



## LETTER

# Identification of the Phosphorylated Residues in TveIF5A by Mass Spectrometry

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## KEYWORDS

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**Abstract** The initiation factor eIF5A in *Trichomonas vaginalis* (TveIF5A) is previously shown to undergo hypusination, phosphorylation and glycosylation. Three different pI isoforms of TveIF5A have been reported. The most acidic isoform (pI 5.2) corresponds to the precursor TveIF5A, whereas the mature TveIF5A appears to be the most basic isoform (pI 5.5). In addition, the intermediary isoform (pI 5.3) is found only under polyamine-depleted conditions and restored with exogenous putrescine. We propose that differences in PI are due to phosphorylation of the TveIF5A isoforms. Here, we have identified phosphorylation sites using mass spectrometry. The mature TveIF5A contains four phosphorylated residues (S3, T55, T78 and T82). Phosphorylation at S3 and T82 is also identified in the intermediary TveIF5A, while no phosphorylated residues are found in the precursor TveIF5A. It has been demonstrated that eIF5A proteins from plants and yeast are phosphorylated by a casein kinase 2 (CK2). Interestingly, a gene encoding a protein highly similar to CK2 (TvCK2) is found in *T. vaginalis*, which might be involved in the phosphorylation of TveIF5A in *T. vaginalis*.

## Introduction

Eukaryotic translation initiation factor 5A (eIF5A) is a polyamine-dependent protein present in all eukaryotic cells [1].

The precursor eIF5A undergoes a specific spermidine-dependent post-translational modification known as hypusination for eIF5A activation [2]. During hypusination, a unique amino acid, Ne-(4-aminobutyl-2-hydroxyl) lysine, called hypusine, is generated by transferring the aminobutyl moiety of spermidine to a specific lysine residue in precursor eIF5A [2]. Both the lysine residue and the flanking residues are conserved among eukaryotes [3]. Hypusine is exclusively synthesized in precursor eIF-5A [3]. Besides hypusination, eIF5A proteins are phosphorylated in *Saccharomyces cerevisiae* (eIF5Ab) [4,5] and maize (ZmeIF5A). The phosphorylation in ZmeIF5A occurs at serine residue 2 (S2) [6]. Interestingly, eIF5A is represented by more than one isoforms in *Plasmodium falciparum*, presumably due to post-translational modifications [7]. Expression of

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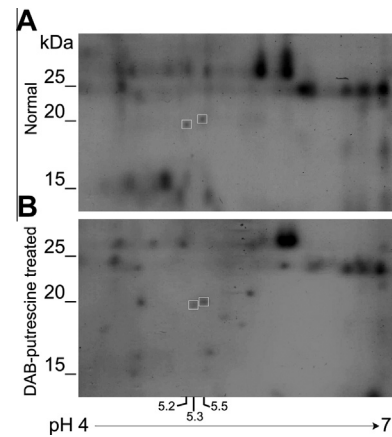
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two eIF5A isoforms was decreased modestly during schizont development, while expression of another isoform with a considerably more acidic isoelectric point (pI) was increased 15-fold and demonstrated a significant fold change during the proteomic analysis of *P. falciparum* at various developmental stages [7].

eIF5A from *Trichomonas vaginalis* (TveIF5A) is of archaeobacterial origin [8]. *T. vaginalis* is the protozoan parasite responsible for trichomonosis, which is the most common sexually transmitted infection. Trichomonosis is associated with an increased risk of human immunodeficiency virus (HIV) infection [9], pelvic inflammatory disease [10] and adverse pregnancy outcomes [11]. Our previous studies reported that two spots with different isoelectric points (pI) (5.2 and 5.5) were detected in the *T. vaginalis* protein extracts. These two spots corresponded to the precursor (pI 5.2) and mature TveIF5A (pI 5.5), respectively [8], and the mature isoform contains the hypusine residue [12]. Interestingly, when putrescine biosynthesis was inhibited in *T. vaginalis* using 1,4-diamino-2-butanone (DAB) and the parasites were then transferred to a medium containing exogenous putrescine, a new TveIF5A spot with a pI of 5.3 was observed [12]. According to sequencing analysis, these three isoforms shared identical primary structures; however, the precursor and intermediate isoforms were 19 kDa, while the mature isoform was 20 kDa, which is phosphorylated and glycosylated [12]. Moreover, in silico analyses of the precursor isoform indicated that serine residues S3, S21, S83 and S153, threonine (T) residues T78 and T114, and tyrosine (Y) residues Y37 and Y96 are potential phosphorylation sites [12]. In this study, we went further to identify the phosphorylated residues in TveIF5A using LC-MS/MS. We found that mature TveIF5A contains four phosphorylated residues, S3, T55, T78 and T82; among which S3 and T82 were also phosphorylated in the intermediary isoforms, whereas no phosphorylated residues were identified in the precursor TveIF5A.

## Results

*T. vaginalis* was grown in TYM-serum medium (control) or transferred into a medium containing exogenous putrescine after DAB treatment (DAB-treated). The extracted proteome was subjected to 2D gel separation and an identical gel was immunoblotted for the identification of the specific spots immunorecognized by the anti-TveIF5A antibodies. In this report, we focused on the 25–15 kDa region to identify phosphorylation sites in the three TveIF5A isoforms (Figure 1). For the parasites grown in the control medium, spots that were immunorecognized by the anti-peptide TveIF5A antibody corresponded to the precursor and mature TveIF5A isoforms with pIs of 5.2 and 5.5, respectively (Figure 1A). As expected, for the DAB-treated parasites that were then transferred into medium containing exogenous putrescine, the intermediary TveIF5A isoform was observed with a pI of 5.3 (Figure 1B). These three spots were excised from the gels and digested with trypsin to identify the protein and its possible phosphorylated residues by MS/MS analysis. According to the MS/MS identification, all the three isoforms, with pIs of 5.5, 5.3 and 5.2, respectively, corresponded to TveIF5A (Table S1). According to previous reports, the pI 5.5 isoform corresponds to mature TveIF5A, whereas the pI 5.3 and 5.2 isoforms correspond to the intermediary and precursor TveIF5A, respectively.



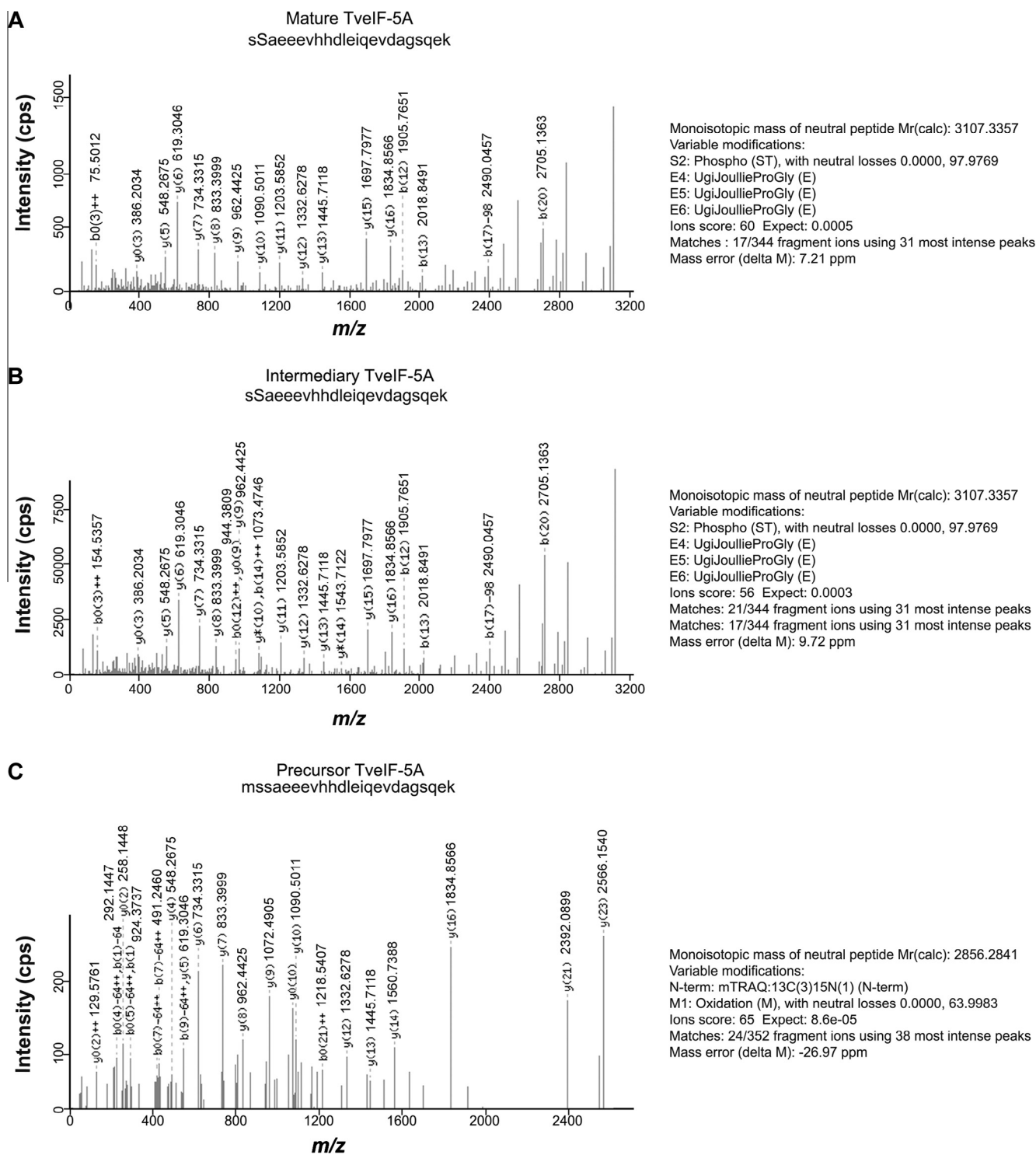
**Figure 1 Identification of the three TveIF5A isoforms**

*Trichomonas vaginalis* was grown under normal conditions (A) or treated with DAB and then cultured in a medium containing exogenous putrescine for 30 min (B). Total proteins were extracted and separated on 15% polyacrylamide 2DE gel. The gel was stained with Sypro-Ruby. Shown here are partial gels containing small proteins (15–25 kDa) including TveIF5A isoforms (boxed). The pI of each TveIF5A spot is indicated at the bottom. Experiments were performed at least in triplicate with identical results.

The MS/MS data were analyzed to identify modifications such as phosphorylated residues. We manually validated the peptides to identify the phosphorylated residues. Figure 2 shows the phospho-site assignment for one of the phosphorylated peptides as an example. The peptide (SSAEDEVHHDLEIQEVDAGSQEK) found in the mature (pI 5.5) (Figure 2A) and intermediary (pI 5.3) (Figure 2B) TveIF5A had a monoisotopic mass of 3107.3357 and the S2 residue of this peptide represented a phosphorylated residue (Table 1). Peptide MSSAEDEVHHDLEIQEVDAGSQEK was identified for the precursor TveIF5A (pI 5.2); however, no phosphorylated residues were revealed (Figure 2C).

The phosphorylation sites were identified by manual validation of the phosphorylated peptides (Tables 1 and S2) using Mascot analysis as described in Material and Methods section. The mature isoform (pI 5.5) contains four phosphorylated residues, S3, T55, T78 and T82, whereas the intermediary isoform (pI 5.3) contains two phosphorylated residues at S3 and T82 (Table 1). No phosphorylated residues were observed in the precursor TveIF5A (pI 5.2).

The position of the phosphorylated residues in the primary sequence of TveIF5A is shown in Figure 3A. Multiple sequence alignment analysis indicated that T55 is highly conserved in all the species detected, while S3 and T78 are replaced with acidic residues D or E in most of the remaining species and T82 often is replaced with S, a residue potentially undergoing phosphorylation. K57, the residue for hypusination in TveIF5A [12], and the flanking sequences are highly conserved across all species examined (Figure S1). TveIF5A share 37% identity with eIF5A from *Leishmania mexicana*. We thus generated a 3D model of TveIF5A using eIF5A from *L. mexicana* (PDB: 1XTD) [13] as the template. According to the 3D model, the phosphorylated residues T55 and T82 are near K57 (Figure 3B).



**Figure 2** Phospho-site assignment and manual validation

The peptides SSAEEEVHHDLEIQEVDAGSQEK in the mature (A) and intermediary (B) TveIF5A and MSSAEEEVHHDLEIQEVDAGSQEK in the precursor TveIF5A (C) were obtained by Mascot analysis and subjected to manual validation for the phosphorylation sites. The S2 in peptide SSAEEEVHHDLEIQEVDAGSQEK from the mature and intermediary TveIF5A isoforms produced a positive signal for phosphorylation, whereas, the corresponding residue is not phosphorylated in the precursor.

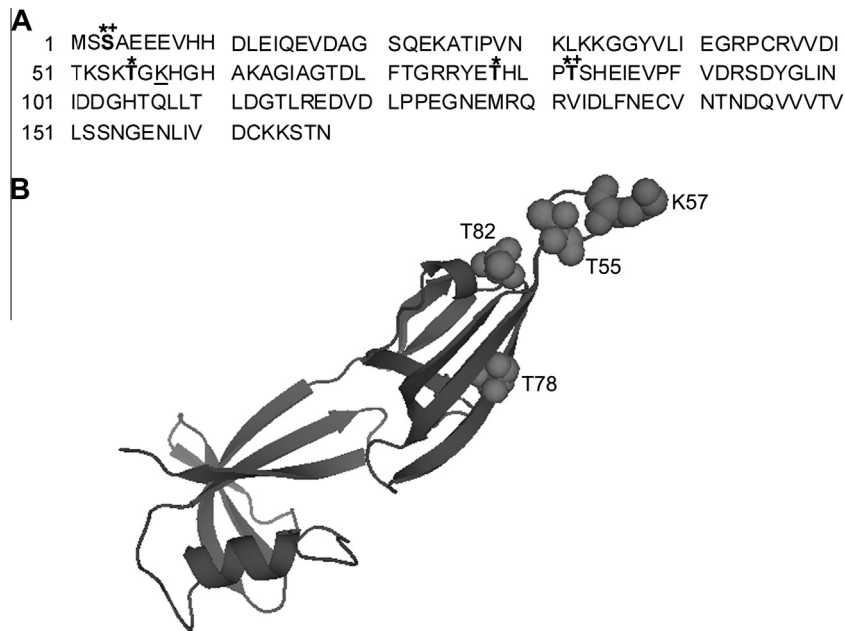
Several eukaryotic translation initiation factors are reported to be phosphorylated by casein kinase 2 (CK2). For example, S2 that is highly conserved in plants and protozoan parasites (Figure S1) is phosphorylated in most plant eIF5A

proteins that have been examined so far [14–16]. In most plant eIF5As, the phosphorylation of S2 is performed by a protein that belongs to the family of CK2 kinases [17]. Given the involvement of CK2 in the phosphorylation of plant eIF5A,

**Table 1** Phosphorylated residues identified in the TveIF5A isoforms

Isoform	pI	MS/MS fragmentation peptide	Monoisotopic mass of the neutral peptide (calc)	Phosphorylated residue and its position in the fragmentation peptide
Mature	5.5	YETHLPTSHEIEVPFVDR	2167.9827	T3
		RYETHLPT <sup>+</sup> SHEIEVPFVDR	2338.0995	T8
		TGKKGHAKAGIAGTDLFTGR	2354.2009	T1
		SS <sup>+</sup> AEEEVHHDLEIQEVDAGSQEK	3107.3357	S2
Intermediary	5.3	SS <sup>+</sup> AEEEVHHDLEIQEVDAGSQEK	3107.3357	S2
		YETHLPT <sup>+</sup> SHEIEVPFVDR	2167.9827	T7

Note: The variable modification for phospho (S/T) is neutral loss of 97.9769.

**Figure 3** TveIF5A phosphorylation sites

**A.** Shown is the location of the phosphorylation sites identified using MS/MS analysis in the primary sequence of TveIF5A. Phosphorylated residues found in the mature isoform are indicated by \* above, whereas phosphorylated residues found in the intermediary isoform are indicated by + above. **B.** TveIF5A model base on the template 1XTD from *Leishmania mexicana* eIF5A. The phosphorylated (T55, T78 and T82) residues and the hypusine-containing residue (K57) are indicated in balls.

we searched for the homologue of plant CK2 in the *T. vaginalis* genome. Our results showed that locus TVAG\_064190 contains a 1023 bp open reading frame (ORF) potentially encoding a protein of the CK2 kinase family (*tvck2*) (Figure S2A). Protein sequence alignment analysis showed that the TvCK2 residues 50–333 correspond to the protein kinase domain containing the ATP-binding region signature (56-VGTGKYSDVfAykgdtk...VAIK-77) and the S/T protein kinase active site signature (161-ImHrDVKplNILF-173) (Figure S2B). In addition, gene expression of *tvck2* was detected in the CNCD147 isolate of *T. vaginalis* by using RT-PCR (Figure S2C).

## Discussion

The highly conserved eukaryotic translation initiation factor 5A (eIF5A) is involved in a broad spectrum of cellular functions. In *Arabidopsis*, three eIF5A isoforms have been

identified including AteIF5A1, AteIF5A2 and AteIF5A3, which play different roles in various biological processes [14–16]. For example, AteIF5A1 is involved in xylem formation, whereas AteIF5A2 participates in cell division, growth and death caused by pathogen infection and AteIF5A3 responds to osmotic and nutrient stresses [14–16].

Three TveIF5A isoforms have been reported with the mature isoform phosphorylated [12]. Using LC–MS/MS, we demonstrated in this study that the precursor TveIF5A is not phosphorylated; the intermediate isoform is phosphorylated at S3 and T82 and the mature isoform is phosphorylated at S3, T55, T78 and T82. These results are in agreement with our previous *in silico* analyses [12].

Despite the absence in mammalian eIF5A [18], N-terminal S2 is highly conserved in plants and protozoan parasites examined, which could potentially be phosphorylated by CK2 [19]. In maize, S2 phosphorylation of eIF5A plays a unique role in regulating the intracellular localization of eIF5A and the nuclear export of mRNAs it binds to [18]. However, no phos-

phorylation was observed at S2 in TveIF5A; instead, phosphorylation was detected at S3 in the mature and intermediary TveIF5A isoforms. Our preliminary data suggest that TveIF5A might bind to certain mRNAs that possess eIF5A consensus-binding elements (unpublished data), suggesting that phosphorylation of mature TveIF5A might participate in specific regulation of this protein in *T. vaginalis* as well. Interestingly, S3 is replaced with acidic residues D/E in most other species. Similar replacement is also observed for T78. TveIF5A is highly similar to its counterparts found in other protozoa, plant and human. According to our previous phylogenetic analyses, TveIF5A is more closely grouped with the bacterial elongation factor P [8]. These suggest an early evolution of TveIF5A and high conservation in the evolutionary scale.

Phosphorylated residues T55 and T82 are located near K57, the site for hypusination in TveIF5A. In particular, the conserved T55 is located in the same loop with K57. In higher eukaryotes, the positively charged hypusine residue in eIF5A facilitates its bind to exportin 4 (Exp4), enhancing the nuclear export of eIF5A [20]. On the other hand, the negative charge in the phosphorylated residues may prevent the interaction of eIF5A with its nuclear receptor, causing an accumulation of eIF5A in the nucleus [18]. Interestingly, human CK2 possesses a nuclear localization signal [21], which might phosphorylate and retain eIF5A in the nucleus [18]. A recent study showed that the phosphorylated proteins might have a crucial function in the morphological transformation of *T. vaginalis* from trophozoite to amoeboid or pseudocyst [22]. Our preliminary study suggests that hypusination may be involved in stabilizing mRNA coding for these proteins (unpublished data). Until now, it remains obscure whether there is a link between hypusination and phosphorylation or whether their proximity has a biological significance. Further studies are needed to elucidate the specific function of mature TveIF5A, the potential involvement of phosphorylation and its association with hypusination in the role of this protein.

CK2 is a ubiquitously expressed serine/threonine protein kinase present in all eukaryotes [23]. Several eukaryotic translation initiation factors are reported to be phosphorylated by CK2. For example, CK2 phosphorylates S2 in eIF2 $\beta$  from human [24], S318 in eIF2a, T52 and T85 in eIF2b, and S209, T240 and S451 in eIF5 from wheat [25].

Our data also show that *T. vaginalis* contains an ORF coding for TvCK2. It was reported that the consensus substrate sequence for CK2 is S/T-x-x-E/D/pS [17]. Interestingly this consensus sequence was found in the phosphorylated S3 residue (MSSAE...) and in the phosphorylated T82 (.TSHE...) in TveIF5A. CK2 $\beta$  is autophosphorylated [26] at S2 (MSSSEEV...) and S3 (MSSSEEV...) [17,26]. Interestingly, TveIF5A also contains S2 and S3 (MSSAEEE...) in its N-terminal domain, suggesting that phosphorylation at S3 of TveIF5A revealed by MS/MS analysis might be performed by CK2 present in *T. vaginalis*. Thus, TvCK2 might phosphorylate the S3 or T82 residues of TveIF5A. However, we cannot rule out the possibility that other kinases are involved in phosphorylation of TveIF5A, since there are ~880 putative kinase genes in *T. vaginalis* genome, which encode distinct protein kinases [27].

In conclusion, LC-MS/MS analysis indicates that in *T. vaginalis*, TveIF5A precursor is a non-phosphorylated isoform and mature TveIF5A is phosphorylated at S3, T55, T78 and T82, whereas the intermediate isoform, which is found under polyamine depletion and upon restoration using exogenous

putrescine, is phosphorylated at S3 and T82. The differential phosphorylation profiles may explain the different pIs of three TveIF5A isoforms. An endogenous CK2 that is expressed in *T. vaginalis* might be involved in the phosphorylation of TveIF5A.

## Materials and methods

### *T. vaginalis* culture

The parasites were grown in Diamond's trypticase-yeast extract-maltose (TYM) medium supplemented with 10% heat-inactivated horse serum (TYM-serum) for 24 h at 37 °C (referred to as normal medium). The polyamine metabolism was inhibited as previously reported [12]. Briefly, the parasites were grown for 24 h at 37 °C in TYM-serum medium supplemented with 20 mM 1,4-diamino-2-butanone (DAB) (Sigma Chemical, St. Louis, MO), washed with sterile phosphate-buffered saline (PBS) and transferred to TYM-serum medium supplemented with 40 mM putrescine for 30 min (Sigma) (referred to as DAB-Putrescine).

### Two-dimensional gel electrophoresis (2DE)

Parasites from *T. vaginalis* ( $1.8 \times 10^8$ ) were collected by centrifugation at 900g for 5 min (4 °C), washed three times with PBS (pH 7.0) and lysed in rehydration solution containing 7 M urea, 4% CHAPS, 70 mM DTT, 2% IPG buffer (pH 4–7) and trace bromophenol blue (Bio-Rad) as previously reported [28]. For the first dimension, 70  $\mu$ g of protein was applied to an IPG strip (17 cm, pH 4–7 linear; Bio-Rad). All isoelectric focusing took place on a Protean IEF system (Bio-Rad). Before the second dimension, the proteins were reduced (10 mg/ml DTT) and alkylated (25 mg/ml iodoacetamide) step-wise. Equilibrated IPG strips were separated on 15% SDS-PAGE gels and were then stained using SYPRO® Ruby Protein Gel stain (Invitrogen) following procedures described by the manufacturer or transferred onto nitrocellulose membranes for 2DE Western blotting. Finally, the stained gels were documented using a Gel Doc EQ (Bio-Rad) system. Image analysis was performed using the PDQuest software (Bio-Rad).

### Western blotting

The 2DE separated proteins were transferred onto nitrocellulose membranes and blocked with 0.5% skim milk in  $1 \times$  PBS with 0.05% Tween-20 (PBS-Tween) for 18 h at 4 °C. The membranes were incubated with anti-TveIF5A mouse serum (1:1000 dilution) [8] for 18 h at 4 °C, washed five times with  $1 \times$  PBS with 0.1% Tween-20 (PBST), incubated with a peroxidase-conjugated secondary antibody (1:3000 dilution; Bio-Rad) for 2 h at 25 °C, and developed by chemiluminescence using an enhanced chemiluminescence ECL Plus Western blotting detection system (GE Healthcare) according to the manufacturer's instructions. Preimmune mouse serum (PI) was used as a negative control (1:1000 dilution).

Finally, the image analysis was performed using the PDQuest software (Bio-Rad) to match the immunorecognized spots with their corresponding protein spots in the gels. These spots were then excised from the gels and digested with trypsin for identification by mass spectrometry.

## Identification of phosphorylated residues

After staining the gels, the spots were excised and digested using trypsin according to a previous protocol [29]. Samples were incubated for trypsin digestion overnight (37 °C), and 100 µl of extraction buffer (1:2 (vol/vol) 5% formic acid/acetonitrile) was then added to each tube. The tubes were incubated for 15 min (37 °C) in a shaker. Samples were centrifuged at 900g for 5 min, and the supernatant was collected in a tube and dried in a vacuum centrifuge. The samples were redissolved in 20 µl of 0.1% (vol/vol) trifluoroacetic acid in a tube, purified using C18 ZipTip pipette tips (Millipore, USA) following the manufacturer's instruction and then injected into the instrument as previously reported [28].

## Search parameters

The default search parameters were such that a Mascot server (Matrix Science, London, UK, available at <http://www.matrix-science.com>, version 2.2; Matrixscience, London, UK) threshold score of 5% indicated that the protein identification was likely incorrect. The Mascot Distiller v2.1 was used for peak list generating software. All samples were searched with the taxonomy filter "Other Eukaryota" (379307 sequences). The database search parameters were set as follows: enzyme (trypsin with a maximum of one missed cleavages); fixed modifications (carbamidomethyl ( $\pm 57.021$  Da at cysteine residue)); variable modifications (deamidated ( $\pm 1$  Da at cysteine residue)) and methionine oxidation ( $\pm 15.995$  Da at methionine residue) Phospho (ST), Phospho (Y); peptide mass tolerance ( $\pm 1.2$  Da); fragment mass tolerance ( $\pm 0.6$  Da); mass tolerance for precursor ions ( $\pm 100$  ppm); and mass tolerance for fragment ions ( $\pm 0.6$  Da). The protein identification reporting criteria included at least two MS/MS spectrum matched at the 95% level of confidence (molecular weight search score  $\pm 25$ ) and the presence of a consecutive  $\beta$  and/or  $\gamma$  ion series of three or more amino acids. Instrument type is ESI-QUAD-LIT.

## In silico analysis

The N-terminal domains of eIF5A from different species were aligned using the Clustal W program (<http://www.ch.embnet.org/software/ClustalW.html>) using gap penalties of 10 to minimize internal gaps. The sequences of *Zea mays* (NP\_001105550.1), *Oryza sativa* (CAC84392.1), *Arabidopsis thaliana* (AEE29088.1), *Toxoplasma gondii* (EEB00280.1), *P. falciparum* (AAM46152.1), *S. cerevisiae* (AAB65008.1), *Schizosaccharomyces pombe* (CAB16195.1), *Homo sapiens* (NP\_01961.1), *Mus musculus* (CAI35154.1), *Drosophila melanogaster* (NP\_611878.1), *Entamoeba histolytica* (XP\_657397.1), *Leishmania infantum* (CAB95733.1), and *T. vaginalis* (ADE62353.1) were used for the alignment.

## TveIF5A modelling

The Swiss-Model Workspace (<http://swissmodel.expasy.org/>) tool was used to model TveIF5A using *L. mexicana* eIF5A (X-ray resolution: 2.70Å) (PDB ID: 1XTD) as the template. The PyMol software (DeLano Scientific LLC v0.99) was used to highlight the phosphorylation sites in TveIF5A.

## Screening of databases and the sequence analysis of CK2 from *T. vaginalis*

The CK2 gene sequences from several species were obtained from the GenBank database (<http://www.ncbi.nlm.nih.gov/Genbank/>). The search for the CK2 gene in the trichDB genome database (<http://www.trichdb.org/trichdb/>) was performed using the consensus sequences of CK2 from *Leishmania major*, *H. sapiens*, *Arabidopsis thaliana* and *S. cerevisiae* as query. The deduced amino acid sequence of CK2 from *T. vaginalis* (TvCK2) was subsequently aligned using the Clustal W program (<http://www.ch.embnet.org/software/ClustalW.html>) and used to determine the percentage of identity and E values for the related proteins by BLAST (<http://www.expasy.org/tools/blast/>). The alignment with ClustalW used gap penalties of 10 to minimize internal gaps.

## Amplification of tvck2 from *T. vaginalis*

*T. vaginalis* isolate CNCD147 was collected by centrifugation at 900g for 5 min at 4 °C (Allegra™ X-22 Centrifuge, Beckman Coulter). The pellet was suspended in 1 ml of TRIzolR reagent (Invitrogen, Life Technologies, Carlsbad, CA). Total RNA was extracted as recommended by the manufacturer and reversely transcribed using the Superscript Reverse Transcriptase kit (Invitrogen) and the oligo-dT (dT18) (10 pmol/µl) primer. PCR amplification was performed using the primers (sense: 5'-GATCGGATCCATGTCGCGTGGAAAGGGCGGTTCAAAG-3'; antisense: 5'-CTGCACTGCAGTTATGGTTCAATTTTCATGTTGCGAACAGAATCG-3'). (GenBank accession number for *tvck2* is KC688695).

## Authors' contributions

LIQG performed the experimental assays. CLC and JFA performed the bioinformatic analysis and GMH performed the mass spectrometry assays. EAS conceived the idea and supervised the project. All authors read and approved the final manuscript.

## Competing interests

The authors declare that they have no competing interests.

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## Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.gpb.2013.07.004>.

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