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A targeted multi-enzyme mechanism for selective microtubule polyglutamylation

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Summary

Polyglutamylases are enzymes that form polyglutamate side chains of variable lengths on proteins. Polyglutamylation of tubulin is believed to regulate interactions of microtubules with microtubule-associated proteins and molecular motors. Subpopulations of microtubules are differentially polyglutamylated, yet only one modifying enzyme has been discovered in mammals. In an attempt to better understand the heterogeneous appearance of tubulin polyglutamylation, we searched for additional enzymes and report here the identification of six mammalian polyglutamylases. Each of them has a characteristic mode of catalysis and generates distinct patterns of modification on microtubules, which can be further diversified by cooperation of multiple enzymes. Polyglutamylases are restricted to confined tissues and subtypes of microtubules by differential expression and localization. In conclusion we propose a multi-enzyme mechanism of polyglutamylation that can explain how the diversity of polyglutamylation on selected types of microtubules is controlled at the molecular level.

Introduction

Posttranslational modifications are a general mechanism for the regulation of protein functions. Modifying enzymes covalently link molecules to well defined amino acid residues of the target proteins. Most modifications are reversible due to the existence of de-modifying enzymes. From a structural point of view, posttranslational modifications can be divided into either mono- or polymodifications. Phosphorylation and acetylation, which add only one molecule to a modification site, are examples of monomodifications. Polymodifications such as polyglutamylation, polyglycylation or ubiquitinylation form side chains of variable length on a single acceptor site.

Protein modifications generate signals, which are binary for monomodifications, as solely an "on" and an "off" mode exist. Polymodifications, on the other hand, generate a range of signals on a single modification site by varying the length of the added side chain. This allows for fine-tuning of the signal that, therefore, encodes fuzzy logic information. Sophisticated enzymatic mechanisms might be necessary to control such complex modification events. Here we address the enzymes and mechanisms involved in the polyglutamylation of microtubules (MTs).

MTs are major constituents of the cytoskeleton. Composed of heterodimers of conserved α- and β-tubulins, MTs might adapt to specific functions by the utilization of different tubulin isotypes as well as by posttranslational modifications. The known modifications of tubulin include monomodifications such as acetylation, phosphorylation and the reversible removal of the C-terminal tyrosine residue; and polymodifications such as polyglutamylation and polyglycylation (Luduena, 1998; Westermann and Weber, 2003). Polymodifications are added onto γ-carboxyl groups of specific glutamate residues within the tubulin tail domains and form peptide side chains of glutamate or glycine (Edde et al., 1990; Redeker et al., 1994). While polyglycylation is mostly restricted to axonemes of motile cilia and flagella (Bre et al., 1996; Iftode et al., 2000; Plessmann and Weber, 1997), polyglutamylation is found on diverse types of MTs (Westermann and Weber, 2003).

Polyglutamylation is abundant in neurons, where most α- and β-tubulins carry side chains of an average length of one to six glutamate residues (Audebert et al., 1994). Even longer side chains are found on axonemal MTs in cilia and flagella (Kierszenbaum, 2002; Lechtreck and Geimer, 2000; Million et al., 1999; Schneider et al., 1997), and also on centrioles, the core components of centrosomes and basal bodies (Bobinnec et al., 1998b). In most proliferating mammalian cells, interphase MTs are polyglutamylated at a very low level, but during mitosis the mitotic spindle accumulates polyglutamylation on β-tubulin (Regnard et al., 1999). It thus appears that MT populations can be distinguished between

different cell types as well as inside a single cell by specific polyglutamylation patterns. The parameters that specify those patterns are the density of the modification (fraction of modified tubulins within a MT), choice of the tubulin subunit (α- or β-tubulin) and isotype, choice of specific glutamate acceptor sites within the tail domains of tubulin and the length of the polyglutamate side chains.

Tubulin polyglutamylation has been proposed to be important for several MT-related processes, such as motility of cilia and flagella (Gagnon et al., 1996; Million et al., 1999), stability of centrosomes (Abal et al., 2005; Bobinnec et al., 1998a) and neurite outgrowth (Audebert et al., 1994; Ikegami et al., 2006). Due to its presence at the major binding domains of MT-associated proteins (MAPs) and MT-based molecular motors (kinesins and dyneins), polyglutamylation could contribute to the regulation of interactions between MTs and these partners (Ikegami et al., 2007, review: Nogales, 2000). In vitro binding experiments suggested that polyglutamylation is indeed such a regulator, since several MAPs and kinesin-1 associated selectively with α- or β-tubulin in some specifically polyglutamylated states (Bonnet et al., 2001; Boucher et al., 1994; Larcher et al., 1996).

The first polyglutamylase enzymes have recently been identified as members of the tubulin tyrosine ligase like (TTLL) protein family (Janke et al., 2005; Ikegami et al., 2006). The goal of the present study was the identification of tubulin polyglutamylases and the characterization of the mechanisms that assure the formation of distinctly polyglutamylated subtypes of MTs.

Results

Sequence analysis of TTLL proteins

Using genomic, cDNA and EST sequences, we reconstructed the most probable coding regions for all mammalian (murine and human) *TTLL* genes. A structural model of murine TTLL1, an α-tubulin preferring polyglutamylase, assigned domains or single amino acid residues to the catalysis of polyglutamylation (Janke et al., 2005). To identify additional polyglutamylases within the group of TTLL proteins, we compared all reconstructed murine *TTLL* sequences (Fig. S1) with *TTLL1*. The sequence alignment shows that the putative catalytic domain of all TTLL proteins, previously referred to as TTL domain, is subdivided into two regions. One part, common to all TTLLs and TTL, is now called "core TTL-domain", whereas a second conserved region upstream of it is now the "extended TTL-domain" (Fig. 1A, S2; Table S1). Within this region, only TTLL1, 2, 4, 5, 6, 7, 9, 11, 13 are highly homologous, while TTLL3, 8, 10, 12 and TTL are distinct.

All elements predicted to be essential for the ATPase reaction (yellow boxes and bold letters in Fig. S2) are found in the core TTL-domain, and are conserved between all TTLLs and TTL. On the other hand, regions of the sequences predicted as important for tubulin and glutamate interactions (green frames in Fig. S2) are localized in both, the core- and the extended TTL-domain. Especially the motifs in the extended TTL-domain distinguish the highly conserved group of TTLL1, 2, 4, 5, 6, 7, 9, 11 and 13 from the remaining TTLL proteins. Because all known mammalian polyglutamylases (TTLL1, 7; Janke et al., 2005; Ikegami et al., 2006) are contained within this group, other members are most likely polyglutamylases as well.

Apart from polyglutamylases, TTL is the only other protein of the TTLL group with a known function, as it catalyzes ligation of tyrosine to tubulin (Ersfeld et al., 1993). The extended TTL-domain of TTL is not conserved with other TTLLs, suggesting that motifs within this domain determine which amino acid is ligated by a particular enzyme. On the other hand, the general homology of all TTLLs in the core TTL-domain is most likely representing the similar reaction mechanisms of all enzymes.

To analyze the phylogenetic relationships among all mammalian TTLL proteins, we submitted the reconstructed TTLL sequences to resemblance analysis. By using three different alignment methods, we obtained a consistent arrangement of the TTLLs and TTL in a family tree (Fig. 1B). As predicted from the above analysis, TTLL3, 8, 10 and 12 as well as TTL are outgroups of the polyglutamylase group.

New active polyglutamylases are identified by overexpression in cell culture

TTLL1, the catalytic subunit of the neuronal polyglutamylase complex, is not active when overexpressed in mammalian cell culture. A similar result was obtained for the orthologous Ttll1p in *Tetrahymena thermophila*; however, another ciliate TTLL protein, Ttll6Ap, greatly increased polyglutamylase activity upon overexpression indicating that it acts in an autonomous manner (Janke et al., 2005).

Interphase MTs in HeLa cells are polyglutamylated at a low level, as revealed by weak labeling with the glutamylation-specific antibody GT335 (Wolff et al., 1992). In order to identify mammalian polyglutamylases that confer high polyglutamylase activity when overproduced, we transfected HeLa cells with plasmid vectors for overexpression of EYFP fusion proteins of all 13 murine TTLLs. GT335 labeling of MTs is increased in HeLa cells overexpressing TTLL4, 5, 6, 7, 11 and 13 (Fig. 2A), which is not the case when the corresponding ATPase-deficient proteins are expressed (data not shown; mutations equivalent to E326G in TTLL1: see Fig. S2, Table S1). This demonstrates that the observed increase in MT polyglutamylation is directly related to the enzymatic activity of the overexpressed TTLLs.

Extracts from HeLa cells overproducing TTLLs were also analyzed by Western blot. A strong increase in GT335-labeling of tubulins is observed after expression of TTLL4, 5, 6 and 11, but not TTLL7 and 13 (Fig. 2B, panel GT335). Since the latter two enzymes are active according to immuno fluorescence (Fig. 2A), the lack of signal on Western blot is most likely related to a low level of activity or transfection. This is supported by the observation that truncated versions of TTLL7 and 13 increase the levels of tubulin polyglutamylation (Fig. 2B; TTLL7S and TTLL13_N549; see also: sequence requirements; Fig. 5A, B).

Substrate specificity of polyglutamylases

Among all active polyglutamylases, only TTLL4 and 5 increase the GT335 labeling of protein species other than those present in the 50 kDa tubulin region, suggesting a multi-substrate specificity of these polyglutamylases (Fig. 2B). As for the 50 kDa region, there are two known substrates of polyglutamylation, tubulin and nucleosome assembly proteins (NAPs; Regnard et al., 2000). In order to identify polyglutamylases involved in the modification of NAPs, we performed in vitro assays using either MTs or recombinant NAP1 and NAP2. Although TTLL4, 5, 6 and 7S are highly active with MTs (Fig. 2C, 3), only TTLL4 is efficiently modifying NAPs (Fig. S3A, 3). In contrast, overexpression of TTLL4 in HeLa cells leads to a strong increase in GT335 labeling of tubulin, but not of NAPs (Fig. S3B). This is most likely related to the high level of basal polyglutamylation already present on endogenous NAPs, as compared to low level of modification on interphase MTs (Regnard et al., 2000). Thus we conclude that the majority of GT335

labeling in the 50 kDa region is associated with an increase in tubulin polyglutamylation (Fig. 2B).

Upon overexpression in HeLa cells, TTLL5, 6, 11 and 13 modify mostly α-tubulin, whereas TTLL7S shows a preference towards β-tubulin. TTLL4 as well as TTLL13_N549 modified both α- and β-tubulin. The in vitro assay (Fig. 2C) confirmed the specificity towards α-tubulin for TTLL5 and 6 and to β-tubulin for TTLL7S. However, TTLL4, which modifies both α- and β-tubulin in vivo (Fig. 2B) is a β-tubulin preferring enzyme in the in vitro test. Based on subsequent experiments showing that high enzymatic activities of certain polyglutamylases can result in the modification of both tubulin subunits (see chapter "Domain requirements for polyglutamylase activity" for more details), we conclude that TTLL4 is a β-tubulin polyglutamylase under non-saturating conditions.

Reaction specificity of polyglutamylases

The polyglutamylation reaction consists of two biochemically distinct steps, initiation and elongation. Initiation is the formation of an isopeptide bond with the γ-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). To determine if different polyglutamylases are involved in initiation or elongation, we used two different polyglutamylation-specific antibodies. GT335 recognizes all forms of polyglutamylated proteins, because it is specific to the branching point of the glutamate side chain (Wolff et al., 1992). On the other hand, the polyE antibody recognizes only long polyglutamylate side chains, probably with a minimum size of 3 glutamate residues (Wloga and Gaertig, personal communication; see also Fig. 4B). Thus, increase in GT335 labeling is indicative of initiating activity, whereas increase in polyE labeling indicates elongation activity.

Tubulins from cells expressing TTLL4, 5, and 7S are strongly labeled with GT335, whereas polyE antibody detects no (TTLL4) or very weak (TTLL5, 7S) bands. Inversely, in cells that express TTLL6, 11 and 13_N549, tubulin bands react much stronger with polyE antibody than with GT335 (Fig. 2B). These results suggest that TTLL4, 5 and 7 are side chain initiating polyglutamylases, whereas TTLL6, 11 and 13 have preference for side chain elongation.

Reaction and substrate specificity of recombinant polyglutamylases

In order to demonstrate that enzymatic characteristics of the mammalian polyglutamylases identified here are intrinsic features of the TTLL proteins, we purified several recombinant TTLLs from bacteria. Not all recombinant proteins are stable after purification, thus we used the most stable and enzymatically active truncated versions

TTLL4_C639, TTLL6_N705 and TTLL7S (Fig. 3A; see Fig. 5A for schematic representation of the truncated protein versions). In addition to the mammalian enzymes we also purified TtTtll6Ap_N655, a truncated version of the *Tetrahymena thermophila* β-tubulin elongating polyglutamylase TtTtll6Ap (Fig. S4; Janke et al., 2005; Wloga and Gaertig, personal communication). The recombinant enzymes were tested for enzymatic activity with different substrates. All four polyglutamylases display high levels of activity with MTs as substrates, but only TTLL4_C639 efficiently modifies NAP1 and NAP2 (Table S2, Fig. 3B). Preferential activities of recombinant enzymes towards α- and β-tubulin are identical to those obtained for enzymes overexpressed in HeLa cells (Fig. 3B, compare to Fig. 2C, 5C) or Tetrahymena cells (Janke et al., 2005). Thus we show that substrate preferences are intrinsic features of autonomously active polyglutamylases.

Initiating and elongating polyglutamylases can be discriminated by their specific activities towards differently pre-glutamylated MTs in vitro. We tested the recombinant TTLLs using highly polyglutamylated MTs from brain and almost unmodified MTs from HeLa cells as substrates in the enzymatic test. An initiating polyglutamylase should modify both MT substrates, whereas an elongating enzyme would preferentially modify brain MTs. TTLL6_N705 and TtTtll6Ap_N655 are highly active on brain MTs as compared to their much lower activities with HeLa MTs (Fig. 3B). This indicates that preexisting polyglutamylation is important for activity of these enzymes and classifies them as elongating polyglutamylases. However, the difference between enzymatic activities on HeLa MTs compared to brain MTs is more profound for TtTtll6Ap_N655 (5%) than for TTLL6_N705 (19%; Fig. 3B). This confirms the in vivo observation that TtTtll6Ap_N655 is an elongation-only polyglutamylase (Fig. S4) whereas TTLL6_N705 can also catalyze the initiation reaction (Fig. 5B). Nevertheless, TTLL6_N705 is more active on brain MTs in vitro, indicating that its preferential activity is side chain elongation.

TTLL4_C639 and TTLL7S are highly active on both MTs substrates, which is indicative of initiating polyglutamylase activity. Further evidence for the autonomous initiating activity of TTLL4_C639 comes from its ability to modify bacterially expressed, non-glutamylated NAPs (Fig. 3B). Taken together these results confirm the in vivo substrate and reaction specificities of several polyglutamylases.

Cooperative effects between different polyglutamylases

In the light of the separation of polyglutamylases into enzymes that have either dominant initiation or elongation activity (Fig. 2B, C, 3B), we tested whether initiating and elongating enzymes cooperate in MT polyglutamylation. We expressed the α-tubulin initiating enzyme TTLL5 together with the α-tubulin elongases TTLL6, 11 and 13. Co-

expression of initiating and elongating enzymes increase the level of long side chains on tubulin above the levels seen when these enzymes were expressed alone (Fig. 4). Long side chains are detected with B3 (only α-tubulin, side chains ≥2 glutamate residue; Gagnon et al., 1996; Kann et al., 2003) and polyE antibodies (Wloga and Gaertig, personal communication; Fig. 4B). The cooperative effect between initiating and elongating enzymes is particularly pronounced for a combination of TTLL5 and 13 detected by polyE antibody (Fig. 4A, bottom panel).

The most striking effect, though, was observed with GT335 antibody, suggesting an increase in the number of side chains above the level that is conferred by the initiating activities alone. As deduced from the Western blots, this increase is much more pronounced than the increase in long side chains and could be explained by a model taking endogenous deglutamylase activity (Audebert et al., 1993) into account (see Discussion). Taken together, these results show that different polyglutamylase subtypes can act in a cooperative manner in cells.

Domain requirements for polyglutamylase activity

Comparing the domain structure of TTLLs that belong to the polyglutamylase branch, it is striking that all enzymes active upon overexpression are relatively large as compared to the inactive TTLL1, 2 and 9 (Fig. 1). This suggested that parts of the less conserved sequences of the larger TTLLs might be required for autonomous polyglutamylase activity. To test this hypothesis, we expressed truncated versions of selected TTLLs.

Truncations did not affect the activity of polyglutamylases as long as tested proteins contained ~100 amino acids upstream of the extended TTL-domain and ~150 amino acids downstream of the core TTL-domain; further truncations yield enzymatically inactive proteins (Fig. 5A, B, C). Truncated TTLLs usually show higher levels of expression as compared to the full-length proteins (Fig. 5B, upper panel), and consistently the polyglutamylase activity levels are increased in the corresponding cell extracts (Fig. 5C). Because TTLL1, 2 and 9 are shorter than the here determined minimal size of active polyglutamylases, they might need cofactors for enzymatic activity. This is consistent with the previous finding that TTLL1 is solely active as part of a multi-protein complex (Janke et al., 2005).

Western blots with GT335 or polyE antibodies show that the preference for initiation or elongation is not altered by truncations (Fig. 5B). In vitro enzymatic assays for TTLL4 and 6 demonstrate further that the tubulin subunit preference of these enzymes remains unaffected (Fig. 5C). However, truncated versions of TTLL4 and 6 modify both tubulin subunits in vivo (Fig. 5B). To investigate why these enzymes behave differently in vitro

and in vivo, we determined α- and β-tubulin polyglutamylation in vivo at different time points following transfection of TTLL4_C639 or TTLL6_N513 expression vectors. In these experiments, the preferred tubulin subunit (deduced from in vitro activities, Fig. 5C) is always modified at first, and only later followed by polyglutamylation of the other subunit (Fig. 5D). This suggests that high polyglutamylase activities inside cells lead to saturation-like effects, which result in similar polyglutamylation levels on preferred and less preferred substrates after longer periods of time.

Localization of TTLLs in ciliated cells

To assure the modification of a selected subpopulation of MTs in a cell, the required polyglutamylases might specifically localize to the modification sites. We studied the subcellular localization of TTLLs in cells with primary cilia, because this allowed for the observation of three different MT species in a single cell: cytoplasmic MTs, basal bodies (structures similar to centrioles) and axonemes. The latter two contain highly polyglutamylated MTs and thus are potential targets for polyglutamylases (Bobinnec et al., 1998b; Million et al., 1999).

EYFP fusion proteins of all TTLLs were expressed in MDCK cells. The cells were grown to confluence and left for another 5-7 days to allow for the formation of primary cilia (Roth et al., 1988). TTLL5, 6 and 7 localize to both, basal bodies and cilia. TTLL1, 9 and 11 specifically label basal bodies while TTLL4 is present in cilia and in some cells also at the basal bodies (Fig. 6). Since TTLL4-EYFP was hardly visible, we expressed a non-tagged version of TTLL4 and visualized the overexpressed protein with anti-TTLL4 antiserum. We also noticed that despite the fact that GT335 labeling of cytoplasmic MTs is increased when active enzymes are overexpressed, none of the TTLLs is strongly associated with these MTs. Similar images of diffuse staining were already observed in transfected HeLa (Fig. 2A). This suggests that association of polyglutamylases with MTs is not the simple result of enzyme-substrate interaction, but is probably mediated by other proteins.

The non-conserved domains that are not required for enzymatic activity of polyglutamylases (Fig. 5) could be involved in intracellular targeting. To test this, we expressed a truncated version of TTLL6 (TTLL6_N705; Fig. 5). Unlike the full-length TTLL6, the truncated protein did not localize to cilia. In some cells, the basal body localization remains, but the labeling is weaker as compared to full-length TTLL6 (Fig. 6). Similar results were obtained for TTLL6_N513 and TTLL7S (results not shown), demonstrating that at least for TTLL6 and TTLL7, domains dispensable for enzymatic activity are important for specific localization of the enzymes.

Tissue-specific expression of TTLLs

Selective expression of TTLLs in different cells or tissues might influence the modification pattern of MTs. To investigate this possibility, we examined TTLL expression in nine different murine tissues. We could not detect mRNAs by Northern blot analysis, most likely due to low levels of expression. Therefore, we amplified TTLL mRNA sequences by RT-PCR. For each TTLL, primer pairs were selected for generation of PCR-products of about 1 kbp, which were verified by Southern blot. PCR products of correct size are recognized with specific probes for each TTLL, yet additional PCR-products are detected for TTLL2, 3, 6, 7, 9 and 10 (Fig. S5). These products are most likely alternatively spliced forms of those transcripts. It is striking that with the exception of testis, where all TTLLs are highly expressed, TTLLs are expressed in a tissue-specific manner. Higher expression levels of polyglutamylases in brain and testis correspond to the important role of polyglutamylation in these organs (neurons, flagella).

Discussion

New tubulin polyglutamylases

Previous biochemical studies using enriched enzyme activities from brain tissue and HeLa cells have shown that polyglutamylases can display different reaction characteristics, and, consequently, produce different polyglutamylation patterns on MTs (Regnard et al., 1999). It remained unclear how these specific activities are generated. One possibility is the existence of a single type of polyglutamylase, which acquires specificity by association with different co-factors. This model is compatible with the previously discovered neuronal polyglutamylase, because the enzyme, TTLL1, is a part of a multi-protein complex that could potentially contain exchangeable regulatory subunits. On the other hand, the identification of a second polyglutamylase, Ttll6Ap from *Tetrahymena thermophila*, showed that autonomously active enzymes exist and suggested that they have intrinsically determined enzymatic specificities.

Since all tubulin polyglutamylases identified so far belonged to the TTL domain protein family, it was likely that other enzymes were found in this group. Database searches followed by sequence reconstructions allowed us to identify 13 *TTLL* coding sequences in mouse. Besides TTLL1, eight other mammalian TTLLs group together in a phylogenetic clade and are characterized by a high degree of sequence conservation in core and extended TTL-domain (Fig. 1B). This clade contains at least six autonomously active polyglutamylases, TTLL4, 5, 6, 7, 11 and 13, which when overexpressed dramatically increase tubulin polyglutamylation in mammalian cells. The remaining TTLLs of this clade (TTLL1, 2, 9) are shorter and most likely lacking domains important for autonomous activity. It is possible that similar to TTLL1 (Janke et al., 2005), TTLL2 and 9 are polyglutamylases that acquire enzymatic activity only in complex with other proteins. Knockout studies in Tetrahymena suggest that TTLL9 is indeed a polyglutamylases (Wloga and Gaertig, personal communication).

In the outgroup of the TTLL-tree, only TTL has a known function: ligation of tyrosine to tubulin (Ersfeld et al., 1993). It is very likely that the remaining TTLL3, 8, 10 and 12 are enzymes that catalyze ligations of other amino acids to tubulin, such as glycine.

Specific properties of polyglutamylase determine the polyglutamylation patterns on MTs

All of the six autonomously active polyglutamylases differ in their preferences for either side chain initiation (TTLL4, 5, 7) or elongation (TTLL6, 11, 13) and also in their preference for α- (TTLL5, 6, 11, 13) or β-tubulin (TTLL4, 7; Table 1). These properties are consistently observed in vivo and in vitro, with enzymes expressed in HeLa cells or in

bacteria, and even with enzymes that are truncated (Fig. 3, 5). Therefore we conclude that the enzymatic characteristics of autonomous polyglutamylases are inherent features of the active domains. This suggests that the determination of specific polyglutamylation patterns on MTs might be a direct consequence of the choice of polyglutamylases. Domains of the enzymes that are not required for catalysis might have other important functions, for instance localization to specific intracellular sites as shown for TTLL6 and 7.

The existence of specialized polyglutamylases for side chain initiation and elongation strongly suggests that these enzymes cooperate in the polyglutamylation of MTs. The simplest cooperative model predicts that elongating polyglutamylases are only active when initiating enzymes have generated the branching point. This is indeed the case for TTLL13 and TtTtll6Ap (Fig. S4; Wloga and Gaertig, personal communication). Another type of elongating polyglutamylases, TTLL6 and 11, is less restricted and can also initiate new side chains.

Yet, in all tested combinations of initiating and elongating polyglutamylases, the most significant effect is the strong increase in the level of very short glutamate side chains (panel GT335, Fig. 4A). Since this result does not follow the simple cooperation model, we propose the involvement of endogenous deglutamylases. Previously described deglutamylase activities removed glutamate from polyglutamate chains in a sequential manner (Audebert et al., 1993). In conditions where only an initiating polyglutamylase is present, a large part of the newly generated branching points would be removed by the deglutamylase. When initiating and elongating enzymes are co-expressed, the long side chains generated by the elongating enzyme could titrate deglutamylase and lead to an almost unopposed initiating activity.

Tubulin subunit preference can be overwritten by saturation

All polyglutamylases described here have a preference for the modification of either α- or β-tubulin. Yet, some enzymes modify both tubulin subunits when overproduced in cell culture, most likely due to high concentration and activity of the enzyme (TTLL4 in Fig. 2B, truncations of TTLL4 and 6 in Fig. 5B). We show that the preferred tubulin subunit is modified at first, only later followed by the less favored one (Fig. 5D). It seems from these experiments that the modification of the less favored tubulin subunit is inflicted by the saturation of the preferred one. Although this was the consequence of overexpression of polyglutamylases, published observations suggest that saturation can also occur under physiological conditions. During neuronal differentiation, the polyglutamylation on α-tubulin precedes the modification on β-tubulin, and in differentiated neurons, both tubulin subunits are similarly modified with long glutamate side chains (Audebert et al., 1993;

Audebert et al., 1994). Since the major polyglutamylase activity in brain comes from the TTLL1 complex that preferentially modifies α-tubulin (Janke et al., 2005), it is possible that side chain elongation on β-tubulin is catalyzed by the TTLL1 complex after saturation of the α-tubulin substrate.

Expression and specific localization of polyglutamylases

We show that specific activities and preferences of polyglutamylases are sufficient for the determination of the polyglutamylation patterns on MTs. Consistent with this idea, polyglutamylases are expressed at higher levels in organs showing strong MT polyglutamylation, as brain and testis. Yet, in most organs a specific selection of TTLLs is expressed, which is most likely indicating the presence of specific MT polyglutamylation patterns in those tissues.

To assure the selective polyglutamylation of different subcellular MT species, intracellular localization of polyglutamylases should be restricted to the target MTs. We show that in ciliated cells, some, but not all polyglutamylases localize to the cilium and the basal bodies. However, they are not found in association with other MTs. This suggests that polyglutamylases have specific localization signals that guide them selectively to their target MTs. We show that these signals can be contained within the proteins, as TTLL6 and 7 have sequences important for ciliary localization. On the other hand, almost all polyglutamylases are found at the basal bodies. A similar tendency of TTLLs to accumulate on centrosomes was observed in other cell types including HeLa cells (Fig. 2A). It seems likely that not all of these enzymes are involved in centriole polyglutamylation, therefore basal bodies and centrosomes might gather polyglutamylases and release them to the target MTs only when they are required.

Conclusion

In the present study we propose a mechanism of MT polyglutamylation that is based on the different enzymatic characteristics of polyglutamylase enzymes. In mammals, nine tubulin polyglutamylases are available for the generation of many different subtypes of MT polyglutamylation. The relatively defined enzymatic characteristics of each polyglutamylase (Table 1) suggest that the choice of enzymes determines the final polyglutamylation pattern on MTs. Accordingly we show that polyglutamylases are expressed in a tissue-specific manner and localize to subsets of MTs within the cells. Specific combinations of different polyglutamylases and deglutamylases are thought to be the major determinants of the final modification patterns, which can further evolve due to saturation effects. In conclusion, we propose that the diversity of MT polyglutamylation in

mammals could be assured by a mechanism that is based on the specific enzymatic characteristics of the polyglutamylases and regulated by differential expression and localization of these enzymes.

Experimental procedures

Sequence determination of mammalian TTLL proteins

We searched NCBI Blast (http://www.ncbi.nlm.nih.gov/BLAST/) for sequences matching to known TTLL proteins. Each sequence was searched for in human, mouse and rat genomic DNA, cDNA and expressed sequence tags (ESTs). Every new TTLL sequence was re-submitted to BLAST. The identified sequences were aligned, corrected and assembled into a final number of 13 different *TTLL* coding sequences. No hints on additional *TTLL* genes are found in mouse and human genomes. Murine and human sequences were compared for further corrections. All murine *TTLL*s were cloned from brain or testis cDNA libraries, and several clones were sequenced, compared and gave rise to consensus sequences by exclusion of PCR-dependent mutations (Fig. S1). Nucleotide sequences were deposited in the EMBL Sequence Database as accession numbers AM690745 (*TTLL2*), AM690746 (*TTLL3*), AM690747 (*TTLL4*), AM690748 (*TTLL5*), AM690749 (*TTLL6*), AM690750 (*TTLL7S*), AM690751 (*TTLL7*), AM690752 (*TTLL8*), AM690753 (*TTLL9*), AM690754 (*TTLL10*), AM690755 (*TTLL11*), AM690756 (*TTLL12*), AM690757 (*TTLL13*).

Sequence analysis

To assign domains, all murine TTLL protein sequences and TTL were aligned with the neighbor-joining method using Clustal X (Chenna et al., 2003). The most conserved parts of the sequences were subdivided into the core- and the extended TTL-domain. A short domain containing the essential residue E326 from TTLL1 (ATP-site) is highly homologous in all TTLLs. Resemblance trees were built from the aligned TTL-domains by the maximum likelihood and parsimony methods using PYLIP (Felsenstein, 1997). Gaps were deleted and the trees were drawn with Treeview (Page, 1996). The bootstrap values are calculated with the neighbor-joining method.

Immunofluorescence and microscopy

HeLa and MDCK cells were cultured on glass coverslips under standard conditions and fixed using a protocol for preservation of cytoskeletal structures (Bell and Safiejko-Mroczka, 1995). Fixed cells were incubated with GT335 (1:3,000; Wolff et al., 1992) or anti-TTLL4 antiserum for 1 hour, followed by 30 min with anti-mouse Alexa 555 (1:1,000; Molecular Probes) or anti-rabbit Alexa 488 (1:500; Molecular Probes). DNA was visualized by DAPI staining (0.02 µg/ml). Coverslips were mounted with MOWIOL mounting medium.

We used DMRA microscopes (Leica, Germany) or LSM510 Meta (Zeiss, Germany) for confocal microscopy. Images were acquired using Metamorph (Universal Imaging Corp., USA) and Axiovision (Zeiss, Germany) software. For deconvolution and 3D-reconstruction, image stacks were processed with Huygens Pro (Scientific Volume Imaging b.v., Netherlands) using MLE algorithms. 3D restored stacks were processed with Imaris (Bitplane, Switzerland) for volume rendering.

Recombinant proteins

TTLL proteins were expressed as GST fusions in bacteria and purified on Glutathione Sepharose according to the manufacturers protocol (GE Healthcare). The identity of the purified proteins was determined by Western blot with anti-GST antiserum. Minor protein bands are also labeled with this antibody, indicating some degradation of the fusion proteins. 6His tagged NAPs were purified on a nickel-loaded Chelating Sepharose resin (GE Healthcare). The proteins were eluted with imidazole.

Polyglutamylase assay

Recombinant TTLL proteins or HeLa cell extracts were used for the determination of polyglutamylase activities as described before (Janke et al., 2005; Regnard et al., 1998). Reaction mixtures (20 μl; 50 mM Tris-HCl pH 9.0, 400 μM ATP, 2.4 mM $MgCl_2$, 500 μM DTT, 4 μM taxotere, 8 μM L–[^{3}H]-glutamate 45-55 Ci/mmol; GE Healthcare) and 0.2 mg/ml taxotere-stabilized MTs were incubated at 30°C for 2 hours. Taxotere-stabilized MTs were prepared from adult mouse brains as described (Regnard et al., 1999). Quantifications were done by scintillation counting of the α- and β-tubulin bands after SDS-PAGE and electro-transfer onto nitrocellulose, as described before (Regnard et al., 1998).

Western blot

Western blots were incubated with GT335 (1:1,000), DM 1A (1:1,000; Sigma), E7 anti-β-tubulin (1:20; Chu and Klymkowsky, 1989), anti-GFP (1:5,000; Torrey Pines Biolabs, Houston, TX), B3 (1:5,000; Abcam) or polyE anti-polyglutamylation (serum 2303; 1:1,000; courtesy of M. Gorovsky). Antisera for TTLL5, 6, 11 and 13 were raised in rabbits with bacterially expressed proteins.

Protein bands were visualized with HRP-labeled donkey anti-rabbit or -mouse Ig 1:10,000 (GE healthcare) or anti-mouse IgM (Jackson Immuno Research Laboratories), followed by detection with chemo luminescence (ECL western blot detection kit, GE healthcare).

Figure legends

Figure 1

The group of TTLL proteins in mouse.

(A) Schematic representation of the 13 murine TTLL proteins and TTL with conserved homology domains (drawing size matches real size in number of amino acids; see also Fig. S1 for sequences, Table S1 for domains and Fig. S2 for sequence alignment). The core TTL-domain is the common hallmark of the whole group of TTLL proteins (yellow box), although less conserved in TTLL12 (Striped boxes visualize low sequence conservation). The extended TTL-domain (orange box), absent in TTL and TTLL12 and poorly conserved in TTLL3, 8 and 10 is characteristic of polyglutamylases (Fig. 1B). The region essential for ATP binding (red bar) has very high sequence homology between all TTLLs. Non-conserved parts of the sequences are drawn in white.

(B) Family tree of murine TTLL proteins and TTL. Only nodes with bootstrap values >900 are considered as significant positions. Enzymes with polyglutamylase activities (Fig. 2; *TTLL1 is active in complex with other proteins; Janke et al., 2005) are outlined in red. TTL, TTLL3, 8, 10 and 12 are outgroups.

Figure 2

TTLL reaction specificity in vivo.

All TTLL proteins were tested for polyglutamylase activity.

(A) Immunofluorescence of HeLa cells transfected with TTLL-EYFP fusion constructs (green) and labeled with GT335 (red) and DAPI (blue). YFP positive cells with increased GT335 staining on MTs indicate that the corresponding TTLL protein (red box) has a polyglutamylase activity upon overexpression. Scale bar is 10 μm.

(B) Western blot of extracts from HeLa cells after expression of non-tagged TTLL proteins (GT335 represents total levels of polyglutamylation while polyE antibody labels proteins with long polyglutamate side chains, see also Fig. 4B). The gel system allows for the separation of α- and β-tubulin (Edde et al., 1987). Both tubulins are separately labeled with DM 1A and E7 anti-β-tubulin ($^{\#}$). Due to very low activity levels of the full-length proteins, truncated versions of TTLL7 (*; TTLL7S, Fig. 5A, S1) and 13 (§; TTLL13_N549, Fig. 5A) are included in the analysis.

(C) In vitro tests for polyglutamylase activity: Taxotere-stabilized MTs from brain were subjected to in vitro polyglutamylation assays with extracts from HeLa cells expressing the

respective TTLL-EYFP proteins. Control reactions were performed with enriched polyglutamylase activity from mouse brain (Janke et al., 2005) and *Xenopus laevis* egg extracts. Incorporation of ^{3}H-glutamate was measured separately for α- and β-tubulin.

Figure 3

TTLL reaction specificity in vitro.

Recombinant TTLL proteins are produced in bacteria and their enzymatic properties are analyzed in vitro.

(A) Purified GST-fusion proteins of truncated murine TTLL4, 6 and 7 and *Tetrahymena thermophila* Ttll6Ap (see Fig. 5A) were run on 8% SDS-PAGE gels and stained with Coomassie brilliant blue. The biggest and most intense bands correspond to non-degraded fusion proteins.

(B) Relative enzymatic activities of the recombinant TTLL proteins. The activities towards different substrates are expressed as percentage of the total activity towards brain MTs (total activities are given in Table S2). NAP1 and NAP2 are recombinant murine proteins produced in bacteria.

Figure 4

Cooperative polyglutamylation.

Analysis of cooperative effects between a polyglutamylase with preferential initiation activity (TTLL5) and different elongases (TTLL6, 11, 13).

(A) Western blot analysis of HeLa cells overexpressing TTLL5, TTLL6, 11 or 13 compared to cells that co-overexpress TTLL5 together with TTLL6, 11 or 13. Protein overexpression is visualized with a mixture of TTLL5, 6, 11 and 13 specific antisera from rabbits. The degree of polyglutamylation of α- and β-tubulin is determined with GT335, B3 and polyE antibodies.

(B) Schematic representation of the modification pattern detected by the respective antibodies. “E” symbolizes the glutamate residues added to tubulin. GT335 detects all polyglutamate side chains independently of their length (green E), B3 detects only side chains ≥2E (yellow) and polyE antibody is specific to long side chains of at least 3E (red).

Figure 5

Minimal sequence requirements for polyglutamylase activities.

Different truncations were generated for selected polyglutamylases and their corresponding enzymatic activities were determined.

(A) Schematic representation of the TTLL constructs used. The sequences expressed for each truncated version are depicted as black bars underneath the pictograms of the full-length TTLL proteins (number indicate length in amino acids). Domains dispensable or required for autonomous activities are shown. Note that TTLL1 is smaller than the minimal size necessary for autonomous polyglutamylase activity (see also Fig. 1A). An N-terminal truncation of TTLL6 is inactive (not included in the analysis below).

(B) Western blot analysis of extracts from HeLa cells after overexpression of EYFP-fusion proteins of full-length and truncated TTLL4, 6, 7 and 13. The level of overexpression is visualized with anti-GFP antibody. Polyglutamylation levels on tubulin are determined with GT335 and polyE antibodies (Fig. 4B). (§ lane is overexposed to visualize low expression of EYFP-fusion proteins; $^{\#}$ only a non-tagged version of full length TTLL13 has detectable activity and is therefore included)

(C) In vitro polyglutamylation test for TTLL4 and 6 and related truncations. Incorporation of ^{3}H-labelled glutamate into α- and β-tubulin is measured separately.

(D) Time course of α- and β-tubulin modification by truncated versions of TTLL4 and 6. Both enzymes modify at first their preferred tubulin isoform (β-tubulin for TTLL4 and α-tubulin for TTLL6) and only later the less preferred tubulin subunit.

Figure 6

Localization of TTLLs in cilia and on basal bodies.

TTLL-EYFP fusion proteins or TTLL4 were expressed in MDCK cells. Polyglutamylated MTs were stained with GT335 that strongly labels the axonemes of the primary cilia. Basal bodies are not detected with GT335 because of the fixation method used. TTLL4 (*) was labeled with anti-TTLL4 antiserum. The localization of EYFP-TTLL fusion proteins or TTLL4 to basal bodies or cilia is documented. Only TTLL proteins with localization to either basal bodies (yellow arrowhead) or cilia (blue arrowhead) are included in the figure (§ TTLL4 localized only in some cells to the basal bodies). Confocal 3D images of the basal bodies and the proximal part of the cilia were deconvolved and projected to 2D images shown here. Scale bar is 2 μm.

Table 1

Specific activities of polyglutamylases (* data from biochemically enriched activities; Regnard et al., 1999; § data not shown)

	substrate specificity			**reaction specificity**	
	α-tubulin	β-tubulin	NAP	initiation	elongation
TTLL4	+	+++	+++	+++	–
TTLL5	+++	+	–	+++	+
TTLL6	+++	+	–	+	+++
TTLL7	+	+++	–	+++	+
TTLL11	++	+	–	+	+++
TTLL13	++	+	–	–	++
TtTtll6Ap	+	+++	–	–	+++
Xenopus egg extract	+	+++	+++	+++§	–§
HeLa cell activity *	+	+++	+++	+++	–
neuronal activity *	+++	++	–	+++	+++

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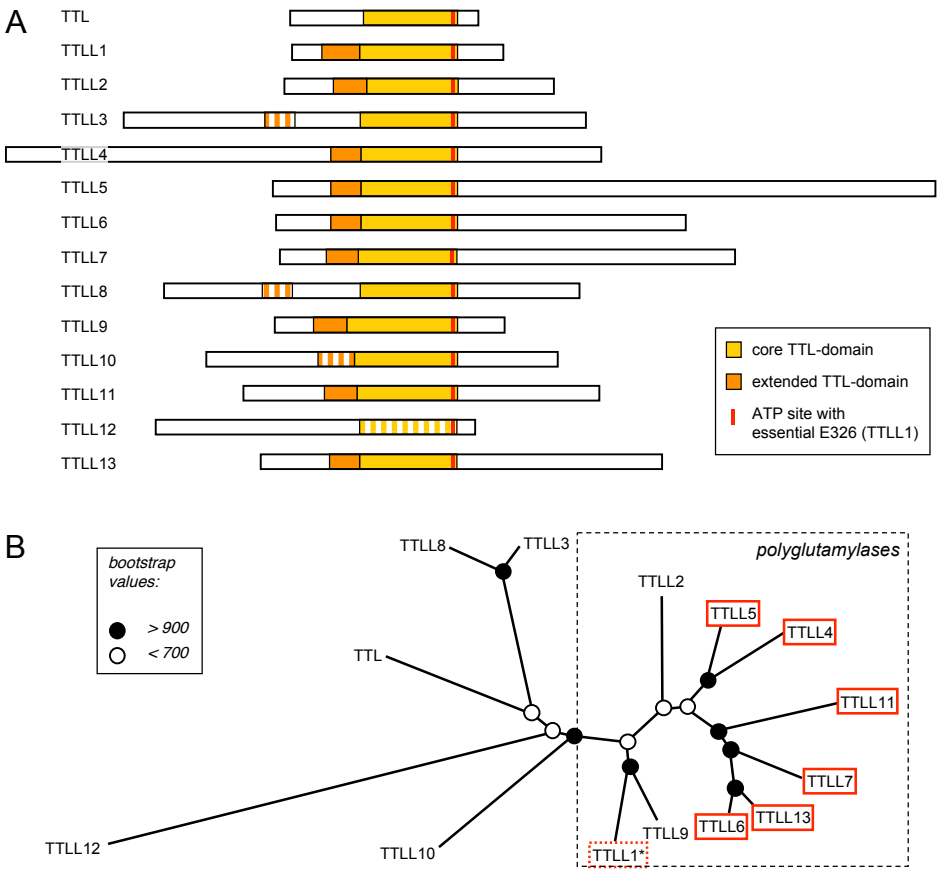

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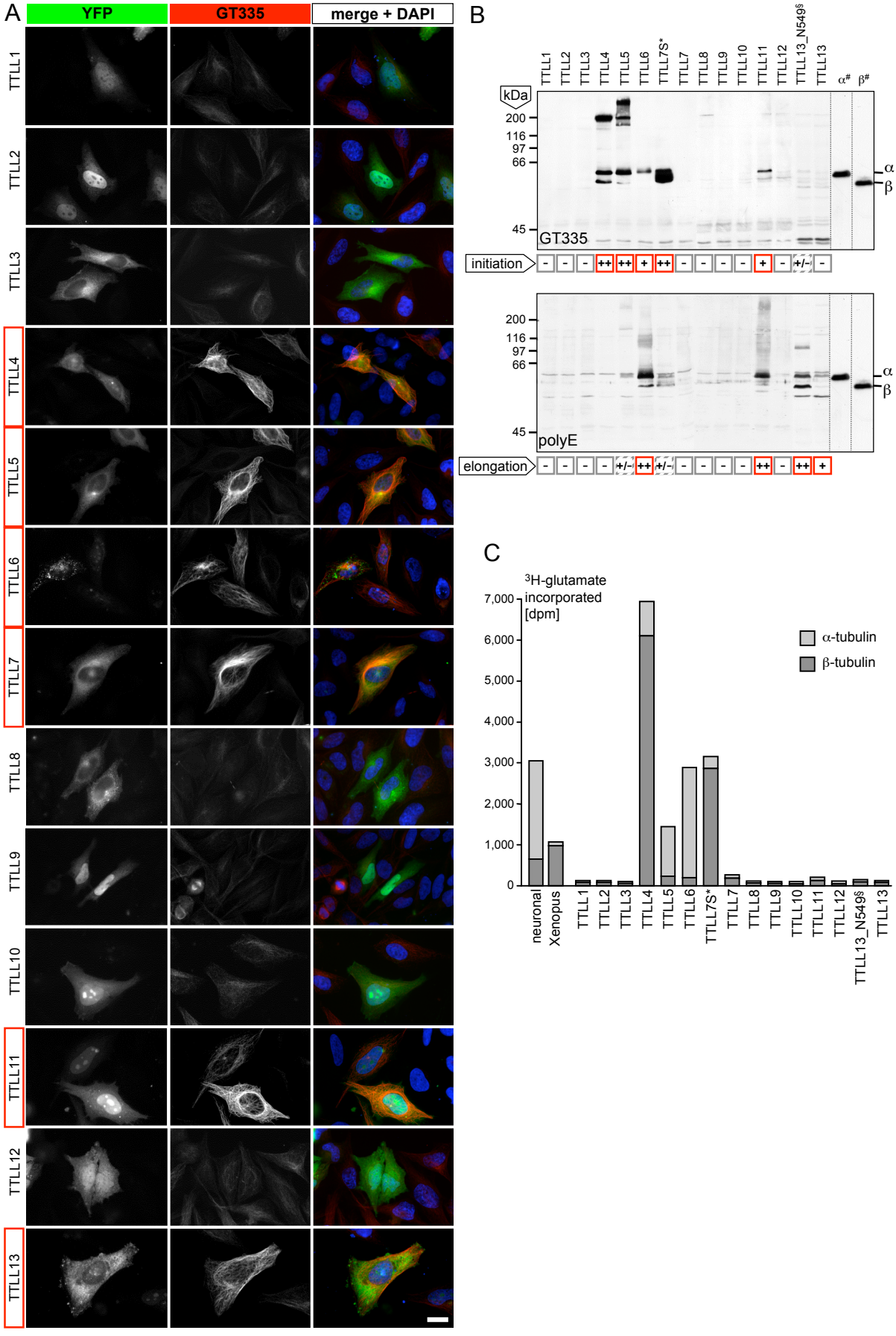

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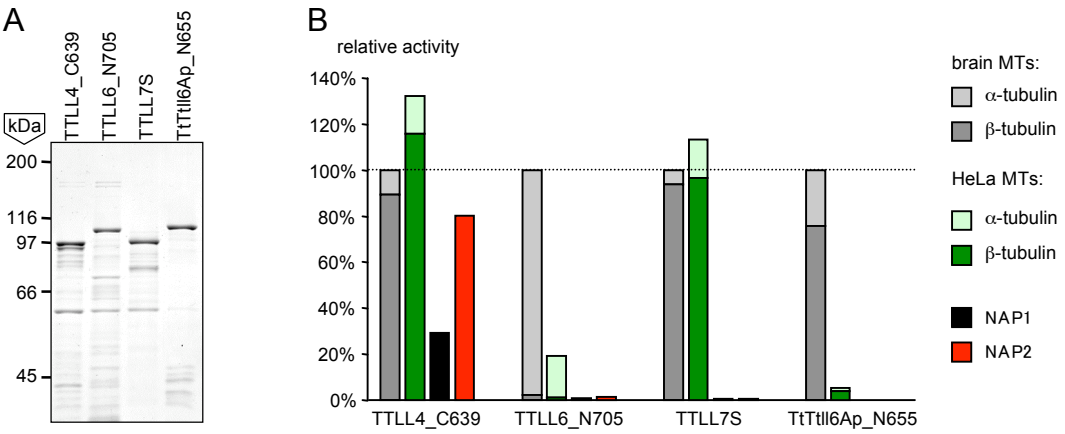

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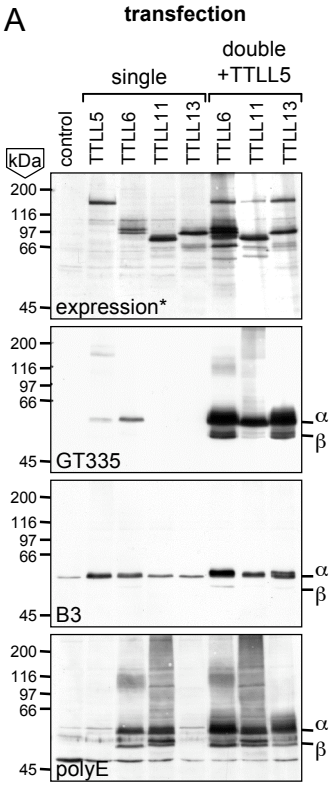

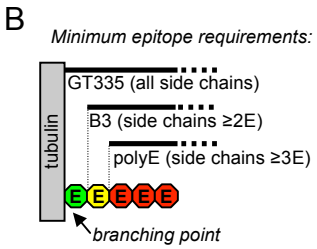

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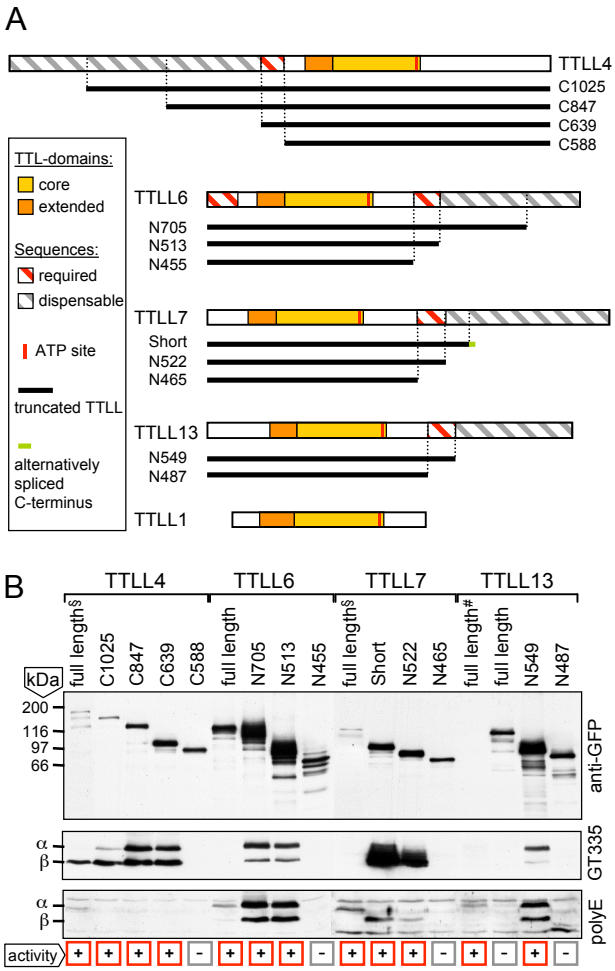

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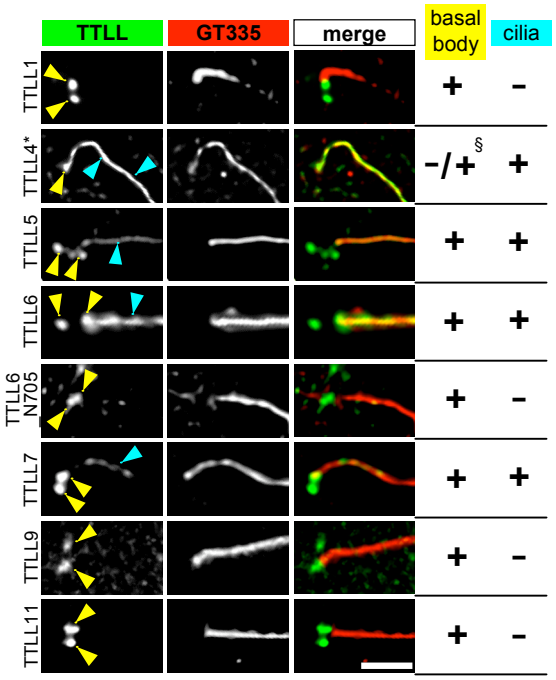

Supplementary material

Table S1: Molecular coordinates of all murine TTLL proteins and TTL (see Fig. S1, S2)

Protein	MW (kDa)	pI	Length (AA)	Start (end) AA extended TTL-domain	Start AA TTL-domain	ATP binding site (essential E)	End AA TTL-domain
TTL	43.1	6.12	377		W 147	E 331	A 337
TTLL1	49.1	8.74	423	Q 60	W 135	E 326	S 332
TTLL2	62.1	9.09	540	Q 99	W 166	E 343	A 349
TTLL3	104.4	8.33	927	Q 282 (N 344)	W 473	E 662	T 668
TTLL4	132.5	9.32	1193	Q 652	W 713	E 901	S 907
TTLL5	147.7	9.12	1328	Q 117	W 177	E 366	S 372
TTLL6	94.5	9.19	822	Q 111	Y 171	E 359	S 365
TTLL7	105.5	9.15	912	Q 93	F 157	E 349	S 355
TTLL8	94.9	9.00	833	Q 198 (S 260)	W 394	E 583	T 589
TTLL9	54.1	9.08	461	V 78	W 146	E 361	S 367
TTLL10	79.3	9.26	704	Q 225	W 298	E 499	A 505
TTLL11	82.4	9.34	727	Q 178	F 243	E 428	S 444
TTLL12	74.0	5.45	639		W 411	E 600	D 606
TTLL13	93.5	8.85	804	Q 139	Y 199	E 389	S 395

Table S2: Enzymatic activity of recombinant polyglutamylases measured by incorporation of ^{3}H glutamate (dpms) into different substrates. Total values shown in this table correspond to relative values represented in Fig. 3B.

			Recombinant enzymes			
			TTLL4 C639	*TTLL6 N705*	*TTLL7S*	*TtTtll6Ap N655*
Substrates	brain MTs	α-tubulin	10,556	10,281	710	14,830
		β-tubulin	89,565	238	10,987	46,352
	HeLa MTs	α-tubulin	16,626	1,914	1,954	751
		β-tubulin	115,983	103	11,308	2,389
	NAP1		29,248	84	77	63
	NAP2		80,430	133	73	87

Supplementary figure legends

Figure S1

Sequences of new murine TTLLs.

Consensus protein sequence of murine TTLL2 to 13 (mus musculus: mm) according to our cloning and sequencing. Clones were obtained from cDNA libraries. The longest identified splicing variant is shown. Sequences have been reconstructed because they were mostly not available from databases. At the 10/10/2006, all sequences were submitted to a Blast search (www.ncbi.nlm.nih.gov/BLAST/Blast.cgi). Only some proteins obtained matches ≥99% (% match and accession numbers).

* short version of mmTTLL7: cloned due to an alternative splicing event at position ~1793 bp of the open reading frame. Alternative 3'-sequence expressed. Version referred to as TTLL7 in Ikegami et al., 2006

alternative spliced exons found by cloning: sequence in bold, italic.

Figure S2

Partial sequence alignment of all murine TTLLs and TTL.

The sequences were aligned with Clustal X and corrected manually for the best fit of all sequences in the conserved domains. Identical residues are shown in pink, similarities in

blue. Major functional and structural elements were assigned according to the modelling of TTLL1, in which α-helices (violet boxes) and β-strands (turquoise arrows) were predicted by using glutathione synthetase as matrix (Janke et al., 2005). Secondary structure elements were corrected (added: white boxes or arrows) considering strong homologies of the present alignment. Secondary structure predication was performed using PredictProtein (http://www.predictprotein.org; Rost and Liu, 2003) and with JPred (http://www.compbio.dundee.ac.uk/~www-jpred; Cuff and Barton, 2000). Domains and residues were labeled according to the TTLL1-model: red numbers indicate the beginning of sub-domains: (1) N-terminal substrate-binding domain, (2) part 1 and (3) part 2 of the ATP-binding domain and (4) the C-terminal region. Residues that interact with ATP and Mg^{2+} are in bold letters and the corresponding domains are outlined in yellow. Predicted substrate interacting domains are outlined in green. The bars above the alignment indicate the localization of the extended TTL-domain (orange; not conserved in TTL and TTLL12) and core TTL-domain (yellow). The essential residues corresponding to E326 (TTLL1) and the surrounding domain are labeled with a red bar (Fig. 1A).

Figure S3

NAP polyglutamylation in vitro and in vivo

(A) Incorporation of radioactive glutamate into NAP1 protein was measured in an in vitro assay. Among all TTLL proteins overproduced in HeLa cells, only TTLL4 incorporated ^{3}H-glutamate into recombinant NAP1 protein. A related activity has been found in Xenopus egg extract. In contrast, brain polyglutamylase is very active for MT polyglutamylation (Fig. 2C) but cannot modify NAP1. (B) The most active truncated version of TTLL4, TTLL4_C639 was overexpressed in HeLa cells and four equivalent samples were analyzed on standard SDS-PAGE (8%, 150 mm run length) for separation of tubulins and NAPs. By separately detecting α-tubulin (DM 1A) and β-tubulin (E7), NAPs (4A8; Fujii-Nakata et al., 1992; Ishimi et al., 1985) and polyglutamylation (GT335), we show that under in vivo conditions, overexpressed TTLL4_C639 is almost exclusively increasing GT335 signals on α- and β-tubulin. Therefore, the GT335 signal in the α-tubulin region of SDS-PAGE gels used in Fig. 2B, 5B and 5D originate mostly from α-tubulin, and to a much lesser extend from NAP1 and NAP2 polyglutamylation in HeLa cells.

Figure S4

TtTtll6Ap_N655 is an elongation-only polyglutamylase.

Western blots of extracts from HeLa cells overexpressing TTLL4_C639 and TtTtll6Ap_N655 were probed with GT335 and polyE antibodies. The initiation-only

polyglutamylase TTLL4_C639 strongly increased GT335 labeling but did not generate a signal with polyE antibody. In contrast, TtTtll6Ap_N655 had no effect on GT335 labeling but generated a polyE signal on tubulins, indicating an exclusive elongating activity. Both enzymes display high enzymatic activities (Table S2, Fig. 5C), which lead to saturation-like effects resulting in the modification of both α- and β-tubulin (Fig. 5D).

Figure S5

Tissue specific expression of TTLLs in mouse.

The figure shows Southern blots of TTLL-specific RT-PCR products detected with TTLL-specific probes. Total RNA was prepared from different mouse organs with TRIZOL® reagent (Invitrogen) according to the manufacturers protocol. Total RNA content was determined at 260 nm and 5 µg of RNA were used for the synthesis of cDNA with the first strand cDNA synthesis kit (GE healthcare) following the manufacturers protocol. PCR reactions were performed with Go-taq (Promega) in 25 µl using 2.5% of the total cDNA synthesis per PCR reaction. Primer pairs were chosen for the generation of approximately 1 kbp products. The size of the PCR-products was verified; a band of the predicted size was present in each reaction. 32 amplification cycles were run for linear detection (conditions have been tested before: the expression level of TTLLs is very low). Actin was included as expression control (*). The PCR-products were transferred in alkaline conditions (0.4 M NaOH) onto Hybond-N+ membrane (GE healthcare) and incubated with ULTRAhyb® (Ambion) hybridization buffer according to the manufacturers protocol. ^{32}P-labeled probes were synthesized with Prime-It® Random primer labeling kit (Stratagene) following the manufacturers protocol, but replacing the random primers by a mixture of TTLL-specific primers.

Strong signals were highlighted in red. ($^{\#}$) For some TTLLs, also smaller RT-PCR products were detected with the specific probes, which is most probably related to alternative splicing of those gene products. (*) For the actin control we showed the ethidium bromide stained gel.

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Rost, B., and Liu, J. (2003). The PredictProtein server. Nucleic Acids Res 31, 3300-3304.

Complete protein sequences of TTLLs

mmTTLL2:

MDGLQSLGDVTAEIFGLPLEGHSVQESKPLKTEDEPQGAPLKPLVFRVDESTPGLVQSVLLERGWDKFDEQRQDVEDW
NLYWRSSSFRRAEYVNVKPWQRLNHHPGMTNLTRKDCLAKHLARMRSRYGESLYEFTPLTFIMPTDYTKFVAKYFKEK
QDLGTKPSYWICKPAELSRGRGIIIFSDIRDLMFKGTYVVQKYICNPLLVGRYKCDLRIYVCITGFKPLTIYMYQEGL
VRFATEKFDLRNLEDYYSHLTNSSINKLGASYQKIKEVVGQGCKWTLSRFFSYLRNWDVDDLLLRQKISHMVILTVLA
MAPSVPVTYNCFELFGFDILIDDNLKPWLLEVNYNPALTLDCSTDESVKRSLVHDVIELLYLNGLRSEEKKCGRTSPG
NSVVSLARSHHHEFCATPNSSSYASLIEFTTGSKSDPAVQNICPKHTRTSQLREMMSRRDRLLTKEAAKSKPRHKAWH
LPRKMVFPYASQSQPHKMKGPAGDLPEAGSTPNDHAGNFVLIFPFNKATFRASRNGLNVKRIIQELQKLMNK.

mmTTLL3:

MQGVSSALLLSAGQLGPGAAWYRQEGSSECSWLRRSQPSELRTNFSSRWPWPRNSESRRSERLQWPGPASAKPEVASC
GDSRRDYSSLPARHLSSARESSMPGALGTVNPQPVRTLVPPTLDEPLPDALRPPDDSLLLWRGLTKGPNHMGRLRNAK
IHVERAVKQKKIFMIHGRYPVIRCLLRQRGWVEKKMVHPPGTALPAPQKDLDSSMLGDSDATEDEDEEENEMFRESQL
LDLDGFLEFDDLDGIHALMSRMVRNETPYLIWTTRRDVLDCRFLSKDQMINHYARAGSFTTKVGLCLNLRNLPWFDEA
DADSFFPRCYRLGAEDDKKAFIEDFWLTAARNVLKLVVKLEEKSQSISIQAREEEAPEDTQPKKQEKKLVTVSSDFVD
EALSACQEHLSSIAHKDIDKDPNSPLYLSPDDWSQFLQRYYQIVHEGAELRYLEVQVQRCEDILQQLQNVVPQLDMEG
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YRDSYIRFSTQPFSLKNLDNSVHLCNNSIQRHLEASCHRHPMLPPDNMWSSQRFQAHLQEVDAPKAWSSVIVPGMKAA
VIHALQTSQDNVQCRKASFELYGADFVFGEDFQPWLIEINASPTMAPSTAVTARLCAGVQADTLRVVIDRRLDRSCDT
GAFELIYKQPAVEVPQYVGIRLLVEGSTIKKPVPVGHRRTGVRSSLPHLLTQQGSGESKDSGSPTHRSASRKNARAES
LEHTEKPEPAAVASVSGKGKKAPFHFPSLHSKAWLPSPRVHRPQGRVLRLQHDQLVGSKALSTTGKALMTLPTAKVLM
SFPPHPDLKLAPSMLKPGKVGFELCCTTWRVVLSGGIGEEGHRQRAAPRPSSAPGKGLSSTEPCSKTET.

mmTTLL4 (100% NP_001014974):

MASAGTEHYSIGLRRGNSFKQRHPSGTVSASPSEKPSEVKVWSQAHQQVKPIWKLEKKHVGTLSAGLGTSFLGVPSQP
AYFLCPSTLCSSGTTAVIAGHSNPCYLQSLPNLFSNTLLYRRTNVRQKPYQQLESFCLRSSPSEKRSFSLPQKGLPVS
VTANKATSSTVFPMAQPMATSPTDPYLSLAAAGENPSRKSLASAISGKIASPLSYKPMLNNNSFMRPNSTKVPLSQAT
DGLKPVSSPKIQPVSWHHSGGTGDCVPQPGDHKVPQNIATVLDDVTAPITPSIPSTLNISTASVTSSQCSQSNFRMEA
HPCGLDENPDSQSATKEVHFTEAVRKLAEKGLEKMPRQGYQFEQACFVNPSFQWGLLNRSRRWKPLMGQRFPQEDIGL
DSAILPGTSDTLGLDSTVFCTKRISIHLLASHVHGLNPSPACGSAVDPQVLGEDRAPVPPSSLQPLGVAEVATRLSSV
HLDQPGKEPEEAKDLNSCTKGGGSATDLQPNQVEPEDTEDELGDGLEDSCSHDENEEEEGDSECSSLSVVSPSESVAL
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KAWESSSRQKWIVKPPASARGIGIQVIHKWSQLPKRRPLLVQRYLHKPYLISGSKFDLRIYVYVTSYDPLRIYLFSDG
LVRFASCKYSPSMKSLSNKFMHLTNYSVNKKNTEYQANADETACQGHKWALKALWNYLSQKGINSDAIWEKIKDVVVK
TIISSEPYVTNLLKLYVRRPYSCHELFGFDIMLDENLKPWVLEVNISPSLHSNSPLDISIKGQMIRDLLNLAGFVLPN
MEDIISSSSSPSSSSGSSTSLPSSPRDKCQMTPEHFTAQKMKKAYYLTQKIPDQDFYASVLDVLTPDDVRVLVEMEDE
FSRRGQFERIFPSRISSRYLRFFEQPRYFNILTTQWEQKYHGNKLKGVDLLRNWCYKGFHTGIVSDSAPLWSLPTSLM
TTSKGDGTPNSASKSRKKSASEGTTLSSEDRSTPKSKKSQAGLSPISRKTLSSRSNENTSKQSKRSTPGLPVLKYSGQ
SSRLSAASASQSVTDSRLTAVSS.

mmTTLL5 (99% Q8CHB8):

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SILTAHGFHEVHPSSTDYNLMWTGSHLKPFLLRTLSEAQKVNHFPRSYELTRKDRLYKNIIRMQHTHGFKAFHILPQT
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LMAHVEDLIIKTIISAELAIATACKTFVPHRSSCFELYGFDVLIDNTLKPWLLEVNLSPSLACDAPLDLKIKASMISD
MFTVVGFVCQDPAQRTSNRSIYPSFESSRRNPFQKPQRTRPLSASDAEMKNLVASAREKVPGKLGGSVLGLSMEEIKV
LRRVKEENDRRGGFIRIFPTSETWEIYGSYLEHKTSMNYMLATRLFQDRGNPRRSLLTGRARVSTEGAPELKVESMNS
KAKLHAALYERKLLSLEVRKRRRRSGRLRAMRPKYPVIAQPAEMNIKTETESEEEEEVGLDNDDEEQEASQEESAGSL
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mmTTLL6:
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GNCDKKKVLEEERQRGRFLQQCPNREIRLEEVKGFQAMRLQKTEEYEKKNCGGFRLIYPGLNLEKYDKFFQDNSSLFQ
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mmTTLL7S* (100% XP_001005472):
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AAVQQEKITDLQNYQRINHFPGMGEICRKDFLARNMTKMIKSRPMDYTFVPRTWIFPSEYTQFQNYVKELKKKRKQKT
FIVKPANGAMGHGISLIRNGDKVPSQDHLIVQEYIEKPFLMEGYKFDLRIYILVTSCDPLKIFLYHDGLVRMGTEKYI
PPNESNLTQLYMHLTNYSVNKHNERFERNETEDKGSKRSIKWFTEFLQANQHDVTKFWSDISELVVKTLIVAEPHVLH
AYRMCRPGQPPGSESVCFEVLGFDILLDRKLKPWLLEINRAPSFGTDQKIDYDVKRGVLLNALKLLNIRTSDKRKNLA
KQKAEAQRRLYGQNPVRRLSPGSSDWEQQRHQLERRKEELKERLLQVRKQVSQEEHENRHMGNYRRIYPPEDKALLEK
YEGLLAVAFQTFLSGRAASFQREMNNPLKKMREEDLLDLLEQCEIDDEKLMGKTGRVRGPKPLCCMPECAEVTKKQKY
YGSSDSSYDSSSSSSNSELDENEKELCQKRLDQVPYSLKHTSHCKIIQQSSKSISPTSLEEEL.

mmTTLL7:
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PDAEELSLYRIFNRVFNRLLWSHGQGLWSCFCDSGSSWESIFSKSPEVVTPLQLQCCQRLVELCKQCLLVVYKYTTET
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mmTTLL8 (100% NP_766406):
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ACKVCQAYLGQLEHEDIDVSEASTEALSEEEWNDLTQQYYLLVHGNASITDSKSYFAQCQALLSKISSVNPQTEIDGI
RNIWIIKPAAKSRGRDIVCMDRVENILSLVAADSQTTKDNKWVVQKYIETPMLIYDTKFDIRQWFLVTDWNPLTIWFY
KESYLRFSTQRFSLDKLDSAIHLCNNSIQRRLKNDKERSPLLPCHNMWTSTRFQEYLQKRGRGGTWGSIIYPSMKRAV
TNAMRVAQDHVEARKNSFELYGADFILGRDFKPWLIEINSSPTMHPSTPVTAQLCAQVQEDTIKVVVDRKLDRNCDIG
NFELLWRQPAVELPPFNGSDLCVEGISVKKAKKQMPPIASVGLSESLLDAPPKVRSARALMETVIRPPRTTVRQDWKR
EEAKVLSTTWSMPVMDAEVRGRAKPIYAFEVNDYQHVDNKSHKSGYTRVQSSKVPGVTLTSAQHPALFAQTMKPTQMT
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mmTTLL9:

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KGIFLFRRLKDIMDWRKGTSGKKPTGVETQPARANMNPSGSHDTRSSDDQKDDLPVENYVAQRYVENPYLIGGRKFDL
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mmTTLL10[#]:

MALHPQAGRPHRDGS***EAQAEAAAQDLGRLPSPSKVGAAVCRIQGL***GHRAARRPRRGIGTTSASRVPRPGALMPATRNR
PRFIHCRGQPPRTRVSSKRSKRSRIHPCHTEVPGWTHEKQMGSSVKERLRPELSQLDQDADDLEEEEAARLPVTSPDG
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LLTTKIGLLSALREHARTLSKARMLPSTQTKVLKMEEFFPETYRLDIRDERQAFFALFDETQMWICKPTASNQGKGIF
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GS.

mmTTLL11[#]:

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LFLDETDNCHVTRII.

mmTTLL13:

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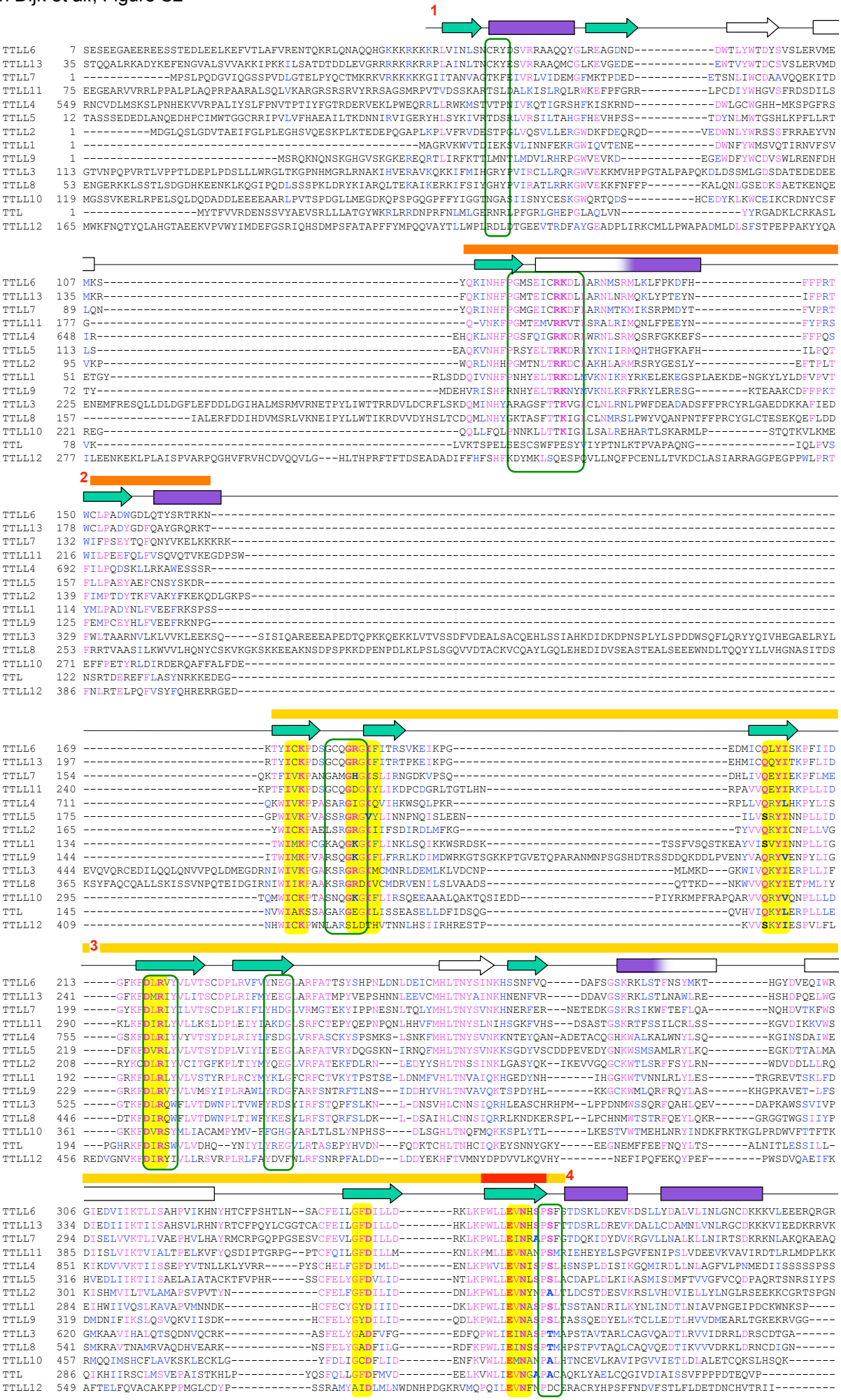
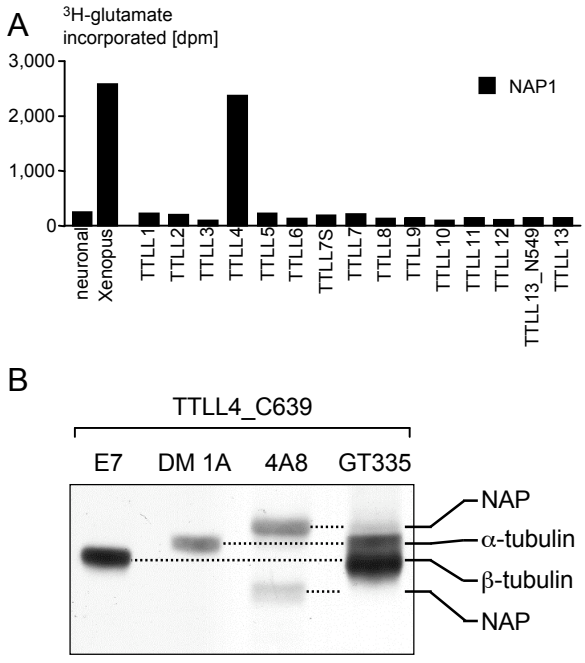
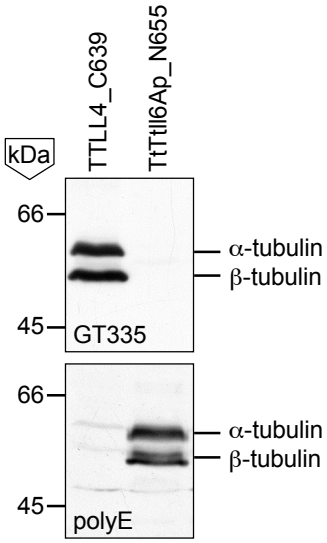
van Dijk et al., Figure S2



van Dijk et al., Figure S3



van Dijk et al., Figure S4



van Dijk et al., Figure S5