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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 and is required for bloodstream trypanosome growth. 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 (Geiss-Friedlander and Melchior, 2007;Ulrich, 2009). First, a SUMO-specific protease (SENP) removes 2-11 amino acids at the SUMO C-terminus, revealing a C-terminal di-glycine motif (Kim and Baek, 2009). Next, SUMO is activated by the SUMO activating complex (E1 complex), which consists of two enzymes, Aos1/SAE1 (budding yeast/human) and Uba2/SAE2 (Desterro et al., 1999;Johnson et al., 1997). 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) (Desterro et al., 1997;Johnson and Blobel, 1997). From the E2

conjugating enzyme, SUMO is transferred to a target lysine residue (Geiss-Friedlander and Melchior, 2007;Ulrich, 2009). This process is assisted by an E3 ligase. SUMO is usually attached as a monomer, although chain formation can occur (Ulrich, 2008). SUMO is removed from its targets by a variety of peptidases called SENPs (Mukhopadhyay and Dasso, 2007). SENP regulation is critical for homeostasis (Au et al., 2009;Drag and Salvesen, 2008;Kim and Baek, 2009;Yeh, 2009) and is also involved in responses to stresses such as heat shock and oxidation (Au et al., 2009;Tempe et al., 2008).

Most studies of SUMO so far have concentrated on multicellular eukaryotes and yeast. Outside these groups, work on *Chlamydomonas* revealed that the abundance of SUMOylated proteins increases during heat shock and osmotic stress (Wang et al., 2008). SUMO was also examined in *Toxoplasma gondii* (Braun et al., 2009) and *Plasmodium falciparum* (Issar et al., 2008): 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.

The amino-acid sequence of *Trypanosoma brucei* SUMO (*Tb*SUMO, 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. It was shown by chemical shift analysis that *Tb*SUMO interacts with human Ubc9 (Shang et al., 2009). RNAi targeting SUMO in procyclic trypanosomes caused growth arrest and cell death, and HA-tagged SUMO was predominantly found in the *T. brucei* nucleus (Liao et al., 2010). Meanwhile, in the kinetoplastid *Trypanosoma cruzi*, possible SUMOylated proteins have been identified by tandem affinity purification and mass spectrometry (Bayona et al., 2011). SUMOylation of one of the identified targets, metacaspase 3, was confirmed by immunoprecipitation (Bayona et al., 2011). PFR1 (also called PAR3) was also identified as a SUMO target by Western blot analysis and *in vitro* SUMOylation (Annoura et al., 2012). *T. cruzi* SUMO itself has a SUMOylation site and is able to polymerize *in vitro* (Annoura et al., 2012).

In this paper we describe preliminary functional characterisation of 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 *Tb*SUMO open reading frame (ORF) was amplified using the following primers fw: 5'-gac aag ctt ccg cca ccg acg aac cca

ctc ata ac-3' rv: 5'-gtc gat atc tca tgt ctg ctc cac cat cgc-3' and cloned into the p2676 vector (Kelly et al., 2007) using *Hind* III and *EcoR* V.

For the N-terminal V5 *in situ* tag, a part of the *Tb*SUMO 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 ccg cgg tgt cct tgt ggt tac gt-3' rv: 5'-gac tct aga aag agg aag tcg ggg ag-3'). The ORF and UTR fragments were cloned into a vector containing the V5-tag and the Blasticidin resistance as described in (Shen et al., 2001) using *Apa* I and *Xho* I for the ORF and *Sac* II and *Xba* I for the UTR fragment.

For the expression of recombinant *Tb*SUMO in E.coli, the ORF was amplified with the following primers: fw: 5'- gag ggt acc gac gaa ccc act cat aac-3'
rv: 5'-ccc aag ctt tca cgc cat gca cca aag-3' 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). Protein expression was done using BL21 cells. Protein purification was done under native conditions, using Ni-NTA agarose beads (Qiagen) according to the Qiagen manual.

For the RNAi constructs, portions of the open reading frames of the targeted genes were amplified and cloned into p2T7TA blue(Alibu et al., 2004). The following primers were used: for *Tb*SUMO fw: 5'-ggg ggt acc gac gaa ccc act cat aac-3' rv: 5'-ccc aag ctt tca cgc cat gca cca aag 3'; for *Tb*09.160.0970 (*Tb*SENP) fw: 5'-cag acg act cac tat cgc ca-3', rv:5'-tgc gct caa atg ttg ttc tc-3' and for *Tb*927.2.2460 (*Tb*UBC9) 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 RNAit (Redmond et al., 2003).

Trypanosome culture

Trypanosoma brucei strain Lister 427 expressing the tet repressor, with or without T7 polymerase, were used throughout (Alibu et al., 2004), with culturing and transfecting of trypanosomes as previously described (van Deursen et al., 2001).

For growth studies, bloodstream-form cells were diluted to a starting concentration of $5x10^4$ cells/ml, with a maximum density of $1.5\text{-}2x10^6$ cells/ml. Procyclics were diluted to $5x10^5$, with a maximum density of $5x10^6$. 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 $1.5\text{-}2x10^6$ 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, H_2O_2 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 approximately $5x10^9$ 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 $2x10^6$ cells/ml, procyclic cells at a density of 5 $x10^6$ 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% IGEPAL, 20mM NEM, one tablet of complete protease inhibitor (without EDTA, Roche) pH=7.8) by passing the cells 20-25 times through a 21 gauge needle. The lysate was spun at 16,200g 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 (Puig et al., 2001). 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

Anti-V5 antibodies (AbD serotech) were diluted in 1ml 1xPBS and incubated with protein A sepharose (1mg antibody per mg beads) for 1h at room temperature. Then the bound antibody was coupled to the column according to (Harlow and Lane, 1999). The beads were washed five times with PBS before use.

2.5-5$x10^9$ procyclic cells a density of approximately 5 $x10^6$cells/ml were harvested, 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% IGEPAL, 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 16,200g 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% IGEPAL, 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 IP100. Elution was done by incubating the beads twice with 125µl

IP buffer mixed with 25µl V5 peptide (2mg/ml) for 30min, then were boiled in 4x Laemmli buffer.
Eluates were TCA-precipitated and loaded on a 15% SDS gel, which was stained with silver stain.

Purification of metabolically labelled proteins

A small scale purification of metabolic labelled proteins was done to test the efficiency of the V5 pull-down. About $6x10^7$ procyclic cells were spun down at 600g, transferred to a 1.5ml Eppendorf tube and washed once with PBS. The cells were resuspended in 400ml labelling medium, as described in (Clayton, 1987). Then 12µl ^{35}S methionine solution (120µCi) was added. The cells were incubated for 1h at 30°C. Then the cells were pelleted and washed twice with PBS containing 10mM NEM and 10mM IAA.
The purification was done according to the already described protocols, with slight modifications. 5µl α-V5-tagged beads were used. Cell lysis was done by keeping the cells on ice for 45min, with occasional vortexing. The samples were incubated on a rotary shaker rotated at 4°C for 3,5h. The beads were spun down and washed five times with 1ml IP100. The supernatant of the V5-tagged cells was given on 5µl new α-V5-tagged beads and rotated at 4°C for additional 1.5h. The proteins were eluted with 5µl V5 peptide (2mg/ml). To yield additional proteins, the beads were boiled in 2x Laemmli buffer.

Results and discussion

Many proteins are SUMOylated in *T. brucei*

To detect SUMOylated proteins, an antibody was raised to His-tagged *Tb*SUMO produced in *E. coli*. 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 (not shown).
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. In addition, many SUMOylated proteins were present, with a prominent band above 100kDa. The pattern was unaffected by heat shock (Figure 1A) or by treatment for 12h or 24h with a sub-lethal level (100 µM) of phloretin (Haanstra et al., 2011) 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). In accordance with this result, a changing SUMOylation pattern during differentiation was found in *T. cruzi* (Annoura et al., 2012). Our experiments only detected the most abundant SUMOylated proteins and it is quite possible that many more, 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 (Shen et al., 2001). 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 (Bossis and Melchior, 2006) 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 (15,6µM or 31,25µM, 1h incubation; Figure 1C, lane 2 and 3). 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* (Zhou et al., 2004), or 10mM in mammalian cells (Bossis and Melchior, 2006;Saitoh and Hinchey, 2000), increase SUMOylation, probably by inhibiting the SENP proteases (Bossis and Melchior, 2006;Xu et al., 2008). Trypanosomes probably react at lower peroxide concentrations because they are much more susceptible to oxidative stress than mammalian cells and yeast: the EC_{50} of hydrogen peroxide for bloodstream *T. brucei* is 223µM (Krieger et al., 2000), 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- and V5-tags and a variety of protocols. As previously reported for *T. cruzi*, all of these attempts failed (Annoura et al., 2012).

First, we attempted tandem affinity purification, as previously successfully applied to the exosome (Estevez et al., 2001). SUMOylation was stable for 2h at 4°C in TAP lysis buffer, as observed by Western blot (not shown). Both protease inhibitor and N-ethyl-maleimide (NEM, 20 mM) were included in the lysis buffer in order to inhibit SUMO proteases. However, NEM was removed by washing before the TEV cleavage step, as it inhibits the TEV protease. Eluates from cells expressing TAP-tagged SUMO gave exactly the same pattern on SDS-PAGE as eluates from cells expressing TAP alone and SUMO was not visible. Further analysis indicated that the majority of TAP-tagged SUMO had bound to the IgG beads, but it was never recovered. Additional bands were however not seen after analysis of the boiled beads.

A one-step immunoprecipitation, using V5 tagged SUMO, also yielded no specific protein pattern. Western blot analysis, however, showed that only about 5% of the V5-tagged SUMO bound to the beads, suggesting that the problem lay with poor binding of the tagged protein to the beads.

One reason for the failure of the purification of SUMO might be that we failed to inhibit SUMO proteases.

As SUMO binds covalently to its targets, it should be possible to purify SUMOylated proteins under denaturating conditions, with all proteases completely inactive during the purification.Therefore, expression of His-tagged SUMO in trypanosomes, would allow purification on nickel agarose beads, in the presence of urea.

Bayona et al. (Bayona et al., 2011) have, in contrast to our experience and that of Annoura et al., reported purification of SUMOylated *T. cruzi* proteins. They started with ten times as many parasites, included TLCK, iodoacetamide, and PMSF as protease inhibitors, and used a tandem affinity approach with His and HA tags, without TEV protease cleavage and with a control extract lacking tagged protein. The list of putatively SUMOylated proteins included most highly abundant proteins, so - although the authors attempted quantitation by comparing spectral counts, and did the experiments in triplicate - it is unclear how many of the identified proteins really are SUMOylated. Nevertheless, they could confirm that a very small proportion of metacaspase 3 is indeed SUMOylated.

Role of SUMOylation in *T. brucei*

To identify enzymes involved in SUMOylation, we performed reciprocal BLASTp searches using yeast and human sequences, as well as comparing the results to the homologues found in *T.cruzi* (Bayona et al., 2011). We found several putative homologues of Uba2 and Aos1, the enzymes forming the E1 complex, and also for 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. The same was reported for *T.cruzi* (Bayona et al., 2011).

RNAi targeting SUMO in bloodstream trypanosomes halted growth 2 days after RNAi induction (Figure 2A). The RNAi was confirmed by Northern blot (not shown). We observed numerous defects in cell division, which is normal in growth-arrested trypanosomes and does not 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); 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 (Liao et al., 2010), in contrast, observed stronger growth inhibition. Thus in trypanosomes, as in *S. cerevisiae* (Johnson et al., 1997), SUMO is essential for growth. In contrast, SUMO is not essential in fission yeast (Tanaka et al., 1999) or *Aspergillus* (Szewczyk et al., 2008).

Next, we targeted the possible E2 conjugating enzyme UBC9 (*Tb*927.2.2460) in procyclic trypanosomes expressing V5-SUMO A *UBC9* RNAi line grew slower than the parent line, even in the absence of tetracycline (not shown). There was only marginal slowing after tetracycline addition and the decrease in SUMOylation was reproducible but moderate (Figure 2B, lanes 5-9), suggesting that substantial levels of UBC9 activity remained.

Finally, we 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 reproducibly a clear increase in the abundance of SUMO modification (Figure 2B, lanes 1-4), confirming that the identified gene indeed is important for SUMO removal in trypanosomes. It is paradoxical that only one SUMO protease was identified, since the activity is needed for both activation and removal of SUMO. Since RNAi targeting SENP resulted in a general 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 the initial SUMO processing than for SUMO removal.

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.

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Tables

Table 1

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
Aos1	E1 complex	Tb11.02.5410	AOS1
Uba2	E1 complex	Tb927.5.3430	UBA2
Ubc9	E2 complex	Tb927.2.2460	UBC9
PIAS4/Siz1	E3 ligase	Tb09.211.2400	
PIAS4/Siz1	E3 ligase	Tb927.2.4420	
PIAS4/Nfi1	E3 ligase	Tb11.01.8710	
PIAS1/Siz1	E3 ligase	Tb927.6.4830	
SUMO1/Ulp2	SENP	Tb09.160.0970	SENP

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 pHD2020. 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) (Queiroz et al., 2009). Then, the culture was centrifuged and resuspended in procyclic-form

medium at 27°C (lanes 7 & 8). The asterisk marks the lane disappearing during the treatment. The arrow marks the lane corresponding to the unblound SUMO protein.
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-*in situ* tag SUMO at the N-terminus (Shen et al., 2001). 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 (AbD Serotech); as a control, a monoclonal antibody to tubulin (KMX1) (from K. Gull) was used.

Figure 2

Roles of SUMO, UBC9 and SENP
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 x 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 (Alibu et al., 2004) 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.

Figure 1

Protein modification by tagged SUMO

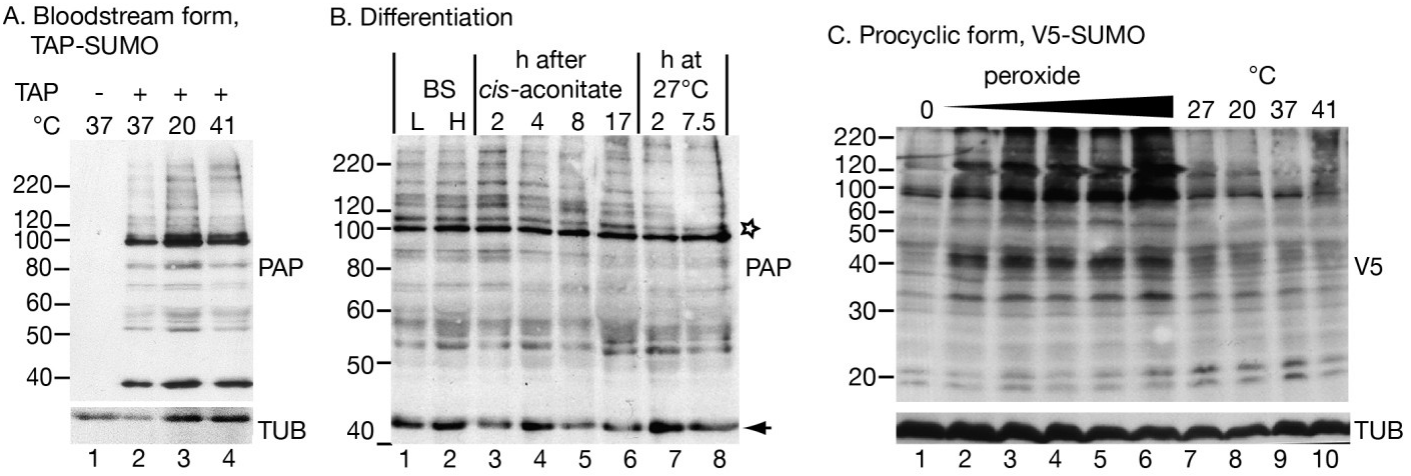

Figure 2

Roles of SUMO, UBC9 and SENP