

1 **Genomic and phenotypic characterization of experimentally selected resistant *Leishmania***
2 ***donovani* reveals a role for dynamin-1 like protein in the mechanism of resistance to a**
3 **novel anti-leishmanial compound**

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20

21 **Abstract**

22 The implementation of prospective drug resistance (DR) studies in the R&D pipelines is a
23 common practice for many infectious diseases, but not for Neglected Tropical Diseases.
24 Here, we explored and demonstrated the importance of this approach, using as paradigms
25 *Leishmania donovani*, the etiological agent of Visceral Leishmaniasis (VL), and TCMDC-
26 143345, a promising compound of the GSK 'Leishbox' to treat VL. We experimentally
27 selected resistance to TCMDC-143345 in vitro and characterized resistant parasites at
28 genomic and phenotypic levels. We found that it took more time to develop resistance to
29 TCMDC-143345 than to other drugs in clinical use and that there was no cross resistance to
30 these drugs, suggesting a new and unique mechanism. By whole genome sequencing, we
31 found two mutations in the gene encoding the *L. donovani* dynamin-1-like protein (LdoDLP1)
32 that were fixed at highest drug pressure. Through phylogenetic analysis, we identified
33 LdoDLP1 as a family member of the dynamin-related proteins, a group of proteins that
34 impacts the shapes of biological membranes by mediating fusion and fission events, with a
35 putative role in mitochondrial fission. We found that *L. donovani* lines genetically engineered
36 to harbor the two identified LdoDLP1 mutations were resistant to TCMDC-143345 and
37 displayed altered mitochondrial properties. By homology modeling, we showed how the two
38 LdoDLP1 mutations may influence protein structure and function. Taken together, our data
39 reveal a clear involvement of LdoDLP1 in the adaptation/resistance of *L. donovani* to
40 TCMDC-143345.

41

42

43 **Importance**

44 Humans and their pathogens are continuously locked in a molecular arms race during which
45 the eventual emergence of pathogen drug resistance (DR) seems inevitable. For neglected
46 tropical diseases (NTDs), DR is generally studied retrospectively, once it has already been
47 established in clinical settings. We previously recommended to keep one step ahead in the
48 host-pathogen arms race and implement prospective DR studies in the R&D pipeline, a
49 common practice for many infectious diseases, but not for NTDs. Here, using *Leishmania*
50 *donovani*, the etiological agent of Visceral Leishmaniasis (VL), and TCMDC-143345, a
51 promising compound of the GSK 'Leishbox' to treat VL, as paradigms, we experimentally
52 selected resistance to the compound and proceeded to genomic and phenotypic
53 characterization of DR parasites. The results gathered in the present study suggest a new DR
54 mechanism involving the *L. donovani* dynamin-1 like protein (LdoDLP1) and demonstrate the
55 practical relevance of prospective DR studies.

56

57 **Introduction**

58 The lifespan of any anti-microbial drug is, unfortunately, limited; its clinical use generally
59 represents a new step in the arms race between human creativity and pathogen
60 adaptability. Sooner or later, drug resistance (DR) or another phenotypic adaptation arises
61 (1). Human countermeasures (such as new therapeutic regimens or combination therapy)
62 can be adopted, but the drug will ultimately have to be replaced by a new compound, if any
63 are available. Understanding the process of DR and developing strategies to counter it are
64 particularly critical for neglected tropical diseases (NTDs), for which there are typically only a
65 few drugs in the therapeutic arsenal and the R&D pipeline (2). It is essential to safeguard
66 existing compounds and to develop new ones. At the same time, studies on molecular
67 mechanisms of resistance are classically applied to understand the mode of action of
68 antimicrobial agents, because of the possibility that the resistance determinants are caused
69 by specific genetic variations resulting in the altered target binding to the drug molecule.

70 For NTDs, DR is generally studied retrospectively, once DR has already been established in
71 clinical settings. In a recent opinion paper, we recommended to keep one step ahead in the
72 arms race and implement prospective DR studies in the R&D pipeline, a common practice for
73 many infectious diseases, but not for NTDs. Two specific recommendations have been given
74 so far (2): (i) exploiting resources of parasite bio-banks to test the efficacy of novel
75 compounds on a wider range of parasites, including recent isolates from clinically relevant
76 settings for the prospective use of the compound -a practice shown to be highly relevant (3)-
77 and (ii) experimentally selecting DR to new lead compounds and characterizing it broadly to
78 assess the adaptive skills of the parasite to the compound, guide further drug development
79 and help countering DR if it develops in clinical practice.

80 Here, using *L. donovani* (the etiological agent of Visceral Leishmaniasis, VL, which is fatal if
81 left untreated) and TCMDC-143345, a promising anti-VL compound of the GSK 'Leishbox' (4)
82 as paradigms, we experimentally selected resistance to the compound and proceeded to a
83 genomic and phenotypic characterization of DR parasites. We found that it took more time
84 to develop resistance to TCMDC-143345 than to other drugs in clinical use and that there
85 was no cross resistance to these drugs, suggesting a new and unique mechanism. Whole
86 genome characterization of independent TCMDC-143345-resistant lines highlighted two
87 mutations in the gene encoding dynamin-1-like protein (LdoDLP1) that were fixed at highest
88 drug pressure. Through phylogenetic analysis, we identified LdoDLP1 as a family member of
89 the dynamin-related proteins (DRPs), a group of proteins that impacts the shapes of
90 biological membranes by mediating fusion and fission events, with a putative role in
91 mitochondrial fission. We genetically engineered our *L. donovani* strain to harbor the two
92 identified LdoDLP1 mutations: parasites were resistant to TCMDC-143345 and displayed
93 altered mitochondrial properties. The results are further supported by homology modeling
94 which provides insights as to how the two LdoDLP1 mutations may influence protein
95 structure and function. Taken together, the data presented in this paper reveal a clear
96 involvement of LdoDLP1 in the adaptation/resistance of *L. donovani* to TCMDC-143345. Our
97 results also demonstrate the practical relevance of prospective drug resistance studies to
98 guide R&D pipeline and future clinical applications of that compound.

99

100 **Materials and Methods**

101

102 ***Parasites***

103 We used the *L. donovani* strain MHOM/NP/03/BPK282/0 clone 4 (further called
104 LdBPK_282 cl4, see growth conditions in suppl. text), originally derived from a Nepalese
105 patient with confirmed VL and cryo-preserved at the Institute of Tropical Medicine, in
106 Antwerp, Belgium. The strain is considered sensitive to antimonials [Sb^{III}], Miltefosine
107 [MIL] and Amphotericin B [AmphoB] and it was used for determining the reference
108 genome of *L. donovani* (5). The strain kept most of its intrinsic phenotypic features, like
109 virulence (5), transmissibility to sand flies (6) and natural drug susceptibility (7), thus
110 constituting a good model for 'real-life' parasites that will be exposed to new drugs in
111 natural conditions.

112

113 ***Selection of resistance and stability of resistance***

114 For the selection of resistance to TCMDC-143345, promastigotes were initially grown in
115 quadruplicates (lines A, B, C, D) and later on because loss of lines A and B, line D was divided
116 in 4, constituting a total of 5 lines (C, D1-D4). MIL was used as positive control for the
117 experimental set-up of drug resistance selection and two duplicates were used (A and B). As
118 negative controls, two additional lines were used: (i) the Wild-Type [WT] line LdBPK_282 cl4
119 maintained during the same passage numbers as the resistant lines but without the drug
120 pressure and (ii) a WT line maintained with DMSO (which was used as solvent for TCMDC-
121 143345). The resistant lines were maintained in the continuous presence of drugs, as
122 described elsewhere (7). Increasing concentrations of drugs were added in a step-wise

123 manner until all lines grew at similar rates as wild-type parasites: (i) for MIL: 0, 2, 10, 15, 60,
124 100 μM , (ii) for TCMDC-143345, line C: 0, 0.2, 1, 2, 4, 5, 6, 8, 12 and 12 μM and for line D: 0,
125 0.2, 1, 2, 4, 5, 6, 10, 18 and 25 μM . Each selection round was approximately 5 weeks (2
126 passages per week) with the IC_{50} measured after each round. The selection flowchart is
127 summarized in figure S1. To test the stability of the TCMDC-143345-resistant phenotype, the
128 resistant line D1 was maintained for 20 weeks without drug pressure, after which the IC_{50}
129 was measured.

130 ***Promastigotes susceptibility tests***

131 Susceptibility tests were performed after each selection round (drug resistance selection) or
132 after parasite engineering (CRISPR-Cas9 or over-expression, see below). The $\text{IC}_{50\text{s}}$ were
133 determined on logarithmic-stage promastigotes after 72 hours of exposure to TCMDC-143345
134 or MIL with a resazurin assay as previously described (8) and summarized in Supplementary
135 text. For the cross-resistance experiments the same protocol was used. The following
136 maximal concentrations were used for the testing of the compounds: 50 μM for TCMDC-
137 143345 and compound Y, 400 μM for MIL, 2 mM for Sb^{III} and 200 μM for AmphoB. Ten
138 points of 1 in 2 dilutions were used per compound. Four independent experiments were run
139 with technical duplicates per experiment.

140 ***Amastigotes susceptibility tests***

141 Phorbol myristate acetate (30 nM) (PMA, Sigma) was added to THP-1 cells (human
142 monocytic leukemia, ATCC-TIB-202, see maintenance conditions in supp. text) at 37°C for 48
143 hours to differentiate these into adherent macrophages. Cells were washed and incubated
144 with complete RPMI medium containing stationary phase (day 6) *L. donovani* promastigotes

145 at a macrophage/promastigote ratio of 1/30. After 5 h incubation at 37°C, non-internalized
146 promastigotes were removed by 3 successive washes with PBS and infected macrophages
147 were incubated with TCMDC-143345 in RPMI medium supplemented by 5% heat inactivated
148 Horse serum for 96 h. TCMDC-143345 was tested with a starting concentration of 25 µM in a
149 3-fold serial dilution. A 3-fold serial dilution of 3 µM amphotericin B was used as positive
150 control. Experiments were done in triplicate with technical duplicates per experiment. For
151 confocal microscopy, infected cells were washed with PBS, fixed for 30 minutes with 4%
152 formaldehyde, rinsed again with PBS and stained with 4',6'-diamidino-2-phenylindole (DAPI
153 300 nM). Images were acquired with an LSM 700 Zeiss confocal microscope. The number of
154 infected macrophages and the number of amastigotes per infected macrophage were
155 determined by manual counting. These numbers obtained from the average of counted wells
156 were used to establish the infection index (% infected macrophages × amastigotes/infected
157 macrophages). IC_{50s} were calculated with GraphPad Prism using a sigmoidal dose-response
158 model with variable slope.

159

160 ***DNA and library preparation for whole genome sequencing***

161 At each round of the resistance selection (5 weeks culture), parasites were harvested from
162 lines C and D and from the two WT controls (maintained without and with DMSO). The list of
163 samples sequenced is summarized in Table S1. DNA isolation was done using QIAamp DNA
164 blood minikit (Qiagen), and the DNA concentration was assessed with the Qubit DNA broad-
165 range DNA quantification kit (Thermo Fisher). Library preparation and sequencing of the
166 different lines of the stepwise selection were performed at the Wellcome Sanger Institute
167 (Hinxton, United Kingdom). Genomic DNA was sheared into 400–600-base pair fragments by

168 focused ultrasonication (Covaris Adaptive Focused Acoustics technology, AFA Inc., Woburn,
169 USA). Amplification-free indexed Illumina libraries were prepared (9) using the NEBNext
170 Ultra II DNA Library Prep kit (New England BioLabs). The libraries were quantified using the
171 Accuclear Ultra High Sensitivity dsDNA Quantitative kit (Biotium) and then pooled in
172 equimolar amounts. Paired end reads of 150-bp were generated on the Illumina HiSeq X10
173 according to the manufacturer's standard sequencing protocol (10). Data Release: raw data
174 was deposited in the European Nucleotide Archive (ENA) with the accession number
175 ERS441806-ERS441816.

176

177 ***Whole genome sequencing data analysis***

178 Somy, single nucleotide polymorphisms (SNPs), local copy number variations (CNVs) and
179 Indels were determined as described elsewhere (5),(11) using the BPK282v2 PacBio
180 reference genome (5); more details can be found in supplementary text. SNPs and small
181 indels were considered significantly different between parasite lines when the allele
182 frequency showed difference of at least 0.25 and Mann–Whitney U test p-value <0.05 (12).
183 Allele frequency shifts larger than 0.80 were considered homozygous variants. We used one
184 criterion to evaluate whether a gene or chromosome copy number difference was
185 biologically meaningful and statistically significant: the absolute difference in
186 gene/chromosome copy number should be at least 0.5 to be significant. Gene ontology
187 analyses were done as explained in supplementary text. Heat maps were created using the
188 heatmap3 package in R (R Development Core Team 2015). Reasoning that mutations away
189 from the sensitive parental strain at causative loci were likely to contribute to resistance but
190 that multiple variants in the same loci could contribute to the resistance phenotype, we

191 adopted a simple approach to identify significant loci informed by burden tests used in rare-
192 variant association studies (13). We summed the non-reference allele frequency of variants
193 at each locus for each sequence sample and tested for association by regression of these
194 total allele frequencies per locus against the measured IC_{50} for that sample.

195

196 ***CRISPR-Cas9 mediated engineering of Leishmania***

197 LdBPK_282 cl4 parasites were transfected with the linearized pTB007 vector, obtained from
198 Dr. Eva Gluenz (University of Oxford, UK) (14). Transgenic parasites were selected with
199 25 μ g/mL Hygromycin starting 24h after transfection and a clone was isolated using a micro-
200 drop method (15). In order to introduce the Ala324Thr or the Glu655Asp mutation using the
201 CRISPR-Cas9 system, the single guide RNAs (sgRNAs) DynMut1-gRNA and DynMut2-gRNA
202 were designed targeting the sequences CAGCAGCTGTGCAGTGGGCT and
203 GGCACTGCTCTCCGAGGCCCCC respectively (sites of mutation are underlined). The sgRNA
204 templates for in vivo transcription were generated by PCR as previously described (14). The
205 sequence of all primers used in this work are provided in Table S2. For each mutation, a
206 double-stranded donor DNA bearing the missense mutation was generated by annealing of
207 synthetic oligos. The DNA repair templates also included synonymous nucleotide
208 substitutions to distinguish the CRISPR-Cas9-mediated mutations from potential naturally
209 occurring mutations. The Ala324Thr and the Glu655Asp mutations were independently
210 recreated by transfecting the respective sgRNA template and donor dsDNA using the Basic
211 Parasite NucleofectorTM Kit 1 (Lonza) with the U-033 program following manufacturer
212 recommendations. Control transfections were made by transfecting either each donor
213 dsDNA without their respective sgRNA templates or by replacing each donor dsDNA by the

214 DynWT1 or DynWT2 dsDNAs, which lack the missense mutation. After 24h post transfection,
215 10^6 parasites of each transfection were transferred to a 24-wells plate in a final volume of
216 1mL/well of HOMEM medium with 20% Fetal Bovine Serum and $9\mu\text{M}$ of TCMDC-143345 or
217 0,1% DMSO (control). Plates were incubated at 26°C for 12 days and cell viability was
218 determined by flow cytometry using the NucRedTM Dead 647 ReadyProbesTM and the
219 VybrantTM DyeCycleTM Green dyes (ThermoFisher). Parasites that survived and grew in the
220 presence of TCMDC-143345 were transferred to culture flasks and kept under pressure with
221 $6\mu\text{M}$ of TCMDC-143345 for 2 passages, when clones were isolated with a microdrop method
222 (15) and grown in absence of drug pressure.

223

224 ***Over-expression of WT LdoDLP1 gene in Lines C and D3***

225 The wild-type LdoDLP1 gene was PCR-amplified from the gDNA of LdBPK_282 cl4 with the
226 primers InF-LdDNM1-F and InF-LdDNM1-R and cloned in the *NotI* and *NcoI* sites of the
227 pLEXSy-Hyg2.1 expression vector (Jena Bioscience). The plasmid was linearized with the *SwaI*
228 enzyme and transfected in parasites of fully resistant Lines C and D3, as well as the standard
229 LdBPK_282 cl4 using the Basic Parasite NucleofectorTM Kit 1 (Lonza) with the U-033 program.
230 The empty, linearized pLEXSy-Hyg2.1 vector was also transfected in each line as control.
231 Parasites were selected and maintained with $50\mu\text{g}/\text{mL}$ Hygromycin after 24h post-
232 transfection.

233

234 ***Phylogenetic analysis***

235 The amino acid sequences of several DRPs (see details in supplementary text) were aligned
236 using MAFFT (16) to generate a sequence alignment from which a rooted phylogenetic tree

237 was constructed through the maximum-likelihood method using PHYML (17). *Escherichia coli*
238 CrfC, which shares features with the DRP family members, was employed as an outgroup to
239 root the phylogenetic tree. The reliability of the tree was verified by performing 1000
240 bootstrap replicates.

241

242 ***Mitochondrial membrane potential and cell viability***

243 [i] Selected resistant lines C and D3, together with DMSO control with same number of
244 passages as well as [ii] CRISPR-engineered mutants, together with the WT Line and the WT
245 transfected with the CRISPR-Cas9 and called pT007 (both used as controls) were cultivated
246 at a density of 1×10^6 without or with 12.5 μM of TCMDC-143345. On days 2, 4 and 7 the
247 mitochondrial membrane potential (MtMP) and cell viability were co-evaluated with the
248 Mitotracker DeepRed and NucGreen respectively (Thermo Fisher Scientific). Briefly, 1
249 volume of parasites was incubated with 2 volumes of a medium containing 0.1 μM of cell
250 tracker Deep Red and 1 drop/mL of NucGreen. The samples were incubated for 15 min at 26
251 $^{\circ}\text{C}$ and subsequently re-pelleted by centrifugation at 1500 g per 5 min. The cells were
252 resuspended in new medium and analysed by flow cytometry (BD FACS Verse) in the
253 medium flow rate mode. An unstained sample was included in each experiment as negative
254 control for the establishment of the autofluorescence and the gates for the selection of the
255 population positive and negative for both fluorochromes.

256

257 ***Homology modeling***

258 A homology model for the LdoDLP1 dimer was generated using MODELLER (18) with the
259 following crystal structures as templates: *Homo sapiens* DLP1 (Uniprot ID O00429, PDB ID
260 4BEJ) (19), *Rattus norvegicus* DNM1 (Uniprot ID P21575, PDB ID 3ZVR)(20), *H. sapiens* DNM3
261 (Uniprot ID Q9UQ16, PDB ID 5A3F) (21) and *H. sapiens* DNM1 (Uniprot ID Q05193, PDB ID
262 3SNH) (22). The homology model has zDOPE and GA341 scores of -0.255 and 1.000,
263 respectively, thereby indicating its reliability. Molecular graphics visualization and analysis
264 were performed with UCSF ChimeraX (23).

265

266 **Results**

267 ***Experimental resistance of promastigotes to TCMDC-143345 takes 50 weeks to establish, is***
268 ***stable, and is maintained in amastigotes.*** To assess the ability of parasites to adapt to
269 TCMDC-143345 we set up an experimental resistance experiment in quadruplicates for
270 TCMDC-143345 (Line A, B, C, and D), starting with 0.2 μM and for comparison we used
271 Miltefosine (MIL) pressure in duplicates (lines A and B) starting with 2 μM . For TCMDC-
272 143345, two lines (A and B) were lost during the selection process after round 4. The
273 selection was continued from round 5 with line C and lines D1-D4, generated by splitting the
274 original line D at this round (Fig. S1, Table S1).

275 The first parameter to be evaluated was the time to resistance (7), which was defined as the
276 time needed for each line to display a wild-type (WT) growth curve in the presence of the
277 highest selection pressure following the stepwise selection process. The selection dynamics
278 for various lines are shown in Figure 1A. It took 10 selection rounds (time to resistance of
279 approximately 50 weeks) for line D1 to reach the highest TCMDC-143345 IC_{50} (55 μM). In
280 comparison, the IC_{50} of TCMDC-143345 for the WT line maintained without drug pressure for
281 the same time was 2-3 μM . The selection dynamics for line C was rather different from line
282 D1 and the resistance selection was not complete even after 10 rounds, thereby implying a
283 longer time to resistance. At round 5 of the selection, line C was not adapting well, which is
284 why the pressure was decreased. The highest IC_{50} achieved was 15 μM at round 9. For the
285 remaining D replicates, line D3 had a similar resistance profile as line D1 while for lines D2
286 and D4 the selection had to be stopped one round earlier (IC_{50} ranging between 16 and 25
287 μM , Fig. S1) due to the limited availability of TCMDC-143345.

288 To place these results into context, the time to resistance for MIL was assessed in parallel,
289 and was about 30 weeks (6 selection rounds), with a shift of the IC_{50} from 13 μ M to 100 μ M.
290 We added for comparison, data from our previously published study with the same
291 LdBPK_282 cl4 strain, in which we showed that the time to resistance to trivalent
292 antimonials was 20 to 5 weeks (depending on the selection protocol) (7). Fig 1B shows
293 clearly that the time to resistance was the longest for TCMDC-143345. The second evaluated
294 parameter was the stability of the resistance phenotype. Both the WT line and Line D1 were
295 maintained for 20 weeks without drug pressure and then challenged again with TCMDC-
296 143345. From Fig. S2A, it can be observed that the withdrawal of the drug pressure for a
297 prolonged period of time did not alter the susceptibility of line D1 to TCMDC-143345 (IC_{50}
298 $>25 \mu$ M). Thirdly, the susceptibility of intracellular amastigotes was assessed. TCMDC-
299 143345 pressure was applied on THP-1 macrophages infected with line D1. Intracellular
300 amastigotes of line D1 showed an IC_{50} of 30 μ M versus 2 μ M for the WT, confirming that the
301 resistance selected at the promastigote stage was maintained at the amastigote stage (Fig.
302 S2B). Fourthly, promastigotes of the resistant line D1, WT and D1-no drug (D1 line
303 maintained for 20 weeks without drug pressure) were tested for their susceptibility to
304 known antileishmanial compounds (MIL, Ampho B and Sb^{III}) as well as one novel compound
305 (Compound Y) that has a chemical structure related to TCMDC-143345. No cross-resistance
306 was observed for Ampho B and Sb^{III} with similar IC_{50} values observed for all lines (Fig. S2C &
307 E). There was increased susceptibility of the TCMDC-143345-resistant lines to MIL (Fig. S2D).
308 Interestingly, all TCMDC-143345-resistant lines showed higher IC_{50} values for compound Y
309 compared to the WT, thereby implying cross-resistance of the TCMDC-143345-resistant lines
310 to compound Y (Fig. S2F). All IC_{50} values are shown in Data S1A. Finally, we looked at the *in*
311 *vitro* fitness of the TCMDC-143345-resistant promastigotes in the absence of the drug. The

312 resistant lines had a moderated but significant lower rate of growth than the wildtype (Fig.
313 S2G and supplementary text): C was the most slowly growing line.

314

315 ***Missense mutations in the gene encoding the dynamin-1 like protein LdoDLP1 can be found***
316 ***in independent selected resistant lines to TCMDC-143345***

317 To identify genetic changes underlying the observed resistance to TCMDC-143345, we
318 applied whole-genome sequencing (WGS) and we characterized genomic changes of nuclear
319 DNA in the control WT and the resistant C and D1 lines at all steps of the selection process
320 (see Fig. S1). First, we analyzed changes at the level of nucleotide sequence, *i.e.* single
321 nucleotide polymorphisms (SNPs), and small insertion and deletions (INDELs). A total of 245
322 SNP variants not present at the start of drug selection were identified in all the lines (Data
323 S1B). Among these, only 2 missense mutations with large and statistically significant changes
324 in allele frequency were observed in line C (Fig. 2A & 2C). The first one appeared at 5 μ M
325 TCMDC-143345 with a gradual increase in the allele frequency from 0 to 1 in
326 LdBPK_290029300, the gene encoding dynamin-1 like protein (LdoDLP1). This C:T missense
327 mutation translates to a change from alanine at position 324 to threonine (Ala324Thr). The
328 second significant change in the allele frequency in line C concerns another missense
329 mutation G:T in chr6; the allele frequency shifts to 0.5 at 4 μ M TCMDC-143345 in a
330 conserved hypothetical protein (LdBPK_060013600) and stabilizes around 0.7 during further
331 selection. Only one missense mutation with a significant change in the allele frequency has
332 been observed in all D lines (Fig. 2B). It starts at 6 μ M TCMDC-143345, changes from 0 to 1
333 and stays stable until the end of the selection pressure. Intriguingly, that mutation is also in
334 the gene encoding LdoDLP1 (LdBPK_290029300), but results in a different change at the
335 amino acid level compared to the LdoDLP1 mutation found in Line C; *i.e.*, glutamate at

336 position 655 changes to aspartate (Glu655Asp). These results were supported by using a
337 testing approach inspired by burden tests for rare disease associations, confirming that the
338 number of mutations in this gene is the most highly correlated with IC_{50} (see supplementary
339 text and Fig. S3). No significant indels were detected in lines C or D(1-4) along the selection
340 pressure.

341 As we previously showed that aneuploidy and local copy number variations (CNVs) occur at
342 an early stage of the selection process (8), we wondered whether it would be case in this
343 experiment. Aneuploidy was already present before selection, but additional aneuploid
344 chromosomes were observed around 4-6 μ M TCMDC-143345 and final patterns of
345 aneuploidy at the end of selection were rather different between Lines C and D1 (Fig. 3). Few
346 CNVs were observed and the most striking were amplifications/deletions of large
347 subtelomeric chromosomal stretches, also line-specific: in chr17 (D1) and chr30 (C) (Fig.S3B-
348 C). More details on the aneuploidy and CNVs can be found in supplementary text.

349

350 ***The CRISPR-Cas9-mediated mutants and overexpression lines confirm the role of LdoDLP1***
351 ***in the resistance to TCMDC-143345***

352 As the only common missense mutation in the two independent resistant lines was found in
353 LdoDLP1, we hypothesized that the genetic variation in this gene is responsible for the
354 TCMDC-143345 resistance. To test this hypothesis, we recreated the identified mutations in
355 wild-type promastigotes by means of a modified CRISPR-Cas9 system described elsewhere
356 (14) and we analyzed their resistance to TCMDC-143345. Detailed results of selection of
357 transfected promastigotes and subsequent controls are shown in Supplementary text. Three
358 clones were derived from each of the selected line (bearing the artificially introduced

359 Ala324Thr or Glu655Asp mutations). The 6 clones were then submitted to a susceptibility
360 test using a resazurin assay. The CRISPR-Cas9 engineered clones displayed a 4 to 5-fold
361 increase in IC_{50} to TCMDC-143345 when compared to the WT LdBPK_282 cl4 or the uncloned
362 parasites from control transfections #2 and #7 with a donor DNA lacking the missense
363 mutations (DynWT1 and DynWT2 respectively - One-way ANOVA, $P < 0.001$), achieving an
364 IC_{50} similar to Line C (Fig. 4A). Both the Ala324Thr (DynMut1 = mutation of line C) and the
365 Glu655Asp (DynMut2 = mutation of line D) mutations had similar impacts on susceptibility to
366 TCMDC-143345. Notably, no mutant clone displayed an IC_{50} similar to Line D.

367 In parallel to the CRISPR-Cas9-induced mutagenesis experiment, we also investigated the
368 effect of over-expressing the wild type LdoDLP1 gene in lines C and D3. The transfection of
369 an over-expression vector containing the wild type form of the gene completely abrogated
370 resistance to TCMDC-143345 in Line C, reducing their IC_{50} to levels similar to the wild type
371 LdBPK_282 cl4 (Fig. 4B - One-way ANOVA, $P < 0.001$). In line D3 however, while a significant
372 reduction in IC_{50} was observed (One-way ANOVA, $P < 0.001$), parasites still demonstrated
373 resistance to the compound, with an average IC_{50} of 29 μ M. Altogether these results indicate
374 that the mutations in the LdoDLP1 constitute the major genetic changes responsible for the
375 observed resistance to TCMDC-143345 in both lines. Growth curves in the absence of drug
376 pressure showed a similar growth rate between DynMut1 and DynMut2 and pT007 control,
377 the WT line, which constitutively expresses Cas9 protein (Fig.S2H and supplementary text).

378

379 ***Phylogenetic analysis suggests that LdoDLP1 plays a role in mitochondrial fission***

380 LdoDLP1 belongs to the family of dynamin-related proteins (DRPs), a group of proteins that
381 impacts the shapes of biological membranes by mediating fusion and fission events. Given

382 the i) clear contributions of the Ala324Thr and Glu655Asp LdoDLP1 mutations to the TCMDC-
383 143345 resistance phenotype and ii) the absence of detailed biochemical studies on
384 leishmanial DRPs, a phylogenetic analysis was performed in order to learn more about the
385 protein's possible biological function. As was reported by Morgan and colleagues in their
386 work on *Trypanosoma brucei* DLP1 (24), a sequence alignment followed by the construction
387 of a rooted phylogenetic tree reveals that DRPs fall into different functional clades.
388 Interestingly, LdoDLP1 clusters together with *T. brucei* DLP1 into the clade of DRPs involved
389 in mitochondrial fission, (Figure S4), suggesting a role for LdoDLP1 in this biological process.

390

391 ***TCMDC-143345 resistant lines exhibit lower mitochondrial membrane potential compared***
392 ***to susceptible, wild-type parasites***

393 It has been well established that mitochondrial dynamics plays a role in the maintenance of
394 normal mitochondrial membrane potential (MtMP) and cellular respiration (25)(26). Given
395 the putative role of LdoDLP1 in mediating mitochondrial fission, we therefore evaluated
396 whether the parasite lines containing LdoDLP1 mutations displayed changes in their
397 mitochondrial activity. The MtMP and cell viability were simultaneously evaluated on
398 logarithmic (day 2), early stationary (day 4) and late stationary promastigotes (day 7). In the
399 presence or absence of TCMDC-143345 the overall trend was that cells with good viability in
400 DR lines had decreased MtMP in comparison to the WT during the three days of testing
401 (Two-way RM ANOVA, $P < 0.001$) (Fig 5A-B). However, the differences between WT and DR
402 lines were bigger in logarithmic parasites (Fisher's LSD test, P values < 0.0001) both in
403 presence or absence of TCMDC-143345. Noteworthy, also on day2 the drug pressure
404 dramatically alters the Mitotracker RFUs in the WT but barely in the case of resistant lines
405 (see scatters plots Fig.5 A). We further evaluated if the CRISPR-engineered lines bearing the

406 mutations Ala324Thr (DynMut1) and Glu655Asp (DynMut2) in LdoDLP1 had also diminished
407 MtMP and it was confirmed for both mutations. Moreover, as with the *in vitro* selected DR
408 lines the differences between LdoDLP mutant lines and the WT were larger during the
409 logarithmic phase (Fig. S5). These results overall confirm that the resistant lines containing
410 LdoDLP1 mutations have higher survival rate with the cost of moderate but significant
411 decrease of the MtMP.

412

413 ***Homology modeling suggests a molecular basis for the putative impact of the Ala324Thr***
414 ***and Glu655Asp mutations on LdoDLP1 function***

415 Given that the introduction of the LdoDLP1 Ala324Thr and Glu655Asp point mutations
416 bestows TCMDC-143345 resistance onto the parasite and leads to an altered mitochondrial
417 membrane potential, a structural model for LdoDLP1 was generated through homology
418 modeling in an attempt to provide a molecular basis for these observations. LdoDLP1
419 contains all structural features characteristic of DRPs (Fig. 6, panel A): a neck domain (aka
420 bundle-signaling element or BSE) consisting of three α -helices, a GTPase domain, an α -helical
421 stalk domain that contains the dimerization interface, and a foot domain (aka 'paddle' or
422 pleckstrin homology or PH domain). In contrast to various other DRPs, LdoDLP1 is devoid of
423 the intrinsically disordered proline-rich domain (PRD).

424 The Ala324Thr mutation maps onto a part of the stalk domain that is closely located to the
425 α 2-helix of the neck domain (Fig. 6B) and is known to be responsible for the higher-order
426 oligomerization of DRP dimers, which is important for DRP function in fission events ((27)
427 (28) (21) (29), more in-depth explanation in the Supplementary text). The Glu655Asp
428 mutation is located in a region known as 'Hinge 1', which connects the stalk domain with the

429 α 3-helix of the neck domain (Fig. 6, panel B). ‘Hinge 1’ confers flexibility to DRPs, which is
430 crucial for the so-called “hydrolysis-dependent powerstroke” underlying protein function
431 ((29) (30) (31), more in-depth explanation in the Supplementary text). Hence, the LdoDLP1
432 mutations contributing to TCMDC-143345 resistance could i) alter the tendency of LdoDLP1
433 dimers to form higher-order oligomers (Ala324Thr) and ii) have a considerable impact on the
434 flexibility of LdoDLP1’s ‘Hinge 1’ region (Glu655Asp), which would in turn be expected to
435 affect protein function.

436

437 **Discussion**

438 Humans and their pathogens are continuously locked in a molecular arms race and human
439 interventions like chemotherapy have a further impact on parasites adaptations and
440 counter-adaptations (1). This is well illustrated by the present study in which we (i) selected
441 in *L. donovani* resistance to TCMDC-143345, a novel and potent anti-leishmanial compound
442 that emerged from the Leishbox library of anti-leishmanial compounds (4) and (ii)
443 characterized the extent of adaptations developed by the parasite.

444 Ten selection rounds (about 50 weeks) were necessary to obtain resistance to TCMDC-
445 143345 in promastigotes, resistance was stable in the absence of the drug and it was also
446 maintained in intracellular amastigotes. The same LdBPK_282 cl4 line was previously used
447 for selecting and characterizing resistance to drugs used in clinical practice, hereby allowing
448 comparisons about development of resistance to different compounds. Overall, it took more
449 time to develop resistance *in vitro* to TCMDC-143345 (10 rounds, 50 weeks) than to MIL
450 (present study, 6 rounds, 30 weeks) and to Sb^{III} (1-4 selection rounds or 5-20 weeks,
451 depending on the protocol used)(7).

452 Whole genome sequencing is a powerful tool to identify molecular changes accompanying
453 DR development in pathogens (7)(8, 32). In this study, in-depth genomic analysis revealed
454 that TCMDC-143345 resistant lines were characterized by several aneuploidy changes, CNVs
455 (see discussion in supplementary text) and SNPs. Especially the SNP analysis during the
456 development of resistance turned out to be very informative. Out of 245 detected SNPs, only
457 2 missense mutations were fixed in 2 independent resistant lines and both are located in the
458 gene encoding *L. donovani* dynamin-1 like protein (LdoDLP1). We used CRISPR-Cas9 to
459 recreate the Ala324Thr or the Glu655Asp mutations in the LdoDLP1 gene of WT LdBPK_282
460 cl4 promastigotes. Hereby, we demonstrated that these mutations independently confer
461 resistance to TCMDC-143345, leading to an IC₅₀ in all mutant clones that is similar to Line C.
462 Consistent with this, the over-expression of the WT LdoDLP1 gene completely abolished
463 resistance to TCMDC-143345 in line C, while it had only a partial impact in the IC₅₀ of line D3.
464 These observations demonstrate that a loss-of-function mutations in LdoDLP1 gene are
465 sufficient to provide a resistance to TCMDC-143345 in both lines. However, in line D this
466 resistance is further increased by other uncharacterized modifiers acting in concert with the
467 mutation in the LdoDLP1. Further work is needed to explore the functional impact of the
468 specific aneuploidy changes, CNVs and SNPs observed in that line.

469 LdoDLP1 belongs to the family of dynamin-related proteins (DRPs) which are involved in
470 several functions(24)(26): i) mitochondrial fusion (fusion of the inner mitochondrial
471 membrane), ii) membrane dynamics of the outer chloroplast membrane, iii) endocytosis and
472 vesicle trafficking in animals, iv) vesicle trafficking in plants, v) mitochondrial fission (fission
473 of the outer mitochondrial membrane), and vi) plate formation and cell division in plants.
474 DRPs cluster in different phylogenetic clades according to these functions(24) and our
475 phylogenetic analysis showed a clear clustering of LdoDLP1 into the DRP clade involved in

476 mitochondrial fission. The combined actions of mitochondrial fission and fusion govern
477 mitochondrial dynamics, which underlie the organisation, copy number, form and function
478 of mitochondria. The rates of these events are regulated according to the metabolic and/or
479 developmental needs of the cell and in response to cellular stress or damage. While
480 mitochondrial dynamics and cell division are not necessarily coupled in eukaryotes, the link
481 between both processes seems to be very stringent in apicomplexan and kinetoplastid
482 parasites. Both Apicomplexans and Kinetoplastids contain a single mitochondrion of which
483 the fission is controlled by a single or a limited number of DLPs (33)(34). *Trypanosoma brucei*
484 harbours two DLP paralogs (TbDLP1 and TbDLP2) (35), of which especially TbDLP1 seems to
485 play a central role in linking the processes of mitochondrial fission, cytokinesis, and
486 distribution of kinetoplastid DNA(24, 36–38). Interestingly, abrogation of TbDLP1 function in
487 *T. brucei* blocks mitochondrial fission and cell division, again leading to parasite fatality(24,
488 36). Similar to other protozoan parasites, *Leishmania* spp. contain a single elongated
489 mitochondrion and harbor a single DLP (24).

490 The clear clustering of LdoDLP1 with *T. brucei* DLP1 into the DRP clade involved in
491 mitochondrial fission hinted towards a role for LdoDLP1 in mitochondrial dynamics, which is
492 why we studied the mitochondrial membrane potential and cell viability of the TCMDC-
493 143345 resistant parasite lines. In accordance with a proposed role of LdoDLP1 in
494 mitochondrial fission, we found that all the TCMDC-143345-resistant lines (including the
495 CRISPR-Cas9 generated LdoDLP1 Ala324Thr and Glu655Asp mutants) showed an altered
496 mitochondrial activity. Interestingly, homology modeling suggests that the LdoDLP1
497 Ala324Thr and Glu655Asp mutations associated with TCMDC-143345 resistance are located
498 in the protein's oligomerisation interface and 'Hinge 1' region, respectively, two regions that
499 are essential for general DRP (and thus LdoDLP1) function. Hence, these mutations are likely

500 to influence protein function, which in turn might explain the observed impact on
501 mitochondrial dynamics within the TCMDC-143345 resistant *L. donovani* parasites.

502 Whether LdoDLP1 is the molecular target of TCMDC-143345 or is part of a coping
503 mechanism without being the direct target of TCMDC-143345 remains to be investigated. In
504 this latter hypothesis, the LdoDLP1 mutations may have arisen to alleviate the drug's
505 detrimental effect on parasite viability. Within this context, it is interesting to note that
506 leishmanial DLP was also proposed to be involved in the resistance profile of antimony- and
507 miltefosine-resistant *Leishmania infantum* (39). In this proteomic study, *L. infantum* DLP was
508 found to be down-regulated in the antimony- and miltefosine-resistant strains, although no
509 information was gathered with regards to possible mutations in the protein. In the former
510 hypothesis (*i.e.*, LdoDLP1 is the molecular target of TCMDC-143345), the Ala324Thr and
511 Glu655Asp mutations provide a direct escape to the drug's deadly mode of action through
512 LdoDLP1 binding. This hypothesis can equally be supported by the MtMP results. As of day 2
513 of drug exposure, we observed a drastic alteration in the Mitotracker RFUs for the WT
514 parasite, whereas the Mitotracker RFUs remained unaffected for the resistant lines. This
515 could be explained by the following scenarios: i) the LdoDLP1 mutations in the DR lines
516 prevent the compound from binding LdoDLP1 (*i.e.*, the mutations are located in the binding
517 site for TCMDC-143345) or ii) they compensate for the effect that drug binding may have on
518 LdoDLP1's function in mitochondrial dynamics (TCMDC-143345 binds another LdoDLP1
519 ligand binding site). Clearly, in this hypothesis, the elucidation of the binding site and
520 molecular interactions responsible for affinity and specific recognition between LdoDLP1 and
521 TCMDC-143345 would shed relevant insights to be exploited in the design of new
522 compounds with optimised potency. This would be especially interesting since DLPs from

523 protozoan parasites are considered as drug targets because of their essentiality with regards
524 to cell division and parasite growth (33).

525 Altogether, the results gathered in present study demonstrate the practical relevance of
526 prospective DR studies. The time-to-resistance here shown for TCMDC-143345 is
527 encouraging in the context of the shelf-life of that compound, but this should be
528 complemented by *in vivo* studies. The demonstrated engagement of the unique leishmanial
529 DLP in the resistance of *L. donovani* to TCMDC-143345 will allow the development of
530 diagnostics targeting that gene to accompany further pre-clinical and clinical studies, if any
531 and it will also guide further investigation on the mode of action. The absence of cross-
532 resistance with other drugs currently used in clinical practice qualifies TCMDC-143345 for
533 future combination therapy if the compound would reach that stage. It is still early to assess
534 whether this mechanism of resistance is relevant against other chemical classes, yet the
535 resistant strains selected become powerful tools to be employed with new chemical classes
536 to ascertain whether they share biological space in terms of mode of action or resistance.

537

538 **Acknowledgements**

539 This study has received funding from the European Union's Horizon 2020 research and
540 innovation programme under the Marie Skłodowska-Curie grant agreement N° 642609 and
541 the Flemish Fund for Scientific Research (12Q8115N). MJ is supported by the Flemish Fund
542 for Scientific Research (postdoctoral fellowship). GN is supported by the Flemish Ministry of
543 Science and Innovation (SOFI Grant MADLEI). JAC, MB and MS are supported by the
544 Wellcome Trust via their core funding of the Wellcome Trust Sanger Institute (grant 206194).
545 The funders had no role in study design, data collection and interpretation, or the decision
546 to submit the work for publication.

547

548

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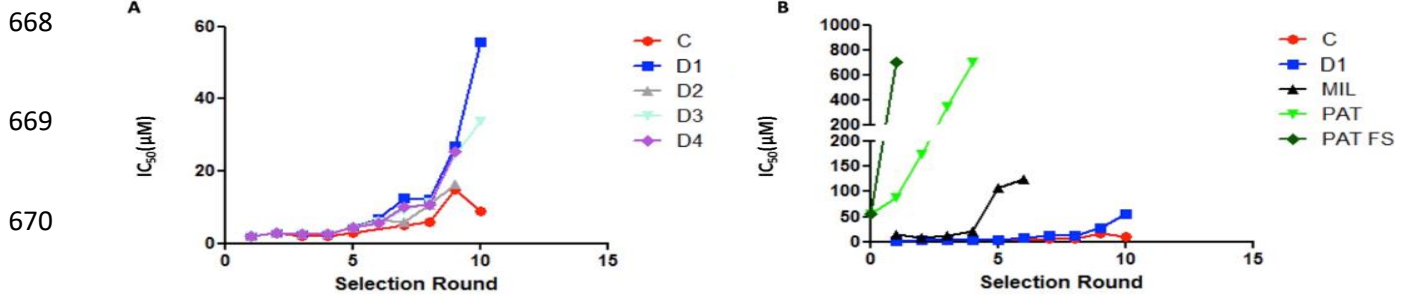
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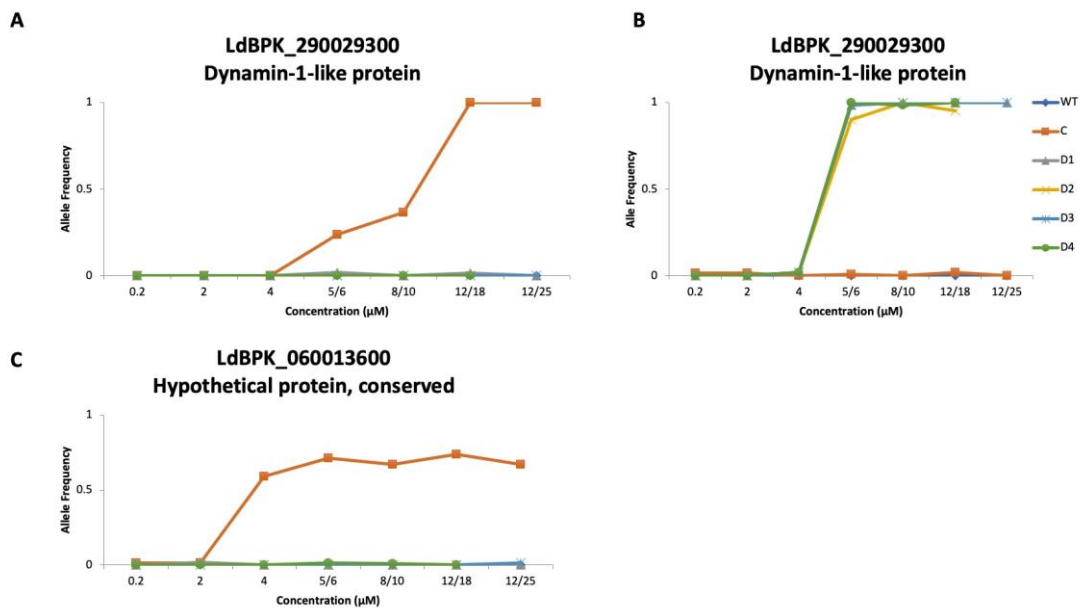
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673 **Fig. 1. Time to resistance during selection experiments.** The IC₅₀ is shown in µM on the Y
674 axis and selection round on the X axis (approx. 5 weeks per round) in (A) the different lines
675 of LdBPK_282 cl4 kept under pressure with TCMDC-143345, in (B) line C and D1 are
676 compared with LdBPK_282 cl4 selected for resistance to different known compounds
677 (Miltefosine, MIL; potassium antimonyl tartrate, PAT); PAT FS, consisting in a 'flash' selection
678 exposing the parasites directly to the highest concentration of the drug (7).

679

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683 **Figure 2. Major SNPs and their allele frequency change observed during selection of**
684 **TCMDC-143345-resistance. (A) C:T missense mutation in position Ld29: 1000024 and (B) C:A**
685 **missense mutation in position Ld29:999029C, both in the gene encoding dynamin1-like**
686 **protein (LdoDLP1, LdBPK_290029300). (C) G:T missense mutation in position Ld06:353587 in**
687 **a conserved hypothetical protein (LdBPK_060013600)**

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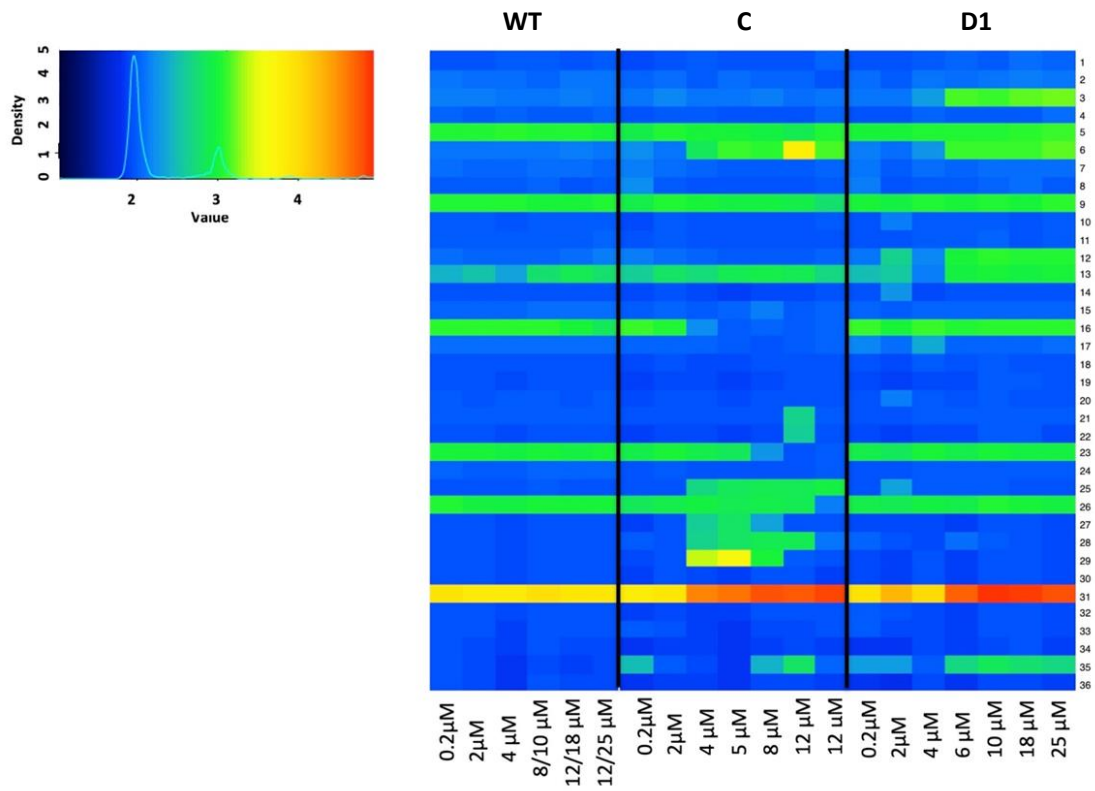
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700 **Fig. 3. Somy changes in TCMDC-143345-resistant lines C and D1 and WT controls.** Heat map

701 representing the karyotype dynamics across the resistance selection of LdBPK_282 cl4 to

702 TCMDC-143345. The color key shows the normalized chromosome read depth (S).

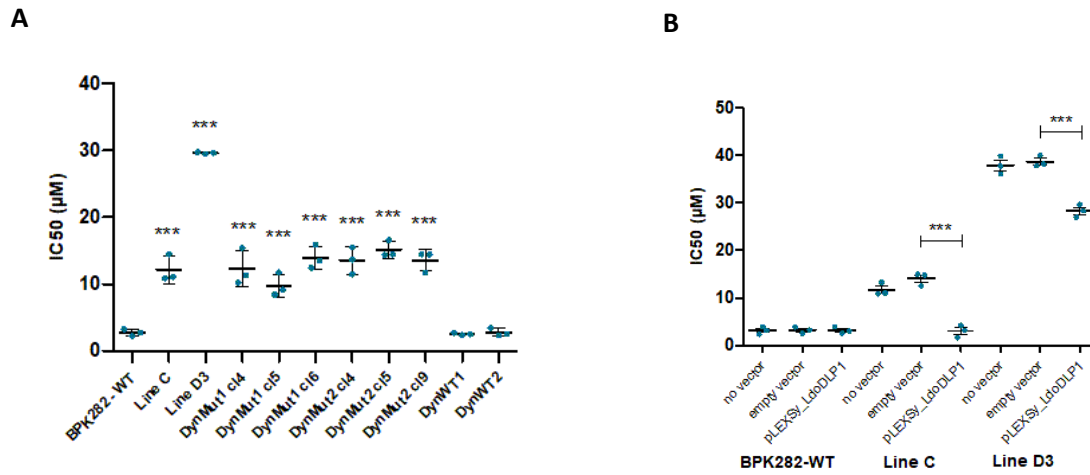
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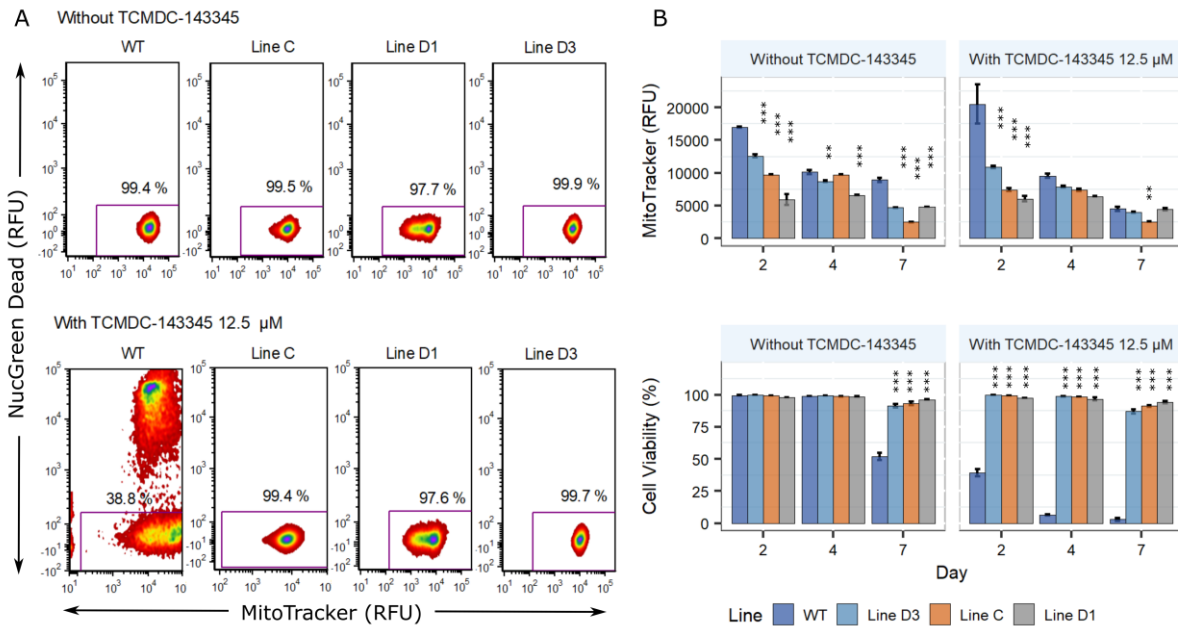
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708 **Fig. 4. A.** Susceptibility of 3 clones with the Ala324Thr (DynMut1 c4, c5 and c6) or the
709 Glu655Asp (DynMut2 c4, c5 and c9) LdDLP1 mutations introduced by CRISPR-Cas9. The
710 WT LdBPK_282 c14 and the unselected parasites transfected with DynWT1 or DynWT2 sgRNA
711 and DNA templates were included as controls. **B.** Susceptibility of Lines C and D when
712 overexpressing the WT LdDLP1 gene. Lines represent the average and standard deviation of
713 the three independent replicates (dots) of each experiment. *** = $p \leq 0.001$ (ANOVA with
714 Bonferroni's Multiple Comparison Test).

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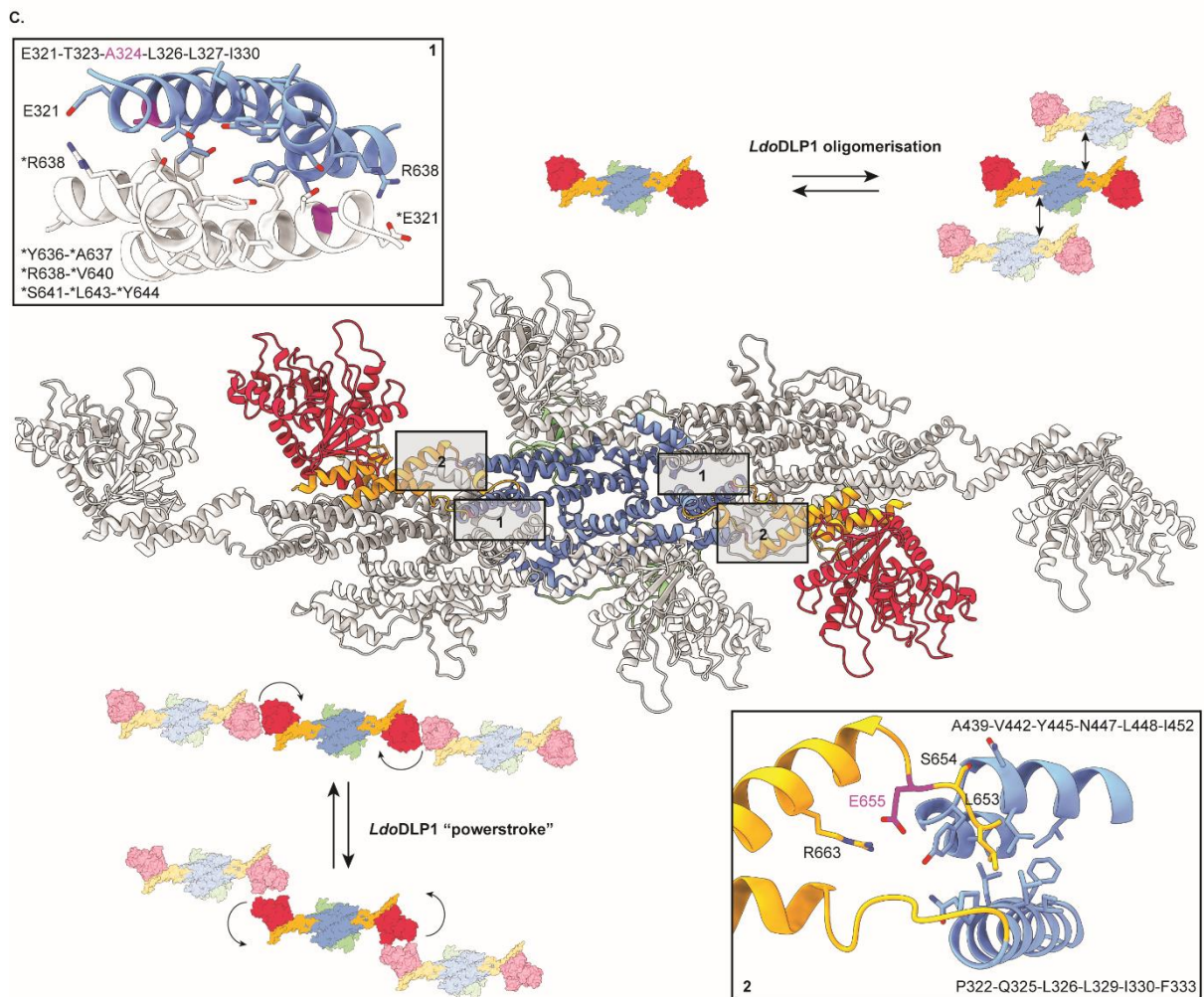
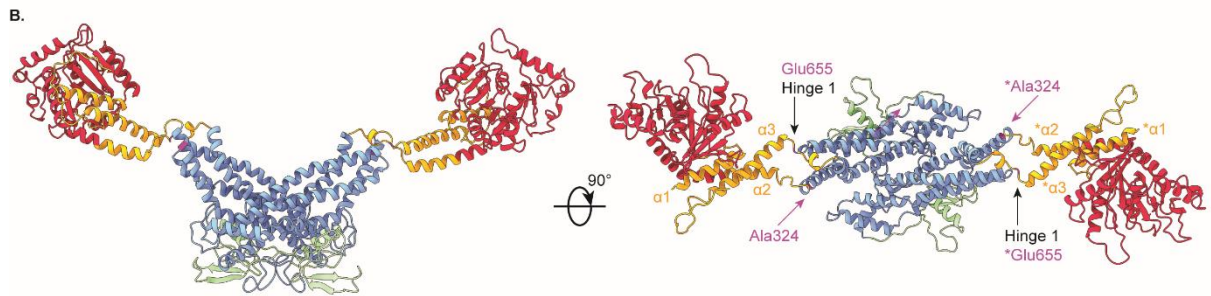
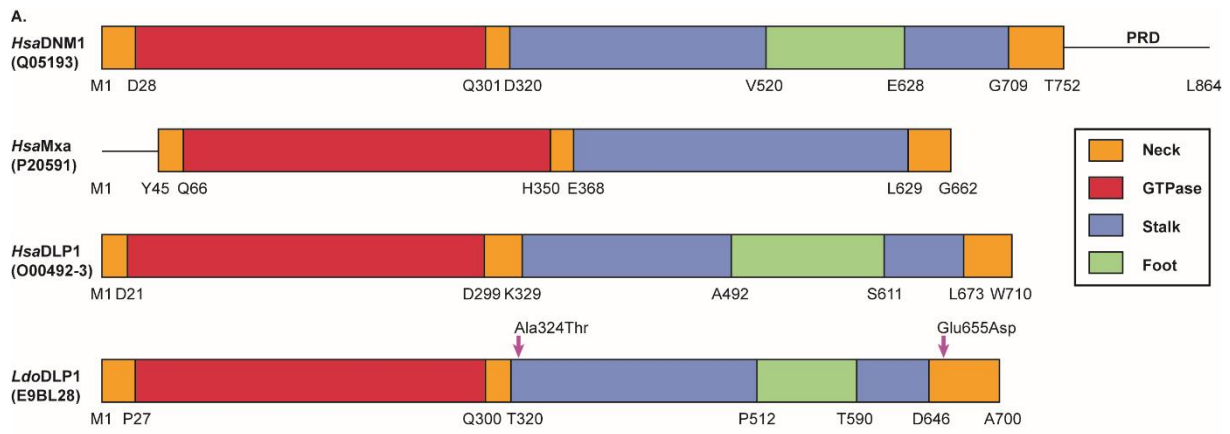
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719 **Figure 5.** Mitochondrial membrane potential (MtMP) and cell viability of TCMDC-143345-
 720 resistant lines in promastigotes. (A) Density plots of the MtMP as measured by the
 721 fluorescence of the MitoTracker DeepRed and the percentage of cell viability as measured by
 722 the fluorescence of the NucGreen Dead. One plot of each line 2 days post treatment with
 723 12.5 μM of TCMDC-143345 and their respective controls are shown. (B) MtMP in WT and
 724 resistant lines on days 2, 4 and 7 post treatment. All the resistant lines had diminished MtMP
 725 in the logarithmic phase of culture (Day 2) in comparison to the wildtype in presence or
 726 absence of TCMDC-143345. The differences between wild type and resistant lines were
 727 smaller in later days of incubation. Each bar represents the mean ± SEM of three biological
 728 replicates. Within each day, the asterisk in the top of the bars represent significant
 729 differences between the resistant line in comparison to the wild type (Fisher's LSD test, $P <$
 730 0.05).

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734 **Figure 6. A homology model of LdoDLP1 suggests a molecular basis for the impact of**
735 **TCMDC-143345 resistance mutations on protein function.** (A.) Schematic representation of
736 the primary sequences of *Homo sapiens* dynamin 1 (*HsaDNM1*), *H. sapiens* myxovirus
737 resistance protein 1 (*HsaMxA*), *H. sapiens* dynamin-like protein 1 (*HsaDLP1*) and LdoDLP1.
738 The Uniprot identifiers are shown as well. The different domains are color-coded and their
739 boundaries have been indicated. The LdoDLP1 Ala324Thr and Glu655Asp mutations
740 associated with TCMDC-143345 resistance are indicated by the magenta arrows. (B.)
741 Cartoon representation of the LdoDLP1 dimer homology model, color-coded as in panel A.
742 The 3 α -helices of the neck domain, ‘Hinge 1’ and the positions of Ala324 and Glu655 have
743 been indicated for convenience. ‘*’ is employed to indicate the second protomer of the
744 LdoDLP1 dimer. (C.) The center displays a model of a higher order oligomer of the LdoDLP1.
745 The central dimer is color-coded as in panel A, whereas the other two dimers are depicted in
746 gray for reasons of clarity. The regions containing the Ala324Thr and Glu655Asp mutations
747 are indicated by boxes ‘1’ and ‘2’, respectively. The insets provide a close-up view of the
748 molecular interactions in these boxed regions. Relevant amino acids are shown in a stick
749 representation. Ala324 (colored magenta) is proposed to be a part of the higher order
750 oligomerization interface of LdoDLP1 (box 1), while Glu655 (colored magenta) is likely to play
751 an important role in LdoDLP1’s “powerstroke” (box 2). ‘*’ is employed to indicate the second
752 protomer of the LdoDLP1 dimer.

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