SUMOylation in *Trypanosoma brucei*

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SUMOylation in *Trypanosoma brucei*

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Running title: SUMOylation in *Trypanosoma brucei*

Key words: SUMO; *Trypanosoma brucei*; Small Ubiquitin-like Modifier; heat shock; oxidative stress.

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Abstract

Small Ubiquitin Like Modifier (SUMO) proteins are involved in many processes in eukaryotes. We here show that *Trypanosoma brucei* SUMO (Tb927.5.3210) modifies many proteins. The levels of SUMOylation were unaffected by temperature changes but were increased by severe oxidative stress. We obtained evidence that trypanosome homologues of the SUMO conjugating enzyme Ubc9 (Tb927.2.2460) and the SUMO-specific protease SENP (Tb927.9.2220) are involved in SUMOylation and SUMO removal, respectively.

Introduction

Small Ubiquitin Like Modifier (SUMO) proteins have been found in almost all eukaryotes. Conjugation of SUMO to target proteins alters their functions in multiple ways, and it is therefore central to a multitude of different cellular processes. Like ubiquitin, SUMO is attached to its targets via 3 enzymatic steps [1, 2]. First, a SUMO-specific protease (SENP) removes 2-11 amino acids at the SUMO C-terminus, revealing a C-terminal di-glycine motif [3]. Next, SUMO is activated by the SUMO activation complex (E1 complex), which consists of two enzymes, Aos1/SAE1 (budding yeast/human) and Uba2/SAE2 [4, 5]. The C-terminal glycine of SUMO forms a thioester bond with a cysteine residue of Uba2. From there, it is transferred to a cysteine residue of the E2 SUMO conjugating enzyme (Ubc9) [6].
From the E2 conjugation enzyme, SUMO binds to a target lysine residue [1, 2]. This process is assisted by an E3 ligase. SUMO is usually attached as a monomer, although chain formation can occur [8]. SUMO is removed from its targets by a variety of peptidases called SENPs [9]. SENP regulation is critical for homeostasis [3, 10-12] and is also involved in responses to stresses such as heat shock and oxidation [10, 13]. SUMO is essential for growth in *S. cerevisiae* [4] but not in fission yeast [14] or Aspergillus [15]. Work on *Chlamydomonas* revealed that the abundance of SUMOylated proteins increases during heat shock and osmotic stress [16]. SUMO was also examined in *Toxoplasma gondii* [17] and *Plasmodium falciparum* [18]: in both cases many SUMOylated proteins were observed and identified by mass spectrometry, but details of the roles of SUMO in specific processes are not yet known.

In the kinetoplastid *Trypanosoma cruzi*, the components of the SUMOylation machinery have been identified by BLAST search. Numerous SUMOylated bands were identified by Western blotting using both anti-SUMO antibody and detection of epitope-tagged SUMO. In addition, 236 potentially SUMOylated proteins were identified by tandem affinity purification and mass spectrometry [19], but unfortunately, a recent careful re-examination of the spectra could unambiguously identify only eight SUMOylated peptides on just seven proteins [20]. One of the targets identified by the tandem affinity purification, metacaspase 3, was confirmed by co-immunoprecipitation [19] although the SUMOylated peptide was not found [20]. The paraflagellar rod protein PFR1 (also called PAR3) was suggested as a SUMO target by Western blot analysis and *in vitro* SUMOylation [21] but again no SUMOylated peptide was found [20]. *T. cruzi* SUMO itself has a SUMOylation site and is able to polymerize [21]. Together these results suggest that many proteins are SUMOylated in *T. cruzi*, but purification is very difficult. Possibly, SUMO protease is very active and persists during purification procedures.

The amino-acid sequence of *Trypanosoma brucei* SUMO (*TbSUMO, Tb927.5.3210*) is 37% identical with that of human SUMO-1 and the 3D structure (solved using NMR) is similar to those of yeast and mammalian SUMO [22]. It was shown by chemical shift analysis that *TbSUMO* interacts with human Ubc9. RNAi targeting SUMO in procyclic trypanosomes caused growth arrest and cell death, and HA-tagged SUMO was predominantly found in the *T. brucei* nucleus [23]. RNA intereference targeting SUMO in *T. brucei* caused growth arrest, followed by death, and antibodies to SUMO in bloodstream-form parasites recognised two prominent bands at around 55 and 60 kDa [24].

In this paper we describe preliminary functional characterisation of further components of the SUMOylation system in *T. brucei* and investigate the effects of various stresses on protein...
SUMOylation.

**Methods**

**Plasmids**

For the N-terminal *in situ* TAP tag, a part of the *TbSUMO* open reading frame (ORF) was amplified using the following primers fw: 5′-gac aag ctt cca cgg acg aac cca ctc ata ac-3′ rv: 5′-gtc gat tca tgt ctc ccc cac cat cgc-3′ and cloned into the p2676 vector [25] using *Hind* III and *EcoR* V.

For the N-terminal V5 *in situ* tag, a part of the *TbSUMO* ORF was amplified (fw: 5′ - gac ctc gag gac gaa ccc act cat-3′, rv: 5′ - gac ggg ccc tca cgc cat gca cca-3′), as well as a part of the 5′ untranslated region (UTR) (fw: 5′- gac cgg cgg tgt cct tgt ggt tac gt-3′ rv: 5′-gac tct aga aag agg aag tgt cgg ag-3′). The ORF and UTR fragments were cloned into a vector containing the V5-tag and the Blasticidin resistance as described in [26] using *Apa* I and *Xho* I for the ORF and *Sac* II and *Xba* I for the UTR segment.

For the RNAi constructs, portions of the open reading frames of the targeted genes were amplified and cloned into p2T7TA blue[27]. The following primers were used: for *TbSUMO* fw: 5′-ggg ggt acc gac gaa ccc act cat-3′; rv: 5′-ccc aag ctt tca cgc cat gca cca aag 3′; for *Tb09.160.0970* (*TbSENP*) fw: 5′-cag acg act cac tgc ca-3′, rv: 5′-tgc gct caa atg ttg ttc tc-3′ and for *Tb927.2.2460* (*TbUBC9*) fw: 5′-tag ctc agt cac gcc tac ga-3′ rv: 5′-aca cac gaa atg gct ctt cc-3′. The primers were designed using RNAi [28].

**Trypanosome culture**

*Trypanosoma brucei* strain Lister 427 expressing the tet repressor, with or without T7 polymerase, were used throughout [27], with culturing and transfecting of trypanosomes as previously described [29].

For growth studies, bloodstream-form cells were diluted to a starting concentration of 5x10⁴ cells/ml, with a maximum density of 1.5-2x10⁶ cells/ml. Procyclins were diluted to 5x10⁵, with a maximum density of 5x10⁶. Tetracycline was added to a final concentration of 0.25µg/ml to induce expression from tetracycline-regulated promoters.

For differentiation, bloodstream-form cells were grown to 1.5-2x10⁶ cells/ml, then cis-aconitate was added to a concentration of 6mM. The cells were grown for 16h at 37°C then transferred to 27°C. Inhibition of glucose transport was achieved by adding phloretin to a concentration of
100µM. For oxidative stress, H₂O₂ was added to procyclic trypanosomes to final concentrations of 250µM, 125µM, 62.5µM, 31.25µM or 15.6µM; the cells were harvested after an incubation time of 1h.

Tandem affinity purification
For each tandem affinity purification 4-5x10⁹ cells were harvested at 4°C and washed twice with ice-cold PBS containing 20mM N-ethyl maleimide (NEM). Bloodstream cells were harvested at a density of 2x10⁶ cells/ml, procyclic cells at a density of 5 x10⁶ cells/ml. The cell pellets were snap-frozen in liquid nitrogen and stored at -80°C. Cell breakage was performed in 6ml breakage buffer (10mM Tris-Cl, 10mM NaCl, 0.1% NP40, 20mM NEM, one tablet of complete inhibitor (without EDTA, Roche) pH=7.8) by passing the cells 20-25 times through a 21 gauge needle. The lysate was spun at 13,000g for 30 min at 4°C to remove the cell debris. Then NaCl was added to a final concentration of 150mM. The purification was done according to [30]. 20mM NEM was added to all the buffers, except during the wash and elution step of the IgG beads and during the TEV cleavage, as NEM inhibits TEV protease.

V5 immunoprecipitation
2.5-5x10⁹ procyclic cells were harvested at a density of approximately 5 x10⁶ cells/ml, washed twice with ice-cold PBS containing 10mM NEM and 10mM iodoacetamide (IAA) and snap-frozen in liquid nitrogen and stored at -80°C. For use, the cell pellet was resuspended in 1ml lysis buffer (10mM Tris-C, 10mM NaCl, 0.1% NP40, 1% SDS, complete protease inhibitor (Roche), 10mM NEM, 10mM IAA, pH=7.5). Cells were passed the cells 20-25 times through a 21 gauge needle. The lysate was spun at 13,000g for 30 min at 4°C to remove the cell debris and was diluted 1:10 in IP100 (10mM Tris-Cl, 100mM NaCl, 0.1% NP40, complete protease inhibitor (Roche), 10mM NEM, 10mM IAA).
Before immunoprecipitation, the lysate was incubated with protein A sepharose for 1 hour on a rotary shaker at 4°C to absorb non-specifically binding proteins. The supernatant was than added to 50µl α-V5 beads (self-made or from Sigma). The lysate was incubated for 3h on a rotary shaker at 4°C. The beads were washed seven times with IP 100. Elution was done by incubating the beads twice with 125µl IP buffer mixed with 25µl V5 peptide for 30min, then boiling in 4x Laemmli buffer.
Results and discussion

Many proteins are SUMOylated in T. brucei

To detect SUMOylated proteins, an antibody was raised to His-tagged TbSUMO produced in E. coli. (For details of all plasmid constructs see Table 1.) The anti-T.brucei SUMO antibody was unfortunately insufficiently specific. Although it recognised purified recombinant SUMO, it detected several bands, but not monomeric SUMO, in bloodstream- and procyclic-form cell extracts and the banding pattern was not affected by SUMO RNAi.

Next, in bloodstream-form trypanosomes, we integrated a sequence encoding a tandem affinity purification tag (TAP-tag) N-terminally in frame with one allele of SUMO. The TAP-SUMO was seen as a ~40kDa band (Figure 1A); the expected size was 33.5 kDa, comprising 12.5 kDa SUMO + 21 kDa tag. Many SUMOylated proteins were present, with a prominent band at 100kDa, which, without the tag, would correspond to an 80kDa SUMOylated conjugate. In T. cruzi also, using either HA-tagged or untagged SUMO, many SUMOylated bands were seen in addition to monomeric SUMO [31]. The pattern that we observed, however, bears no resemblance to the two prominent bands at 55 and 60 kDa that were previously described for bloodstream-form T. brucei using an anti-SUMO antibody [24]. There are several possible explanations for the discrepancy. Our TAP-SUMO may have impaired function due to the tag, or the 100kDa band could actually be equivalent to the 55KDa band (but with very aberrant migration). Alternatively the previously-published pattern could have been affected by antibody cross-reactions with abundant proteins. Since, in the published results, the antibody staining was abolished after 72h RNAi, the last possibility seems unlikely. It cannot, however, be ruled out since no evidence was presented that the antibody recognised native monomeric SUMO on the Western blots. Also the RNAi had depleted the SUMO RNA within 24h but the signal on the blots was unchanged after 48h RNAi despite extensive morphological degeneration [24].

Our pattern of TAP-SUMO-conjugated proteins was unaffected by heat shock (Figure 1A) or by treatment for 12h or 24h with a sub-lethal level (100 µM) of phloretin [32] to partially inhibit glucose import (not shown). The bloodstream forms used for these experiments are not able to differentiate into growing procyclic forms, but can undergo some early steps of differentiation after addition of cis-aconitate and transfer to 27°C. When we did this in the TAP-SUMO-expressing line, the banding pattern remained largely unchanged, but one band migrating at 90 kDa reproducibly disappeared (Figure 1B, marked with a star). In accordance with this result, a changing SUMOylation pattern during differentiation was found in T. cruzi [21]. Our
experiments only detected the most abundant SUMOylated proteins and it is quite possible that
less abundant proteins show regulated SUMOylation.

In procyclic forms (Figure 1C), we integrated a sequence encoding a V5 epitope tag upstream of
the open reading frame [26]. We expected monomeric V5-SUMO to migrate at 13 kDa. This was
not reproducibly seen, but we did sometimes see a band or bands running at 20 kDa (Figure 1C).
In contrast, slower mobility bands were always present, in particular a prominent double band
just below 100 kDa. Comparison of the patterns from bloodstream and procyclic forms (by
manipulating the photographs to allow for the sizes of the tags, not shown) suggested that the
patterns of abundant SUMOylated proteins were similar in both forms. The SUMOylation pattern
in procyclics was unaffected by temperature changes (1h incubations, Figure 1C lanes 7-10).

**SUMOylation increases after oxidative stress**

In mammalian cells, peroxide concentrations of 1mM and lower inhibit SUMOylation [33]
through formation of a disulfide bond between the catalytic domains of the E2 enzyme Ubc9 and
the E1 complex subunit Uba2. In contrast, in trypanosomes, oxidative stress increased the
abundance of SUMOylated protein, even at relatively low peroxide levels (32 µM, 1h incubation;
Figure 1C). We do not know the reason for this discrepancy: the trypanosome E1 and E2
enzymes may differ such that the dimerization cannot occur, or the dimerization in mammalian
cells may be caused by a specific regulatory process that is absent in trypanosomes. Peroxide
concentrations above 1 mM in *Saccharomyces cerevisiae* [34], or 10mM in mammalian cells [33,
35], increase SUMOylation, probably by inhibiting the SENP proteases [33, 36]. Trypanosomes
probably react at lower peroxide concentrations because they are much more susceptible to
oxidative stress than mammalian cells and yeast: the EC\textsubscript{50} of hydrogen peroxide for bloodstream
*T. brucei* is 223µM [37], and we found that procyclic trypanosomes were killed by concentrations
above 250µM.

**Failure to purify SUMOylated proteins from *T. brucei* extracts**

We made multiple attempts to purify the SUMOylated proteins from trypanosome extracts, using
TAP-, His- and V5 tags and a variety of protocols. As previously reported for *T. cruzi*, all of these
attempts failed [21].

First, we attempted tandem affinity purification. SUMOylation was stable for 2h at 4°C in the
lysis buffer, in which standard protease inhibitor mix and N-ethyl-maleimide (20 mM, NEM)
were included in order to inhibit SUMO proteases. Although NEM was removed before the TEV
protease cleavage step, we were unable to elute the tagged proteins from the column. A one-step
immunoprecipitation, using V5-tagged SUMO, also yielded no specific protein pattern because
only 5% of the V5-tagged SUMO bound to the beads. As SUMO binds covalently to its targets, it
might be better to use His-tagged SUMO so that SUMOylated proteins can be purified under
denaturing conditions.

**Role of SUMOylation in *T. brucei***

Reciprocal BLASTp searches using yeast and human sequences, and comparison with *T. cruzi*
[19] revealed several putative homologues of Uba2 and Aos1, the enzymes forming the E1
complex, and also of the E2 enzyme Ubc9 (Table 2). Since SUMO E1 and E2 enzymes resemble
those for ubiquitination, the specificities of these proteins is unclear. There were four possible E3
ligases, consistent with the need to regulate SUMOylation of different targets separately.
However, only one SENP was found. This is surprising, given that the function of SENPs include
both the processing and the removal of SUMO, but the same was reported for *T. cruzi* [19].

As previously reported [24], RNAi targeting SUMO in bloodstream trypanosomes halted growth
2 days after RNAi induction (Figure 2A). We too observed numerous defects in cell division,
which is normal in growth-arrested trypanosomes and does not by itself constitute evidence of a
role of SUMO in regulating the cell cycle. RNAi in procyclic forms expressing V5-SUMO gave
only a transient decrease in V5-SUMO (on day 2 after induction) although the RNA was clearly
decreased; in two independent clones, the doubling time increased from 12.6 h to 14.5 h and 15.1
h (not shown). Liao et al [23] observed stronger growth inhibition.

We first targeted the putative SUMO protease, SENP (Tb09.160.0970/ Tb927.9.2220). RNAi had
hardly any effect on cell growth (doubling time increase of only 0.3 h, not shown), but there was
a strong increase in the abundance of SUMO modification (Figure 2B, lanes 1-4), confirming that
the Tb927.9.2220 protein is important for SUMO removal in trypanosomes. Given this increase
in SUMOylation, we speculate that a different enzyme might be involved in the activation of
SUMO prior to transfer to the E1 conjugating enzyme. Alternatively, much lower levels of SENP
activity may be needed for initial SUMO processing than for SUMO removal.

Next, we targeted the possible E2 conjugating enzyme UBC9 (*Tb927.2.2460*) in procyclic
trypanosomes expressing V5-SUMO. A *UBC9* RNAi line grew slower than the parent line, even
in the absence of tetracycline, and there was only marginal slowing after tetracycline addition
(not shown). We did not check the mRNA levels: the mRNA is present at less than one copy per
cell so it would be difficult to detect even before RNAi [38]. However, there was a reproducible
decrease in SUMOylation (Figure 2B, lanes 5-9) after RNAi.

Conclusions
We confirmed the functions of the trypanosome SENP and UBC9 genes, and could show that SUMO modifies many trypanosome proteins. The pattern of SUMOylation was surprisingly unresponsive to stress and also appeared not to be strongly developmentally regulated.

Acknowledgements
We thank Keith Gull (University of Oxford) for the anti-tubulin antibody, J. Haanstra and B. Bakker (Utrecht) for communicating phloretin results and Frauke Melchior (ZMBH) for advice. DD was supported by Sonderforschungsbereich 544 of the Deutsche Forschungsgemeinschaft.

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Table Legends

Table 1
Plasmids used in this work (not all results described in text)

Table 2
Trypanosome genes potentially involved in SUMOylation. Genes were identified by reciprocal BLASTp. Only genes giving a yeast SUMO pathway enzyme as the best match are included. The putative PIAS homologues each have the expected RING domain and the single SENP has a cysteine protease domain.

Figure legends

Figure 1
Protein modification by tagged SUMO
A. Effect of temperature on the pattern of modification with TAP-SUMO in bloodstream trypanosomes containing the plasmid pH2020. Lane 1: cells without TAP-SUMO. Lanes 2,3,4: The cells were incubated for 1 h at the indicated temperatures. The antibody used for detection was PAP: peroxidase anti-peroxidase antibody (binds to the IgG-binding domain of the TAP tag).
B. Effect of differentiation conditions on the pattern of TAP-SUMOylated proteins. Bloodstream trypanosomes were isolated at 6 x 10⁵ cells/ml (L, lower density, lane 1) or 2.5 x 10⁶ cells/ml (H,
higher density, lane 2). Cis-aconitate was added to the higher-density cells and the culture incubated at 37°C for 17h (lanes 3-6) [39]. Then, the culture was centrifuged and resuspended in procyclic-form medium at 27°C (lanes 7 & 8).

C. Effect of oxidative stress and temperature stress on the pattern of V5-SUMO modification in procyclic trypanosomes. Parasites were transfected with pHD2021 to V5-\textit{in situ} tag SUMO at the N-terminus [26]. Lanes 1 and 7 are controls. Lanes 2-6: Cells with V5-\textit{in situ}-tagged SUMO were incubated for 1 h with 15.6, 31.2, 62.5, 125 or 250 µM hydrogen peroxide. Lanes 8-10: incubation for 1 h at the indicated temperatures. Proteins were detected with anti-V5; as a control, a monoclonal antibody to tubulin (TUB) (from K. Gull) was used.

**Figure 2**

A. Effect of RNAi targeting \textit{SUMO} on growth of bloodstream-form trypanosomes. RNAi was induced by addition of tetracycline and growth followed daily, with dilution as required to keep the cell density below 1 x 10^6/ml.

B. The effect of RNAi targeting \textit{TbUBC9} and \textit{TbSENP} on SUMOylation in procyclic trypanosomes. Trypanosomes expressing T7 polymerase and the \textit{tet} repressor [27] were transfected with pHĐ 2021 and pHĐ2038 or pHĐ2037. RNA interference was induced with tetracycline (100 ng/ml in the absence of other selective drugs) for the times shown and the patterns of SUMOylation assayed by Western blotting.
Protein modification by tagged SUMO. A. Effect of temperature on the pattern of modification with TAP-SUMO in bloodstream trypanosomes containing the plasmid pH2020. Lane 1: cells without TAP-SUMO. Lanes 2, 3, 4: The cells were incubated for 1 h at the indicated temperatures. The antibody used for detection was PAP: peroxidase anti-peroxidase antibody (binds to the IgG-binding domain of the TAP tag). B. Effect of differentiation conditions on the pattern of TAP-SUMOylated proteins. Bloodstream trypanosomes were isolated at 6 x 10^5 cells/ml (L, lower density, lane 1) or 2.5 x 10^6 cells/ml (H, higher density, lane 2). Cis-aconitate was added to the higher-density cells and the culture incubated at 37°C for 17h (lanes 3-6) [39]. Then, the culture was centrifuged and resuspended in procyclic-form medium at 27°C (lanes 7 & 8). C. Effect of oxidative stress and temperature stress on the pattern of V5-SUMO modification in procyclic trypanosomes. Parasites were transfected with pH2021 to V5-in situ tag SUMO at the N-terminus [26]. Lanes 1 and 7 are controls. Lanes 2-6: Cells with V5-in situ-tagged SUMO were incubated for 1 h with 15.6, 31.2, 62.5, 125 or 250 µM hydrogen peroxide. Lanes 8-10: incubation for 1 h at the indicated temperatures. Proteins were detected with anti-V5; as a control, a monoclonal antibody to tubulin (TUB) (from K. Gull) was used.
Figure 2

A. Effect of RNAi targeting SUMO on growth of bloodstream-form trypanosomes. RNAi was induced by addition of tetracycline and growth followed daily, with dilution as required to keep the cell density below $1 \times 10^6$/ml. B. The effect of RNAi targeting $TbUBC9$ and $TbSENP$ on SUMOylation in procyclic trypanosomes. Trypanosomes expressing T7 polymerase and the tet repressor [27] were transfected with pHD 2021 and pHD2038 or pHD2037. RNA interference was induced with tetracycline (100 ng/ml in the absence of other selective drugs) for the times shown and the patterns of SUMOylation assayed by Western blotting.
Table 1 (on next page)

Table 1

Plasmids used in this work (not all results described in text).
| pH D2 | Description | Cloning strategy |
|-------|-------------|------------------|
| 02    | SUMO /TAP in-situ tag (N-termina1) | A part of the SUMO ORF was amplified using the following primers: <br>fw: 5'-gac aag ctt ccg cca ccg aac cca ctc ata ac-3' <br>rv: 5'-gtc gat atc tca tgt ctg ctc cac cat cgc-3' <br>and cloned into the p2676 [25] using Hind III and Eco RV |
| 01    | SUMO /V5 in-situ tag (N-termina1) | The SUMO ORF was amplified using the following primers: <br>fw: 5' - gac ctc gag gac gaa ccc act cat-3' <br>rv: 5'- gac ggg ccc tca cgc cat gca cca-3'. <br>A fragment of the UTR was amplified using the following primers: <br>fw: 5'- gag ccc cgg tgt cct tgt ggt tac gt-3' <br>rv: 5'-gac tct aga aag agg aag tgc ggc ag-3'. <br>The ORF was cloned into the Bla V5 vector [26] using Apa I and Xho I, the 5' UTR using Sac II and Xba I. |
| 02    | SUMO knock-out 2 | A fragment of the SUMO 3' UTR was amplified using the following primers: <br>fw: 5'- gac tct aga cat aag tgc ggc tag tgg-3' <br>rv: 5'-gtc ccc cgg gca aac gac cgc aga agt-3'. <br>A 5'-UTR fragment was amplified using the following primers: <br>fw: 5'-cac tgg ctc gct atc cac atc ctc a-3' <br>rv: 5'-gtc aag ctt cgt ggg ctc aga aat gaa-3'. |
The 3'-UTR fragment was cloned into pH1748 (Blasticidin resistance cassette in polylinker) using *Xba* I and *Sac* II, the 5' UTR fragment using *Xho* I and *Hind* III.

| pH  | SUMO knock-out | The Blasticidin resistance was removed from pH2022 using *Hind* III and *Eco* RI and replaced by a Puromycin resistance which was acquired by digesting pH1747 with the same enzymes. |
|-----|----------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| pH  | SUMO RNAi      | The SUMO ORF was amplified using the following primers: **fw**: 5'-ggg ggt acc gac gaa ccc act cat aac-3'  
| D2  | rv: 5'-ccc aag ctt tca cgc cat gca cca aag -3'  
| 02  | and cloned into p2T7™blue [27]                                                                  |
| pH  | SUMO His-      | The SUMO ORF was amplified using the following primers: **fw**: 5'- gag ggt acc gac gaa ccc act cat aac-3'  
| D2  | SUMO/His       | rv: 5'-ccc aag ctt tca cgc cat gca cca aag-3'  
| 02  | in-situ tag    | and cloned into pQEA38 using *Kpn* I and *Hind* III. pQEA38 is an expression vector with ten His tags and a TEV cleavage site, modified from pQTEV (AY243506), from the lab of D. Görlich (then at ZMBH). |
| pH  | SUMO/His       | A 5' UTR fragment was amplified using the following primers:  
| D2  | in-situ tag    | **fw**: 5'- gac ccg cgg tgt cct tgt ggt tac gt-3'  
| 02  | (N-termina1)   | **rv**: 5'- gag ctg tgt ggt acg tag gtc ggg ag-3'  
| 6   | l)             | and cloned into the Bla V5 vector using *Sac* II and *Xba* I. Then the V5 tag was removed using *Eco* NI and *Xho* I.  
|     |                | The SUMO ORF and the His-tag sequence were cut out of pHD 2025 using *Eco* RI and *Hind* III.  
|     |                | The vector and the tagged ORF were blunted using the Klenow fragment and ligated. |
| pH  | *TbSEN* P      | A fragment of the SENP ORF was amplified using the following primers:  
| D2  | RNAi           | **fw**: 5'-cag acg act cac tat cgc ca-3'  
| 03  | rv:5'-tgc gct caa atg tgt ttc tc-3'  
| 7   | and cloned into the p2T7™blue vector | |
| pH  | *TbUB*         | A fragment of the *UBC9* ORF was amplified using the following |
D2  C9  RNAi  primers  
03  
8  fw: 5'-tag ctc agt cac gcc tac ga-3'
rv:5'-aca cac gaa atg gct ctt cc-3'
and cloned into the p2T7TAblue vector
Table 2

Trypanosome genes potentially involved in SUMOylation. Genes were identified by reciprocal BLASTp. Only genes giving a yeast SUMO pathway enzyme as the best match are included. The putative PIAS homologues each have the expected RING domain and the single SENP has a cysteine protease domain.
| Enzyme     | Function | Tb homologue | Name |