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Short communication

Spontaneous excision and facilitated recovery as a control for phenotypes arising from RNA interference and other dominant transgenes

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ABSTRACT

An essential control for genetic manipulation of microbes is the regeneration of the wild-type state and phenotype to validate that any mutant phenotypes are 'on target'. For *Leishmania* gene knockouts, this is often done by re-expression of the target gene from episomal vectors, often bearing counter-selectable markers. Methods for similarly validating the outcomes from dominant mutations such as those arising from RNA interference (RNAi) are needed. We present here such an approach, relying on facilitated recovery after spontaneous excision – or 'popouts' – of dominant transgenes stably inserted into the ribosomal RNA array, utilizing GFP as a marker and single cell sorting to recover regenerated WT controls. We validate its utility using RNA interference knockdowns of the paraflagellar rod gene *PFR2* of *L. (Viannia) braziliensis*. The method yields stably modified lines suitable for long term studies of *Leishmania* virulence, relies solely on host rather than introduced genetic machinery, and is thus readily applied in many species and circumstances including functional genetic testing.

Genetic tools in *Leishmania* have focused largely on methods yielding stably modified lines suitable for analysis of virulence in animal models, experiments which can often take more than 3 months. As in all other microbes, growth of parasites in culture can lead to unanticipated changes, including loss of virulence [1] and the procedures inherent to genetic manipulation can induce unplanned and/or off-target alterations as well [2]. In *Leishmania*, the significant potential for 'off-target' effects can confound the interpretation of genetic alterations, and thus reconstitution of the wild-type genotype and phenotype are considered vital controls to establish that any effects seen in engineered mutants are genetically on target [3].

In *Leishmania*, gene knockout complementation controls are typically performed by introduction of an episomal plasmid expressing an ectopic gene copy, either directly after ablation of all chromosomal copies, or prior to ablation of the chromosomal copies followed by chromosomal ablation and plasmid segregational loss [see 4, 5 for examples]. Several commonly used episomal vectors bear counter selectable drug resistance or fluorescent markers, such as thymidine kinase or GFP, which additionally facilitate tests of gene essentiality [6,7]. As episomal vectors can be lost at varying rates in the absence of continuous drug pressure [8], or show expression levels or patterns different from the endogenous gene, the ectopic gene copy can also be introduced into chromosomal locations such as the 18S ribosomal RNA locus or its native location.

While the approaches above address the requirements for recessive 'loss of function' mutations, there is a need for similar controls for stable dominant genetic alterations, such as those arising from RNA interference (RNAi) or dominant negative point mutations, where the high level of expression frequently required is often obtained through integration into the ribosomal RNA locus [9]. Moreover, episomal vectors may function poorly in *Leishmania* bearing an active RNAi pathway, as in *Viannia* sp. such as *L. braziliensis* and *L. guyanensis*; this pathway has been lost in most other *Leishmania* species [9].

To address this need, we developed a quasi-segregational approach similar to that used for episomal positive/negative vectors such as pXNG [6]. In this facilitated excisional or 'popout' approach, dominant-acting transgenes are first integrated along with a GFP marker into the genome and their phenotype assessed. Then, lines that have spontaneously excised the dominant transgene from the genome are recovered, in order to establish that they recapitulate the wild-type starting point. To avoid the need for additional machinery, such as enzymes mediating site-specific recombination/excision, we capitalized on the intrinsic genetic dynamism of repetitive arrays such as the ribosomal RNA locus. At some frequency, recombination and/or gene conversions amongst genes within such loci will spontaneously yield construct-free variants, which can be recovered by appropriate

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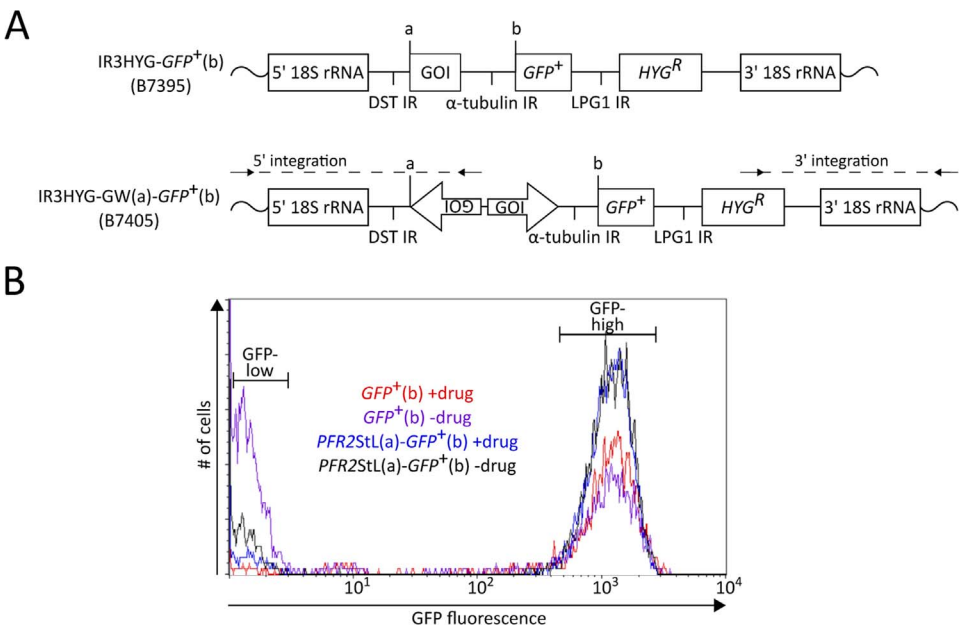

Fig. 1. Generation of *PFR2StL*(a)-*GFP*$^+$(b) parasites. (A) Schematic of IR3-*GFP*$^+$(b) (B7395) and IR3-GW (a)-*GFP*$^+$(b) (B7405) constructs carrying resistance to hygromycin B (*HYG*^{R}). Constructs carry flanking sequences from the 18S rRNA locus that enables integration into the parasite genome by homologous recombination and intergenic regions (IR) carrying splice acceptor sites that permit proper *trans*-splicing and polyadenylation of transcribed RNA. The *GFP* ORF is inserted into the 'b' cloning site, while the 'a' site remains available for cloning of a gene of interest (GOI) or an RNAi transgene targeting the GOI. Plasmid sequences have been deposited in GenBank under the accession numbers MG725339 (pIR3HYG), MG735671 (pIR3HYG-*GFP*$^+$(b)), MG735672 (pIR3HYG)-GW(a), and MG737713 (pIRHYG-GW(a)-*GFP*$^+$(b)). Small black arrows indicate the location of genotyping primers and dashed lines denote PCR amplicons used to confirm loss of excision of construct. Primers used were: 5′ integration, 5′-ACATCA GACGTAATCTGCCGC/5′-CGCTAGTCTAGATTACGCTACGCAGAAAGAGAAG; and for 3′ integration, 5′-CGCTAGTCTAGAGCCGTCC TCCACCTCCTCCGCG/5′-CGACTTTTGCTTCCTCTA TTG. Note that the 5′ integration reverse primer shown was chosen to be specific for the gene of interest (*PFR2* here). PCR products were run on 1% agarose gel and visualized with ethidium bromide staining (not shown). (B) Withdrawal of drug selection from cells containing an integrated *GFP*$^+$(b) or *PFR2*StL(A)-*GFP*$^+$(b) construct allows for the accumulation of a GFP-negative population. *L. braziliensis* strain M2903 (MHOM/BR/75/M2903) was grown in fresh Schneider's Insect Medium supplemented with 10% heat-inactivated fetal bovine serum, 100 μM adenine, 5×10^{-5}% hemin, 2 μg/mL biopterin, 2 mM L-glutamine, 500 units/ml penicillin and 50 ug/mL streptomycin. Transfections of promastigote cells were performed as described previously [9,11] and cells were plated on semisolid media composed of 1% agar in M199 containing 15 μg/mL hygromycin B (Gold Biotechnologies). Clonal lines were passaged in media containing 10 μg/mL hygromycin B. To allow deletion of the popout constructs, cells were passaged in Schneider's medium lacking selection and GFP expression was monitored by flow cytometry. Parallel cultures under drug selection were used as a comparison. Shown is one representative clone out of three examined for each construct after two passages (12 cell doublings) following withdrawal of drug selection. Clonal lines were obtained by single-cell sorting. Log-phase cells were resuspended in phosphate-buffered saline, passed through a CellTrics 50 μm filter (Partec) to remove clumps, and single cells were recovered on the basis of GFP expression using a Beckman Coulter MoFlo cell sorter. Individual cells were placed into wells of a 96-well plate containing 200 μL of Schneider's Insect Medium containing no selective antibiotic (GFP-negative cells) or 10 μg/mL hygromycin B (GFP-positive cells) and incubated at 27 °C for 10 days before parasite growth was scored. Wells were expanded to 5 mL with or without antibiotic, as appropriate, and passaged thusly.

selections [10]. In our studies we have favored the use of green fluorescent protein (GFP) markers and fluorescence-activated cell sorting (FACS) recovery of single cells, which is rapid, yields clonal lines directly, and avoids treatment with potentially mutagenic nucleoside analogs targeting several counter-selectable markers, such as thymidine kinase.

As proof of principle, we introduce and illustrate the use of the vector pIR3HYG-*GFP*$^+$(b) for 'popouts' of RNAi knockdowns of the paraflagellar rod protein 2 *(PFR2,* LBRM_16_1480), confirming first both loss of *PFR2* expression and paraflagellar rod structure, and the re-emergence of wild-type phenotypes following selection of popouts exhibiting construct loss.

We generated a modified version of the SSU rRNA integrating vectors pIR1/2 [9,11] termed pIR3HYG (strain B7093). As for other pIR vectors, pIR3 bear two expression sites (a and b) and a drug resistance marker, flanked by ~1 kb of SSU rRNA sequence, whose termini are exposed by *Swa*I digestion to promote homologous integration into any *Leishmania* species (Fig. 1A). pIR3 differs from the related pIR1 and pIR2 vectors by the presence of a full length α-tubulin intergenic region of *L. braziliensis* M2903 separating the two expression sites. In other experiments not shown, we found that the level of expression of reporter constructs integrated into the 'a' or 'b' sites was comparably high in *Viannia* as well as *Leishmania* species.

pIR3HYG-GW(a) (B7381) was constructed by inserting the full-length *L. braziliensis* α-tubulin intergenic region between the two expression sites of pIR2HYG-GW(a). This plasmid contains a "GW" segment that enables rapid cloning of an inverted repeat (stem-loop, StL) from a starting donor vector utilizing Gateway™ site-specific recombination technology (Invitrogen, Thermo Fisher Scientific). We previously used this strategy successfully to trigger transgene-driven RNAi in *Leishmania (Viannia)* species [9,12] and here used it to generate pIR3HYG-*PFR2StL*(a)-*GFP*$^+$(b) (B7608). pIR3HYG-*GFP*$^+$(b) (B7395) and pIR3HYG-GW(a)-*GFP*$^+$(b) (B7405) were constructed by insertion of the GC-rich *GFP*$^+$ ORF [13] into the "b" cloning site of pIR3HYG and pIR3HYG-GW(a), respectively. Following *Swa*I digestion, pIR3HYG-*GFP*$^+$(b) or pIR3HYG-*PFR2StL*(a)-*GFP*$^+$(b) were electroporated into *L. braziliensis* M2903, cells were plated on semisolid media containing hygromycin B (see Fig. 1 legend), and transfectant colonies emerged within 2 weeks, in similar numbers.

GFP flow cytometry showed similarly high levels of expression in all *SSU:IR3HYG-GFP*$^+$(b) and *SSU:IR3HYG-StL*(a)-*GFP*$^+$(b) lines examined (Fig. 1B) and the cell pellets were visibly green. As expected, quantitative RT-PCR tests showed that *PFR2* mRNA expression was greatly reduced in the *PFR2* StL transfectants (Fig. 2A). Similarly, these showed alterations in paraflagellar rod formation and structure seen in previous RNAi and gene knockout studies (Fig. 2B), and severe alterations in swimming behavior [9,14]. Specifically, the bulk of the paraflagellar rod structure was absent from both longitudinal and transverse sections (9/10 and 11/11 cells examined, respectively) of the flagellum, and cells were unable to swim in a forward direction, instead "tumbling" in place.

*PFR2*StL(a)-*GFP*$^+$(b) or *GFP*$^+$(b) transfectant lines were then grown without drug pressure for 2 passages (roughly 12 cell doublings) to allow emergence of cells showing spontaneous loss of the *SSU:IR3HYG* constructs from the ribosomal gene array. In cultures in which drug selection was maintained, 81–98% of cells expressed high levels of GFP, whereas in cultures in which drug selection was withdrawn, only 40–96% of cells expressed high levels of GFP. Fig. 1B shows representative traces, with selective drug-containing cultures containing 97% and 94% GFP-positive cells and drug-free cultures containing 62% and 81% GFP-high cells for *GFP*$^+$(b) and *PFR2StL*(a)-*GFP*$^+$(b) populations, respectively. From both construct transfectants, we gated for 'high' or 'low' GFP expression (Fig. 1b) and sorted single cells into individual 96 well microtiter plate wells containing Schneider's media,