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Tricks in *Plasmodium*'s molecular repertoire – Escaping 3′UTR excision-based conditional silencing of the chloroquine resistance transporter gene

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ABSTRACT

In the human malaria parasite *Plasmodium falciparum*, the major determinant of chloroquine resistance, *P. falciparum* chloroquine resistance transporter (*pfcrt*), likely plays an essential role in asexual blood stages, thus precluding conventional gene targeting approaches. We attempted to conditionally silence the expression of its ortholog in *Plasmodium berghei* (*pbcrt*) through Flp recombinase-mediated excision of the 3′untranslated region (UTR) during mosquito passage. However, parasites maintained *pbcrt* expression despite 3′UTR excision. Characterisation of these *pbcrt* mRNAs, by 3′rapid amplification of cDNA ends, identified several replacement 3′UTR sequences. Our observations demonstrate the astounding genetic plasticity of this parasite when faced with the loss of an essential gene.

Mutations in the *Plasmodium falciparum* chloroquine resistance transporter (*pfcrt*; PlasmoDB ID: MAL7P1.27) play a central role in resistance of the human malaria parasite, *P. falciparum*, to several antimalarial drugs, most notably chloroquine (Petersen et al., 2011; Roepe, 2011; Ecker et al., 2012; Summers et al., 2012). *pfcrt* encodes an integral membrane protein that localises to the parasite's digestive vacuole, the site of haemoglobin digestion and chloroquine action. Early secondary structure predictions and homology modelling predicted that PfCRT is a transporter (Fidock et al., 2000; Martin and Kirk, 2004), and recent biochemical and pharmacological studies lend support to the hypothesis that mutant PfCRT can transport chloroquine out of the digestive vacuole (Summers and Martin, 2010; Baro et al., 2011; Sanchez et al., 2011; Griffin et al., 2012; Papakrivos et al., 2012). Nevertheless, more than a decade after its discovery, the native function of PfCRT remains unknown. Failed gene targeting attempts in *P. falciparum* and the mouse malaria parasite, *Plasmodium berghei*, suggest that this function is essential, at least in the parasite's asexual blood stages (Waller et al., 2003; Ecker et al., 2011). Intriguingly, PfCRT peptides have also been detected by mass spectroscopy in sporozoites (Florens et al., 2002), an extracellular stage that neither possesses a digestive vacuole nor digests haemoglobin.

To gain insight into the functional requirement for CRT across the parasite lifecycle, we made use of a conditional knockout (cKO) system recently optimised for *P. berghei* by the Ménard laboratory (Combe et al., 2009; Lacroix et al., 2011). This method is based on the Flp recombinase/Flp recognition target (Flp/*FRT*) site-specific recombination system from yeast, involving Flp recombinase-mediated recognition of two 34 bp *FRT* sites leading to excision of the intervening DNA (referred to as the *FRT*ed sequence). For *P. berghei*, this cKO approach begins by replacing the endogenous 3′untranslated region (UTR) of the targeted gene with a *FRT*ed sequence comprising a 3′UTR sequence from the thrombospondin related adhesive protein (*trap*; PlasmoDB ID: PBANKA_134980) gene followed downstream by the human dihydrofolate reductase (h*dhfr*) selectable marker. This double crossover homologous recombination event is undertaken in parasite strains that harbour an integrated copy of Flp recombinase that is expressed under a mosquito stage-specific promoter such that passaging of these parasites to *Anopheles* mosquitoes initiates excision of the *FRT*ed 3′UTR. This leaves the gene of interest with no 3′UTR, thereby destabilising transcripts and generating a functional KO. The power of this cKO system was initially demonstrated by Combe et al. (2009) who used it to silence *msp1* (essential for parasite invasion of erythrocytes) and uncovered a second role for this gene in the formation of merozoites in infected hepatocytes. More recently this system was applied to conditionally silence

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the parasite invasion molecules AMA1 and RON4, revealing that the former is important only for merozoite invasion of erythrocytes whereas RON4 is required for sporozoite invasion of hepatocytes (Giovannini et al., 2011). This system was also used to conditionally inactivate the *P. berghei* cGMP-dependent protein kinase (PKG), leading to the developmental arrest of late liver stage parasites and the demonstration that these stages can induce potent immunity against sporozoite challenge (Falae et al., 2010).

To conditionally silence the *P. berghei* ortholog of *pfcrt* (known as *pbcrt*; PlasmoDB ID: PBANKA_121950), we first engineered the p*Pbcrt*-cKO transfection plasmid to replace the endogenous *pbcrt* 3′UTR with the *FRT*ed *trap* 3′UTR and h*dhfr* marker (Fig. 1A). Briefly, the last 0.6 kb of the *pbcrt* coding sequence was PCR-amplified using primers p1 + p2 (listed in Table 1); and the first 0.6 kb of *pbcrt* 3′UTR was amplified using primers p3 + p4. These fragments were cloned into p3′TRAP_h*dhfr_FRT* (Combe et al., 2009) via *Sph*I and *Not*I (for the coding sequence) or *Hin*dIII and *Sph*I (for the 3′UTR). Notably, our p*Pbcrt*-cKO plasmid unintentionally lacked the first 12 nucleotides of the *trap* 3′UTR compared with the plasmid used by Combe et al. (2009) (see below). *Sph*I-linearised plasmid was then electroporated into asexual blood stage parasites of two "deleter" strains, TRAP/FlpL or UIS4/Flp. These recipient strains express either Flp recombinase or the thermolabile version FlpL (Lacroix et al., 2011). In the TRAP/FlpL strain, recombinase expression is driven by the *trap* promoter, which becomes active in maturing oocysts and peaks in salivary gland sporozoites (Rosinski-Chupin et al., 2007). The UIS4/Flp deleter strain controls Flp expression using the *uis4* promoter, which becomes highly upregulated in salivary gland sporozoites (Matuschewski et al., 2002).

Deleter strain parasites electroporated with the p*Pbcrt*-cKO plasmid were inoculated into CD-1 (Charles River) mice and subjected to two rounds of in vivo drug pressure with the parasite

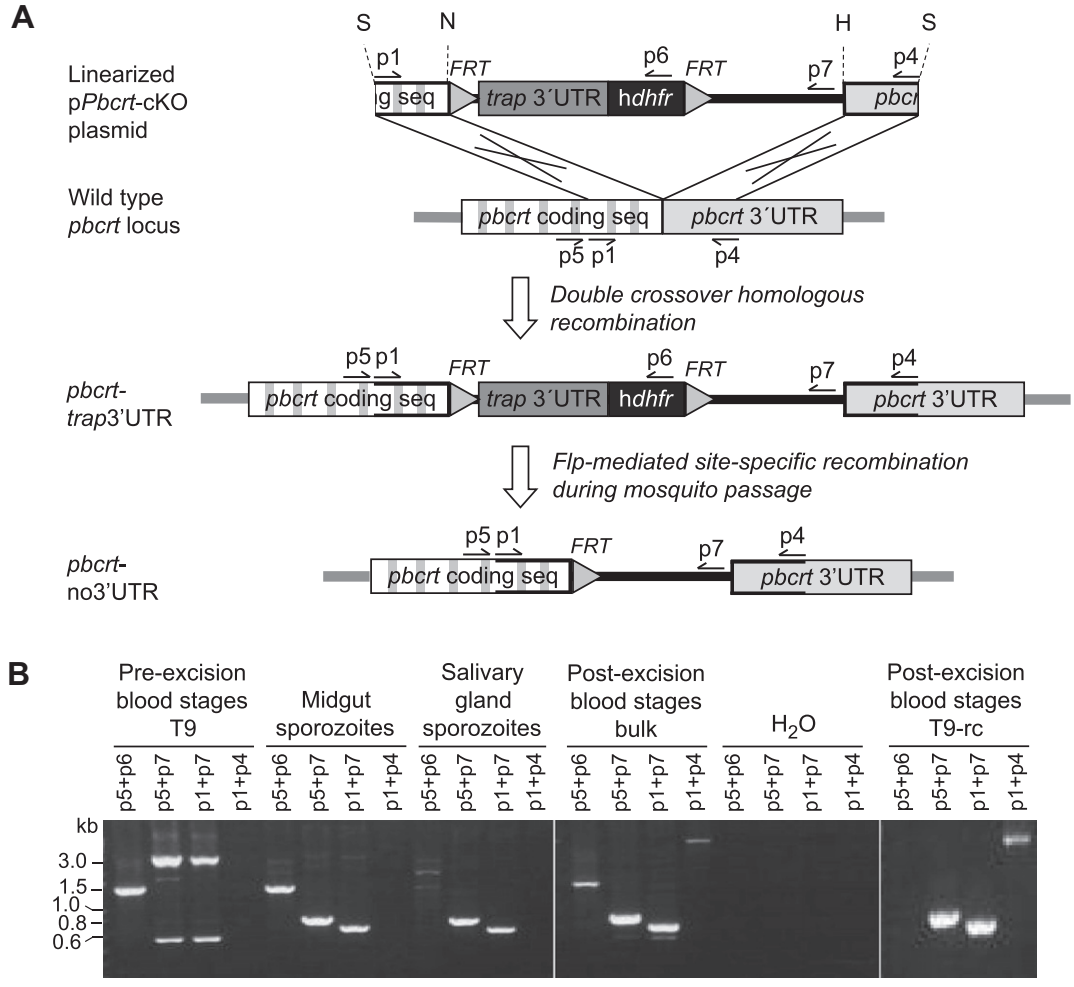

Fig. 1. Generation of *Plasmodium berghei* chloroquine resistance transporter (*pbcrt*) conditional knockout (cKO) parasites via 3′untranslated region (UTR) excision mediated by Flp/*FRT* site-specific recombination. (A) Schematic of the double crossover event between the linearised transfection plasmid and the endogenous *pbcrt* locus, resulting in the pre-excision *pbcrt-trap* 3'UTR locus and the post-excision *pbcrt*-no3'UTR locus. The structure of the 13-exon *pbcrt* gene is shown stylistically, with introns in grey and exons in white. The thick black line denotes the plasmid backbone. Flp, Flp recombinase; FRT, Flp recognition target; *trap*, thrombospondin related adhesive protein; h*dhfr;* human dihydrofolate reductase selectable marker. H, *Hin*dIII; N, *Not*I; S, *Sph*I. (B) PCR analysis of the *pbcrt* locus pre- and post-excision. The non-excised locus was detected using primers p5 + p6 (that yield a 1.5 kb band). The excised locus, post mosquito passage, was detected using primers p5 + p7 (yielding a 0.86 kb product), and primers p1 + p7 (0.73 kb). These primer pairs can also amplify the non-excised locus, yielding 3.1 and 3.0 kb products, respectively, in the pre-excision parasites (the presence of the 0.6 kb band in those lanes and in the post-excision bulk blood stages was determined by sequencing to be off-target amplification of the mouse integrin α-8 gene). Absence of the wild-type *pbcrt* locus was confirmed in all recombinant parasites using primers p1 + p4 (that yield a 1.2 kb product in wild-type parasites; data not shown). These primers yielded a large (> > 3 kb) product spanning the plasmid backbone in the *pbcrt*-no3′UTR locus in post-excision blood stage parasites (this product would theoretically also be present in sporozoites with an excised locus, but it is difficult to amplify a product of this size from the low yield of parasite genomic DNA that can be obtained from infected mosquitoes). Excision of the *trap* 3′UTR was not observed in blood stage parasites prior to mosquito transmission, but was evident in midgut and salivary gland sporozoites. Following transmission to naïve mice, most but not all blood stage parasites had excised the *FRT*ed *trap*3′UTR locus. The re-cloned post-excision parasites T9-rc and U4-rc harbour the excised locus (*pbcrt*-no3′UTR) exclusively.