REVIEWS

PROTOZOMICS: TRYPANOSOMATID PARASITE GENETICS COMES OF AGE

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Trypanosomatid protozoans cause important diseases of humans and their domestic livestock. Various molecular genetic tools are now allowing rapid progress in understanding many of the unique aspects of the molecular and cell biology of these organisms. Diploidy and the lack or difficulty of sexual crossing has been a challenge for forward genetics, but powerful selections and functional complementation have helped to overcome it in *Leishmania*. RNA interference has been adapted for forward genetics in trypanosomes, in which it is also a powerful tool for reverse genetics. Interestingly, the efficacy of different genetic tools has steered research into different aspects of the biology of these parasites.

ANTIGENIC VARIATION
The changing of the surface antigens by a pathogen to evade the immune response of the host.

SPECTRAL DISEASE
Infections that can manifest in several forms, often varying greatly in severity and symptoms in different individuals, probably reflecting differences in the immune response of the host.

PHAGOLYSOSOME
A vacuole in a cell in which a phagocytosed particle is digested.

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Trypanosomatid protozoans are important parasites of humans and their domestic animals (TABLE 1). Species of African trypanosomes — the agents of sleeping sickness in humans (*Trypanosoma brucei rhodesiense* and *T. b. gambiense*) — also have a severe impact on the cultivation of domestic livestock in wide areas of the continent; these species being *T. b. brucei*, *T. congolense* and *T. vivax*. They live freely in the blood and lymph of mammals and evade the immune response of the host primarily because of ANTIGENIC VARIATION[1], which was first described nearly a century ago. The South American trypanosome *T. cruzi*, which is the agent of Chagas disease, afflicts 16 million people predominantly in less-developed areas. After invasion of host vertebrate cells, trypanosome parasites escape to the cytoplasm and ultimately induce a debilitating response, which is often associated with cardiac and other organ system failures.

Leishmaniasis is a SPECTRAL DISEASE, manifesting in pathologies that range from mild to disfiguring to fatal; the outcome is determined in part by the species of *Leishmania* and in part by host factors that are associated with the innate and acquired immune responses. In mammals, this parasite differentiates to a stage that is adapted for survival in the PHAGOLYSOSOME of the macrophage, the cell that is normally responsible for pathogen elimination.

How parasites carry out the developmental transitions that are necessary for survival and transmission among their insect and mammalian hosts and the mechanisms that they use to resist, often for decades, a tremendously hostile array of defences are of interest to biologists and clinicians who seek to control these pathogens. As TABLE 1 indicates, at present, there are no effective strategies for chemotherapy and vaccination against these diseases, despite considerable evidence that these are attainable goals. Genetic tools promise to radically advance our ability to probe the biological questions; however, the commitment of governments and corporations to the development of potential therapeutic treatment to control these diseases has been questioned, given that the diseases are often restricted to relatively poor countries. There are signs that this situation is improving, however, with the recent emergence of several new initiatives, such as that sponsored by the Gates Foundation (see online link).

In the past 12 years, various genetic tools have been introduced that allow manipulation of trypanosomatid genomes[2,3] (TABLE 2). Many of these will be familiar to those who work with model organisms, and the task for molecular parasitologists has been to get them to work in parasites that pose unique challenges. These methods constitute a powerful genetic 'toolkit' that allows experimenters to investigate gene function by both gain- and loss-of-function strategies, and to study the localization

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Table 1 | **Diseases caused by trypanosomatid protozoan parasites***

Parasitic species	Disease	Insect vector	People exposed (×10^6)	People infected (×10^6)	Chemotherapy‡	Vaccination
African trypanosomes *Trypanosoma brucei rhodesiense* or *gambiense*	Human: sleeping sickness	Tsetse fly	60	0.5	Available: arsenicals and DFMO	No: might not be feasible
T. brucei and other species	Animal: ngana	Tsetse fly	—	—	Isometamidium	NA
S. American trypanosome *T. cruzi*	Chagas disease	Reduviid bug	120	16	No	No: some evidence of feasibility
Leishmania spp. (tropical and semi-tropical regions)	Leishmaniasis (visceral, cutaneous and mucocutaneous)	Phlebotomine sand fly	350	12	Available: antimonials and miltefosine	No: clearly feasible

*Data and references are from the WHO Special Programme for Research and Training in Tropical Diseases (see online link). ‡Only the chief compounds used clinically are provided here; all of the available drugs have limitations arising from availability, cost or efficacy and new ones are urgently required. There is also evidence from laboratory studies and, in some cases, in field studies for resistance to all of the drugs currently used. DFMO, difluoromethyl-ornithine; NA, not applicable.

of their protein products using various *in vivo* tags, such as the green fluorescent protein (GFP). Complementing our ability to carry out reverse genetic manipulations has been the development of methods for 'forward' genetics, including functional genetic rescue, transposon tagging and, more recently, the introduction of RNA interference (RNAi)-based mutant screens. Trypanosomatid protozoans have joined many other microbes in entering the era of genome science with rapidly progressing genome projects that include both genome and EST (expressed sequence tag) data becoming available[4]. When completed, the genome sequences will provide tremendous new opportunities for probing gene function in the context of biology, disease and the control of disease.

In this review, I briefly summarize the genetic 'toolkit' that is available for the study of trypanosomatid protozoans and that is, in most respects, now nearly complete. Although the literature has focused on genetic tools for the study of African trypanosomes (mostly *T. brucei*) and *Leishmania*, it seems that, almost all of the tools that are available for the South American trypanosome *T. cruzi* work similarly. Particular attention is given to some of the challenges and opportunities now facing workers in this field: the strategies for forward genetic analysis and their importance; and the impact of RNAi technology on the study of gene function in African trypanosomes. It is clear that the differences in the genetic tools that are available for the different parasites are having a marked impact on the kinds of question that experimenters are now pursuing in these organisms.

Introduction to trypanosomatid protozoans

The family Trypanosomatidae (order Kinetoplastida) diverged early in eukaryotic evolution, near the base of the evolutionary tree, before the emergence of several protozoan lineages and well before the separation of the 'crown' metazoan lineages that comprise plants, animals and fungi[5]. Trypanosomatids encompass numerous protozoan genera, none of which are free living. Some (for example, *Leptomonas*, *Herpetomonas* and *Crithidia*) are associated with, and transmitted exclusively by, insects, whereas others (such as *Phytomonas*) are found associated with plants. Some species harbour prokaryotic ENDOSYMBIONTS, and it has been proposed that some trypanosomatid genes were acquired after an ancient endosymbiotic event that involved an algae-like ancestor[6]. At least three times during evolution, trypanosomatids independently colonized mammalian hosts, giving rise to the ancestors of the South American trypanosome *T. cruzi*, the African trypanosomes (*T. brucei* ssp.) and *Leishmania*[7,8]. Notably, these have been associated with different insect vectors, different survival strategies in the vertebrate host and, of course, radically different disease pathology.

Given their evolutionary antiquity, trypanosomatid parasites have been invaluable for the discovery and the study of evolutionary novelty at the molecular, biochemical and cellular levels (for example, see REF. 9). At the molecular level, this includes antigenic variation through programmed DNA rearrangements in African trypanosomes, POLYCISTRONIC TRANSCRIPTION and *trans*-splicing to generate mature mRNAs, BENT DNA (in the KINETOPLAST DNA minicircles), circular amplifications that mediate drug resistance and templated mitochondrial RNA editing. At the biochemical level, these parasites rely heavily on GPI (glycosylphosphatidylinositol)-anchored surface molecules (which bear protein and/or carbohydrate moieties) to assemble their surfaces, and they have unusual enzyme cofactors, such as trypanothione rather than glutathione. At the cellular level, they contain novel organelles, such as the GLYCOSOME, the ACIDOCALCISOME, and the unusual mitochondrion that contains the kinetoplast DNA network with extensively bent DNAs and that is the site of RNA editing. Trypanosomatids also have specialized endocytic and exocytic pathways that are active exclusively in a remarkable compartment known as the FLAGELLAR POCKET.

Genetic crossing: to mate or not to mate?

The nature and the extent of genetic exchange vary considerably in trypanosomatids. It is well established that African trypanosomes undergo genetic exchange in the insect host phase of the infectious cycle, and there is good evidence that this occurs in the wild, although it is equally clear that genetic exchange is not obligatory[10]. Conversely, sexual exchange has not been shown experimentally in *Leishmania*, and the available data indicate that, in the wild, genetic exchange might be infrequent

ENDOSYMBIONT
An organism which lives in the cells of a host organism in a mutualistic relationship or while doing no apparent harm.

POLYCISTRONIC TRANSCRIPTION
The transcription of two or more adjacent open reading frames after a single transcription initiation event. In trypanosomatids, this might extend more than 60 kb and encompass dozens of open reading frames.

BENT DNA
Intrinsically bent DNA arises from a series of 8–10 adenine residues on the same strand that is thought to assist in packaging it into a tight network.

KINETOPLAST
An organelle in the mitochondrion that consists of a large concatenated DNA network of both minicircles (each with an intrinsically 'bent' sequence and guide RNAs required for RNA editing) and maxicircles (which encode genes that are typically associated with mitochondrial DNA in other organisms). It is a defining feature of the protozoan order Kinetoplastida, to which the trypanosomes belong.

GLYCOSOME
A subcellular compartment, related to the peroxisome, originally named because it contains glycolytic enzymes, although other enzymes have subsequently been found there.

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ACIDOCALCISOME
An organelle that contains high concentrations of calcium and polyphosphates. Its role is poorly understood, but it might function as a reservoir for calcium in intracellular signalling and for energy.

FLAGELLAR POCKET
A 'pocket-like' invagination of the cellular membrane from which the flagellum arises in trypanosomatids.

EPISOME
A replicon that can exist either extrachromosomally or when integrated into the bacterial chromosome.

at best[11]. So, sexual crosses and positional cloning approaches for identifying phenotypes of interest are feasible in trypanosomes, albeit subject to the limited availability of tsetse fly colonies, but are poorly suited to applications in *Leishmania*. It should also be noted that, even in species for which there is clear evidence of genetic exchange, there are data indicating that clonal evolutionary propagation might be the predominant mode in many lineages[12].

The toolkit for reverse genetics

Various reverse genetics tools are available for trypanosomatids (TABLE 2). DNA can be introduced transiently with >90% efficiency in trypanosomes[13] and with up to 30% efficiency in *Leishmania*, whereas stable transfection can be achieved at high efficiencies in both, with a frequency of up to 5% in *Leishmania* (K. Robinson and S.M.B., unpublished observations). Transcription of stable transfection vectors is typically driven by endogenous RNA polymerases when integrated into the chromosome or by 'trapping' on circular EPISOMES in *Leishmania*; transient transfection vectors contain endogenous (RNA polymerase I) or heterologous (phage T7) promoters, with the latter being typically stronger[2,14–16] (K. Robinson and S.M.B., unpublished observations). Several positive and negative selectable markers, such as thymidine kinase and cytidine deaminase, are available. Due to the challenges posed by diploidy and the difficulty or impossibility of sexual crosses, dominant drug-resistance markers are the most useful. Expression vectors that allow the expression of heterologous proteins in various settings are available, including circular episomes and linear artificial chromosomes (ACs) in trypanosomes and in *Leishmania* (TACs and LACs, respectively)[17,18]. Most trypanosomatid species grow rapidly in standard culture media (with doubling times typically of 6–10 h), and colonies can be obtained by plating on semi-solid media, albeit with varying degrees of difficulty — *Leishmania* perhaps being the most adept. So, several rounds of genetic manipulation can be carried out in a reasonable amount of time (at least by somatic-cell genetic standards, if not by those of bacteriologists).

One interesting complexity of trypanosomatids that affects reverse genetics methods at many fundamental levels involves peculiarities in the mechanisms of gene expression that are used by this group. As in other eukaryotes, trypanosomes have three RNA polymerases with typical functions: RNA polymerase I transcribes ribosomal DNA, polymerase II transcribes protein-encoding genes and polymerase III transcribes small RNAs such as tRNAs and small nuclear RNAs[14,19]. However, in trypanosomatids, RNA polymerases I and II have taken on additional roles that are associated with, or required for, polycistronic transcription[20,21]. Typically, trypanosomatid genomes are organized into long arrays of open reading frames (ORFs)[4], which are transcribed into long polycistronic pre-mRNAs. These are then processed by coupled *trans*-splicing and polyadenylation reactions to generate mature monocistronic mRNAs, each of which bears at its 5′ end a common 39-nucleotide (nt) mini-exon[19]. The mini-exon is transcribed by RNA polymerase II, yielding an ~110–140-nt donor for the *trans*-splicing reaction. Uniquely, in African trypanosomes, the abundant blood-stream-stage variant surface glycoproteins (VSGs) and the tsetse-fly stage procyclins are transcribed by RNA polymerase I, possibly because the responsibility for capping and start site selection are taken over by the RNA-processing apparatus in trypanosoids[22].

By contrast, the promoters of all other protein-encoding genes have yet to be identified, despite considerable effort by many laboratories[14]. Given that two principal roles of promoters in other eukaryotes (capping and mRNA start site selection) are instead handled by the trypanosomatid *trans*-splicing machinery, the role of promoters seems to have been de-emphasized in these organisms. Here, RNA polymerase II need not initiate at a particular site as long as it is sufficiently processive to transcribe long stretches of the genome. Remarkably, in trypanosomatids, this has reached the point at which promoters are not necessary and possibly might not exist for RNA-polymerase-II-transcribed protein-encoding genes[14]. Because the regulatory needs of these parasites are considerable as they undergo their complex life cycle developmental transitions, their needs must be accomplished through downstream mechanisms that involve RNA and protein regulatory processes[14,19].

Table 2 | **A summary of genetic tools in trypanosomatid protozoans***

	Trypanosoma brucei	*Trypanosoma cruzi*	*Leishmania*
In vitro culture models	++‡	++	+++
In vivo disease models	++	++	+++
Transient transfection	+++	+	++
Stable transfection	+++	++	+++
Expression vectors:			
Episomal	+	++	+++
Integrating	+++	+	+++
Regulatable	+++	–	+
Selectable markers:			
Positive	6	3	8
Negative	1	1	2
Gene knockouts	+++	++	+++
Sexual crossing	+	ND	–
Positional cloning	Possible	ND	–
RNAi	+++	ND	–
Functional rescue	+	ND	+++
Transposon mutagenesis	+	–	++
Genome size (Mb)	35	40	35
Number of chromosomes	11 + mini-chromosomes	>30	~36

*For references, please see the appropriate sections of the text; for reviews, see REFS 2,11. ‡The '+' symbol indicates that a given method has been established, the number of + symbols reflects an approximate assessment of how well the method works and/or the extent of its use in a given species. For example, although genetic crosses work well in African trypanosomes, the need for a specialized tsetse fly (an obligate vector) colony has hindered widespread use of this method. Similarly, although good models for various developmental transitions in trypanosomes exist, usually a given strain can only execute a subset of these[78]. ND, not determined.

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Therefore, to obtain the basal expression of episomal constructs in *Leishmania*, the only *cis*-acting element that is required is a splice acceptor — which can be completely synthetic — located 5′ of the ORF of interest[23]. This can also provide the signal for polyadenylation once transcription around the circular template is complete[24]. By contrast, episomal DNA is rare in trypanosomes, and although the signal requirements of RNA processing resemble those of *Leishmania*, expression vectors are typically designed to integrate into chromosomes where they are subsequently transcribed[2]. As discussed below, the rarity of episomes in trypanosomes might be a consequence of differences in RNA processing that are related to RNAi, rather than from the basic mechanisms of transcription.

Homologous gene replacement or targeting works particularly well in trypanosomatids, with few if any convincing reports of non-homologous recombination[25]. Although two rounds of targeting are required to obtain null mutants, they are readily generated for non-essential genes. In *Leishmania* and possibly trypanosomes, attempted knockouts of essential genes led to a remarkable emergence of ANEUPLOID and POLYPLOID parasites, an unusual outcome that is now used as a criterion for whether the targeted gene is essential[26]. In trypanosomes, conditional knockouts of essential genes have been achieved after placement of an integrated gene copy under the control of the tetracycline-inducible system[27]. This system has proved exceedingly useful and broadly applicable, especially for the evaluation of essential genes. Occasionally, problems that are associated with leakiness in the regulatory system emerge and, under some conditions, regulation breaks down giving rise to revertants, such as that seen with trypanothione reductase in *T. brucei*[28].

All is silence?

Recently, gene silencing or RNAi technology has risen to prominence in trypanosomes. The effectiveness of RNAi in *T. brucei* was first shown for α-tubulin, by expressing a stem–loop or double-stranded RNAs (dsRNAs) after transfection or by direct transient introduction of dsRNAs[29,30]. Placement of integrated RNAi constructs under control of the *tet* repressor system provides a rapid and powerful method to probe gene function in trypanosomes, which allows investigators to evaluate quickly the importance of dozens of genes in a single study[13,31–33]. Antisense approaches and RNAi are particularly well suited to the study of trypanosomatids, as transcripts from both alleles (or from tandemly repeated gene families) are downregulated, and so the problems of diploidy and the lack of, or difficulties with, a sexual cycle are conveniently overcome. The success and utility of antisense strategies was prominent at the recent Woods Hole Molecular Parasitology Meeting, with more than 50 abstracts devoted to the application of this remarkable technology (see online link). In many respects, RNAi has become the tool of choice for probing gene function in trypanosomes[30].

Remarkably (and disappointingly to many), RNAi approaches seem to function poorly and perhaps not at all in *Leishmania* (K. Robinson and S.M.B., unpublished observations), and the absence of RNAi applications in this parasite was conspicuous at the aforementioned conference. Similarly, on the basis of our experiences with various genes and expression vectors in *Leishmania*, antisense approaches work poorly, showing only small effects at best[34–36]. An exception is the *L. donovani A2* gene, the strong antisense inhibition of which led to reduced parasite virulence[37]. Although many workers now believe that antisense effects often arise from RNAi mechanisms, in the *A2* study it was concluded that inhibition was achieved through non-RNAi mechanisms[38]. The apparent absence of RNAi activity in *Leishmania*, despite many attempts to show it, is remarkable in light of our increasing recognition of the importance of RNAi-like processes. Many cellular functions have been shown to incorporate one of the defining features of RNAi activity, namely small complementary RNAs (known as small interfering RNAs in RNAi studies); these roles include chromatin structure, DNA rearrangements, RNA processing and stability, and translation[39–41]. With the exception of altered mRNA stability, the role of small complementary RNAs in these processes has not been established.

RNAi pathways have been implicated in the control of the spread of 'genomic parasites', such as transposable elements and viruses[41,42]. This makes it particularly paradoxical that trypanosomes have an abundant number of mobile element families, whereas *Leishmania* noticeably lacks them (for example, see REF. 43 and references therein). However, the lack of RNAi activity in *Leishmania* is consistent with several studies that show antisense transcription and/or transcripts in this organism, which implies significant natural levels of dsRNA[44,45] (N. Fasel, personal communication). This has been most thoroughly described for episomal cDNAs, including gene amplifications that often occur in natural and drug-resistant lines[46–50]. Curiously, episomal cDNAs are scarce in trypanosomes, and those described so far have unusual properties[51]. Could this reflect a need to avoid the generation of dsRNA in trypanosomes but not in *Leishmania*, making this species permissive for episomal gene amplification because of its tolerance to dsRNA? Nonetheless, experimental studies of RNAi and transcription in *Leishmania* indicate that it is probably remarkably tolerant to the presence of dsRNA, and that a significant level of endogenous dsRNA might be the 'norm'. This might not bode well for the future of RNAi tools in this parasite. For example, it predicts that attempts to restore the RNAi pathway to *Leishmania*, through the introduction of relevant genes from trypanosomes (or other species) might fail because of the destruction of essential mRNAs that might normally be associated with dsRNA.

Although the loss of such an important pathway would be remarkable, the well-studied *Saccharomyces cerevisiae* (budding yeast) lacks genes that are required for RNAi activity, whereas its relative *Schizosaccharomyces pombe* (fission yeast) retains them[52]. However, the RNAi

ANEUPLOID
Having an unbalanced chromosome number. An example is trisomy.

POLYPLOID
Having more than two complete sets of chromosomes (two sets being the prevalent diploid state).

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pathway in *S. pombe* shows some differences compared with those pathways in other eukaryotes[53]. Furthermore, seemingly healthy mutants that are deficient in RNAi activity have been obtained in several species including *Caenorhabditis elegans*[54]. The explanation of the forces that lead to the loss of RNAi pathways in nature is speculative at best. Curiously, both *Leishmania* and *Saccharomyces* are host to RNA viruses, and RNAi is thought to feature prominently in the resistance of eukaryotic cells to these parasites[55]. Could the loss of RNAi in these lineages be a reflection of a successful viral attack on these organisms at some point in their evolutionary past? Interestingly, one *Leishmania* strain that carries an RNA virus has a growth advantage under certain conditions[56]. Regardless, so far, RNAi approaches have not been rewarding to investigators who pursued them in *Leishmania*, whereas in African trypanosomes, their riches have been abundantly forthcoming.

Forward genetics by complementation

When mutants are available, genetic rescue can be achieved through transfection of wild-type DNA libraries, by using vectors that integrate into the genome (in trypanosomes) and by using episomal shuttle cosmid vectors (in *Leishmania*) (FIG. 1). The challenge is to overcome the 'asexual diploid' problem that is associated with the generation of mutants — two independent mutations or a mutation followed by a loss-of-heterozygosity (LOH) event are required to obtain a loss-of-function mutant[57,58]. In *Leishmania*, the frequency of mutations and LOH has been estimated

LOSS OF HETEROZYGOSITY (LOH). A loss of one of the alleles at a given locus as a result of a genomic change, such as mitotic deletion, gene conversion or chromosome mis-segregration.

PANNING A technique for isolating parasite subpopulations; monoclonal antibodies or lectins are attached to solid supports over which parasite populations are passed and allowed to attach; after washing, bound parasites are eluted and recovered.

FLUORESCENCE ACTIVATED CELL SORTING (FACS). The separation of cells or chromosomes by their fluorescence and light-scattering properties, which are measured as the particles flow in a liquid stream past laser beams. The stream is then broken into droplets, and selected droplets are electrically charged and deflected into collection vessels as they pass through an electric field.

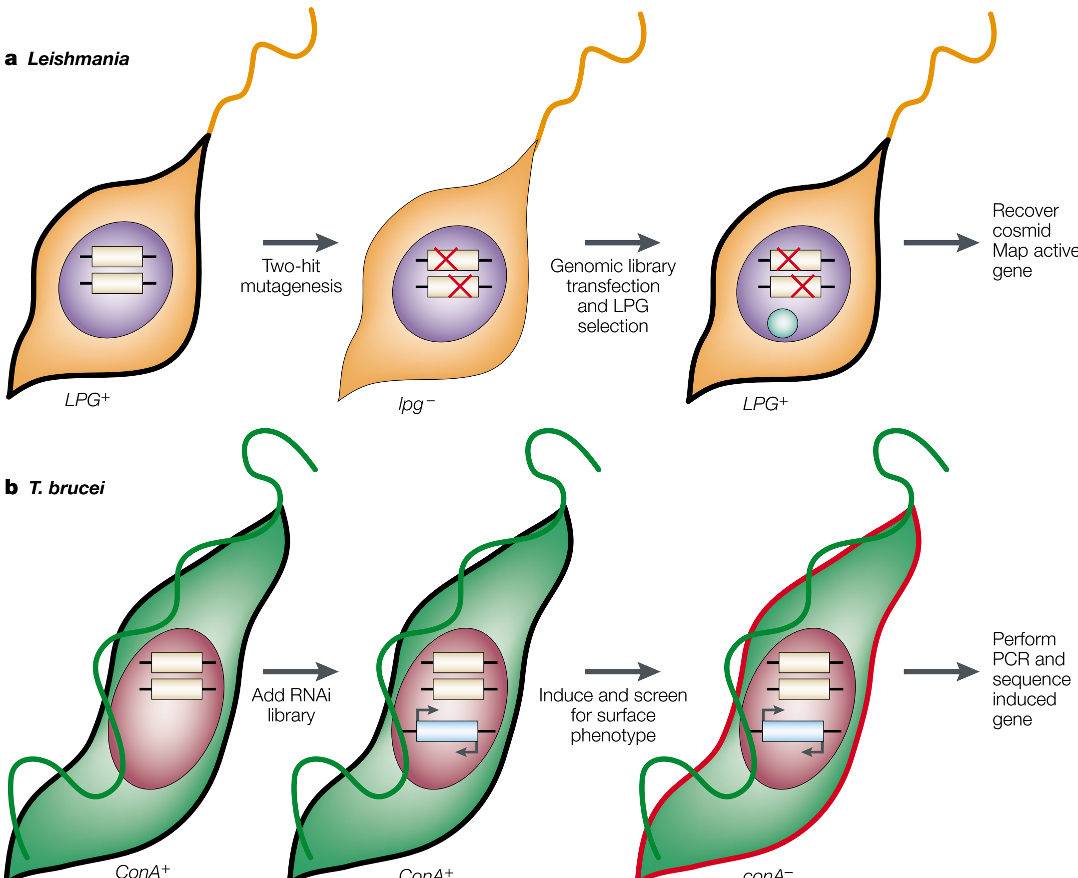

Figure 1 | **Comparison of forward genetics in *Leishmania* and *Trypanosoma brucei*. a** | *Leishmania* parasites are mutagenized heavily to generate cells that carry mutations in both alleles (two hits; red crosses) or one allele followed by a LOSS OF HETEROZYGOSITY (not shown). Mutants (here, those with affected synthesis of the major surface glycoconjugate lipophosphoglycan, LPG) are then recovered (here, by loss of the ability to agglutinate with LPG-specific lectins (green circle) or monoclonal antibodies), and then transfected with a wild-type genomic DNA library in a shuttle cosmid vector. Parasites in which LPG synthesis has been restored are recovered by PANNING or FLUORESCENCE-ACTIVATED CELL SORTING, the episomal cosmid is recovered and the active gene is identified by transposon mutagenesis or deletion analysis. A thick outer line represents the wild-type cell surface; a thin line represents the mutant cell surface. **b** | In the pioneering work by Englund and colleagues[69], wild-type trypanosome parasites that express T7 RNA polymerase and the *tet* repressor are first transfected with an RNA interference (RNAi) library to yield a large population of independent transfectants. The RNAi library included opposing *tet* repressor-regulatable T7 RNA polymerase promoters, between which a library of random genomic DNA fragments was inserted (turquoise rectangle). After tetracycline induction, parasites that had altered or mutant phenotypes were identified (in this case, mutants with an abnormal cell surface (red) were identified by a failure to react with concanavalin A (ConA)), and the DNA segment responsible between the T7 promoters was identified by PCR.

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at ~10^{-6} and 10^{-5}, respectively, making the recovery of mutants that are defective in both alleles relatively rare ($<10^{-6}$ even after mutagenesis)[57]. So, to identify mutants, powerful screens or selections are required. This poses a major obstacle to the recovery of mutants that affect virulence, as their rarity precludes the use of brute-force screening, and the inability of avirulent mutants to survive in their host precludes this selective method.

In *Leishmania*, forward mutagenesis approaches have been particularly useful in the study of genes that affect the parasite surface. Powerful selections that use lectins or antibodies that are reactive to abundant surface glycoconjugates, such as lipophosphoglycan (LPG), have been developed[59]. Various mutants (such as *r2d2*, *c3po*, *ob1*, *jedi* and *spock*) have been isolated by using this strategy, and the genes that are responsible for their defects have been identified[59,60]. These include glycosyltransferases and proteins, such as activated sugar transporters and chaperone-like molecules, that participate in assembly and compartmentalization in the secretory pathway[59]. Remarkably, most of the genes that were identified in these studies are new and, in some cases, they defined protein families after mining of the nascent genome sequence databases.

As well as defining the complex pathway for LPG and its related glycoconjugate synthesis, mutants allow assessments of the biological functions of LPG in the context of the parasite itself. This has been crucial in the study of LPG, as many of the LPG domains are shared in exact or analogous forms with other parasite glycoconjugates. This makes the assignment of function to specific molecules (or domains) a complex and often contradictory task for standard biochemical or cell-biological assays[61]. For these studies, investigators create complete knockouts by removing the whole protein-encoding region, and generated complementing derivatives that restore the biological functions of the knockout to that of the wild type. These precautions of using complementing derivatives guard against the concerns that are associated with heavy mutagenesis or loss of virulence during culture or transfection, which is a common occurrence in some strains. The value of the genetic approach was immediate: for example, it turned out that, although LPG and related glycoconjugates clearly have principal roles in steps that are essential for mammalian virulence of *L. major*, their role in *L. mexicana* virulence is minimal at best[61–63]. The resolution of this surprising finding might lie in the different ways that these two seemingly similar parasites interact with the host immune system[63].

Forward genetics has been used in *Leishmania* in various gain-of-function settings, in schemes that capitalize on natural variation. In the case of LPG, cross-strain or species transfections that were modelled on the LPG mutant studies have been used to identify genes that mediate the LPG side-chain modifications implicated in developmental and species-specific modifications (D. E. Dobson, S. J. Turco and S.M.B., unpublished data). These, in turn, have been implicated in sand-fly tropisms[64]. This extends parasite genetics to both of the hosts that are required for completion of the infectious cycle. Notably, transmission is often the 'weakest link' in arthropod-borne diseases, making the study of insect-stage survival and virulence particularly important. Cross-strain or cross-species transfections have been used to identify loci that are associated with the ability of *L. donovani* to spread to internal organs after cutaneous infection[65]. Finally, the dominant nature of many drug-resistance mutations has made them especially amenable to forward genetics approaches, in particular to those that involve natural or artificially engineered cDNA amplifications that carry genes the overexpression of which confers resistance[47,66,67]. This strategy can be modified to identify genes that mediate resistance to chemical stress, such as to oxidants, that are associated with the infectious cycle[68]. So, although asexual diploidy is a limitation, there are several methods that include forward genetics approaches that promise to yield a rich harvest in the future.

Forward RNAi genetics: silence speaks volumes

In trypanosomes, the 'well-behaved' RNAi technology has recently been applied to forward genetic analysis (FIG. 1). Trypanosomes that had been previously engineered to express T7 RNA polymerase and the tetracycline repressor were transfected with an RNAi library that contained random segments of genomic DNA, transcription of which was under the control of opposing T7 RNA polymerase promoters regulated by *tet* operator elements[69]. After induction with tetracycline, synthesis of dsRNA that corresponded to the inserted DNA in each trypanosome transfectant was predicted to lead to loss of mRNA expression of the cognate gene. In this study, RNAi 'knockdown' parasites that had altered expression of the surface protein EP-procyclin were sought. EP-procyclin can be detected because it reacts with the lectin concanavalin A (conA), and sequencing of the introduced DNA that was present in several lines with reduced conA reactivity identified the trypanosome hexokinase gene. It was then shown directly that hexokinase inactivation led to a shift in expression from glycosylated EP-procyclin to unglycosylated GPEET-procyclin. This unanticipated finding was confirmed by 'reverse genetic' RNAi silencing of other glycolytic pathway genes. These data indicated that trypanosomes might somehow monitor glucose levels through the glycolytic pathway and modulate the expression of surface proteins accordingly. So, as in *Leishmania* and countless other organisms, forward genetics yielded an unanticipated result that led to an improved understanding of parasite biology. Although not all trypanosome genes are likely to yield phenotypes through RNAi approaches alone, it is already clear that a large fraction will[32], and plans for genome-wide scans are therefore already under way. These studies are just an initial foray into a bold new era of trypanosome genetics, exploiting the potential of 'forward' RNAi technology.

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Genetics with transposable ‘genomic parasites’

The power of insertional mutagenesis is compromised in asexual diploid organisms, but correspondingly its use in generating gene fusions that are suitable for probing protein localization and regulation in biological contexts is increased, as heterozygous insertions are frequently phenotypically silent. The fact that DNA integration occurs predominantly by homologous recombination in trypanosomatids is an obstacle to insertional mutagenesis strategies that have been successful in other organisms. Although they undoubtedly occur at a low frequency, non-homologous events have not yet been described in wild-type parasites. So, insertional mutagenesis has been achieved by importing heterologous transposons. Expression of the *Drosophila mariner* transposase in *Leishmania* enabled transposition of engineered *mariner* derivatives *in vivo*, and the ‘trapping’ of parasite genes[70], whereas electroporation of a pre-formed Tn5 transposase–transposon complex yielded random genomic insertions in trypanosomes[71]. So, the genomes of both organisms are now amenable to large-scale identification and trapping strategies, and to the systematic identification of proteins that target the unusual organelles of these organisms (and perhaps some yet to be identified).

One virtue of transposons is that they can be engineered to trap and/or report exclusively on protein levels. Such fusions are ideally suited for organisms such as trypanosomatids in which protein-based regulatory mechanisms might be a dominant regulatory force. An example of these is a *mariner* ‘sandwich’ transposon that can yield protein fusions that retain both amino- and carboxy-terminal information[72]. Such transposons can be used in shuttle mutagenesis strategies, in which transposition is achieved *in vitro* or in *Escherichia coli*, or after introduction of active transposase and transposons into parasites *in vivo*[70,73]. The use of translational gene fusions to probe protein levels, without the aid (or expense) of two-dimensional gels or MASS SPECTROMETRY, has been dubbed ‘proteogenomics’[74].

MASS SPECTROMETRY
A technique that provides accurate information about the molecular mass of complex molecules. It can identify extremely small amounts of proteins by their mass-fragment spectra.

A fork in trypanosomatid research directions

Although, obviously, there is considerable overlap in the questions, interests and methodologies that are used to study the trypanosomatid pathogens, differences in the types of tools and the available biological systems for their study are increasingly leading to a divergence in research emphasis. In African trypanosomes, the ability to generate, rapidly and systematically, conditional knockdowns of many genes through the use of regulatable RNAi technology provides a powerful tool for both forward and reverse genetics. So, when studying processes that are common to all trypanosomatids, such as kinetoplast DNA replication, *trans*-splicing and the novel cellular architecture, African trypanosomes provide a powerful model, effective at serving as the ‘yeast’ for basic shared trypanosomatid biology. These studies capitalize on the well-developed cell and molecular biology of these parasites[75].

Many of the processes that can be studied in trypanosomes differ in *Leishmania*, of course, where they are amenable to study by various genetic tools other than RNAi. However, attention in *Leishmania* has increasingly focused on the opportunities to study drug resistance, parasite virulence throughout the infectious cycle and the immunological aspects of host defences to intracellular parasites, for which well-characterized mouse models are used[76]. Notably, the ability to cultivate all stages of the *Leishmania* infectious cycle *in vitro*, and the viability of parasites that lack key virulence determinants during *in vitro* culture, facilitate the generation and testing of mutants that affect pathways essential for virulence (as opposed to viability). Because parasitic diseases such as leishmaniasis typically involve long-term chronic infections, the genetic tools for the study of these processes must be particularly robust and able to withstand long periods in the host. Here, the classic gene-knockout approach is well suited, as there is a tendency for the available conditional knockout or knockdown systems to break down over long periods of time (and especially in the face of strong selective pressures from the host)[28].

The future: protozomics

Although this review has focused on genetic tools and approaches, the impact of the emerging trypanosome and *Leishmania* genome projects cannot be ignored and, indeed, will lead to an explosion of functional genomic studies. Bioinformatic approaches and database mining will reveal many genes with specific expected functions in these parasites. However, because of the unique requirements of the infectious cycle, the demands that are placed by the parasite on many products of these genes that are necessary for its survival will differ and therefore warrant further study. Moreover, what is not present in the parasite genome might be as informative; parasites are notorious for discarding pathways when those of the host will do or when they have elected to carry out essential functions in unique ways. For example, database mining has yet to reveal convincing examples of RNA polymerase II transcription factors, other than the components of the basal transcriptional apparatus[77]; this is in keeping with the general de-emphasis of the transcriptional regulatory steps that are described above.

As noted earlier, a cautionary note emerges from forward genetic screens, which turned up many genes the function of which could not have been predicted from sequence data alone. As in other organisms, genome sequences will facilitate cosmid rescue and transposon trapping strategies, and in trypanosomes systematic tests of gene function through RNAi can be contemplated, in a manner familiar to those who study model organisms such as yeast or *C. elegans*. Second- and third-generation strategies, such as microarrays and proteomics, will undoubtedly rise to prominence in the near future.

Most exciting will be the joining of the riches of the genome with the genetic toolkit now available, and its application to unravelling the fascinating peculiarities of these organisms, as well as the ways in which they go about infecting their hosts and causing disease. We are still faced with inadequate chemotherapy and no effective vaccines, but the tools and resources that emerge from parasite genetics and genomics promise to alleviate this problem in the near future.

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