PL-ISSN 0015-5497 (print), ISSN 1734-9168 (online)
Folia Biologica (Kraków), vol. 63 (2015), No 3
doi:10.3409/fb63_3.195

The ts111 Mutation of *Paramecium tetraurelia* Affects a Member of the Protein Palmitoylation Family*

Małgorzata PRAJER and Sebastian TARCZ

Accepted June 26, 2015

PRAJER M., TARCZ S. 2015. The ts111 mutation of *Paramecium tetraurelia* affects a member of the protein palmitoylation family. Folia Biologica (Kraków) **63**: 195-213.

The thermosensitive ts111 mutant of *Parameciun tetraurelia* carries a recessive mutation which causes cell death after 2-8 divisions at the restrictive temperature of 35°C. Expression at 35°C induces disassembly of the infraciliary lattice (ICL). In this study, we found that the ts111 mutation also results in significant abnormalities in the number and structure of contractile vacuole complexes (CVCs) and in their functioning at the restrictive temperature. In order to characterize the *ts111* gene, the complementation cloning was performed by microinjection into the macronucleus of an indexed genomic DNA library. The mutation was complemented by a sequence of 852 bp, which differed from the mutant sequence by a single nucleotide substitution. The deduced protein sequence is 284 amino acids long. It contains a domain referred to as the DHHC domain, associated with 2 trans-membrane helices. The DHHC proteins belong to the Palmitoyl-Acyl Transferases (PATs) protein family, which is implicated in the protein palmitoylation process playing the role in protein addressing. The ts111 mutation induces the amino acid change, localized before the first membrane helix. Transformation of ts111 mutant cells with the *TS111-GFP* gene fusion showed the expected reparation restoring thermoresistance and also demonstrated a localization of the protein in contractile vacuoles, but not in the ICL. The entire gene silencing in wild type cells at restrictive temperature caused the same effect as the expression of a point mutation in ts111 mutant. The authors propose the following hypotheses: (i) function of CVCs at the restrictive temperature depends in *Paramecium* on the TS111 protein – a member of the PAT family, and the primary effect of the termosensitive ts111 mutation are morphological abnormalities and dysfunction of CVCs, (ii) disassembly of the ICL is a secondary effect of the ts111 mutation, which results from disturbed regulation of the intracellular concentration of Ca^{+2} ions caused by the abnormal functioning of CVC.

Key words: Palmitoylated protein, contractile vacuole, infraciliary lattice, *Paramecium.*

Małgorzata PRAJER, Sebastian TARCZ, Department of Experimental Zoology, Institute of Systematics and Evolution of Animals. Polish Academy of Sciences, Sławkowska 17, 31-016 Kraków, Poland.
E-mail: prajer@isez.pan.krakow.pl

The ts111 mutant of *Paramecium tetraurelia* has been first described as a temperature sensitive mutant, the cells of which died after 2-8 divisions at 35ºC, while the wild type cells survive and grow at this restrictive temperature (BEISSON & ROSSIGNOL 1969).

The ts111 mutation had a dual effect on the *Paramecium* cell phenotype at the restrictive temperature:

(i) According to BEISSON & ROSSIGNOL (1969) the ts111 mutation also induced the disassembly of the infraciliary lattice (ICL).

(ii) According to our study, the ts111 mutation affected also the morphology and function of contractile vacuole complexes (CVCs).

The infraciliary lattice in *Paramecium* is a contractile network of cytoskeletal filaments in the cortex (GARREAU DE LOUBRESSE *et al.* 1988). Its

*Supported by Grant No NN 303 352135 from the Polish Ministry of Science and Higher Education /National Science Centre. Scientific stays (M.P.) at Centre de Génétique Moleculaire (CNRS – Gif sur Yvette, France) were supported by the GDRE "Paramecium Genome Dynamics and Evolution" program.

main components are centrins, which are highly conserved cytoskeletal proteins and centrin-binding proteins (KLOTZ *et al.* 1997; BEISSON *et al.* 2001; GOGENDEAU *et al.* 2007, 2008). Centrins constitute a distinct family within the superfamily of EF-hand calcium modulated polypeptides (MADEDDU *et al.* 1996). Centrins are implicated in Ca^{+2} regulated processes responsible for cortical contraction (GOGENDEAU *et al.* 2007). However, centrin-based contractile subcortical structures are only found in protists (in Alveolates) (ZHANG & HE 2012). Our observation of the phenotype of the mutant ts111 at the restrictive temperature confirmed the depolymerisation of the ICL. Our study has also revealed that the heat shock induces abnormalities in the structure and function of the CVCs in mutant cells. The CVCs are specific organelles responsible for osmoregulation in protozoa and freshwater sponges by extrusion of water and excess of ions, mainly Ca^{+2}. Proton gradient drives chemiosmotically the transfer of water and ions into the contractile vacuole using the V-type H^{+}-ATPase as an active transporter (FOK *et al.* 1995; 2002). They then expel its contents periodically by exocytosis through the vacuole pores. In *Paramecium,* this CVC is composed of a central vacuole, which is connected via ampoules to 5-10 radial collecting canals during diastole. During systole they are transiently disconnected (ALLEN 2000). Each collecting canal is surrounded by a branched network of membranous tubules forming a smooth spongiome, which is connected to the decorated spongiome that bears the pegs on cytosolic surface of their membranes. The pegs represent the V-ATPase complex and act as a proton pump (FOK *et al*. 1995, 2002; WASSMER *et al*. 2006). The smooth spongiome is a flexible membrane reservoir that allows swelling during diastole (ALLEN 2000; PLATTNER 2013). CVC functioning depends on a number of transient, rhythmic and orchestrated fusions of membranous structures.

The aim of this study was to clone and sequence the *TS111* gene, as well as to predict the structure and function of the protein coded by this gene. In order to identify and characterize the *ts111* gene, and taking advantage of the easy observation of the ICL network, the complementation cloning of an indexed genomic DNA library (KELLER & COHEN 2000) by microinjection into the macronucleus was performed. The *TS111* gene was cloned and sequenced. The deduced protein sequence presents a domain referred to as the DHHC domain, which is associated with 2 trans-membrane helices. This DHHC protein family is known to be implicated in the protein palmitoylation process, playing a role in protein addressing. S-palmitoylation is one of the most frequent post-translational modifications of proteins and recently been intensively studied (CHAMBERLAIN *et al.* 2013; FRÉNAL *et al.* 2014; HORNEMANN 2015; LIN & CONIBEAR 2015; OESTE *et al*. 2014). Proteins bearing characteristic DHHC domain belong to the Palmitoyl-Acyl Transferases (PATs) protein family. Among others, PAT enzymes are involved in the vesicular neuronal transport as well as in the targeting and trafficking of proteins in neurons (BRIGIDI & BAMJI 2013; YOUNG *et al.* 2012). Several proteins containing DHHC domains have been implicated in human diseases (CHAVDA *et al.* 2014; ROLDAN *et al.* 2015; XU & ZHONG 2015).

Material and Methods

Strains and culture conditions

The ts111 mutant of *Paramecium tetraurelia* used in this study and wild type (WT) strain d4-2 of *P. tetraurelia* (SONNEBORN 1974) were derived from the stock collection of the Centre de Génétique Moléculaire in Gif-sur-Yvette, France. The ts111 mutant was obtained by mutagenesis with UV irradiation treatment of the d4-2 stock of *P. tetraurelia* (BEISSON & ROSSIGNOL 1969). Cells were cultured at either 28°C or 35°C, according to the SONNEBORN's (1970) standard method in medium with lettuce or in a wheat grass powder infusion (BHB, L'arbre de vie, Luçay Le Male, France), inoculated with *Klebsiella pneumoniae* and supplemented with 0.4 μg/ml β-sitosterol (Merck).

Cloning with the DNA complementation method using the microinjection technique

Functional complementation cloning of an indexed genomic DNA library of *Paramecium tetraurelia* (KELLER & COHEN 2000) was performed using the sib selection screening method and the DNA microinjection technique, as previously described (SKOURI & COHEN 1997; FROISSARD *et al*. 2001). The rescuing pool of macronuclear DNA was divided into smaller and smaller pools until the unique rescuing pool had been identified and then the gene of interest was isolated. Each DNA sample was injected into 15-20 cells. The effect of the injections was tested by culturing the injected cells at a restrictive temperature of 35°C and surviving and dividing cells were assessed as rescued ones. Finally, the following primers were used to amplify the *TS111* gene:
5'-TTAAACTAGTATGGCAGTGCCATCATAAGTGTATAA;
3'-TTAACTCGAGAACAACAGGAGTAATATTTGTTATC.

Genomic DNA extraction

Total genomic DNA for sequencing the DNA fragments of interest was prepared from 200 ml of a log-phase cell culture, both wild type and mutant cells using the DNAzol reagent (Life Technologies), according to the supplied protocol.

Plasmid construction

To express the GFP-tagged TS111 protein, two fusion constructs were prepared into which the entire sequence of *TS111* gene was introduced. In the first pPXV-GFP plasmid with the GFP open reading frame designed for expression in *Paramecium* (HAUSER *et al.* 2000), the insert was cloned at the 5' end of the GFP in pPXV-GFP into the SpeI and XhoI restriction sites. To construct the second plasmid, the insert was amplified from genomic DNA by PCR, using primers into which a linker containing the specific sequence of the KpnI restriction enzyme was added. After the digestion with the KpnI this fragment was cloned into the KpnI restriction site located at the 3' end of the GFP synthetic gene designed by E. Meyer and J. Cohen (unpublished data), and which had been introduced into the pPXV vector, the recombinant gene being under the control of the *Paramecium* calmodulin regulators (HAYNES *et al.* 1995). The cloned genes were entirely sequenced to ensure that no error was introduced during amplification. Eventually, both DNA constructs for microinjection were prepared with a Plasmid Midi Kit (QIAGEN) according to the protocol provided by the manufacturer. Both plasmids were linearized with the SfiI restriction enzyme.

For gene silencing the entire *TS111* gene was amplified by PCR and cloned into the SpeI and XhoI site of the L4440 feeding vector (GALVANI & SPERLING 2002). The gene paralog amplified by PCR was cloned in the Litmus 28 vector (NEB), wherein the EcoR1 site was replaced by Srf1 site. The entire gene that had been amplified by PCR was cloned into this Sfr1 site according to the protocol of the pPCRscriptTM Cloning Kit (Stratagene). These two vectors allowed for the synthesis of double-stranded RNA corresponding to the cloned genes ligated into double T7 promoters (TIMMONS & FIRE 1998).

Cell transformation using the microinjection technique

Plasmids pPXV-GFP, containing an insert of interest after precipitation were resuspended in sterile water at a concentration of 5 μg/μl. Microinjections of DNA solutions were performed into the macronuclei of vegetative mutant ts111 cells, which derived from young clones that had undergone four to six divisions after autogamy as was previously described (RUIZ *et al.* 1998). Each plasmid was injected into 15 cells. Transformation was screened for the ability of injected cells to survive at the restrictive temperature as well as for the intensity of fluorescence signal of the GFP tagged protein. Microinjection was performed under an inverted Nikon Phase-contrast microscope equipped with Narishige micromanipulation devices and an Eppendorf air pressure microinjector.

Gene silencing using the RNAi feeding method

The feeding plasmid was used to transform the HT115 strain of *E. coli,* which is RNase III-deficient and has IPTG-inducible T7 polymerase activity (SAMBROOK *et al.* 1989). Transformation of bacteria was performed using the electroporation method. For gene silencing wild type *Paramecium* cells were incubated in the feeding medium that contained bacteria expressing the double-stranded RNA (GALVANI & SPERLING 2002) according to a standard protocol (BEISSON *et al.* 2010). Then the viability of the cells at the restrictive temperature was observed over successive days through their daily transfer into freshly-induced feeding bacteria. For negative controls, *Paramecia* were fed with HT115 bacteria carrying the L4440 plasmid without the insertion.

Immunofluorescence microscopy

Immunolabelling was essentially carried out according to standard protocols (BEISSON *et al.* 2010), except for the GFP-labelled structures, where a few modifications replaced the fixation in paraformaldehyde. Cells were fixed in methanol at -20ºC for 17 min. Then PHEM Tween 1% were added on the surface of methanol and the cells were transferred into the PHEM Tween 1% for 3-5 min. Permeabilization was performed for 2 min in a PHEM buffer (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 2 mM $MgCl_2$) (SCHLIWA & VAN BLERKOM 1981) containing 1% Triton X-100. After two rinses in PBS BSA 3%, the cells were incubated with the anti-GFP polyclonal antibody for 30 min, and rinsed twice again in PBS BSA 3% before being incubated with the secondary antibody. Then cells were washed twice in PBS BSA 3%, and the final wash was complemented with Hoehst (Sigma-Aldrich). Finally, cells were mounted in Citifluor (Citifluor LTD, Canterbury, England). Two antibodies were used: the monoclonal antibody IA9 raised against the *Paramecium* infraciliary lattice (BEISSON *et al.* 2001) diluted 1:200, and the polyclonal anti-GFP antibody (Interchim, France) diluted 1:300. Appropriate secondary anti-mouse or anti-rabbit antibodies: Alexa 594 or Alexa 488

(Jackson ImmunoResearch Labs (West Grove, PA)) - were diluted 1:250.

Cells were observed under an Axioskop 2-plus fluorescence microscope equipped with a Coolsnap-CF video camera system (Zeiss, Germany) with GFP filters. Images were processed using Metamorph software (Universal Imaging, Downington, PA).

In vivo observations of contractile vacuoles

To observe the contractile vacuoles in living cells and to record the GFP-expression in living transformed cells, they were washed in Dryl's buffer supplemented with 0.2% BSA. Then individual cells in a small drop of this solution were transferred onto a coverslip. To perform longer observations of living cells, they were immobilized by putting them into a small drop of suspension of polystyrene microparticles with a diameter of either 15 μm or 20 μm (Fluka Chemie GmbH). Pulsations (contractions) of the CVs were counted for 1 min in wild and mutant cells at the log-phase of growth cultured at 28°C and 35°C. Because thermosensitive cells die the next day after individual isolation into a small volume of medium, the effect of restrictive temperature was observed in exponentially growing cells in a mass culture volume of 25 ml, which were exposed to the restrictive temperature of 35°C for about 10h before measurement. CVC pulsations in non-transformed cells were examined under a microscope equipped with phase-contrast objective. CVC pulsations in cells transformed with GFP-TS111 fusion were observed under an Axioskop 2-plus fluorescence microscope.

Cell fractionation, lysis procedures and protein analysis

In order to further protein characterization, the conditions of its extraction were determined along the procedure of post-microsomal protein preparation. Different aliquots were electrophoretically separated, blotted and the presence of pTS111 was revealed by treatment with the anti-GFP antibody. For protein extraction mutant cells not transformed and transformed by microinjection of the pPXV-GFP plasmid with the TS111 insert were used. Two liters of exponentially growing cultures were washed in Dryl's buffer and centrifuged at low speed. From this step all subsequent treatments were performed at 4°C. The resulting cell pellet (about 0.2 ml), was suspended with five volumes of lysis buffer: 0.25 M sucrose, 50 mM potassium phosphate, pH 7.0. The cells were mechanically broken in a potter homogenizer. They were supplemented with an anti-protease mixture as described in KLOTZ *et al.* (1997). The cell lysate was centrifuged for 15 min at 15 000x rpm to eliminate insoluble proteins. An aliquot of the supernatant and the pellet were kept for electrophoretic analysis. The supernatant was centrifuged at 100 000x rpm for 1h, yielding a post-microsomal supernatant and a microsomal pellet (Pm). SDS-PAGE was performed as previously described (KLOTZ *et al.* 1997) Upon completion of migration, proteins were electrophoretically transferred onto a nitrocellulose membrane (Schleicher & Schüel, Dassel, Germany) in 0.05M Tris-borate buffer, pH 7.0, for 2 h at 35 V. The quality of the transfer was tested by staining the proteins with 1% Ponceau S Red in 7% acetic acid. The filters were saturated with 5% non-fat dry milk in 0.02 M Tris-HCl, pH 7.4, 0.15 M NaCl, 0.1% Tween-20. All subsequent incubations and washes were done in the same solution. Incubation with the rabbit anti-GFP antibody (dilution 1:250) was carried out overnight at 4°C with mild agitation. Washing (3 times 15 min) and incubation with the secondary anti-rabbit antibody was performed at room temperature for 30min.

Sequence analysis and prediction programs

The initial characterization of the DNA and protein sequences was made using the DNA STRIDER program (MARCK 1988). Sequence comparisons with public databases were performed using the BLAST program (ZHANG & MADDEN 1997) and for sequence alignments, the CLUSTAL W (THOMPSON *et al.* 1994) and the Muscle programs (EDGAR 2004) were used. For the prediction of protein localization and transmembrane topology, Phobius software (KÄLL *et al.* 2007) and CBS Prediction Servers at www.cbs.dtu.dk were used. The CSS-Palm Site was used for palmitoylation sites prediction (REN *et al.* 2008). Web service Phylogeny.fr (DEREEPER *et al.* 2008, 2010) was used to analyze the phylogenetic relationships.

Results

The ts111 mutant has been characterized for the first time by disassembly of infraciliary lattice and death at the restrictive temperature of 35°C after a few cell divisions, while the wild type cells survive and grow at this restrictive temperature (Fig. 1) as was described by BEISSON and ROSSIGNOL (1969). In this study, we also observed impaired function and abnormal morphology of the contractile vacuoles complex in the ts111 mutant cells at the restrictive temperature, which had not been reported previously. The wild type cells grow at 35°C without disassembly of infraciliary lattice or abnormalities in the CVs.

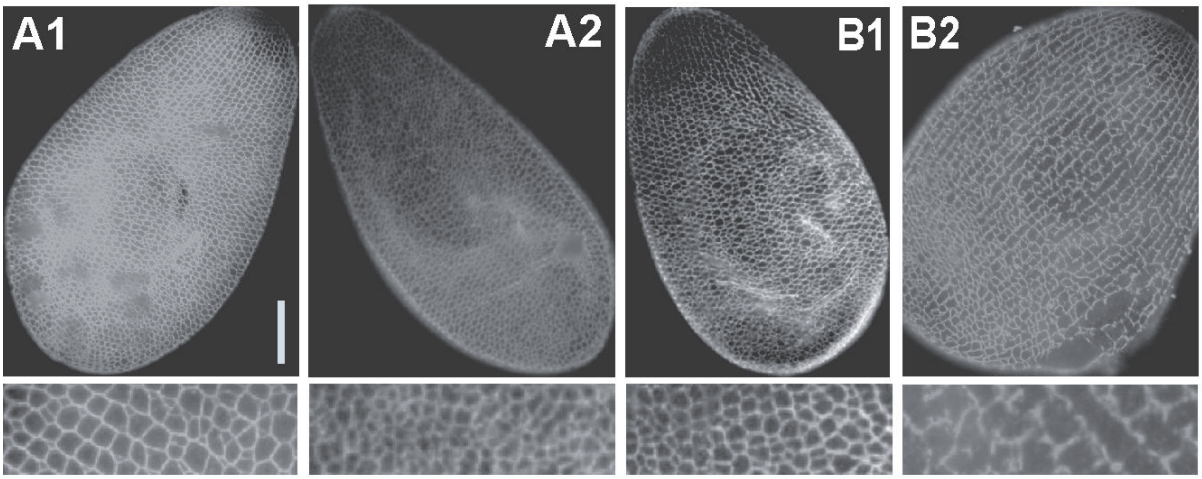

Fig. 1. Infraciliary lattice in wild type cells and mutant cells growing at 28°C and at restrictive temperature. Wild type cells: A1 – 28°C; A2 – 35°C. Mutant ts111: B1 – 28°C; B2 – 35°C. Early temperature shock effect – ICL depolymerization is still incomplete, while the rate of CVC contractions is already slower and anomalies are visible. The ICL decorated with the IA9 antibody. Scale bar: 10 μm.

Complementation cloning and general characteristics of the *TS111* gene

Complementation cloning of the wild type *TS111* gene was done using microinjection of the ts111 mutant cells with an indexed *Paramecium* genomic library followed by sib-selection according to SKOURI and COHEN (1997). The rescued cells displayed the wild type morphology and fission rate at the restrictive temperature of 35°C, whereas the ts111 mutant cells died after a few divisions. The size of the insert complementing mutation was 10 kb of genomic DNA in plasmid (fraction P, subfraction XXVII, plaque 133, fraction A, subfraction M4): Scaffold_80, positions 244,001 to 254,000 (Paramecium DB http://paramecium.cgm.cnrs-gif.fr/; ARNAIZ *et al.* 2007; ARNAIZ & SPERLING 2011). From this region, the gene: GSPATG00023862001 was chosen as a hypothetical *TS111* gene since it was the only one in the region without a paralog issued from the last whole genome duplication (AURY *et al.* 2006). Indeed, such paralogs often complement mutations in a gene and prevent the detection of any phenotype. The gene was sequenced as part of the large insert. Study based on the functional complementation method revealed a single point mutation of the *TS111* gene. The open reading frame was 852 bp long, without introns. The *ts111* mutant gene differed from wild type allele by one single nucleotide substitution of guanine by adenine at position 188 (Fig. 2). One paralog of the *TS111* gene: GSPATG000345550001 resulting from the second Whole Genome Duplication exists in the *Paramecium* genome showing 46.5% sequence identity with the *TS111* gene. The *TS111* gene was then cloned in the plasmid pPXV-GFP. This construct was microinjected into the macronuclei of the ts111 mutant giving transformed cells thermoresistant i.e. they were rescued at the restrictive temperature of 35°C.

The nucleotide sequence of the *TS111* gene corresponds to the gene annotated as GSPATG00023862001 in the *Paramecium* genome project (AURY *et al.* 2006) and is accessible at Paramecium DB (http://paramecium.cgm.cnrs-gif.fr/ (ARNAIZ *et al.* 2007; ARNAIZ & SPERLING 2011).

Function of the predicted protein coded by the *TS111* gene

The predicted *TS111* gene product is a protein of 283 amino acids. In the ts111 mutated protein glycine at position 63 is replaced by glutamic acid. The protein sequence contains the Asp-His-His-Cys (DHHC) motif within a Cystein-Rich Domain (CRD) which is an integral part of the catalytic site of palmitoyl acyl transferase (PAT) (Fig. 3). Hence, the TS111 protein belongs to the PAT family proteins. The reaction catalyzed by the PAT enzymes is a post-translational modification of proteins by attaching the C16 saturated fatty acid palmitate to cysteine residues via a thioester linkage. This enzyme adds a palmitoyl group to other proteins in order to anchor them to cell membranes (CHAMBERLAIN *et al.* 2013). It can also be the target of autopalmitoylation. The TS111 protein shows a theoretical isoelectric point of 5,99 and a theoretical molecular mass of 33,378 Da (Phobius software (KÄLL *et al.* 2007) and CBS Prediction Servers at www.cbs.dtu.dk). Immunoblot analyses of the different fractions obtained along the procedure of cell extraction showed that the GFP-TS111 protein from wild type or mutant cells were localized in the microsomal pellet (Fig. 4).

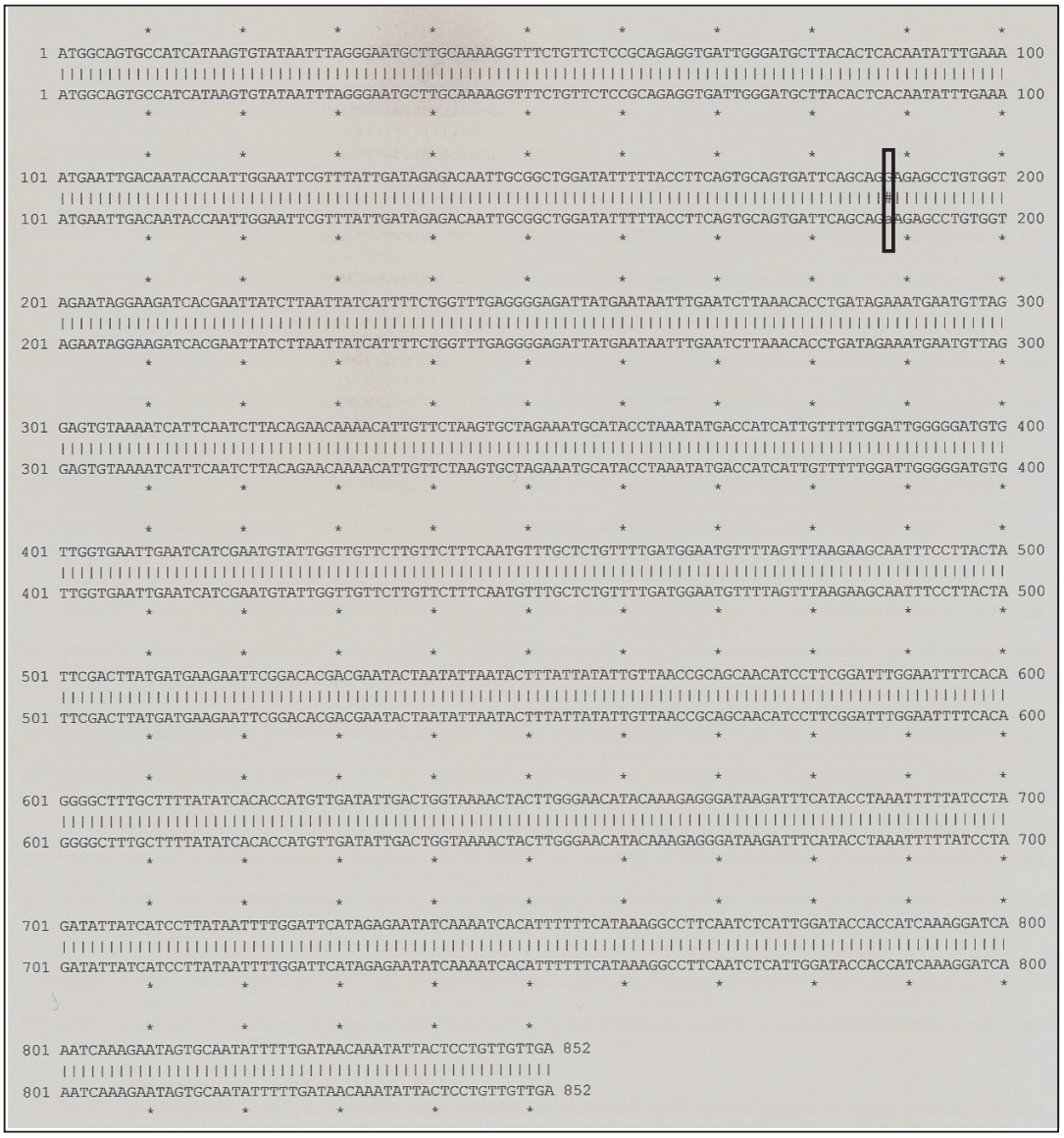

Fig. 2. Alignment of the DNA sequences of *TS111* and *ts111*. The localization of the mutation G to A is marked in the black rectangle.

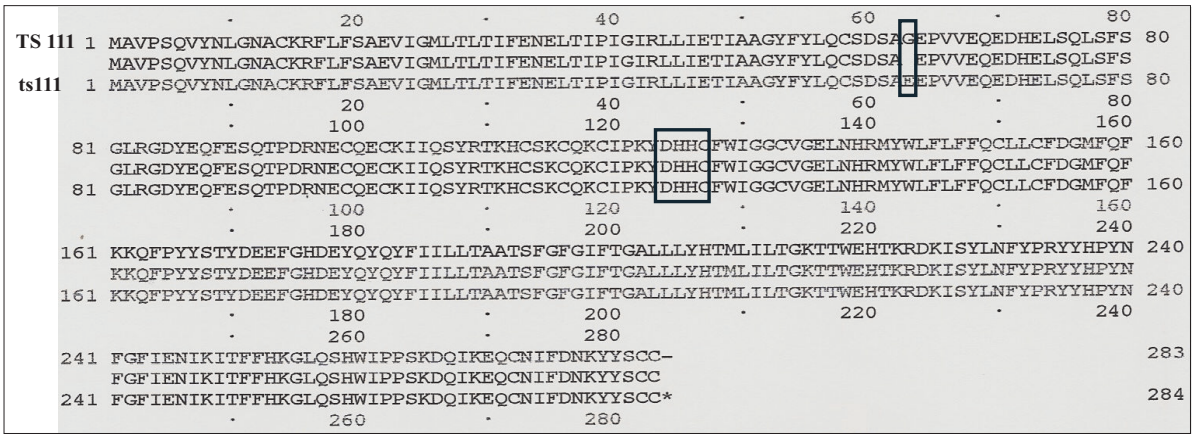

Fig. 3. Protein alignment of TS111 and ts111. Localizations of the site of mutation and position of the domain DHHC are marked in the black rectangles.

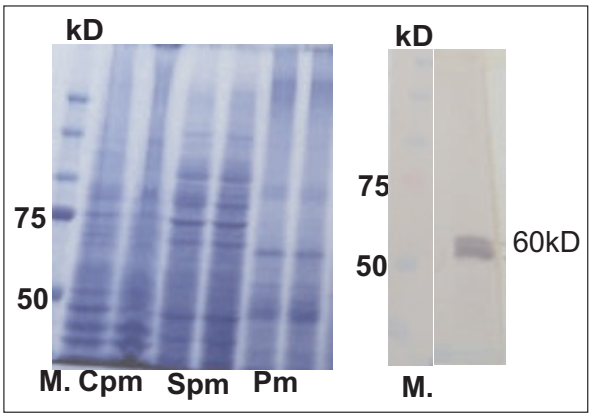

Fig. 4. Electrophoregram and blot of proteins from mutant ts111 cells and from cells transformed by microinjection of the pPXV-GFP-T111. Pm – microsomal pellet. M – molecular mass marker. The expected molecular mass of the protein labelled with GFP was about 60 kD (33kD pTS111 + 27kD pGFP).

Rescue of the mutant using microinjection of the pPVX GFP-TS111 and localization of the TS111 protein

In order to check whether the *TS111* gene would rescue the ts111 mutant, a sequence encoding a GFP tag was fused to the entire ORF of the *TS111* gene. Two constructs were prepared: the first one coded the TS111 protein fused at its N-terminal side with pGFP, and the second one coded the TS111 protein fused at its C-terminal site with pGFP. Two vectors of pPXV containing these constructs were used to transform both wild type and mutant cells using the microinjection technique. Both constructs were effective, but the GFP fluorescence signal was stronger in cells injected with the pPVX GFP-TS111. Transformed mutant cells were thermoresistant at 35°C. At this temperature, the rescued cells revealed the normally developed ICL as was shown by immunolabelling with the anti-centrin antibody (Fig. 5 A). Localization of the GFP fusion protein in the pores and radial canals of the contractile vacuole complexes, was observed both in the cells immunolabelled with the anti-GFP antibody (Fig. 5 B1, B2) as well as in living cells (Fig. 5 B3). In rescued living cells, no abnormalities were observed in the CVCs' structure or function at the restrictive temperature.

Gene silencing and functional analysis of the TS111 protein

The RNA interference adapted for *Paramecium* research as a feeding technique is an easy method of gene silencing. To investigate the role of the TS111 protein, its encoding gene was silenced in wild type cells by feeding them with transformed HT115 bacteria, producing double stranded RNA homologous to the target sequence. The *TS111* gene was silenced in both wild type cells and mutant ts111 cells. *TS111*-silenced wild type cells grew normally at 28°C and had normal rate of CV contractions. However, in some cells, an enlarged cell volume was observed without any effect on ICL or CV functioning. Most likely this effect was caused by *Klebsiella pneumoniae* replacing with *E. coli* in the feeding medium. At the restrictive temperature, *TS111*-silenced WT cells died after one or two divisions when they were isolated individually into a small volume (0.3 ml) of feeding medium i. e. containing transformed HT115 bacteria. The effect of gene silencing was weaker when 5 or 10 cells were isolated into the same volume of feeding medium. Then the cells survived but generation time was prolonged. In such cells, ICL disassembly (Fig. 6) and anomalies in the CVCs were observed. The RNAi effect could be reversed by transferring the cells into a standard medium. These cells became thermore-

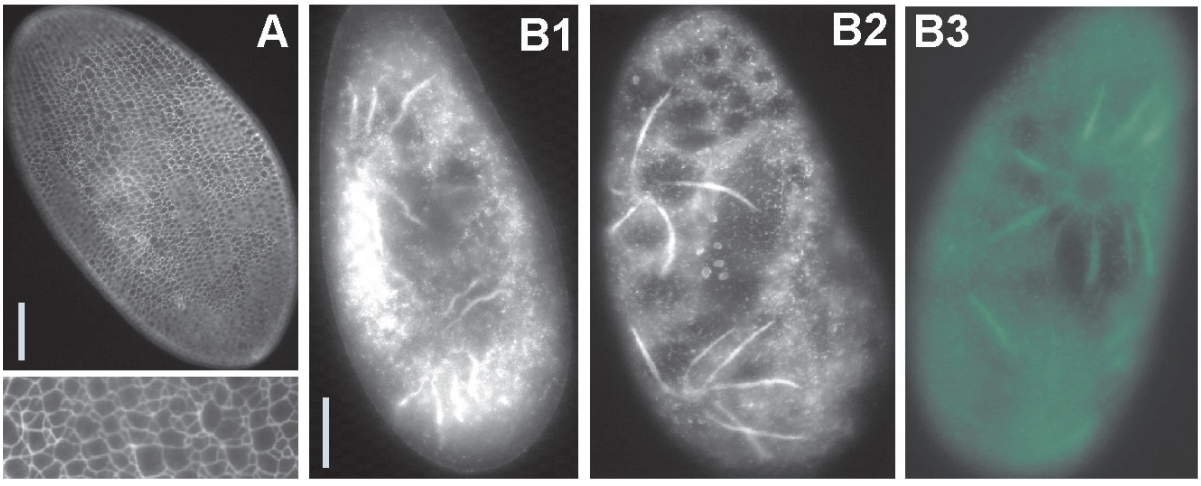

Fig. 5. Mutant ts111 cells transformed by microinjection of pPXV-GFP-TS111 observed at restrictive temperature. A – normal ICL, labeled with the IA9 antibody. Contractile vacuoles are labeled with an anti-GFP antibody: B1 – fixed with methanol, B2 – fixed with osmic acid, B3 – in living cells. Scale bar: 10 μm.

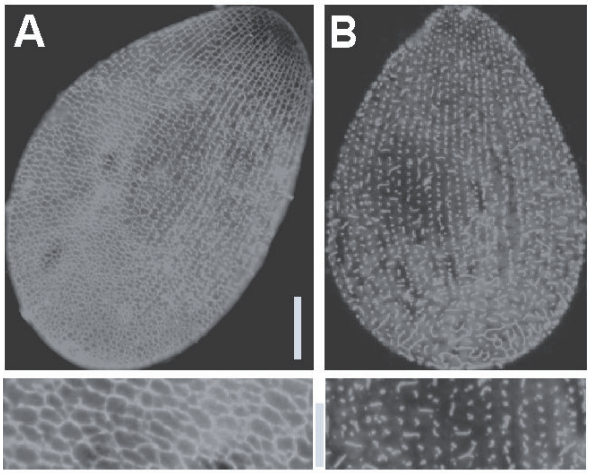

Fig. 6. Effect of *TS111* gene silencing on the ICL. A – at 28°C the ICL is normally developed. B – disassembly of the ICL at restrictive temperature. The ICL is decorated with IA9 antibody. Scale bar: 10 μm.

sistant. In mutant cells, the feeding medium induced cell mortality at the restrictive temperature after 1-2 divisions. Frequent enlarged cell volumes and prolonged generation times were observed at permissive temperature of 28ºC without any effect on the ICL or the CVCs' morphology. Silencing of the paralog GSPATG00034550001 did not affect the *Paramecium* phenotype. Wild type cells fed with transformed bacteria were thermoresistant at the restrictive temperature. Their shape and generation time were not changed. Mutant cells cultured in the same medium expressed thermosensivity at 35°C. Simultaneous silencing of the *TS111* gene and its paralog GSPATG00034550001, by double feeding applied to wild type cells resulted in enlarged cell volumes and slower generation times (1-2 divisions/24h) at the permissive temperature of 28ºC, versus 3-4 cell divisions/24h in control cells. In this experiment the control cells were the WT cells fed with bacteria transformed by pPD plasmid without any insert. This result suggests some pleiotropy and epistasis of *TS111* gene and its paralog, but again it could be due to difference in bacterial food in the medium. Such cells were isolated again into the freshly prepared double silencing feeding medium and cultured at the restrictive temperature. By the next day, nearly 20% of the cells had died, while the remaining cells mostly divided only 1 to 2 times and showed enlarged cell volumes. In some of these (nearly 20%) the beginning of ICL disassembly was observed. This result can be explained by a not fully effective *TS111* gene silencing, due to dilution of the effective *TS111* feeding medium by the bacteria producing double stranded RNA corresponding to the paralog gene sequence. Mutant cells exposed to double feeding did not show any anomalies at 28°C and were thermosensitive at 35°C.

Restrictive temperature affects contractile vacuoles function

In vivo observations of the CV pulsations (contractions) in ts111 mutant cells cultured under restrictive temperature conditions reveal an abnormal functioning of the CVCs. Wild type cells under the same conditions did not show any abnormalities in either the function or the structure of CVCs (Fig. 7). In mutant cells cultured at 35°C, the rate of contraction was twice slower than in the wild type cells cultured at the same temperature (Table 1). One of the two contractile vacuole pores (CVPs) was frequently enlarged and working very slowly, and there was a reduced number and length of

Table 1

Comparison the frequency of CV contractions at permissive and restrictive temperature in: wild type, wild type with *TS111* silenced gene, ts111 mutant, and ts111 mutant rescued cells derived from feeding and microinjection experiments

Temperature	Number of Contractile Vacuole Contractions/1 min									
	Wild type						Mutant		Mutant injected with TS111-GFP	
	Medium:						Standard medium			
	Standard[1]		Feeding[2]		Feeding Control[3]					
	Mean value n=15	±SD	Mean value n=15	±SD	Mean value n=15	±SD	Mean value n=15	±SD	Mean value n=15	±SD
28°C	11.1	1.8	9.9	1.4	9.8	1.4	11.4	1.2	11.1	1.8
35°C	10.8	1.5	4.4	0.9	8.2	1.4	5.0	0.9	11.0	1.7

[1] Standard growth medium inoculated with *Klebsiella pneumoniae*. [2] Medium inoculated with transformed HT115 *E. coli* containing pPD for silencing *TS111* gene (see text for details). [3] Medium inoculated with HT115 *E. coli* transformed with pPD without insert used as a control medium for the gene silencing by Feeding method.

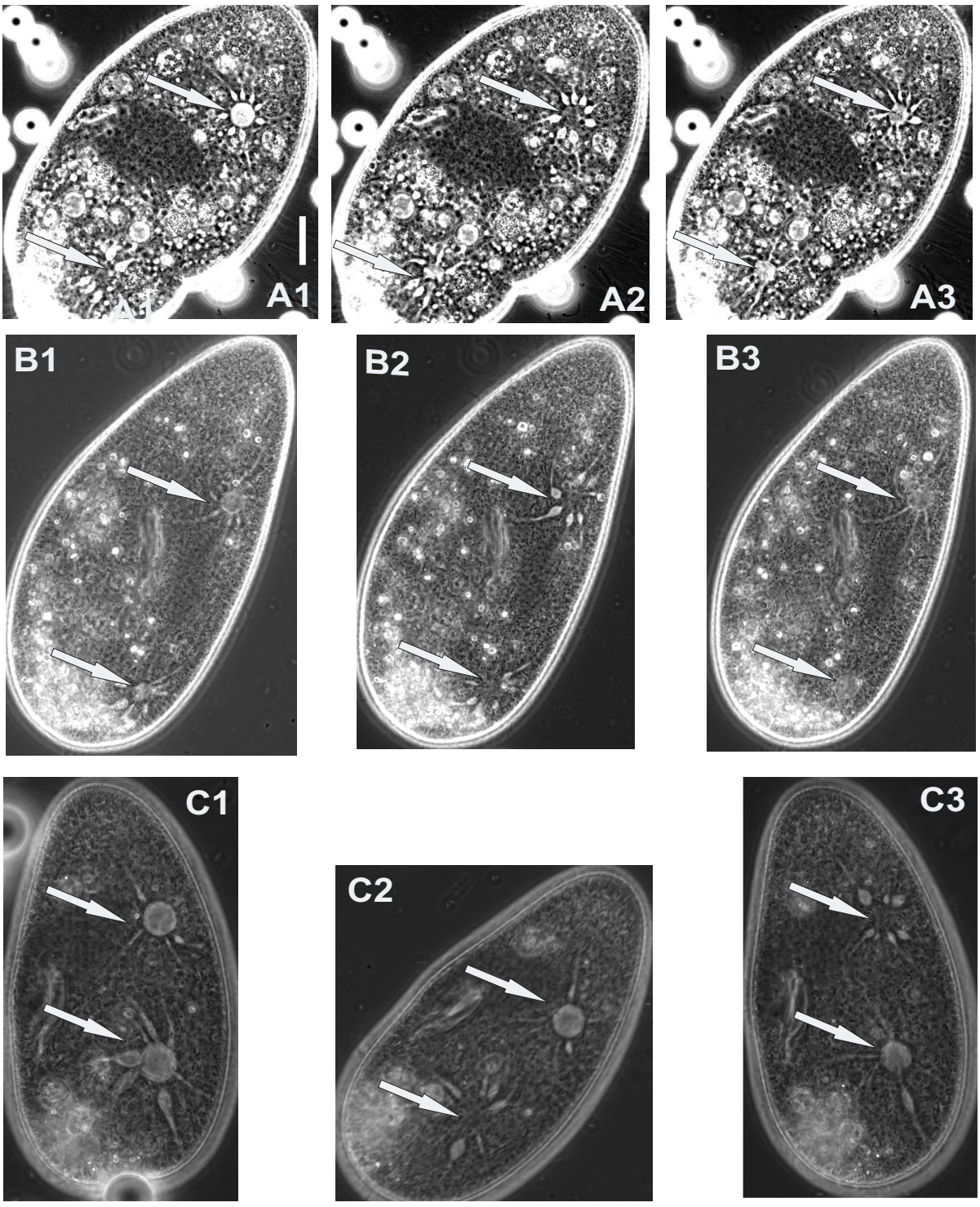

Fig. 7. CVCs in wild type cells and mutant ts111 at 28°C and 35°C – *in vivo* observations of successive stages of the cycle of CVC functioning. A1-A3: Wild type cell at 28°C. A1: Pore of the posterior vacuole with ampoules, anterior vacuole with ampoules. A2: Small posterior vacuole with radial canals and ampoules, pore of anterior vacuole with ampoules. A3: Both anterior and posterior vacuoles with radial canals. B1-B3: Wild type cells at 35°C. B1: Two vacuoles with radial canals. B2: Pores of both posterior and anterior vacuoles with radial canals and ampoules. B3: Posterior vacuole and anterior vacuole with radial canals. C1-C3: Mutant ts111 cells at 28°C. C1: Posterior vacuole with radial canals and ampoules, anterior vacuole with radial canals. C2: Pore of posterior vacuole with radial canals and one ampoule, anterior vacuole with radial canals. C3: Posterior vacuole with radial canals, anterior vacuole with ampoules. Arrows indicate CVC; scale bare: 10 μm.

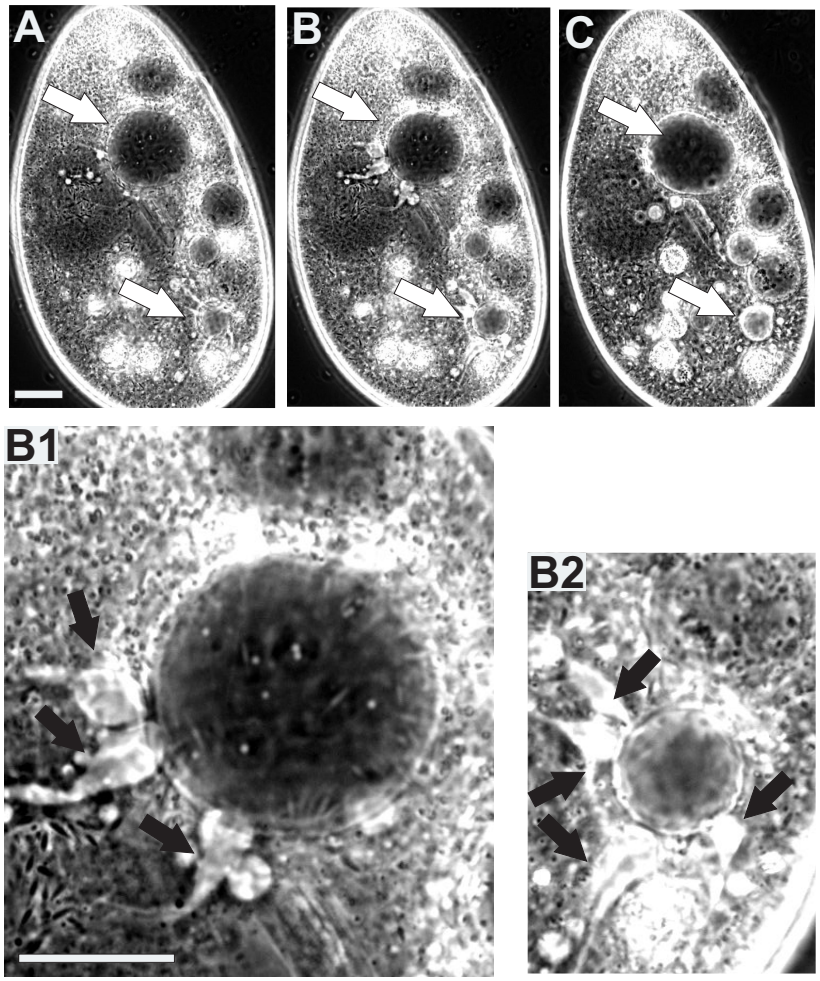

Fig. 8. Anomalies of CVC in mutant ts111 cell at 35°C – *in vivo* observations. A, B, C: successive stages of CVC functioning cycle. Anterior vacuole abnormally enlarged. Reduced number of radial canals and ampoules in both anterior (B1) and posterior (B2) vacuoles. White arrows indicate CVC; black arrows – ampoule; scale bare: 10 μm.

functional radial canals (Fig. 8). The reduced number of CVCs and an asynchrony in their contraction were also observed (Fig. 9). The restrictive temperature did not affect CVC function in wild type cells cultured in the standard culture medium. The rate of pulsation at the restrictive temperature in the wild type cells was only slightly lower than at the standard culture temperature (Table 1). Our observations revealed that in the mutant cells submitted to heat shock at 35°C, abnormal function of CVCs had already preceded disassembly of the ICL. In the mutant cell mass culture (of the volume about 20-50 ml) the disassembly of ICL was observed after ca one division following the first observations of CVC anomalies. Transformation of the mutant cells by microinjection of the pPXV-GFP with the TS111 sequence restored their normal cell growth and function of the CVCs at the restrictive temperature i.e. their normal appearance and the same rate of contractions at both temperatures.

Discussion

In this study we isolated, amplified and sequenced *TS 111* gene and predicted amino acid sequence of the TS111 protein. According to our analysis the TS111 protein belongs to a family of the Palmitoyl-Acyl Transferases (PAT proteins), because it has a characteristic DHHC domain present within a Cystein-Rich Domain, which is an integral part of the catalytic site of the PAT enzymes. It is the first PAT protein found in Ciliates.

The PAT gene family is conserved in eukaryotes. Protein palmitoylation was first discovered in yeast where 7 PAT genes are expressed (ROTH *et al.* 2002; LOBO *et al.* 2002). In *Caenorhabditis elegans* 15 PAT genes were recently identified and characterized (EDMONDS & MORGAN 2014), in *Drosophila* 22 PAT genes are present (BANNAN *et al.* 2008) and in mammals 23 PAT genes (FUKATA *et al.* 2004). In plant *Arabidopsis thaliana* 24 PAT family members were described (BATISTIĆ 2012). The BLAST search in the *Paramecium tetraurelia* ge-

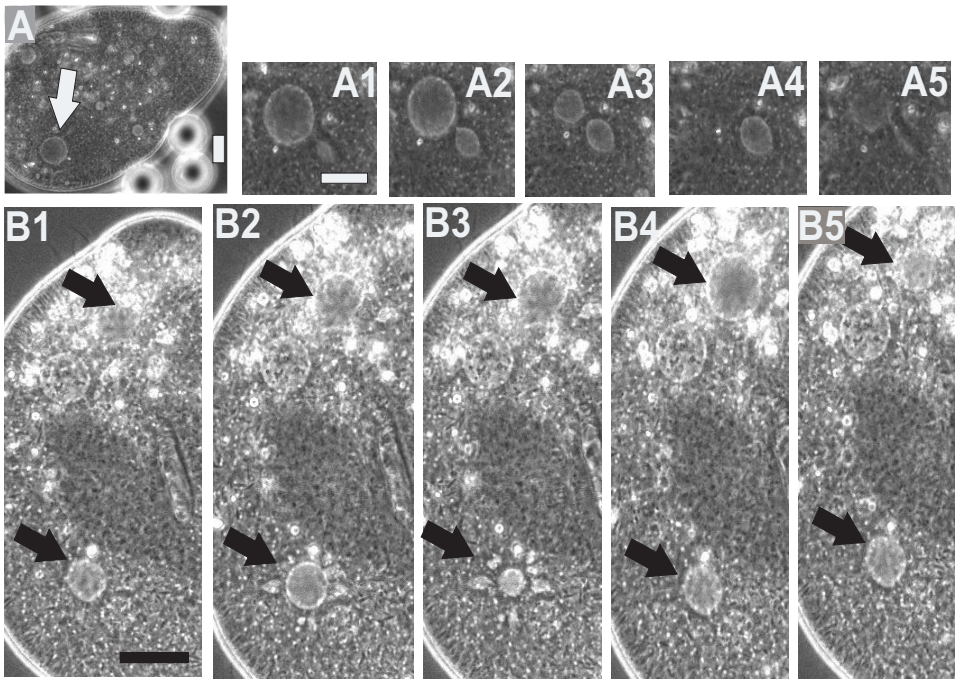

Fig. 9. Anomalies of CVC in mutant ts111 cells at 35°C – *in vivo* observations. A: Cell with only one functional posterior vacuole which has only one ampoule. A1-A5: Successive stages of this abnormal vacuole functioning. B1-B5: Successive stages of CVC functioning in the cell in which anterior vacuole works two times slower than the posterior one. Lack of ampoules and radial canals in this vacuole. Arrows indicate CVC; scale bare: 10 μm.

nome database revealed 52 genes coding distinct PAT proteins with the DHHC motif. The TS111 protein described in our study has the particularity of the presence of double C in the C-terminal side. This motif also exists in several plants and other organisms (Fig. 10; Suplementary material). The mutation present in the *ts111* gene results in the substitution of glycine with glutamic acid in posi-

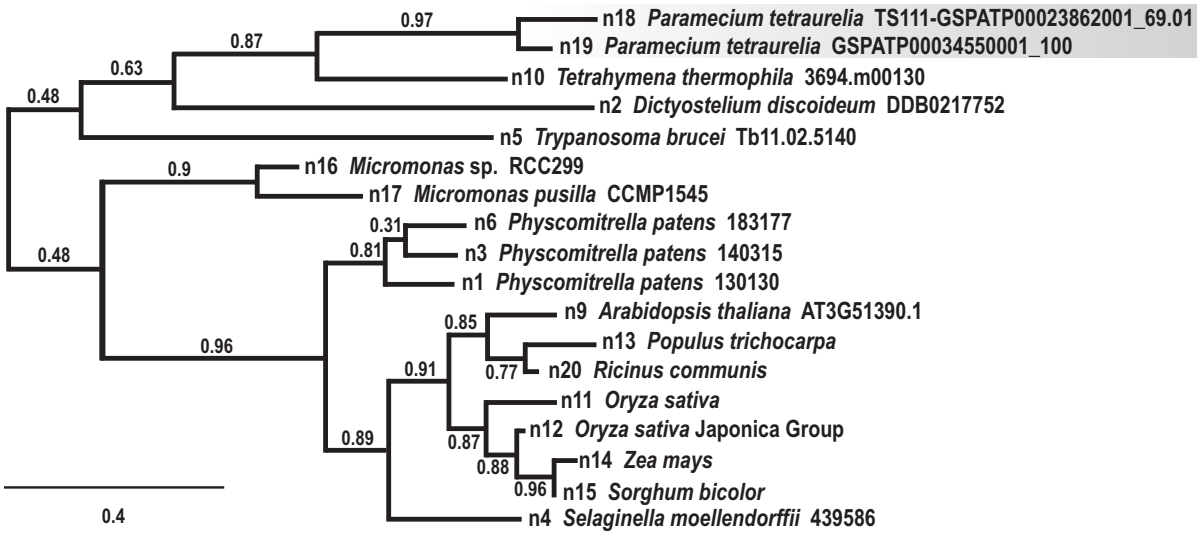

Fig 10. The phylogenetic tree of the palmitoyl transferase sequences characterized by the presence of the double C in their terminal part (see: Suplementary material) shows relationships among the plants and other organisms representing Ciliophora (*Paramecium tetraurelia* and *Tetrahymena termophila*) as well as Mycetazoa and Euglenozoa. Protein sequences were aligned using the CLUSTAL FORMAT: MUSCLE (3.7) multiple sequence alignment. Tree was constructed using the service Phylogeny.fr and the neighbor-joining algorithm with 1000 bootstrap replicates. Evolutionary distances are indicated with the scale bar.

tion 63 of the protein sequence. The alignment of sequences shows that this glicine (63) is a conserved amino acid in zDHHC proteins (see: Supplementary material). This position is close to the C-Rich-DHHC domain, which starts at the position 98 and forms the enzymatic site of PAT. In the *Paramecium* genome there are only two proteins characterized by CC-terminal part among 52 proteins containing the DHHC domain (after the removal of brothers) – this is *TS111*gene and its paralog. However, the results of the gene silencing experiments show that the function of the gene paralog is not similar to the function of the *TS111* gene. In *Tetrahymena* only one protein with a double C in the C terminal part is present among 33 proteins containing the DHHC domain.

S-palmitoylation is a reversible post-translational protein lipid modification that results in the addition of a C16-carbon saturated fatty acyl chain to the cytoplasmic cysteine residues through thioester linkage. This modification is mediated by Palmitoyl-Acyl Transferase (PAT) and reversed by Protein Palmitoyl Thioesterase (PPT). The core DHHC motif is particularly conserved, and is essential for the catalytic function. PATs can undergo auto-palmitoylation on the active site cysteine of the DHHC motifs. The autopalmitoylated enzyme is competent to transfer of palmitate to the protein substrate. Therefore, the mutation of this active site cysteine abolishes palmitoylation of both the enzyme and substrate (MITCHELL *et al.* 2010). S-palmitoylation is one the most frequent posttranslational modifications of proteins. The PATs are polytopic transmembrane proteins that are found along the endomembrane system of eukaryotic cells and mediate palmitoylation of peripheral and integral membrane proteins. Protein palmitoylation can affect the interaction of the palmitoyl protein with membrane lipids/proteins, and the reversibility of palmitoylation regulated by protein acyltransferase and protein acyl thioesterase allows protein trafficking between membrane compartments. It contributes to membrane association, protein sorting and regulates the functional activities of integral and peripheral membrane proteins (CHAROLLAIS & VAN DER GOOT 2009; IWANAGA *et al.* 2009). It is likely that the TS111 protein contributes to membranes fusions and separations during CVC contractions. It is mainly localized in the CVC as was demonstrated in GFP chimeras. This is a transmembrane protein with two predicted transmembrane helices (Fig. 11). It has also two predicted sites of palmitoylation on the cysteines 282 and 283, with a probability of 0.992 and 0.995 respectively. The CVCs in transformed cells were decorated with TS111 GFP tagged protein only after osmium or methanol fixation, but not after triton fixation method. This result was probably due to membrane degradation by triton and was consistent with hypothesis that pTS111 is a transmembrane protein, that is palmitoylated at the C-terminal cysteines. The TS111 protein is located in radial canals and in the pores of the CVC. It is likely that this protein with the PAT enzyme function is implicated in the functioning of the ATPase pump. The mutated protein ts111 is functional in *Paramecium* cells at the standard temperature of 28°C but at the restrictive temperature of 35°C, its function is impaired. Dysfunction of the ts111 protein is responsible for the CVC anomalies that were observed at the restrictive temperature, both in ts111 mutant cells as well as in wild type cells that were subjected to *TS111* gene silencing. At 35°C, such cells revealed a rate of CVC contraction that was nearly two times slower than in the standard temperature of 28°C. Nevertheless, in cells expressing a slower rate of contraction, the ICL was not initially impaired in most of the mass culture cells, but it was disassembled after some delay before the cell death. The pores of the CVs and the CVs were frequently enlarged and not functional. Hence, it is likely that the disassembly of the ICL observed at restrictive temperature is a secondary effect of abnormal CVC function. The functioning of the ICL is dependent on the concentration of Ca^{+2} ions (GOGENDEAU *et al*. 2007). In *Paramecium* cells, the CVC is responsible for osmoregulation and regulation of concentration of ions, especially Ca^{+2} (ISHIDA *et al.* 1993; STOCK *et al.* 2001; LADENBURGER *et al.* 2006; PLATTNER 2013; 2015). Therefore, we propose the hypothesis that disassembly of the ICL in the *ts111* mutant cells at the restrictive temperature is induced by failure of the Ca^{+2} regulation by impaired CVC function. This hypothesis is consistent with the following data. The transfer of water and ions in *Paramecium* cells is regulated by the vacuolar-proton-ATPase pump

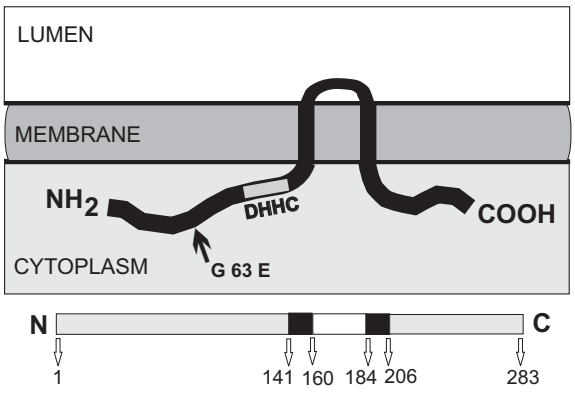

Fig. 11. Scheme of hypothetic localization of the domain DHHC in the protein ts111 with two transmembrane helices. Predicted localization of aminoacids: 1-140 cytoplasmic, 141-160 transmembrane, 161-179 non cytoplasmic, 180-206 transmembrane, 207-283 cytoplasmic. According to Phobius prediction.

(FOK *et al.* 2002). The vacuolar-proton-ATPase is a multi-subunit enzyme complex that is able to transfer protons through membranes against an electrochemical potential under ATP hydrolysis. It is also responsible for the active transport of ions. The V-ATPase is composed of two subcomplexes: the cytosolic V_1-sector, and the transmembranous V_0-sector. Genes encoding the V-ATPase subunits were identified in *Paramecium tetraurelia*. Initially, the six genes encoding c-subunits of V_0 sector and two genes encoded F-subunit of V_1-sector were investigated. Proteins of both subunits were localized, using GTP fusion, in the radial arms of contractile vacuoles as well as in the food vacuoles and in the trichocysts. Silencing of c-subunits of V_0 or the F-subunits of V_0 caused defects in the cycle of contractile vacuole complex and general prolongation of frequency of pulsation (WASSMER *et al.* 2005). This result was very similar to the effect of silencing of *TS111* gene coding palmitoylated transmembrane protein. Then, 17 genes encoding the large a-subunit of V_1-sector of the VATase was studied. Two of these genes encoded proteins located at the radial canals of contractile vacuoles (WASSMER *et al.* 2006). None of the tested genes of V-ATPase subunits encode palmitoylated proteins, as was shown by their sequence analysis. Our study on PAT gene and protein function in *Paramecium* may be relevant for future human biomedical research. Analysis of phylogenetic relationships among sequences of 45 amino acids of the CRC-site DHHC has revealed that the nearest to the TS111 protein is human protein DHH12 encoded by *ZDHH12* gene. This protein is localized to the Golgi apparatus as well as to the endoplasmic reticulum. It is composed of 265 amino acids. In the protein sequence next to the DHHC domain four transmembrane domains are present. The size of the *ZDHHC12* gene is 3.26 Kb and it is the shortest one among DHHC family of human palmitoyltransferases. This protein serves to tether amyloid precursor protein (APP) in the Golgi apparatus and suppresses the generation of APP-containing vesicles, which in turn suppresses the trafficking of APP-containing vesicles into the late secretory pathway. DHHC12 palmitoyltransferase can regulate APP trafficking and metabolism in multiple ways (KORYCKA *et al.* 2012) and it is implicated in the neuropsychiatric diseases along with several human PATs (YOUNG *et al.* 2012). Recently, it has been discovered that DHHC-12 plays an important role in the posttranslational control of synaptic plasticity. Of the 23 known human palmitoyltransferases that catalyze the palmitoylation of proteins, pDHHC12 was identified as one enzyme that specifically modifies gephrin, playing the role of the regulator of neuronal function (DEJANOVIC *et al.* 2014).

TS111 protein is the first protein belonging to the family of palmitoylated proteins, that has been identified and described in protozoan ciliate *Paramecium tetraurelia*. Based on our studies, it can be concluded that this protein is implicated in osmoregulation processes and in the regulation of intracellular ion Ca^{+2} concentration. It is located in CVC and it can affect the functioning of the ATPase proton pump. Further studies are needed in order to know the possible relationship between the V-ATPase proteins and the PAT protein coded by *TS111* gene. In conclusion of our study we propose two hypotheses: (i) CVC functioning of in *Paramecium* depends on TS111 protein and at restrictive temperature the primary effect of the thermosensitive ts111 mutation or *TS111* gene silencing are morphological abnormalities and dysfunction of CVCs, (ii) disassembly of the ICL is a secondary effect of the ts111 mutation, which results from disturbed regulation of the intracellular concentration of Ca^{+2} ions caused by abnormal functioning of CVC.

Acknowledgements

We greatly thank Janine BEISSON and Jean COHEN (CNRS, Gif sur Yvette, France) for donating a mutant ts111 of *P. tetraurelia* to our study and for encouraging us to take on the topic and stimulating discussions while the research was performed. We also thank Catherine KLOTZ for her help with cell fractioning and Western Blotting.

References

ALLEN R.D. 2000. The contractile vacuole and its membrane dynamics. BioEssay **22**: 1035-1042.

ARNAIZ O., CAIN S., COHEN J., SPERLING L. 2007. Paramecium DB: a community resource that integrates the *Paramecium tetraurelia* genome sequence with genetic data. Nucleic Acids Res. **35**: D439-444.

ARNAIZ O., SPERLING L. 2011. *Paramecium*DB in 2011: new tools and new data for functional and comparative genomics of the model ciliate *Paramecium tetraurelia.* Nucleic Acids Res. **39**: D632-6.

AURY J.-M., JAILLON O., DURET L., NOEL B., JUBIN C., PORCEL B. M., SÉGURENS B., DAUBIN V., ANTHOUARD V., AIACH N., ARNAIZ O., BILLAUT A., BEISSON J., BLANC I., BOUHOUCHE K., CŔMARA F., DUHARCOURT S., GUIGO R., GOGENDEAU D., KATINKA M., KELLER A.-M., KISSMEHL R., KLOTZ C., KOLL F., LE MOUËL., LEPÈRE G., MALINSKY S., NOWACKI M., NOWAK J. K., PLATTNER H., POULAIN J., RUIZ F., SERRANAO V., ZAGULSKI M., DESSEN P., BÉTERMIER M., WEISSENBACH J., SCARPELLI C., SCHÄCHTER V., SPERLING L., MEYER E., COHEN J., WINCKER P. 2006. Global trends of whole-genome duplications revealed by the ciliate *Paramecium tetraurelia.* Nature **444**: 171-178.

BANNAN B.A., VAN ETTEN J., KOHLER J.A., TSOI Y., HANSEN N.M., SIGMON S., FOWLER E., BUFF H., WILLIAMS T.S., AULT J.G., GLASER R.I., KOREY C.A. 2008. The Drosophila protein palitoylome: characterizing palmitoyl-

thioesterases and DHHC palmitoyl-transferases. Fly (Austin) **2**: 198-214.

BATISTIĆ O. 2012. Genomics and localization of the *Arabidopsis* DHHC-Cystein-Rich-Domain S-acyltransferase protein Family. Plant Physiol. **160**: 1597-1612.

BEISSON J., BÉTERMIER M., BRÉ M.H., COHEN J., DUHARCOURT S., DURET L., KUNG C., MALINSKY S., MEYER E., PREER J.R. Jr, SPERLING L. 2010. *Paramecium tetraurelia:* the renaissance of an early unicellular model. Cold Spring Harb. Protoc. 2010 Jan; 2010(1):pdb.emo140. doi: 10.1101/pdb.emo140.

BEISSON J., CLEROT J.C., FLEURY-AUBUSSON A., GARREAU DE LOUBRESSE N., RUIZ F., KLOTZ C. 2001. Basal body-associated nucleation center for the centrin-based cortical network in *Paramecium.* Protist **152**: 339-354.

BEISSON J., ROSSIGNOL M. 1969. The first case of linkage in *Parameciumaurelia.* Genet. Res. **13**: 85-90.

BRIGIDI G.S., BAMJI S.X. 2013. Detection of protein palmitoylation in cultured hipocampal neurons by immunoprecipitation and acyl-biotin exchange(ABE). J. Vis. Exp. Feb 18,(72). pii: 50031. doi. 10.379?500031.

CHAMBERLAIN L.H., LEMONIDIS K., SANCHEZ-PEREZ M., WERNO M.W., GERLEKU O.A., GREAVES J. 2013. Palmitoylation and the trafficking of peripheral membrane proteins. Biochem. Soc. Trans. **41**: 62-66.

CHAROLLAIS J., VAN DER GOOT F.G. 2009. Palmitoylation of membrane proteins. Mol. Membr. Biol. **26**: 55-66.

CHAVDA B., ARNOTT J.A., PLANEY S.L. 2014. Targeting protein palmitoylation: selective inhibitors and implications in disease. Expert Opin. Drug Discov. **9**: 1005-1019.

DEJANOVIC B., SEMTNER M., EBERT S., LAMKEMEYER T., NEUSER F., LÜSCHER B., MEIER J.C., SCHWARZ G. 2014. Palmitoylation of gephyrin controls receptor clustering and plasticity of GABAergic synapses. PLoS Biol. **12**: e1001908. doi: 10.1371/journal.pbio.1001908. eCollection 2014 Jul.

DEREEPER A., GUIGNON V., BLANC G., AUDIC S., BUFFET S., CHEVENET F., DUFAYARD J.F., GUINDON S., LEFORT V., LESCOT M., CLAVERIE J.M., GASCUEL O. 2008. Phylogeny.fr: robust phylogenetic analysis for the non-specialist. Nucleic Acids Res. **36**: W465-469.

DEREEPER A., AUDIC S., CLAVERIE J.M., BLANC G. 2010. BLAST-EXPLORER helps you building datasets for phylogenetic analysis. BMC Evol. Biol. **10**: 8. doi: 10.1186/1471-2148-10-8.

EDGAR R.C. 2004. Muscle: multiple sequence alignement with high accuracy and high throughput. Nucl. Acids Res. **32**: 1792-1797.

EDMONDS M.J., MORGAN A. 2014. A systematic analysiss of protein palmitoylation in *Caenorabditis elegans.* BMC Genomics **15**: 841.

FOK A., AIHARA M., ISHIDA M. NOLTA K.V., STECK T.L., ALLEN R.D. 1995. The pegs on the decorated tubules of the contractile vacuoles complex of *Paramecium multimicronucleatum.* J. Eukaryot. Microbiol. **55**: 481-491.

FOK A., YAMAUCHI K., ISHIHARA A., AIHARA M.S., ISHIDA M., ALLEN R.D. 2002. The vacuolar-ATPase of *Paramecium* multimicronucleatum: gene structure of the B subunit and the dznamics of the V-ATP-rich osmoregulatorz membranes. J. Eukaryot. Microbiol. **49**: 185-196.

FRÉNAL K., KEMP L., SOLDATI-FAVRE D. 2014. Emerging roles for protein S-palmitoylation in *Toxoplasma* biology. Intern. J. Parasit. **44**: 121-131.

FROISSARD M., KELLEr A.-M., COHEN J. 2001. ND9, a novel protein with *Armadillo*-like repeats involved in exocytosis: physiological studies using allelic mutants in *Paramecium*. Genetics **157**: 611-620.

FUKATA M., FUKATA Y., ADESNIK H., NICOLI R.A., BREDT D.S. 2004. Identification of PSD-95 palmitoylated enzymes. Neuron **44**: 987-96.

GALVANI A., SPERLING L. 2002. RNA interference by feeding in *Paramecium.* Trends Genet. **18**: 11-12.

GARREAU DE LOUBRESSE N., KERYER G., VIGUES B., BEISSON J. 1988. A contractile cytoskeletal network of *Paramecium*: the infraciliary lattice. J. Cell Sci. **90**: 351-364.

GOGENDEAU D., BEISSON J., GARREAU DE LOUBRESSE N., LE CAER J.P., RUIZ F., COHEN J., SPERLING L., KOLL F., KLOTZ C. 2007. An Sfi1p-like centrin-binding protein mediates centrin-based Ca^{2+} -dependent contractility in *Paramecium tetraurelia*. Eukaryot. Cell. **6**: 1992-2000.

GOGENDEAU D., KLOTZ C., ARNAIZ O., MALINOWSKA A., DADLEZ M., GAREAU DE LOUBRESSE N., RUIZ F., KOLL F., BEISSON J. 2008. Functional diversification of centrins and cell morphological complexity. J. Cell Sci. **121**: 65-74.

HAUSER K., HAYNES W. J., KUNG C., PLATTNER H., KISSMEHL R. 2000. Expression of the green fluorescent protein in *Paramecium tetraurelia*. Eur. J. Cell Biol. **79**: 144-149.

HAYNES W.J., LING K.Y., SAIMI Y., KUNG C. 1995. Induction of antibiotic resistence in *Paramecium tetraurelia* by the bacterial gene APH-3'-II. J. Euk. Microbiol. **42**: 83-91.

HORNEMANN T. 2015. Palmitoylation and depalmitoylation defects. J. Inherit. Metab. Dis. **38**: 179-86.

ISHIDA M., AIHARA M.A., ALLEN R.D., FOK A.K. 1993. Osmoregulation in *Paramecium:* the locus of fluid segregation in the contractile vacuole complex. J. Cell Sci. **106**: 693-702.

IWANAGA T., TSUTSUMI R., NORITAKE J., FUKATA Y., FUKATA M. 2009. Dynamic protein palmitoylation in cellular signaling. Prog. Lipid Res. **48**: 117-127.

KÄLL L., KROGH A., SONNHAMMER E. 2007. Advantages of combined transmembrane topology and signal peptide prediction – the Phobius web serwer. Nucleic Acids Res. **35**: W429-432.

KELLER A. M., COHEN J. 2000. An indexed genomic library for *Paramecium* complementation cloning. J. Eukaryot. Microbiol. **47**:1-6.

KLOTZ C., GARREAU DE LOUBRESSE N., RUIZ F., BEISSON J. 1997. Genetic evidence for a role of centrin-associated proteins in the organization and dynamics of the infraciliary lattice in *Paramecium.* Cell Motil. Cytoskeleton **38**: 172-186.

KORYCKA J. ŁACH A., HEGER E., BOGUSŁAWSKA D.M., WOLNY M., TOPORKIEWICZ M., AUGOFF K., KORZENIEWSKI J., SIKORSKI A.L. 2012. Human DHHC proteins: A spotlight on the hidden player of palmitoylation. Europ. J. Cell Biol. **91**: 107-117.

LADENBURGER E.M., KORN I., KASIELKE N., WASSMER T., PLATTNER H. 2006. An Ins(1,4,5)P_3 receptor in *Paramecium* is associated with the osmoregulatory system. J. Cell Sci. **119**: 3705-3717.

LOBO S., GREENTREE W.K., LINDER M.E., DESCHENES R.J. 2002. Identification of a Ras palmitoyltransferase in *Saccharomyces cerevisiae*. J. Biol. Chem. **277**: 41268-73.

LIN D.T., CONIBEAR E. 2015. Enzymatic protein depalmitoylation by acyl protein thioesterases. Biochem. Soc. Trans. **43**: 193-198.

MADEDDU L., KLOTZ C., LE CAER J.P., BEISSON J. 1996. Characterization of centrin genes in *Paramecium.* Eur. J. Biochem. **238**: 121-8.

MARCK C. 1988. 'DNA Strider': a 'C' program for the fast analysis of DNA and protein sequences on the Apple Macintosh family of computers. Nucleic Acids Res. **16**: 1829-1836.

MITCHELL D.A., MITCHELL G., LING Y., BUDDE C., DESCHENES R. 2010. Mutational analysis of *Saccharomyces cerevisiae* Erf2 reveals a two-step reaction mechanism

for protein palmitoylation by DHHC enzymes. J. Biol. Chem. **285**: 38104-38114.

OESTE C.L., PINAR M., SCHINK K.O., MARTINEZ-TURRION J., STENMARCK H., PENALVA M.A., PEREZ-SALA D. 2014. An isoprenylation and palmitoylation motif promotes intraluminal vesicle delivery of proteins in cells from distant species. PLOS One **9**(9): e107190. doi: 10.1371.

PLATTNER H. 2013. Contractile vacuole complex – its expanding protein inventory. Int. Rev. Cell Mol. Biol. 3**06**: 371-416.

PLATTNER H. 2015. molecular aspects of calcium signaling at the crossroads of unikont and bikont eukaryote evolution – The ciliated protozoan *Paramecium* in focus. Cell Calcium **57**: 174-185.

REN J., WEN l., GAO X, JIN C., XUE Y., YAO X. 2008. CSS-Palm 2.0: an updated software for palmitoylation sites prediction. Protein Eng. Des. Sel. **21**: 639-644.

ROLDAN N., GOORMAGHTIGH E., PÉREZ-GIL J., GARCIA-ALVAREZ B. 2015. Palmitoylation as a key fator to modulate SP-C-lipid interactions in lung surfactant membrane multilayers. Biochim. Biophys. Acta 1848: 184-191.

ROTH A.F., FENG Y., CHEN L., DAVIS N.G. 2002. The yeast DHHC cysteine-rich domain protein Akr1p is a palmitoyl transferase. J. Cell Biol. **159**: 23-8.

RUIZ F., VAYSSIE L., KLOTZ C., SPERLING L., MADEDDU L. 1998. Homology–dependent gene silencing in *Paramecium*. Mol. Biol. Cell **9**: 931-941.

SAMBROOK J., FRITSCH E.F., MANIATIS T. 1989. Molecular Cloning: a Laboratory Manual. Cold Spring Harbor, New York.

SCHLIWA M., VAN BLERCOM J. 1981. Structural interaction of cytoskeletal components. J. Cell Biol. **90**: 222-235.

SKOURI F., COHEN J. 1997. Genetic approach to regulated exocytosis using functional complementation in *Paramecium*: identification of the *ND7* gene required for membrane fusion. Mol. Biol. Cell **8**: 1063-1071.

SONNEBORN T.M. 1970. Methods in *Paramecium* research. (In: Methods in Cell Physiology, vol. 4. D. M. Prescott ed. Academic Press, New York, London): 241-339.

SONNEBORN T.M. 1974. *Paramecium aurelia*. (In: Handbook of Genetics, vol. 2. R.C. King ed. Plenum New York): 469-594.

STOCK C., ALLEN R.D., NAITOH Y. 2001. How external osmolarity affects the activity of the contractile vacuole complex, the cytosolic osmolarity and the water permeability of the plasma membrane in *Paramecium multimicronucleatum.* J. Exp. Biol. **204**: 291-304.

THOMPSON J.D., HIGGINS D.G., GIBSON T.J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucl. Acids Res. **22**: 4673-80.

TIMMONS L., FIRE A. 1998. Specific interference by ingested dsRNA. Nature **395**: 854.

WASSMER T., FROISSARD M., PLATTNER H., KISSMEHL R., COHEN J. 2005. The vacuolar proton-ATPase plays a major role in several membrane-bounded organelles in *Paramecium.* J. Cell Sci. **118**: 2813-2824.

WASSMER T., KISSMEHL T., COHEN J., PLATTNER H. 2006. Seventeen a-subunit isoforms of *Paramecium* V-ATPase provide high specialization in localization and function. Mol. Biol. Cell **17**: 917-930.

YOUNG F.B., BUTLAND S.L., SANDERS S.S., SUTTON L.M., HAYDEN M.R. 2012. Putting proteins in their place: palmitoylation in Huntington disease and other neuropsychiatric diseases. Prog. Neurobiol. **97**: 220-238.

XU Z., ZHONG L. 2015. New insights into the posttranslational regulation of human cytosolic thioredoxin by S-palmitoylation. Biochem. Biophys. Res. Commun. 4: 949-956.

ZHANG J., MADDEN T.L. 1997. PowerBLAST: a new network BLAST application for interactive or automated sequence analysis and annotation. Genome Res. **7**: 649-656.

ZHANG Y., HE C.Y. 2012. Centrins in unicellular organisms: functional diversity and specialization. Protoplasma **249**: 459-467.

Supplementary Material

The alignment of sequence of palmitoylated proteins, of several plants and other organisms, characterized by the presence a double C in C terminal part. It shows that G63 in TS111 protein, that is changed in ts111 protein to A, is a conserved amino acid in zDHHC proteins. This position is close to the C-Rich-DHHC domain which starts at position 98 and forms the enzymatic site of PAT.

[Ricinus communis]]gi|255564059|ref|XP_002523028.1|
[Arabidopsis thaliana]-AT3G51390.1
[Ttermophila3694].m00130
[Oryza sativa (japonica cultivar-group)]gi|38345855|emb|CAE01851.2| OSJNBa0084K11.19
[Populus trichocarpa]gi|224133934|ref|XP_002327715.1|
[Zea mays]gi|212723844|ref|NP_001131661.1|
[Sorghum bicolor]gi|242066424|ref|XP_002454501.1|
[Micromonas sp. RCC299]gi|255073083|ref|XP_002500216.1|
[Dictyostelium discoideum] AX4]gi|66819453|ref|XP_643386.1|
[Physcomitrella patens-jgi|Phypa1_1|130130|e_gw1.80.60.1

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CLUSTAL FORMAT: MUSCLE (3.7) multiple sequence alignment

n9_Athalia      ------------------------------------------------------------
n13_Populu      ------------------------------------------------------------
n20_Ricinu      ------------------------------------------------------------
n11_Oryza       ------------------------------------------------------------
n12_Oryza       ------------------------------------------------------------
n14_Zea_ma      ------------------------------------------------------------
n15_Sorghu      ------------------------------------------------------------
n4_Smoelle      ------------------------------------------------------------
n6_Ppatens      ------------------------------------------------------------
n1_Ppatens      ------------------------------------------------------------
n3_Ppatens      ------------------------------------------------------------
n5_Tbrucei      ------------------------------------------------------------
n16_Microm      ------------------------------------------------------------
n17_Microm      ------------------------------------------------------------
n2_Ddiscoi      ------------------------------------------------------------
n10Ttermop      MIFMIQEFKIRRVVVFKEETRQTIVLKEAKVSTTRGKQIIESEDEEDIQSFPQQALLQHQ
n18Ptetrau      ------------------------------------------------------------
n19Ptetrau      ------------------------------------------------------------

n9_Athalia      -----------MGVCCPFLQPW-------DRARDQCLLNLPCLSDP-VRRSSLLLKLALV
n13_Populu      ---------------MIFLPSMSIF--------NFCHRFVPCLADP-ATRSSLGLKAALV
n20_Ricinu      -----------------MPMTSVFRESCHGALHGCYRLFPCLADP-ARRSALGLKVALV
n11_Oryza       -------------------------MPRCGAGGPCVWIRALSQPQRHGRKPWRGVRVVVL
n12_Oryza       ------------------------------MASTSAAEPGVRFSDRAARRSSLGLRSMVL
n14_Zea_ma      ------------------------------MASSSAVEPSFRLSDR-ERRSSLGLRFTVL
n15_Sorghu      ------------------------------MASSSAAEPSSRLSDR-AKRSSLGLRFMVL
n4_Smoelle      -------------------------------------MRCFTSDACGSCSTGFGGKICWF
n6_Ppatens      ---------------------------------------------MRSNVWQIGWRVKVMWV
n1_Ppatens      ---------------------------------------------MRPNVWHIGWGVKLMWV
n3_Ppatens      ---------------------------------------------MGPNAWHTGWGVRVMWV
n5_Tbrucei      ---------------MPVLPPW-------RQRTSIFHRSEGRVQSVDATRISLVAAAGLV
n16_Microm      ---------------MPPPRKFRPREPAVLVSQTAGCFWWREEVRRKHGSTPWIPKIFWV
n17_Microm      ------------------------------------------------------------
n2_Ddiscoi      ----------MKLNSNINNNINNSSNSNNNFDAKNIIVDTITPPDPSVEFERKLAKSIFC
n10Ttermop      YHSQIMANINQPINNQDSQINQQVGSSINNQANNILARPSLHQMKQSVKDALCGSRVFYV

n18Ptetrau      ------------------------------------------MAVPSQVYNLGNACKRFLF
n19Ptetrau      ------------------------------------------MVVPNEVKDIGIACKRFLF

n9_Athalia      ALHLVFIGFLFLFDAEFIEKTKRDPW--YMGCYILLFSATLLQYF--VTSGSSPGYVV--
n13_Populu      VLHIVYAGILFLFDSDLIEKTKQEPWS-YTGLYLLLFVATLIQYF--VASYSSPGYVL--
n20_Ricinu      MLHLIYVGILFIFDDDLIEKARQEPW--YIVLYLLLFVATLVQYF--ATACSSPGYVL--
n11_Oryza       LLHALFIGAVFLLDPTLQRQIHEAKW--YIILYGLLVLLTLVQYL--YTATSSPGYLP--
n12_Oryza       LMHVVFVGAVFLLDPTLDRRIREEPW--YIGAYGALVLIALVQYF--YTAGSSPGYVL--
n14_Zea_ma      LMHVMFVGAVFILDPTLDWRIHEEPW--YIGLYGVLVLLTLVQYF--YTAGSSPGYVI--
n15_Sorghu      LMHLLFVGAVFILDPTLDWRIHEEPW--YIGLYGVLVLLTLVQYF--YTAGSSPGYVV--
n4_Smoelle      LAHAVGVGLLILLYEELQHQIFSYTW--YALLYLVLIVVTVIQYY--RVAGSSPGYVE--
n6_Ppatens      LVHTVGVGGLLFLDSNLYRNTVAYSW--WAGSYYVLLLVVVIQYC--CTAGSSPGYLV--
n1_Ppatens      LVHAVGVSGLFFLDSDLYRYTTTYSW--WAGSYFTLLLIVVIQYC--CTAGSTPGYLA--
n3_Ppatens      LVHAVGVGGLLFLDSDLRTLSLAFSW--WAGSYYLLFLAVVIQYC--RTAGSTPGYLV--
n5_Tbrucei      ASQSATFTVLYLFLPEG-----------LAGLTHLMVAAVLFNAFMLWNWGSDPGFVT--
n16_Microm      FFHVLFIVLLFQFDNDMTDGGAG-----YQTGFAFACLLNLYAFL--CVANSNPGYVS--
n17_Microm      ---------------MTDGGDA-----YKTGVAFSLIVNAAFYL--ALANSNPGYVE--
n2_Ddiscoi      LVHFIVYCVIIFRKGTILDQAFKDKDYFYLIWTHCVFFFAIGTYF--LISSKRPGFVS--
n10Ttermop      SMTVLSLCMQLIYGNDLRDEVLEGE---LDPFIYLFLVLLSAYYF--WTCGKNPGYAPFE
n18Ptetrau      SAEVIGMLTLTIFENELTI---------PIGIRLLIETIAAGYFY--LQCSDSAGEPV--
n19Ptetrau      TTEFLGFLILTLFQNEMVL---------PIGIRLLIEAILAAYFY--INCSDSPGSPL--
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```
n9_Athalia      ----------------------------------DAMRDV------------------
n13_Populu      ----------------------------------DAMREV------------------
n20_Ricinu      ----------------------------------DAMRGL------------------
n11_Oryza       ----------------------------------DMLTAG------------------
n12_Oryza       ----------------------------------DAMKAG------------------
n14_Zea_ma      ----------------------------------DVMRAG------------------
n15_Sorghu      ----------------------------------DVMRAG------------------
n4_Smoelle      ----------------------------------DLEND-------------------
n6_Ppatens      ----------------------------------DMLSED------------------
n1_Ppatens      ----------------------------------DVLSED------------------
n3_Ppatens      ----------------------------------DALSED------------------
n5_Tbrucei      ------------------------------------------------------------
n16_Microm      ----------------------------------EQERCP------------------
n17_Microm      ----------------------------------EDEKAQ------------------
n2_Ddiscoi      ----------------------------------LSNQNLNNNNNNNGSSNKFILEDSM
n10Ttermop      ADDIELQNTQLREQSSSDNQNYQEIQNSQDRHDNYDLQSNKSQRSSNNQNLIPQNSLALA
n18Ptetrau      ----------------------------------VEQEDH------------------
n19Ptetrau      ----------------------------------SGDDDQ------------------

n9_Athalia      ----------CEASAMYRNPSTT--SIQHASRKS-------------------------
n13_Populu      ----------SEKNSLFRKASML--SKQPASSKN-------------------------
n20_Ricinu      ----------NDKNAAFAKASIP--SKQPASSKN-------------------------
n11_Oryza       ----------SRMHATFINTTTL--SKQANSKS-------------------------
n12_Oryza       ----------STMHATYINTATL--SKQSSPNN-------------------------
n14_Zea_ma      ----------SMMHATFVNTAAL--SKQSNSRN-------------------------
n15_Sorghu      ----------SMMHATFVNTTAL--SKQSNSRN-------------------------
n4_Smoelle      ----------PEF-----------------------------------------------
n6_Ppatens      ----------AEYEIRAKAAVHG--NSRASQSVV-------------------------
n1_Ppatens      ----------AGFEARAKQALNGGISRTSSQAAA-------------------------
n3_Ppatens      ----------ANCESFAKSAAAG--SCRSSQSAA-------------------------
n5_Tbrucei      ------------------------------------------------------------
n16_Microm      ----------EDQEDVYREQDRIRRERIAARVKAKRERERH------------------
n17_Microm      ----------AAEFLSVQRNVASLNAPGGDVESGAGGG----------------------
n2_Ddiscoi      GCIPQLNINPTPNYSKISNIKRKLKNSSGDITKNQEN----------------------
n10Ttermop      ASIEQQNSDGRKSSSKQFQQDDYDEIDEKQNKQDQNFNRVCTKIGKRHSDIVYSSNQYSQ
n18Ptetrau      ------------------------------------------------------------
n19Ptetrau      ------------------------------------------------------------

n9_Athalia      --------ESVVVNVEGGSA----------------------SCPRR---------PPTP
n13_Populu      --------GSLVITVEGSQSE--------------------RNIPES---------NVTS
n20_Ricinu      --------GSLIITVDESRSG--------------------KTSSGS---------NITS
n11_Oryza       --------GSLNSAMSRSKID--------------------QQNPQS---------TTAL
n12_Oryza       --------GSLNSPLSLPQQQ--------------------KLNPTT---------STST
n14_Zea_ma      --------GNTNSPTSHAQLQ--------------------KLSAMT----------PTS
n15_Sorghu      --------GNTSSPTSRAQLQ--------------------KLSTMT---------PTSS
n4_Smoelle      --------EAGIKAVAGST-----------------------------------------
n6_Ppatens      --------EKGC-SSYGSFNS--------------------SESTRT---------ASRI
n1_Ppatens      --------EAGR-SIYGSFSE--------------------SSGGSS---------RASR
n3_Ppatens      --------ESGRKNVKGGYAP--------------------VSEASPLLMNSINVKRESD
n5_Tbrucei      --------SSEEPSTARERAI---------------------------------------
n16_Microm      --------AAEAEDVAEDDEGGDDAELPDLESGGGDRTTDRTTSPRGEDEGTGGLLGAPG
n17_Microm      --------GGGTTRPAGGRAS----------------------SSRDLLGPSYIDSSNAE
n2_Ddiscoi      --------EDLVPLMEISKNIDEDSINDDTITTTTTTTTTTTSTSTIPEISNDDDDNNNEN
n10Ttermop      KNHINNSDNQNYHPIVNQEDDSIQHNTKNSSGPRNHSASELSQEYRNELQQETNSFNTSG
n18Ptetrau      --------ELSQLSFSGLRGD---------------------------------------
n19Ptetrau      --------ELSQFSISTSQRD---------------------------------------
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n9_Athalia      WGKLVLDLY--PPGTSI---------RNLTCGYCHVEQPPRTKHCHDCDRCVLQFDHHCV
n13_Populu      WTKLVLDMY--PPGTSV---------RTLSCTFCNVEQPPRAKHCHDCDRCVLQFDHHCV
n20_Ricinu      WTKIVLDMY--PPGTSV---------RTLTCSYCNVEQPPRAKHCHDCDKCVLQFDHHCV
n11_Oryza       LLQQTMDLY--PPGTST---------RDFTCSYCRLIQPPRTKHCHDCDKCVLQFDHHCV
n12_Oryza       WLQRVAELY--PPGSSS---------RDWTCTYCRVIQPPRTRHCHDCDKCVLQFDHHCV
n14_Zea_ma      WAQMVVDLY--PPGSNS---------RDWTCTHCRVVQPPRTRHCHDCDKCVLQFDHHCI
n15_Sorghu      WAQMVMDLY--PPGSSS---------RDWTCTYCRVVQPPRTRHCHDCDKCVLQFDHHCI
n4_Smoelle      --------------------------PFSHCSTCRVVQPPRTKHCHDCNKCVLRFDHHCV
n6_Ppatens      LTENGKAPN--SLSLVC---------NADRCLYCQHWQPLRVKHCHDCDKCVLRFDHHCV
n1_Ppatens      ENAKVRASN--VSSLSS---------HTGRCPYCGLWQPLRTKHCHDCDKCVLRFDHHCV
n3_Ppatens      GCHSTRGSA--HSLLSI---------STGFCAYCGYLQPLRTKHCHDCDKCVLRFDHHCV
n5_Tbrucei      --------------------------RRW-CSVCRLLQPLRTKHCDKCGRCVRKYDHHCY
n16_Microm      GASNSSDL---ATSSSFVGADNDEPPVGQYCKHCKAWQGLRTKHCHDCGRCVRRFDHHCF
n17_Microm      ILTREIPTYGEDDGGDDDGDDDTPMPVGQNCKHCDAWQGLRTKHCHDCGRCVRKFDHHCF
n2_Ddiscoi      NNDNVNNRNNNNSNGEKEDNDIDKLKNHYFCKKCLVDIPLRTKHCVKCNRCVLKYDHHCV
n10Ttermop      SNSQRMSSN--TNEESENNEQLDAAAQLFYCHICKRHQPFRSKHCDDCGRCICKFDHHCF
n18Ptetrau      --YEQFESQ--TPDRNE------------CQECKIIQSYRTKHCSKCQKCIPKYDHHCF
n19Ptetrau      --NSQQESQ--IPERNV------------CQECKIIQPFRTKHCQKCKKCIPKYDHHCF

n9_Athalia      WLGTCIGQKNHSKFWWYICEETTLCIWTLIMYVDYL--------SNVAKPWWKNAIIILL
n13_Populu      WLGACIGWGNHCRFWWYIFEETALCIWTGILYITSL-------KANISRAWWKDVIMILL
n20_Ricinu      WLGTCIGQGNHCRFWWYICEETTLCLWTGILYIAYL-------KANITRAWWKDAIMIIL
n11_Oryza       WLGTCIAKRNYCRFWWYIFEQTVLTVWTVAFYIQFF-------YLGIVVSWWKFAIGIVL
n12_Oryza       WLGTCIGKKNHCRFWWYIFEETILSIWTVALYIDSI-------RLDVDKAWWKDFIGVIL
n14_Zea_ma      WLGTCIGKKNHCRFWWYISEETILCIWTAVLYIESL-------RLDVDKAWWKDFVGVIL
n15_Sorghu      WLGTCIGKKNHCRFWWYIFEETILCIWTAALYIESL-------RLDMDKAWWKDFVGVIL
n4_Smoelle      WLDTCIGQYNHRRFWWYVFLETFLCIWSTVLYFLAF-------HLQKSSA-WPQNLLLLV
n6_Ppatens      SLGTCVGQRNHRKFWWYIFYETVLVMWTIVRYISAF-------GRNTGSSSLLEKIAVLV
n1_Ppatens      WLGTCVGQKNHRKFWWYIFYEAALVMWSIVWYIRAF-------RRSIGHTWWVEESIVML
n3_Ppatens      WLGTCVGQRNHRKFWWYIFCETALVMWTLVSYIRAF-------GSSIGSTTLLEGLAVLL
n5_Tbrucei      CIGGCVGEFNHVRFVLTLASAVPYFVLLPPALLKCFSLG---DIIDLDRV-ISRNIVPFI
n16_Microm      WVGTCVGEKNHARFVWYLVAQTALIVWAFHVSNSGW------KYADTFHELFEINAGPVC
n17_Microm      WVGSCVGEKNHARFTSYLATETACVIWALHISGTGL------RYHDTFGELFAKNAGPLF
n2_Ddiscoi      FIGGCVGLNNHKNFLLFLLAESLLLLLGLRIIVTGF------VRENSIKEWIFSNIAIIP
n10Ttermop      WIGGCVGELNHRKFWFFLLLQSIVIFWTFMNSLNALDRY-ISVNNQGEESYSQEYGAFAV
n18Ptetrau      WIGGCVGELNHRMYWLFLFFQCLLCFDGMFQFKKQFPYYSTYDEEFGHDEYQYQYFIILL
n19Ptetrau      WVGGCIGELNHRTFWLFLFFQCLLCFDGLFQFNKQLGLYSIYEEEQSDNKYKYQYFMILL

n9_Athalia      LVILAIS-LIFVLLLLIFHSYLILTNQSTYELVR-RRRIPYMRNIPGR-VHPFSRGIRRN
n13_Populu      LVTLSFA-VIFLLLLLIFHSYLILTNQTTYELIR-RRRIPYLRGIPER-VYPFSEGVCRN
n20_Ricinu      LVTLSIA-SIFLLLLLLFHSYLILTNQTTYELVR-RRRIPYLRGIPER-VYPFSKGACRN
n11_Oryza       LVALILI-LVVLLPLLIFHAYLALTNQTTYEIAR-RKRISYLREVPSR-VHPFSKGICRN
n12_Oryza       LAVLIFI-LIFLLLLLIFHTYIALTNQTTYEVAR-RKRIFYMRGIPDK-VHPFSKGICRN
n14_Zea_ma      LAVLIFI-LIFLLLLWLFHSYIAVTNQTTYEVAR-RKRIFYLRGVPER-VHPFSRGICRN
n15_Sorghu      LAVLIFI-LIFLLLLWLFHSYIALTNQTTYEVAR-RKRIFYLRGVPER-VHPFSRGICRN
n4_Smoelle      TFVGLLCCSIFLTTLLVFHSYLVLTNQTTYEKTR-RTRIPYLRNLPKD-AHPFSKGGCGN
n6_Ppatens      LIIGLISTECFLVTLLLFHSFLILTNQTTYETTR-RHRIPYLRTLPEN-VHPFSKGMDAN
n1_Ppatens      VILGLIITECFLITLFLFHSYLIITNQTTYELTR-RRRIPYLRMLPEK-VHPFNRGMDVN
n3_Ppatens      LILGLITAECFLMSLLVFHSFLVLTNQTTYELTR-RRRIPYLRTLPEK-VHPFSKGMDAN
n5_Tbrucei      FVAYTTIQLVLVLSLLGLHCTLLLNNKTTWELSS-RGRITYLD--SRA-ANPFNKGIVQN
n16_Microm      MSIALFIFALFVGSLLGFHVYLIVTNQTTWEVSS-RDKISYLAGVPHN-VYAFSRGPMRD
n17_Microm      LCVFLFFFALFVGSLLGFHAYLIVTGQSTWEVSSGRDKVSYLRGVPKN-VYPFSLGPVKN
n2_Ddiscoi      PTLLIFGGLCMPFALFCFHSFLILTNQSSWEFNK-YQRITYLKPFSKRGINPFNKGPWNN
n10Ttermop      LSFVLFILFLFTGVLCAYHTFLILTNQTTWEHVK-KNKISYIAKMPKG-FYPFNQGVFQN
n18Ptetrau      TAATSFGFGIFTGALLLYHTMLILTGKTTWEHTK-RDKISYLNFYPRY-YHPYNFGFIEN
n19Ptetrau      FGTISFGFGIFTGALFLYHTMLIITGQTTWEHTK-RDKITYLKFYPKF-YHPYNYGLIKN
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n9_Athalia      LYNVCCGN---YNLDSLPTAFELEDRSRPY----TCIDMLKCRCC------
n13_Populu      LYKFCCARSSIYSLERLPTAMELEDKSRPY----TCLGFLTCRCC------
n20_Ricinu      LYEFCCVRSSLYNLERLPTPIELEEKSRPY----TCLDFLTCRCC------
n11_Oryza       LYDLCISKQRGFFLEAVPPLEVLQARARPY----TCRDVISCRCC------
n12_Oryza       IYVFCFSRQKGYDLEEVPPLEELEARAAPY----TCRDIICCRCC------
n14_Zea_ma      LYDFCCSSQKGYILEAVPPREELEARAARY----TCRDVICCRCC------
n15_Sorghu      LYDFCCSSQKGYILEAVPPSEELEARAARY----TCRDVICCRCC------
n4_Smoelle      VTEFCCASQP-YRFYNLPPDHEAAGPCLAARNATESIT-KSTKCCSWWR--
n6_Ppatens      LSSFCCSPSSEYPIYVLPSREELQNMVHPL----CCFQL------------
n1_Ppatens      LYSFCCSRSSEYPIYVLPSPEELEDMARPT----SCFNCKYFRCC------
n3_Ppatens      LYSFCCSSSSEHPIYVLPSPEELENMARPT----SCFSCKYFCYCGFCYHC
n5_Tbrucei      VYFLFRRK----PINWYSVLEEDE--------------------CALV---
n16_Microm      AREFCCSPPP--PRYTLRSFEWMREWSRTE----TIWENKYYVCC------
n17_Microm      VMRTCCEPPP--PRYEMRPMKWYRAESKRE----TIWENRYWVCC------
n2_Ddiscoi      LKKFLKGDENP-SDWILLSKYEVDQMKKKEENTFNIWNNKYYSCCG-----
n10Ttermop      IKLIFFHDNQ-LRDWQVPTLREAQQRYQNT---FNIFENEYYNCC------
n18Ptetrau      IKITFFHKGLQ-SHWIPPSKDQIKEQC-------NIFDNKYYSCC------
n19Ptetrau      IRLLFFHRNVQ-SHWIPPQKDQIQEQC-------NIFDNKYYSCC
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