Microtubule network, an integral part of the eukaryotic cytoskeleton, plays crucial roles in cell division, differentiation, the maintenance of cell shape and polarization, motility and intracellular trafficking as well as in pathological inclusion body formation (Conde and Caceres, 2009; de Forges et al., 2012). Dynamic instability is a characteristic behavior of microtubules, it is central to their functions allowing them to rapidly reorganize, differentiate spatially and temporally in accordance with environmental signals/factors (Mitchison and Kirschner, 1984). The stability and dynamics of the microtubule network are regulated by post-translational modifications (such as acetylation) as well as by static and transient associations with different interacting partners (Microtubule Associated Proteins (MAPs) and cytosolic enzyme/proteins) (de Forges et al., 2012).

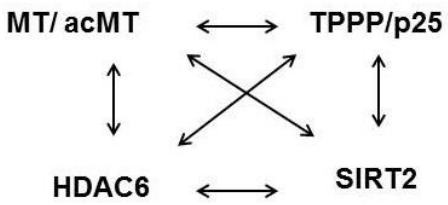
The Tubulin Polymerization Promoting Protein (TPPP/p25) was identified in Prof. Judit Ovádi' research group (CellArch) in the Institute of Enzymology, RCNS as a MAP (Hlavanda et al., 2002). TPPP/p25 is a disordered protein with extended unstructured N- and C- terminal segments straddling a flexible core region (Zotter et al., 2011). It regulates the stability and dynamics of the microtubule network via its tubulin polymerization, acetylation promoting and microtubule bundling activities (Hlavanda et al., 2002; Tőkési et al., 2010). This protein is predominantly expressed in oligodendrocytes and crucial for their differentiation (Lehotzky et al., 2010). The non-physiological level of TPPP/p25 is coupled with distinct central nervous system diseases such as synucleinopathies (Kovacs et al., 2004). TPPP/p25 and alpha-synuclein (SYN) are co-enriched and co-localized within the neurons (Lewy bodies) and oligodendrocytes (glial cytoplasmic inclusions) in the cases of Parkinson's disease (PD) and Multiple System Atrophy (MSA), respectively (Kovacs et al., 2004). The interface of the SYN-TPPP/p25 complex has been validated as an anti-Parkinson drug target (Szunyogh et al., 2015; Szénási et al., 2017).

The histone deacetylase 6 (HDAC6) is a unique member of the HDAC histone deacetylase family; it is localized within the cytoplasm and displays substrate specificity for non-histone proteins such as α-tubulin. The ubiquitously expressed HDAC6 is considered the major tubulin deacetylase (Hubbert et al., 2002; Hammond et al., 2008; Li and Yang, 2015). The tubulin deacetylase sirtuin-2 (SIRT2) is predominantly expressed in oligodendrocytes and implicated in their differentiation (Southwood et al., 2007; Harting and Knöll, 2010). The inhibitory effect of TPPP/p25 on the activity of HDAC6 due to their direct association was demonstrated with recombinant human proteins, and the enhancement of the acetylation of the microtubule network in HeLa cells expressing TPPP/p25 ectopically was visualized by immunofluorescence microscopy using specific acetyl-tubulin antibody (Tőkési et al., 2010). The TAU protein, a key MAP, is also an interacting partner of HDAC6 (Noack et al., 2014 and references therein). The acetylated TAU was detected in neuronal and glial inclusions in the case of Alzheimer's disease and other tauopathies (Cohen et al., 2011).

The objective of my OTKA PD-124061 project entitled "Potential role of TPPP/p25 in the ultrastructural organization of the multifunctional microtubular network" is the characterization of the multiple associations of TPPP/p25 with other microtubule regulatory proteins involved in both physiological and pathological processes. The interactions of TPPP/p25 with tubulin/microtubules, tubulin deacetylases, SYN and TAU as well as their functional consequences have been studied at molecular and cellular levels.

RESULTS

Effect of TPPP/p25 on the associations of HDAC6 and SIRT2 to tubulin/microtubules (MTs)



The quantification of the pair-wise interactions by ELISA was established as follows: SIRT2 was immobilized on the plate, then TPPP/p25 or tubulin was added at various concentrations and their bindings to SIRT2 were detected by specific TPPP/p25 or tubulin antibodies. The apparent dissociation constant for the binding of TPPP/p25 to SIRT2 (Kd = 32.4 ± 4.6 nM) was significantly lower than that of SIRT2-tubulin (Kd ≥ 2 μM) (Szabó et al., 2017). The functional consequences of these heteroassociations were studied by turbidity measurement, pelleting experiment and Western blot analysis using acetyl-tubulin antibody. The results revealed that SIRT2 impedes the TPPP/p25-promoted microtubule assembly independently whether it was present in its active (NAD^+) or inactive form. However, the TPPP/p25-assembled tubulin ultrastructures displayed resistance against the deacetylase activity of SIRT2. In addition, we showed that TPPP/p25 counteracted the SIRT2-derived tubulin deacetylation due to their direct association producing enhanced microtubule acetylation. The inhibition of the SIRT2 deacetylase activity by TPPP/p25 was evolved by the assembly of these tubulin binding proteins into a ternary complex. Co-localization of the SIRT2-TPPP/p25 complex on the microtubule network was visualized in HeLa cells by immunofluorescence microscopy using Bimolecular Fluorescence Complementation (BiFC) (Szabó et al., 2017, Figure 1), while SIRT2 did not co-localized with the microtubule network in HeLa cells. A significant feature of the tubulin acetylation-promoting system has been identified, TPPP/p25, as an interacting partner of both tubulin and SIRT2, can effectively promote the binding of SIRT2 to tubulin likely in a piggy-back manner ensuring a fine-tuning mechanism for the regulation of the acetylation level of the microtubule network. This control could be mediated via conformation changes within the ternary complex leading to the attenuation of SIRT2 deacetylase potency.

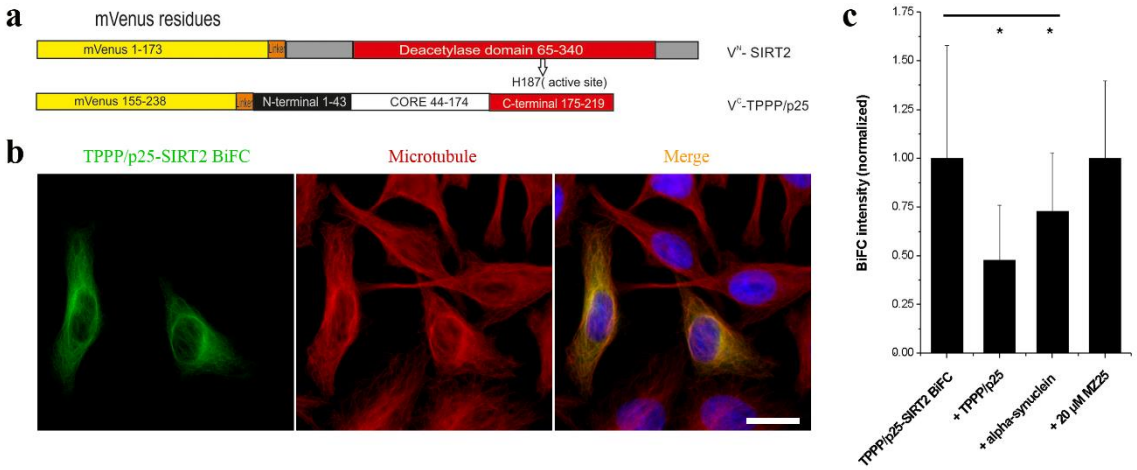
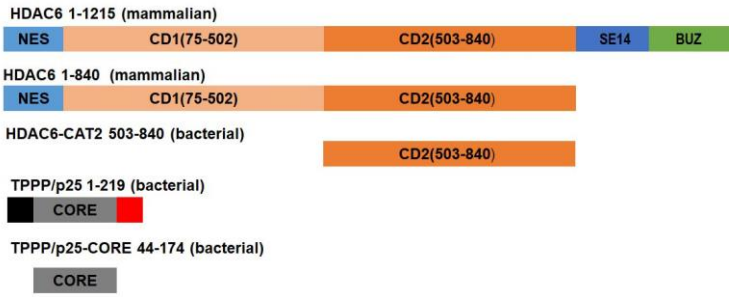


Figure 1. Interaction and localization of TPPP/p25 and SIRT2 in living HeLa cells as detected by immunofuorescence microscopy coupled with BiFC technology. (a) Scheme of the applied BiFC constructs. (b) Co-localization of the TPPP/p25-SIRT2 complex (green) with the MT network (red). MT network was stained with Alexa546, nuclei was counterstained with DAPI (blue). Scale bar: 10 μm. (c) BiFC signal (green) of the assembly of VenusN-TPPP/p25 and VenusC-SIRT2 and the effect of unlabelled TPPP/p25, α-synuclein and MZ25.

LC8-2 as a new binding partner of HDAC6 and TPPP/p25

In order to monitor the interactions of TPPP/p25 with HDAC6 and other microtubule-related proteins in living HeLa cells, the hetero-assotiation of these proteins was investigated and constructs have been successfully produced for these studies. The results obtained by applying the BiFC technology using the constructs of HDAC6 and DYNLL2/LC8-2, as a new interacting partner of TPPP/p25, have been published (Oláh et al., 2019). In conclusion, LC8-2 does not influence the acetylation of the microtubule network directly, however, the binding of LC8-2 to TPPP/p25 reduced the inhibitory potency of TPPP/p25 on HDAC6 counteracting the TPPP/p25-derived hyperacetylation of the microtubule network. On one hand, TPPP/p25 promotes the association of LC8-2 to the microtubule network that could be related to its dynamics/stabilization. On the other hand, the association of LC8-2 to HDAC6, a newly discovered heteroassociation, is promoted by TPPP/p25 suggesting the formation of a ternary complex. The formation of the binary and ternary HDAC6 complexes could have epigenetic function by modulating the intracellular localization of HDAC6, a key histone deacetylase enzyme. The multiple interactions of the microtubule-related proteins, TPPP/p25, HDAC6 and LC8-2, may display a role in the fine-tuning of the dynein-derived trafficking process partly by the modulation of the acetylation level of the microtubule network (Oláh et al., 2019).

Identification of the binding domains involved in the HDAC6-TPPP/p25 complex



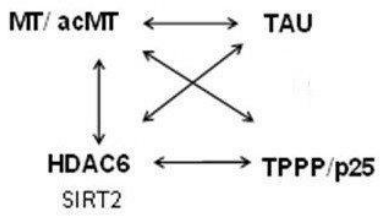
TPPP/p25 inhibits the activity of HDAC6, resulting in the hyperacetylation of the microtubule network (Tőkési et al., 2010). HDAC6 is unique among all histone deacetylases with its tandem catalytic domains designated CD1 and CD2; the catalytic activity of CD1 is highly specific for substrates bearing C-terminal acetyl-lysine residues, while CD2 is probably involved in the deacetylation of tubulin (Hai and Christianson, 2016). TAU, as a MAP protein, inhibits HDCA6 and interacts with it by the SE14 domain (Ding et al. 2008); however, the binding domains involved in the HDAC6-TPPP/p25 complex has not been investigated yet.

For the identification of the binding domain involved in HDAC6-tubulin and HDAC6-TPPP/p25 complexes, the mammalian constructs, HALO-HDAC6 2-1215 (Skultetyova et al., 2017), HA-HDAC6 1-840-FLAG and the bacterial construct pET28a-HDAC6-catalytic domain 2 (CD2 503-840) have been used (Salemi et al., 2017). The quantification of the pair-wise interactions of HDAC6 constructs was performed by ELISA experiments as follows: TPPP/p25 was immobilized on the plate, HA-HDAC6 1-840-FLAG transfected HeLa cell extract was added at various concentrations and the binding to this HDAC6 species was detected by HA-specific antibody. These studies showed that the full lenght HDAC6 and the HDAC6 1-840 constructs interact with TPPP/p25.

The further quantification of the pair-wise interactions by ELISA was established as follows: HDAC6-CD2 was immobilized on the plate, then TPPP/p25 or tubulin was added at various concentrations and their binding to HDAC6-CD2 was detected by specific TPPP/p25 or tubulin antibodies. The apparent dissociation constants for the binding of TPPP/p25 (Kd = 100-150 nM) and for that of the tubulin (Kd ~ 1 μM) to HDAC6-CD2 were determined by non-linear curve fitting. These data indicate that both protein can bind to HDAC6-CD2 providing direct evidence that CD2 is involved in the deacetylation of tubulin and for the possible binding domain for TPPP/p25 within HDAC6. The CORE segment of

TPPP/p25 binds to HDAC6-CD2 (Kd = 75-100 nM) and these data indicate that the CORE region is involved in the interaction and it is a possible binding region for HDAC6 within TPPP/p25.

Mutual effects of TAU and TPPP/p25 on their associations with the microtubule network and new interacting partners of TAU



My research also focused on the characterization of the interrelationship of the TAU protein with other microtubule regulatory proteins. These studies were carried out with ELISA using human recombinant proteins as well as with BiFC technology in living HeLa cells. These methods allowed the quantification of the interactions and the visualization of the heteroassociations of the TAU-related associations. For the isolation of TAU protein, I designed and prepared the bacterial expression construct such as His-tagged TAU T40 isoform containing residues of 1-441. In the ELISA experiments, TAU was immobilized on the plate, then TPPP/p25 or tubulin or SIRT2 were added at various concentrations and their binding to TAU was detected by specific antibodies. These studies showed that the human recombinant TAU interacts with tubulin according to the expectation, with SIRT2 and SYN, but not with TPPP/p25. In fact, the direct interaction of TAU with the C-terminal region of SYN has been recently observed (Dasari et al., 2019, Lu et al 2020).

The tubulin polymerization/aggregative potencies of both TAU and TPPP/p25 were investigated by pelleting experiment to determine the partition of the monomeric and polymerized forms of tubulins in the supernatant and pellet fractions, respectively and by turbidity measurements. Both proteins alone induced the polymerization of tubulin into microtubules, as expected. These studies also showed that the addition of TAU to the pelleting or the polymerization assay counteracted the polymerization potency of TPPP/p25 indicating their competition for tubulin binding. The binding of TAU to the C-terminal tail of microtubules exposed on the surface has been recently supported as well (Kellog et al. 2018).

The BiFC consructs related to project

In order to monitor the interactions of proteins in living cells, the following constructs have been successfully produced (see Table 1). The BiFC FRET assays are suitable for the detection of possible ternary complexes, because the mCerulean and mVenus (complemented) fluorescence proteins are a donor/acceptor pair for fluorescence resonance energy transfer (Shyu et al., 2008). The multicolor BiFC analysis provides information for the alternative interactions of a protein of interest, which can be visualized simultaneously in living cells (Kerppola, 2013). This assay is capable of parallel visualization of protein associations, the VN-CerC protein complex signal is green and CerN-CerC protein complex signal is blue and their merge is purple. For these multicolor assays, I produced mCerulean 1-158 (CerN) and mCerulean 159-238 (CerC) vector constructs such as CerN-SIRT2, CerC-Tau or CerC-TPPP/p25 constructs to test the occurrence of simultaneous interaction of these microtubule regulatory proteins. Several planned BiFC constructions were produced successfully (Szabó et al., 2017; Oláh et al., 2019; Table 1).

Table 1

	N-terminal fragments Fluorescence Protein	C-terminal fragments Fluorescence Protein	Fluorescence signal	Successful and pulished result
BiFC	VN-HDAC6	VC-SIRT2	yes, localized on MT	yes, unpublished
	VN-HDAC6	VC- TPPP/p25	yes, localized on MT	yes, unpublished
	VN-SIRT2	VC-TPPP/p25	yes, localized on MT	yes, Szabó et al., 2017
	VN-HDAC6	VC- LC8-2	yes, localized on MT and nucleus	yes, Oláh et al., 2019
	VN-TPPP/p25	VC- LC8-2	yes, localized on MT	yes, Oláh et al., 2019
	VN-HDAC6	VC-TAU	yes, diffuse distribution within cells	yes, unpublished
	VN-SIRT2	VC-TAU	yes, localized on MT	yes, unpublished
	VN- TPPP/p25	VC-TAU	no	
	VN-SIRT2	VC-TAU	yes, localized on MT	yes, unpublished
	VN-SYN	VC-TAU	yes, diffuse distribution within cells	yes, unpublished
FRET BiFC	VN-HDAC6	VC-TPPP/p25	430/535 nm FRET	yes, unpublished
	CerFL-SIRT2			
	VN-SIRT2	VC-TPPP/p25	430/535 nm FRET	yes, unpublished
	CerFL-tubulin			
	VN-HDAC6	VC-TAU	430/535 nm FRET	yes, unpublished
	CerFL-SIRT2			

I am a co-author of a review paper published in MDPI Cells focusing on the physiological and pathological functions of TPPP/p25 (Oláh et al., 2020). This review focuses on the TPPP/p25, a new microtubule associated protein, on its "regulatory functions by day and pathological functions at night". Physiologically, the moonlighting TPPP/p25 modulates the dynamics and stability of the microtubule network by bundling microtubules and enhancing the tubulin acetylation due to the inhibition of tubulin deacetylases. The optimal endogenous TPPP/p25 level is crucial for its physiological functions, to the differentiation of oligodendrocytes, which are the major constituents of the myelin sheath. Pathologically, TPPP/p25 forms toxic oligomers/aggregates with α-synuclein in neurons and oligodendrocytes in Parkinson's disease and Multiple System Atrophy, respectively; and their complex is a potential therapeutic drug target. TPPP/p25-derived microtubule hyperacetylation counteracts uncontrolled cell division. All these issues reveal the anti-mitotic and α-synuclein aggregation-promoting potency of TPPP/p25, consistent with the finding that Parkinson's disease patients have reduced risk for certain cancers. I am also a co-author of a bookchapter entitled "Dementia in Parkinson's disease" edited by IntechOpen focusing on the drug target discovery for parkinsonism.

Publications related to the PD-OTKA project

1. Szabó A, Oláh J, Szunyogh S, Lehotzky A, ***Szénási T***, Csaplár M, Schiedel M, Lőw P, Jung M, Ovádi J. (2017) Modulation of Microtubule Acetylation by the Interplay of TPPP/p25, SIRT2 And New Anticancer Agents with Anti-SIRT2 Potency. Sci Rep. 7(1): 17070 IF:4.122 Independent citation: 6
2. Oláh J, Szunyogh S, ***Szénási T***, Szaniszló T, Szabó A, Lehotzky A, Berki T, Nyitray L, Ovádi J. (2019) Interactions between two regulatory proteins of microtubule dynamics, HDAC6, TPPP/p25, and the hub protein, DYNLL/LC8. Biochim Biophys Acta Mol Cell Res. 1866(12):118556. IF:4.105 Independent citation: 1
3. Oláh J, Lehotzky A, Szunyogh S, ***Szénási T***, Orosz F, Ovádi J. (2020) Microtubule-Associated Proteins with Regulatory Functions by Day and Pathological Potency at Night. Cells. 4;9(2):357. IF:4.366 Independent citation: 2
4. Oláh J, Lehotzky A, ***Szénási T***, Ovádi J. (2021) A potential innovative therapy for Parkinson's disease: Selective destruction of the pathological assemblies of alpha-synuclein In: Lin, Zhang (Editor) Dementia in Parkinson's Disease
   London, Egyesült Királyság / Anglia : IntechOpen Publishing Company

Submitted to publication

Lehotzky A; Oláh J; Fekete JT; ***Szénási T***; Szabó E, Győrffy B, Várady G, Ovádi J. Co-transmission of alpha-synuclein and TPPP/p25 inhibits their proteolytic degradation in human cell models. Frontiers in Molecular Biosciences, submitted to publication, 2021 February

References

Cohen TJ, Guo JL, Hurtado DE, Kwong LK, Mills IP, Trojanowski JQ, Lee VMY. The acetylation of tau inhibits its function and promotes pathological tau aggregation, Nature Communications (2011), vol. 2, no. 1, article 252.

Conde C, Cáceres A. Microtubule assembly, organization and dynamics in axons and dendrites. Nat Rev Neurosci (2009) 10: 319-332

Dasari AKR, Kayed R, Wi S, Lim KH. Tau Interacts with the C-Terminal Region of α-Synuclein, Promoting Formation of Toxic Aggregates with Distinct Molecular Conformations. Biochemistry. (2019) 58(25):2814-2821.

de Forges H, Bouissou A, Perez F. Interplay between microtubule dynamics and intracellular organization. Int J Biochem Cell Biol. (2012) 44: 266-274

Ding H, Dolan PJ, Johnson GV. Histone deacetylase 6 interacts with the microtubule-associated protein tau. J Neurochem. (2008) 106(5):2119-30.

Hai Y, Christianson DW. Histone deacetylase 6 structure and molecular basis of catalysis and inhibition. Nature Chemical Biology, (2016) 12:741–747.32

Hammond JW, Cai D, Verhey KJ. Tubulin modifications and their cellular functions. Curr Opin Cell Biol. (2008) 20: 71-6.

Harting K, Knöll B. SIRT2-mediated protein deacetylation: An emerging key regulator in brain physiology and pathology. Eur J Cell Biol. (2010) 89: 262-9.

Hlavanda E, Kovács J, Oláh J, Orosz F, Medzihradszky KF, Ovádi J. Brain-specific p25 protein binds to tubulin and microtubules and induces aberrant microtubule assemblies at substoichiometric concentrations. Biochemistry (2002) 41: 8657-64

Hubbert C, Guardiola A, Shao R, Kawaguchi Y, Ito A, Nixon A, Yoshida M, Wang XF, Yao TP. HDAC6 is a microtubule-associated deacetylase. Nature (2002) 417: 455-8.

Kellogg E.H., Hejab N.M.A., Poepsel S., Downing K.H., DiMaio F., Nogales E. Near-atomic model of microtubule-tau interactions. Science (2018) 360 (6394), pp. 1242-1246.

Kovács GG, László L, Kovács J, Jensen PH, Lindersson E, Botond G, Molnár T, Perczel A, Hudecz F, Mezo G, Erdei A, Tirián L, Lehotzky A, Gelpi E, Budka H, Ovádi J. Natively unfolded tubulin polymerization promoting protein TPPP/p25 is a common marker of alpha-synucleinopathies. Neurobiol Dis. (2004) 17: 155-62.

Kerppola TK. Multicolor bimolecular fluorescence complementation (BiFC) analysis of protein interactions with alternative partners. Cold Spring Harb Protoc (2013) 9:798-803.

Lehotzky A, Lau P, Tőkési N, Muja N, Hudson LD, Ovádi J. Tubulin polymerization-promoting protein (TPPP/p25) is critical for oligodendrocyte differentiation. Glia (2010) 58: 157-68

Li L, Yang XJ. Tubulin acetylation: responsible enzymes, biological functions and human diseases. Cell Mol Life Sci. (2015) 72: 4237-55.

Lu J, Zhang S, Ma X, Jia C, Liu Z, Huang C, Liu C, Li D. Structural basis of the interplay between α-synuclein and Tau in regulating pathological amyloid aggregation. J Biol Chem. (2020) 22;295(21):7470-7480.

Mitchison T, Kirschner M. Dynamic instability of microtubule growth. Nature (1984) 312: 237-242

Noack M, Leyk J, Richter-Landsberg C. HDAC6 inhibition results in tau acetylation and modulates tau phosphorylation and degradation in oligodendrocytes. Glia (2014) 62:535–547.

Salemi LM, Maitland MER, Yefet ER, Schild-Poulter C. Inhibition of HDAC6 activity through interaction with RanBPM and its associated CTLH complex. BMC Cancer. 2017 1 17 1:460.

Shyu YJ, Suarez CD, Hu CD. Visualization of ternary complexes in living cells by using a BiFC-based FRET assay. Nat Protoc. (2008) 3 11:1693-702.

Skultetyova L, Ustinova K, Kutil Z, Novakova Z, Pavlicek J, Mikesova J, Trapl D, Baranova P, Havlinova B, Hubalek M, Lansky Z, Barinka C. Human histone deacetylase 6 shows strong preference for tubulin dimers over assembled microtubules. Sci Rep. (2017) 14;7 1:11547.

Southwood CM, Peppi M, Dryden S, Tainsky MA, Gow A. Microtubule deacetylases, SirT2 and HDAC6, in the nervous system. Neurochem Res. (2007) 32: 187-95.

Szénási T, Oláh J, Szabó A, Szunyogh S, Láng A, Perczel A, Lehotzky A, Uversky VN, Ovádi J. Challenging drug target for Parkinson's disease: Pathological complex of the chameleon TPPP/p25 and alpha-synuclein proteins. Biochim Biophys Acta. (2017) 1863:310-323.

Szunyogh S, Oláh J, ***Szénási T***, Szabó A, Ovádi J. Targeting the interface of the pathological complex of α-synuclein and TPPP/p25. Biochim Biophys Acta. (2015) 1852: 2653-61.

Tőkési N, Lehotzky A, Horváth I, Szabó B, Oláh J, Lau P, Ovádi J. TPPP/p25 promotes tubulin acetylation by inhibiting histone deacetylase 6. J Biol Chem. (2010) 285: 17896-906

Zotter A, Bodor A, Oláh J, Hlavanda E, Orosz F, Perczel A, Ovádi J. Disordered TPPP/p25 binds GTP and displays Mg2+-dependent GTPase activity. FEBS Lett. (2011) 585: 803-8