



Identification and confirmation of SUMOylation-modified proteins in *Giardia* trophozoites



Hye Rim Yeo , Mee Young Shin , Juri Kim* , Soon-Jung Park*

Department of Tropical Medicine, Institute of Tropical Medicine, Yonsei University College of Medicine, Seoul 03722, Korea

Abstract

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*Correspondence

jk, zhuri@yuhs.ac
sjp, sjpark615@yuhs.ac

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Posttranslational modification by the small ubiquitin-related modifier (SUMO) is one of the crucial cellular processes in *Giardia lamblia*, a protozoan pathogen. In this study, 5 candidate SUMO substrate proteins of *G. lamblia* trophozoites were chosen based on their enrichment through affinity chromatography using a SUMO-interaction motif: never in mitosis A-related kinase (NEK), aminoacyl-histidine dipeptidase (AHD), protein disulfide isomerase 2 (PDI2), alcohol dehydrogenase 3, and ornithine carbamoyltransferase. Transgenic *Giardia* trophozoites expressing 1 of the 5 candidate SUMO substrate proteins were constructed, and their expression was confirmed by western blot using hemagglutinin-tag. Arginine deiminase (ADI) protein was expressed in *Giardia* trophozoites as a positive control. Cell extracts were processed for affinity chromatography using SUMO-interaction motif resin. As expected, the SUMOylated form of ADI was detected in the affinity chromatography extracts of ADI-expressing cells. Among the 5 candidate proteins, SUMOylated forms of NEK, AHD, and PDI2 were identified in the affinity chromatography extracts. These results suggest that NEK, AHD, and PDI2 activity is modulated via SUMOylation in *Giardia* trophozoites.

Keywords: *Giardia lamblia*, SUMOylation, posttranslational modification

The posttranslational modification (PTM) of proteins is a dynamic process that efficiently modulates cellular response to various stimuli in eukaryotes. Ubiquitination is one of the main PTMs involved in controlling proteasome-mediated degradation of various proteins, such as cell cycle-related components, and coordinating the localization and activity of diverse proteins. As an alternative mode of ubiquitination, the covalent and reversible attachment of small ubiquitin-like modifier (SUMO) to lysine residues of target proteins regulates cellular functions such as nuclear-cytoplasmic transfer, DNA repair, cell cycle regulation, and transcriptional regulation [1].

Giardia lamblia, a pathogen causing diarrheal disease, is present as 2 distinct forms, trophozoites and cysts. Trophozoite is a multiplying form found in the host's small intestine, whereas cyst is an infective form found outside the host. Interestingly, *Giardia* lacks the anaphase-promoting complex required for the ubiquitination-dependent degradation of cell cycle components [2]. Therefore, SUMOylation is considered an important PTM involved in regulating *Giardia* trophozoites division.

The *G. lamblia* genome contains genes encoding SUMO, SUMO-activating enzyme, SUMO-conjugating enzyme, and SUMO nuclease, 2 genes have been predicted as encoding SUMO ligases [3]. Two known substrates of *Giardia* SUMO are α -tubulin [4] and arginine deiminase (ADI), a metabolic enzyme that converts arginine to citrulline [5].

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Author contributions

Conceptualization: Kim J
 Formal analysis: Kim J
 Funding acquisition: Park SJ
 Investigation: Yeo HR, Shin MY
 Methodology: Yeo HR, Shin MY
 Supervision: Kim J, Park SJ
 Visualization: Kim J, Park SJ
 Writing – original draft: Kim J, Park SJ
 Writing – review & editing: Kim J, Park SJ

Conflict of interest

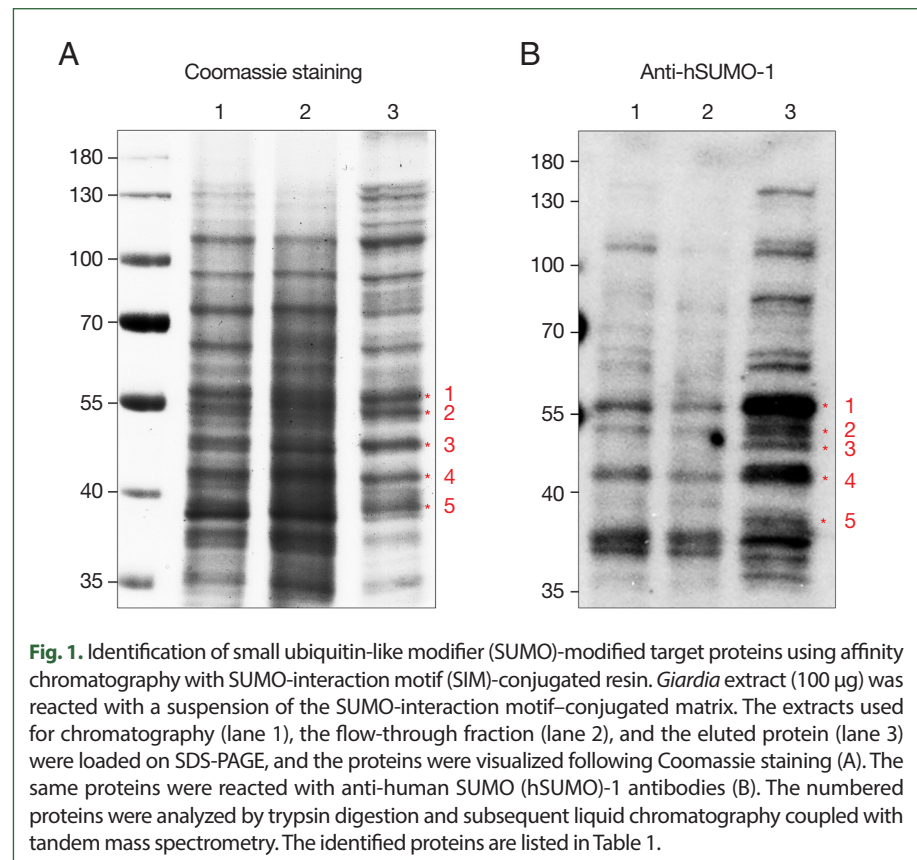
The authors declare no conflict of interest related to this study.

ORCID

Hye Rim Yeo
<https://orcid.org/0009-0003-2704-5874>
 Mee Young Shin
<https://orcid.org/0000-0003-3676-2683>
 Juri Kim
<https://orcid.org/0000-0001-8270-7584>
 Soon-Jung Park
<https://orcid.org/0000-0002-0423-1944>

We aimed to define the role of SUMOylation in *Giardia* by identifying SUMOylation-modified proteins. *G. lamblia* trophozoites (ATCC 30957; American Type Culture Collection, Manassas, VA, USA) were cultured at 37°C in modified TYI-S-33 medium supplemented with 10% heat-inactivated calf serum (Gibco, Rockville, MD, USA), and 0.75 mg/L bovine bile (Sigma-Aldrich, St. Louis, MO, USA). Approximately 4×10^7 *Giardia* trophozoites were harvested by centrifugation at $1,900 \times g$ for 20 min at 4°C. After washing twice with $1 \times$ phosphate-buffered saline. One hundred microliters lysis buffer (150 mM NaCl, 200 mM iodoacetamide, 50 mM Tris-HCl (pH 7.5)) with a $1 \times$ protease inhibitor cocktail (GenDEPOT, Katy, TX, USA) was added, and the cells were broken down using a sonicator (Qsonica, Newtown, CT, USA).

The resultant extracts were processed via an affinity chromatography using a SUMO-QAPTURE-T kit (Enzo, Farmingdale, NY, USA). Briefly, 100 µg of protein was mixed overnight at 4°C with 40 µl of SUMO-interaction motif (SIM)-conjugated matrix suspension. After 2 washes with binding buffer (50 mM Tris-HCl [pH 7.5]), the bound proteins were eluted with $5 \times$ SDS-PAGE loading buffer (250 mM Tris-Cl (pH 6.8), 15% SDS, 50% glycerol, 25% β -mercaptoethanol, 0.01% bromophenol blue). The eluted proteins, along with the extracts used for chromatography and the flow-through fraction, were separated on a 12% SDS-PAGE and then visualized with Quick Coomassie Stain (Protein Ark, Rotherham, UK) (Fig. 1A). These proteins were also reacted with anti-human SUMO-1 antibodies (Fig. 1B). Among the immunoreactive protein bands in the eluted fraction (Fig.



1B), 5 upregulated immunoreactive proteins were excised from the corresponding gel stained with Coomassie blue (Fig. 1A) for subsequent identification at PROTIA (Seoul, Korea). The trypsin-digested products of the 5 chosen proteins were processed via liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) and underwent UniProt (<https://www.uniprot.org/>) analyses based on the *G. lamblia* database (GiardiaDB, <https://giardiadb.org/giardiadb/app>). For each sample, proteins with a Mascot ion score greater than 25 and comparable molecular weight were selected to determine whether they were modified by SUMO (Table 1). The analysis showed the proteins in decreasing size, serially numbered and presented with the GiardiaDB ID members, putative protein functions, predicted molecular weights, and isoelectric points, and information related to LC-MS/MS analysis (Mascot score, matches, sequences, and coverages). The proteins were identified as never in mitosis A-related kinase (NEK) 15409, aminoacyl-histidine dipeptidase (AHD), protein disulfide isomerase (PDI) 2, alcohol dehydrogenase (ADH) 3, and ornithine carbamoyltransferase (OCT).

To determine whether the identified proteins underwent SUMOylation in vivo, i.e., within *Giardia* trophozoites, transgenic *Giardia* were constructed in which 1 of the 5 proteins was expressed as a hemagglutinin (HA)-tagged form following transfection with an expression plasmid. Trophozoites expressing HA-tagged ADI were included as controls. Table 2 presents a list of primers and plasmids used in this study [6]. To construct the plasmid expressing ADI, an ADI DNA fragment with its own promoter (GL50803_112103) was created using PCR with *Giardia* genomic DNA (gDNA) and primers t112103-F-infusion and t112103-R-infusion. The PCR amplicon was subsequently cloned into pKS-3HA. NEO digested with *NotI* using an In-Fusion HD Cloning Kit (Takara, Shiga, Japan), generating pADI-HA.NEO. A 1,545-bp DNA fragment encoding the NEK15409 open reading frame (GL50803_15409) and its 200-bp promoter was amplified from *Giardia* gDNA by PCR using primer pair t15409-F/r15409-R. A DNA fragment containing the promoter and open reading frame of PDI2 (GL50803_9413) was also prepared by PCR amplification using the primer pair t9413-F/r9413-R. The resultant NEK15409 and PDI2 DNA fragments were cloned into the *HindIII* and *XhoI* sites of pKS-3HA.NEO to produce p15409-HA.NEO and p9413-HA.NEO, respectively. DNA fragments encoding AHD (GL50803_15832) and ADH3 (GL50803_3593) were generated by PCR using the primer pair t15832-F/t15832-R and t3593-F/t3593-R, respectively. The resultant DNA fragment was inserted into the *SacII* and *NotI* sites of pKS-3HA.NEO to generate p15832-HA.NEO and p3593-

Table 1. Proteins Identified as candidate small ubiquitin-related modifier substrates using liquid chromatography coupled with tandem mass spectrometry

Protein name (GiardiaDB ID)	Molecular weight	pI value	Score	Matches	Sequences	Coverage (%)
Kinase, NEK (GL50803_0015409)	57,771	4.91	433	25	7	17.7
Aminoacyl-histidine dipeptidase (GL50803_0015832)	56,158	5.55	295	8	3	8.1
Protein disulfide isomerase 2 (GL50803_009413)	50,832	8.51	356	15	4	10.7
Alcohol dehydrogenase 3 (GL50803_003593)	45,423	5.96	1,060	36	5	13
Ornithine carbamoyltransferase (GL50803_0010311)	37,268	6.6	10,705	455	16	51.4

NEK, never in mitosis A-related kinase; pI, isoelectric point.

Table 2. Primers and plasmids used in this study

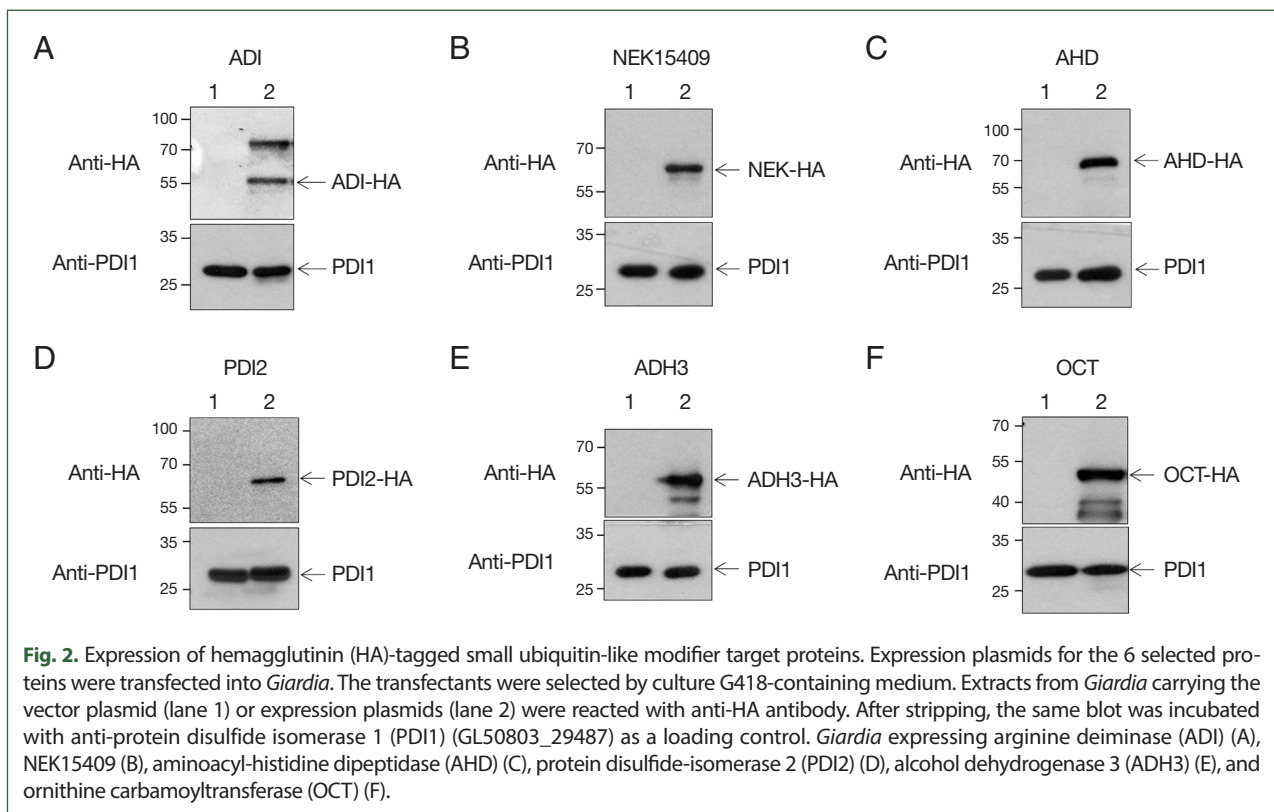
Name	Sequence (5' to 3')	
Primers		
t112103-F-infusion	ttggagctccaccgcggtggcgccACTGTGGTCTGTAAGTGTAGATCCAC	
t112103-R-infusion	gggatccactagttagagcgccCTTGATATCGACGAGATGTCAGC	
t15409-F	GCATAAAGCTTCCCGTCTCCCTACGCACACGTC	
r15409-R	ACCGCCTCGAGCAGGTTACAGGACCTCAGCACTC	
t9413-F	GCATAAAGCTTCGACAGTCAGTATGTCAGTATC	
r9413-R	ACCGCCTCGAGCTTCTTGCGCTTCTCTGCGCAGG	
t15832-F	ATAAGCCGCGGTGGATTGGCGGCGCCAAGTTC	
t15832-R	ATAAGAATGCGGCCGCGCTGGAGGGTGGCAATCTCCGAGAC	
t3593-F	ATAAGCCGCGGCTGTAATGGCATCAACCAA	
t3593-R	ATAAGAATGCGGCCGCGCTGTAGAAGGACTCGGAGAAA	
t10311-F-infusion	ttggagctccaccgcggtggcgccGTCAGCTTCATTTTATTATCGAATAACATCATTGGAG	
t10311-R-infusion	gggatccactagttagagcgccCTCCATCTTGCAAGTCAAGAAAG	
Plasmids		
pKS-3HA.NEO	Shuttle vector, Amp ^R , encoding <i>neo</i>	[6]
pADI-HA.NEO	pKS-3HA.NEO, 200-bp promoter region and 1,740-bp encoding <i>adi</i> (GL50803_00112103)	This study
p15409-HA.NEO	pKS-3HA.NEO, 200-bp promoter region and 1,545-bp encoding <i>nek</i> (GL50803_0015409)	This study
p15932-HA.NEO	pKS-3HA.NEO, 200-bp promoter region and 1,527-bp encoding <i>ahd</i> (GL50803_0015932)	This study
p9413-HA.NEO	pKS-3HA.NEO, 200-bp promoter region and 1,347-bp encoding <i>pdi2</i> (GL50803_009413)	This study
p3593-HA.NEO	pKS-3HA.NEO, 200-bp promoter region and 1,221-bp encoding <i>adh3</i> (GL50803_003593)	This study
p10311-HA.NEO	pKS-3HA.NEO, 200-bp promoter region and 981-bp encoding <i>oct</i> (GL50803_0010311)	This study

Restriction enzyme sites are underlined. Lowercase letters indicate mutated nucleotides.
 HA, hemagglutinin; NEO, neomycin; Amp, ampicillin; ^R, resistant.

HA.NEO, respectively. A DNA fragment encoding OCT (GL50803_10311) was obtained from *Giardia* gDNA by PCR using the primers t10311-F-infusion and t10311-R-infusion and then ligated with pKS-3HA.NEO using an In-Fusion HD Cloning Kit.

These plasmids were transfected into *Giardia* trophozoites as previously described [6]. Transfected *Giardia* cells were selected in TYI-S-33 medium supplemented with 600 µg/mL G418. The expression of HA-tagged proteins was confirmed by western blot using anti-HA antibodies. The expression of HA-tagged ADI was detected in extracts of *Giardia* carrying the expression plasmid for ADI. One of the 2 immunoreactive bands was 57 kDa, the expected size of HA-tagged ADI. Additionally, a larger band (~72 kDa) was consistently observed (Fig. 2A), and it was thought that this may be SUMOylated ADI, as detected in a previous study [5]. The *Giardia* trophozoites harboring the empty vector showed no immunoreactive bands with anti-HA antibodies. In contrast, the amounts of *G. lamblia* protein disulfide isomerase 1 (PDI1, GL50803_29498) were equivalent in cells with the vector or ADI-expression plasmid. *Giardia* harboring the expression plasmid for NEK15409, AHD, PDI2, ADH3, and OCT were examined for the expression of the HA-tagged protein via western blotting using anti-HA antibodies (Fig. 2B, C, D, E, and F, respectively). The expression of these proteins was absent in the cells carrying the empty vector. The amount of PDI1 was monitored as a loading control for relative protein expression in the transgenic *G. lamblia*.

Giardia expressing 1 of the 6 HA-tagged proteins (ADI, NEK15409, AHD, PDI2, ADH3, and OCT) were used as input samples for affinity chromatography conjugated with SIM.



Flow through and bound proteins were monitored as unbound and eluent samples, respectively, for further studies. Western blot analysis using anti-HA antibodies revealed that the fractions of HA-tagged ADI-expressing *Giardia* contained both modified and unmodified ADI (Fig. 3A). The SUMOylated form of the protein was larger than the unmodified protein. Interestingly, an immunoreactive band with a higher molecular weight than the unmodified protein was detected in *Giardia* expressing HA-tagged NEK15409, AHD, and PDI2 (Fig. 3B, C, and D, respectively). In contrast, no immunoreactive bands were observed in the experiments involving ADH3 and OCT (Fig. 3E and F, respectively).

SUMOylation is one of the PTMs present in *G. lamblia*. Because this organism lacks anaphase-promoting complex [2], it is more likely that SUMOylated proteins play a main role in diverse cellular processes, including cell cycle control. RNA interference-mediated depletion of SUMO causes defects in the shape of *Giardia* trophozoites [4]. Two centrins of *G. lamblia* have been identified as SUMO substrates, and defects in SUMOylation resulted in the arrest of cell cycle progression and conformational changes in *Giardia* (Yeo et al., unpublished result). In *Aspergillus*, a mutation in UbaB, a SUMO-activating enzyme, resulted in abnormal chromatin bridges indicating the role of SUMO in chromosome segregation [7]. In budding yeast, SUMOylation of septin is critical for recruiting Fir1, a cytokinesis checkpoint protein, at the hourglass stage of septin prior to its transformation into the double-ring form [8]. Mutagenesis of nuclear and spindle-associated protein 1 in humans revealed that 2 independent SUMOylation events affected its nuclear localization and activity by causing differential association with RANBP2, a nuclear porin [9].

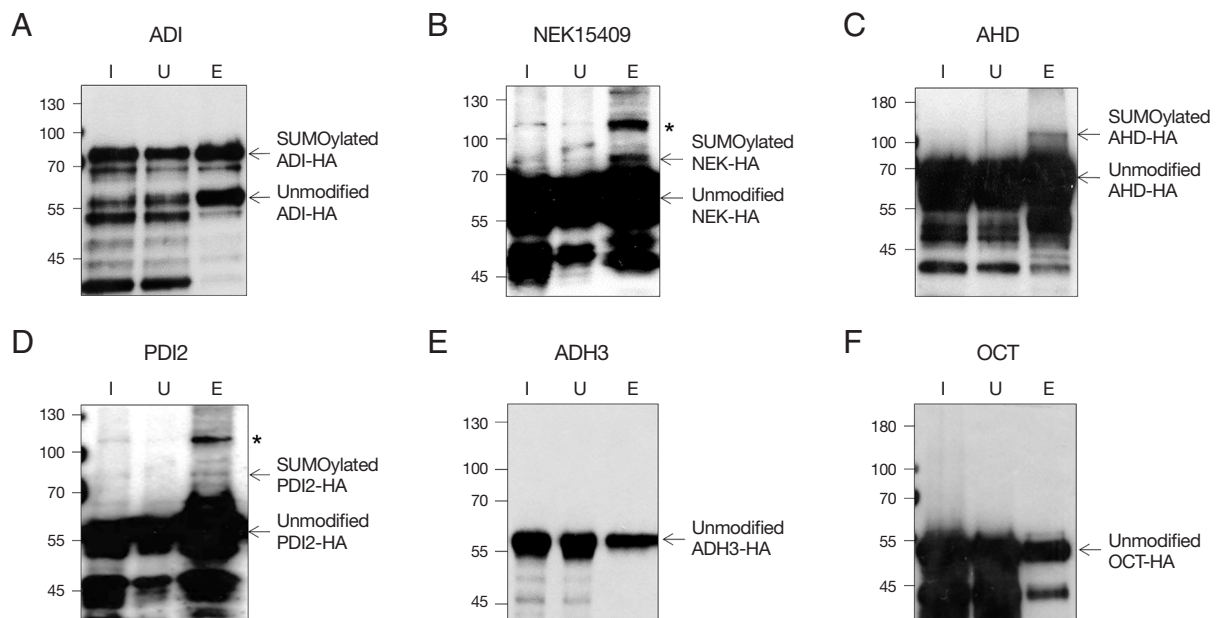


Fig. 3. Confirmation of proteins modified by SUMOylation via an affinity chromatography of transgenic *Giardia* expressing candidate proteins. *Giardia* extract expressing hemagglutinin (HA)-tagged protein was reacted with a SUMO-interaction motif-conjugated matrix suspension. Fractions for input extract (lane I), unbound (lane U), and eluent (lane E) were loaded onto SDS-PAGE and reacted with anti-HA antibody. Arginine deiminase (ADI) (A), NEK15409 (B), aminoacyl-histidine dipeptidase (AHD) (C), protein disulfide-isomerase 2 (PDI2) (D), alcohol dehydrogenase 3 (ADH3) (E), and ornithine carbamoyltransferase (OCT) (F). Asterisks indicate nonspecific bands in the elution fraction.

Among the known SUMO substrates in *Giardia*, ADI was included as a control in the SUMOylation assay (Fig. 3A). As expected, formation of the larger immunoreactive HA-tagged ADI (modified ADI) was the most obvious compared with other candidate SUMO substrates, namely NEK15409, AHD, and PDI2 (Fig. 3B, C, and D, respectively). ADI is a multifunctional protein. As a metabolic enzyme, it is involved in the arginine dihydrogenase pathway with carbamate kinase and OCT. This pathway is important not only for energy generation but also for immune evasion of this protozoan against host cells [5]. Interestingly, we identified OCT as a SUMO substrate when performing affinity chromatography using SIM (Fig. 1A; Table 1). However, SUMOylation of OCT was not verified through subsequent detection from *Giardia* expressing HA-tagged OCT (Fig. 3F). Therefore, it is unlikely that SUMOylation modulates the activity of this pathway. Besides its metabolic role, ADI plays a multifaceted role via its peptidyl-ADI activity, and it modifies the conserved CRGKA tail of variant-specific surface proteins [5].

One of the SUMO substrates identified in this study was NEK15409 (Fig. 3B). NEK is a conserved cell cycle-related kinase in eukaryotes. Interestingly, the *Giardia* genome has almost 200 NEKs, but information on their expression, activity, and function is lacking in most cases. Two NEKs, NEK1/NEK92498 (GL50803_92498) and NEK2/NEK5375 (GL50803_5375), have been investigated as the main NEKs, demonstrating roles in morphogenesis, cytokinesis, and excystation of *G. lamblia* [10]. Four NEKs (NEK1, NEK2, NEK16279, and NEK101534) were identified as components of basal bodies, adhesive disc or flagella of *Giardia* trophozoites [11]. RNA-seq analysis of G1/S-arrested versus G2/M-arrested *Giar-*

dia revealed that 3 NEKs (NEK1, NEK5999, and NEK95593) were upregulated at the G2/M phase [12]. Furthermore, several transcriptomic analysis have shown the upregulation of 8 NEKs (NEK8350, NEK11364, NEK91451, NEK95593, NEK3957, NEK114307, NEK15409, and NEK133701) during encystation [13]. In addition, defects in NEK8445 altered microtubule polymerization, eventually leading to structural malformation of flagella and adhesive discs in *Giardia* trophozoites [14]. NEK15409, one of the encystation-induced NEKs was identified as a SUMO substrate in this study, suggesting that PTM modulates its activity and possibly localization.

PDI is a representative marker protein of the endoplasmic reticulum. Its thioredoxin-like domain contains a cysteine redox-active site, facilitating the formation of disulfide bonds in proteins, resulting in the refolding of secretory or denatured proteins [15]. Five PDIs have been identified in *Giardia*, all which have a single thioredoxin-like domain in the N-terminus region: PDI1 (GL50803_29487, 26 kDa), PDI2 (GL50803_9413, 50 kDa), PDI3 (GL50803_14670, 13 kDa), PDI4 (GL50803_103713, 39 kDa), and PDI5 (GL50803_8064, 15 kDa) [16]. In *Giardia*, PDI2 appears to play a major role in disulfide bond reduction, as only PDI2 was able to complement the activity of the knockout mutant *Escherichia coli* [16]. Observations of PDI1, 2, and 3 in the endoplasmic reticulum and encystation specific vesicles suggest their role in the assembly and release of cysteine-rich variable surface proteins in trophozoites and cyst wall formation during encystation [17]. Interestingly, PDI4 and PDI2 bind to nitazoxanide, suggesting their potential as drug targets [18].

The last candidate SUMO substrate is AHD (GL50803_9413) (Fig. 3C). AHD is a metalloproteases performing various functions including protein degradation, protein maturation, and cell cycle control. In *Spironucleus vortens*, a diplomonad fish parasite closely related to *Giardia*, AHD was found to bind to nitroimidazole, causing redox imbalance due to antioxidant failure [19]. This enzyme is secreted from *Giardia* trophozoites upon exposure to human intestinal epithelial cells [20]. Our finding of AHD as a SUMO substrate (Fig. 3C) suggests that secretion of AHD is regulated by SUMOylation.

The identification of NEK15409, AHD, and PDI2 as candidate SUMO substrates indicates that their activity and localization are modulated via SUMOylation in *Giardia* trophozoites. The roles of these components in division or/and pathogenesis will be upcoming issues in study on *G. lamblia*.

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