Supplementary information for

Multifactorial basis for *in vitro* acquisition of miltefosine resistance in *Leishmania donovani*: a genomic appraisal

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Running title: NGS of miltefosine resistant *Leishmania*

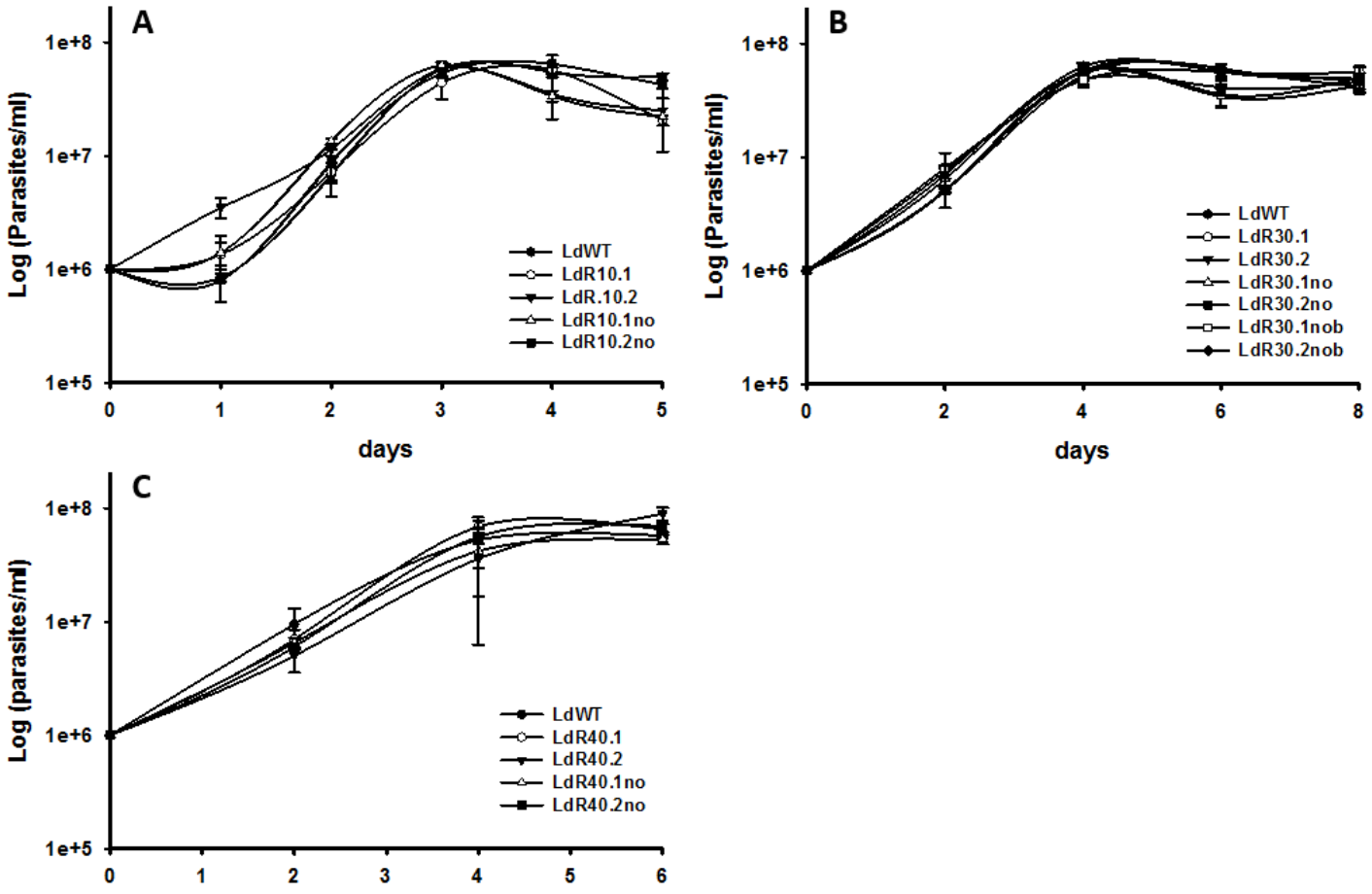

Figure S1. Growth curves of *L. donovani* promastigotes cultured in the presence or absence of HePC. Growth rate was determined by microscopical counting in triplicate and compared to WT strain. Results represent the average of three independent experiments and error bars indicate standard deviation. Parasites step-wise selected with 10 µM (A), 30 µM (B) or 40 µM (C) of HePC and subsequently cultivated with or without the drug pressure (LdR##no).

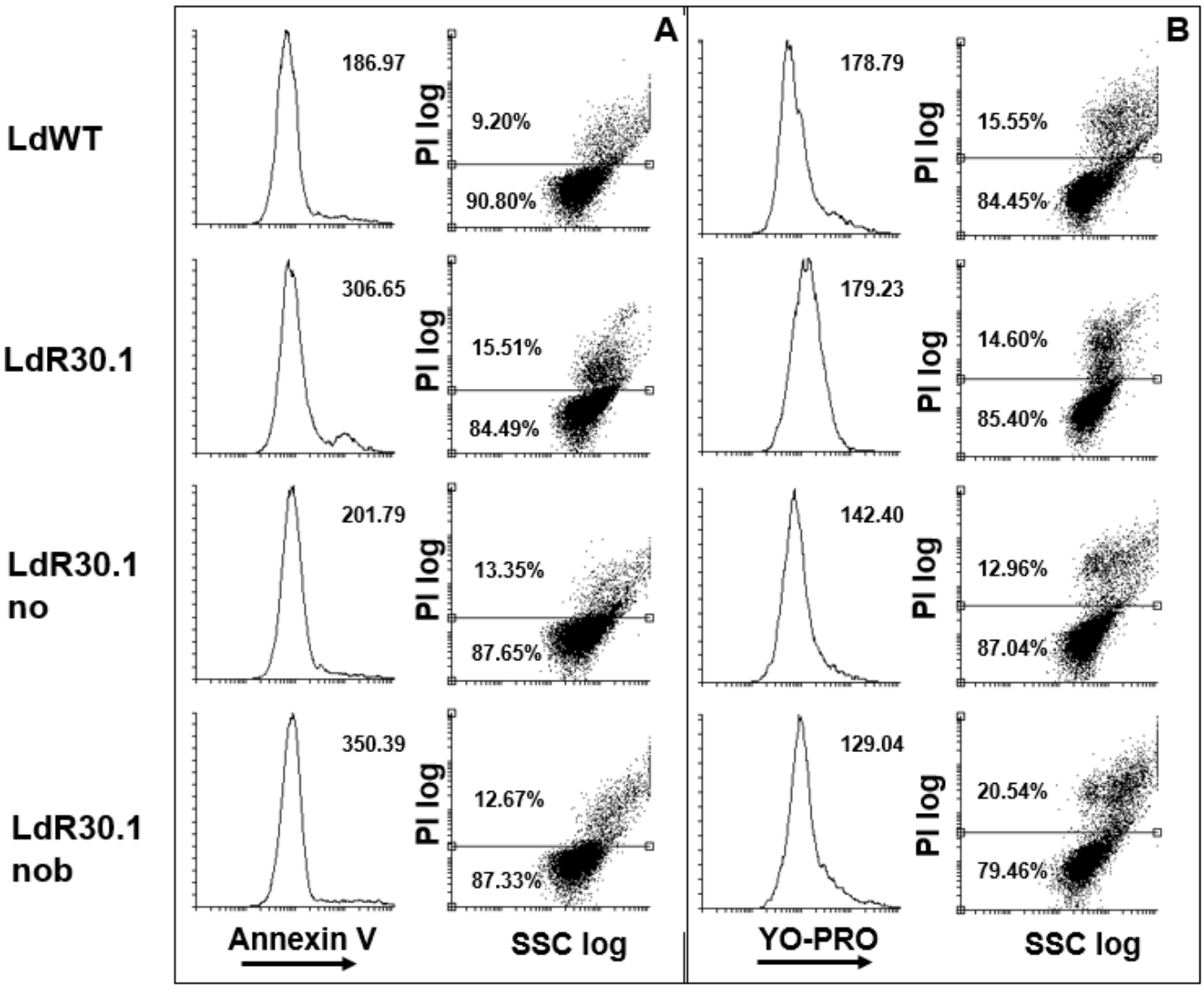

Figure S2. Viability and apoptosis induction by HePC selection. Flow cytometry analysis of HePC-resistant *L. donovani* promastigotes: WT, R30.1, R30.1no and LdR30.1nob using two different apoptotic markers: Annexin V (panel A) and YO-PRO (panel B). 10,000 events per sample were recorded.

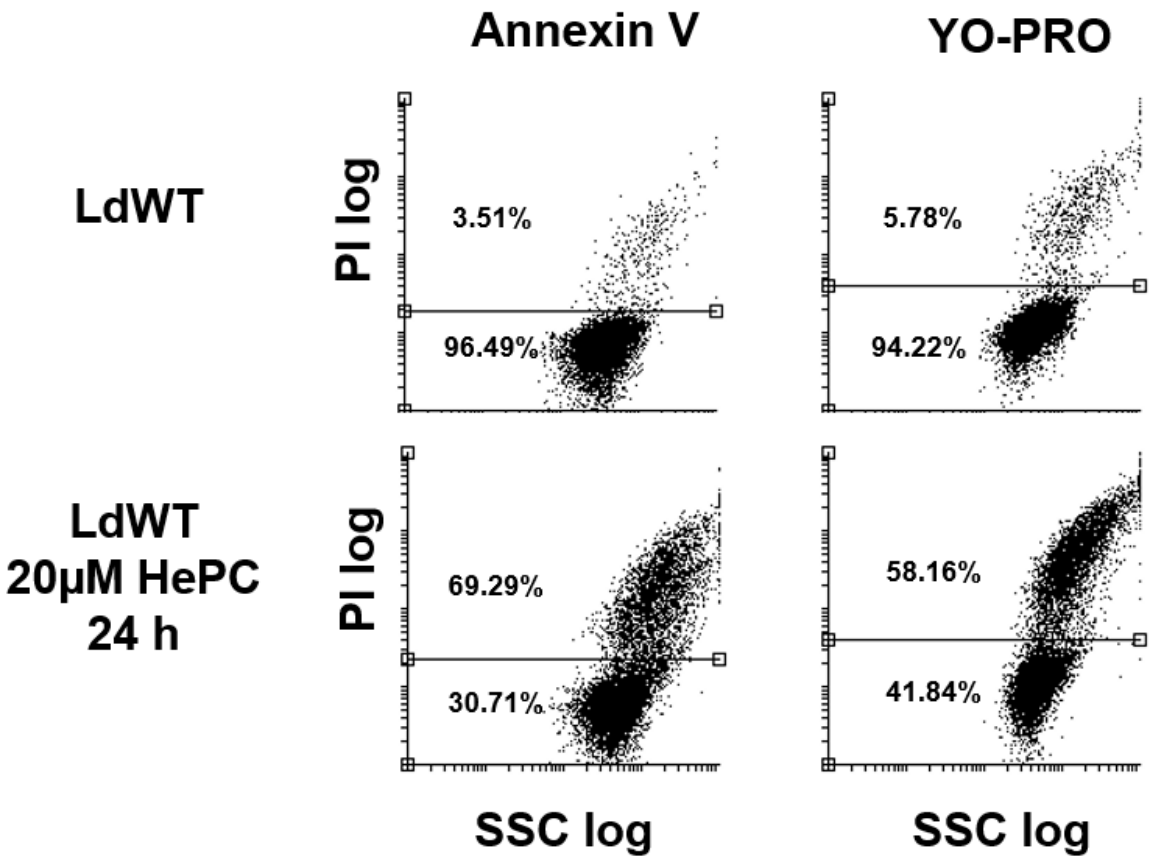

Figure S3. Flow cytometry analysis of *L. donovani* promastigotes treated with HePC. WT parasites were incubated with or without 20 μM HePC for 24 h and subsequently analyzed by flow cytometry using the fluorescent markers YO-PRO and Annexin-V. Death extent was quantified by measuring percentage of cells positive for propidium iodide in treated and non-treated parasites. Dot plots show 10,000 events per sample.

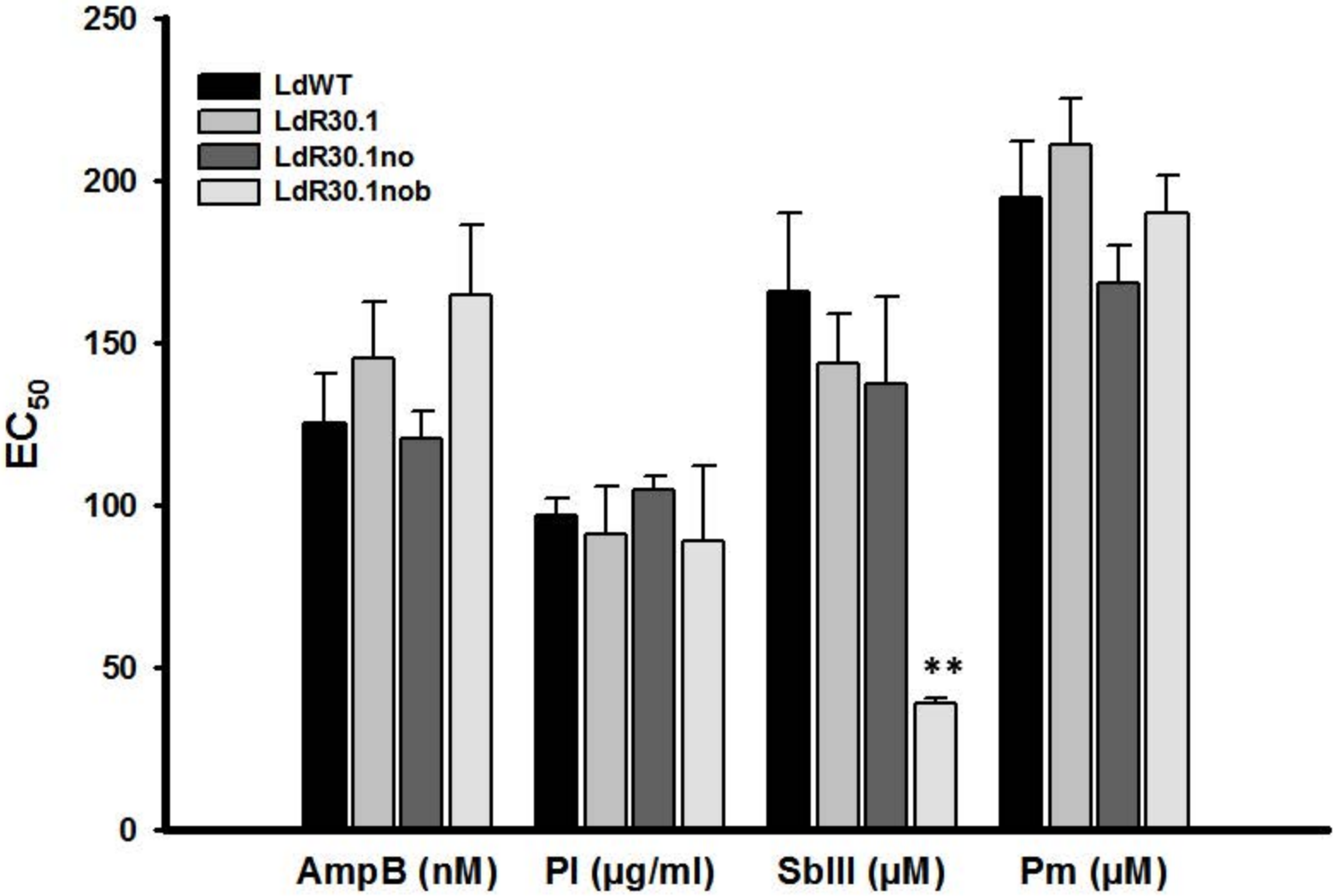

Figure S4. Cross-resistance to reference compounds. HePC-resistant promastigotes do not present cross-resistance to alternative anti-leishmanial agents as compared to the WT. In one case (LdR30.1nob) an increased susceptibility to SbIII was observed (**$p<0.05$). Results represent the average of three independent experiments and error bars indicate standard deviation.

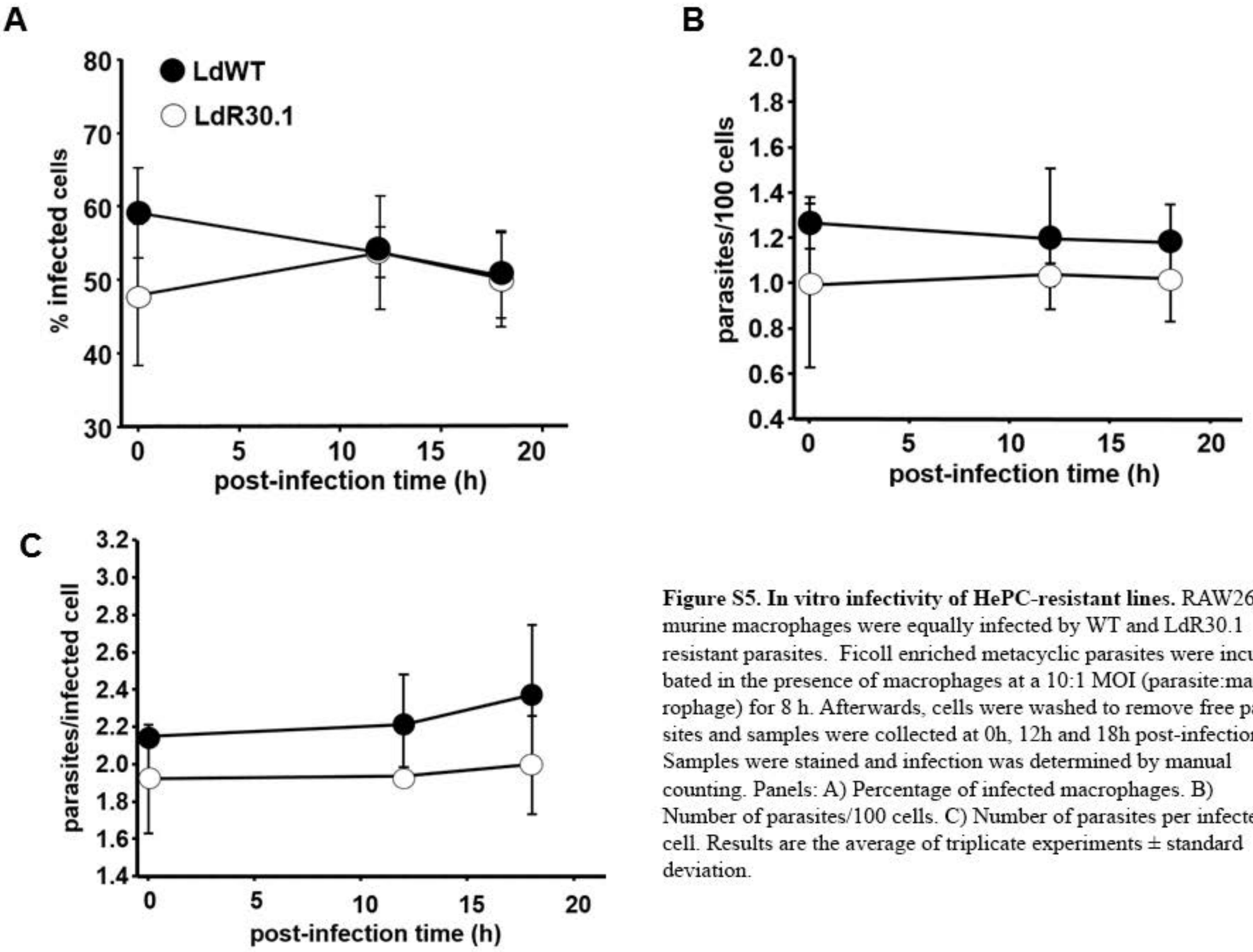

Figure S5. In vitro infectivity of HePC-resistant lines. RAW264.7 murine macrophages were equally infected by WT and LdR30.1 resistant parasites. Ficoll enriched metacyclic parasites were incubated in the presence of macrophages at a 10:1 MOI (parasite:macrophage) for 8 h. Afterwards, cells were washed to remove free parasites and samples were collected at 0h, 12h and 18h post-infection. Samples were stained and infection was determined by manual counting. Panels: A) Percentage of infected macrophages. B) Number of parasites/100 cells. C) Number of parasites per infected cell. Results are the average of triplicate experiments ± standard deviation.

Figure S6. **Heatmap based on chromosome copy number comparing WT, R30.1, R30.1no and R30.1nob.** Read depth was calculated, and normalized to a value of 2 for disomic chromosomes.