Joining forces: first application of a rapamycin-induced dimerizable Cre system for conditional null mutant analysis in Leishmania.

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Joining forces: First application of a rapamycine-induced dimerizable CRE system (DiCre) for conditional null mutant analysis in *Leishmania*

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Abstract. Reverse genetics in *Leishmania* spp has gained importance beyond basic research as efforts increase to discover and validate new drug targets. Often, the most promising targets are essential for viability of the parasites, defying a genetic analysis by current gene replacement strategies. In this issue of Molecular Microbiology, Duncan et al. demonstrate the applicability of DiCre recombination in *Leishmania* for induced replacement of the kinase CRK3 gene in promastigotes. DiCre gene replacement leads to the rapid loss of the gene and allows monitoring the phenotypic effects of the loss of function, eliminating the need for prolonged cultivation and selection. Implementation of the DiCre approach will allow functional genetics of the most important of *Leishmania* genes and is likely to boost genetic research and drug target discovery.

Challenges in *Leishmania*. Of the over 8000 predicted genes in the *Leishmania genome*, over 40% code for hypothetical proteins with unknown function. Other genes are annotated based on conserved functional domains or homology to orthologous genes from other organisms. Given the evolutionary distance of early branching trypanosomatids to crown group eukaryotic model organisms, such sequence-based predictions of gene function require experimental

validation, typically by reverse genetics where a null mutant of the gene of interest (GOI) will have a defined phenotype (Cruz et al., 1991). However, in spite of its enormous impact on our understanding of *Leishmania* biology and infectivity, the null mutant approach is strongly has important limitations. First, the genome of *Leishmania* spp is highly unstable and undergoes frequent gene and chromosome amplification (Haimeur et al., 2000, Singh et al., 2001, Leprohon et al., 2009, Rogers et al., 2011, Mannaert et al., 2012, Ubeda et al., 2014) that may compensate null mutant phenotypes thus masking the true phenotype. Second, null mutant studies fail to inform on the functions of essential genes as their deletion causes a lethal phenotype. In addition parasites often develop compensatory gene amplification and heteroploidity when essential genes are targeted for replacement (Mottram et al., 1996, Hassan et al., 2001a, Hassan et al., 2001b, Agron et al., 2005). Unlike in *T. brucei*, where essential gene function can be directly investigated through conditional RNA interference analysis (Bastin et al., 2000, Ngo et al., 1998, Shi et al., 2000), the lack of RNAi key enzymes precludes this approach in Old World leishmaniae. Also the absence of robust and tightly controlled inducible expression systems in *Leishmania* so far precluded the study of essential genes and novel drug targets by conditional null mutant analysis. A variety of alternative molecular, chemical-genetics and pharmacological approaches was developed to overcome these limitations.

Molecular genetics approaches. Gene replacement by homologous recombination (Figure 1A) was first established in *Leishmania* spp by the laboratory of Steve Beverley (Cruz et al., 1991). Deletion of both alleles of a GOI was achieved by sequential electroporation of linear constructs comprising two different antibiotic resistance genes flanked by the 5' and 3' untranslated regions, and subsequent *in vitro* selection for successful recombination events in antibiotic-supplemented medium. Since then this technology has been widely applied in all major protozoan parasites, and has significantly advanced our understanding of suspected *Leishmania* transporters, signalling proteins, chaperones, or virulence factors (Turco et al., 2001, Mottram et al., 2004, Wiese, 2007, Ommen & Clos, 2009).

This approach often fails to generate null mutants, suggesting that the GOI may be essential, e.g. with the *L. mexicana* MAPKs LmxMPK4 and LmxMPK6, for which gene replacement could only be achieved in the presence of an episome expressing the GOI (Wang et al., 2005,

Wiese et al., 2009). However, this approach (Figure 1B) in itself does not conclusively prove the essentiality of the GOI since transgenic over expression of the GOI may simply increase fitness and facilitate growth *in vitro*.

To establish a better genetic proof of essentiality, a facilitated knock system has been introduced that is based on the episomal expression of the GOI from a vector that carries the *Herpes simplex* virus thymidine kinase (*HSV*-TK) for counter selection (Figure 1C) under the anti-viral drug ganciclovir (GCV) (LeBowitz et al., 1992, Muyombwe et al., 1997, Morales et al., 2010, Feng et al., 2013). Essentiality of the GOI is established by the stability of the transgene under GCV selection (Dacher et al., 2014). Using a plasmid shuffle approach this system even allowed a structure function analysis revealing two essential phosphorylation sites in the *L. donovani* co-chaperone STI1 (Morales et al., 2010) and parasite-specific sequence elements in the *L. major* MAP kinase MPK4 (Dacher et al., 2014). However, the persistence of GOI expression despite negative selection usually precludes the development of a null mutant phenotype for essential genes.

Chemical genetics approaches. When targeting multi-copy GOIs, homologous recombination is often not feasible, but a pharmacological knock-down may be considered if inhibitors are available for the protein of interest (POI). This approach is feasible in all life cycle stages and even permits gradual deactivation of a POI in dose response experiments. The resulting phenotypes are conditional and reversible. Examples of inhibitor-induced phenotype analysis targeted the *Leishmania* arginase (Iniesta et al., 2001), the heat shock protein 90 (Wiesgigl & Clos, 2001) and the MDR1 P-glycoprotein (Perez-Victoria et al., 2006). To establish the specificity of inhibitors, an over expression of the GOI and a concomitant reversion of the inhibitor-induced phenotype can be attempted (Wiesgigl & Clos, 2001), a strategy shared with homologous recombination-based strategies.

Specificity can be controlled in cases where the GOI is rendered inhibitor-sensitive by targeted mutagenesis and expressed in place of the natural gene. The inhibitor-sensitised transgene can then be compared with the wild type, e.g.in the analysis of the *L. mexicana* mitogen-activated protein kinase 1 (Melzer, 2007).

Another approach is the expression of a inhibitor-resistant transgene. The specificity of the HSP90-specific inhibitor radicicol was shown by expressing a resistant variant, HSP90rr, in *L. donovani*. Under challenge with radicicol, only parasites expressing HSP90rr showed growth (Hombach et al., 2013), thus excluding off-target interactions as cause for drug-induced growth arrest and stage conversion. This system also allowed the expression and conditional phenotype analysis of additional mutations with HSP90rr.

The expression of a fusion protein of POI and a destabilising domain (ddFKBP) allows a conditional depletion of the POI. The ddFKBP promotes proteasomal degradation of the fusion protein (Banaszynski et al., 2006), but this effect is much reduced in the presence of the rapamycin analogue Shld1. A double-allelic replacement of an essential gene can be performed in the presence of a POI-ddFKBP-coding transgene and in the presence of Shld1. Withdrawal of Shld1 will unmask the ddFKBP and the fusion protein will be degraded. This conditional knock-down will then allow to observe the effects of POI depletion. The ddFKBP-mediated degradation depends on the structure of the POI and the position – N-terminal or C-terminal – of the ddFKBP. If (over)expressed from an episomal transgene, even the reduced POI abundance after Shld1 withdrawal may exceed that of an endogenously coded protein. Lastly, the functionality of the fusion protein may be compromised by the ddFKBP addition, in particular when function resides within the N- or C termini of the POI.

The CRE/Lox system. First described in the late 1980s (Sauer, 1987, Sauer & Henderson, 1988), the Cre-Lox system has been used *in vitro* and *in vivo* to facilitate efficient and inducible recombination between two Lox sequence elements. The gene for the recombinase, Cre, is placed under an inducible promoter to achieve ligand-controlled or tissue/development stage-specific recombination for inducible gene deletion. However, the leakiness and/or cell type restriction of promoters limited the usability of this system. The advent of the DiCre technology eliminated most of these shortcomings. Splitting the Cre protein into its two functional domains and fusing them to the FKBP12 (FK506-binding protein) and FRB (binding domain of the FKBP12-rapamycin associated protein) respectively results in two inactive domains. These separate domains can be joined and activated by adding rapamycin or one of its analogues,

resulting in Cre-dependent recombination between LoxP sequence elements (Jullien et al., 2003).

The DiCre system has been successfully employed in the apicomplexan parasite *Plasmodium falciparum* (Collins et al., 2013) where a high efficiency (100%) was achieved. Another example was the conditional expression of an apical membrane protein (PfAMA1), showing 80% reduced expression within a single intra-erythrocytic amplification cycle, and demonstrating its crucial role in the merozoite stage (Yap et al., 2014). Cre-Lox-dependent recombination was also applied successfully in another apicoplexan, *Toxoplasma gondii* (Andenmatten et al., 2013, Bouchut et al., 2014).

In this issue of Mol. Microbiol. (Duncan et al.), a first report of a DiCre-mediated conditional gene replacement in a *Leishmania* parasite is presented. *Leishmania* with its lack of defined promoters and transcriptional regulators (Clayton, 2002) did not lend itself to the application of classic Cre-Lox recombination. The approach (Figure 1D) requires very low background expression of Cre to prevent unwanted, low level recombination from occuring (Jullien et al., 2003). Tetracycline-dependent expression of intrachromosomal or episomal transgenes was reported for *L. infantum* (*L. chagasi*) and for *L. mexicana* (Yao et al., 2007, Kraeva et al., 2014), but their usefulness for controlling Cre expression has not been explored so far.

In the paper by Duncan et al., the authors target a protein kinase gene, Cdc2-related kinase 3 (CRK3), a gene that was previously shown to be essential for cell cycle progression using homologous recombination mediated gene replacement (Hassan et al., 2001b). One natural CRK3 allele is replaced with a DiCre expression cassette, including an antibiotic resistance marker gene. The second allele is then replaced with CRK3::GFP fusion gene flanked by LoxP sequence elements ($CRK3^{Flox}$). In the absence of rapamycin, the resulting gene replacement mutants showed normal in vitro proliferation. As expected, the rapamycin-induced dimerisation of the Cre moieties (DiCre) caused a rapid cessation of proliferation, a G2 cell cycle arrest and increased hypodiploidity, the latter indicating DNA degradation. The analysis was also carried out in the presence of wild type and mutant CRK3 transgenes, showing that only the wild type transgene could restore growth under rapamycin.

The potential of the diCRE system and its limitations. The impact of the report by Duncan et al. is less about the function of CRK3, which was expected based on previous analyses (Grant et al., 2004), but more about the establishment, validation, and successful application of this novel and very useful tool for the phenotypic analysis of essential genes. In addition, this system holds the promise to study the stage-specific function of essential genes given that excision of the GOI can be achieved in amastigotes ex vivo. However, despite the potential of the DiCre approach, the broad applicability of the DiCre system will depend on how frequently the phenotypes of inducible null mutants will point at gene function when genes of unknown or unconfirmed function are examined. Second, the DiCre approach is likely not usable for some of the biologically most important genes which are organised in multi-copy arrays (Rogers et al., 2011). Third, use of DiCre will not prevent compensatory genetic reorganisation when the null mutants are studied in the longer term. And finally, rapamycin inhibits TOR kinases that have been implicated in *Leishmania* cell viability, acidocalcisome biogenesis, and infectivity (Madeira da Silva & Beverley, 2010), and thus may cause secondary phenotypic effects in null mutants that may not observable in the wild-type control. Nevertheless the DiCre system published in this issue by Duncan et al. represents an important milestone for reverse genetics of essential *Leishmania* genes. Combining this conditional null mutant system with new gene editing possibilities provided by the CRISPR/cas9 system that has been recently applied to *Leishmania* (Sollelis et al., 2015, Zhang & Matlashewski, 2015) will pave the way for improved functional genetics of these important human pathogens.

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Figure Legend:

Fig. 1: Schematic representation of different *Leishmania* knock out strategies. The endogenous alleles of the gene of interest (GOI) are sequentially replaced by targeting constructs containing

two different antibiotic resistance cassettes (black rectangles, *ab1* and *ab2*) that are flanked by 5' and 3' UTR regions of the GOI (not indicated) allowing for homologous recombination. Deletion of essential genes causes lethality (indicated by dotted outline of a parasite in a). Null mutant survival can be rescued by over-expression of the GOI from an episomal vector (b and c). The herpes simplex virus thymidine kinase (TK) allows for negative selection against the episome that can be monitored following the levels of GFP expression (c). For conditional null mutant analysis by Dimerizable Cre (DiCre, d), the first allele of the GOI is replaced by a targeting construct comprising the two inactive Cre moieties fused to FKBP12 (FK506-binding protein) and FRB (binding domain of the FKBP12-rapamycin associated protein), and a first selectable antibiotic resistance cassette (*ab1*). The second allele is replaced by a targeting construct containing the GOI and a second selectable antibiotic resistance cassette (*ab2*) that is flanked by loxP sites to allow for Cre-mediated excision. Hetero-dimerization of FKBP12/FRB in the presence of rapamycin reconstitutes active Cre leading to excision of the second allele of the GOI and establishment of the homozygous null mutant.