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## Characterisation of polyglutamylases in trypanosomatids



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### ABSTRACT

Microtubules are subject to post-translational modifications, which are thought to have crucial roles in the function of complex microtubule-based organelles. Among these, polyglutamylase was relatively recently discovered, and was related to centrosome stability, axonemal maintenance and mobility, and neurite outgrowth. In trypanosomatids, parasitic protozoa where microtubules constitute the essential component of the cytoskeleton, the function of polyglutamylated microtubules is unknown. Here, in order to better understand the role of this conserved but highly divergent post-translational modification, we characterised glutamylase and putative polyglutamylases in these parasites. We showed that microtubules are intensely glutamylated in all stages of the cell cycle, including interphase. Moreover, a cell cycle-dependent gradient of glutamylase was observed along the cell anteroposterior axis, which might be related to active growth of the microtubule 'corset' during the cell cycle. We also identified two putative polyglutamylase proteins (among seven analysed here) which appeared to be clearly and directly involved in microtubule polyglutamylase in *in vitro* activity assays. Paradoxically, in view of the importance of tubulins and of their extensive glutamylase in these organisms, RNA interference-based knockdown of all these proteins had no effect on cell growth, suggesting either functional redundancy or, more likely, subtle roles such as function modulation or interaction with protein partners.

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### 1. Introduction

Microtubules (MTs) are highly conserved dynamic structures made of  $\alpha$ - and  $\beta$ -tubulin heterodimers that are encoded by a family of polymorphic genes and are involved in a variety of essential cell processes such as intracellular transport, mitosis and mobility. In addition to their length and structure variations, MTs can be subject to a series of reversible post-translational modifications (PTMs) (acetylation, phosphorylation, polyglutamylase, polyglucylation, palmitoylation and detyrosination), some of which are very unusual (reviewed in Wloga and Gaertig (2010) and Janke and Bulinski (2011)). Polyglutamylase is a PTM that generates glutamate side-chains of variable length on the gamma-car-

boxyl group of glutamate residues within the primary sequence of the target protein (reviewed in Janke et al. (2008)). It occurs in a variety of proteins, essentially  $\alpha$ - and  $\beta$ -tubulins (Eddé et al., 1990; Rüdiger et al., 1992), but also the nucleosome assembly proteins NAP1 and NAP2 (Regnard et al., 2000) and other nucleo-cytoplasmic shuttling proteins (van Dijk et al., 2008) that, in higher eukaryotes, are proposed to function in a broad array of physiological activities, through molecular mechanisms as diverse as phosphatase inhibition, chromatin regulation, caspase activation and intracellular transport (Okada et al., 2011; Reilly et al., 2011; Kuryan et al., 2012). The modification is generated by relatively recently discovered polyglutamylating enzymes, members of the Tubulin Tyrosine Ligase-Like (TLL) family (Janke et al., 2005). TLLs constitute a large family of proteins that share a homology domain with TTL, another tubulin-modifying enzyme, and whose members can catalyse the ligation of glutamate amino acids to tubulins or other substrates (van Dijk et al., 2008). In mammals, 13 TLL enzymes exist, among which nine are glutamylases (van Dijk et al., 2007) and three are catalysing a related modification, glycylation (Rogowski et al., 2009; Wloga et al., 2009). Each enzyme displays defined reaction preferences for modifying the  $\alpha$ - or  $\beta$ -tubulin and for generating short or long sidechains

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(van Dijk et al., 2007; Rogowski et al., 2009). Both the polyglutamyl- and the polyglycylation reactions consist of two biochemically distinct steps: initiation and elongation, which are often mediated by distinct TTLs. Initiation is the formation of an isopeptide bond with the gamma-carboxyl group of the glutamate acceptor site, whereas side chain elongation consists of the formation of regular peptide bonds (Redeker et al., 1991; Wolff et al., 1994). It has been proposed that the range of signals generated by the variable length of the added sidechain may allow a fine tuning of the function of MTs and of their interactions with protein partners (Lacroix and Janke, 2011) (reviewed in Janke et al. (2008) and Janke and Kneussel (2010)). Thus, tubulin PTMs, and more specifically polymodifications, are thought to have crucial roles in the assembly, maintenance and function of complex MT-based organelles (reviewed in Janke and Bulinski (2011)).

Polyglutamyl- and polyglycylation is an ancient phenomenon evolutionarily conserved from protists to mammalian cells; it is present in sperm flagella of mammals, in sea urchins as well as in several protists including *Giardia*, *Tetrahymena*, *Crithidia* and *Trypanosoma* (Eddé et al., 1990; Seebeck et al., 1990; Bré et al., 1994; Rüdiger et al., 1995; Moulay et al., 1996; Plessmann and Weber, 1997; Schneider et al., 1997; Weber et al., 1997; Westermann et al., 1999). In mammalian cells, tubulin polyglutamyl- and polyglycylation is related to centrosome stability, axonemal maintenance and mobility in cilia and flagella, and neurite outgrowth (Gagnon et al., 1996; Bobiniec et al., 1998; Million et al., 1999; Westermann and Weber, 2003; Janke et al., 2005; Ikegami et al., 2006; Pathak et al., 2007; Vogel et al., 2010) (reviewed in Janke et al. (2008)). In protists, knowledge of the biological function of polyglutamylated MTs is limited (Wloga et al., 2008; Wloga and Gaertig, 2010) and, in view of the extreme genetic and biological diversity encountered in these organisms (Berriman et al., 2005), probably much more diverse than in mammals.

*Trypanosoma* and *Leishmania* are flagellated parasitic protozoa of the Trypanosomatidae family. They have a simple but precisely ordered cytoskeleton, primarily made of stable MTs (Schneider et al., 1997) and, with MTs constituting the most abundant part of their cytoskeleton, they show a reduced dependence on the acto-myosin network (Kohl and Gull, 1998; Berriman et al., 2005). MTs constitute four sub-structures in trypanosomatids: the mitotic spindle, the flagellar axoneme, the basal body of the flagellum and, most importantly, the subpellicular 'corset'. This corset is made exclusively of a dense network of MTs cross-linked to each other and to the plasma membrane, forming a helical pattern along the long axis of the cell (reviewed in Robinson et al. (1995)). It is responsible for the cell shape and plays a major role in events such as positioning of organelles, mitosis and cytokinesis (Sasse and Gull, 1988).

Similar to other organisms, MTs in these parasites are subject to a series of PTMs (dephosphorylation, acetylation, polyglutamyl- and polyglycylation) (Wolff et al., 1992; Gull, 1999). Using mass spectrometry analysis, trypanosomatid tubulin has been shown to be extensively glutamylated and non-glutamylated tubulins were reported to be almost absent (Schneider et al., 1997). In contrast, the closely related modification glycylation was not detected in *Trypanosoma brucei* (Schneider et al., 1997). The discovery that TTL proteins catalyse tubulin glutamyl- and polyglycylation (Janke et al., 2005; van Dijk et al., 2007) led us to identify eight TTL genes in silico in the genome of *T. brucei*. These enzymes belong to five of the diverse subtypes of this protein family (TTL1, TTL4, TTL6, TTL9 and TTL12). To understand the role of this evolutionarily conserved but highly divergent PTM (Wloga and Gaertig, 2010) and to better characterise tubulin glutamyl- and polyglycylation in trypanosomatids, the distribution of polyglutamylated MTs during the cell cycle in *Leishmania major* and *T. brucei* was examined using specific antibodies (Abs). The putative TTL genes of *T. brucei* and *L. major* were then cloned

and the subcellular localisation of the gene products determined using GFP-fused recombinant proteins; their enzymatic activities were analysed in vitro and an insight into their putative biological function obtained using RNA interference (RNAi).

## 2. Material and methods

### 2.1. Parasites

*Leishmania major* 'Friedlin' promastigotes (MHOM/IL/81/Friedlin) were grown as previously described (Dubessay et al., 2004). Procytic forms of the 29–13 line of *T. brucei* were grown at 27 °C in SDM 79 (Sigma®, MO, USA) supplemented with FCS (10%), hemin (7 µg/ml), hygromycin (30 µg/ml) and geneticin (10 µg/ml) for continuous culture, plus phleomycin (5 µg/ml) for RNAi experiments.

### 2.2. Bioinformatics

The sequences of genes encoding putative TTL proteins in *L. major* and *T. brucei* were searched in the MapView database (<http://www.genedb.org/genedb/leish/>). The corresponding trypanosomatid protein sequences were aligned using Clustal X and corrected manually as described previously (Wloga et al., 2008).

### 2.3. Construction of *L. major* cell lines expressing GFP fused proteins

The genes encoding putative TTLs were PCR-amplified from genomic DNA. The PCR products were cloned into a pGEM-T-Easy vector (Promega®, WI, USA) and then inserted into the expression vectors pTH6cGFPn and pTH6nGFPc (Dubessay et al., 2006) which place the GFP fusion at the N-terminus and C-terminus, respectively. The presence of the reading frame of the recombinant proteins was confirmed by sequencing.

Transfection in *L. major* was performed as described previously (Casanova et al., 2009). Briefly,  $5 \times 10^7$  cells with 80 µg of plasmid DNA were electroporated using a Bio-Rad GENE Pulser II and a Pulse Controller (Bio-Rad, Hercules, USA) at 25 µF and 1500 V with two pulses of 0.5 ms each and 10 ms between each pulse. Hygromycin B (Sigma®) was added at 30 µg/ml for selective pressure.

### 2.4. Microscopy and immunofluorescence imaging

For the intracellular localisation of GFP-fused proteins or for immunofluorescence of whole cells, transfected cells grown to mid-log phase were fixed in 4% paraformaldehyde (PFA) and air-dried on microscope fluorescence slides (Dubessay et al., 2006). For immunofluorescence analysis of cytoskeletons, cells were washed in PBS and deposited on 8-well slides. Cytoskeletons were extracted in 0.25% Nonidet P40 (NP40), 100 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES), 1 mM MgCl<sub>2</sub>, pH 6.9, for 5 min at room temperature (Pradel et al., 2006) and then fixed in 4% PFA for 20 min for *L. major* or for 5 min for *T. brucei*.

In both immunofluorescence preparations (whole cell or cytoskeleton), fixed cells were incubated with either the GT335 (1:10,000, Adipogen®, CA, USA), the PolyE (1:10,000 for *L. major* and 1:5,000 for *T. brucei*, made by the authors) or the YL1/2 (1:200, Santa Cruz Biotechnology®, CA, USA) Abs for 45 min, followed by 45 min with anti-mouse Alexa 546 or 488 (1:500, Molecular Probes®, OR, USA), anti-rabbit Alexa 488 (1:500, Molecular Probes®) or anti-rat conjugated with TRITC (1:500; Santa Cruz Biotechnology®) Abs, respectively.

To study the mitochondrion, cultivated cells were primarily incubated for 10 min at 27 °C with Mitotracker Red CMXRos (500 nM, Molecular Probes®) and washed in culture medium without

FCS. After a second wash in culture medium with FCS, the cells were fixed as described above.

DNA was visualised by DAPI staining (0.01 µg/ml). Slides were mounted with Mowiol mounting medium (Calbiochem®, CA, USA) and viewed by phase contrast and immunofluorescence microscopy using appropriate filters on a Zeiss® Axioplan 2 microscope with a 100× objective. Digital images were captured using a Photometrics CoolSnap CDD camera (Roper Scientific®, FL, USA) and processed with Metaview Software (Universal Imaging Corporation®, PA, USA).

## 2.5. Northern blot and western blot

### 2.5.1. Northern blot

Total RNA from promastigote forms of *L. major* and procyclic forms of *T. brucei* was extracted using a RNeasy extraction kit (Qiagen®, Netherlands) and denatured in a solution of 2.5 × 3-(*N*-morpholino)propanesulfonic acid (MOPS), 9.25% formaldehyde and 50% formamide. RNAs were then incubated for 10 min at 65 °C and 5–10 min on ice, before being separated on an agarose gel (1.4% agarose, 6% formaldehyde and 1× MOPS). RNAs were transferred to a nylon membrane (Amersham Hybond-N+, GE Healthcare®, UK) and hybridized with a specific DNA probe radio-labelled with α<sup>32</sup>P-dCTP by random priming. The GPI-anchor transamidase subunit 8 gene (*GPI8*; *LmjF.18.0360* and *Tb10.6.1.3060*) which is constitutively expressed, was used as a control.

### 2.5.2. Western blot

A total of 2 × 10<sup>8</sup> *L. major* recombinant cells were centrifuged at 600g for 10 min and washed with a protease inhibitor cocktail (complete EDTA-free protease inhibitor cocktail tablets, Roche®, Switzerland). Cells were then lysed for 10 min at 100 °C in a loading buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 0.01% bromophenol blue, 25% glycerol, 5% β-mercaptoethanol). Lysates were separated on a 10% SDS-PAGE gel and blotted onto a Polyvinylidene difluoride (PVDF) membrane (Bio-Rad®) saturated with PBS, 5% skimmed milk and 0.05% Tween 20 for 1 h. Different primary Abs were used: a mouse anti-GFP Ab (1:1,000, Roche®), the mouse anti-α-tubulin monoclonal Abs (mAbs) '12G10' (1:1,000) and GT335 (12G10 was developed by J. Frankel and M. Nelson and obtained from the Developmental Studies Hybridoma Bank, University of Iowa, USA). Immunoprecipitation was revealed with anti-mouse alkaline phosphatase conjugate Ab (1:7500, Promega®). The membranes were developed using the nitro-blue tetrazolium/5-bromo-4-chloro-3'-indolylphosphate (NBT/BCIP) reaction.

## 2.6. In vitro polyglutamylase assays

*Leishmania major* expressing recombinant TLL proteins and *L. major* Friedlin promastigote cells were lysed in 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES)/NaOH pH 6.8, 2 mM EGTA, 1 mM MgCl<sub>2</sub>, 0.2% NP40 and protease inhibitors (complete EDTA-free protease inhibitor cocktail tablets, Roche®). Tubulin glutamylase activity was determined as described by Regnard et al. (1998) and Janke et al. (2005). Reaction mixtures (20 µl) containing 50 mM Tris-HCl pH 9, 400 µM ATP, 2.4 mM MgCl<sub>2</sub>, 500 µM DTT, 4.6 µM taxol, 8 µM L-[<sup>3</sup>H]-glutamate (45–55 Ci/mmol, GE Healthcare®), 0.2 mg/ml of taxol-stabilized MTs and *L. major* extracts were incubated at 30 °C for 2 h. The taxol-stabilized MTs were prepared from adult mouse brain MTs or HeLa cells (Regnard et al., 1999). Mice used in this study were maintained in the animal facility of the Centre National de la Recherche Scientifique (CNRS) in Montpellier, France. Procedures involving mice were performed in compliance with national animal welfare laws, guidelines and policies, under the supervision of the regional ethical committee. Quantifications were done by scintillation counting of the α- and

β-tubulin bands after loading of the samples on a 10% SDS-PAGE and electro-transfer onto nitrocellulose, as previously described (Regnard et al., 1998). Polyglutamylase activity was also determined using ANP32B and NAP1 as substrates, at 0.2 mg/ml.

## 2.7. RNAi in *T. brucei*

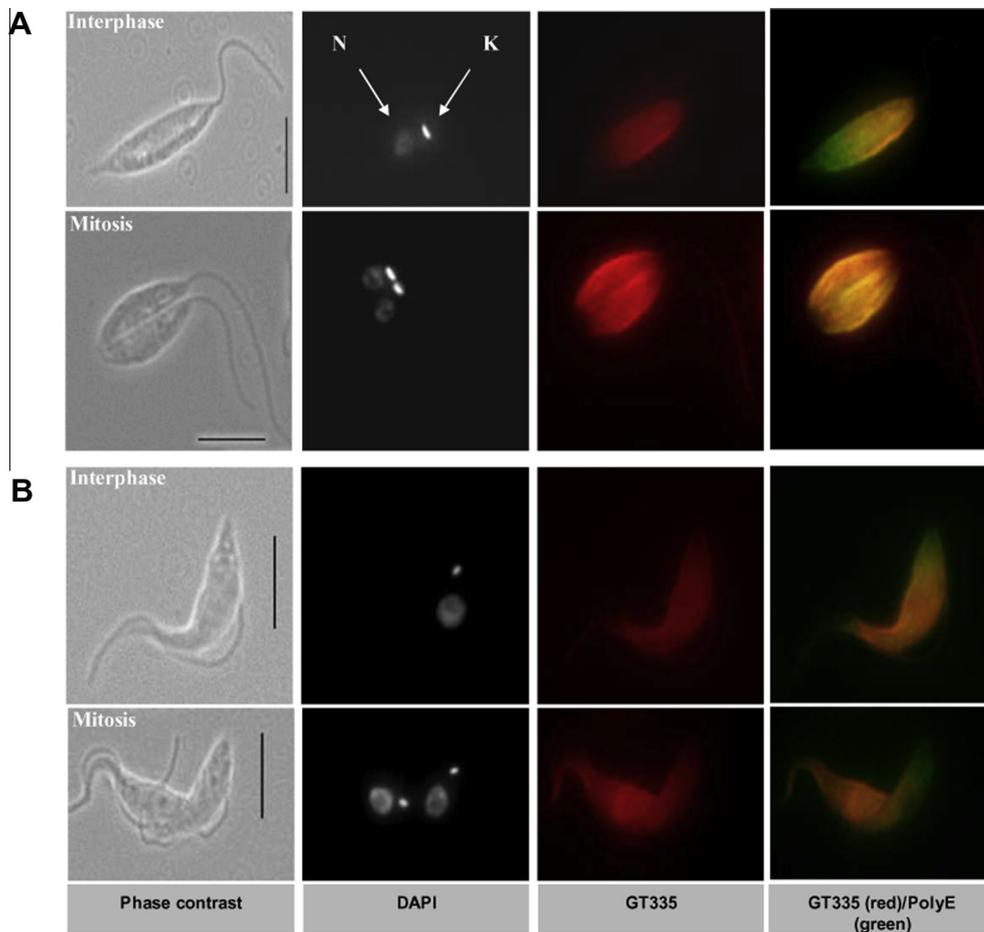
Amplification of parts of *T. brucei* TLL genes was performed using genomic DNA with the primers listed in Supplementary Table S1. The PCR products including the HindIII (AAGCTT) and SacI (CCGCGG) restriction sites were cloned into a pGEMT-Easy vector (Promega®) and into a p27T7tiB/GFP vector (trypanoFAN: <http://trypanofan.path.cam.ac.uk/trypanofan/main>). Linearised plasmid DNA (10 µg) was transfected into 3 × 10<sup>7</sup> *T. brucei* procyclic forms using an exponential protocol (1500 V, 25 µF) on a Bio-Rad® Gene Pulser Xcell™ electroporation system. Transfectants were grown under drug selective pressure (phleomycin, 5 µg/ml) for 15–20 days prior to induction by tetracycline (1 µg/ml) as previously described (Blaineau et al., 2007). Cell type labelling (GT335-positive or -negative) was estimated in the presence and absence of tetracycline as described in Section 2.4. Approximately 800 cells were counted.

## 3. Results

### 3.1. Distribution of glutamylated and polyglutamylated tubulin in *L. major* and *T. brucei*

While MT polyglutamylation has been reported as extensive in *T. brucei* from mass spectrometry data (Schneider et al., 1997), no data are available for *Leishmania*. To visualise glutamylation in trypanosomatid cells, we performed immunofluorescence in *L. major* and *T. brucei* using two Abs directed against polyglutamylated tubulin: GT335 is a mAb that recognises all forms of polyglutamylated tubulin independently of the length of the polyglutamate side chain (Wolff et al., 1992), whereas PolyE is a polyclonal Ab that is specific to tubulin modified by the presence of side chains at least three glutamates long (Rogowski et al., 2009). Contrary to the low level of polyglutamylated tubulin in interphase MTs, which is observed in most proliferating mammalian cells (Regnard et al., 1999), the trypanosomatid interphasic cells appeared intensely stained with both antibodies (Fig. 1). Moreover, in *L. major* cells, GT335 labelling was clearly more intense in G2/M cells (as defined from the appearance of the nascent flagellum) than in non-mitotic cells (Fig. 1, Supplementary Fig. S1). This shows (and confirms in *T. brucei*) that MTs are highly polyglutamylated at all stages of the cell cycle. Interestingly, the posterior third of the cell body lacked GT335 labelling in interphasic cells of both species, which was clearly confirmed by the merging of the fluorescence of both antibodies (Fig. 1). However, during the G2/M phase, this relative reduction was no longer observed in *L. major* promastigotes (Fig. 1A, Supplementary Fig. S1), although it persisted in *T. brucei* procyclic forms (Fig. 1B). This specific immunofluorescence pattern was confirmed using detergent-extracted cytoskeletons in both species (Supplementary Figs. S1 and S2). This experiment also confirmed that polyglutamylated tubulin is located in the cortex of MTs and not inside the cell body. Finally, the flagellum was always decorated with the antibodies, although less intensely than the rest of the cell (Supplementary Figs. S1–S3). In contrast with mammalian cells (Bobiniec et al., 1998), the centrioles of the basal body, as well as the mitotic spindle, were not distinctly labelled by the antibodies.

The differential distribution of GT335 labelling being reminiscent of the distribution of (de)tyrosinated α-tubulin (Sasse and Gull, 1988), we wanted to see if there was a correlation between



**Fig. 1.** Localisation of glutamylated microtubules in *Leishmania major* and *Trypanosoma brucei* during the cell cycle. (A) *Leishmania major* promastigote forms; (B) *Trypanosoma brucei* procyclic forms. For each organism, non-mitotic and G2/M cells are shown (here termed 'interphasic' and 'mitotic', respectively, for convenience). Each representative cell is viewed in phase contrast microscopy and in fluorescence microscopy using DAPI, the GT335 monoclonal antibody which labels all glutamate side chains (red), the PolyE monoclonal antibody (which labels long glutamate side chains) (green) and GT335–PolyE merging. The nucleus (N) and the single dense, intensely stained, mitochondrial DNA (K for kinetoplast) are stained with DAPI. Bar = 5 µm.

the distribution of glutamylated and tyrosinated MTs. As expected (Sherwin et al., 1987), the tyrosination-specific antibody YL1/2 clearly labelled the basal body in both species (Figs. 2 and 3). It also intensely decorated the cleavage furrow in *L. major* mitotic cells (Fig. 3). Moreover, also as previously described (Sasse and Gull, 1988), YL1/2 labelling was distributed over the whole cell body, with an increase in the posterior third of the cell in *T. brucei* (Fig. 2), i.e. the cell region where GT335 labelling was partially deficient. This observation was also made in non-mitotic cells of *L. major* (Fig. 3).

### 3.2. Identification of the genes encoding putative TLL proteins in *L. major* and *T. brucei*

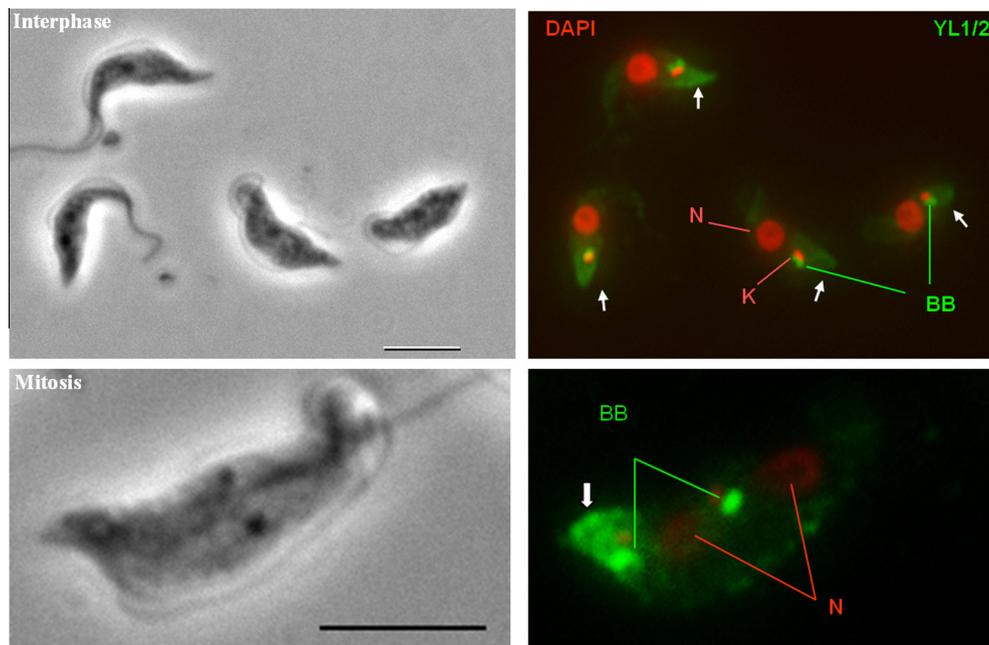
Using bioinformatics, we identified nine and 10 genes encoding proteins with a TTL domain in the *L. major* and *T. brucei* genomes, respectively. Two genes in *L. major* and one in *T. brucei* code for proteins with a high sequence homology to TTL (Table 1, Supplementary Table S1). Following phylogenetic studies described in Janke et al. (2005), the trypanosomatid TLL proteins are classified into five families: TLL1, TLL4, TLL6, TLL9 and TLL12. The analysis of the predicted domains of most of these putative TLLs identified a core TTL domain with an ATP binding site, a domain which is the common hallmark of TLL proteins, and an extended domain which is characteristic of the polyglutamylase activity (Fig. 4). Two proteins, classified as TLL12 and present only in *T. brucei*, did not

exhibit an extended domain and their homologue in mammals has previously been shown to not act as a tubulin glutamylating enzyme (van Dijk et al., 2007). Thus the seven remaining putative polyglutamylase proteins were examined in the two species (Table 1).

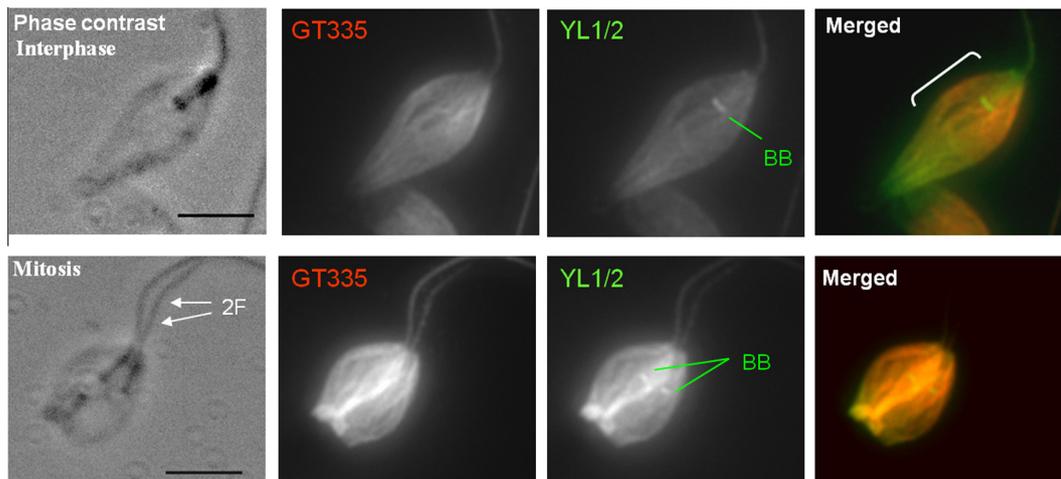
mRNA levels of the putative polyglutamylase genes were evaluated by northern blot analysis of the promastigote forms of *L. major* and procyclic forms of *T. brucei*. All of the genes were clearly transcribed compared with *GPI8*, a constitutively expressed gene (Supplementary Fig. S4). Using this crude measurement, *tll4a* seems to show high expression levels in both species, and *tll6b* seems to be expressed at very high levels in *L. major*, while *tll6a* and *tll9* appeared to be expressed at low levels in *T. brucei*. These results are actually corroborated by the comprehensive RNA-seq study in *L. major* by Rastrojo et al. (2013), which showed RNA levels of *tll4a* and *tll6b* threefold higher than those of most the other genes.

### 3.3. Subcellular distribution of GFP-fused putative polyglutamylases

We constructed *L. major* recombinant strains expressing GFP-fused TLL proteins. The expression of the GFP-fused proteins in recombinant cells was verified by western blot using an anti-GFP antibody (Supplementary Fig. S5) and their subcellular localisation was analysed using fluorescence microscopy. In order to rule out any bias due to the tag insertion, the GFP tags were introduced



**Fig. 2.** Localisation of tyrosinated microtubules in *Trypanosoma brucei*. G1/S (interphase) and G2/M (mitosis, indicated by the presence of two nuclei (N) and two basal bodies (BB)) cells are viewed in phase contrast microscopy and in fluorescence microscopy. A merged fluorescence of DAPI-stained DNA (red) and YL1/2 monoclonal antibody (green), which detects tyrosinated  $\alpha$ -tubulin, is shown. In non-mitotic cells as well as mitotic cells, tyrosinated  $\alpha$ -tubulins are essentially present at the basal body (BB) and the posterior third of the cell (white arrows). K, kinetoplast. Bar = 5  $\mu$ m.



**Fig. 3.** Localisation of glutamylated microtubules and tyrosinated microtubules on detergent-extracted cytoskeletons of *Leishmania major*. G1/S cells (interphase) and G2/M cells (mitosis, indicated by the presence of two flagella (2F)), are shown. The monoclonal antibody YL1/2, which is specific for tyrosinated  $\alpha$ -tubulin, decorates the basal body (BB), the whole of the cell body and, in mitotic cells, the cleavage furrow. In non-mitotic cells, more glutamate side chains, revealed by the monoclonal antibody GT335, are present at the anterior part of the cell (brackets). By contrast, mitotic cells are intensely labelled by both antibodies. Bar = 5  $\mu$ m.

at the N- and at the C-terminal ends in independent experiments: both constructs (LmTLL-GFPn/LmTLL-GFPc) gave identical results for all genes except for TLL4B (see below), indicating that the GFP tag did not modify the localisation of these proteins.

Four of the recombinant TLL proteins (LmTLL1, LmTLL6A and 6B, and LmTLL9) showed a diffuse distribution in the cell body but were excluded from the nucleus (Fig. 5). In addition, LmTLL6B-GFP exhibited an intensely fluorescent dot at the very posterior end of the cell. LmTLL4A-GFP showed a diffuse localisation and was particularly concentrated in the nucleus (Fig. 6). Surprisingly, LmTLL4B-GFPc was located exclusively in the mitochondrion, as confirmed by co-localisation with the mitochondrial marker Mitotracker™ (Fig. 6). Accordingly, an in silico analysis of the sequence of LmTLL4B revealed a mitochondrial

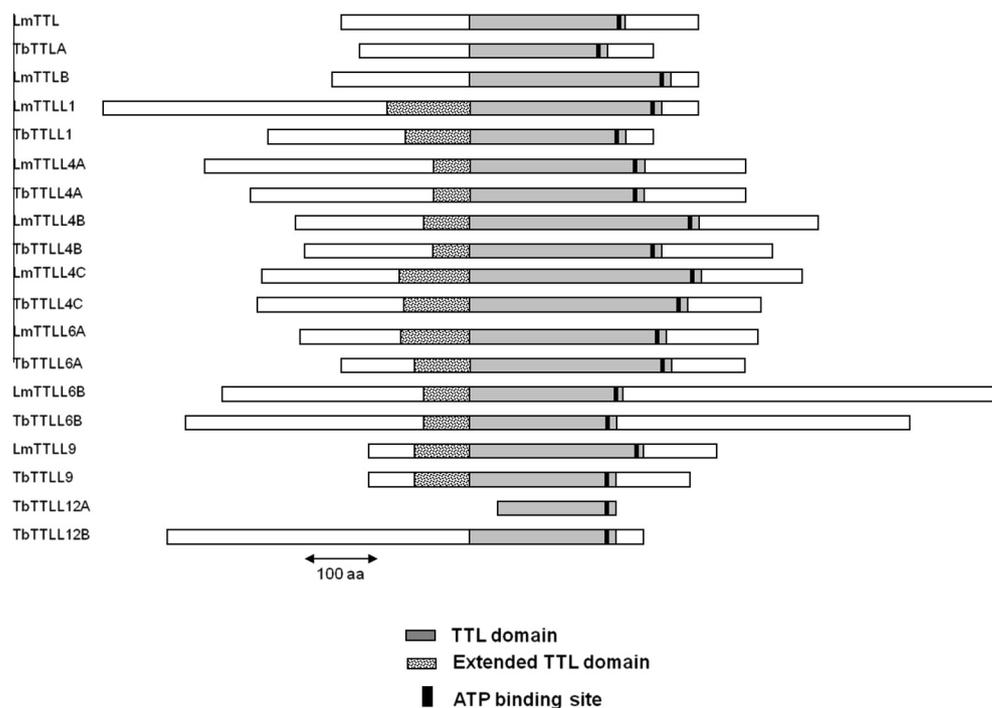
addressing signal at the N-terminus of the protein, which also explained why the N-terminally tagged protein did not localise to the mitochondrion. Finally, LmTLL4C-GFP was exclusively seen in the nucleus, with a pronounced accumulation at the mitotic spindle in G2/M cells, and at the midbody in anaphase (Fig. 6).

### 3.4. In vitro polyglutamylation activity and substrate specificity of the *L. major* recombinant TLL proteins

In vitro assays with taxol-stabilised MTs (Regnard et al., 1998; van Dijk et al., 2007) were performed in order to test the tubulin polyglutamylase activity of recombinant TLL proteins from GFP-TLL expressing *L. major* cells. To determine whether these were involved in the initiation or the elongation of glutamate side

**Table 1**Identification of the genes encoding putative Tubulin Tyrosine Ligase and Tubulin Tyrosine Ligase-Like proteins in *Leishmania major* (*Lm*) and *Trypanosoma brucei* (*Tb*).

TTL code	Reference number of gene sequence <sup>a</sup>		Annotation in GeneDB ( <i>Lm</i> , <i>Tb</i> ) <sup>a</sup>
	<i>L. major</i>	<i>T. brucei</i>	
TTLA	LmjF.27.2490	Tb927.2.5250	Tubulin Tyrosine Ligase-Like protein
TTLB	LmjF.32.2930	–	Tubulin Tyrosine Ligase-Like protein
TTL1	LmjF.16.0380	Tb927.5.3860	Tubulin Tyrosine Ligase, putative
TTL4A	LmjF.35.4930	Tb927.9.9580	Tubulin tyrosine ligase, putative
TTL4B	LmjF.30.2280	Tb927.6.3570	Tubulin Tyrosine Ligase-Like protein
TTL4C	LmjF.20.0790	Tb927.1.1550	Tubulin tyrosine ligase, putative
TTL6A	LmjF.29.0260	Tb927.3.5380	Hypothetical protein, conserved
TTL6B	LmjF.11.0400	Tb927.11.6810	Tubulin Tyrosine Ligase-Like protein
TTL9	LmjF.18.0370	Tb927.10.13870	Tubulin Tyrosine Ligase putative
TTL12A	–	Tb927.11.2390	Hypothetical protein, conserved
TTL12B	–	Tb927.11.2420	Hypothetical protein, conserved

LmjF, *Leishmania major* Friedlin strain; Tb927, *Trypanosoma brucei brucei* 927 strain.<sup>a</sup> <http://www.genedb.org>.**Fig. 4.** Schematic representation of the *Leishmania major* and *Trypanosoma brucei* proteins identified from their Tubulin Tyrosine Ligase domain. Non-conserved sequences are drawn in white. The respective positions of the various domains are detailed in Supplementary Table S1. aa, amino acids.

chains, we used two kinds of substrates: highly polyglutamylated MTs from brain tissue and almost unmodified MTs from HeLa cells. While both initiating and elongating enzymes can act on the brain MTs by either generating new side chains or increasing the length of the pre-existing side chains, HeLa MTs are solely modified by enzymes able to initiate the formation of side chains (van Dijk et al., 2007).

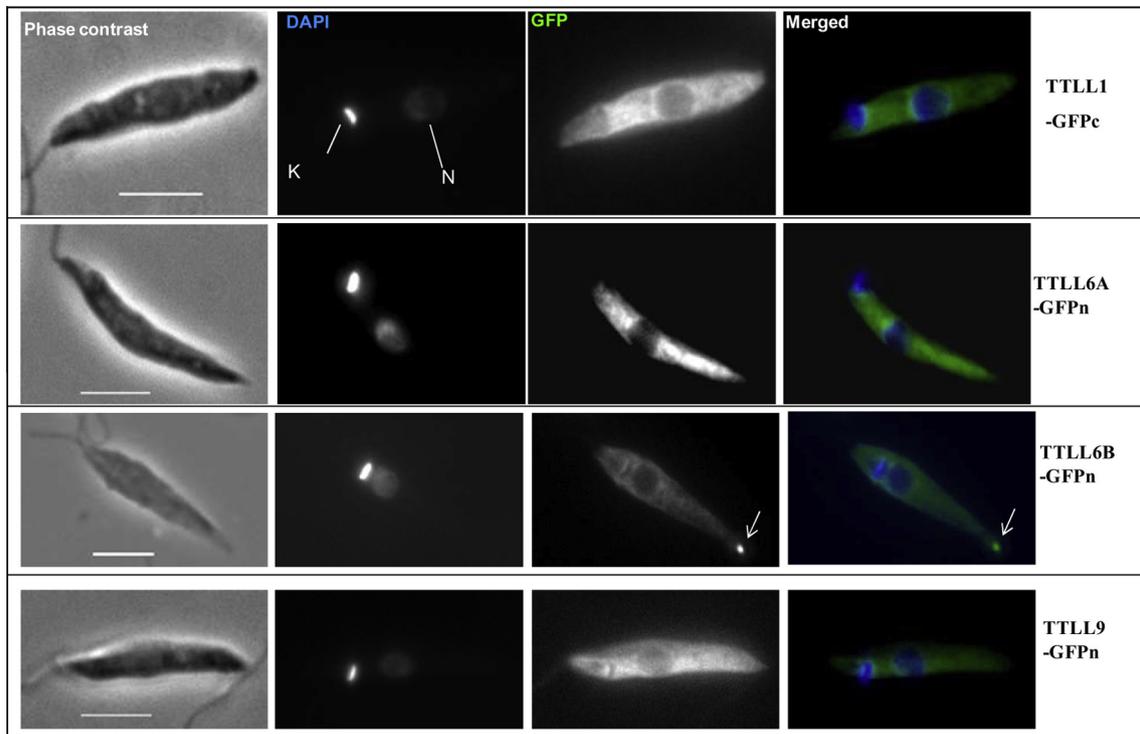
LmTTL4A and LmTTL6B were found to be active on both MT substrates. Their ability to modify HeLa tubulin demonstrates that these enzymes possess a chain-initiating polyglutamylase activity (Fig. 7A). LmTTL6B preferentially modifies  $\beta$ -tubulin subunits, and can therefore be classified as a  $\beta$ -tubulin chain-initiating polyglutamylase.

In order to study the glutamylase activity towards substrates other than the tubulins, we performed the same in vitro assays for LmTTL4A, LmTTL4B, LmTTL4C, LmTTL6A and LmTTL6B using recombinant NAP1 and ANP32B, two non-tubulin substrates strongly modified by some of the mammalian TTLs (Regnard et al., 2000; van Dijk et al., 2008). Again, LmTTL4A and LmTTL6B

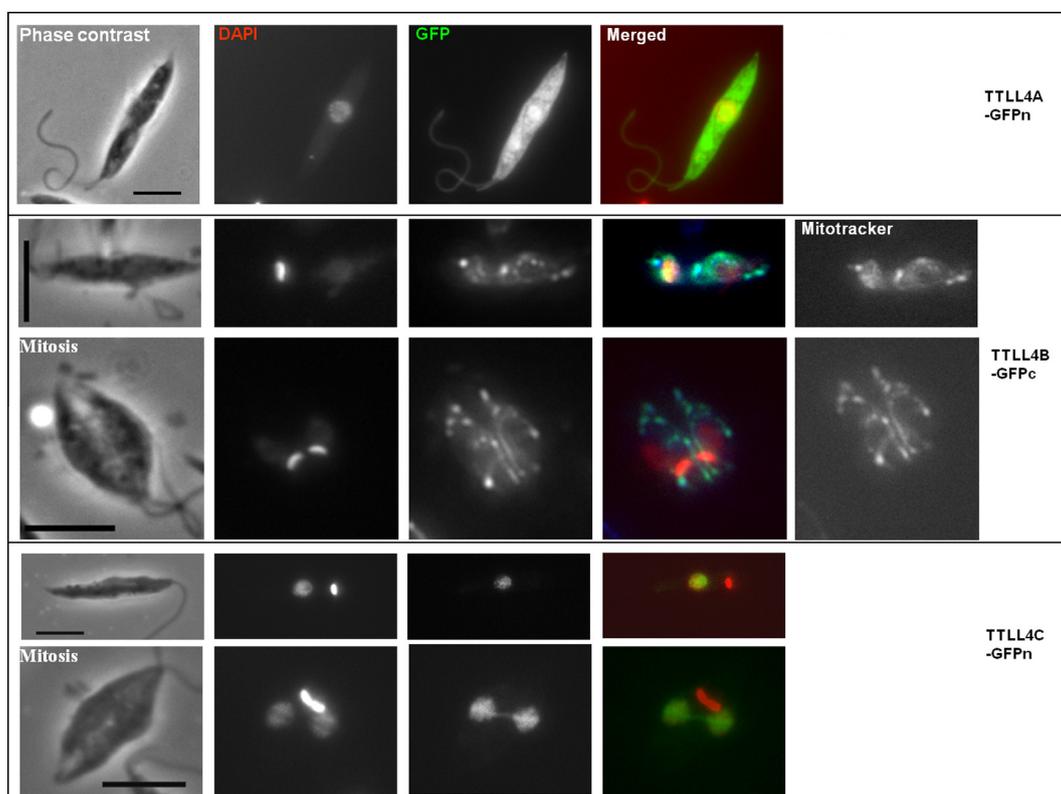
proved to be the only clearly active enzymes: LmTTL6B presented a high glutamylase activity on NAP1 and ANP32B, whereas LmTTL4A presented activity on ANP32B only (Fig. 7B and C). Very slight activity was seen on NAP1 only for LmTTL4C and LmTTL6A, while LmTTL4B exhibited no significant glutamylase activity on either of the substrates (Fig. 7B and C).

### 3.5. Functional analysis of trypanosomatid TTL proteins by RNAi in *T. brucei*

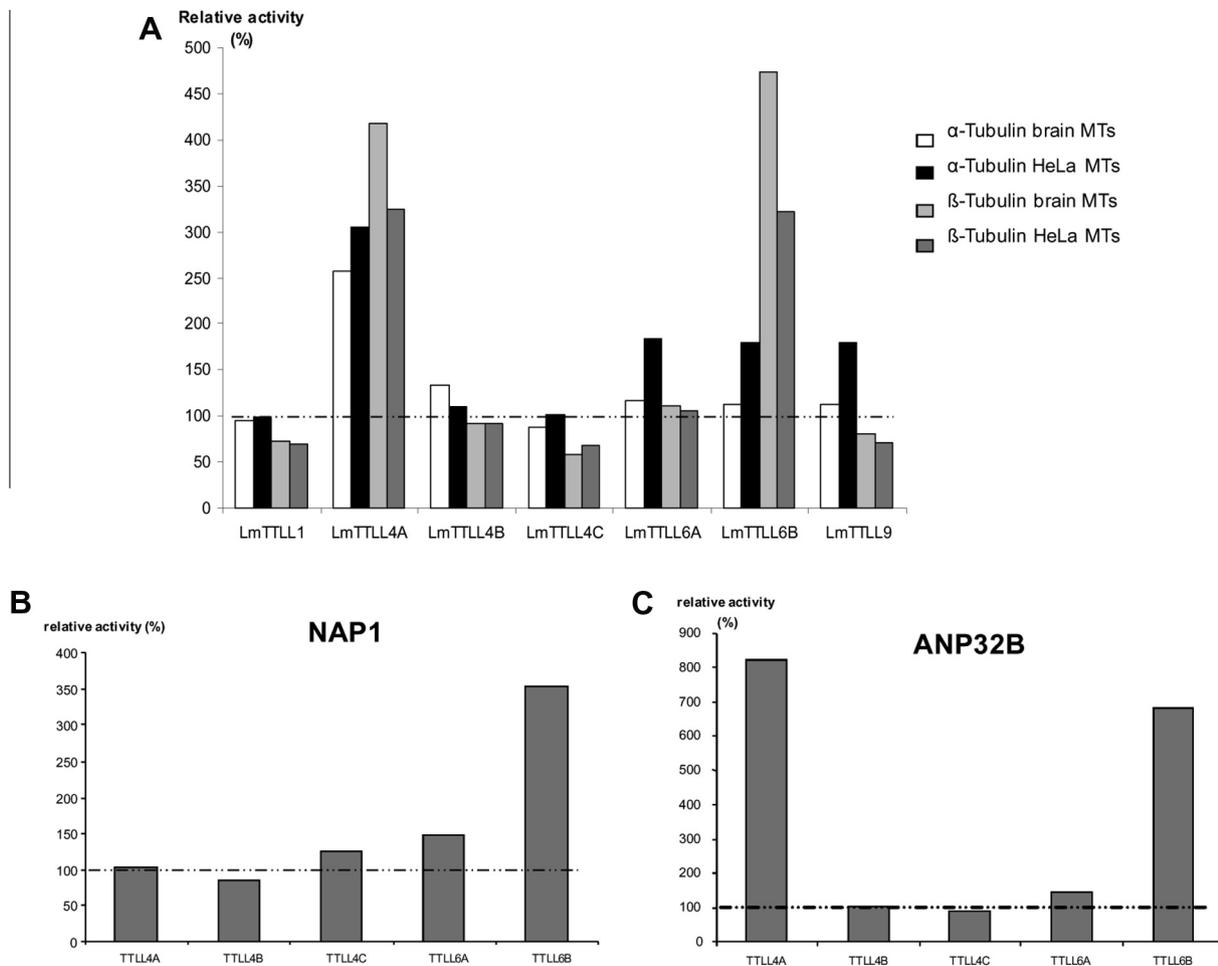
Studying loss-of-function phenotypes by gene knockout is usually cumbersome in *Leishmania* due to a 'mosaic aneuploidy' that leads to frequent generation of extra chromosomal copies in this organism (Dubessay et al., 2002; Sterkers et al., 2011). Moreover, RNAi is not functional in *L. major* due to the absence of proteins of the interference pathway (Robinson and Beverley, 2003), but can be used in *T. brucei* (Shi et al., 2000). We therefore independently transfected RNAi vectors targeting all seven TTL genes into *T. brucei* procyclic forms, and subsequently studied the RNAi-



**Fig. 5.** Subcellular localisation of GFP-fused versions of Tubulin Tyrosine Ligase-Like proteins, LmTTLL1, LmTTLL6A, LmTTLL6B and LmTTLL9 in *Leishmania major* promastigotes. Phase-contrast microscopy, fluorescence microscopy using DAPI, GFP fluorescence, and merged fluorescence with DAPI in blue and GFP in green are shown. The results for each of the above proteins are displayed. The GFP tags were fused at the N- (LmTTLL-GFPn) and at the C-terminal ends (LmTTLL-GFPc) of the proteins. The recombinant Tubulin Tyrosine Ligase-Like proteins essentially show a diffuse localisation on the cell body, with LmTTLL6B-GFPn also exhibiting a strong dotted location at the posterior end of the cell (white arrow). N, nucleus; K, kinetoplast. Bar = 5  $\mu$ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



**Fig. 6.** Subcellular localisation of GFP-fused versions of Tubulin Tyrosine Ligase-Like proteins LmTTLL4A, LmTTLL4B and LmTTLL4C in *Leishmania major* promastigotes. The GFP tags were fused at the N-terminal end (LmTTLL-GFPn) and at the C-terminal end (LmTTLL-GFPc) of the proteins. Phase-contrast images, fluorescence microscopy using DAPI, GFP, DAPI-GFP merged fluorescence with DAPI in red and GFP in green, and Mitotracker™ (mitochondrion-specific marker) fluorescence are shown. Bar = 5  $\mu$ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



**Fig. 7.** In vitro glutamylase activity and substrate specificity of *Leishmania major* recombinant Tubulin Tyrosine Ligase-Like (LmTTL) proteins. (A) Microtubules (MTs) from brain and HeLa cells, (B) NAP1, and (C) ANP32B substrates were subjected to an in vitro glutamylation assay using extracts of *L. major* cells expressing the recombinant Tubulin Tyrosine Ligase-Like proteins. The controls were wild-type *L. major* cells, not transfected with Tubulin Tyrosine Ligase-Like proteins, from which the reference “100%” activity (dotted line) was defined. Incorporation of  $^3\text{H}$  glutamate was determined by scintillation counting. The relative activity of each protein is expressed as a percentage over the total activity in the control reaction.

induced phenotypes such as cell growth, cytokinesis and glutamylated tubulin distribution.

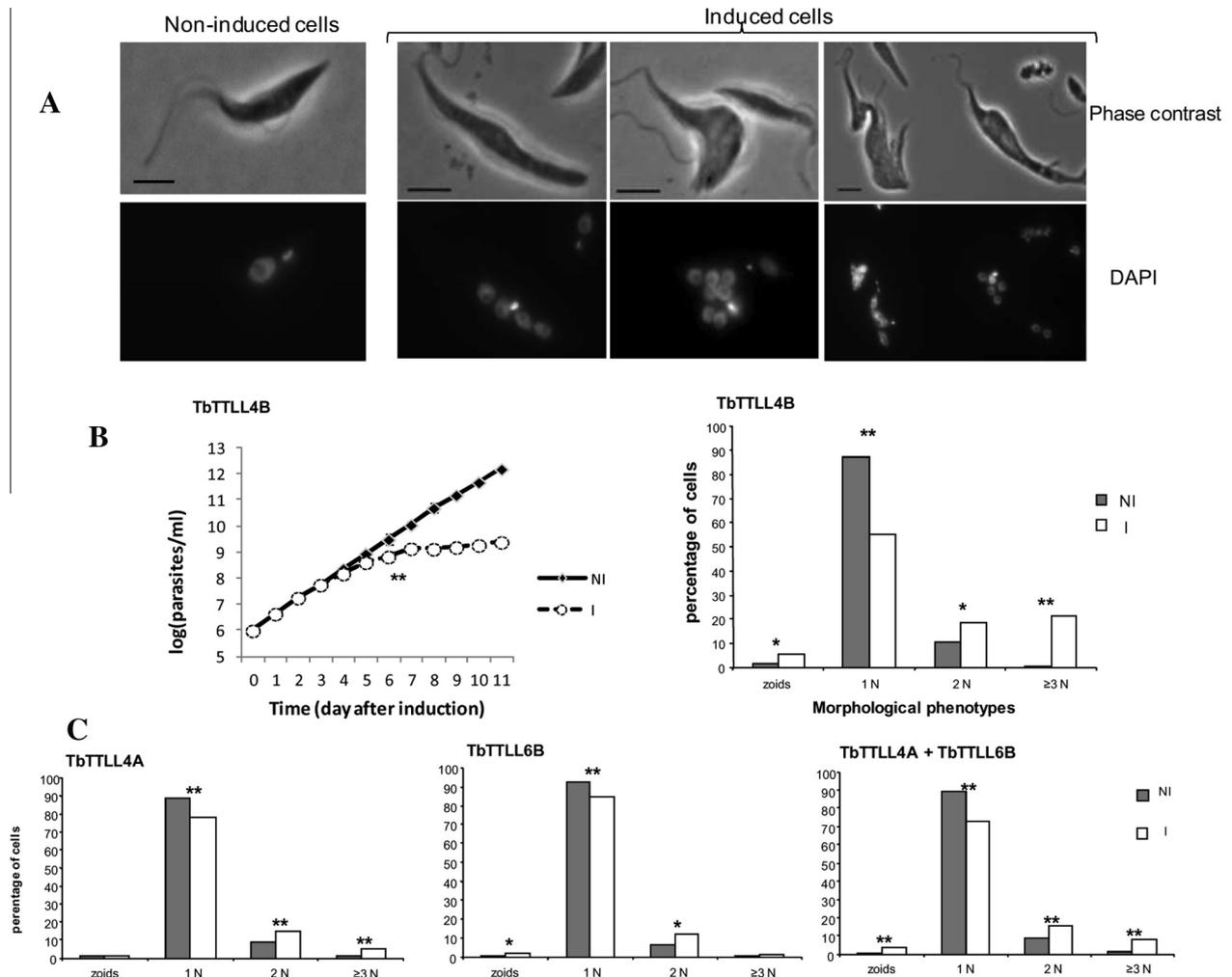
RNAi against TbTTL4B, the TLL protein identified as mitochondrial in *L. major* and with a similar mitochondria-addressing signal in *T. brucei*, induced a significant cell growth defect 5 days after induction, which was accompanied by a blockage of cytokinesis. The cytokinesis phenotype was revealed by a significant increase in the numbers of multinucleated ( $\geq 3$  nuclei) and anucleated cells termed ‘zoids’ (Robinson et al., 1995), contrasting with a low number of kinetoplasts (Fig. 8A and B).

In contrast, RNAi targeting the remaining TbTTL genes had no effect on growth, although all of those efficiently depleted their respective targets as shown by northern blots (Supplementary Fig. S6). With respect to cell morphology, a moderate but statistically significant increase in multinucleated cells and in zoids was noted in TbTTL4A- and TbTTL6B-RNAi cell lines, respectively, as well as a reduction in mononucleated cells (Fig. 8C). Since both proteins act as initiating polyglutamylases in vitro, we hypothesised that they could have redundant functions in vivo. A double RNAi for TbTTL4A and TbTTL6B was performed; induced cells exhibited no growth defect (Supplementary Fig. S7), but the cell cycle disruption appeared even more significant (Fig. 8C, Supplementary Table S2), suggesting a possible synergy between both proteins.

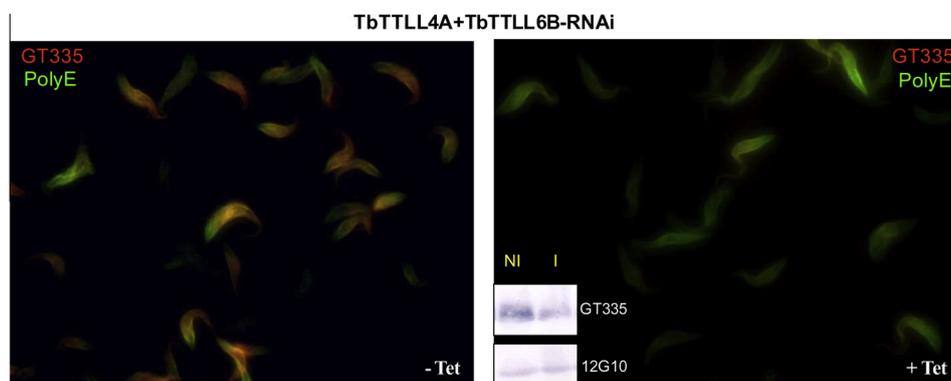
We then studied the effect of RNAi on glutamylation of MTs in TbTTL4A-RNAi, TbTTL6B-RNAi and the corresponding double RNAi cell lines, using the GT335 and PolyE Abs (Fig. 9). Cells labelled by GT335 were counted and the mean percentage of GT335-stained cells in the population determined in all lines at day 5 after tetracycline induction. After TbTTL4A RNAi induction, the fluorescence of PolyE observed by microscopy appeared not to be affected by RNAi, suggesting a compensating elongation of the side chains by other enzymes. By contrast, the proportion of GT335-labelled cells was reduced from 93.7% to 63.5% (non-induced and tetracycline-induced populations, respectively). No difference was observed after the TbTTL6B-RNAi. However, following the double TbTTL4A + TbTTL6B RNAi (Fig. 9), the reduction in the proportion of GT335-labelled cells was much greater, from 89.2% to 47.6% in non-induced and tetracycline-induced cells, respectively. The reduction in GT335-tubulin staining following double RNAi could also be seen using western blots (Fig. 9).

#### 4. Discussion

In trypanosomatids, MTs constitute a particularly essential component of the cytoskeleton, as other classical cytoskeletal components such as actin have a minimal function. As major determinants of flagellar length dynamics (Blaineau et al., 2007) and



**Fig. 8.** Phenotypes induced by RNA interference (RNAi) targeting the putative polyglutamylases TbTTL4A, -4B and -6B in *Trypanosoma brucei*. (A) Microscopic observation of TbTTL4B-RNAi *T. brucei* procyclic forms without tetracycline induction and on day 8 after tetracycline induction: phase-contrast and DAPI. Multinucleated cells became visible after induction. (B) Growth curves of non-induced (NI; closed lozenges) and tetracycline-induced (I; open circles) TbTTL4B-RNAi *T. brucei* procyclic cell lines (means  $\pm$  S.D.;  $n = 3$ ).  $**P < 0.01$  ( $t$ -test). Cell growth decreased from day 6 after tetracycline induction. Distribution of morphological phenotypes induced by TbTTL4B-RNAi induction shows an accumulation of multinucleated cells from day 7 (grey: NI; white: I). Significant differences compared with NI cell lines are indicated as follows ( $t$ -test,  $**P < 0.01$ ,  $*P < 0.05$ ). (C) Phenotype analysis after induction of TbTTL4A-, TbTTL6B- and TbTTL4A + TbTTL6B-RNAi. Five hundred to 600 cells were counted in each cell line. The number of zoids (anucleated cells) and multinucleated cells ( $\geq 3$  N) increased, especially after performing the double RNAi. Significant differences compared with NI are indicated as follows ( $t$ -test,  $**P < 0.01$ ;  $*P < 0.05$ ).



**Fig. 9.** Effect of RNA interference (RNAi) targeting putative polyglutamylases on the distribution of glutamylated microtubules (MTs) in *Trypanosoma brucei*. The distribution of glutamylated MTs was studied in TbTTL4A + TbTTL6B-RNAi cell lines (-Tet: RNAi-non-induced; +Tet: RNAi-induced) using the monoclonal antibodies GT335 (red) and PolyE (green). Only merged fluorescence images are shown. Insert: the reduction of glutamylated in the TbTTL4A + 6B double RNAi cells was also visible using western blot with GT335 and 12G10 (anti-tubulin control antibody) monoclonal antibodies (NI: non-induced cells; I: induced cells; Tet, tetracycline). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

morphogenesis (Gull, 1999), they are essential for parasites that display transformations in cell shape and organelle positioning during their life cycle (Robinson et al., 1995). Finally, and classically, they participate in intracellular transport, mitosis and cell division. Due to the particular structure of MTs, assembled from heterodimers of  $\alpha$ - and  $\beta$ -tubulin, PTMs along these structures are likely to encode novel information for the cell, both linked to the nature, length and spacing patterns of these modifications. Indeed, the modified tubulin subunits are non-uniformly distributed along MTs and, as stated previously, “analogous to the model of the ‘histone code’, diverse PTMs are proposed to form a biochemical ‘tubulin code’ that can be ‘read’ by factors that interact with microtubules” (Verhey and Gaertig, 2007). Polyglutamylation indeed affects processes such as the interaction of MTs with kinesins, MT-associated proteins (MAPs) or MT-severing factors through a modulation of affinity depending on the polyglutamate chain length (Boucher et al., 1994; Wolff et al., 1994; Larcher et al., 1996; Bonnet et al., 2001; Lacroix et al., 2010). Here, we sought to explore the extension and relevance of this process in trypanosomatids. This report presents an updated insight into MT glutamylation and is, to our knowledge, the first published study of putative glutamylases in these parasites.

Contrasting with what is known in mammalian proliferative cells, the study of the distribution of polyglutamylation confirmed that interphasic cells are highly glutamylated in trypanosomatids. As glutamylation often accumulates in stable MTs, this is consistent with the extreme stability of the cytoskeleton of trypanosomatids (Sasse and Gull, 1988), in contrast to the highly dynamic MTs in interphasic mammalian cells. Furthermore, we observed a distinctive labelling of the posterior third of the cells, which was decorated by the Ab PolyE but not by GT335. The simplest explanation for this finding would be that fewer glutamylated tubulins are present at the posterior end of the cell but they carry, on average, longer side chains. An alternative hypothesis might be that GT335 is not as insensitive to the side chain organisation as claimed. In any case, this differential distribution of polyglutamate side chains is strikingly reminiscent of the distribution of detyrosinated  $\alpha$ -tubulin. Indeed, stable MTs also often accumulate detyrosinated tubulins, whereas tyrosinated  $\alpha$ -tubulin is a marker for newly assembled MTs; these were shown, in *T. brucei*, to be more present and to elongate at this same posterior end, termed ‘dynamic’, during the G1/S phase (Robinson et al., 1995; Sherwin et al., 1987; Sherwin and Gull, 1989; our data). The differential staining between GT335 and YL1/2, which recognises tyrosinated tubulins, is in agreement with the idea that MTs are more dynamic in the posterior third of the cell. Thus, our data show a differential and dynamic pattern of MT glutamylation in the posterior end of the cell, which we hypothesise is related to active growth of the MT corset during the cell cycle.

The differential pattern of glutamylation is modified at the G2/M stage in *L. major* since, at this stage, we constantly observed intensification, as well as extension, of both GT335 and YL1/2 labelling over the whole cell body (Figs. 1 and 3). Both were particularly intense on the cytokinesis furrow during cytokinesis. Surprisingly, this was not observed in *T. brucei*, suggesting differences in the use of PTMs of MTs between both trypanosomatids, which might be related to the major differences (e.g. the symmetric/asymmetric nature) in cell division between both organisms (Wheeler et al., 2011).

Among the seven TLL proteins studied here two, LmTLL4A and LmTLL6B, appear clearly and directly involved in MT glutamylation in vitro. This function is further supported by the marked reduction in GT335 labelling in vivo when the levels of both proteins were jointly reduced. It can be stressed that both enzymes are expressed at high levels in vivo in *L. major* and *T. brucei* (Supplementary Fig. S4). In vitro glutamylation assays further

allowed classification of LmTLL6B as a  $\beta$ -tubulin initiating glutamylase. It is noteworthy that the inhibition of TbTLL4A and/or TbTLL6B had little effect on growth curves; nevertheless, zoids and multinucleated cells increased, particularly after double RNAi, thereby suggesting that polyglutamylation might (directly or indirectly) play a role in mitosis/cytokinesis. Finally, when we tested the in vitro polyglutamylation of two other well-known substrates of polyglutamylases, NAP1 and ANP32B, only LmTLL4A and LmTLL6B demonstrated clear activity on these nuclear substrates, of which two orthologues (*Lmjf.31.1750/Tb927.9.5730* and *Lmjf.19.0440/Tb927.10.15180*) are annotated in the genome sequence.

Different hypotheses could explain the failure to observe a clear in vitro glutamylation activity for the remaining recombinant LmTLL proteins: expression at low levels relative to the endogenous proteins, inhibition of the protein expression in the particular life cycle stage (promastigote) used for this study, inadequate substrates used to test for these proteins, or the lack of an activation step or cofactors for enzyme activity (Janke et al., 2005; van Dijk et al., 2007). In particular, TLL1 in higher eukaryotes is known to be active only as part of a multiprotein complex (Janke et al., 2005). Therefore, the in vitro polyglutamylation assay used here does not allow us to rule out enzymatic activity of the remaining TLL proteins.

Two putative polyglutamylases, LmTLL4A and LmTLL4C, were partially or exclusively (respectively) located at the nucleus. As seen above, LmTLL4A was strongly active on ANP32B and MTs. LmTLL4C, which is exclusively nuclear, showed weak activity on NAP1, whereas mammalian TLL4 has very strong activity on NAP1 (van Dijk et al., 2007). As a consequence, the correct substrate for LmTLL4C might be a nuclear protein that has not been tested here. Since inhibition of the expression of TLL4C did not cause any notable growth reduction, we hypothesise that this action might take place at more subtle levels of regulation or differentiation, and/or that redundancy between TLLs might occur.

Finally, LmTLL4B exhibited a highly peculiar localisation, being exclusively present in the single mitochondrion of *L. major*. This putative polyglutamylase showed no activity on either MTs or NAP1/ANP32B. It is likely that the substrates of this enzyme, which were not searched for in this study, are mitochondrial proteins. Furthermore, the inhibition of this enzyme induces growth arrest and, specifically, a blockage of cytokinesis, which is induced by its expression inhibition. In trypanosomatids, cytokinesis is directly dependent upon smooth progress of the part of the cell cycle associated with the kinetoplast, the single and complex mitochondrial DNA (Robinson et al., 1995). The identification of the substrate(s) of this enzyme should assist in clarifying the complex mechanisms regulating this original step in the cell cycle (Hammarton, 2007).

In conclusion, PTMs are essential regulators of protein function and they could be particularly important in organisms such as trypanosomatids where RNA pol II promoters are nearly absent. This study about putative polyglutamylases in trypanosomatids provides insights into an original PTM, polyglutamylation, which is extensively present in these parasites. Our data strongly suggest that LmTLL4A and LmTLL6B are bona fide MT polyglutamylases. The putative role of the other TLLs is not understood. It is paradoxical, in view of the importance of tubulins in these organisms and their extensive glutamylation, that the inhibition of most TLLs had no effect on cell growth or the cell cycle. This may suggest redundancy between polyglutamylases, as indicated here by the increased effect of the double TLL4A + TLL6B RNAi. Also, the role of polyglutamylation may be more subtle than can be shown here and/or limited in time: for example, as shown in several organisms, it may help to stabilise or, conversely, destabilise MTs; or it may produce varying signals for MAPs, MT-severing fac-

tors or molecular motors (reviewed in Janke and Bulinski (2011)). Here, as in most cases, PTMs appear not to induce a loss/gain of function but rather a subtle modulation of this function, thereby likely playing a much larger and more complex role than presently suspected, yet difficult to grasp.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ijpara.2014.09.005>.

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