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HELMINTHOLOGY - ORIGINAL PAPER

Screening and verification for proteins that interact with leucine aminopeptidase of *Taenia pisiformis* using a yeast two-hybrid system

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Abstract

Leucine aminopeptidase of *Taenia pisiformis* (TpLAP) belonging to the M17 peptidase family has been implicated as a stage-differentially expressed protein in the adult stage of *T. pisiformis*. In order to further dissect the biological functions of TpLAP in the growth and development of adult worms, TpLAP-interacting partners were investigated. In this study, a yeast two-hybrid (Y2H) cDNA library from adult *T. pisiformis* was constructed. Using pGBKT7-TpLAP as bait, proteins interacting with TpLAP were screened by Y2H system and positive preys were sequenced and analyzed using the Basic Local Alignment Search Tool (BLAST). Our results showed that six genuine TpLAP-interacting proteins, including LAP, dynein light chain (DLC), SUMO-conjugating enzyme (UBC9), histone-lysine n-methyltransferase, trans-acting transcriptional, and one unknown protein, were identified via Y2H assay. Furthermore, the interaction between TpLAP and UBC9 of *T. pisiformis* (TpUBC9), an important protein involved in SUMOylation pathway, was further validated by one-to-one Y2H assay, co-immunoprecipitation, and confocal analysis. These findings provide a deeper understanding of the biological functions of TpLAP and offer the first clue that TpLAP may act as a novel SUMOylated substrate, suggesting that the SUMO modification pathway plays an important role in regulation of adult worm growth and development.

Keywords *Taenia pisiformis* · Yeast two-hybrid · Interacting proteins · TpLAP · TpUBC9 · Co-immunoprecipitation

Introduction

Taenia pisiformis is a globally distributed parasite that causes serious diseases in its hosts and considerable economic losses in the rabbit breeding industry. *T. pisiformis* has a complex life cycle requiring a definitive host (commonly dogs) and an intermediate host (commonly rabbits) (Loos-Frank 2000; Toral-Bastida et al. 2011). The adult tapeworms have been highly adapted to the intestinal environments for growth, development, and survival in the definitive host. Successful invasion, immune escape, strobilae formation, and egg production are critical events during the long-term coexistence between worm and host, and some critical molecules are needed in order for tapeworms to develop survival strategies in their host. Recent evidence indicates that the living parasite can secrete proteases to perform various pathophysiological functions, including protein degradation, nutrient acquisition, and tissue invasion (Monteiro et al. 2017; Victor et al. 2012; Zhang et al. 2018). The tapeworms can secrete a number of peptidases, such as aminopeptidase, serine, and cysteine endopeptidases, which are responsible for worm pathogenesis due to its ability to cleave host macromolecules into short peptides (Li et al. 2006; Rascon Jr. and McKerrow 2013; Sako et al. 2007; White Jr. et al. 1996; Zimic et al. 2007). The parasite-derived proteases have been considered as attractive vaccine candidates and drug targets based on their key functions in parasite biology (McKerrow et al. 2006; Tsubokawa et al. 2017; Yang et al. 2015). However, more precise characterizations of tapeworm proteases are required

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to reveal their biological functions in cestodiasis progression and to develop efficient prevention methods.

In a previous study, we identified leucine aminopeptidase from *T. pisiformis* (TpLAP), a member of M17 peptidase family, overexpressed in the adult stage of *T. pisiformis* (Zhang et al. 2018). Nevertheless, the physiological and biological roles of TpLAP, especially in worm growth and development, remain poorly understood. It was reported that M17 LAPs from animals, plants, and microbes showed multiple functions ranging from MHC I antigen presentation, DNA binding, site-specific recombination to transcriptional regulation (Moore 2004; Reijns et al. 2005; Woolwine et al. 2001). In other parasites, M17 LAPs were involved in amino acid regulation, egg hatching, and encystation (Lee et al. 2015; McCarthy et al. 2004; Stack et al. 2007). TpLAP may also exhibit secondary activities in various processes by certain pathways and identifying its interactions with proteins may provide additional clues to its functions.

In the present study, we used the full-length cDNA of TpLAP as a bait to screen a cDNA library from adult *T. pisiformis* using a yeast two-hybrid screening (Y2H) system. This analysis resulted in the identification of several TpLAP-interacting genes, including LAP, dynein light chain (DLC), SUMO-conjugating enzyme (UBC9), histone-lysine n-methyltransferase, trans-acting transcriptional, and one unknown protein (GenBank: ERG09889). Moreover, we further confirmed the interaction between TpLAP and TpUBC9 in vitro by a special Y2H assay and co-immunoprecipitation (Co-IP). Based on bioinformatics analysis and Y2H verification, these candidate genes are expected to play essential functions during the development of adult *T. pisiformis*.

Materials and methods

Parasite, cell lines, and primers

The proglottid tissues of adult *T. pisiformis* and HEK293 human embryonic kidney cells were stored at the Key Laboratory of Veterinary Parasitology of Gansu Province, P.R. China. Yeast strain Y2HGold and reagents for the yeast two-hybrid assays were purchased from Clontech Co. (Mountain View, CA, USA). Gene-specific primers used in this study are listed in Table 1 and the target fragments inserted into every vector were validated by DNA sequencing.

Construction of Y2H cDNA library of adult *T. pisiformis*

Total RNA was extracted from adult *T. pisiformis* with Trizol reagent (Invitrogen, Carlsbad, CA, USA) and mRNA was purified using a FastTrack MAG mRNA Isolation Kit (Invitrogen). The integrity of total RNA was assessed using an Agilent 2100 Bioanalyzer with RNA integrity number (RIN) value. A cDNA library for the Y2H assay was constructed using a CloneMiner II cDNA Library Construction Kit (Invitrogen). Briefly, the first-strand cDNA was synthesized using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) and amplified into double-stranded cDNAs (dscDNAs). The attB1 adapter was ligated to the 5′ end of dscDNAs. After fractioning the cDNA by column chromatography, the purified dscDNAs were cloned into the pDONR 222 vector to generate a Gateway entry library by BP recombination. The resulting constructs were electrotransformed into *Escherichia coli* DH10B competent cells and spread onto LB agar plate (containing 50 μg/ml kanamycin). To generate the secondary library, the plasmid DNA was extracted from the primary cDNA library with the PureLink Midiprep Kit (Invitrogen) and transferred into the pGADT7-DEST AD vector by LR recombination. All steps were performed in strict accordance to the manufacturers' guidelines. The library titer and capacity were determined as follows: titer of cDNA library (CFU/ml) = (total colonies on plate × dilution factor)/volume plated (ml). Capacity (CFU) = CFU/ml × total bacteria volume (ml). To determine the recombination rate and inserted fragment sizes, 48 colonies were randomly selected from the cDNA library to amplify with library universal primers (Table 1) under the following PCR conditions: 94 °C for 5 min, 94 °C for 30 s, 58 °C for 30 s, 72 °C for 2 min, 25 cycles, and 72 °C for 5 min. The PCR products were analyzed using 1.2% agarose gel electrophoresis.

Generation of pGBKT7-TpLAP bait plasmid and auto-activation tests

Before performing the Y2H screen, the bait plasmid pGBKT7-TpLAP (BD-TpLAP) was constructed and tested for auto-activation. In brief, the full-length coding sequence (CDS) of TpLAP gene (GenBank accession no: MG366593) was amplified and inserted into the pGBKT7 vector via the *Nde* I and *Pst* I restriction endonucleases (New England Biolabs, Ipswich, MA, USA). After sequence confirmation, the plasmid BD-TpLAP (100 ng) was gently mixed with carrier DNA (20 μl) and 1× TE/LiAc/PEG4000 (500 μl), then transformed into Y2HGold competent yeast cells (50 μl) following the protocol described in the Yeastmaker Yeast Transformation System 2 User Manual (Clontech). The plasmids pGBKT7, pGBKT7-Lam plus pGADT7, and pGBKT7-53 plus pGADT7 were included to serve as negative and positive controls, respectively. The transformants were grown on SD/-Trp/X-α-Gal (SDO/X) or SD/-Leu-Trp/X-α-Gal (DDO/X) plates (Clontech) and incubated for 3–5 days at 30 °C. TpLAP expression in Y2HGold yeast was tested via western blot using anti-TpLAP monoclonal antibody (1:1000 dilution) as a probe (Zhang et al. 2018). The lack of auto-activation and toxicity of BD-TpLAP was determined if the white colonies grew well on SDO/X plates.

Table 1 PCR primers for TpUBC9 and TpLAP

Primer name	Primer sequences (5′ to 3′)	Sites	Vectors
Library universal primers			
pGADT7-F	TAATACGACTCACTATAGGGCGAGCGCCGCCATG	–	pGAD7-DEST
pGADT7-R	GTGAACTTGCGGGGTTTTTCAGTATCTACGATT	–	pGAD7-DEST
Bait plasmid PCR			
BD-TpLAPF	GGAATTCCATATGATGGGGGACCAGGGCATCAA TTTCG	*Nde* I	
BD-TpLAPR	AACTGCAG TCAGAGACGAGGGAGGACGTACATGC	*Pst* I	
TpUBC9 full length PCR			
TpUBC9F	ACATGGGGGGAGTAATGGGTGAT	–	pMD19-T
TpUBC9R	TTGGGGACGAATGCACATATTGG	–	pMD19-T
Prey plasmid PCR			
AD-TpUBC9-F	CCGGAATTCATGGGGGGAGTAATGGGTG	*EcoR* I	pGADT7
AD-TpUBC9-R	CACGAGCTCTTAAGATAGATTTGGGTTACG	*Sac* I	pGADT7
Tagged plasmids PCR			
Flag-TpUBC9F	GCCCAAGCTTATGGGGGGAGTAATGGGT	*Hind* III	pCMV-N-Flag
Flag-TpUBC9R	CCGGAATTCTTAAGATAGATTTGGGTTACG	*EcoR* I	pCMV-N-Flag
HA-TpLAPF	CGGAATTCAAATGGGGGACCAGGGCATC	*EcoR* I	pCMV-HA
HA-TpLAPR	ACGCGTCGACTCAGAGACGAGGGAGGACG	*Sal* I	pCMV-HA

Note: Underlined sequences indicate the sites of restriction endonucleases

Y2H screening

To identify that interacted with TpLAP, we screened TpLAP against the Y2H cDNA library from adult *T. pisiformis*. Y2H screening was performed using the Matchmaker Two Hybrid system (Clontech). Briefly, the BD-TpLAP (bait, 3 μg) and cDNA library plasmids (prey, 10 μg) were co-transformed into Y2HGold competent yeast cells. Co-transformation of pGBKT7-53 plus pGADT7 was used as a positive control and pGBKT7-Lam plus pGADT7 was set as a negative control. After transformation, the co-transformants were plated onto SD/-Leu/-Trp/X-α-Gal/AbA (DDO/X/A) agar plates and incubated for 3–5 days at 30 °C. Blue colonies were selected and plated onto higher stringency SD/-Ade/-His/-Leu/-Trp/X-α-Gal/AbA (QDO/X/A) plates for testing reporter gene expression.

Rescuing the prey plasmids and confirmation of the interactions

Positive clones were further confirmed by prey plasmid rescue and one-to-one Y2H verification. To rescue prey plasmids from yeast, plasmids were extracted from each of the blue colonies using a Yeast Plasmids Mini Preparation kit (Tiangen, Beijing, China). The plasmids were further purified by transformation of *E. coli* DH5α competent cells (TransGen, Beijing, China) and selection on LB agar plate (containing 100 μg/ml ampicillin) to enrich prey plasmids for sequencing using universal primers (Table 1). The obtained sequences were blasted against National Center for Biotechnology Information (NCBI) databases to annotate the function of the corresponding genes. To further confirm the genuine positive interactions, the purified prey plasmids plus BD-TpLAP plasmid were co-transformated into Y2HGold cells and the clones selected on QDO/X/A agar plates. The protocols for yeast transformation, plasmid isolation, and interaction test processes followed the methods described in the Yeast Protocols Handbook manual (Clontech). The same positive and negative controls used in the Y2H screening were included in these experiments.

Verification of TpLAP and TpUBC9 interactions using Y2H assay

The Y2H technique was performed to validate the predicted interaction of TpUBC9 with TpLAP in yeast. In order to generate pGADT7-TpUBC9 (AD-TpUBC9), the full-length CDS of TpUBC9 (Supplementary file 1: Fig. S1) was amplified by RACE-PCR and inserted into the pGADT7 vector using *EcoR I* and *Sac* I restriction endonucleases. All constructs were confirmed by DNA sequencing. BD-TpLAP and AD-TpUBC9 were co-transformed into Y2HGold strains and cultured on DDO and QDO/X/A agar plates for 3–5 days at 30 °C. In this experiment, co-transformations of pGBKT7 plus AD-TpUBC9 or BD-TpLAP plus pGADT7 were used to detect further the auto-activation of TpUBC9 and TpLAP genes.

Enzymatic activity assay of HA-TpLAP in HEK293T cells

Prior to co-immunoprecipitation analysis, the presence of TpLAP activity in the HEK293T cells was determined using L-leucine-p-nitroaniline (Leu-pNA, Sigma, St. Louis, MO, USA) as a substrate based on the procedure as described in previous study (Zhang et al. 2018). Briefly, HEK293T cells were cultivated in cell flask with DMEM medium (containing 8% fetal bovine serum and 1.0% antibiotic agent; Gibco, Grand Island, NY, USA) as previously described (Takahashi 2015). The plasmid pCMV-HA-TpLAP was constructed and transfected into the HEK293T cells using Xfect™ Transfection Reagent (Clontech) according to the manufacturers′ protocol. The cells transfected with pCMV-HA vector were used as the negative control. After 72 h, the cells were harvested, resuspended in ice-cold PBS (pH 7.4), and treated with freeze-thaw in thrice. Cell lysates were then centrifuged at 12,000×*g* for 20 min at 4 °C and the target proteins were purified from the cell supernatants by using anti-HA magnetic beads (Pierce, Rockford, IL, USA). The enzymatic reaction was performed by adding 2 μg of purified HA-TpLAP and 2 μl of Leu-pNA (200 mM) to a final 200 μl volume of assay buffer (50 mM Tris-HCl, 1 mM $MnCl_2$, pH 8.0) and incubated for 6 h at 45 °C. The reaction was terminated by cooling the plate on ice for 5 min and the release of pNA was determined spectrophotometrically at 405 nm using a microplate reader (Bio-Rad, Hercules, CA, USA). Blank incubations were carried out in parallel by incubating Leu-pNA in protein-free wells and the products purified from cells transfected with pCMV-HA was used as a negative control. The LAP from porcine kidney (SLAP; Sigma) was applied as a positive control and evaluated the relative activity of TpLAP. All enzymatic assays were detected by triplicate.

Co-immunoprecipitation

For Co-IP analysis, the plasmid pCMV-N-Flag-TpUBC9 was constructed and co-transfected with pCMV-HA-TpLAP into the HEK293T cells as above. After 48 h, the cells were harvested and lysed in 200 μl RIPA buffer containing 1 mM PMSF (Beyotime, Shanghai, China) for 30 min on ice. Cell lysates were centrifuged at 12,000×*g* for 20 min at 4 °C. The supernatants were collected for western blot and Co-IP analysis.

The Co-IP assay was performed using the Protein A/G Agarose system (Beyotime) according to the manufacturer's instructions. Briefly, the supernatant was incubated with 10 μg of anti-FLAG antibody and constantly mixed with end-over-end rotation at 4 °C overnight. Protein A/G agarose slurry (100 μl) was added to the mixture and incubated with gentle rotation for 5 h at 4 °C. After centrifugation, the complex was washed several times with cold PBS and the harvested pellets were boiled with 2× loading buffer for immunoblot analysis. Rabbit IgG was used as the negative control and the input supernatant was used as the positive control. The bound TpUBC9 was detected with rabbit anti-FLAG antibody (Sigma, 1:100 dilution) and the immunoprecipitated TpLAP was detected by mouse anti-TpLAP antibody (1:500 dilution). Human β-actin was included as an internal control and detected by a mouse monoclonal antibody (Beyotime).

Confocal analysis

Sections with HEK293 cells co-transfected with the pCMV-HA-TpLAP and pCMV-N-Flag-TpUBC9 were fixed in 4% paraformaldehyde and treated with permeability agent (Beyotime) for 30 min. HEK293 cells co-transfected with the pCMV-HA and pCMV-N-Flag were used as a negative control. The sections were incubated with a mixture of mouse anti-TpLAP (1:200 dilution) and rabbit anti-FLAG (1:100 dilution) overnight at 4 °C. The sections were rinsed thrice with PBS and incubated with Alexa Fluor 488 goat anti-rabbit IgG (1:500 dilution) and Cy3 goat anti-mouse IgG antibody (Sigma, 1:100 dilution) at 37 °C for 30 min. The sections were washed again with PBS and DAPI was used to stain the cell nuclei for 10 min at room temperature. Finally, the cells were imaged with a laser confocal microscope (Leica, Buffalo Grove, IL, USA).

Results

Y2H *Taenia pisiformis* cDNA library construction

Our results showed that the RIN value and OD_{260}/OD_{280} ratio of total RNA were 7.4 and 1.99, respectively, which indicated high integrity of the total RNA. Based on the LB agar plate clone numbers, the library capacity was calculated to be 5.4×10^6 CFU. In order to confirm the recombination rate and insert size of the cDNA library, 48 positive clones were randomly selected and confirmed by PCR from 0.25 to 2.0 kb in size with an average length > 1 kb, and the recombination rate was 100% (Fig. 1). These results indicated that this high-quality cDNA library was sufficient for the subsequent application to Y2H screening.

Autoactivation, toxicity, and expression of TpLAP in Y2HGold strain

The positive bait plasmid BD-TpLAP, containing the full-length CDS of TpLAP (residues 1–522, Fig. 2a), was identified by PCR and double restriction enzyme digestion by *EcoR* I/*BamH* I (Fig. 2b). Western blot

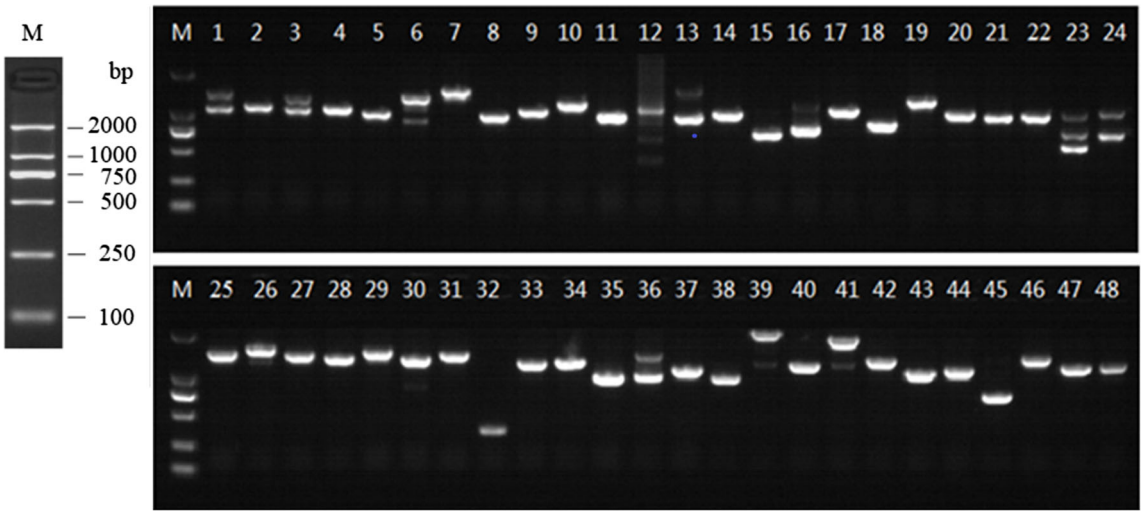

Fig. 1 Identification of recombination rate and insert size of cDNA library. Partial PCR products of randomly selected clones from the cDNA library were analyzed by 1.2% agarose gel electrophoresis to determine the recombination frequency and fragment size. Lane M, DL2000 DNA marker; lane 1–24, PCR products from the primary library; lane 25–48, PCR products from the secondary library

analysis showed that the TpLAP protein was expressed successfully in the Y2HGold cells. The molecular weight of the fusion protein was about 78 kDa, composed of TpLAP protein (~ 57 kDa) and DNA-BD protein (~ 21 kDa), which was consistent with the results by western blot detection (Fig. 2c). Moreover, the auto-activation and toxicity tests indicated that the BD-TpLAP transformants grew very well on SDO/X plates without any blue colonies (Fig. 2d). The negative control (PGBKT7-Lam+PGADT7-T) grew on DDO/X

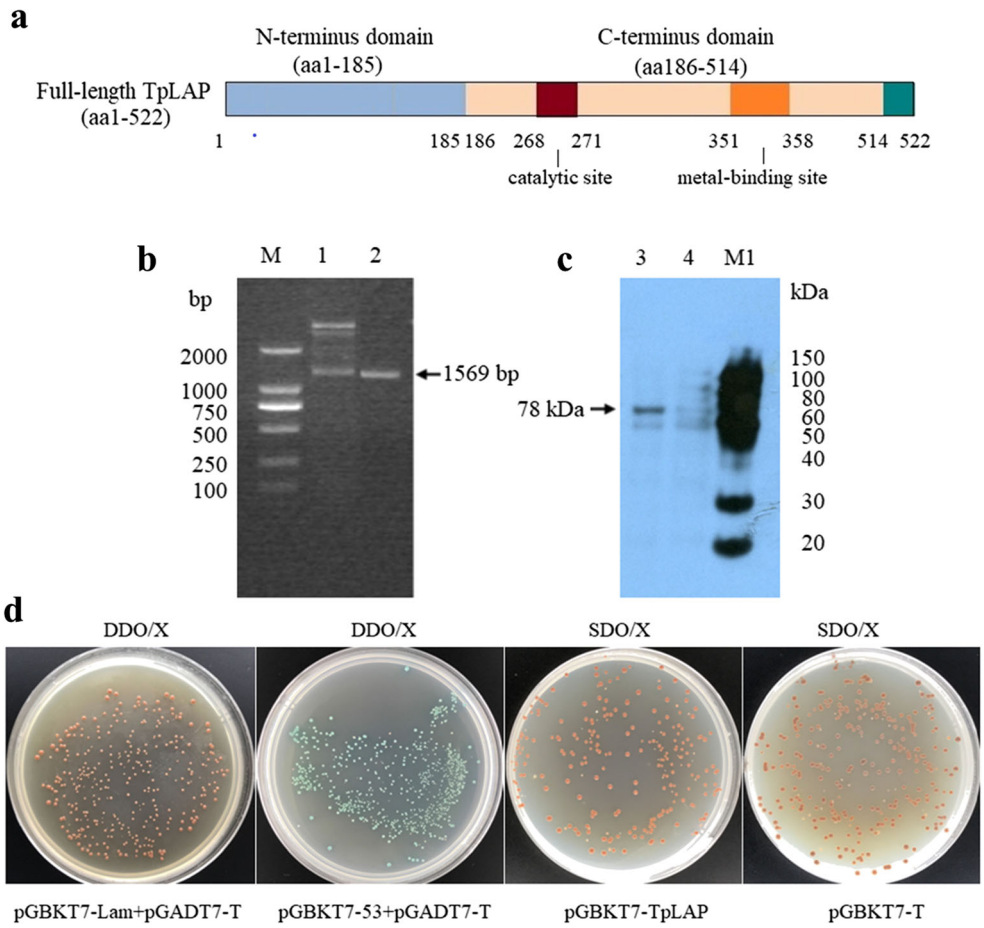

Fig. 2 Expression, auto-activation, and toxicity tests for BD-TpLAP in Y2HGold yeast cells. **a** Schematic illustration of full length and different domains of TpLAP. **b** Gel electrophoresis of BD-TpLAP plasmid. M, DL2000 DNA marker; lane 1, BD-TpLAP digested by *Nde* I/*Pst* I; lane 2, PCR product of BD-TpLAP. **c** Western blot detection of fusion proteins in Y2HGold yeast using anti-TpLAP antibody. Lane 3, Y2HGold/BD-TpLAP; lane 4, Y2HGold/pGBKT7; M1, protein ladder. **d** Determination of the auto-activation and toxicity of the BD-TpLAP using SDO/X and DDO/X indication plates. The pGBKT7-Lam plus pGADT7-T plasmids and pGBKT7-53 plus pGADT7-T were included to serve as negative and positive controls, respectively

plates with white colonies, while the positive control (pGBKT7-53+pGADT7) grew on DDO/X plates with blue colonies (Fig. 2d). Taken together, these findings confirmed that the bait plasmid BD-TpLAP did not autonomously activate the reporter genes (Mel1and ADE) or produce toxicity when expressed in Y2HGold yeast cells, suggesting that the BD-TpLAP plasmid was suitable for use in Y2H screening.

Screening for proteins interacting with TpLAP proteins via Y2H assay

Sixty-three blue colonies were obtained through Y2H screening (Fig. 3a). Plasmids in the positive colonies were extracted and amplified by PCR using pGADT7 specific primers (Table 1) to estimate the sizes of the inserts. Sequencing and BLAST analysis showed that 63 positive colonies corresponded to seven novel prey genes, including LAP, DLC, histone-lysine n-methyltransferase, SUMO-conjugating enzyme (UBC9), trans-acting transcriptional, NG dimethylarginine, and one unknown clone (Table 2). Gel electrophoresis results indicated that the positive colonies contained 1668, 340, 1015, 701, 964, 834, and 936 bp inserts (Fig. 3b). Among them, LAP, DLC, histone-lysine n-methyltransferase, and UBC9 appeared with more blue colonies (≥ 4), suggesting that the four proteins interact genuinely with TpLAP.

Rescuing the prey plasmids and confirmation of the interactions

To further verify the positive results of these seven clones, prey plasmids extracted from these clones and BD-TpLAP were individually co-transformed into Y2HGold cells and plated on DDO/X and QDO/X/A indication plates, respectively. Growth of blue colonies on the nutrient-deficient selective agar plates implied corresponding protein interactions. Of these seven independent prey plasmids, only NG dimethylarginine was a false-positive interactor, while remaining six preys did interact with TpLAP, evidenced by the strong blue colonies observed on both DDO/X and QDO/X/A plates

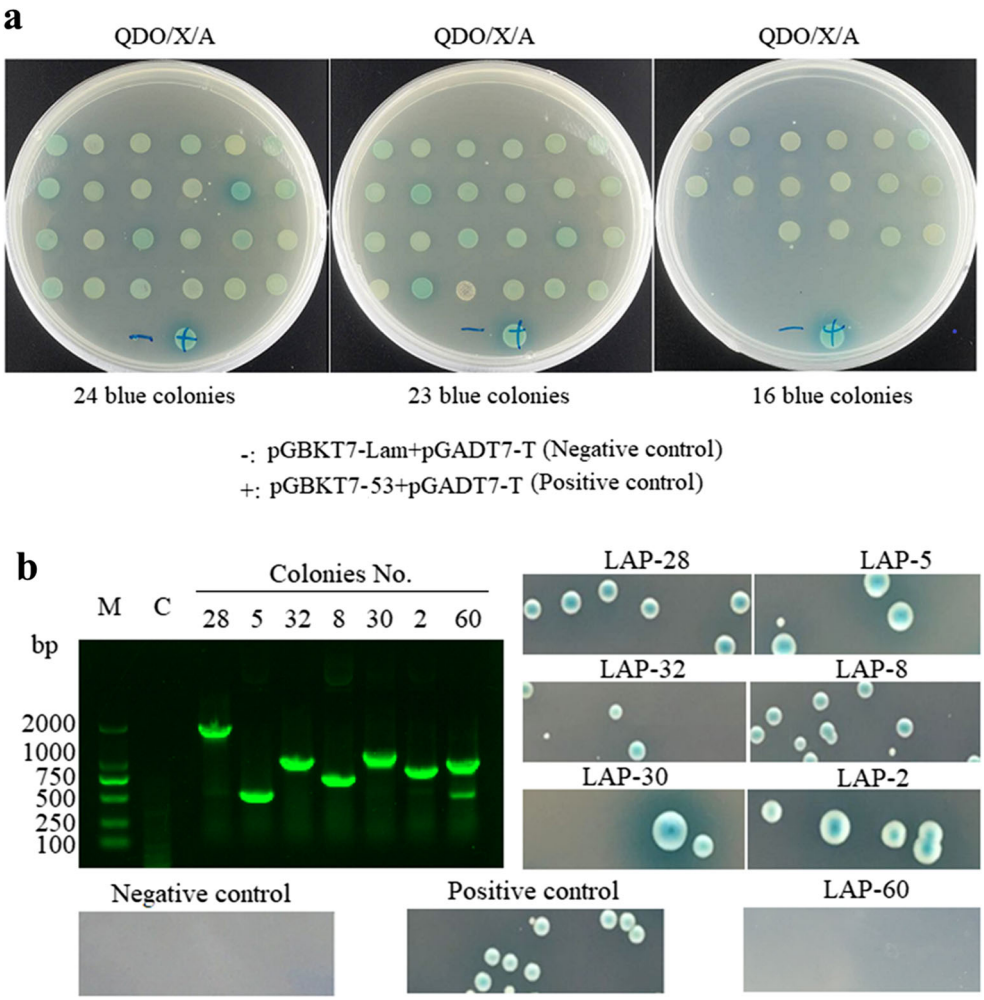

Fig. 3 Screening of TpLAP-interacting proteins in yeast two-hybrid assay. **a** Sixty-three positive colonies on QDO/X/A plates. Blue colonies indicate positive results, while absence of colonies indicates negative results. "+" and "−" indicate positive and negative controls, respectively. **b** PCR amplification of the inserts in seven prey plasmids and the interaction between BD-TpLAP and each putative prey plasmid in Y2H assay. M, DNA marker; C, negative PCR control. The genuine positive interactions were indicated on DDO/X/A plates by blue colonies

Table 2 List proteins interacting with TpLAP identified by the yeast two-hybrid system

Gene no.	Clone no.	Gene description	Gene accession no.	ORF	Pairwise	Inserts (bp)
LAP-28	33	Leucyl aminopeptidase	CDS 42600.1	+	Blue	1668
LAP-5	9	Dynein light chain	CDS 22858.1	+	Blue	340
LAP-32	13	Histone-lysine n-methyltransferase	EUB62179.1	+	Blue	1015
LAP-8	4	UBC9	EUB62720.1	+	Blue	701
LAP-30	1	Unknown	EUB55256.1	+	Blue	964
LAP-2	2	Trans-acting transcriptional	EUB60577.1	+	Blue	834
LAP-60	1	NG dimethylarginine	EUB62919.1	+	–	936

Note:"+" indicates PCR sequencing contained ORF fragments. "–" indicates no blue colony growth

for one-to-one partners (Fig. 3b), which indicated that six preys interacting with full length TpLAP were genuinely positive.

Confirmation of the interaction between TpLAP and TpUBC9 by specific Y2H assay

Considering the vital roles of UBC9 in the SUMOylation pathway, a TpLAP-interacting partner, TpUBC9, was selected for further verification of the interaction between TpUBC9 and TpLAP by a one-to-one Y2H assay. As shown in Fig. 4, all transformants grew well on DDO plates with obvious white colonies. The negative control (pGBKT7-Lam+ pGADT7) did not grow on QDO/X/A selection plates. In contrast, the positive control (pGBKT7-53+pGADT7) grew on QDO/X/A selection plates with blue colonies. Meanwhile, Y2HGold yeast cells co-transfected with BD-TpLAP and AD-TpUBC9 grew on QDO/X/A plates, producing visible blue colonies. While the Y2HGold yeast cells containing pGBKT7+AD-TpUBC9 or empty pGADT7+BD-TpLAP did not grow on QDO/X/A plates, failing to form obvious colonies. These results indicated that neither BD-TpLAP nor AD-TpUBC9 were auto-activated and indeed interacted with each other in Y2H Gold yeast cells.

Validation of the TpLAP-TpUBC9 interaction in vitro

HA-TpLAP protein was successfully produced in HEK293 cells with approximately 58.6 kDa of predicted molecular mass. The purified HA-TpLAP presented well enzymatic activity with 79.1% relative activity against SLAP using Leu-pNA as substrates when assayed under the standard conditions described in the "Materials and methods" section. The physical interactions of TpLAP with TpUBC9 in HEK293 cells were further confirmed by Co-IP and confocal analysis. As shown in Fig. 5 a, anti-FLAG and anti-TpLAP antibodies specifically recognized the corresponding fusion protein in western blots with the expected size of 21.3 kDa for Flag-TpUBC9 and 58.6 kDa for HA-TpLAP. In the Co-IP experiments, cell lysates containing HA-TpLAP and FLAG-TpUBC9 proteins were incubated with protein A/G resin that was covalently coupled with anti-FLAG antibody. HA-TpLAP also could be detected in the precipitation complex by anti-TpLAP antibody. In contrast, when anti-FLAG antibody was replaced by IgG in the control test, two proteins were not detected in the precipitation. Confocal microscopy indicated strong fluorescent signals were detected in HEK293 cells, green for FLAG-TpUBC9 and red for HA-TpLAP, indicating that the two proteins were simultaneously over-expressed in vitro. The FLAG-TpUBC9 with HA-TpLAP proteins was primarily colocalized in the cytosol of HEK293 cells (Fig. 5b). Based on these data, we concluded that TpUBC9 interacts directly with TpLAP in HEK293 cells.

Discussion

Identification of functional protein-protein interactions is fundamental for better understanding the molecular mechanisms of protein function in many biological processes. To date, several methods have been used commonly to analyze protein interactions, including Y2H techniques, bimolecular fluorescence complementation (BiFC), co-immunoprecipitation, and pull down experiments (Lai and Chiang 2013; Lin and Lai 2017; Louche et al. 2017; Paiano et al. 2018). Among them, the classical Y2H technique is popularly applied in parasite studies to seek and identify bait-prey interactions because of its low cost and reliable detection (Buro et al. 2017; Wang et al. 2016). Indeed, Y2H screening systems can enable researchers to easily find new interacting partners for a protein of interest, or to confirm interaction between two known proteins (Wagemans and Lavigne 2015).

In the present study, to further dissect the biological functions of TpLAP, a cDNA library from adult

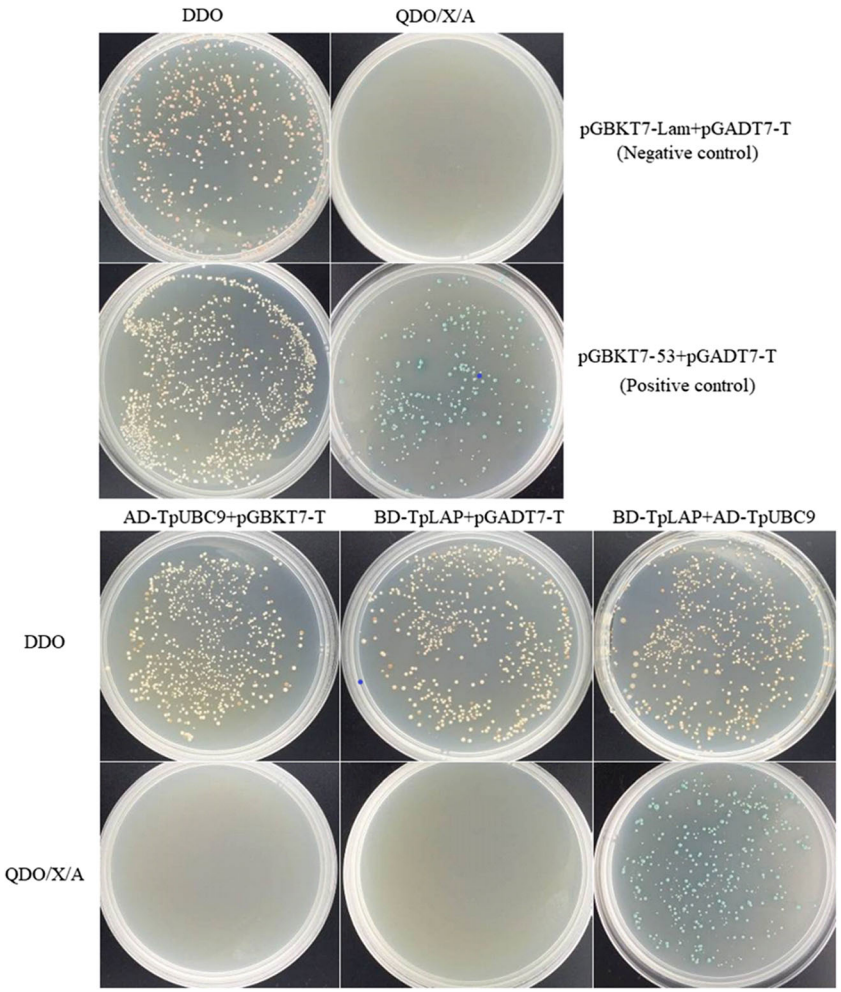

Fig. 4 Confirmation of the interaction between TpLAP and TpUBC9 by one-to-one Y2H assay. BD-TpLAP and AD-TpUBC9 plasmids containing full length CDS were co-transformed into Y2HGold cells. Co-transformants grew on DDO/X and QDO/X/A plates to monitor protein interactions with visible blue colonies

T. pisiformis was constructed and Y2H assays were used to identify candidate TpLAP-interacting partners. Before performing the YH2 analysis, auto-activation of the bait protein was used to evaluate whether or not it is suited for subsequent screening. We constructed a bait plasmid with the full-length CDS of TpLAP to avoid protein misfolding by truncation, which may affect the results of protein interactions. At the same time, we adopted the co-transformation method, which can reduce or eliminate the toxicity of fusion protein expression on fresh yeast cells and improve the efficiency and positive rate of library screening. We found that the full-length TpLAP does not autonomously activate the reporter genes or produce toxicity. Six candidate genes were identified as interacting with TpLAP, including LAP, DLC, UBC9, histone-lysine n-methyltransferase, transacting transcriptional, and one unknown protein (GenBank: ERG09889), implicating that TpLAP is very active in *T. pisiformis* and with multiple functions. Among these six positive partners, LAP was identified as most positive with 33 clones, which might be affected by LAP protein structures because M17LAP could form a hexamer consisting of six identical monomers. In this regard, it was speculated that the LAP subunits were directly aggregated into the hexameric complex in vivo and possessed protease hydrolytic properties, which was consistent with previous published results about LAP crystal structure of known species (Kim et al. 2013; Rawlings and Barrett 1995; Rawlings et al. 2006).

DLC is a subunit of the dynein motor complex and plays crucial roles in mediating dynein complex assembly and transporting various cargos (Hays and Karess 2000; Kamal and Goldstein 2002; Kirkham et al. 2016; Li et al. 2015). Y2H studies have shown that DLC interacts with various enzymes. For example, DLC1 has been identified as an interactor and inhibitor for neuronal nitric oxide synthase (Jaffrey and Snyder 1996). The interactions of DLC1 with p21-activated kinase 1 (PAK1) might play fundamental roles in cell survival (Lu et al. 2005). In addition, the interactions of DLC with phosphatase 1 contribute to the regulation of the microtubule dynamics and sperm motility (Korrodi-Gregorio et al. 2013). Although

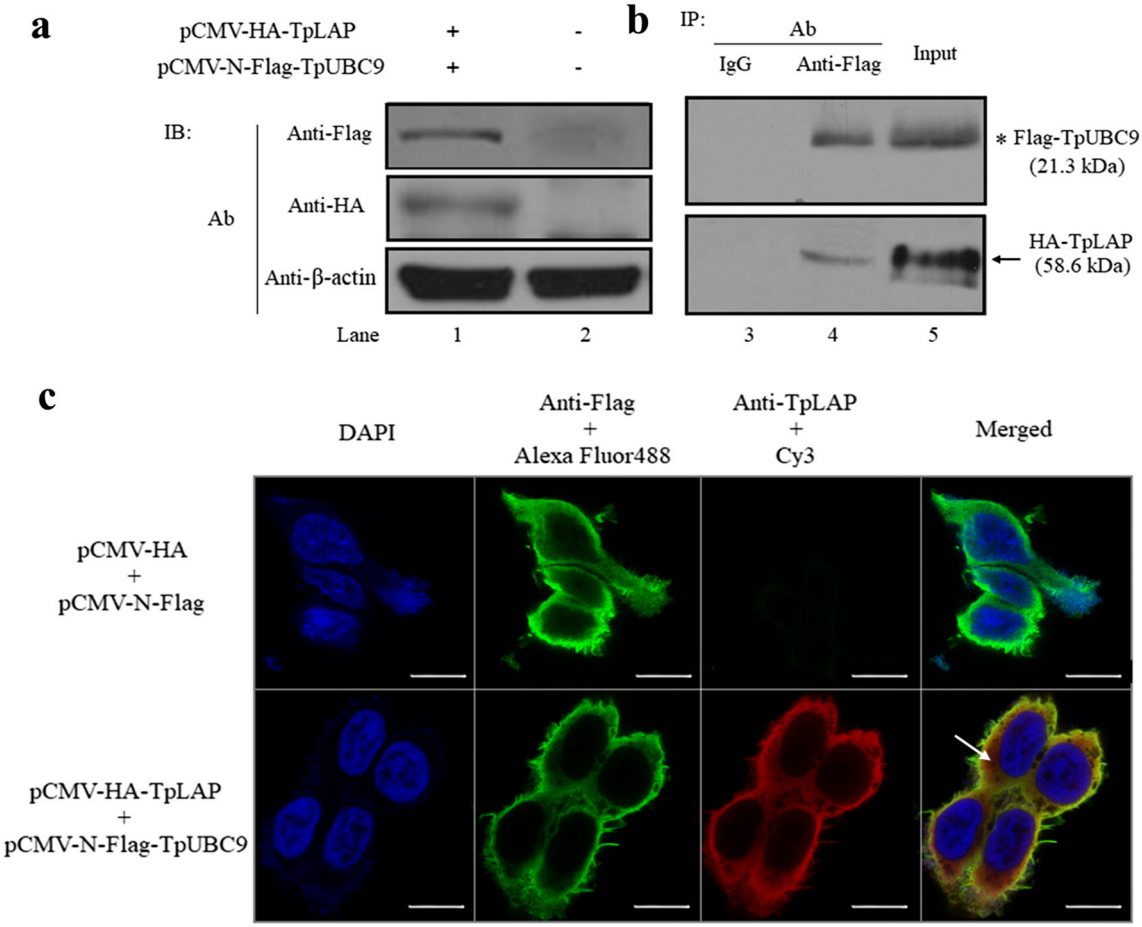

Fig. 5 Confirmation of the interaction between TpLAP and TpUBC9 in HEK293T cells. **a** HEK293T cells were co-transfected with pCMV-HA-TpLAP and pCMV-N-Flag-TpUBC9 plasmids. Expression of target proteins was probed with anti-TpLAP antibody, followed by anti-FLAG tag antibody and anti-β-actin (internal control) by immunoblotting (IB). **b** Cell lysates were immunoprecipitated (IP) with an anti-FLAG antibody for TpUBC9 (21.3 kDa) and detected using an anti-TpLAP antibody for the precipitation of TpLAP (58.6 kDa). **c** Co-localization of TpLAP and TpUBC9 in HEK293 cells is represented by yellow (arrowed) in the merged image. Nuclei were stained with DAPI. Scale bars represent 10 μm. All the experiments were performed three times with similar results

the basis for DLC requirement in parasites remains quite obscure, it has become apparent that DLC play numerous roles for efficient parasite growth. In protozoa, DLC is essential for asexual development in erythrocytic stages of *Plasmodium falciparum* by interacting with myosin A and actin 1 (Daher et al. 2010). Partial loss of DLC gene function in *Trypanosoma brucei* leads to flagellar motility defects and cell death (Baron et al. 2007; Kirkham et al. 2016). Zheng and colleagues identified 48 DLC members in the *Echinococcus granulosus* genome and speculated their functions were associated with transforming growth factor (TGF)-β signaling (Zheng et al. 2013). However, no previous report has suggested any molecular cooperation between DLC and enzymes in parasites. In the present study, we show for the first time that DLC is a putative interacting partner of TpLAP in tapeworm. It is speculated that DLC protein might affect the functions of TpLAP via a direct binding way, but the biological significance of the DLC-TpLAP interaction needs to be investigated in subsequent studies. Interestingly, one unique E2-conjugating enzyme in the SUMOylation pathway, UBC9, was identified by Y2H assay in the present work. SUMO modification is catalyzed by an enzymatic cascade composed of an activating enzyme (E1), conjugation enzyme (E2), and ligase (E3). UBC9 is the only known E2-conjugating enzyme for SUMOylation to date, which possesses binding interfaces for E1, substrates, E3, and SUMO (Pichler et al. 2017). SUMO modification has been studied well in humans and several model species (Smith et al. 2012). However, there is a lack of knowledge on SUMOylation system and processes in tapeworms. The present study represents the first to identify interactions between the proteolytic enzyme (TpLAP) and major components in the SUMO pathway (UBC9) in adult tapeworms. Because of the critical role of UBC9 in the SUMOylation process, its proper function is essential for normal growth and development of several model systems, including *Caenorhabditis elegans*, *Drosophila*,

zebrafish, and mice (Deyrieux and Wilson 2017). For example, the loss of UBC9 function yielded developmental defects with severe abnormalities in the pharyngeal muscle, vulva, and tail in nematodes (Broday 2017; Jones et al. 2002; Roy Chowdhuri et al. 2006). Similarly, the mutation of UBC9 orthologs gene blocked nuclear import of bicoid during early *Drosophila* embryogenesis and resulted in multiple defects in anterior segmentation (Epps and Tanda 1998). A critical role for UBC9 was likewise evident during mouse development where UBC9 defects broadly cripple nuclear function in stem cell differentiation and leads to apoptosis and early embryonic lethality (Kuehn 2005; Nacerddine et al. 2005; Tahmasebi et al. 2014). SUMOylation has been reported to participate in protein-protein interactions, subcellular ,localization and protein stabilization (Eifler and Vertegaal 2015; Heun 2007; Johnson 2004; Wilson 2017). As a novel TpLAP-interacting partner, TpUBC9 may be involved in regulating the activity and stability of TpLAP, which is closely related to maintaining the normal physiological function of TpLAP in worms. Further studies are required to identify the core components of the SUMOylation pathway in *T. pisiformis* and its exact functions during parasite growth and survival. The TpUBC9-interacting domain and sites of TpLAP will remain under study and need to be validated.

Conclusions

In summary, this work explored potential interacting partners of TpLAP by Y2H assay and six potential binding proteins were obtained. Among these proteins, TpUBC9, an E2-conjugating enzyme, had further demonstrated its physical interaction with TpLAP via the Y2H and Co-IP methods. The present results provided the first evidence that TpLAP activity may require SUMOylation and may lead to discovering novel molecular mechanisms of the TpLAP protein interaction network, as well as uncovering more clues for researchers to dissect its multifunctional effects in *T. pisiformis*.

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Compliance with ethical standards

Conflicts of interest The author declares that no competing interests exist.

Ethics approval Not applicable.

Consent for publication Not applicable.

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