementation was not
819 provided.

820 *XL-BioID*

821 BDF5 and KKT19 were N-terminally tagged with BirA*⁴² and then cultures were grown to mid-
822 log phase. For the final 18h of growth biotin was added to the medium at 150 μM, 4 x 10⁸
823 parasites were washed twice in PBS and limiting cross-linking was performed with 1 mM
824 dithiobis(succinimidyl propionate) (DSP) for 10mins at 26 °C in PBS. After quenching with
825 20mM Tris pH 7.5 for 5mins, cell pellets were washed in PBS and then lysed in RIPA buffer
826 supplemented with protease inhibitors followed by Benzonase treatment and sonication.
827 Biotinylated and cross-linked proteins were purified using Streptavidin magnetic beads
828 (MagResyn) which were washed with a series of harsh washes before cross-linker reversion
829 and on bead digest with Trypsin Lys-C (Promega). Peptides were then desalted and prepared
830 for mass spectrometry. For a full protocol see *Geoghegan et al.*⁵¹

831

832 Hypothetical proteins were screened for remote structural homology using HHPRED³¹ and
833 Phyre2⁷⁵ to identify putative domains in these proteins.

834

835 *Co-Immunoprecipitation*

836 Co-immunoprecipitation to confirm BioID hits was performed by tagging candidate proteins
837 with 3xHA::mCherry PURO using an adapted pPLOT vector (gifted by Ewan Parry, Walrad Lab.)
838 in the *L. mexicana* T7/Cas9 3xMYC::mNG::BDF5 strain previously generated. Correct
839 integration of the tag was confirmed by western blotting for the HA epitope. For pulldowns,
840 30 ml of mid-log cultures (~1.5 x 10⁸ cells) were harvested, by centrifugation at 1200 x g for
841 10 minutes and resuspended in PBS. DSP reversible cross-linker (dithiobis(succinimidyl
842 propionate) (Thermo) was added to 1 mM and incubated for 10 mins at 26 °C. Cross-linking
843 was quenched by the addition of Tris pH7.5 to 20 mM and parasites washed with PBS. The
844 cells were then lysed using 1x RIPA buffer (Thermo) supplemented with 3x HALT Protease
845 inhibitors (Thermo) and 1 x PhosSTOP (Roche), 2 μl (500 Units) BaseMuncher Endonuclease
846 (Abcam). The lysate was sonicated 3 x 10 seconds at 40% amplitude using a probe sonicator
847 (Sonics Vibra-Cell) and then clarified by centrifugation 10, 000 x g for 10 mins at 4 °C. HA-
848 tagged bait proteins were then immunoprecipitated by the addition of 30 μl anti-HA magnetic
849 beads (Pierce) incubated for 2 hours with rotation at 4 °C. The beads were then washed 3
850 times using the supplemented RIPA lysis buffer and proteins eluted from the beads using 40
851 μl 1x LDS buffer supplemented with 250 mM DTT and heating to 60°C for 10 mins. The eluted
852 fractions were analysed for presence of the BDF5 prey protein and intended bait proteins by
853 western blotting for the MYC and HA epitopes respectively.

854

855 *ChIP-Seq*

856 BDF5 ChIP-seq was performed using a modification of a protocol previously optimised for *T.*
857 *brucei*⁶⁰ and the ChIP-it Express Enzymatic Kit (Active Motif). *Lmx DiCre BDF5::6xHA*^{-/+flx}
858 parasites were grown to 5 x 10⁶ cells ml⁻¹ in sufficient volume to collect 3 x 10⁸ cells per ChIP

859 replicate. Cells were fixed with 1% formaldehyde for 5 mins then quenched with 1x of the
860 included glycine solution. Fixed cells were Dounce homogenised until only nuclei were visible
861 by microscopy. Following enzymatic digestion of purified nuclei, the mix was sonicated 3 x 10
862 seconds at 40% amplitude using a probe sonicator (Sonics Vibra-Cell) to increase recovery of
863 mono- to tetra-nucleosomes⁵⁴. Chromatin fractionation and release was checked by agarose
864 gel electrophoresis before immunoprecipitation using anti-HA magnetic beads (Thermo) for
865 2 h at 4 °C. Beads were washed and the cross-linking was reversed following manufacturer's
866 instructions. Liberated DNA and the retained input samples were purified and concentrated
867 using ChIP-cleanup mini-columns (Zymogen). This DNA was quantified using a Qubit (Qiagen)
868 High Sensitivity DNA kit and sent for library preparation. Library generation was performed
869 on a minimum of 5 ng DNA using (TF KIT) in the Genomics Laboratory of University of York
870 Bioscience Technology Facility. Sequencing was conducted at the University of Leeds. Reads
871 were quality checked and trimmed using FastQC version 11.0.5 and Cutadapt version 2.5,
872 respectively. This was followed by alignment to the *L. mexicana* T7/Cas9 genome using BWA-
873 MEM (version 0.7.17). Paired ChIP-seq and input alignment files were normalised to each
874 other using deepTools' bamCompare (version 3.3.1) with SES normalisation and bin size of
875 500. Bigwig files were converted to wig files with UCSC's bigWigToWig tool, and the resulting
876 3 files were combined by taking the mean. Peaks were filtered to only include those with a
877 mean log2 ratio greater than 0.5 and peaks that were less than 5 kb apart were merged.
878 Strand switch regions were defined as regions between the end of a CDS on one strand and
879 the beginning of CDS on the other strand. Data were visualised using IGV (Broad Institute) and
880 Circa software (OMGenomics).

881

882 *Flow cytometry*

883 Flow cytometry of fixed and live cells treated with propidium iodide for cell cycle and
884 live/dead analysis was conducted as previously reported⁴⁶. For determination of total RNA
885 levels by SYTO RNASelect staining, 1 ml of culture was treated with 500 nM SYTO RNASelect
886 for 20 mins at 25 °C. Cells were collected by centrifugation 1200 x g for 10 mins and washed
887 with PBS before resuspension in PBS 10 mM EDTA pH 7.4. Cells were analysed using a
888 Beckman Coulter Cyan ADP flow cytometer with detection of the stained RNA in the FL1
889 channel.

890

891 *Stranded ERCC Controlled RNA-seq*

892 Cultures of promastigote *Lmx DiCre BDF5::6xHA*^{-/+flx} were treated with DMSO or 300 nM
893 rapamycin for 48 h then passaged to a density of 2 x 10⁵ cells ml⁻¹ for another 48 h. At this
894 point 2 x 10⁷ cells were collected, washed in PBS and processed for total RNA extraction. Total
895 RNA was extracted using Monarch Total RNA Miniprep kit (NEB) as per the manufacturer's
896 instructions with the exception of the addition of ERCC Synthetic RNA Transcripts (Ambion)
897 to the RNA extraction buffer used to lyse the cells. This was added to a final concentration of
898 1/1000 from the manufacturers stock solution. In addition to the on-column Dnase digest, an
899 additional treatment of the eluted RNA was performed with TURBO DNA Free kit as per the

900 manufacturer's protocol. RNA was processed by Novogene using Illumina Ribo Zero method
901 and NEBNext® Ultra™ Directional RNA Library Prep Kit to generate libraries which were
902 sequenced on Illumina NovaSeq 6000 S4 flowcell with PE150. Reads were processed with
903 FASTQC Groomer before mapping with HiSAT. BAM files were converted to bigwig format
904 using bamCoverage (DeepTools) with a scaling factor applied to normalise the total reads to
905 the median ERCC read values. Metaplots were generated using deepTools computematrix
906 and plotProfile tools for dSSR and cSSR in reference-point mode (centre point of the SSR). PTU
907 metaplots were generated using the same tools in scale-regions mode.

908

909 *Splicing RT-PCR*

910 Analog-sensitive CRK9 (LmxM.27.1940) mutants were generated using Cas9 to perform
911 precise genome editing to replace the codon encoding methionine at the gatekeeper position
912 (M501) with a glycine or alanine residue (protocol adapted from⁶⁸). Oligos sequences
913 provided in **Table S1**. The validation of the CRK9 mutants was performed by sequencing using
914 OL11605 and a dose response curve, set at 2.5×10^4 cells ml⁻¹ treated with the bulky kinase
915 inhibitors (BKIs: PP1 (1-(1,1-dimethylethyl)-3-(4-methylphenyl)-1H-pyrazolo[3,4-d]pyrimidin-
916 4-amine), 1NM-PP1 (1-(1,1-dimethylethyl)-3-(1-naphthalenylmethyl)-1H-pyrazolo[3,4-
917 d]pyrimidin-4-amine) and 1NA-PP1 (1-(1,1-dimethylethyl)-3-(1-naphthalenyl)-1H-
918 pyrazolo[3,4-d]pyrimidin-4-amine)) in a range concentration varying from 0 to 120 μ M. The
919 viability of treated and untreated control was assessed after 72h, using Alamar blue at
920 0.0025% (w/v). The parental T7/Cas9 cell line was used as control for the analog-sensitive
921 CRK9 lines. The inhibition profile was analysed by nonlinear regression using Prism Version 9
922 (GraphPad).

923

924 Cultures of promastigote *Lmx DiCre BDF5::6xHA^{-/+flx}* were treated with DMSO or 300 nM
925 rapamycin for 48 h then passaged to a density of 2×10^5 cells ml⁻¹ for another 48 h. Positive
926 controls for cis- and trans- splicing defects were provided by treating *L. mexicana* T7/Cas9
927 CRK9^{M501G} with 30 μ M 1NM-PP1 for 3 h (15x the EC90 at 72h) . Total RNA was purified using
928 NEB Monarch Total RNA MiniPrep Kit. cDNA was synthesised using NEB ProtoScript II with
929 random hexamers. Triple-primer PCR was conducted to determine the trans-splicing of the SL
930 RNA to LmxM.25.0910 with OL12370, OL12371 and OL12372. cis-splicing of the intron in
931 polyA-polymerase (LmxM.08_29.2600) was detected using OL OL12342 and OL12343. PCRs
932 were performed with PCR Bio Ultra Red Mix.

933

934 *Statistics*

935 For routine statistical analyses data were analysed with Prism Version 9 (GraphPad). Western
936 blot quantitation was performed using BioRad ImageLab software using the Stain-Free Total
937 Protein Channel as the normalisation channel.

938

939

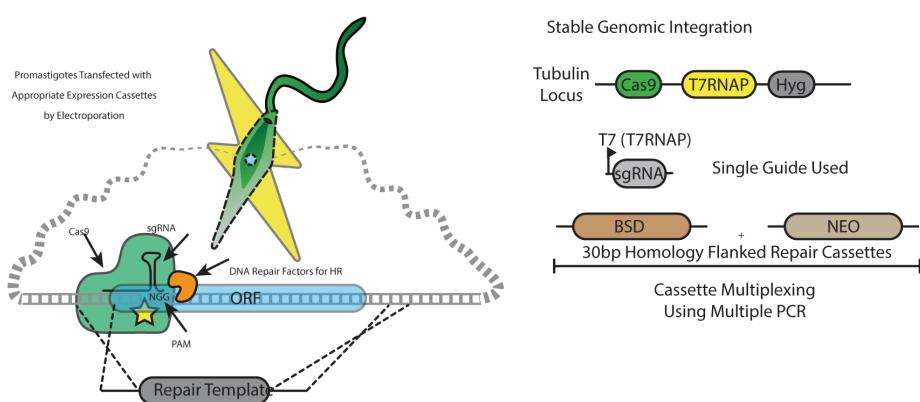
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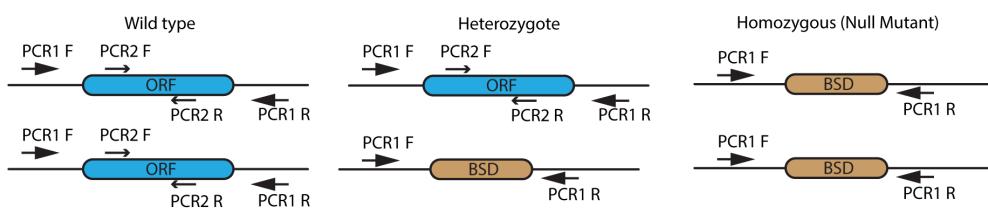
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943 **Supplemental Figures**

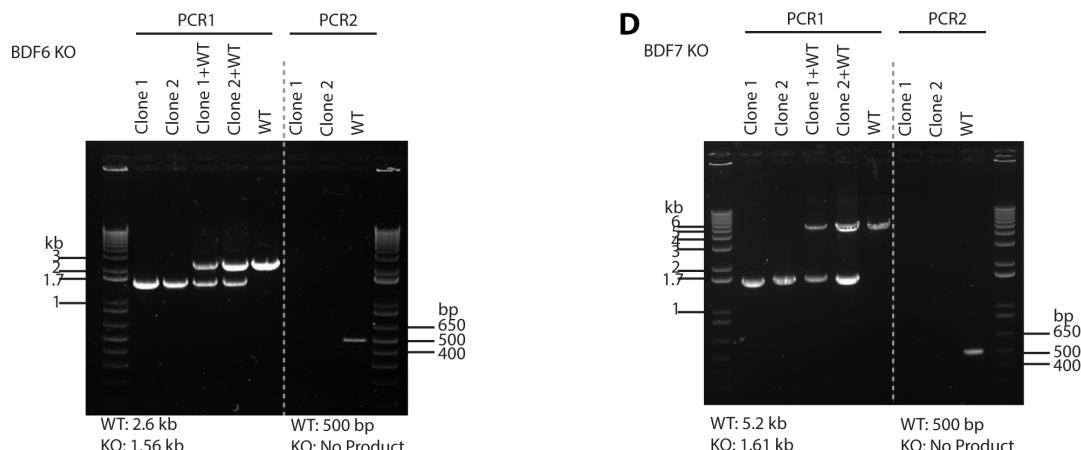
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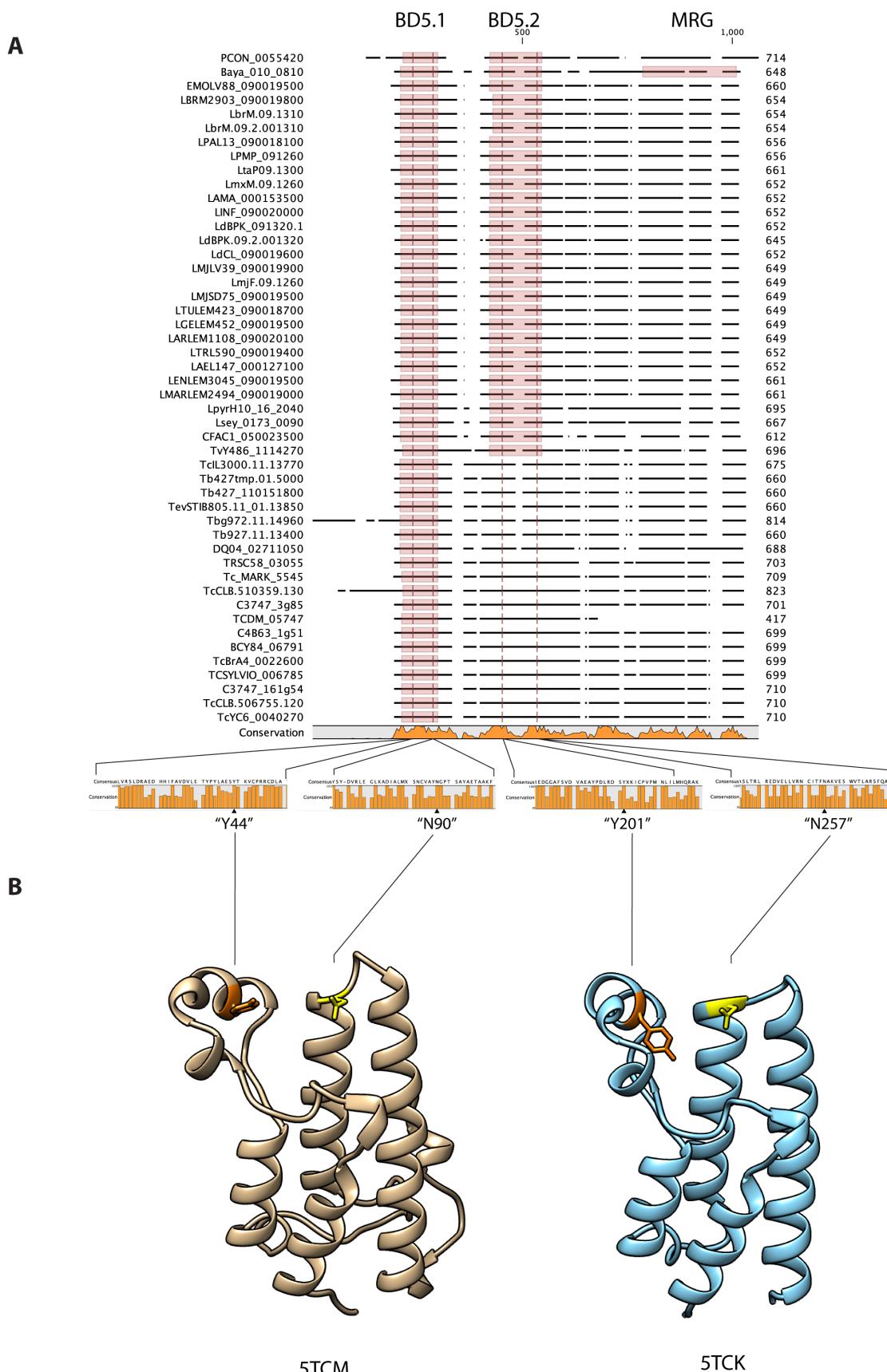
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944

945

946 **Figure S1: CRISPR/Cas9 screening to identify non-essential bromodomain factors.** A. Cartoon depicting the experimental strategy. B. Cartoon depicting the PCR strategy to define gene knockouts isolated from CRISPR/Cas9 screening. C. Agarose gel PCR validation of *BDF6* null mutants. D. Agarose gel showing PCR validation of *BDF7* null mutants.

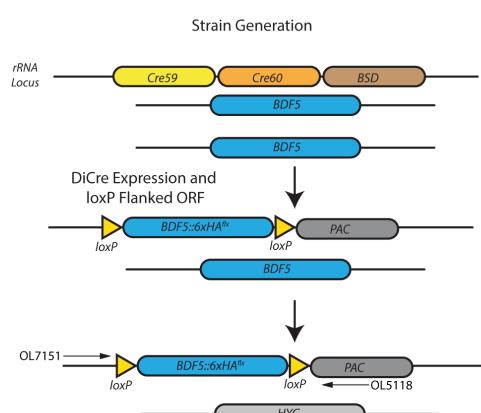


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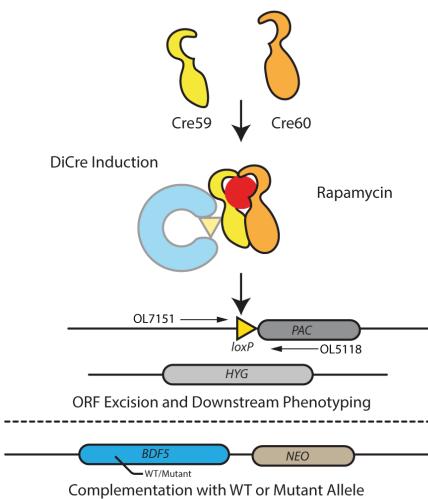
Figure S2: CLUSTAL alignment of kinetoplastid BDF5 proteins. A. Amino acid sequences of BDF5 syntetic orthologues were aligned using the Clustal Omega plugin for CLC. Domains that were

953 readily identifiable using the PFAM search plugin are annotated by shaded boxes, conserved
 954 tyrosine and asparagine residues are annotated by red lines within the shaded BD5.1 and
 955 BD5.2 domains. **B.** X-ray crystal structures of LdBDF5 bromodomains generated by the SGC and
 956 deposited at the PDB, conserved tyrosine residues coloured orange and conserved asparagine
 957 residues in yellow.

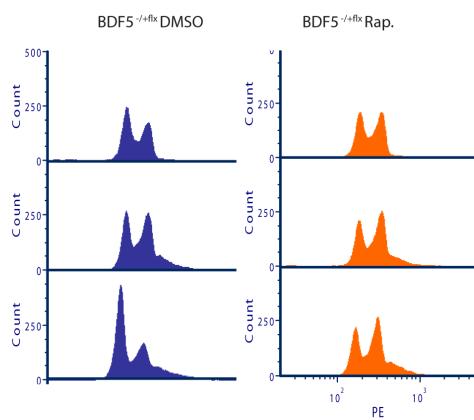
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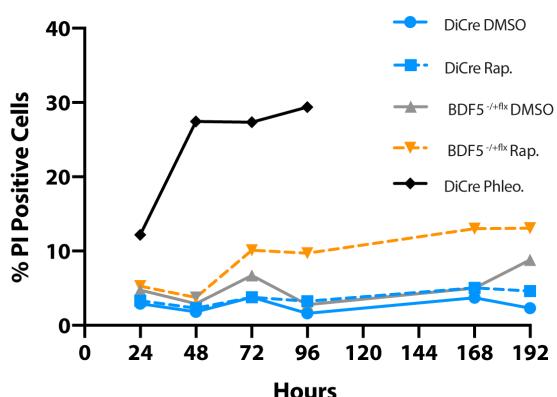
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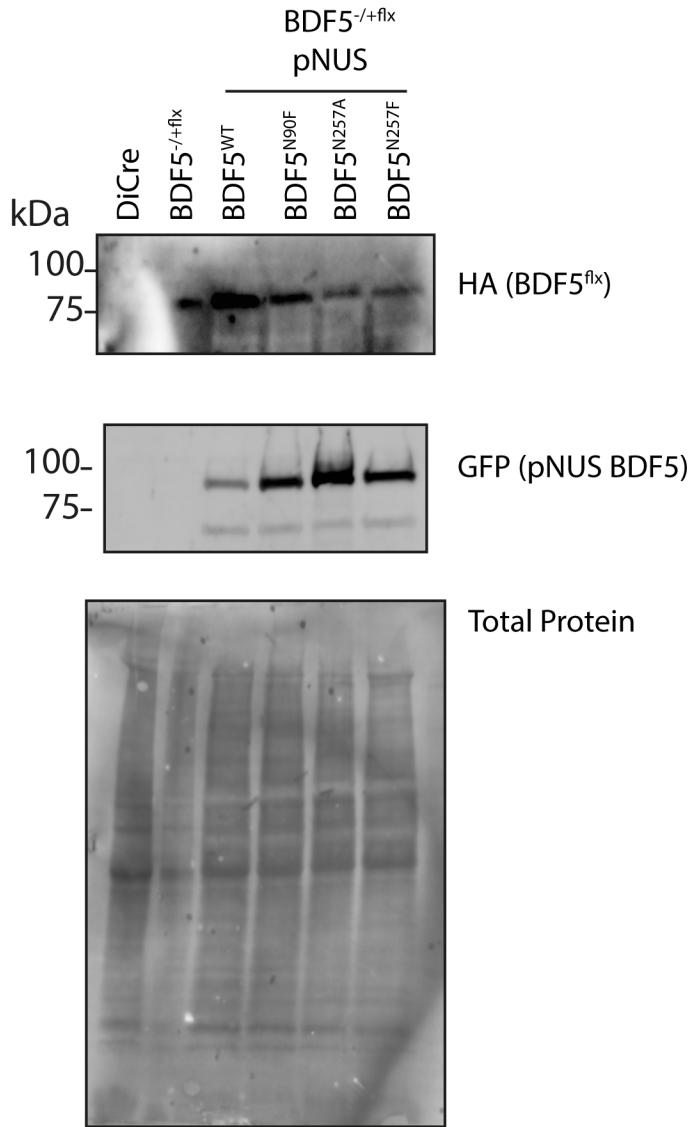
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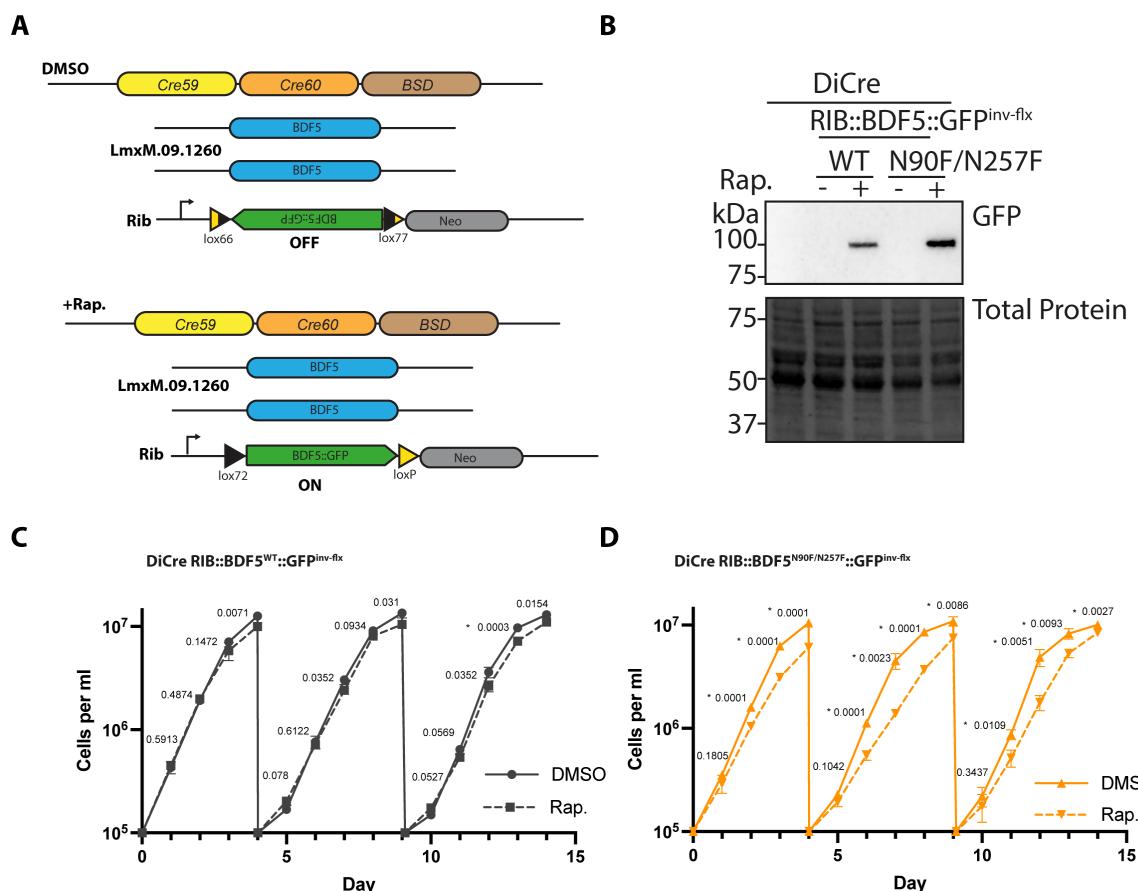
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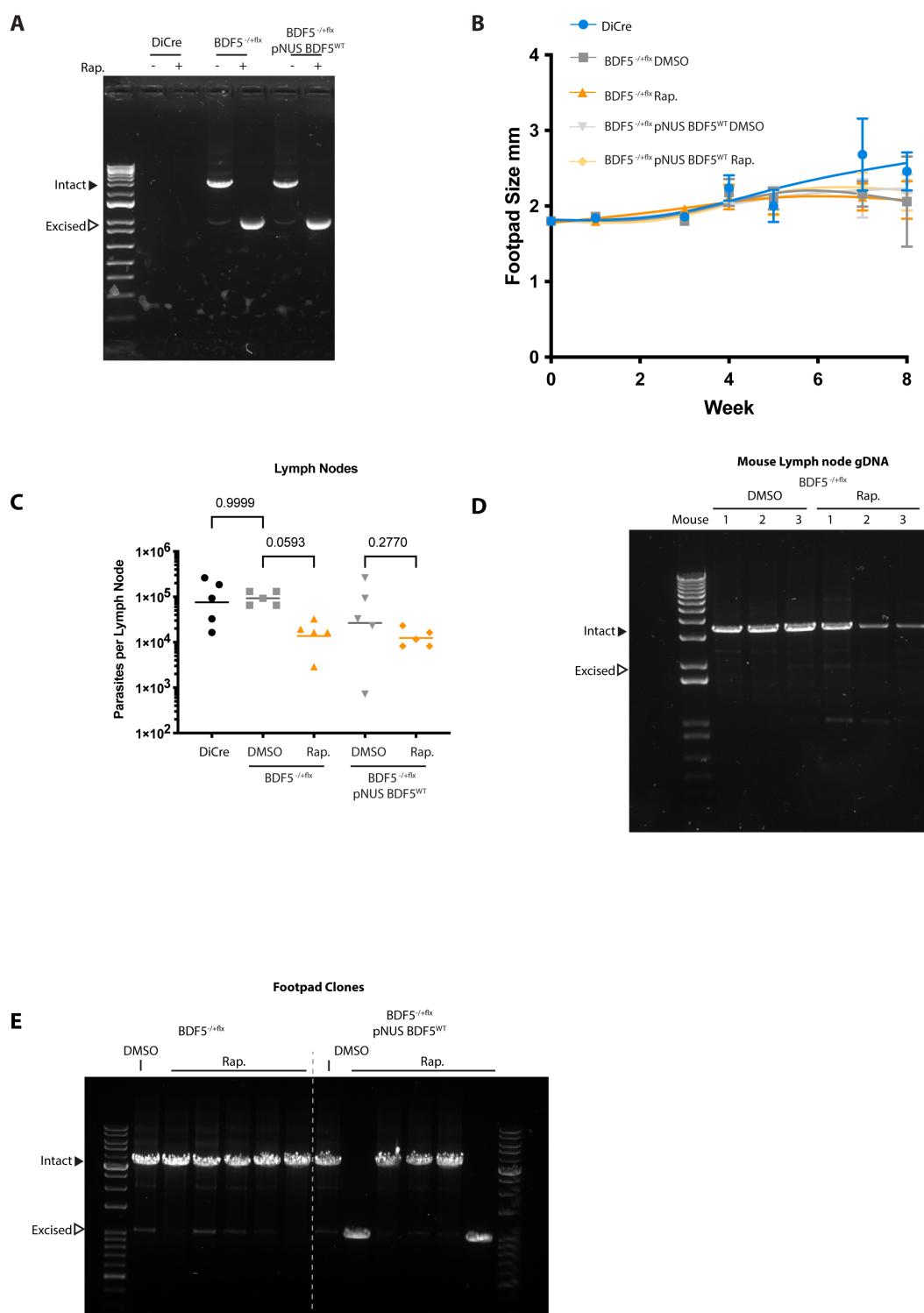
958
 959 **Figure S3: Characterisation of BD5 using DiCre inducible gene deletion in promastigotes.**
 960 **A.** Cartoon representation of workflow to generate *Lmx*
 961 *::DiCreΔbdf5::HYG/Δbdf5::BDF5::6xHA*^{flx}. **B.** Cartoon representation of floxed allele excision
 962 using rapamycin to dimerise the split Cre recombinase, exemplifying the ability to introduce
 963 add-back alleles for functional genetics. **C.** Flow cytometry of methanol fixed, RNase A treated,
 964 propidium iodide stained promastigote cultures to characterise the effects of BDF5 knockout
 965 on the cell cycle over a 72 h timecourse N=20,000 events. **D.** Live/dead analysis using flow
 966 cytometry of non-fixed, propidium iodide treated promastigote cultures following BDF5
 967 knockout. A 1 µg/ml phleomycin control was included. Points and error bars indicate mean ±
 968 standard deviation, N=20,000 events.



969
970 **Figure S4: Episomal Expression of BDF5 mutant alleles.** Western blot analysis of cell lines
971 expressing BDF5::GFP mutant alleles from pNUS episome vectors in the *Lmx*
972 ::*DiCreΔbdf5::HYG/Δbdf5::BDF5::6xHA^{flx}* background.
973



974
975 **Figure S5: Rapamycin inducible overexpression of BDF5 alleles.** A. Cartoon representing the
976 experimental set-up for expression of an extra BDF5 allele. *BDF5*^{N90F/N257F}::GFP was cloned into
977 pRIB in an inverted orientation and flanked by directional loxP sites⁵⁰ yielding
978 *pRIB::BDF5*^{N90F/N257F}::GFP^{inv}. After integrating this into *Lmx*::*DiCre*, clones were isolated and
979 treated with 300 nM rapamycin to induce expression of the *BDF5*^{N90F/N257F}::GFP mutant
980 protein. A control strain was also generated to express a wild-type *BDF5* allele in the same
981 manner B. Western blot analysis of cultures from C and D at the 48 h timepoint of the second
982 growth cycle. C. Growth curve of promastigote cultures of *DiCre*::*BDF5*::GFP^{inv-flx} treated with
983 rapamycin or DMSO. Data points represent mean values \pm standard deviation. N=3 D. Growth
984 curve of promastigote cultures of *DiCre*::*BDF5*^{N90F/N257F}::GFP^{inv-flx} treated with rapamycin or
985 DMSO. Data points represent mean values \pm standard deviation N=3. For C and D repeated t-
986 tests were performed using Prism (GraphPad), p-values are annotated and those marked *
987 were defined as significant.
988

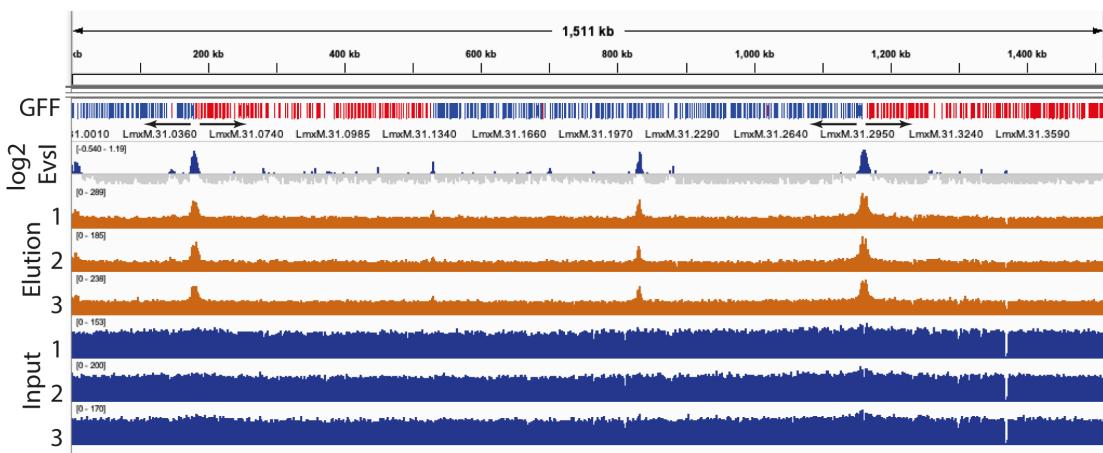


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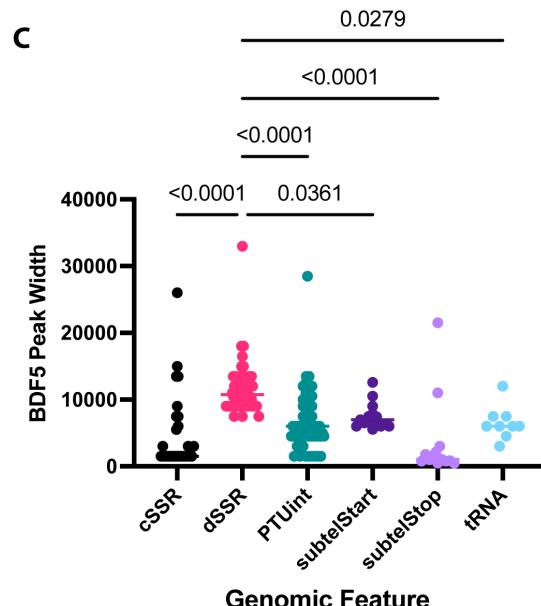
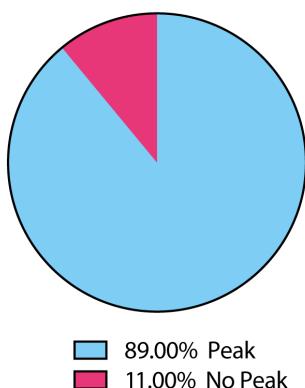
990 **Figure S6: Characterisation of BDF5 using DiCre inducible gene deletion in murine infection. A.**
991 PCR and agarose gel analysis of stationary phase cultures used to infect mice. **B.**
992 Measurements of footpad lesion size of mice infected with indicated parasite strains. Points
993 and error bars indicate mean \pm standard deviation, N=5. **C.** Parasite burdens from infected
994 mouse popliteal lymph nodes determined by limiting dilution, individual points for each mouse
995 with median values indicated by line. Comparisons of Kruskal-Wallis test with Dunn's
996 correction indicate by lines, associated p-values written above, n=5. **D.** Agarose gel
997 exemplifying PCR analysis of genomic DNA extracted from popliteal lymph nodes of mice

998 infected with the BDF5::6xHA^{-/+flx} cultures that were treated with rapamycin or not, indicating
999 retention of the BDF5 allele at 8-weeks post-infection. **E.** PCR and agarose gel analysis
1000 exemplifying clones surviving as promastigotes following clonogenic assay to detect *BDF5*^{-/+flx}
1001 allele.

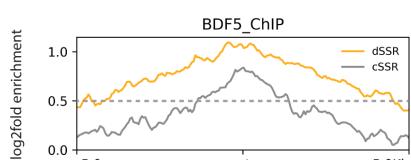
A *Lmx Chr31*



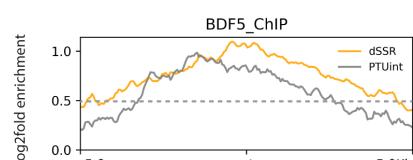
B **Proportion of SSRs with Called Peak**



D

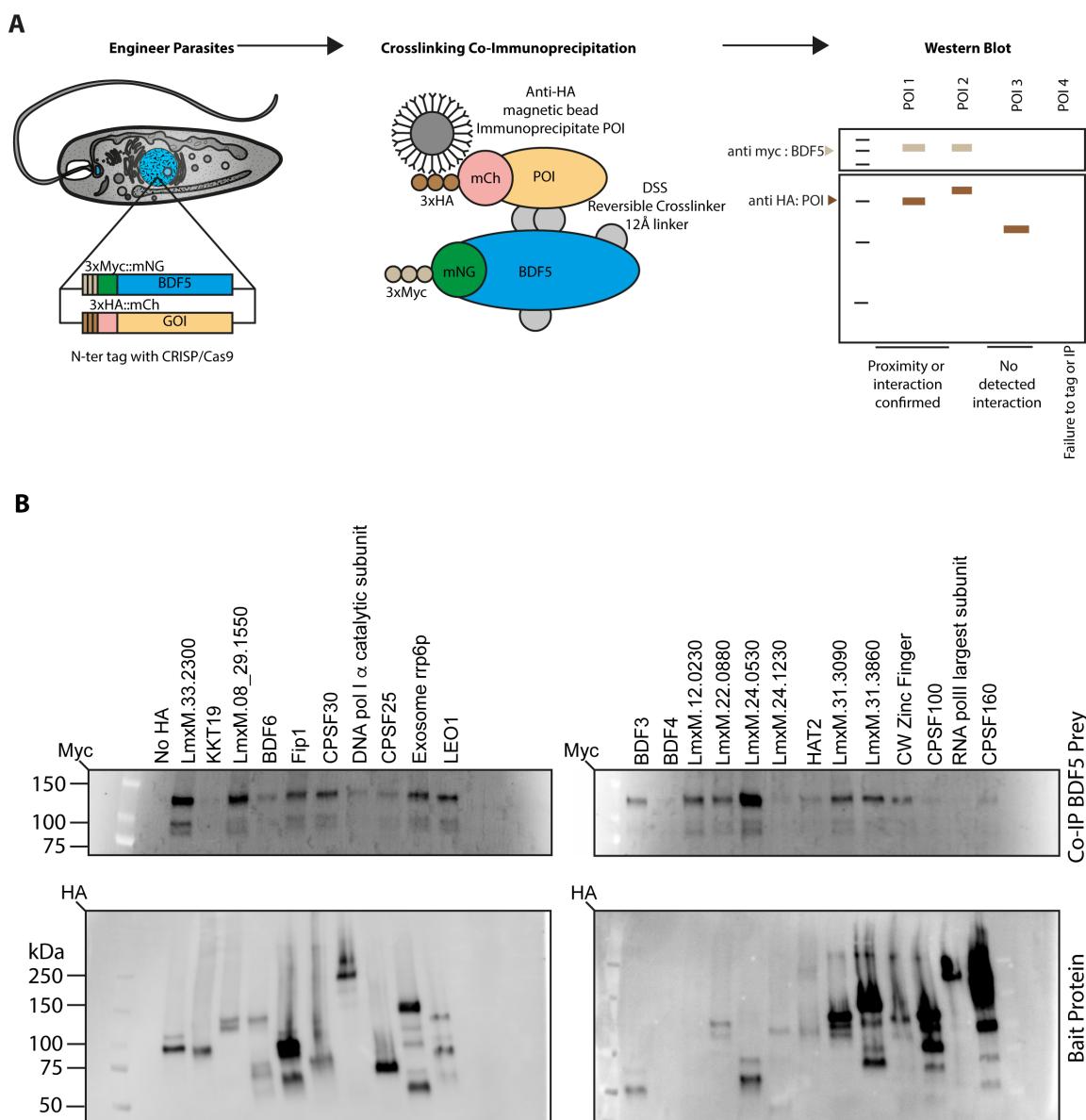


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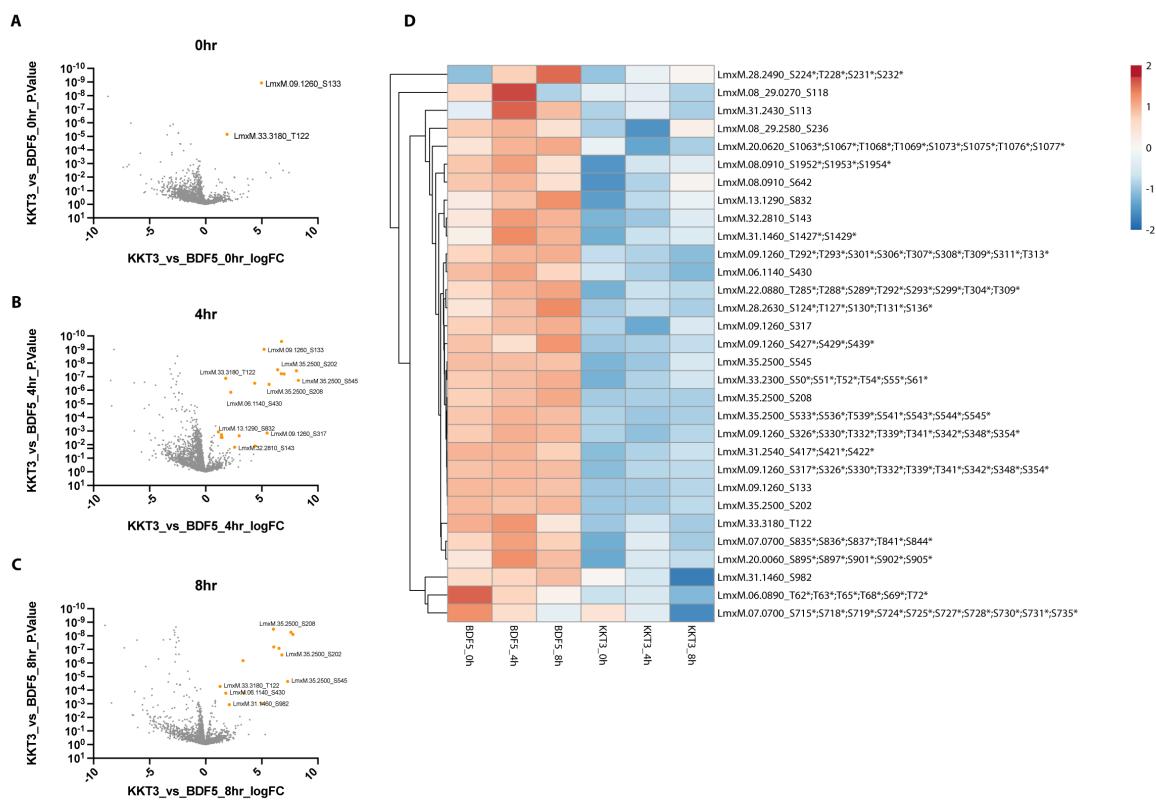


1002
1003 **Figure S7: ChIP-seq analysis of BDF5 distribution on chromatin. A.** Example of IGV genome
1004 browser view of chromosome 31 indicating the genes in polycistronic transcription units
1005 (colour and arrow coded by direction), the read depth for input and eluted sample of the
1006 ChIP-seq (N=3) and the enrichment of BDF5 on a log2 fold scale, GFF (gene feature file)
1007 indicates gene CDS coloured by strand (red+, blue-). **B.** Pie chart indicating the proportion of
1008 SSRs with a BDF5 enriched peak. **C.** BDF5 peak size at different genomic regions as defined by
1009 MACS2 algorithm to call the enriched peaks. cSSR (convergent strand switch region), dSSR
1010 (divergent strand switch region), PTInt (internal PTU peak), subtelStart (subtelomeric peak)

1011 consistent with PTU transcriptional start), subtelStop (subtelomeric peak consistent with PTU
1012 transcriptional stop), tRNA (tRNA gene located away from any of the other features). Values
1013 above denote p-value from Kruskal-Wallace test to compare samples. **D.** Metaplot of average
1014 BDF5 fold enrichment at dSSR (n=60) and cSSR (n=40) regions. **E.** Metaplot of BDF5 average
1015 BDF5 fold enrichment at dSSR (n=60) and PTU internal peaks (n=56).
1016

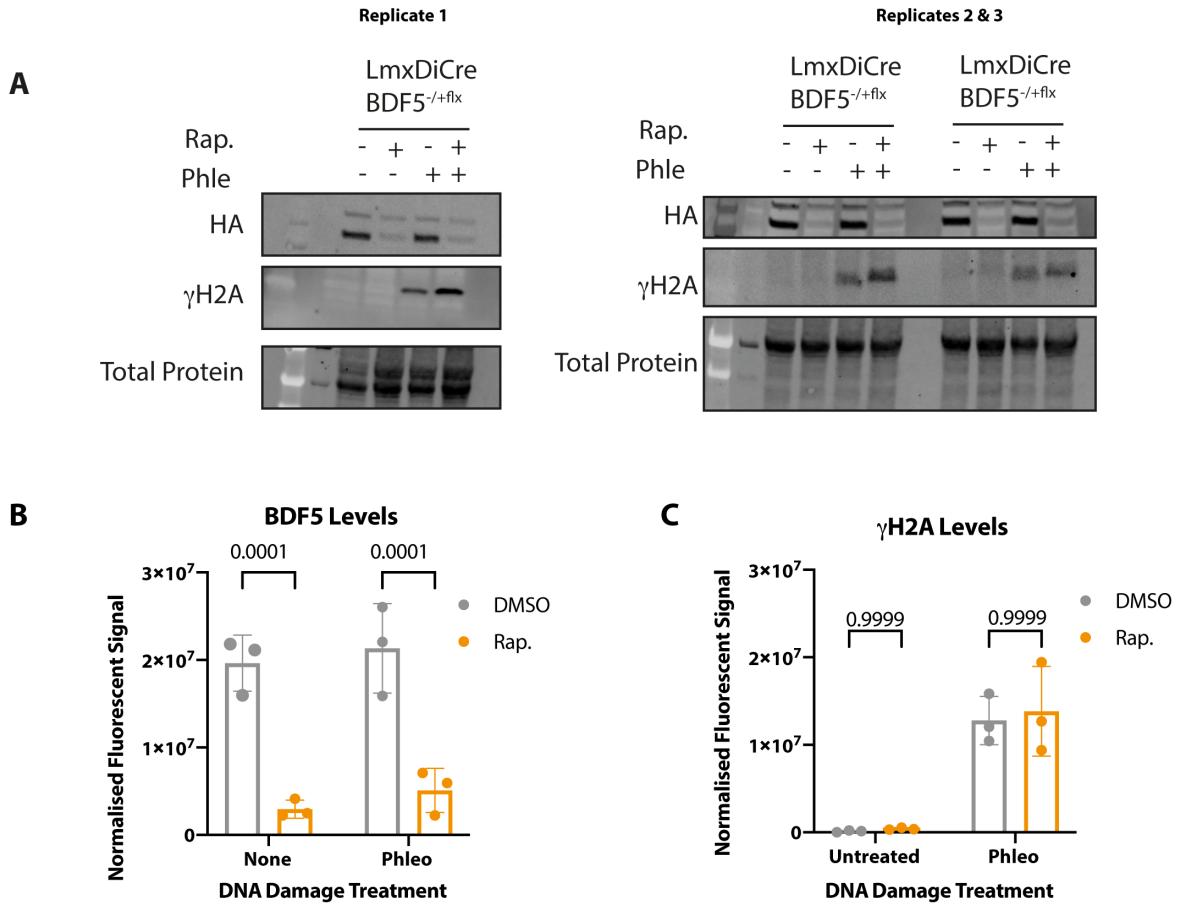


1017
1018 **Figure S8: Co-immunoprecipitation analysis of BDF5-proximal proteins.** **A.** cartoon of
1019 experimental workflow. BDF5-proximal proteins identified by XL-BioID proteins were HA-
1020 tagged in the *LmxT7/Cas9 3xmyc::mNG::BDF5* strain to generate a panel of cell lines containing
1021 both a HA-tagged protein of interest (POI) and myc-tagged BDDF5. **B.** The HA-tagged proteins
1022 were used as bait in an anti-HA immunoprecipitation and co-precipitating (co-IP) myc-tagged
1023 BDF5 protein was is detected using western blot with anti-myc (Upper Panel). Confirmation of
1024 precipitation of the bait protein was confirmed by western blot (lower panel). The presence of
1025 BDF5 co-precipitating with a bait POI confirms the XL-BioID observations as being robust.
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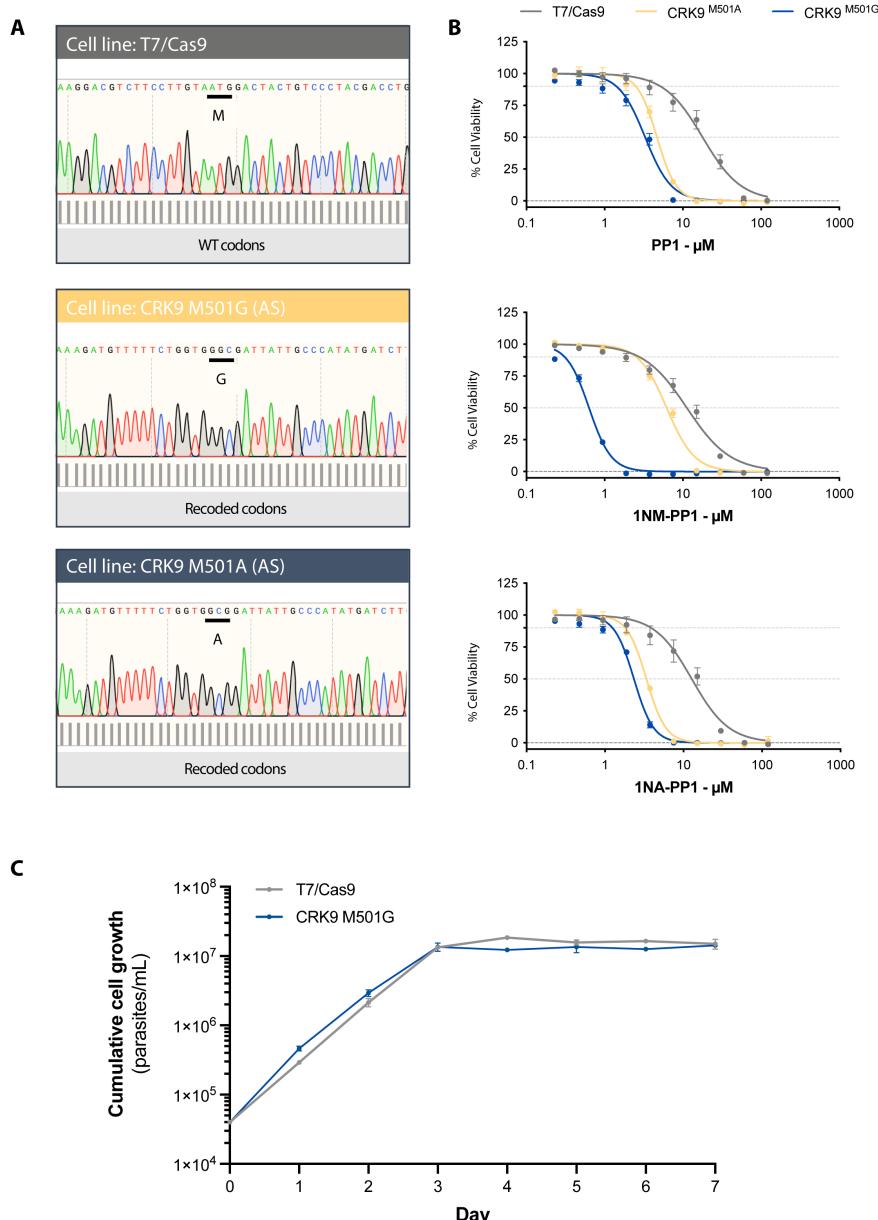


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1029 **Figure S9: Proximity phosphoproteomic analysis of BDF5 across the cell cycle. A, B and C.**
1030 Volcano plots of phosphopeptide enrichment and confidence over 0 h, 4 h and 8 h release
1031 from hydroxyurea synchronisation. Ambiguous phosphosite localisations are denoted with a
1032 *. D. Heatmap of proximal phosphosites represented by median values of 5 replicates after
1033 log₂+1 transformation and data centring. Samples depicted are the BDF5 and KKT3 0 h, 4 h
1034 and 8 h timepoints.

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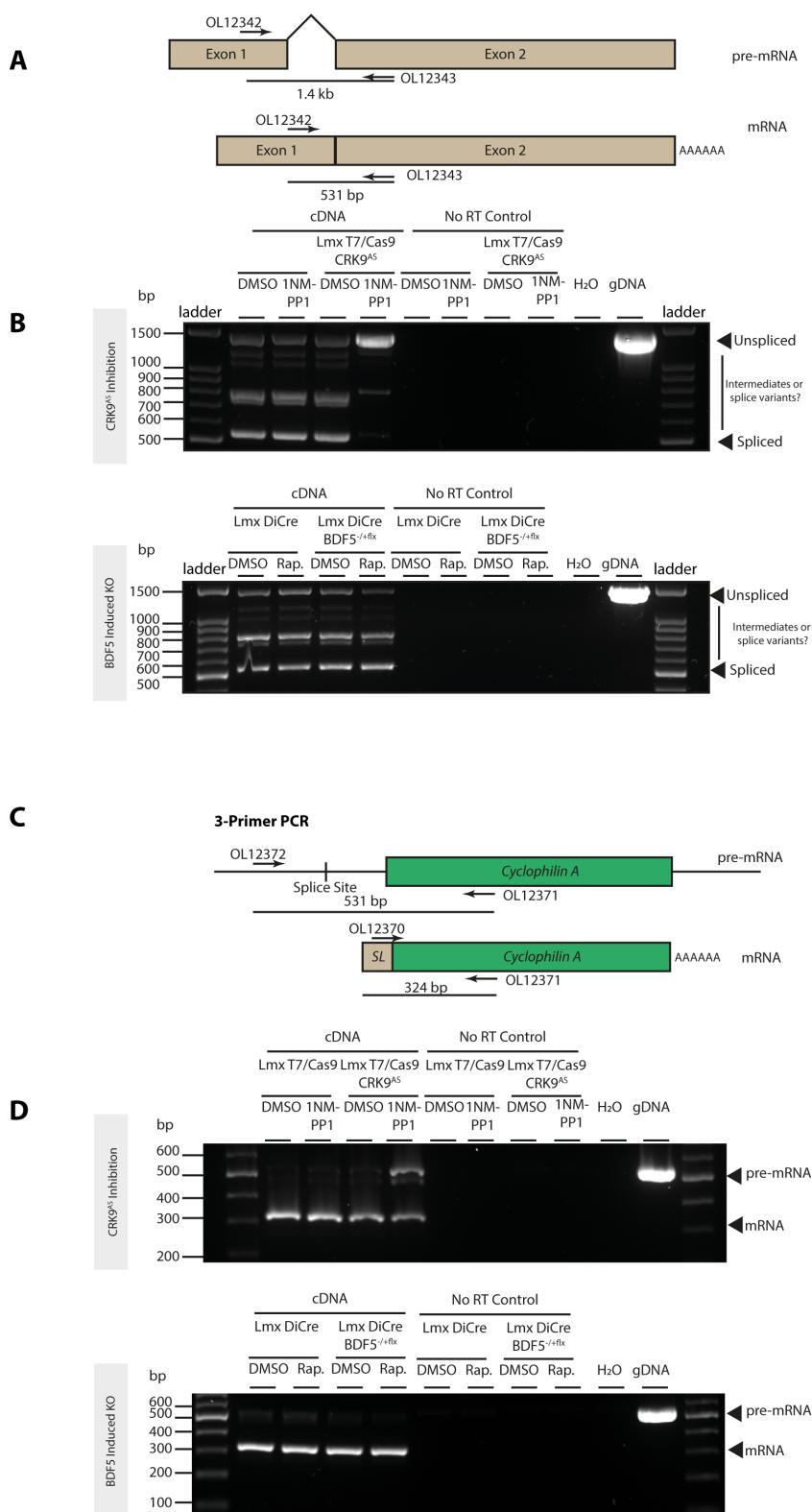


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1038 **Figure S10: DNA damage response in BDF5 depleted cells.** A. Western blot analysis of cultures
1039 treated with DMSO or Rapamycin to delete the floxed allele of *BDF5* in *BDF5*^{-/+flx} for 48 h
1040 followed by passaging to 2×10^5 cells ml^{-1} , another 48 h of rapamycin treatment with the final
1041 24 h including or not 1 $\mu\text{g ml}^{-1}$ of phleomycin (Phle). BDF5::6xHA levels and γ H2A levels were
1042 assessed by Western blot with anti-HA and Anti- γ H2A antibodies respectively. (N=3). B.
1043 Normalised quantification of chemiluminescent signal of BDF5 bands. Data points and bars
1044 indicate mean \pm SD, N=3. C. Normalised quantification of chemiluminescent signal of γ H2A
1045 bands. Data points and bars indicate mean \pm SD, data compared by 2-way ANOVA, p-values
1046 indicated above. N=3.
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Figure S11: Generation of CRK9 analog-sensitised strains. A. Sanger sequencing of *CRK9* in *L. mexicana* T7/Cas9 and precision-edited mutants showing homozygous strains encoding small gatekeeper mutations M501G and M501A. **B.** Dose-response curves of promastigote cell viability after 72 h treatment in varying concentrations of bulky-kinase inhibitors PP1, 1NM-PP1 and 1NA-PP1, measured by alarm blue method, mean \pm SD, n=3. **C.** Growth curve of promastigote cultures of T7.Cas9 and the CRK9^{M501A} strain indicating the small gatekeeper residue does not impact growth of promastigotes, mean \pm SD, n=3.



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1062 inhibition or BDF5 deletion. cDNA prepared using random hexamers was used to prime the
1063 assay, thus capturing the pre-mRNA and mRNA. *L. mexicana* T7/Cas9 was used as the control
1064 strain for the CRK9 analog-sensitised strain. Accumulation of the pre-mRNA is only observed
1065 when CRK9^{AS} is inhibited with 1NM-PP1. No-RT controls were included to exclude gDNA
1066 contamination. H₂O indicates a water control to exclude master mix contamination, and
1067 gDNA was used as a positive control to exemplify the unspliced band size. **C.** Cartoon showing
1068 the strategy of the triple-primer RT-PCR assay to detect trans-splicing of Cyclophilin A mRNA
1069 (LmxM.25.0910) after CRK9 inhibition or BDF5 deletion. **D.** Agarose gel of triple-primer RT-
1070 PCR assay to detect trans-splicing of Cyclophilin A mRNA (LmxM.25.0910) after CRK9
1071 inhibition or BDF5 deletion. cDNA prepared using random hexamers was used to prime the
1072 assay, thus capturing the pre-mRNA and mRNA. No-RT controls were included to exclude
1073 gDNA contamination. H₂O indicates a water control to exclude master mix contamination,
1074 and gDNA was used as a positive control to exemplify the unspliced band size. Accumulation
1075 of the pre-mRNA is only observed when CRK9^{AS} is inhibited with 1NM-PP1.

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