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Ubiquitin and ubiquitin-like conjugation systems in trypanosomatids

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In eukaryotic cells, reversible attachment of ubiquitin and ubiquitin-like modifiers (Ubls) to specific target proteins is conducted by multicomponent systems whose collective actions control protein fate and cell behaviour in precise but complex ways. In trypanosomatids, attachment of ubiquitin and Ubls to target proteins regulates the cell cycle, endocytosis, protein sorting and degradation, autophagy and various aspects of infection and stress responses. The extent of these systems in trypanosomatids has been surveyed in recent reports, while in *Leishmania mexicana,* essential roles have been defined for many ubiquitin-system genes in deletion mutagenesis and life-cycle phenotyping campaigns. The first steps to elucidate the pathways of ubiquitin transfer among the ubiquitination components and to define the acceptor substrates and the downstream deubiquitinases are now being taken.

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Introduction

Ubiquitin is a single-domain protein of 76 residues with a β-grasp fold. Its sequence is highly conserved with just 2–4 residue differences between the ubiquitins of *Trypanosoma* species and their mammalian hosts [1]. Its attachment to target proteins is a widespread post-translational modification controlling molecular fate and cellular processes. Ubiquitination is mediated by tripartite enzyme systems comprising ubiquitin-activating (E1), ubiquitin-conjugating (E2) and ubiquitin-ligation (E3) components (Figure 1). Cellular homoeostasis is ensured by deubiquitinases (DUBs), which return the ubiquitin to the cellular pool (Figure 1). Ubiquitin can be attached in multiple copies and in different linkages to target proteins, giving rise to enormous complexity in what has been termed the Ubiquitin Code [2]. For example, Lys48-linked ubiquitination is associated with proteasomal protein degradation, while Lys-63-linked ubiquitination plays a role in protein recruitment to sites of DNA damage. This complexity is augmented by an array of structurally similar, but sequence-divergent, ubiquitin-like modifiers (Ubls), which have cognate E1, E2 and E3 components and Ubl-specific proteases. In trypanosomatids, hundreds of proteins are predicted to be involved in the attachment and removal of ubiquitin and Ubls [3]. Here, we review recent progress in defining and functionally characterising the ubiquitination systems of trypanosomatids to complement an excellent review of the ubiquitination system as a drug target in trypanosomatid diseases [4].

Ubiquitination and deubiquitination components in trypanosomatids

A survey of ubiquitination-system genes in *Leishmania mexicana* has been presented in recent papers [5–7]. These analyses identified 2 ubiquitin-activating (E1), 13 ubiquitin-conjugating (E2) and 79 ubiquitin-ligating (E3) enzymes together with 20 family C12, C19 and C65 cysteine protease deubiquitinases, 5 C97 PPPDE1-containing proteins that may have deubiquitinase activity and 3 JAMM (JAB1/MPN/MOV34) metallopeptidase DUBs. Deletion mutagenesis was used to generate a set of 58 null mutant parasites, each possessing a unique barcode for subsequent life-cycle phenotyping experiments. Null mutants of the E1, UBA1a, the E2s, UBC3, UBC7, UBC12 and UBC13, and the DUBs, DUB1, DUB2, DUB12 and DUB16, were not obtained, implying essential promastigote roles for these factors [5,6]. Twenty-three null mutants were pooled as procyclic promastigotes and grown to stationary phase before differentiation was induced and the survival of the parasites as axenic or intra-macrophage amastigotes, and in mouse footpads, was determined [5,6].

Among the ubiquitination components, the Δ*ubc2* and Δ*uev1* mutants exhibited the most severe loss-of-fitness

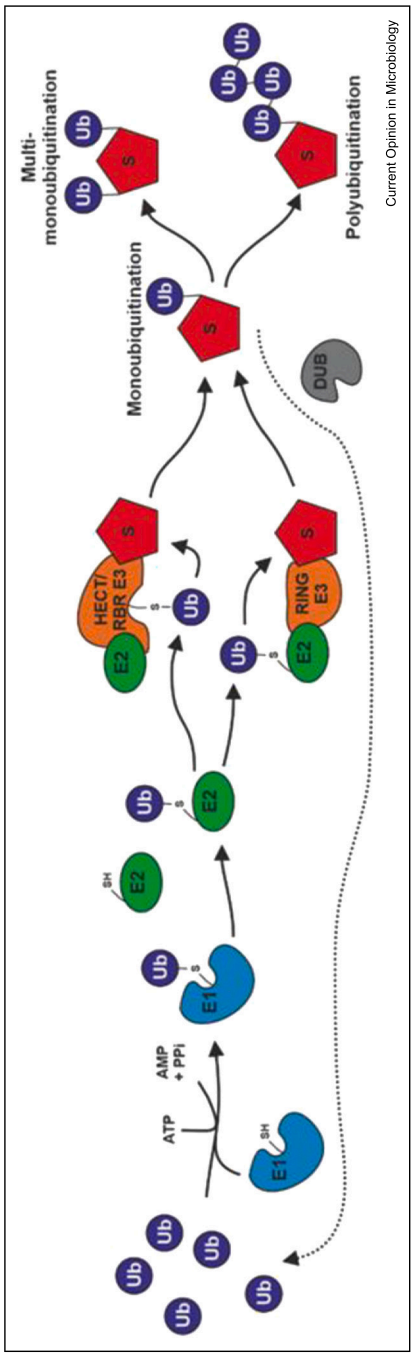

Figure 1

Ubiquitination and deubiquitination. Ubiquitination is brought about by the sequential action of E1, E2 and E3 enzymes. An E1 ubiquitin-activating enzyme catalyses the reaction of the C-terminal carboxylate of ubiquitin (Ub) and ATP to form a ubiquitin-adenylate intermediate. This reacts with a conserved cysteine to form an E1-Ub thioester. The ubiquitin is next transferred to an E2 ubiquitin-conjugating enzyme in a transthioesterification step. Finally, an E3 ubiquitin ligase transfers Ub from the E2 onto (typically) a lysine residue of an acceptor substrate (S) with formation of an isopeptide bond. For HECT and RBR-type E3s (upper pathway), a covalent E3-Ub thioester intermediate is formed. For the RING E3 ligases (lower pathway), the E3 facilitates direct transfer of the Ub from the E2 to the substrate. Additional Ubs may be added to other sites on S (multimonoubiquitination) or to an already-attached Ub (polyubiquitination). A DUB cleaves S–Ub or Ub–Ub bonds to return Ub to the cellular pool.

during differentiation [6]. Knockdown of the UBC2 orthologue in *T. brucei* using RNA-interference (RNAi) also leads to a severe reduction in viability [8]. The crystal structure of a complex of UBC2 and UEV1, a ubiquitin E2 variant lacking the conserved cysteine required for catalytic activity, revealed a heterodimeric structure and an interface that is conserved in human and yeast orthologues. Ubiquitination assays showed that in the presence of *Leishmania* UBA1a and the human E3s RNF8 or BIRC2, UBC2 promoted the formation of polyubiquitin chains on proteins present in the assay. The inclusion of UEV1 restricted the products to K63-linked ubiquitin dimers [6].

The life-cycle phenotyping assay emphasised the important role played by DUBs in *L. mexicana* with three of the deubiquitinases required for transformation to amastigotes and seven more required for proliferation in mice [5]. These findings are consistent with the extensive protein turnover that accompanies the changes in cell structure during differentiation. DUB2, which was further characterised, exhibits broad linkage specificity in cleaving diubiquitin substrates [5]. This suggests that a broad repertoire of ubiquitin linkages may be formed in trypanosomatids, but so far, direct evidence is lacking.

Ubiquitination in the cell cycle of trypanosomatids

The levels of many cell-cycle regulators oscillate during the eukaryotic cell cycle because of periodic ubiquitination and proteolysis. Two key multisubunit E3 complexes important for cell-cycle progression are the anaphase-promoting complex or cyclosome (APC/C) and the SKP1–CULLIN1–F-box (SCF) complex. APC/C controls chromosome segregation and exit from mitosis by triggering ubiquitination and degradation of cell-cycle regulators. Depletion of key components of the APC/C leads to metaphase arrest in *T. brucei* procyclic forms and anaphase arrest in bloodstream forms [9,10], suggesting that mitosis is regulated in a stage-specific manner. Depletion of another APC/C component, AP2, results in mitotic arrest of procyclic forms and stabilisation of a potential substrate, the mitotic cyclin CycB2/cyc6 [11]. Interestingly, deletion of only three of the 10 identified core APC/C components produces a phenotype, showing that the *T. brucei* APC/C has a smaller group of core components than the corresponding complex in *S. cerevisiae*. A further four non-core APC/C-associated proteins were later shown to play essential roles in regulating cell-cycle progression [12]. All are annotated as regulatory subunits of the proteasome, effectively coupling the ubiquitination of cell-cycle regulators in *T. brucei* to their proteolytic degradation [12].

Meanwhile, depletion of components of a putative *T. brucei* SCF complex revealed a conserved role for SKP1 in the G1/S transition, a possible role for RBX1 in kinetoplast DNA replication and a role for the E2 ubiquitin-conjugating enzyme CDC34 in cytokinesis [13]. CDC34 is essential for infection progression in mice, consistent with the rapid growth arrest observed in cells in which CDC34 is depleted. Interestingly, no phenotype was observed following depletion of CULLIN1, suggesting redundancy in cullin function in *T. brucei* or incomplete knockdown [13].

Roles for ubiquitin in trypanosomatid endocytosis

In eukaryotes, ubiquitination plays an important role in receptor endocytosis and the sorting of proteins through the endosomal system [14]. In *T. brucei*, the glycosomal matrix receptor PEX5 is ubiquitinated by the E2 ubiquitin-conjugating enzyme PEX4, which also localises to the glycosome. This function of PEX4 is at least partially redundant, however, since PEX5 remains ubiquitinated in a Δ*pex4* background [15]. Furthermore, two ubiquitin-associated (UBA) domains in *Leishmania* myosin XXI are required for normal endocytic trafficking, although the underlying mechanism is not known [16].

A high-throughput RNAi screen implicated genes encoding the DUBs, Usp7 and Vdu1 in the susceptibility of *T. brucei* to suramin [17]. Usp7 regulates expression of the invariant-surface glycoprotein ISG75, which is involved in suramin uptake and sensitivity, and ubiquitination of its cytoplasmic domain leads to its internalisation and degradation [18,19]. Cycloheximide-chase experiments showed that knockdown of Usp7 and Vdu1 destabilised ISG75 by increasing the rate of its turnover, presumably by blocking ISP75 deubiquitination [20]. It was proposed that silencing of the DUBs diverts ISG proteins from the endosome-recycling pathway into its degradative arm [20]. Aquaglyceroporin 2, which contributes to the sensitivity of *T. brucei* to pentamidine and melarsoprol, is also directed to the lysosomal-degradation pathway following its ubiquitination [21].

Ubiquitination and infection

During the acute phase of *T. cruzi* human infection, the host E3 ubiquitin ligase GRAIL (gene related to anergy in lymphocytes), a negative regulator of CD4 T-cell responsiveness, is upregulated. This is achieved through disruption of the Akt–mTOR pathway, resulting in the downregulation of otubain-1, a human DUB that negatively regulates GRAIL function [22,23]. During later stages of infection, GRAIL expression is downregulated as otubain-1 expression is upregulated [23]. Elsewhere, recombinant *L. infantum* otubain was reported to stimulate lipid-droplet biogenesis and cytokine secretion in macrophages [24]. While this hints at a role for this DUB in the pro-inflammatory response of macrophages during infection, secretion of the parasite otubain into host cells is yet to be demonstrated.

A clearer example of parasite ubiquitination-system components modifying host-cell targets is the SPRING (secretory protein with a RING finger domain) E3 ligase of *T.* cruzi [25]. Amastigotes secrete SPRING into host cells where it becomes localised to the nucleus. A possible target is breast cancer-associated protein 3 (BCA3), which coimmunoprecipitates with SPRING following their coexpression in wheat germ lysates, and which is ubiquitinated by SPRING *in vitro* [25].

A screen of a bloodstream-form *T. brucei* RNAi library for sensitivity to recombinant apolipoprotein-L1 (apoL1), a component of the TLF1 and TLF2 trypanolytic complexes found in human serum, identified six putative ubiquitin-system components among 63 hits [26]. These included two RING E3 ligases (Tb927.10.12940 and Tb927.11.4860), an E2 ubiquitin-conjugating enzyme (Tb927.9.8000), two DUBs (Tb.927.9.5520 and Tb.927.9.14470) and a p97/cdc48 cofactor (Tb927.11.6340). The most prominent hit was Tb927.10.12940, a putative lysosomal RING E3 ubiquitin ligase, whose knockdown led to a dramatic reduction in parasite sensitivity. Contrastingly, knockdown of Tb927.10.12940 enhanced parasite sensitivity to human serum in which TLF1 is the dominant trypanolytic complex, suggesting that other components shape the response to apoL1. Follow-up analysis suggested that the activity of Tb927.10.12940 was dependent on the function of Tb927.9.8000, the E2 ubiquitin-conjugating enzyme identified in the screen, though the target(s) of ubiquitination have yet to be identified. These observations may help to explain the susceptibility of different African trypanosome species to lysis by human serum [26].

Ubiquitination in translation and protein quality control

The RNA helicase DDX3 is important for optimal elongation of translating ribosomes and for stimulating the dissociation and recycling of ribosomes that have arrested. Prolonged ribosome stalling in *L. infantum* cells lacking DDX3 induces quality-control responses, including recruitment of ubiquitination and proteasome components that degrade nascent polypeptides [27]. Among the components found to be enriched at stalled ribosomes are a HECT (homologous to E6AP C terminus) family E3 ubiquitin ligase (LINF_350029800), subunits APC3, APC6 and APC10 of the APC/C complex, the E2 factor, UBC2, the ubiquitin C-terminal hydrolase DUB15 and the proteasomal ubiquitin receptor Rpn13. Interestingly, the deubiquitinase DUB10 is upregulated in cells depleted of DDX3 [27].

A quality-control system has recently been described in *T. brucei* that removes mistargeted aggregation-prone mitochondrial proteins that accumulate in the cytosol [28]. Its components include an E3 ubiquitin ligase (TbE3HECT1), TbUbl1, which harbours a ubiquitin-like protein domain, a mitochondrial protein of unknown function (Tb927.9.7200) and the proteasome. The system is triggered by ablation of ATOM69, a component of the atypical outer membrane translocase and the mitochondrial import receptor for hydrophobic proteins [28]. Upon triggering of the pathway, TbUbl1, a normally nuclear protein, is released into the cytosol, where it binds to mislocalised mitochondrial proteins and, by mechanisms yet to be elucidated, facilitates their ubiquitination and degradation by the proteasome.

Ubiquitin-like modifiers in trypanosomatids

Excellent reviews of ubiquitin-like modifiers in protozoan parasites have been published [29,30]. In *Leishmania*, the best-characterised Ubls are Atg8 and Atg12, which play roles in parasite autophagy [31,32]. In *Leishmania*, conjugation of ATG12–ATG5 is promoted by the E1-like enzyme ATG7 and the E2-like enzyme ATG10. ATG12–ATG5 is required for phagophore development, including the attachment of ATG8-phosphatidylethanolamine to autophagic membranes [32]. In *L. donovani*, where Atg8 was observed to accumulate around the damaged mitochondrion, deletion of *Atg8* compromised parasite differentiation and infectivity [33]. A functional ATG8-conjugation system has also been found in *T. brucei* and *T. cruzi* [34,35].

A SUMO (small ubiquitin-like modifier) -conjugation system has been characterised in *T. brucei*. Aos1/Uba2 (heterodimeric SUMO E1), Ubc9 (SUMO E2) and SIZ1 (SUMO E3 ligase) play a role in SUMO conjugation. A SUMO-specific protease, sentrin-specific protease (SENP), has also been described [36–38]. SUMO, which has a nuclear localisation, is required for cell-cycle regulation, expression of variant-surface glycoprotein (VSG) genes and chromatin organisation [38–40]. Additionally, the RNA pol-I complex, which is responsible for VSG transcription, is SUMOylated in bloodstream forms by SIZ1, demonstrating one way in which SUMOylation could contribute towards VSG expression [38]. SUMOylation of Centrin, which plays an important role in cell motility, has also been observed *in vitro* [36,41]. These studies point towards nuclear and non-nuclear SUMO functions. In *L. mexicana*, a SUMO orthologue has been identified together with a putative E1 catalytic subunit, UBA2 and an E2 enzyme, UBC9 [6]. SUMO and an associated SENP are constitutively expressed [42]. SENP null mutants are viable albeit with reduced vitality, while SUMO null mutants were not obtained, pointing to an essential role. In the SENP null mutants, C-terminal processing of SUMO precursors is disrupted and covalent SUMO attachment to proteins is prevented, as is the translocation of SUMO to the nucleus. Curiously, *in vitro* infectivity is not affected by loss of SENP-mediated SUMO processing, leading these authors to conclude that functions of unprocessed SUMO are critical for viability [42].

In *T. brucei*, Nedd8 and its putative E2-conjugating enzyme, Ubc12, are enriched in the nucleus and flagellum. Depletion of Nedd8 by RNAi led to reduced levels of protein ubiquitination and caused DNA rereplication in post-mitotic cells [43]. It also impaired spindle assembly and compromised the flagellum-attachment zone filament, leading to flagellum detachment. Six cullins, TbCUL1–TbCUL6, were identified as substrates of TbNedd8 [43]; neddylation is known to positively regulate the activity of Cullin-RING ubiquitin ligases [44]. In *L. mexicana*, E1 catalytic (UBA3) and E2 (UBC12) neddylation components have been proposed based on orthology [6].

The structures of *T. brucei* URM1 and UFM1 have been determined using NMR spectroscopy [45,46]. In *L. donovani*, UFM1 conjugation involves the action of UBA5, an E1 enzyme, and UFC1, an E2 enzyme. UFM1, UBA5 and UFC1 localise to the mitochondrion and ufmylation is important for β-oxidation and amastigote growth in macrophages [47,48]. *L. donovani* Urm1, which is associated with early endosome proteins, and its E1, LdUba4, have also been identified [49].

Conclusions

A current challenge is to elucidate the pathways of ubiquitin transfer among the E1s, E2s and E3s of trypanosomatids and link these to the acceptor substrate proteins and downstream DUBs. This in turn will illuminate cellular function and regulation in these parasites. Many of these functions will be common to model organisms, but given their ancient evolutionary character and often-divergent biochemistry, insights into parasite-specific processes can be anticipated.

Structural biology will contribute to this understanding with accurate models of individual components predicted on deep-learning platforms such as AlphaFold2 [50] and RoseTTAFold [51]. Structure predictions for proteins from trypanosomatids have to date tended to be of lower confidence. The cause is the poor sampling of this branch of life in the protein-sequence databases that underpin structure prediction. By gathering openly available protein-sequence data for species from this lineage, significant improvements to protein- structure

prediction have been demonstrated [52]. This will enable description of the structural context of ubiquitination on a proteome-wide scale [53].

The proteasome is a validated therapeutic target in the treatment of leishmaniasis, Chagas disease and sleeping sickness, and potent and selective inhibitors of high efficacy have been developed [54,55]. Since many components of the trypanosomatid ubiquitin and Ubl-modification systems belong to the ubiquitin-proteasome pathway, they also have great potential as drug targets in neglected tropical diseases.

CRediT authorship contribution statement

Rebecca Burge: Writing – original draft, **Jeremy Mottram**: Writing – review & editing, **Tony Wilkinson**: Writing – review & editing.

Conflict of interest statement

The authors declare no competing interests.

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