Trends in Parasitology

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Opinion

Trypanosomes as a magnifying glass for cell and molecular biology

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The African trypanosome, *Trypanosoma brucei*, has developed into a flexible and robust experimental model for molecular and cellular parasitology, allowing us to better combat these and related parasites that cause worldwide suffering. Diminishing case numbers, due to efficient public health efforts, and recent development of new drug treatments have reduced the need for continued study of *T. brucei* in a disease context. However, we argue that this pathogen has been instrumental in revolutionary discoveries that have widely informed molecular and cellular biology and justifies continuing research as an experimental model. Ongoing work continues to contribute towards greater understanding of both diversified and conserved biological features. We discuss multiple examples where trypanosomes pushed the boundaries of cell biology and hope to inspire researchers to continue exploring these remarkable protists as tools for magnifying the inner workings of cells.

Highlights

Trypanosoma brucei has unique features, a critical phylogenetic position, and is experimentally tractable, allowing considerable contributions to biology.

We focus on eight outstanding topics: glycosylphosphatidylinositol (GPI)-anchor; endocytosis; RNAi; genetic code variations; tRNA import; RNA editing; glycosome; alternative oxidase.

These showcase trypanosomatids critically inform molecular and cellular biology.

Some of these examples overturned molecular dogma based on understanding derived mainly from animals and fungi.

Public health campaigns and new drugs have all but eliminated the threat of African trypanosomiasis to health and economic wellbeing.

This endangers ongoing support for research into these flagellates, which, we believe, should continue: more surprises likely await, while the tractability of *T. brucei* is important to understanding other trypanosomatids that remain a global threat.

Trypanosomes: from disease to discovery

African trypanosomes are unicellular parasites of medical and veterinary importance severely affecting human and livestock populations in sub-Saharan Africa. These parasites are mainly restricted to a region of 10 million km^2 due to almost exclusive transmission by the tsetse fly insect vector. *Trypanosoma brucei* f. *gambiense* and *T. brucei* f. *rhodesiense* are *T. brucei* ecotypes that cause human African trypanosomiasis (HAT), a fatal disease that is historically of major proportions. The earliest epidemics documented by European workers date to the late 19th century and claimed up to 500 000 lives, forcing the colonial powers to call on medical scientists to develop a cure. This effort coincided with the dawn of modern pharmacology driven primarily by Paul Ehrlich and colleagues, and synthesis of the first synthetic drug, salvarsan, which dramatically changed the lives of those infected with *Treponema pallidum* (syphilis), but, unfortunately, failed to act against HAT [1,2]. The impact of trypanosome infections has, however, been understood by the inhabitants and travelers to Africa for centuries [3]. The 20th century witnessed two major HAT epidemics, at least partially controlled with effective, but toxic and cumbersome to administer, drugs [4]. Presently the number of new cases of HAT has been driven to a historic low, in the main by effective public health interventions, in hopes of meeting WHO projections for elimination as a public health problem by 2025 [5]. New drugs have also been developed, and efforts towards understanding disease mechanisms, transmission dynamics, and development of new therapeutic modalities are less pressing than has been the case for many decades [6]. Nevertheless, trypanosomes have provided notable insights beyond those directly related to disease mechanisms and therapies, which have had a significant impact.

Parasites are often taken as examples of organisms that depart from the standard textbook view of molecular and cellular biology and biochemistry, a model based on, amongst other organisms,

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fungi, animals, and vascular plants, as eukaryote exemplars, and bacteria such as *Escherichia coli* and *Bacillus subtilis* for prokaryotes. There are, obviously, many excellent reasons for this apparent viewpoint bias: animals, including ourselves, are inherently of high priority for understanding health and disease mechanisms, plants are agriculturally essential, yeasts have been employed for millennia in diverse roles, and certain species of bacteria, plants, fungi, and animal cells are nonpathogenic. Moreover, selected organisms within these domains are also favored due to experimental malleability, while parasites are challenging due to complex life cycles, difficulty in culturing and/or genetic manipulations, and present inherent safety concerns, albeit that many of these issues have been overcome, and with the fast-growing cohort of fully sequenced parasitic and free-living protists a vast new resource is emerging [7]. Appreciation of diversity, ecology, and the contributions of eukaryotic microbiology highlights the importance of select parasites as models to sit alongside *Saccharomyces*, *Drosophila*, mammals, *Arabidopsis*, and others [8].

However, this is still not the case, and cellular and molecular features originally discovered in parasites are frequently seen as oddities associated with separate evolutionary histories and/or the parasitic lifestyle, accompanied by the never-ending arms race with host immunity and streamlining associated with exploiting host resources. This goes with the mostly unarticulated, nevertheless palpable, view that the molecular aspects and structures studied have relatively little relevance for free-living organisms. Yet trypanosomes have contributed extensively towards understanding many broadly distributed biological phenomena originally considered parasite-specific, or at least taxonomically restricted, and such discoveries have revealed features generally applicable across eukaryotes. To remind readers, these features include *trans*-splicing, **RNA editing** (see Glossary), glycosylphosphatidylinositol (GPI) anchoring, organelle repurposing, and alterations of the genetic code (Figure 1). In many instances, similar pathways or features have been subsequently identified in numerous lineages, including humans, although frequently not in such an extreme form. Hence, trypanosomes have played, and hopefully will continue to play, a valuable role in revealing novel phenomena of fundamental importance to cellular and molecular biology.

While the aforementioned argument could be applied to numerous unicellular and multicellular parasitic organisms, *T. brucei* features most prominently among the better-studied parasites, thanks to decades of intense and dedicated research. A wide spectrum of methods for forward and reverse genetics, and increasingly rich proteogenomic datasets, are helping to address questions of general biological interest. For example, *T. brucei* recently joined an elite club – with human cells and two yeast species – for which the majority of proteins have been localized within the cell (http://tryptag.org/) [9]. This resource allowed for the most comprehensive categorization of proteins into mitochondrial subcompartments to date [10] and will doubtless be powerful for detailed dissection of other cellular components [11]. Due to space, we focus on selected molecular and cellular processes discovered in trypanosomes and related flagellates, concisely describe how these key discoveries have shaped current understanding of a typical eukaryotic cell, and broadly compare each of these examples between trypanosomes and other (multicellular) eukaryotes.

RNA editing: a new paradigm

An exemplary case of trypanosomes illuminating molecular biology is the discovery of RNA editing (Figure 1). Initial findings that post-transcriptionally inserted nucleotides within mRNA occurred in the mitochondrion-encoded cytochrome *c* oxidase subunit II of an obscure fly parasite, *Crithidia fasciculata*, and subsequently in its relative, *T. brucei* [12], established a whole new field of RNA biology. This modest editing example, insertion of four uridines, was quickly followed by observations of elaborate editing involving a multitude of uridine insertions and deletions in other mitochondrial transcripts in additional trypanosomatids. These discoveries prompted comparative

analyses of DNA and RNA sequences from various organisms spanning all domains of life, which unearthed multiple examples and types of RNA editing [13], most commonly involving base conversions rather than the insertion/deletion editing found in trypanosomes [14]. One might argue that the discovery of four inserted uridines to correct a frameshift in the reading frame of a mitochondrial mRNA, leading to the inception of the term RNA editing, was a conceptual advance that allowed for the later discovery of different forms of RNA editing in other eukaryotes.

The research on RNA editing also led to a paradigm shift regarding the existence of noncoding small RNAs. The transformative discovery of guide RNAs as editing templates [15] motivated an intense search for similar RNA species in other organisms, and especially in plants, where editing is particularly extensive, but these efforts were ultimately futile, as distinct mechanisms are operating. Although of a different extraction, guide RNA-(like) molecules have appeared with a vengeance, playing key roles in CRISPR/Cas bacterial immunity and genome editing biotechnology [16]. Some early hypotheses based on the presence of guide RNA molecules, for instance that trypanosomes might have retained remnants of an early, simpler, RNA world [17], turned out to be wrong [1]. Quite the opposite, the non-ancestral, highly complex, editing machinery of trypanosomes, composed of dozens of specialized proteins and hundreds of guide RNA molecules [18,19], is unparalleled in its complexity.

An unexpected alteration of the 'universal' genetic code

The genetic code, which defines the three-letter combination of nucleotides corresponding to a particular amino acid or a stop signal, is considered a universal feature of all life, although at least 32 variants, frequently rare or restricted in their taxonomic distribution, have been described (https://www.ncbi.nlm.nih.gov/Taxonomy/Utils/wprintgc.cgi). Record holders for **non-standard genetic codes** are organellar and protist, in particular ciliate, genomes [20]. Belatedly, trypanosomatids of the genus *Blastocrithidia* have joined this disparate group, recoding all three stop codons into sense codons, using UAA as both coding (tryptophan) and the only remaining stop codon (Figure 2) [21]. Although this is one of the most extreme deviations described so far, it is not unprecedented, with a similar case reported for a ciliate [22]. However, failure to find a tRNA recognizing UGA in this ciliate was interpreted as a result of poor genome assembly [22], an understandable argument considering the notoriously complex ciliate genomes. Such reasoning could not apply to a high-quality assembly of the compact *Blastocrithidia nonstop* genome, with almost all of its 7259 protein-coding genes containing the UGA stop codon in-frame. The absence of the canonical UGA tRNA led to the discovery of a highly unusual UGA tRNA [21]. This tRNA displays an anticodon stem that spans only four rather that five canonical base pairs allowing for significant changes in codon recognition. This sets a precedent, since as of now, tRNA codon specificity cannot be unambiguously assigned based solely on the anticodon [23], and is likely to have wide-reaching consequences, as thousands of truncated anticodon stem tRNAs are found in bacterial and eukaryotic (including human) genomes. These tRNAs have been discarded as nonfunctional, but this new evidence from trypanosomes suggests that many may have a function in diversifying the **standard genetic code**.

The essentiality of tRNA import for mitochondria

In all organisms, genetic code redundancy is accommodated by single tRNAs decoding multiple synonymous codons with identical amino acids. In the case of mitochondrial genomes, a minimal set of 21 different tRNAs is sufficient to handle translation of its protein-coding genes. However, detection of nuclear-encoded tRNAs in the mitochondrion of the protist *Tetrahymena* [24] led to the proposal that (some) tRNAs can be imported from the cytosol. Obligatory mitochondrial tRNA import became obvious with the seminal discovery that *T. brucei* mitochondrial genomes lack tRNA genes, with the organelle importing all tRNAs from the cytosol [25]. This led to the

Glossary

Alternative oxidases (AOXs): mitochondrial di-iron carboxylate enzymes oxidizing ubiquinol instead of cytochrome *c*, in the absence of proton pumping. They are found in many highly diverse eukaryotic lineages.
Catalase: a characteristic enzyme of peroxisomes that detoxifies hydrogen peroxide (H_2O_2), by catalyzing its breakdown into water and oxygen ($2\ H_2O_2 \rightarrow 2\ H_2O + O_2$).
Glycosomes: organelles, so far restricted to trypanosomatids and diplonemids, derived from peroxisomes and so named because they house the majority of the enzymes making up the glycolytic pathway.
GPI-anchor: a glycosylphosphatidylinositol-group attached via an amide linkage to a protein's C-terminal carboxyl group, which allows anchoring to a cell membrane.
Non-standard genetic code: over 32 different alterations of the genetic code, in which one or more codons have been reassigned to encode an amino acid differing from the one specified by the standard genetic code. Such variants occur very rarely.
Phosphofructokinase (PFK): an enzyme that catalyzes the third, ATP-consuming step of glycolysis. In trypanosomatids, this enzyme is not inhibited or activated by any of the multiple metabolites known to act on PFKs of other organisms, except for *Leishmania* species where AMP acts as an inhibitor.
RNA editing: post-transcriptional sequence alteration in RNA molecules that changes coding information, particularly prevalent in organellar transcriptomes.
Standard genetic code: a set of extremely conserved rules used by a wide majority of extant cells to translate information from nucleic acids into amino acids.
Trypanothione: an antioxidant, unique to trypanosomatids, comprised of two molecules of glutathione joined by a spermidine linker. This makes trypanothione more reactive than the commonplace intracellular antioxidant, glutathione, which acts via two *cis*-acting molecules.
Variant surface glycoprotein (VSG): surface molecule of African trypanosomes covering the cell with a

descriptions of new tRNA import mechanisms in a variety of eukaryotes, most prominently *Saccharomyces cerevisiae* [26] (Figure 3). Mitochondrial tRNA import in trypanosomes remains incompletely characterized and may, or may not, require membrane potential, may be directly, or indirectly, aided by components of the protein import pathway and may even require cytosolic factors [27]. Despite these significant lacunae, trypanosomes were a major driver in the tRNA import field and facilitated studies in other organisms, including humans [28], for example, in the context of exploiting 'healthy designer RNAs' to address incurable mitochondrial myopathies

dense coat. VSG switching is effectively used for evasion of the vertebrate host immune system.

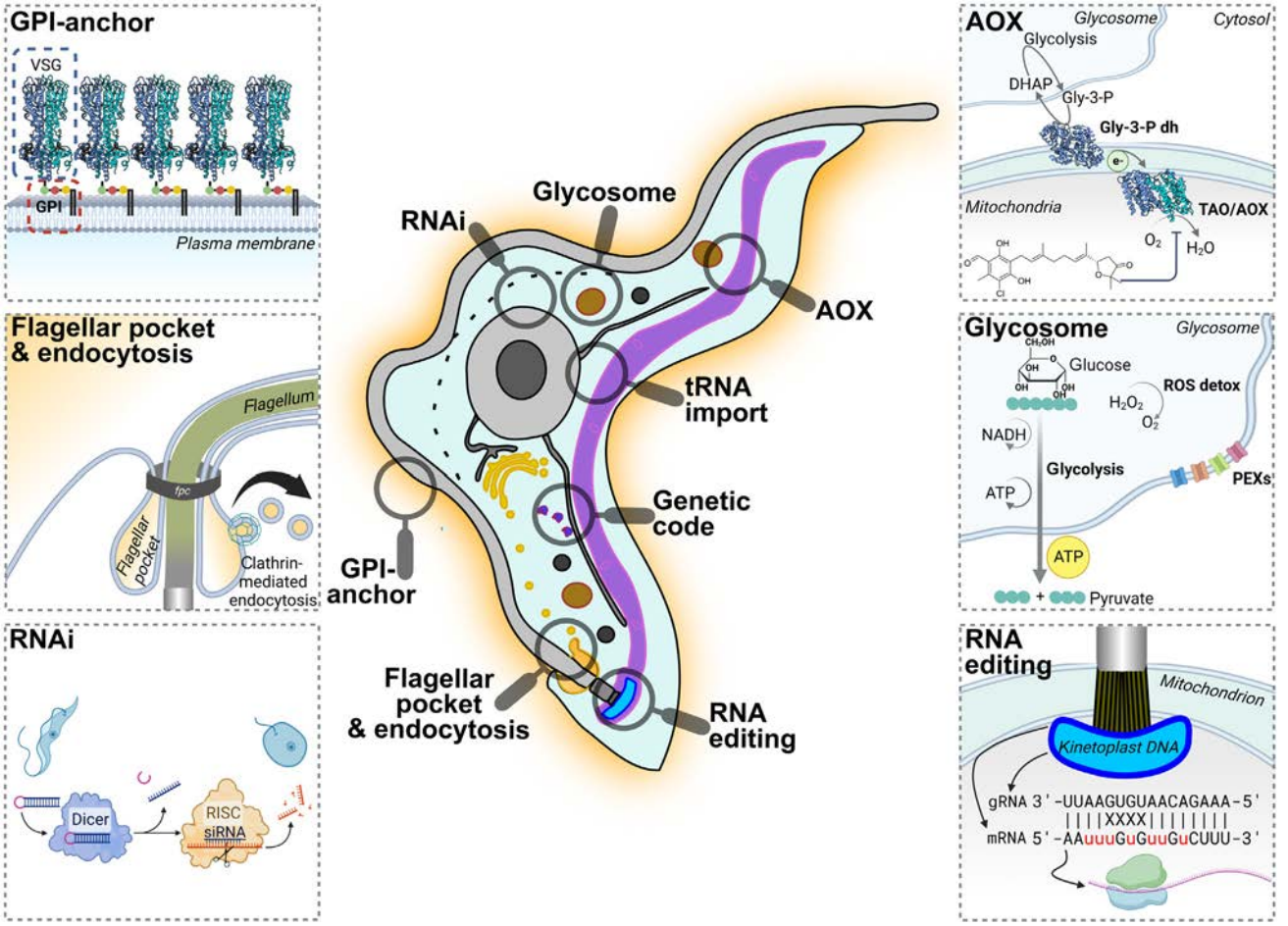

Figure 1. A subset of seminal discoveries made in trypanosomes with a broader impact on cell and molecular biology. Some selected highlights are elaborated upon in the surrounding insets, which are explained here in a clockwise order. AOX (alternative oxidase) maintains redox in concert with the glycerol-3-phosphate (Gly-3-P) shuttle; the chemical structure of the AOX inhibitor ascofuranone is shown. Glycosomes compartmentalize most of the enzymes involved in glycolysis (the pathway that catabolizes 6-carbon glucose to 3-carbon pyruvate, the latter of which is formed in the cytosol) by maintaining redox ($NADH/NAD^+$) and energetic (ATP/ADP) balance; ATP for the cell is generated by the final steps of glycolysis that take place in the cytosol (yellow circle). RNA editing involves the post-transcriptional insertion of uridines (symbolized by red 'u') into mRNA as directed by guide (g) RNA via canonical and noncanonical (X) base pairing. Mitochondrial biogenesis relies on tRNA import via still unknown protein complexes (see Figure 3 in the main text). A reassigned genetic code thanks to the ability of modified 4-base pair (bp) tRNAs able to decode the canonical UGA stop codon as tryptophan (see Figure 2 in the main text). RNAi (RNA interference) was serendipitously discovered when expression of a hairpin, double-stranded RNA complementary to β-tubulin mRNA caused the latter's degradation, resulting in 'fat' trypanosomes. The flagellar pocket (FP) is a specialized part of the plasma membrane where very rapid, clathrin-mediated endocytosis occurs. GPI (glycosylphosphatidylinositol)-anchored proteins coat the surface of *Trypanosoma brucei*, such as the depicted variable surface glycoprotein (VSG). Abbreviations: dh, dehydrogenase; DHAP, dihydroxyacetonephosphate; e-, electron; fpc, flagellar pocket collar; PEXs, peroxins; RISC, RNA-induced silencing complex; siRNA, small interfering RNA. Protein Data Bank (PDB) accession numbers of structures shown: VSG: 5LY9; AOX/TAO: 3VV9; Gly-3-P dh: 1EVY.

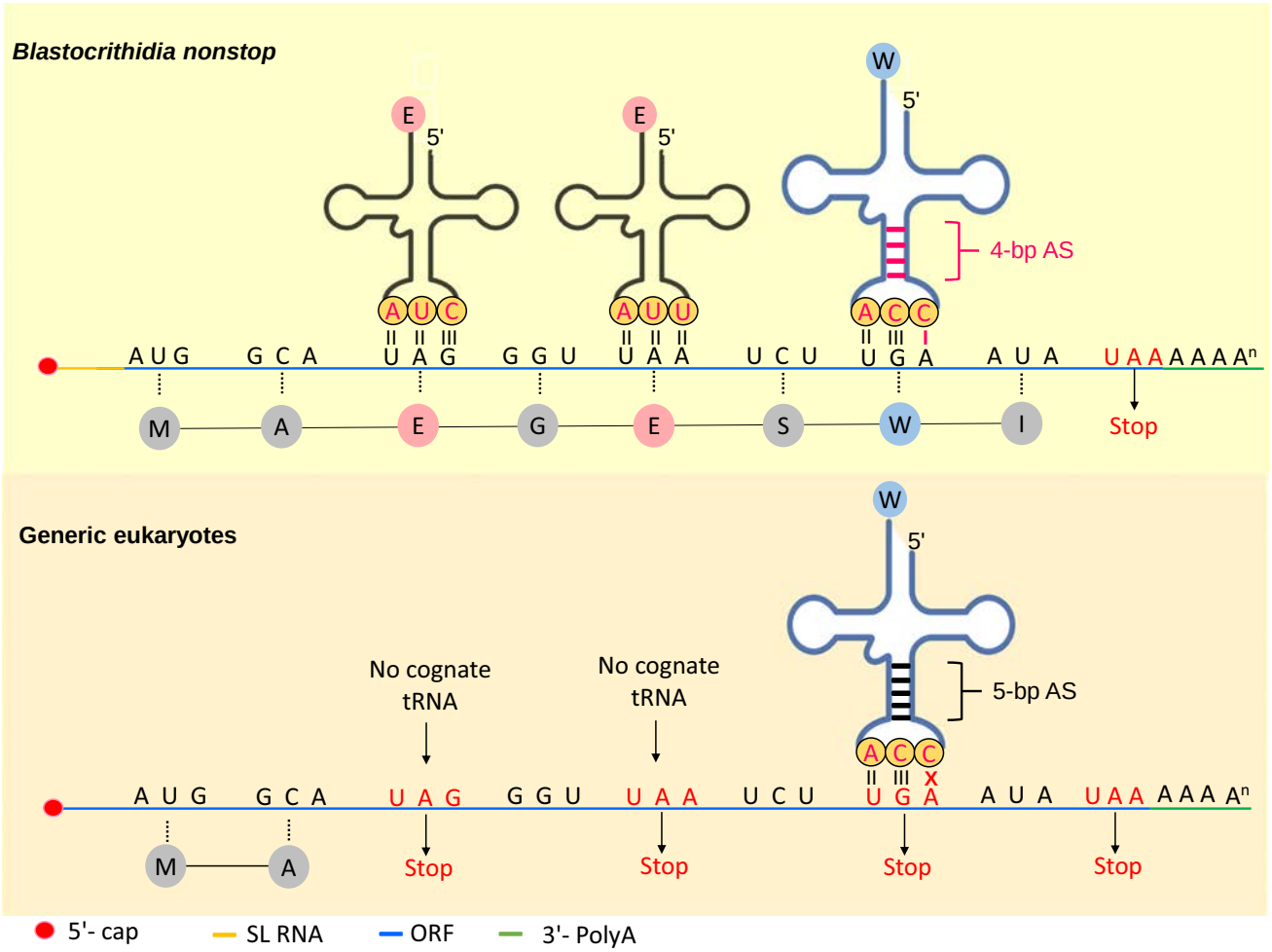

Figure 2. The extensively reassigned genetic code of the trypanosomatid *Blastocrithidia nonstop*. A representative coding sequence containing reassigned stop codons UAG and UAA recognized by fully cognate glutamine (E) tRNAs. Note that the third canonical stop codon UGA is decoded by a uniquely altered tRNA that specifies tryptophan (W). The anticodon stem (AS) of this tRNA is shortened from the canonical 5 bp to 4 bp, allowing it to wobble base pair with UGA. The UAA codon has dual meaning, that is, it also serves as the only remaining stop in *B. nonstop*. How homologous sequences would be translated in generic eukaryotes is shown for comparison. Abbreviations: 5′-cap, uniquely modified 5′ end of mRNA; SL RNA, splice leader RNA; ORF, open reading frame; 3′-polyA, polyadenylated 3′ end of mRNA.

[29]. While the full mechanisms and components of tRNA import remain to be elucidated, the trypanosome tRNA import system likely has more to contribute, undoubtedly providing fertile ground for further investigation and advancing fundamental understanding of the dynamics and mechanisms of tRNA import.

GPI anchors and adaptations within the endocytic apparatus

The cell surface of most trypanosomatids is dominated by GPI-anchored macromolecules; for the African trypanosome this is the **variant surface glycoprotein** (**VSG**), the first protein to have a **GPI-anchor** structure elucidated [30], albeit rapidly followed by Thy-1, the first mammalian structure [31]. The extensive similarity between the VSG and Thy-1 GPI-anchor structures indicated an early origin within the last eukaryotic common ancestor (LECA), along with a biosynthetic pathway which is also extremely well conserved [32]. Earlier work

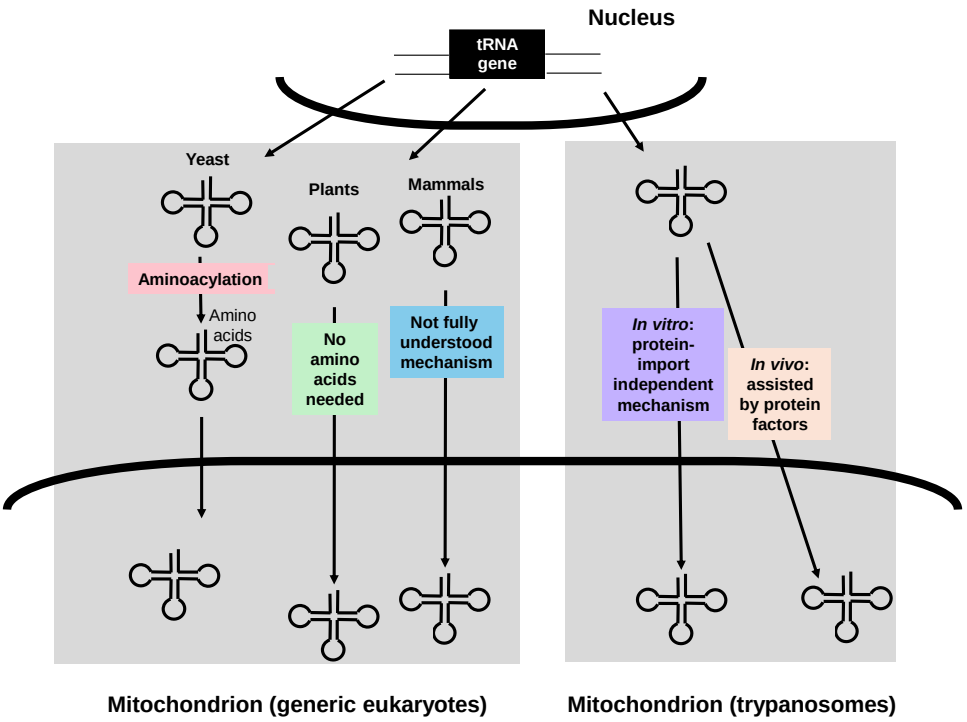

Figure 3. tRNA import into mitochondria in trypanosomes and other eukaryotes. In generic eukaryotes, such as yeast and plants, mitochondrial tRNA import is influenced by cytosolic factors. tRNA import into the organelle has also been described in mammals, but mechanisms remain unknown. Although protein factors may also be involved in trypanosomes, *in vitro* tRNA import efficiently occurs in the absence of added proteins and/or cytosolic factors, suggesting that multiple mechanisms may be at play.

demonstrated the presence of an unusual lipid modification responsible for anchoring VSG to the cell membrane, and it is clear that the huge VSG abundance was an important factor in enabling characterization of the GPI-anchor, and hence a paradigm for additional characterizations [33] (Figure 1).

Many trypanosome surface molecules exploit this mode of membrane attachment, including the mucins of *Trypanosoma cruzi*, lipophosphoglycan of *Leishmania*, and a variety of glycolipids; a considerable number of these molecules are directly involved in pathogenic mechanisms [32]. Variations in GPI-structure do occur, for example, replacement of the glycerolipid backbone by a ceramide-based lipid in some species. GPI-anchored protein functions are diverse and include cell adhesion, signal transduction, and enzymatic activity, with differential sorting and packaging within cell membranes likely one major function of the GPI-anchor [34]. GPI-anchoring is a component of the targeting machinery in polarized cells (e.g., neurons and epithelial cells), while an association with a highly specific lipid microenvironment can, in some cases, differentiate the functions of GPI-anchored and *trans*-membrane domain variants of the same protein [35]. Moreover, cleavage of the C-terminal GPI-processing signal acts as a quality control mechanism in both trypanosomatids and other eukaryotic lineages [36]. The only obvious feature differentiating GPI-anchored proteins in trypanosomatids and elsewhere is their dominance in terms of abundance over *trans*-membrane domain proteins at the cell surface.

African trypanosomes have extremely high rates of endocytic activity, linked to clearance of immune effectors from the cell surface, providing an efficient mechanism preventing recognition of surface determinants by antibodies and additional immune effectors [37]. The high flux is achieved by streamlining conventional clathrin-mediated endocytosis, reflected by the loss of dynamin and a selective cargo receptor. Significantly, these losses correlate with the presence of VSGs, consistent with adaptation to an extracellular niche [38]. Modifications in endocytic machinery composition are now known to be extensive across eukaryotes, with a high rate of secondary losses for the adaptin cargo receptors in particular [39].

Finally, in this regard, a prominent feature of the trypanosome is the flagellar pocket (FP), a specialized surface region where the flagellum enters the cell body, and which encompasses the flagellum with a ring-like structure, the FP collar. This structure creates a discrete plasma membrane subdomain with distinct protein composition and specialized roles in membrane transport. The prominent, easily recognizable structure and the exclusive presence of transport vesicles budding from/docking with the FP in trypanosomes made the specialized functions of this subdomain of the cell surface obvious [40]. The FP is structurally and functionally unusual in possessing the FP collar and a huge flux of membrane traffic, but membrane regions surrounding the base of the flagellum had been recognized earlier or suggested as having distinct functions more widely across eukaryotes [41]. The significance of the FP and broader similarity with cilia/flagellar proximal membrane subdomains became appreciated some time ago and required the advent of more sophisticated imaging and genetic modification systems, but it is now well recognized as a general feature across eukaryotes, encompassing protists such as *Tetrahymena* as well as metazoan cells [42].

Glycosomes: insights into radical remodeling of organelles

Trypanosomatids have repeatedly shown how much a eukaryotic organelle can be transformed beyond the accepted definitions derived from a handful of model organisms. A prominent example of an organelle evolving a new function in a specific lineage is the **glycosome**, an incarnation of peroxisomes, the ubiquitous single-membrane organelle (Figure 1). Peroxisomes are named for their production of the reactive oxygen species (ROS) hydrogen peroxide (H_2O_2) by FAD-linked oxidases, a property connected to two other defining pathways [43]. ROS produced by β-oxidation of (very) long-chain fatty acids is neutralized by the hallmark peroxisomal enzyme, **catalase**.

Unlike plant-seed peroxisomes, initially considered their own organelle class [44], trypanosomatid glycosomes are very radical departures from the canonical organelle, and the majority of glycolysis enzymes are located within the organelle, hence the name [45]. This phenomenon both transformed the organelle and relocated an ancient metabolic pathway. The textbook allosteric inhibition of **phosphofructokinase** (**PFK**) activity by ATP, presumed to reduce futile glycolysis under high energy conditions, is lost in the organelle because of its stable ATP/ADP balance [46]. In *Leishmania*, PFK is inhibited by AMP to act as a switch between glycolysis and gluconeogenesis, demonstrating how ancient, deep-rooted enzymes can adapt in the small confines of the glycosome [47].

Another surprising departure is seen in the glycosomes of dixenous trypanosomatids, which have lost catalase, the signature peroxisomal enzyme, despite retaining the classical β-oxidation pathway, and thus producing H_2O_2 [48]. This might allow regulation of adaptation to different life cycle stages via ROS [49]. To deal with this loss, tryparedoxin peroxidase has been recruited to inactivate H_2O_2, with its cofactor tryparedoxin being oxidized during this process [50]. Next, another unique feature of kinetoplastids, **trypanothione**, a more reactive permutation of the common antioxidant glutathione, reduces tryparedoxin.

These examples illustrate the capacity of classical organelles to be transformed by evolution, utilizing other unique adaptations, but also how these lead to alterations in ancient eukaryotic pathways. But what properties link glycosomes to peroxisomes? For one, the biogenesis of both relies on peroxins, forming complexes within the organelle's membrane [51,52]. Furthermore, glycosomes of monoxenous trypanosomatids retained catalase [53], further evidence of their relationship to peroxisomes. While the adaptive advantage of the peroxisome–glycosome transformation is still debated [51,54,55], trypanosomatids once again have led the way: showing that, under the right circumstances, some organelles can change more radically than textbooks suggest.

Alternative oxidase: a gateway to extreme metabolic alteration

The uniqueness of the *T. brucei* bloodstream form (BF) mitochondrion was recognized as early as the 1960s when it was shown that the parasite is completely dependent on glycolysis for its energy supply, excluding its mitochondrion as the cellular powerhouse [56]. Moreover, the BF mitochondrion contains no cytochrome-mediated respiration yet consumes oxygen at a high rate [57]. The enzyme responsible for this high respiration was originally named glycerol-3-phosphate oxidase (GPO) and localized in the parasite mitochondrion [58]. GPO consists of two components, FAD-dependent glycerol-3-phosphate dehydrogenase and **alternative oxidase** (**AOX**), linked by ubiquinone. Its activity is critical for maintaining glycolytic redox balance by reoxidizing glycolytically produced NADH, donating its electrons to oxygen [59,60] (Figure 1).

Mitochondrial AOXs are di-iron carboxylate enzymes with ubiquinol oxidase activity that occur in most kingdoms of life, with prominent examples identified in free-living and pathogenic protists (including trypanosomes), higher plants, algae, fungi, slime molds, and nematodes [61]. AOXs are usually coexpressed alongside the canonical cytochrome-containing electron transport chain (ETC), allowing considerable metabolic flexibility to respond to a wide range of environmental or developmental conditions. The widespread AOXs therefore regulate cellular redox balance, protect against ROS formation, especially upon ETC inhibition, and contribute towards metabolic and signaling homeostasis, particularly during stress [62].

BF trypanosomes represent an extreme deviation from the other organisms mentioned, as they are dependent on AOX in the absence of the canonical ETC. Consequently, AOX is essential for this infectious stage, and due to its absence from humans, AOX is a potential therapeutic target [63]. The trypanosome enzyme provided the first AOX crystal structure at 2.3 Å resolution, with and without ascofuranone, a potent inhibitor *in vivo* and *in vitro* [64]. This enabled better structural predictions for additional AOXs, paving the way for structure-based drug discovery initiatives for trypanosomes, pathogenic fungi, and amoebae.

Since AOX is absent from complex metazoans, there is optimism that xenotopic expression of AOX could contribute towards therapy for numerous pathologies associated with mitochondrial dysfunction, including Alzheimer's and Parkinson's disease [65]. However, in numerous disease models, xenotopic AOX expression has shown different effects: curative, none, or exacerbating. In most of these studies, the AOX used was from a simple metazoan, the sea squirt *Cioana intestinalis*, the organism closest to humans, possessing AOX [66], raising the question of whether this AOX is the best option, as it has low ubiquinol oxidation activity. Since trypanosomal AOX has the highest reported activity, it may represent a superior option for treating human diseases.

RNA interference: trypanosomes 'almost' first

RNA interference (RNAi) is the process by which antisense RNA induces sequence-specific suppression of gene expression, following the formation of double-stranded (ds) RNA, containing the

antisense strand [67]. RNAi has emerged as an extremely powerful tool, greatly accelerating the functional analysis of eukaryotic genomes. In the mid-1990s two groups generated serendipitous, yet critical, morphological mutants in *T. brucei* that presaged the discovery of the RNAi mechanism. An attempt to construct GFP-expressing trypanosomes, linked to an already available inducible expression system, using one specific construct, produced fluorescent cells that exhibited a rounded morphology, so-called 'fat' cells [68] (Figure 1). Further analysis revealed the presence of an antisense 5′-untranslated region of α-tubulin in the construct as the key factor. The second serendipitous discovery involved transfection using long dsRNA homologous to α-tubulin, which downregulated tubulin synthesis, affecting cell morphology [68]. These experiments indicated that RNAi, reported a few months earlier in *Caenorhabditis elegans* [69], was operational in *T. brucei*.

Concurrent attempts used antisense RNA to downregulate PFR2, a major protein of the paraflagellar rod, targeting the antisense construct to the procyclin locus. This construct indeed led to downregulation of PFR2 protein and flagellar paralysis as expected, albeit with the mutant viable. However, further analysis revealed that PFR2 ablation had been achieved by fortuitous reverse insertion of the construct into the central region of one PFR2 locus. It also acted *in trans* on transcripts from the second PFR2 locus; this, as we now know, was the result of dsRNA production and RNAi. The mutant and background were published in early 1998 [70], predating by a few weeks the key publication from the Fire and Mello laboratories [69], for which they were awarded the Nobel Prize in Physiology and/or Medicine in 2006.

Concluding remarks

By no means are our examples an exhaustive sampling of trypanosome contributions to the wider body of biological knowledge, and how their specific biology acted to magnify processes and hence make them visible. Some trypanosome-initiated discoveries were later recognized as more universal, including tRNA import into mitochondria, degradation of cognate mRNAs by RNAi, GPI anchors as critical alternate mechanisms of membrane protein attachment, and divergent tRNA anti-codon stems facilitating readthrough of in-frame stop codons. Others are perhaps more conceptual, including functional repurposing of ubiquitous organelles, and the post-transcriptional RNA modifications. Last, some of these properties are not only of academic interest but can have practical purpose, such as the use of AOX and truncated tRNAs to treat human diseases [65,71].

In closing, due to their unusual biology, trypanosomes have led to vital scientific contributions. The absence at present of a direct threat to human wellbeing has perhaps made these organisms less attractive for winning competitive and limited research resources. We argue that, while this may indeed be the case, the true value of these organisms has almost always lain elsewhere: in the realm of basic discoveries in biology. Here, the contributions have been extensive, and all indications are that the opportunities for unveiling truly fascinating and insightful biology remain as appealing as ever. Some of these opportunities might revolve around issues we discussed: for example, did recoding of *B. nonstop* come about as a viral protection mechanism? Could trypanosome AOX mitigate malfunctioning human mitochondria (see Outstanding questions)? We hope that a new generation of researchers will be inspired by our examples to take full advantage of the powerful tools available in trypanosomes and to continue the tradition of these organisms being at the forefront of molecular and cell biology.

Outstanding questions

How widespread is the N^6-methyladenosine mRNA stabilization as recently reported for VSG mRNA in *Trypanosoma brucei*?

Does recoding of stop codons in *Blastocrithidia nonstop* protect from viral infections, and what additional advantages are conferred?

How frequent are tRNAs with truncated anticodon stems?

What are the adaptive advantages of RNA editing and glycolytic compartmentalization?

What adaptations in dixenous trypanosomatids facilitated loss of catalase, a peroxisome hallmark?

Why has RNA interference been retained in *T. brucei* when it is lost in closely related *Trypanosoma cruzi* and *Leishmania* species?

What is the nature of the tRNA import complex?

Can trypanosome AOX be used to treat human mitochondrial dysfunction?

How extensive are changes to the architecture and machinery of membrane trafficking in trypanosomes?

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Declaration of interests

The authors have no interests to declare.

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