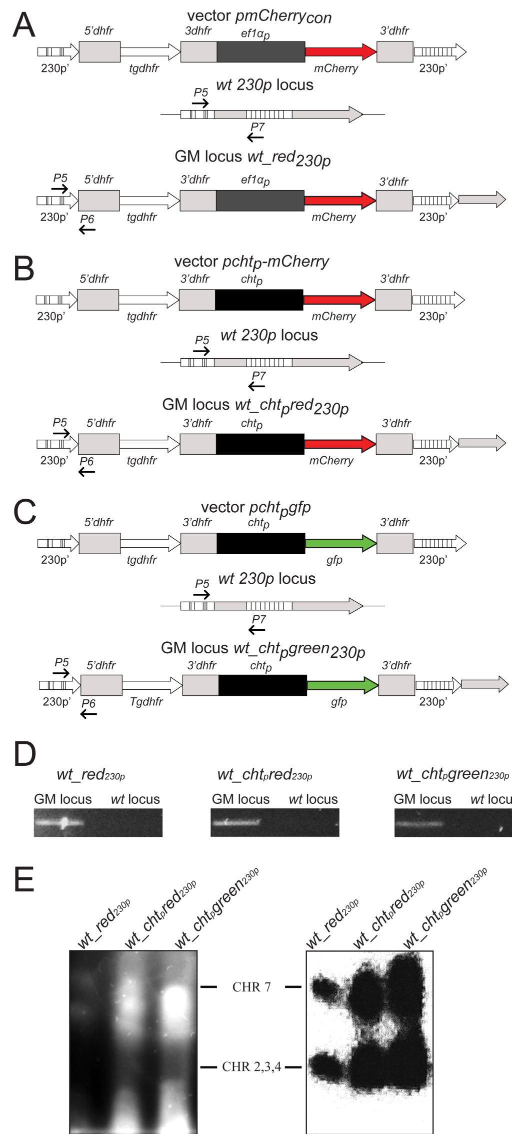

Fig. S1. Generation of wt_red_{230p}, $wt_cht_{p}red_{230p}$ and $wt_cht_{p}green_{230p}$ transgenic parasites. Schematic representation of the $pmCherry_{con}$ (A), $pcht_{p}mCherry$ (B) and$pcht_{p}gfp$ (C) expression cassettes that are inserted into the *230p* locus via double crossover homologous recombination resulting in the loss of a 1 kb region of the native locus. D. Diagnostic PCR of clonal parasites corroborating successful integration of the aforementioned expression cassettes. E. Southern blot analysis of clonal parasites corroborating successful integration of the expression cassettes. The positions of the PCR primers used for the diagnostic PCR reactions are shown (P5, P6 and P7).

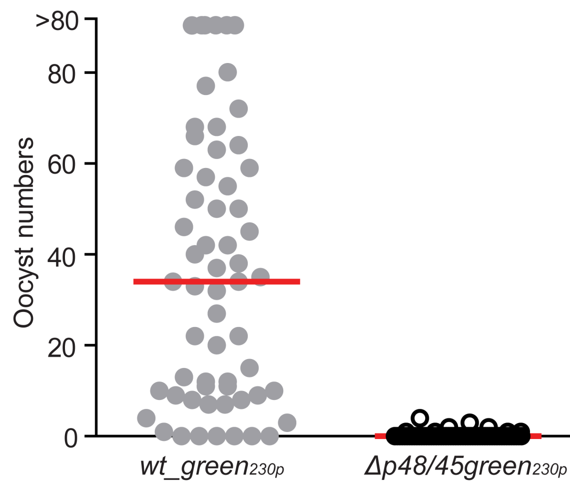

Fig. S2. Oocyst load in *A. gambiae* mosquitoes infected with the *Δp48/45green$_{230p}$*parasite. The incomplete defective phenotype of the male gamete defective*Δp48/45green$_{230p}$* transgenic parasite results in the escape of very few male gametes that are able to fertilize the normal *Δp48/45green$_{230p}$* female gametes to form few ookinetes and thus the few oocysts observed in *A. gambiae* mosquitoes.

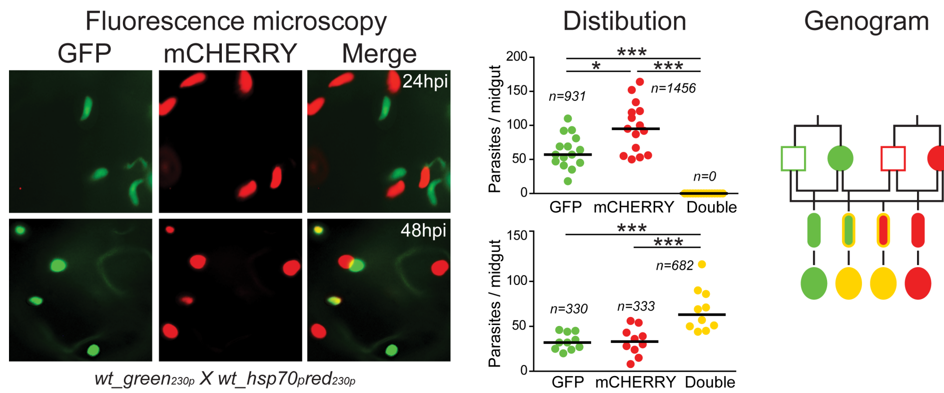

Fig. S3. Allelic expression of fluorescent reporters placed under the control of constitutive gene promoters. *In vivo* cross-fertilization assays in *A. gambiae* mosquitoes directly fed on mice infected with equal numbers of the transgenic parasite lines wt_green_{230p} (*ef1α* gene promoter) and $wt_hsp70_{p}red_{230p}$. The first composite panel includes representative fluorescence microscopy pictures of *A. gambiae* midguts fed on mice co-infected with two transgenic parasite lines as indicated at the bottom of each part, taken at 24 and 48 hpi respectively. The GFP, mCHERRY and a combination of the two channels (merge) are shown. The second panel is a graph showing the distribution and median number of GFP-positive, mCHERRY-positive and GFP/mCHERRY double-positive parasites per midgut at 24 and 48 hpi. The collective results from three biological replicates are shown, where *n* is the total number of parasites counted. The third panel shows a genogram summarizing the results of these cross-fertilization experiments. Squares correspond to male gametocytes, circles correspond to female gametocytes, small ellipses correspond to ookinetes and large ellipses correspond to early stage oocysts. The colour of the outline indicates the genotype, whereas the fill-in colour indicates the phenotype. Yellow outline indicates*gfp/mCherry* heterozygotes, whereas yellow fill-in colour indicates GFP/mCHERRY double-positive parasites. Black lines indicate the crosses and the resulting progeny. Horizontal black lines indicate the median parasite number. Stars indicate statistical significance determined with the Mann–Whitney *U*-test (***$P < 0.001$)

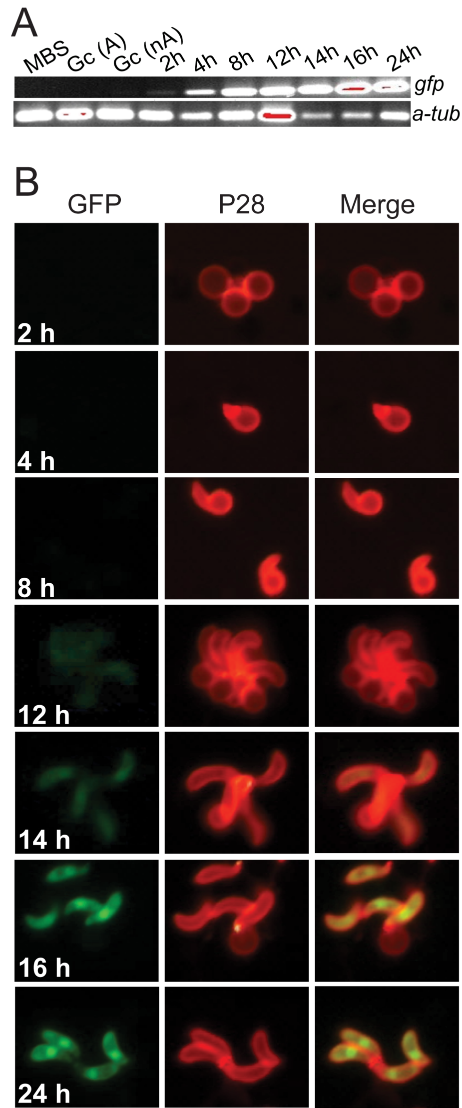

Fig. S4. GFP expression in the *wt_cht$_p$green$_{230p}$* parasite line. A. RT-PCR analysis of *gfp* transcripts from mixed blood stages, activated (A) and non-activated (nA) gametocytes, and parasites purified from *in vitro* ookinete cultures at 2, 4, 8, 12, 14, 16 and 24 h post-fertilization. *Tubulin* transcripts served as a loading control. B. Fluorescence microscopy analysis of *wt_cht$_p$green$_{230p}$* parasites purified from *in vitro*ookinete cultures at 2, 4, 8, 12, 14, 16 and 24 hpa. Parasites were also stained with a Cy3 conjugated α-P28 antibody. Images were taken at ×40 magnification.

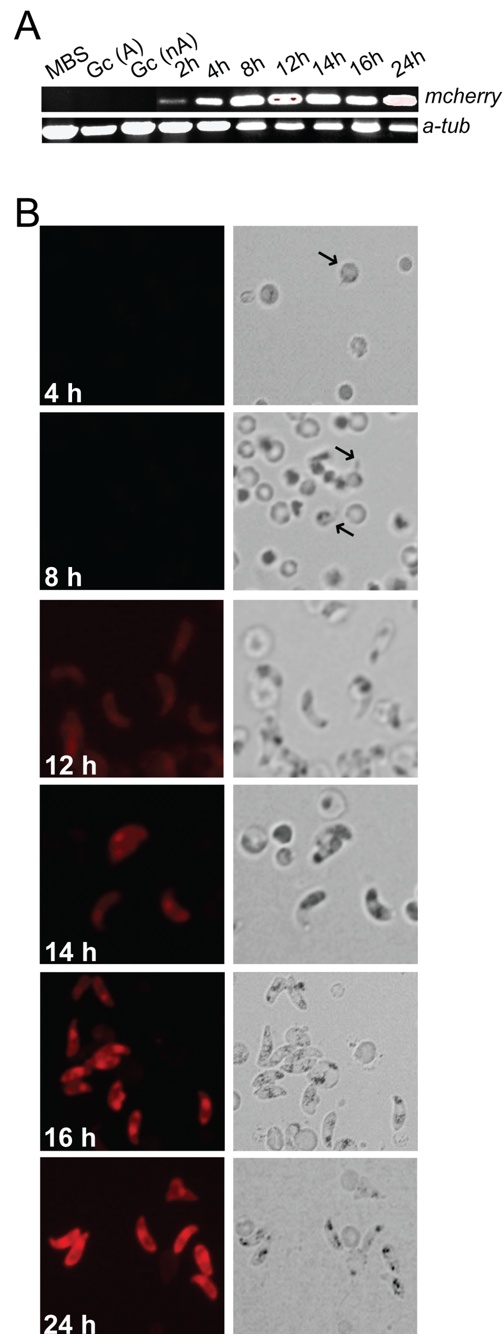

Fig. S5. Expression of mCHERRY in the *wt_cht$_p$red$_{230p}$* parasite line. A. RT-PCR analysis of *mCherry* transcripts from mixed blood stages, activated (A) and non-activated (nA) gametocytes, and parasites purified from *in vitro* ookinete cultures at 2, 4, 8, 12, 14, 16 and 24 h post-fertilization. *Tubulin* transcripts served as a loading control. B. Fluorescence microscopy analysis of *wt_cht$_p$red$_{230p}$* parasites purified from *in vitro*ookinete cultures at 4, 8, 12, 14, 16 and 24 hpa. Bright field images are also shown. Images were taken at ×40 magnification.