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Review

Recent advances in *Leishmania* reverse genetics: Manipulating a manipulative parasite

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ABSTRACT

In this review we describe the expanding repertoire of molecular tools with which to study gene function in *Leishmania*. Specifically we review the tools available for studying functions of essential genes, such as plasmid shuffle and DiCre, as well as the rapidly expanding portfolio of available CRISPR/Cas9 approaches for large scale gene knockout and endogenous tagging. We include detail on approaches that allow the direct manipulation of RNA using RNAi and protein levels via Tet or DiCre induced overexpression and destabilization domain mediated degradation. The utilisation of current methods and the development of more advanced molecular tools will lead to greater understanding of the role of essential genes in the parasite and thereby more robust drug target validation, thereby paving the way for the development of novel therapeutics to treat this important disease.

1. Introduction

Genetics is a powerful tool for understanding the molecular mechanisms that regulate *Leishmania* life cycle progression and host-parasite interactions [1]. Despite some notable exceptions [2], forward genetic approaches have proved challenging in *Leishmania* due to supernumerary chromosomes [3,4] and the complexities of carrying out sexual crosses within the sandfly [5], which has hindered positional cloning as a method for characterising genes generating specific phenotypes. In contrast, the availability of *Leishmania* genome sequences [6,7], readily searchable using the online resource TritrypDB [8] and a decent toolkit for reverse genetic manipulation of the parasite [1,9] has enabled the study of non-essential genes encoding proteins that facilitate infection and identified essential genes that regulate fundamental cellular processes. For the most part these studies have focussed on loss of function analysis using genetic manipulation of individual genes by exploiting the inherent propensity of *Leishmania* to undergo homologous recombination to insert transgenes into the genome. Gene replacement is performed by transfection of procyclic promastigotes with a linear vector containing a drug resistance cassette flanked by arms of homology for the target gene locus [10]. For single copy genes on disomic chromosomes two rounds of transfection with independent drug selectable markers are generally required to generate a null mutant [10], although loss of heterozygosity can result in a null mutant in one round of transfection [11]. Gene deletion mutants can then be evaluated for their ability to replicate in culture as procyclic promastigotes, differentiate to metacyclic promastigotes and axenic amastigotes or infect macrophages, animals or sandflies. Direct transfection of axenic amastigotes is a feasible but rarely performed method [12].

Despite the efficacy of *Leishmania* gene replacement with transgenes containing flanks homologous to the endogenous gene locus, the approach is limited to 'proof of principle' when replacing genes that are essential to promastigote viability. In such instances the genomic plasticity of *Leishmania* can result in the selection of aneuploidy, or cells with extrachromosomal elements that retain a copy of the target gene despite replacement [13,14]. The selection of such cells provides supporting evidence of an important role for the gene, but is not sufficient to prove essentiality or to study the function of essential genes. Expression of an ectopic copy of the target gene from an episome, or via integration into the genome, enables replacement of endogenous gene copies without the selection of clones with altered ploidy, retention of this episome in the absence of selection can provide evidence that a gene is required for proliferation. Episomal complementation can also be used as an elegant means to probe essentiality *in vivo* as recovery of parasites retaining the episome after murine infection evidences the selective pressure exerted on amastigotes to express the essential gene from the plasmid [15,16]. A number of vectors are available for overexpression that either integrate into the ribosomal locus and provide constitutive expression of a gene of interest [17,18], or function as episomes [19]. Overexpression of a gene can also give key insights as to

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the biological role of an essential gene when dominant negative phenotypes arise. Overexpression may also be applied to mode of action studies, for example where increased protein levels render cells susceptible to a drug by increased activation of a prodrug to a cytotoxic metabolite, such as the bio-activation of bicyclic-nitro drugs by the *L. donovani* nitroreductase NTR2 [20]. Equally, overexpression can enable the assessment of drug resistance mechanisms, and has recently been employed to elucidate targets conferring resistance to a variety of anti-leishmanial compounds following expression of *Leishmania* genes from cosmids [21].

In recent years methods for reverse genetic modification of *Leishmania* for elucidating gene function have expanded, primarily due to the adaption of existing systems previously utilised in model organisms such as yeast for regulating essential gene, transcript and protein expression. This review will evaluate these new methods and speculate how future advances will enhance our ability to investigate these organisms further.

2. New tools for assessing if *Leishmania* genes are essential for cellular proliferation

Since the establishment of transfection and homologous recombination to conduct gene replacement in *Leishmania*, proteins have been identified which are essential to the survival of the parasites. Essentiality can manifest itself in a variety of ways; a lethal phenotype in all life-cycle stages [22,23], a conditional lethal phenotype where cell survival is dependent on growth conditions such as nutrient availability [24], or essentiality in one life cycle stage but not another [24–26]. The inability to generate a null mutant can imply essentiality and has been interpreted as the encoded protein being a genetically validated drug target, however by adhering to these criteria a gene can only be provisionally identified as a drug target. Functional analysis of proteins encoded by essential genes through conditional regulation of gene expression is therefore highly desirable in *Leishmania* to facilitate the phenotypic validation of proteins as drug targets, particularly if utilised in a disease context such as murine infection or infection of human macrophage. Until recently the prospect for performing such studies in *Leishmania* were severely limited; fortunately the molecular toolkit for regulating gene, transcript and protein expression in *Leishmania* has expanded in recent years. This is particularly important for the genetic drug target validation.

2.1. Plasmid shuffle

A more refined approach to test if a gene is essential has been adapted from fission yeast for use in *Leishmania*. This 'plasmid shuffle' methodology expands on episomal complementation to confer negative selection against expression of the transgene; expression in tandem with a thymidine kinase (TK) 'suicide' cassette confers sensitivity to ganciclovir (GCV) [27] (Fig. 1A). Active TK synthesises GCV into the toxic metabolite GCV triphosphate which inhibits DNA synthesis, thereby exerting strong selective pressure for the loss of the plasmid. The inclusion of positive marker cassettes for drug resistance and green fluorescent protein (GFP) facilitate clonal selection and downstream gene expression analysis. By this method, a negative selection line can be generated by episomal complementation and subsequent endogenous gene replacement. Both the *L. major DHCH* gene encoding a dual-function protein involved in 10-Formyl-THF metabolism [27] and the *L. donovoni* CYP51 genes have been identified as essential to promastigote survival by their retention on a *TK* expression plasmid in the presence of GCV treatment [28].

A further development of this technique enables the generation of 'partial' essential gene null mutants for functional analysis of protein domains by complementation by loss of the TK plasmid and subsequent retention of a second, TK absent plasmid encoding a mutated copy of the gene [29,30] (Fig. 1A). The application of plasmid shuffle for the identification of residues essential for protein function represents an important advance. In the first of these studies [29] phosphoproteomic analysis identified a complex implicated in the regulation of the heat-shock response elicited when promastigotes are inoculated into the mammalian host. An amastigote specific chaperone complex STI1/HOP was identified and subsequently manipulated by endogenous *STI1* deletion in the presence of an episomal copy in array with the TK and GFP cassettes. After negative selection with GCV the plasmid was retained and the encoded protein established as essential. Further complementation of the plasmid shuffle line was conducted with a repertoire of secondary episomes containing different *STI1* genes that had been mutated at potential phosphorylation sites. Negative selection following complementation with five such mutated sequences enabled the identification of two phosphoserine residues essential for the function of the encoded chaperone. Plasmid shuffle was also applied to show that *L. major* map kinase 4 (MPK4), a protein kinase which is implicated in mediating differentiation through the life cycle, is essential [30]. By further complementation of this line with *MPK4* ATP binding site mutants, partial *null* mutants were assessed by GCV treatment to establish clones which replicated in the presence of negative selection, but had altered protein kinase activity in subsequent differentiation assays. Inducible complementation allowed the identification of the K59R residue *MPK4* expressing mutants which underwent increased metacyclic differentiation *in vitro* but had reduced intracellular macrophage survival [30].

The rapid adoption of plasmid shuffle is testament to the novel biological insights that can be uncovered for a target gene. Its application to a variety of genes in different contexts represents the flexibility of plasmid shuffle to probe gene function.

2.2. DiCre recombinase

Genome engineering by Cre recombinase mediated excision of sequences flanked by locus of crossover of bacteriohage P1 (loxP) sites has been utilised in mammalian systems for over twenty years, but until recently had not been applied to *Leishmania*, possibly because of the lack of a tightly regulated system for induction. One method for regulating Cre expression utilizes a split Cre enzyme fused as two inactive fragments with a FK506-binding protein (FKBP12) and the binding domain of the FKBP12-rapamycin associated protein (FRB) [31]. Treatment with rapamycin dimerises the Cre (DiCre) subunits to reconstitute recombinase activity, and the expression of Cre as non-functional subunits is an elegant method to alleviate any side-effects of overexpression of active, potentially cytotoxic Cre.

This approach was applied to the study of the *L. mexicana* cdc-2 related protein kinase 3 (CRK3), which had previously been suggested to be essential using gene replacement approaches [22], The strategy involved replacing the first *CRK3* allele with the DiCre sequence and the second with a loxP flanked ('floxed') copy of the gene (*CRK3* ^{Flox}), thereby generating a cell line in which gene deletion could be induced upon activation of DiCre by treatment with rapamycin (Fig. 1B) [23]. By conditional gene deletion CRK3 activity was identified as essential for the transition of procyclic promastigotes through G2/M leading to growth arrest, aberrant cells and cell death. A conditional complementation approach was also employed whereby the generation of a cell line expressing a T-loop residue *CRK3*^{T178E} mutant refractory to DiCre mediated excision failed to recover normal cellular function, demonstrating the essentiality of this residue in regulating CRK3 activity. A benefit of this approach when compared with mutant transgene complementation by plasmid shuffle is that the resulting mutant expressing predominantly the mutated transgene in the absence of the floxed copy to enable the resulting phenotype to be directly assessed. In addition the DiCre complementation approach necessitates one less round of transfection compared with the four rounds necessary for plasmid shuffle.

Attempts to ablate genes in the amastigote life cycle stage resulted

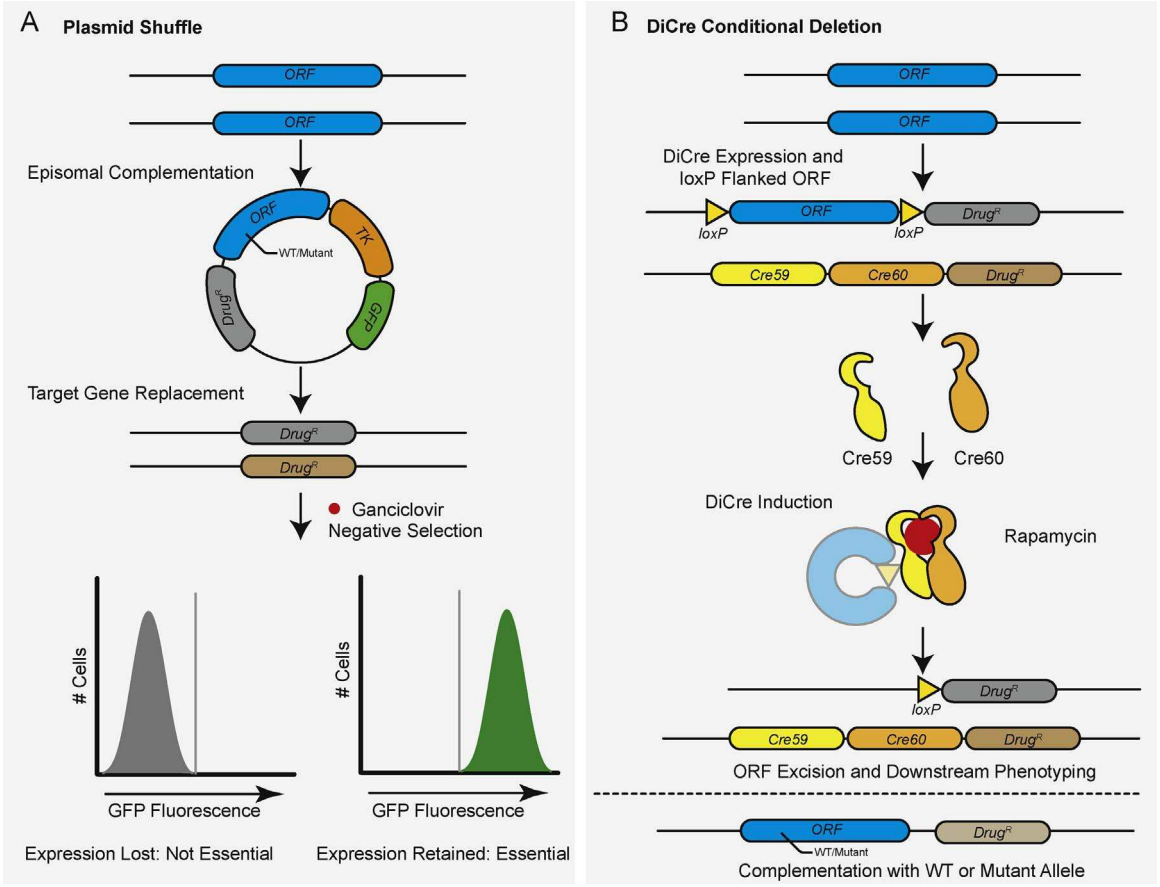

Fig. 1. The current molecular toolkit for analysis of essential *Leishmania* genes A. Plasmid shuffle utilises episomal expression of a target gene open reading frame (ORF) with the negative selectable thymidine kinase (TK) and positive markers for green fluorescent protein (GFP) and drug resistance ($Drug^R$) [30]. Transgene complementation enables deletion of endogenous gene copies and ganciclovir (GCV) treatment induces selection for plasmid retention and the expression of GFP if essential or loss of expression if non-essential. A fourth round of complementation with a mutated version of the target gene ORF enables the identification of active residues within the encoded protein. By GCV negative selection, retention of the ORF and GFP expression is indicative of a deleterious mutation which prevents active protein expression. In contrast, loss of the non-mutated ORF by inducible complementation the mutant gene identifies a functional mutated protein. B. Dimerizable Cre recombinase (DiCre) enables the generation of conditional *null* mutants [23]; the first allele of the ORF is replaced by a targeting construct comprising the two inactive Cre subunits (Cre59 and Cre60) each fused to FKBP12 (FK506-binding protein) and FRB (binding domain of the FKBP12-rapamycin associated protein) together with a drug resistance marker. The second allele is replaced with a loxP flanked (floxed) ORF copy expressed together with a second drug resistance cassette. Treatment with rapamycin causes the subunits to form an active Cre recombinase leading to excision of the floxed ORF to enable downstream phenotyping of *null* mutants. Complementation can be carried out with wild type of mutant transgenes.

in gene loss but were hindered by the increased sensitivity of this life cycle stage to rapamycin treatment, potentially as a result of rapamycin inhibiting the activity of *Leishmania* target of rapamycin (TOR) proteins [32]. The use of alternative dosage strategies and rapamycin analogues with reduced binding to *Leishmania* TORs may circumvent such constraints and as a consequence of the existing limitations with regulating amastigote gene expression the analysis of conditionally *null* amastigotes is a highly desirable tool to probe the function of essential genes in the mammalian life cycle stage. To circumvent the inherent sensitivity of amastigotes to rapamycin, *CRK3* loss was induced for 24 h in bioluminescent, stationary phase promastigotes and assessed for their proliferation in mice. *CRK3* deficient parasites were significantly reduced in their ability to proliferate *in vivo*, indicating the importance of *CRK3* for establishing infection.

The future application of DiCre to murine and macrophage infections will be important for the identification of *Leishmania* drug targets and alternatives to the rapamycin inducible DiCre system exist, where recombinase activity can be induced by different conditions, such as photo-inducible Cre [33] or a homo-dimerisable Cre that is activated by ligands with more beneficial pharmacokinetic properties and reduced binding to endogenous FKBP [34]. Such alternatives could facilitate conditional gene deletion during murine infection or *in vitro* macrophage infection. Further optimisation of the Cre recombinase methodology holds great promise for investigating *Leishmania* essential genes in more biologically relevant contexts.

2.3. CRISPR/Cas9

The CRISPR/Cas9 DNA genome editing methodology has been applied successfully to *Leishmania* and is set to revolutionize the way the parasite is genetically manipulated [35–37]. Cas9 is an RNA dependent DNA endonuclease and the system requires the expression of the Cas9 enzyme concurrently with a single guide RNA composed of a sequence complementary to the target site (marked by a conserved PAM sequence), fused to a scaffold RNA (Fig. 2A). In *Leishmania*, CRISPR-Cas9 induced double stranded breaks can be repaired by homologous recombination if a DNA template is provided, or by micro-homology mediated end joining (MMEJ). Non-homologous end joining (NHEJ) is thought to be absent in *Leishmania* [38,39]. The application of this technology in *Leishmania* has enhanced gene deletion and endogenous tagging capability [35–37] (http://www.leishgedit.net/Home.html).

Several different approaches have been taken for Cas9 editing in *Leishmania* promastigotes. Sollelis and co-workers [36] generated a Cas9 expression plasmid that was stably maintained in *L. major* allowing subsequent transfection with a linearized sgRNA expression cassette that also contained a large homology-flanked drug resistance marker to completely delete the gene of interest (the PFR2 gene array) (Fig. 2B). The sgRNA was transcribed under the control of the PolIII U6

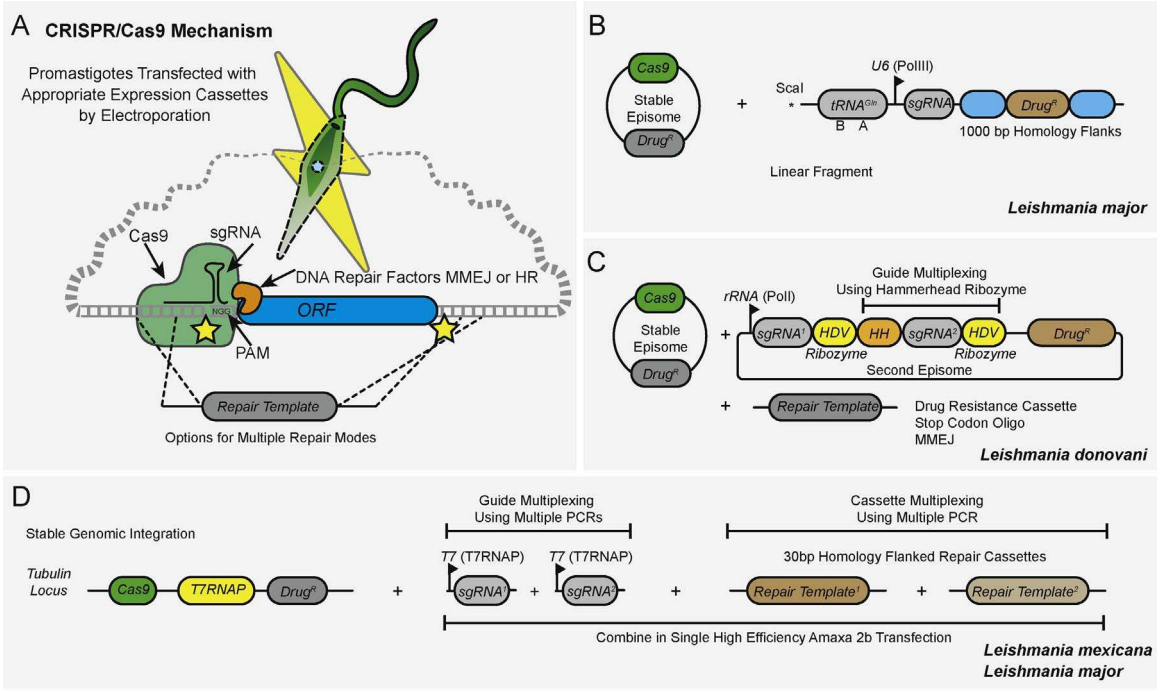

Fig. 2. CRISPR/Cas9 in *Leishmania*. A. Expression of Cas9 and single guide RNAs (sgRNAs) in *Leishmania* promastigotes, allows the sgRNA to guide Cas9 to the specified target location, adjacent to a protospacer–adjacent motif (PAM, in this instance NGG or NAG). Cas9 then induces a double strand break (DSB), which can be repaired by several mechanisms such as microhomology mediated end joining (MMEJ) or homologous recombination (HR). A variety of systems have been developed to achieve this result. B. The approach by Sollelis et al. [36] used an *L. major* cell line that constitutively expressed Cas9 from an episome, the sgRNA was expressed from a linear cassette under control of the *L. major* U6 promoter. The long homology arms of the drug resistance cassette were 1 kb in size and were contained in the same construct as the sgRNA, which was linearized with *Sca*I prior to transfection. C. Zhang et al. [35] generated a strain of *L. donovani* that also expressed Cas9 from a stable episome, and used a second episome to express the sgRNAs. This episome used the rRNA promoter to initiate transcription of the sgRNA and the hepatitis delta virus (HDV) ribozyme to terminate transcription, guides could be multiplexed using the Hammerhead ribozyme (HH) to process arrayed guide cassettes. A variety of repair templates could be used to disrupt genes of interest, including MMEJ, and short-homology flanked oligos or PCR products. This system was later developed into a single plasmid method [37]. D Beneke et al. [41] generated strains of *L. mexicana and L. major* that expressed Cas9 and T7 RNA polymerase (T7RNAP) from the tubulin locus, sgRNAs and repair cassettes were provided as linear PCR products. sgRNAs were expressed under the control of a T7 RNAP promoter and repair cassettes were flanked with 30 bp homology regions, multiplexing of guides and repair cassettes was conducted by combining PCR products and achieving high transfection efficiencies using the Amaxa 2b Nucleofector system.

promoter and both the Cas9 expression plasmid and modification were selected for using separate antibiotics. Off-target effects of CRISPR/Cas9 in *Leishmania* should be low, due to the small genome size and the selection of appropriate guide sequences with online resources such as EuPaGDT [40] and indeed that appears to be the case [36]. Zhang and Matlashewski [35] conducted their study in *L. donovani*, and used resistance to miltefosine as a readout of editing efficiency. Cas9 and sgRNAs were encoded on separate plasmids but the authors varied the mode of gene editing (Fig. 2C). Initially this study targeted the *LdMT* gene and relied on the MMEJ pathway to create point mutations and deletions in the *LdMT* gene. This disruption was also conducted by co-transfecting with a ssDNA oligonucleotide containing homology to the *LdMT* gene flanking an array of stop codons to disrupt the gene. Insertion of a short homology flanked, drug resistance cassette was also demonstrated into two further genes (both essential and non-essential). The ability to tag endogenous genes was also demonstrated by fusing the *LdMT* gene with a homology flanked GFP. Insertional disruption in *LdMT* conferred functional resistance to miltefosine but complete deletion was only achieved using a dual guide system that created double stranded breaks at the 5′ and 3′ flanks of *LdMT* thus deleting the entire locus. Unusually in this study, the sgRNAs were expressed under the control of the rRNA promoter and used a ribozyme sequence to terminate sgRNA transcription, making it different from most other CRISPR/Cas9 systems. The same group refined their system further to include all the elements required for CRISPR/Cas9 editing on a single plasmid, in theory making this applicable to different *Leishmania* species [37]. This study also demonstrated the ability of the system to disrupt multicopy tandem gene arrays, as well as separate loci on different chromosomes to induce chromosome translocations; the ability to edit loci without including selectable markers was also used by generating point mutations in the *RAD51* gene. In an informative experiment different promoters for guide RNA generation were assessed for efficiency, demonstrating that the PolI driven rRNA promoter is more efficient than the PolIII driven U6 promoter, allowing for optimal design of future systems.

Another recent and promising use of CRISPR/Cas9 technology in *Leishmania* sp. is exemplified by the work done in the laboratory of Eva Gluenz [41]. *L. mexicana* and *L. major* cell lines expressing T7 RNA polymerase and Cas9 were transfected with PCR generated DNA cassettes that expressed sgRNAs and PCR generated repair cassettes, simplifying the process of generating mutants (Fig. 2D). This was highlighted by the transfection of parasites with sgRNA cassettes and two different repair cassettes to edit two alleles of the *PF16* gene simultaneously in an endogenous tagging experiment, and to delete both copies of the *PF16* and *LPG1* genes in separate experiments, leading to functional defects in non-clonal populations. Presented as a resource this study also contains a library of template plasmids to allow generation of gene deletion and tagging cassettes; a primer design tool is included at an accompanying website (http://www.leishgedit.net/Home.html). The technique is not inducible, so only non-essential genes can be fully deleted in a manner that gives meaningful information on the phenotype of the null mutant. However, the flexibility, rapidity and efficiency of this system means it is likely to become a method of choice for the initial characterization of gene families in *Leishmania*.

The utilization of CRISPR/Cas9 in *Leishmania* is still in the early stage of development; therefore it will be beneficial to employ a variety

of strategies to establish the optimal approach for genome editing of this organism. For studies that aim to understand parasite virulence *in vivo* efficient techniques are required to rapidly edit genetic loci in species and strains that lose virulence after prolonged culture or extensive modification *in vitro,* mitigating the difficulties that slower, traditional techniques might present. A beneficial property of CRISPR/Cas9 is the ability to ablate multiple alleles of a gene in one round of selection; this has important implications for the study of *Leishmania* as many potentially important genes are encoded on supernumerary chromosomes [3] rendering their manipulation difficult by current gene replacement methods. The study of gene dosage, a phenomenon in which *Leishmania* alter gene expression by copy number variation is implicated in drug resistance [42], virulence and the rapid adaptation of promastigotes and amastigotes to survival in culture media or *in vivo* is an exciting area of *Leishmania* biology which would benefit greatly from CRISPR/Cas9 mediated manipulation. A current limitation is the lack of an inducible CRISPR/Cas9 that could be used to delete essential genes. Such limitations could be circumvented by the conditional activation of CRISPR/Cas9, for example using a split-Cas9 [43] or tetracycline inducible expression. Further development and optimization of the technology is going to be needed to facilitate genome-wide studies on *Leishmania* to identify cohorts of essential genes that encode promising drug targets.

3. New tools for regulating gene expression

The ability to regulate the level of gene expression enables in-depth studies into the function of the expressed protein. The ability to reduce or up-regulate gene expression to alter protein concentration is important for drug target validation, target deconvolution and identifying mechanisms of resistance; increased protein expression confers drug resistance if they specifically target the activity of the up-regulated protein by sequestering the compound, whilst reducing gene expression will result in increased susceptibility as protein levels are reduced. The ability to regulate gene expression in a conditional manner is chiefly important when low levels of expression are insufficient to perform their biological function, or if levels rise above a threshold that causes dominant-negative effects that impair cell viability. In such instances the results of altered expression can be very informative, particularly when expressing mutated genes to infer functions of individual amino acids, such as active site residues, within a protein. In addition, increasingly high-throughput and practical methods for endogenous gene tagging enable the localisation of their products and enable expression levels to be quantified. The repertoire for conducting such work has expanded in recent years, thereby advancing our ability to assess gene function by altering expression levels.

3.1. RNAi

RNA interference (RNAi) is an established and tractable tool for down regulation of gene expression. The RNAi pathway functions by the expression of Dicer-like enzymes DCL1 and 2 which process long double stranded (ds) RNA into small, double stranded duplexes. dsRNA is 'sliced' into single stranded siRNA and loaded as a guide sequence by Argonaute enzyme mediated processing, forming the RNA-inducing silencing complex (RISC) to degrade full length transcript [44] (Fig. 3A). RNAi as a method for functional analysis of genes in parasite species such as *L. major* and *L. donovani* is non-functional due to the evolutionary loss of Dicer-like and Argonaute proteins [45]. In contrast, the retention of such cellular machinery in the *L.* (*Viannia*) enables RNAi as a method for functional analysis of genes in parasite species such as *L. braziliensis*. Functional RNAi was established in this species by electroporation of promastigotes with dsRNA, yielding variability in the efficacy of this method by low levels of knockdown against mediators of LPG expression (*LPG1, LPG2, LPG3*) but efficient reduction of paraflagellar rod protein (PFR1 and PFR2) expression.

The highly variable levels of RNAi knockdown elicited for non-essential transcript is challenging, whilst the absence of an inducible method for dsRNA generation limits the approach to non-essential targets. Yet the approach is useful to reduce the expression of proteins which are encoded as multi-copy genes whereby gene replacement is unfeasible. A recent study by utilised RNAi in *L. braziliensis* to knock down expression of amastin which is encoded by 52 genes across 9 different chromosomes [46]. The method for RNAi in *L. braziliensis* relies on the transfection of dsRNA or a stem-loop construct to initiate gene silencing, however as both approaches lack temporal regulation of the generation of dsRNA and render the method unfeasible for essential genes.

New technologies may emerge that can inducibly deplete mRNA levels, for example components of CRISPR/Cas systems can include enzymes with alternative substrate specificity, such as C2c2, which can degrade RNA [47]. Development of an inducible C2c2 system would ideally allow for specific depletion of a chosen mRNA, although applying this system in a meaningful manner is currently hindered by significant off target mRNA degradation.

3.2. Tetracycline inducible gene expression

Induction of gene expression by tetracycline treatment has been a highly desirable tool in *Leishmania* due to the widespread utilisation of this approach in other trypanosomatids. This system requires the expression of T7 RNA polymerase and tetracycline repressor (TetR) proteins in conjunction with an expression cassette containing a T7 promoter sequence downstream of a TetR binding operon (TetO) and a site for cloning in a gene of interest. Attempts to regulate gene expression in *L. donovani* were previously hindered by high background activity and variable levels of expression depending on the RNA polymerase mediating gene expression [48]. Promisingly, the recent development of a transgenic *Leishmania mexicana* cell line expressing the TetR and T7 polymerases from the ribosomal locus has enabled the development of a system for conditional gene expression [49] (Fig. 3B). The reverse orientation of this cassette reduces background expression, and the authors demonstrate no detectable expression of a reporter gene in the absence of tetracycline by immunoblotting. This system has the potential to be used to generate conditional null mutants, where cells contain a tetracycline inducible ectopic copy of a gene of interest and both allelic copies are replaced by drug resistance genes, as described in *Trypanosoma brucei* [50]. Removal of tetracycline exerts TetR function to ablate gene expression and allow a null phenotype to be assessed.

The ability to regulate *Leishmania* gene expression by tetracycline treatment is highly desirable during murine or hamster infection. *S*tudies into genes required for infection of the mammalian host are possible as the orally bioavailable, tetracycline analogue doxycycline is widely used. Such studies would require sufficient expression of the exogenous TetR and T7 Pol genes in intracellular amastigotes, but a recent study into the expression of T7 Pol revealed dramatically reduced levels of protein expression in both metacyclic promastigotes and amastigotes [51], so optimisation is required.

3.3. DiCre recombinase inducible gene expression

The flexibility of loxP recombination, in addition to the tight regulation of DiCre activity, opens up the possibility of achieving inducible gene expression mediated by DiCre recombinase. Inducible expression requires an antisense gene of interest to be flanked with *cis* rather than *trans* orientated loxP sites. Upon Cre activation, loxP recombination induces the inversion of the antisense sequence to enable RNA polymerase mediated transcription (Fig. 3C), whilst the use of left and right element (LE/RE) mutated lox sites prevents continual recombination and inversion. This 'flip' methodology has recently been validated in *L. major* procyclic promastigotes by conditional expression of the DNA damage response protein Rad9 by diCre recombinase-mediated gene

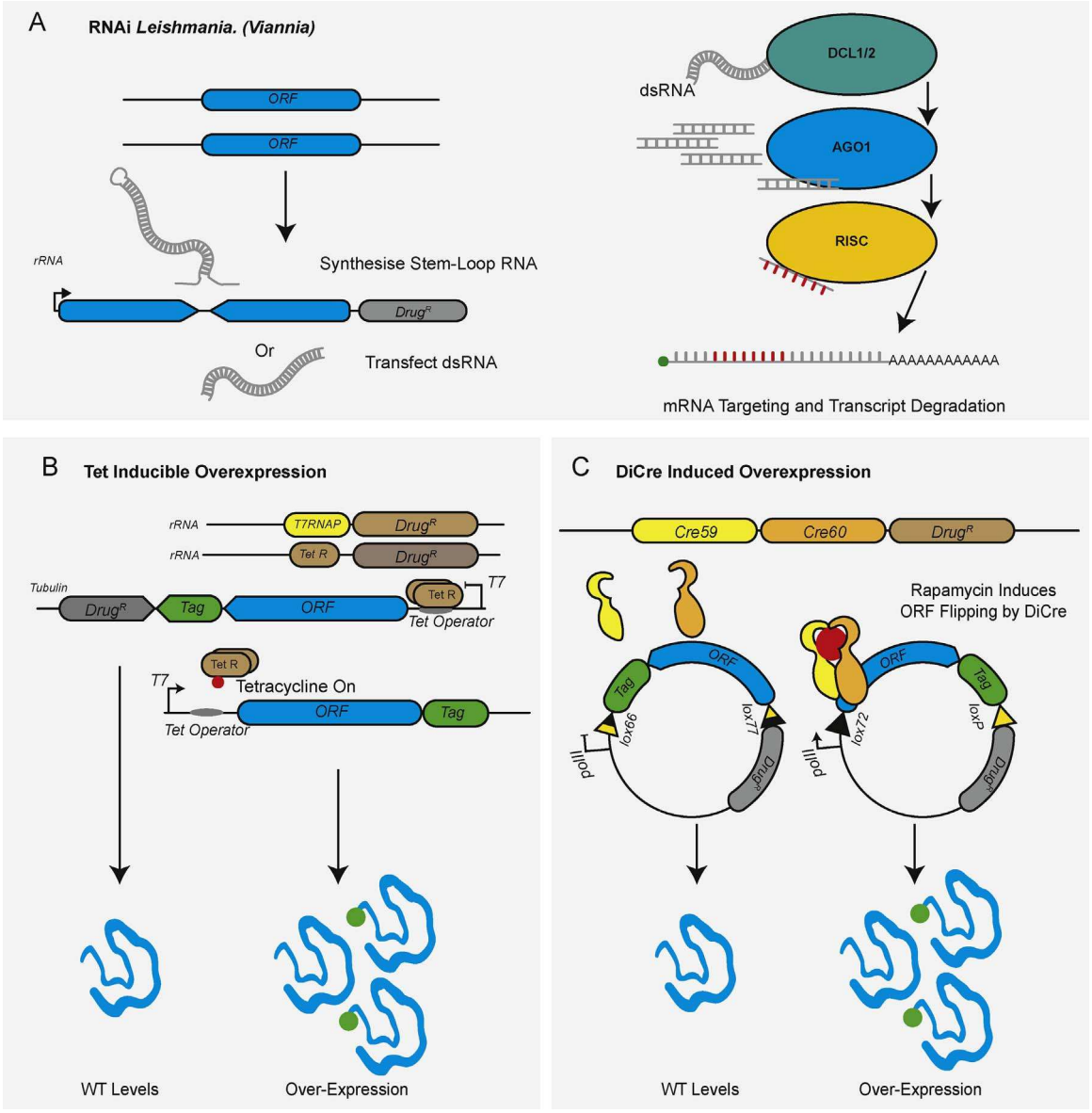

Fig. 3. Regulation of gene transcription. A. Functional RNAi pathway in *Leishmania. L. braziliensis* enables targeting of mRNA by integration of an RNAi targeting sequence into a ribosomal small subunit (SSU) integrative construct [45]. Transfection of the stem-loop construct generates dsRNA by transcription from the ribosomal RNA (rRNA) promotor. Dicer-like (DCL) enzymes process the dsRNA into siRNA duplexes that are processed into single stranded siRNA by Argonaute (AGO1) and loaded to form the RNA-induced silencing complex (RISC). B. Tetracycline overexpression system [49]. The T7 RNA polymerase and Tet Repressor elements are integrated into the rRNA locus. A gene under control of the tet operator is expressed from the tubulin locus in the opposite direction to normal transcription to prevent leaky expression (denoted by the opposing arrowheads on *ORF, Tag* and *Drug*^{R} elements). Tetracycline is added to release inhibition of the cassette by the tet repressor and T7 dependent transcription of the gene proceeds. C. DiCre ORF Flipping Overexpression [52]. An episomal or integrated (not shown) construct can be prepared where the ORF is opposing the promoter and is floxed by the inverted, directional lox66 and lox77 sites. DiCre induction by rapamycin induces flipping of the ORF into the correct orientation for transcription but cannot be flipped back due to the formation of lox72 and loxP sites, leading to inducible expression of the GOI.

inversion [52]. This methodology opens up the possibility of performing conditional mutant overexpression to induce dominant negative phenotypes in promastigotes, but it has the same limitations for amastigotes as described above for diCRE mediated gene deletion.

4. New tools for endogenous protein tagging and regulating protein levels

The ability to regulate protein levels is complementary to genetic based approaches because of differing kinetics of protein loss or gain. This can result in the rapid manifestation of phenotypes dependent on protein half-life opposed to slower onset phenotypes that are buffered by existing cellular mRNA and protein content following inducible gene deletion. However, a constraint of protein manipulation is the requirement for tagging of the protein; this can be problematic if tagging prevents protein function. As such, studies directly manipulating protein stability in *Leishmania* are less common compared with genetic techniques, yet manipulation of protein levels can be a very powerful and robust method.

4.1. Endogenous tagging

Expression of epitope tagged proteins in *Leishmania* has typically been carried from episomal plasmids, which is effective but can give variable levels of expression of the fusion protein in individual cells [19]. An alternative is C- or N- terminal tagging of endogenous genes, which requires long homology arms for successful integration, but which has been simplified with a fusion PCR approach [53] (Fig. 4A).

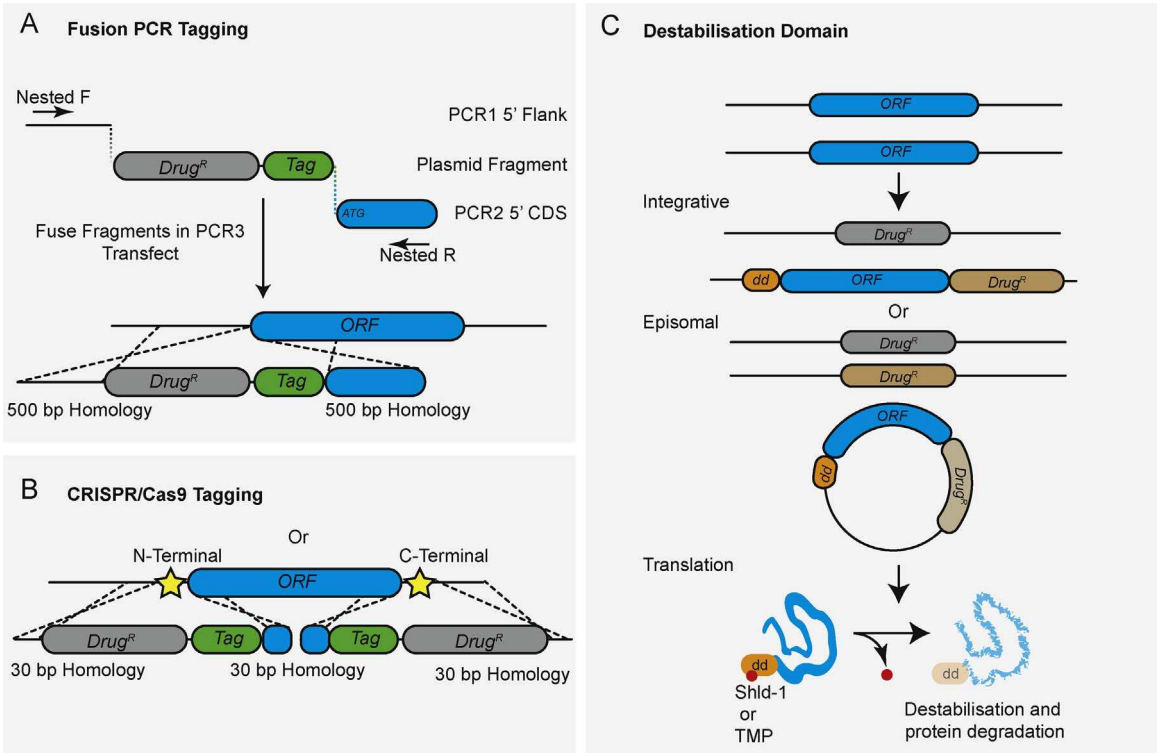

Fig. 4. Protein tagging and manipulation. A. Epitope tagging can be effectively performed by transfecting a 500 bp homology flanked cassette containing a tag in frame with the gene of interest. These cassettes are efficiently constructed by a 3-fragment fusion PCR, and can be used in unmodified *Leishmania* strains. Panel A depicts N-terminal tagging but this strategy can be applied to C-terminal or internal contexts [53]. B. CRISPR/Cas9 mediated epitope tagging is more rapid and efficient as the epitope tag can be generated by PCR with short homology PCR but necessitates a strain expressing functional CRISPR/Cas9 system [41]. C. Destabilization domain tagging of proteins. An allele of a gene can be tagged with a destabilization domain using the approaches in panel A or B, and the remaining WT allele removed by replacement with a drug selectable marker (or double knockout in the presence of an episomal complementation vector). Protein of interest fused to the dd tag is stabilized by the addition of the Shield-1 or trimethoprim ligand, removal of the ligand triggers protein destabilization, and consequent degradation, allowing downstream phenotyping.

This approach does not involve cloning therefore it is rapid and scalable. Unfortunately *Leishmania* genes cannot be tagged using cassettes flanked by short homology (80–100 bp) contained in oligonucleotide primers used to make the cassettes [53]. This "long primer" approach is the basis for the tagging of almost the entire proteome in the TrypTag project [54], and would be welcome in the *Leishmania* field as it is simpler, cheaper and more rapid than the fusion PCR method. Despite short-homology flanked cassettes not integrating efficiently into wild-type *Leishmania*, the application of CRISPR/Cas9 has revolutionised the field, allowing for 30 bp flanked cassettes to efficiently integrate close to a Cas9-induced, double stranded break [41] (Fig. 4B). The flexibility of this system has been increased by the development of a series of vectors allowing genes to be tagged with elements encoding proteins of different function; these include BirA* for proximity dependent biotinylation, NanoLuc and HaloTag for probing protein:protein interactions by BRET assay, and various epitope and fluorescent markers such as mNeonGreen. These can be applied to both N- and C-terminal tagging. By combining the rapid process of the T7/Cas9 system with new, highly advanced protein tags such as mNeonGreen and BirA* the possibility of proteome-wide localisation and interactome projects has now become technically feasible in *Leishmania*.

4.2. Conditional protein destabilisation

An approach to regulate protein expression by conferring inducible stability involves the linkage of a target protein to a regulatory, destabilisation domain (dd) derived and modified from an FKBP domain. The ddFKBP domain leads to proteasome-mediated degradation when the stabilising ligand is absent. Protein stability and therefore activity is conferred by treatment with the synthetic ligand Shld-1 (Fig. 4C) and was utilised effectively in *L. major* promastigotes to stabilise UDP-galactopyranose mutase (ddUGM), an enzyme involved in the biosynthesis of lipophosphoglycan (LPG) [55]. By integration of a *ddGLF* construct into a homozygous, *glf*$^{-}$ null mutant *L. major* line, stability of a dd conjugated UDP-galactopyranose mutase (ddUGM) was conferrable by Shld-1 treatment, with removal of Shld-1 rapidly resulting in the inducible susceptibility to complement mediated lysis due to expression of LPG truncated in the glycan region [55]. The system has also been used to study the dynamics of *Leishmania* flagellum proteins during differentiation [56].

The utilisation of dd to study the function of *Leishmania* proteins held great promise, however it was not until recently that the system was used to regulate the stability of an essential protein [57]. Previous work using individual UDP-sugar pyrophosphorylase (USP) and UDP-Glc pyrophosphorylase (UGP) null mutants implicated their encoded proteins in sugar salvage and the biosynthesis UDP-sugars [58,59]. Individual *UGP* and *USP* null mutants were viable, but double null mutants could not be generated thereby implicating the requirement for at least one enzyme for sufficient sugar nucleotide synthesis for adequate protein glycosylation and parasite viability. To further elucidate the role of USP a cell line was generated expressing a single copy of the UDP-sugar pyrophosphorylase (USP) conjugated to the destabilisation domain in a cell line deficient in the enzyme UDP-Glc pyrophosphorylase (UGP). ddUSP expression in the presence of the stabilising ligand FK506 was sufficient to maintain the pools of sugar nucleotides UDP-Glc and UDP-Gal but in the absence of ligand the resulting loss of ddUSP led to the depletion of these sugars and cell death. This elegant study demonstrates the potential for dd-tagging of essential proteins in *Leishmania* and the ability to combine destabilisation domain tagging with CRISPR/Cas9 approaches may allow more rapid generation of mutants where both alleles are dd-tagged in a single transfection, increasing the potential throughput.

Another inducible protein destabilisation system based on the *Escherichia coli* dihydrofolate reductase destabilizing domain (ecDHFR) that uses a different ligand, trimethoprim, has recently been reported in *Leishmania* [60]. This ligand is cheaper than Shld-1 and provides an alternative to the ddFKBP system.

5. Concluding remarks

Reviewed here is the rapidly expanding repertoire of molecular tools with which to study essential regulators of *Leishmania*. It is hoped that the utilisation of current methods and the development of ever more advanced molecular tools will enhance the identification and validation of genes encoding essential, druggable targets thereby paving the way for the development of novel therapeutics to treat this neglected disease.

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References

[1] S.M. Beverley, Protozomics: trypanosomatid parasite genetics comes of age, Nat. Rev. Genet. 4 (2003) 11–19, http://dx.doi.org/10.1038/nrg980.
[2] S.M. Beverley, S.J. Turco, Lipophosphoglycan (LPG) and the identification of virulence genes in the protozoan parasite *Leishmania*, Trends Microbiol. 6 (1998) 35–40, http://dx.doi.org/10.1016/S0966-842X(97)01180-3.
[3] M.B. Rogers, J.D. Hilley, N.J. Dickens, J. Wilkes, P.A. Bates, D.P. Depledge, et al., Chromosome and gene copy number variation allow major structural change between species and strains of *Leishmania*, Genome Res. 21 (2011) 2129–2142, http://dx.doi.org/10.1101/gr.122945.111.
[4] Y. Sterkers, L. Crobu, L. Lachaud, M. Pagès, P. Bastien, Parasexuality and mosaic aneuploidy in *Leishmania*: alternative genetics, Trends Parasitol. 30 (2014) 429–435, http://dx.doi.org/10.1016/j.pt.2014.07.002.
[5] N.S. Akopyants, N. Kimblin, N. Secundino, R. Patrick, N. Peters, P. Lawyer, et al., Demonstration of genetic exchange during cyclical development of *Leishmania* in the sand fly vector, Science 324 (2009) 265–268, http://dx.doi.org/10.1126/science.1169464.
[6] A.C. Ivens, C.S. Peacock, E.A. Worthey, L. Murphy, G. Aggarwal, M. Berriman, et al., The genome of the kinetoplastid parasite, *Leishmania major*, Science 309 (2005) 436–442, http://dx.doi.org/10.1126/science.1112680.
[7] C.S. Peacock, K. Seeger, D. Harris, L. Murphy, J.C. Ruiz, M.A. Quail, et al., Comparative genomic analysis of three *Leishmania* species that cause diverse human disease, Nat. Genet. 39 (2007) 839–847, http://dx.doi.org/10.1038/ng2053.
[8] M. Aslett, C. Aurrecoechea, M. Berriman, J. Brestelli, B.P. Brunk, M. Carrington, et al., TriTrypDB: a functional genomic resource for the Trypanosomatidae, Nucleic Acids Res. 38 (2010) D457–62, http://dx.doi.org/10.1093/nar/gkp851.
[9] G.F. Späth, J. Clos, Joining forces: first application of a rapamycin-induced dimerizable Cre system for conditional null mutant analysis in *Leishmania*, Mol. Microbiol. 100 (2016) 923–927, http://dx.doi.org/10.1111/mmi.13374.
[10] A. Cruz, S.M. Beverley, Gene replacement in parasitic protozoa, Nature 348 (1990) 171–173, http://dx.doi.org/10.1038/348171a0.
[11] F.J. Gueiros-Filho, S.M. Beverley, Selection against the dihydrofolate reductase-thymidylate synthase (DHFR-TS) locus as a probe of genetic alterations in *Leishmania major*, Mol. Cell. Biol. 16 (1996) 5655–5663.
[12] D. Sereno, G. Roy, J.L. Lemesre, B. Papadopoulou, M. Ouellette, DNA transformation of *Leishmania infantum* axenic amastigotes and their use in drug screening, Antimicrob. Agents Chemother. 45 (2001) 1168–1173, http://dx.doi.org/10.1128/AAC.45.4.1168-1173.2001.
[13] A.K. Cruz, R. Titus, S.M. Beverley, Plasticity in chromosome number and testing of essential genes in *Leishmania* by targeting, Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 1599–1603.
[14] J.C. Mottram, B.P. McCready, K.G. Brown, K.M. Grant, Gene disruptions indicate an essential function for the LmmCRK1 cdc2-related kinase of *Leishmania mexicana*, Mol. Microbiol. 22 (1996) 573–583.
[15] J. Faria, I. Loureiro, N. Santarém, P. Cecílio, S. Macedo-Ribeiro, J. Tavares, et al., Disclosing the essentiality of ribose-5-phosphate isomerase B in Trypanosomatids, Sci. Rep. 6 (2016) 26937, http://dx.doi.org/10.1038/srep26937.
[16] Q. Wang, I.M. Melzer, M. Kruse, C. Sander-Juelch, M. Wiese, LmxMPK4, a mitogen-activated protein (MAP) kinase homologue essential for promastigotes and amastigotes of *Leishmania mexicana*, Kinetoplastid. Biol. Dis. 4 (2005) 6, http://dx.doi.org/10.1186/1475-9292-4-6.
[17] A. Misslitz, J.C. Mottram, P. Overath, T. Aebischer, Targeted integration into a rRNA locus results in uniform and high level expression of transgenes in *Leishmania* amastigotes, Mol. Biochem. Parasitol. 107 (2000) 251–261.
[18] R. Soysa, K.D. Tran, B. Ullman, P.A. Yates, Integrating ribosomal promoter vectors that offer a choice of constitutive expression profiles in *Leishmania donovani*, Mol. Biochem. Parasitol. 204 (2015) 89–92, http://dx.doi.org/10.1016/j.molbiopara.2016.01.008.
[19] E. Tetaud, I. Lecuix, T. Sheldrake, T. Baltz, A.H. Fairlamb, A new expression vector for *Crithidia fasciculata* and *Leishmania*, Mol. Biochem. Parasitol. 120 (2002) 195–204.
[20] S. Wyllie, A.J. Roberts, S. Norval, S. Patterson, B.J. Foth, M. Berriman, et al., Activation of bicyclic nitro-drugs by a novel nitroreductase (NTR2) in *Leishmania*, PLoS Pathog. 12 (2016) e1005971, http://dx.doi.org/10.1371/journal.ppat.1005971.
[21] É. Gazanion, C. Fernández-Prada, B. Papadopoulou, P. Leprohon, M. Ouellette, Cos-Seq for high-throughput identification of drug target and resistance mechanisms in the protozoan parasite *Leishmania*, Proc. Natl. Acad. Sci. U. S. A. 113 (2016) E3012–21, http://dx.doi.org/10.1073/pnas.1520693113.
[22] P. Hassan, D. Fergusson, K.M. Grant, J.C. Mottram, The CRK3 protein kinase is essential for cell cycle progression of *Leishmania mexicana*, Mol. Biochem. Parasitol. 113 (2001) 189–198.
[23] S.M. Duncan, E. Myburgh, C. Philipon, E. Brown, M. Meissner, J. Brewer, et al., Conditional gene deletion with DiCre demonstrates an essential role for CRK3 in *Leishmania mexicana* cell cycle regulation, Mol. Microbiol. 100 (2016) 931–944, http://dx.doi.org/10.1111/mmi.13375.
[24] J.M. Boitz, C.A. Gilroy, T.D. Olenyik, D. Paradis, J. Perdeh, K. Dearman, et al., Arginase is essential for survival of *Leishmania donovani*promastigotes but not intracellular amastigotes, Infect. Immun. 8 (2017) 5, http://dx.doi.org/10.1128/IAI.00554-16.
[25] M.P. Barrett, J.C. Mottram, G.H. Coombs, Recent advances in identifying and validating drug targets in trypanosomes and leishmanias, Trends Microbiol. 7 (1999) 82–88, http://dx.doi.org/10.1016/S0966-842X(98)01433-4.
[26] H.P. Price, D. Paape, M.R. Hodgkinson, K. Farrant, J. Doehl, M. Stark, et al., The *Leishmania major* BBSome subunit BBS1 is essential for parasite virulence in the mammalian host, Mol. Microbiol. 90 (2013) 597–611, http://dx.doi.org/10.1111/mmi.12383.
[27] S.M.F. Murta, T.J. Vickers, D.A. Scott, S.M. Beverley, Methylene tetrahydrofolate dehydrogenase/cyclohydrolase and the synthesis of 10-CHO-THF are essential in *Leishmania major*, Mol. Microbiol. 71 (2009) 1386–1401, http://dx.doi.org/10.1111/j.1365-2958.2009.06610.x.
[28] L.-I. McCall, A. El Aroussi, J.Y. Choi, D.F. Vieira, G. De Muylder, J.B. Johnston, et al., Targeting Ergosterol biosynthesis in *Leishmania donovani*: essentiality of sterol 14 alpha-demethylase, PLoS Negl. Trop. Dis. 9 (2015) e0003588, http://dx.doi.org/10.1371/journal.pntd.0003588.
[29] M.A. Morales, R. Watanabe, M. Dacher, P. Chafey, J. Osorio y Fortéa, D.A. Scott, et al., Phosphoproteome dynamics reveal heat-shock protein complexes specific to the *Leishmania donovani*infectious stage, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 8381–8386, http://dx.doi.org/10.1073/pnas.0914768107.
[30] M. Dacher, M.A. Morales, P. Pescher, O. Leclercq, N. Rachidi, E. Prina, et al., Probing druggability and biological function of essential proteins in *Leishmania* combining facilitated null mutant and plasmid shuffle analyses, Mol. Microbiol. 93 (2014) 146–166, http://dx.doi.org/10.1111/mmi.12648.
[31] N. Jullien, F. Sampieri, A. Enjalbert, J.-P. Herman, Regulation of Cre recombinase by ligand-induced complementation of inactive fragments, Nucleic Acids Res. 31 (2003) e131, http://dx.doi.org/10.1093/nar/gng131.
[32] L. Madeira da Silva, S.M. Beverley, Expansion of the target of rapamycin (TOR) kinase family and function in *Leishmania* shows that TOR3 is required for acidocalcisome biogenesis and animal infectivity, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 11965–11970, http://dx.doi.org/10.1073/pnas.1004599107.
[33] S.E. Schindler, J.G. McCall, P. Yan, K.L. Hyrc, M. Li, C.L. Tucker, et al., Photo-activatable Cre recombinase regulates gene expression in vivo, Sci. Rep. 5 (2015) 13627, http://dx.doi.org/10.1038/srep13627.
[34] N. Jullien, I. Goddard, S. Selmi-Ruby, J.-L. Fina, H. Cremer, P. Herman J-, Conditional transgenesis using dimerizable cre (DiCre), PLoS One 2 (2007) e1355, http://dx.doi.org/10.1371/journal.pone.0001355.
[35] W.-W. Zhang, G. Matlashewski, CRISPR-Cas9-mediated genome editing in Leishmania donovani, MBio 6 (2015) e00861, http://dx.doi.org/10.1128/mBio.00861-15.
[36] L. Sollelis, M. Ghorbal, C.R. MacPherson, R.M. Martins, N. Kuk, L. Crobu, et al., First efficient CRISPR-Cas9-mediated genome editing in *Leishmania*parasites, Cell. Microbiol. 17 (2015) 1405–1412, http://dx.doi.org/10.1111/cmi.12456.
[37] W.-W. Zhang, P. Lypaczewski, G. Matlashewski, Optimized CRISPR-Cas9 genome editing for *Leishmania* and its use to target a multigene family, induce chromosomal translocation, and study DNA Break repair mechanisms, mSphere 2 (2017) http://dx.doi.org/10.1128/msphere.00340-16.
[38] D.G. Passos-Silva, M.A. Rajão, P.H. Nascimento de Aguiar, J.P. Vieira-da-Rocha, C.R. Machado, C. Furtado, Overview of DNA repair in *Trypanosoma cruzi*, *Trypanosoma brucei*, and *Leishmania major*, J. Nucleic Acids 2010 (2010) 840768, http://dx.doi.org/10.4061/2010/840768.
[39] Laffitte M-CN, P. Leprohon, M. Hainse, D. Légaré, J.-Y. Masson, M. Ouellette, Chromosomal translocations in the parasite *leishmania* by a MRE11/RAD50-independent microhomology-mediated end joining mechanism, PLoS Genet. 12 (2016) e1006117, http://dx.doi.org/10.1371/journal.pgen.1006117.
[40] D. Peng, R. Tarleton, EuPaGDT: a web tool tailored to design CRISPR guide RNAs for eukaryotic pathogens, Microb. Genomics 1 (2015) e000033, http://dx.doi.org/10.1099/mgen.0.000033.
[41] T. Beneke, R. Madden, L. Makin, J. Valli, J. Sunter, E. Gluenz, A CRISPR Cas9 high-throughput genome editing toolkit for kinetoplastids, R. Soc. Open Sci. 4 (2017) 170095, http://dx.doi.org/10.1098/rsos.170095.
[42] P. Leprohon, C. Fernandez-Prada, Gazanion É, R. Monte-Neto, M. Ouellette, Drug

resistance analysis by next generation sequencing in *Leishmania*, Int. J. Parasitol. Drugs Drug Resist. 5 (2015) 26–35, http://dx.doi.org/10.1016/j.ijpddr.2014.09.005.

[43] D.P. Nguyen, Y. Miyaoka, L.A. Gilbert, S.J. Mayerl, B.H. Lee, J.S. Weissman, et al., Ligand-binding domains of nuclear receptors facilitate tight control of split CRISPR activity, Nat. Commun. 7 (2016) 12009, http://dx.doi.org/10.1038/ncomms12009.

[44] N.G. Kolev, C. Tschudi, E. Ullu, RNA interference in protozoan parasites: achievements and challenges, Eukaryot. Cell 10 (2011) 1156–1163, http://dx.doi.org/10.1128/EC.05114-11.

[45] L.-F. Lye, K. Owens, H. Shi, S.M.F. Murta, A.C. Vieira, S.J. Turco, et al., Retention and loss of RNA interference pathways in trypanosomatid protozoans, PLoS Pathog. 6 (2010) e1001161, http://dx.doi.org/10.1371/journal.ppat.1001161.

[46] R.M.C. De Paiva, V. Grazielle-Silva, M.S. Cardoso, B.N. Nakagaki, R.P. Mendonça-Neto, A.M.C. Canavaci, et al., Amastin knockdown in *Leishmania braziliensis* affects parasite-macrophage interaction and results in impaired viability of intracellular amastigotes, PLoS Pathog. 11 (2015) e1005296, http://dx.doi.org/10.1371/journal.ppat.1005296.

[47] O.O. Abudayyeh, J.S. Gootenberg, S. Konermann, J. Joung, I.M. Slaymaker, D.B.T. Cox, et al., C2c2 is a single-component programmable RNA-guided RNA-targeting CRISPR effector, Science 353 (2016) aaf5573, http://dx.doi.org/10.1126/science.aaf5573.

[48] S. Yan, P.J. Myler, K. Stuart, Tetracycline regulated gene expression in *Leishmania donovani*, Mol. Biochem. Parasitol. 112 (2001) 61–69.

[49] N. Kraeva, A. Ishemgulova, J. Lukeš, V. Yurchenko, Tetracycline-inducible gene expression system in *Leishmania mexicana*, Mol. Biochem. Parasitol. 198 (2014) 11–13, http://dx.doi.org/10.1016/j.molbiopara.2014.11.002.

[50] M.D. Urbaniak, D.C. Turnock, M.A.J. Ferguson, Galactose starvation in a bloodstream form *Trypanosoma brucei*UDP-glucose 4′-epimerase conditional null mutant, Eukaryot. Cell 5 (2006) 1906–1913, http://dx.doi.org/10.1128/EC.00156-06.

[51] A. Ishemgulova, N. Kraeva, D. Faktorova, L. Podesvova, J. Lukes, V. Yurchenko, T7 polymerase-driven transcription is downregulated in metacyclic promastigotes and amastigotes of*Leishmania mexicana*, Folia Parasitol. 6 (2016) 3, http://dx.doi.org/10.14411/fp.2016.016.

[52] R.E.R.S. Santos, G.L.A. Silva, E.V. Santos, S.M. Duncan, J.C. Mottram, J.D. Damasceno, L.R.O. Tosi, A DiCre recombinase-based system for inducible expression in Leishmania, Mol. Biochem. Parasitol. (2017), http://dx.doi.org/10.1016/j.molbiopara.2017.06.006.

[53] S. Dean, J. Sunter, R.J. Wheeler, I. Hodkinson, E. Gluenz, K. Gull, A toolkit enabling efficient, scalable and reproducible gene tagging in trypanosomatids, Open Biol. 5 (2015) 140197, http://dx.doi.org/10.1098/rsob.140197.

[54] S. Dean, J.D. Sunter, R.J. Wheeler, TrypTag.org: a trypanosome genome-wide protein localisation resource, Trends Parasitol. 33 (2017) 80–82, http://dx.doi.org/10.1016/j.pt.2016.10.009.

[55] L. Madeira da Silva, K.L. Owens, S.M.F. Murta, S.M. Beverley, Regulated expression of the *Leishmania major*surface virulence factor lipophosphoglycan using conditionally destabilized fusion proteins, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 7583–7588, http://dx.doi.org/10.1073/pnas.0901698106.

[56] R.J. Wheeler, E. Gluenz, K. Gull, Basal body multipotency and axonemal remodelling are two pathways to a 9 + 0 flagellum, Nat. Commun. 6 (2015) 8964, http://dx.doi.org/10.1038/ncomms9964.

[57] S. Damerow, C. Hoppe, G. Bandini, P. Zarnovican, F.F.R. Buettner, C.G.K. Lüder, et al., Depletion of UDP-glucose and UDP-galactose using a degron system leads to growth cessation of *Leishmania major*, PLoS Negl. Trop. Dis. 9 (2015) e0004205, http://dx.doi.org/10.1371/journal.pntd.0004205.

[58] S. Damerow, C. Hoppe, G. Bandini, P. Zarnovican, F.F.R. Buettner, M.A.J. Ferguson, et al., *Leishmania major*UDP-sugar pyrophosphorylase salvages galactose for glycoconjugate biosynthesis, Int. J. Parasitol. 45 (2015) 783–790, http://dx.doi.org/10.1016/j.ijpara.2015.06.004.

[59] A.-C. Lamerz, S. Damerow, B. Kleczka, M. Wiese, G. van Zandbergen, J. Lamerz, et al., Deletion of UDP-glucose pyrophosphorylase reveals a UDP-glucose independent UDP-galactose salvage pathway in *Leishmania major*, Glycobiology 20 (2010) 872–882, http://dx.doi.org/10.1093/glycob/cwq045.

[60] L. Podešvová, H. Huang, V. Yurchenko, Inducible protein stabilization system in *Leishmania mexicana*, Mol. Biochem. Parasitol. 214 (2017) 62–64, http://dx.doi.org/10.1016/j.molbiopara.2017.03.008.