INTERACTIONS OF THE DISORDERED TPPP/p25
WITH PHYSIOLOGICAL AND PATHOLOGICAL PARTNERS

Sándor Szunyogh

Eötvös Loránd University
Biology Ph. D. School (School Leader: Prof. Anna Erdei)
Molecular Cell and Neurobiology Program (Program Leader: Prof. Miklós Sass)

Supervisor: Prof. Judit Ovádi, D. Sc.
Professor Emerita
Laureata Academiae

Institute of Enzymology
Research Centre for Natural Sciences
Hungarian Academy of Sciences

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### 1. ABBREVIATIONS

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BiFC</td>
<td>Bimolecular fluorescence complementation</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>CD</td>
<td>Circular dichroism</td>
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<tr>
<td>cELISA</td>
<td>Cellular enzyme linked immunosorbent assay</td>
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<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle medium</td>
</tr>
<tr>
<td>DTE</td>
<td>Dithioerythritol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced green fluorescent protein</td>
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<tr>
<td>ERK2</td>
<td>Extracellular signal-regulated kinase 2</td>
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<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<tr>
<td>FL</td>
<td>Full length</td>
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<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
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<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>IDP</td>
<td>Intrinsically disordered protein</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani medium</td>
</tr>
<tr>
<td>MAP</td>
<td>Microtubule associated protein</td>
</tr>
<tr>
<td>ME</td>
<td>2-mercaptoethanol</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-morpholino)ethanesulfonic acid</td>
</tr>
<tr>
<td>MSA</td>
<td>Multiple system atrophy</td>
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<tr>
<td>MT</td>
<td>Microtubule</td>
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<td>MTOC</td>
<td>Microtubule organizing center</td>
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<td>NMP</td>
<td>Neomorphic moonlighting protein</td>
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<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
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<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>NMWL</td>
<td>Nominal molecular weight limit</td>
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<tr>
<td>OLG</td>
<td>Oligodendrocyte</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>PD</td>
<td>Parkinson’s disease</td>
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<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
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<tr>
<td>SNARE</td>
<td>Soluble N-ethylmaleimide-sensitive factor attachment protein receptor</td>
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<tr>
<td>SYN</td>
<td>α-synuclein</td>
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<tr>
<td>TPPP/p25</td>
<td>Tubulin Polymerization Promoting Protein/p25</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>ΔC TPPP/p25</td>
<td>C-truncated TPPP/p25 (Δ175-219)</td>
</tr>
<tr>
<td>ΔN TPPP/p25</td>
<td>N-truncated TPPP/p25 (Δ3-43)</td>
</tr>
<tr>
<td>CORE TPPP/p25</td>
<td>Terminally double truncated TPPP/p25 (Δ3-43/ Δ175-219)</td>
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2. INTRODUCTION

Moonlighting proteins comprise proteins with multiple, independent functions that do not originated from alterations of the genetic code, splice variants or post-translational modifications (Jeffery 1999). There are multifunctional proteins which physiological functions can be switched, converted into a pathological one, described as neomorphic moonlighting proteins (NMP, Jeffery 2011). Their interactions with distinct protein partners could lead to the formation of protein aggregates that form brain inclusions. Such destructive potential is characteristic for the intrinsically unstructured proteins (IDP) which do not have well-defined three-dimensional structure, yet fulfill essential functions linked to their structural states; however, their macromolecular assemblies frequently result in pathological inclusions (Uversky 2014). Tubulin Polymerization Promoting Protein/p25 (TPPP/p25) (Hlavanda et al. 2002) is a prototype of the NMPs (Ovádi 2011, Oláh et al. 2013): physiologically it modulates the dynamics and stability of the microtubule (MT) network via its tubulin polymerization and acetylation promoting, MT bundling activities (Hlavanda et al. 2002, Tirián et al. 2003, Tőkési et al. 2010); but it also plays a role in different neurological disorders due to its interactions with α-synuclein (SYN) and β-amyloid, which promote aggregation (Kovács et al. 2004, Oláh et al. 2011).

TPPP/p25 is an IDP, its extended unstructured N- and C-termini are straddling a flexible core region (Orosz et al. 2004, Zotter et al. 2011a). Among the binding segments, the guanosine triphosphate (GTP, Zotter et al. 2011a) and Zn²⁺ (Zotter et al. 2011b) are well characterized, while the tubulin binding motif was predicted based upon its homology with the tubulin binding motif of tau protein (Hlavanda et al. 2007). It has been shown that Zn²⁺ binds to a specified segment of the middle, flexible region of TPPP/p25 resulting in alteration in the secondary but not in the tertiary structure (Zotter et al. 2011b); however, the physiological relevance of the binding was not investigated in detail.

In normal brain, TPPP/p25 is predominantly expressed in oligodendroglial cells (Takahashi et al. 1993, Skjoerringe et al. 2006); where it is indispensable for the differentiation of the progenitor cells by its role in the rearrangement of the MT network in the course of the projection elongation necessary to axon ensheathment (Lehotzky et al. 2010). The non-physiological TPPP/p25 expression can generate distinct central nervous system (CNS) diseases: i) the loss of TPPP/p25-positive oligodendrocytes (OLGs) in demyelinated lesions with a
concomitant enrichment of TPPP/p25 in the cerebrospinal fluid of patients is characteristic for multiple sclerosis (Höftberger et al. 2010, Vincze et al. 2011); ii) the lack of TPPP/p25 expression in OLGs is typical in glioma (Preusser et al. 2007); iii) the abnormal co-enrichment of TPPP/p25 with SYN in the inclusion bodies of OLGs and in neurons is representative for multiple system atrophy (MSA) and Parkinson’s disease (PD) (Kovács et al. 2004), respectively, highlighting TPPP/p25 as a powerful diagnostic marker and a potential drug target.

SYN, also a disordered NMP (Uversky 2003), is an extensively studied hallmark protein of synucleinopathies, such as dementia with Lewy bodies, PD and MSA; it is the major component of inclusion bodies in these diseases (Stephanis 2012, Vieira et al. 2015, Kim et al. 2014). SYN is a neuronal protein that under physiological conditions is localized preferentially to presynaptic terminals (Iwai et al. 1995, Murphy et al. 2000), but its exact function is still unclear. Recently it has been reported that its small, soluble oligomeric forms are the fatal species in the development of diseases (Dehay et al. 2015, Roberts et al. 2015, Winner et al. 2011). Nevertheless, no effective and specific drug has been developed for the therapeutic treatment of PD and other synucleinopathies.

The major objective of my PhD thesis was to characterize the interactions of the disordered TPPP/p25 with its physiological partners such as tubulin/MT and the bivalent Zn$^{2+}$ cation as well as with its pathological partner, SYN; and to establish the functional consequences of these interactions at molecular and cellular levels. In addition, I aimed to search for a potential drug target in order to specifically destruct the pathological assembly of TPPP/p25 and SYN.
3. SCIENTIFIC BACKGROUND

3.1. The cytoskeleton of eukaryotes

The cytoskeletal network of most eukaryotic cells comprises three different types of complex ultrastructural filamentous networks: actin filaments, intermediate filaments and the MT network, which display multifarious functions. In the course of growth, proliferation or differentiation the cytoskeletal network has to be rearranged derived by its highly dynamic structure undergoing constant and rapid reorganization (Alberts 2014 and references therein). In an animal cell, actin filaments are found in the cell cortex, which is the meshwork of membrane-associated proteins that supports and strengthens the plasma membrane, providing strength and shape necessary for the locomotion of the cell. It also allows cells to hold certain shapes, such as microvilli, lamellipodia and filopodia; as well as it is involved in contractile ring formation, which creates the cleavage furrow during mitosis. Intermediate filaments are made of a large and heterogeneous group of subunits, which form generally strong ropelike structures, providing mechanical support. Some cells have multiple types of intermediate filaments and some are associated with specific cell types. For example, keratins are found specifically in epithelial cells, neurofilaments help to extend the long axons in neurons; while lamins are found in all cell types, providing structural reinforcement to the cell nucleus. The MT system provides platforms for intracellular transport, including the movement of motor proteins with macromolecular assemblies, vesicles and also organelles; it forms the mitotic spindle, which helps the rearrangement of the dividing cell; and it is also the main constituent of the organelles cilia and flagella responsible for cellular movement (Alberts 2014).

3.1.1. The microtubule network

Microtubules are polymers of two closely related globular proteins, \( \alpha\)- and \( \beta\)-tubulin, which associate spontaneously into a functional subunit. Tubulin polymers assemble into protofilaments and 13 protofilaments form the MT. It is the largest type of filaments with a
diameter of about 25 nm. The MT is a hollow structure; its inner diameter is about 12 nm. In eukaryotic cells, the polymerization of tubulin dimers typically starts in the microtubule-organizing center (MTOC) in the centrosome, attached to a γ-tubulin ring complex. The tubulin dimer assembles into MT with its α-tubulin part to the β part of another tubulin already incorporated into the MT, thus the polymerization is a polar process, which reflects not only the polarity of the tubulin dimer, but dictates the polarity of the MT as well (Howard et al. 2003). The end of the MT attached to the centrosome is called the minus end, while the other, where both the growth and the shrinkage are fast, is called the plus end (Alberts 2014). Both α- and β-tubulin bind GTP, but it is only exchangeable in β-tubulin, which possesses a GTPase activity by hydrolyzing it to guanosine diphosphate (GDP). The presence of GTP or GDP on β-tubulin influences the stability of the dimer in the MT: if β-tubulin is bound to GTP, the tubulin dimer tends to assemble into MTs, while if β-tubulin is bound to GDP, the microtubule tends to fall apart, since the GTP hydrolysis weakens the binding affinity of tubulin for adjacent tubulin dimers. Therefore, GTP hydrolysis is a main factor in the dynamic instability of the MTs. As long as GTP-bound tubulin dimers are added more rapidly than GTP is hydrolyzed, a cap of GTP-bound tubulin exists at the plus end, protecting the growing MT from disassembly. Without the sufficient rate of incorporation, the GDP-bound tubulin rapidly depolymerizes, thus the MT occasionally switches to rapid shrinkage, which is called MT catastrophe, then dynamically it can be rescued by switching back from shrinkage to growth. MT catastrophe is a major mechanism for MT length regulation (Gardner et al. 2013).

### 3.1.2. Microtubule associated proteins

There are a number of protein families which regulate the stability of the MT. Microtubule associated proteins (MAPs) were originally identified as proteins co-purified with MTs during repeated cycles of MT assembly and disassembly, meaning the tightly associated (static) MAPs only (Olmsted 1986); however, this definition has been broadened to involve the dynamically associated proteins as well (Mandelkow et al. 1995). MAP activity is controlled by kinases and phosphatases (Cassimeris et al. 2001). Well known proteins belong to static MAPs: MAP1a and 1b, MAP2a, 2b, and 2c, MAP4, tau protein, 205 kDa MAP, and the isoforms of these proteins that
are often generated by alternative splicing (Mandelkow et al. 1995). These structural MAPs stabilize and promote the assembly of MTs in various ways, they attach to each other to form scaffolds or bundles the MTs (Alberts 2014), these MAPs organize the individual filaments into long lived and well organized tracks (Richter-Landsberg 2008) and protect the MT against disassembly (Denarier et al. 1998). The MAP2/Tau family possesses repeated tubulin binding domains, which allows them to associate with more than one heterodimer, holding the protofilaments together (Dehmelt et al. 2005). Another protein family, the stable tubule only (STOP) proteins may contribute to the cold and drug stability of MTs (Slaughter et al. 2003).

Not all MAPs stabilize the MT: catastrophe factors destabilize the tubulin polymer by increasing the frequency of catastrophes such as Kip3 (Varga et al. 2009) and MCAK (Ritter et al. 2015) or inhibiting nucleation (Wieczorek et al. 2015). Microtubule-severing enzymes are also important MT regulators (Sharp et al. 2012). Katanin is capable of disrupting contacts between α-β-tubulins in the wall of MTs (Zhang et al. 2007). Spastin (Roll-Mecak et al. 2008) and fidgetin (Mukherjee et al. 2012) could release MT minus ends from centrosomes; spastin forms a hexameric ring and pulls the C-terminus of tubulin through its central pore, therefore destabilizes the MT lattice (Roll-Mecak et al. 2008). The regulation of the MT network is a complex process due to the interplay of many proteins, which function by a variety of mechanisms.

Other proteins often found to be associated and even co-purified with MTs, although they are usually not considered as MAPs (Mandelkow et al. 1995), e.g. kinases (protein kinase A, GSK-3 and c-mos), MT motor proteins (kinesin, dynein), proteins involved in biosynthesis (elongation factor EF-la, purinosome complex) (Durso et al. 1994, An et al. 2010) and glycolytic enzymes (glyceraldehyde-3-phosphate dehydrogenase, aldolase, phosphofructokinase) (Knoll et al. 1992, Lehotzky et al. 1993, Vértesy et al. 1997).

3.2. The Tubulin Polymerization Promoting Protein/p25

A protein with random coil structure was isolated from bovine brain by the research group of Prof. Judit Ovádi and was denoted Tubulin Polymerization Promoting Protein (TPPP/25) on the basis of its molecular mass and in vitro function: it polymerizes tubulin into intact and aberrant
MTs and also bundles them as shown by electron microscopic images (Fig. 1, Hlavanda et al. 2002 and 2007).

Fig. 1. Transmission electron microscopy images of the TPPP/p25-induced tubulin assemblies (A, B). A and B, bundles of MTs sectioned at different angles and thread-like oligomers aggregated into dense knobs are shown. The MTs are frequently covered by tiny projections and periodically arranged dense particles (B), which form cross-links between MTs (see inset in panel B). Bars, 200 nm in A, 100 nm in B and 50 nm in the inset. (Hlavanda et al. 2007)

3.2.1. The disordered nature of TPPP/p25

There are several algorithms (PONDR: Romero et al. 1997; IUPred: Dosztányi et al. 2005; FoldIndex: Prilusky et al. 2005, Uversky et al. 2000) which predict a protein to be structurally disordered based partially or completely on amino acid composition and properties (charge, hydrophobicity, accessibility by the solvent).

Using the sequence of TPPP/p25, the Predictor of Natural Disordered Regions (PONDR) algorithm was used to characterize the disordered state of the protein (Fig. 2). The PONDR VLEXT (Romero et al. 1997) considers a region to be disordered if there is at least a 40 aa long
continuous sequence above the threshold of 0.5 (Romero et al. 2001). The graph shows the predicted disorder scores as the function of sequence. This value can vary between 0 and 1 according to the probability that a given amino acid is a part of a disordered region. The 52 aa long region in the N-terminus of TPPP/p25 is considered to be disordered (PONDR score ~1), while the algorithm shows that the middle part of the protein is more ordered, approximately 40 residues are definitely below the threshold (PONDR score ~0). Clear conclusion cannot be drawn from the values of the C-terminus, the shorter sections alternate. Overall, PONDR predicts TPPP/p25 as disordered (45.66%). TPPP/p25 was characterized as an IDP (Hlavanda et al. 2002, Orosz et al. 2004).

Fig. 2. PONDR VL-XT prediction of structural disorder of TPPP/p25. Disorder prediction values (PONDR scores) for a given residue are plotted against the residue number. The significance threshold is shown (0.5), above which residues are considered to be disordered.

Different experimentally proven characteristics of TPPP/p25 (heat stability, high pI, proteolytic sensitivity) also suggested that this protein belongs to the group of IDPs. Circular dichroism (CD) revealed largely random coil conformation with some α-helix content (Hlavanda et al. 2002); Dichroweb (Whitmore et al. 2004) analysis of the spectrum (Fig. 3) showed 14% α-helix, 26% β-sheet and 57% disordered region in TPPP/p25.
Fig. 3. Dichroweb plot for TPPP/p25. Measured circular dichroism (CD) spectrum of TPPP/p25 (green), reconstructed data (blue), and the difference (magenta) are shown. For the analysis, SELCON3 was used with protein data set SET7 (http://dichroweb.cryst.bbk.ac.uk/html/home.shtml).

These data were further corroborated by multinuclear nuclear magnetic resonance (NMR) analysis, which showed that extended unstructured segments of TPPP/p25 are localized at the N-(45 aa) and C-termini (44 aa) straddling a flexible region (130 aa) (Zotter et al. 2011a).

3.2.2. Segments of TPPP/p25 involved in its interactions

TPPP/p25 can be phosphorylated in vitro on multiple sites by the Cdk5, ERK2 and PKA kinases (Fig. 4) (Hlavanda et al. 2007). It was identified from bovine brain extract as an ERK2 binding protein. The kinase regulates TPPP/p25 activity via the phosphorylation of Thr14 and/or Ser18 localized in the unfolded N-terminal tail: the phosphorylation inhibits its tubulin polymerization promoting activity, which resulted in aggregates of tubulin instead of intact-like MTs (Hlavanda et al. 2007).
Potential binding segments of TPPP/p25 have been predicted and identified. A GTP binding motif on TPPP/p25 was predicted in silico: TPPP/p25 contains four GTP binding motifs, three are localized within the flexible region (G^{68}-(X)_{4}GK, D^{82}-(X)_{10}-T, E^{46}XSAL) and one (D^{181}XXG/D^{197}XXG) within the C-terminus; the GTP binding was supported experimentally by size exclusion chromatography and NMR studies (Zotter et al. 2011a). TPPP/p25 displays a GTPase activity, it hydrolyses GTP specifically in a magnesium dependent manner (Zotter et al. 2011a). The protein also has a zinc finger motif (His_{2}Cys_{2}) (His^{61}-Cys^{83}) and the binding of Zn^{2+} causes a structural alteration inducing the formation of a molten globule (Zotter et al. 2011b). Zn^{2+} as a specific bivalent cation enhances the TPPP/p25-promoted tubulin polymerization (Zotter et al. 2011b).

### 3.2.3. Dimerization potency

TPPP/p25 has a molecular mass of 23.7 kDa. This disordered protein can be detected as a single band at ~ 25 kDa in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 5.) under reducing circumstances (in the presence of 2-mercaptoethanol (ME)). However, it was an early recognition that at non-reducing conditions, another band at ~ 50 kDa can also be observed on the gel.
The protein has three cysteine residues (Fig. 4.), rendering it possible to form both intra- and intermolecular disulfide bonds. Indeed, it has been proved that TPPP/p25 forms dimers, stabilized by intermolecular disulfide bridges, which can be diminished by the addition of reducing agents (Fig. 1Fig. 5) (Oláh et al. 2012).

The dimerization of TPPP/p25 is promoted not only by the elevation of protein concentration, but also by the addition of GTP (Oláh et al. 2012) or zinc ions (Lehotzky et al. 2015). Biophysical measurements showed that the monomers undergo a conformational change, a partial folding in the course of their dimerization: the likely unfolded monomer becomes an apparently more folded, compact dimer (Oláh et al. 2012). Chemical cross-linking of SH groups revealed that the stabilized dimeric form displays enhanced tubulin polymerization promoting activity as detected by turbidity measurements (Fig. 6) (Oláh et al. 2012).
Fig. 6. The effect of dilution on the TPPP/p25-induced tubulin polymerization. The polymerization was induced by the addition of an aliquot of TPPP/p25 stock solution (400 μM) to tubulin (bold line) or by the addition of tubulin to TPPP/p25 incubated in the cuvette (solid line). Final concentration of TPPP/p25 was 3 μM, while that of tubulin was 7 μM (modified from Oláh et al. 2012).

3.2.4. Intracellular localization

TPPP/p25 was described to be expressed predominantly in the OLGs, neuropil in rat brain (Takahashi et al. 1993, Skjoerringe et al. 2006) and in human brain as well (Fig. 7) (Kovács et al. 2004, Höftberger et al. 2010).
The expression of TPPP/p25 is crucial for the differentiation of OLGs (Fig. 8) as demonstrated by means of siRNS as well as specific microRNA (mir206), which down-regulate its expression (Lehotzky et al. 2010). TPPP/p25 is able to stabilize the MT network during the elongation of projections in the course of differentiation, which is an essential process, since the differentiated OLGs are the major constituents of the myelin sheath around the axons.

When TPPP/p25 is expressed ectopically in HeLa cells, which is a cell line derived from human cervical adenocarcinoma, the protein is aligned along the MT network resulting in extensive MT bundling; the MT system becomes resistant against anti-MT treatment (nocodazole or cold treatment, Lehotzky et al. 2004). Despite its stabilization effect, the association of
TPPP/p25 to the bundled MTs is highly dynamic, as demonstrated by fluorescence recovery after photobleaching (Lehotzky et al. 2004).

As a disordered protein, TPPP/p25 is degraded by the proteasome machinery as demonstrated in HeLa cells transfected with enhanced green fluorescent protein fused with TPPP/p25 (EGFP-TPPP/p25) (Lehotzky et al. 2004). The overall fluorescence intensity of the labelled protein was quantified in the absence and presence of MG132, a proteasome inhibitor (Carbobenzoxy-Leu-Leu-Leu-al). In the presence of the inhibitor, significant increase of the EGFP-TPPP/p25 level was detected, which is indicative for the role of proteasome in the proteolytic degradation of TPPP/p25 (Goldbaum et al. 2008).

3.2.5. TPPP/p25 in pathological context

The intracellular TPPP/p25 level affects the structural organization of the MT system (Lehotzky et al. 2004): at low expression level the protein aligns along the MT network (Fig. 9., cell marked 1), while its overexpression promotes the formation of two distinct pathological ultrastructures: the perinuclear cage formed by bundled MTs around the nucleus (Fig. 9., cell marked 2) and the aggresome at the centrosomal region (Fig. 9., cell marked 3).

Fig. 9. HeLa cells transfected with pEGFP-TPPP/p25. (A) The representative image shows three TPPP/p25-transfected cells with distinct morphologies in the same microscopic field. Note the co-localization (orange) of EGFP-TPPP/p25 (green) with the MT network (red) at low expression levels (cell marked 1). The other two cells with bright fluorescence show an aberrant MT network and morphological changes with co-localization (marked 2 and 3) (Lehotzky et al. 2004).
The aggresome mimics the pathological inclusions of the brain (Kopito 2000). Aggresome formation may serve as a protective mechanism against the small toxic aggregates (Kawaguchi et al. 2003), which are located at the cell peripheries; these toxic aggregates are transported to the perinuclear aggresome, which is a site for the recruitment of the ubiquitin proteasome system components (Corboy et al. 2005).

The multifunctional TPPP/p25 is involved in different pathological processes. Its non-physiological level in human brain is coupled with the development of distinct CNS diseases (Fig. 10.).

Fig. 10. Consequences of the distinct expression of the NMP TPPP/p25.

In the case of oligodendroglialoma, the lack of TPPP/p25 was observed in the human brain (Preusser et al. 2007). The division of the progenitor cells is extensive in glioma, while the presence of TPPP/p25 would block mitotic spindle formation and help differentiation (Tirián et al. 2003). In multiple sclerosis, which is an idiopathic chronic inflammatory demyelinating disease of the CNS, altered TPPP/p25 levels were found in the affected regions of the brain (Höftberger et al. 2010) coupled with elevated TPPP/p25 levels in the cerebrospinal fluid (Vincze et al. 2011). TPPP/p25 interacts with β-amyloid, producing aggregates that has been detected in a phenotype of diffuse Lewy body dementia with Alzheimer’s disease (Oláh et al. 2011).
The pathological interaction of TPPP/p25 with SYN, the hallmark protein of synucleinopathies has been studied most extensively. Substoichiometric TPPP/p25 concentration induces the formation of SYN fibers (Lindersson et al. 2005). The co-enrichment and co-localization of the two proteins were found in neuronal inclusions of PD and diffuse Lewy body disease, as well as in glial inclusions in MSA (Kovács et al. 2004), and hallmarking feature of TPPP/p25 as marker of synucleinopathies was reported (Kovács et al. 2004).

3.2.6. The moonlighting feature of TPPP/p25

This protein belongs to the group of moonlighting proteins that perform multiple functions without any alterations at genetic level (Jeffery 1999). Despite the one gene-one function paradigm, these proteins display distinct functions due to changes in their cellular localization, cell type, conformations, oligomeric states or hetero-associations (Jeffery 1999). Protein moonlighting differs from simple protein multifunctionality, in which different domains of the same protein display different functions because of gene fusion or exon shuffling (Piatigorsky et al. 1989). Moonlighting is characteristic for many IDPs (Sickmeier et al. 2007), since these proteins do not have a stable 3D structure and can undergo disorder-to-order transitions upon binding (Fink 2005, Tompa et al. 2005).

TPPP/p25 is not only known as a modulating and stabilizing factor of the MT network, it promotes tubulin acetylation by inhibiting the activity of tubulin deacetylases such as sirtuin2 (SIRT2) and histone deacetylase 6 (HDAC6) (Tőkési et al. 2010, Mangas-Sanjuan et al. 2015).

TPPP/p25 is the prototype of the NMPs (Ovádi 2011, Oláh et al. 2013), which display distinct functions under physiological and pathological conditions due to their interactions with distinct protein partners (Jeffery 2011). Its main physiological partner is the tubulin/MT, while under pathological conditions it interacts with SYN resulting in aggregation, which ultimately leads to cell death.
3.3. A hallmark of synucleinopathies: α-synuclein

SYN (Weinreb et al. 1996) is a small protein (140 aa long) that is expressed predominantly in the neurons of the CNS and in red blood cells (Barbour et al. 2008). It is highly soluble, found mainly in the cytosol and the presynaptic terminals near synaptic vesicles; it makes up 0.5%-1.0% of the total cytosolic proteins in brain homogenates (Iwai et al. 1995). SYN is an IDP, although it can adopt various conformations under both physiological and pathological conditions, including induced folding in lipid membranes, self-aggregation and fibril formation (Silva et al. 2013). This protein is the hallmark of synucleinopathies comprising PD, Lewy body dementia as well as MSA.

3.3.1. High conformational plasticity of α-synuclein

Structural disorder predicting algorithms indicate that the C-terminal segment of SYN is disordered. PONDR analysis shows 37% overall disorder for the protein. Its amino acid composition is characteristic for IUPs (complete lack of Trp and Cys, very high Glu content), along with its low aromaticity and high net charge (-10) (Orosz et al. 2004 and references therein). It has a long, fully disordered segment on its C-terminus, as well as a minor region on the N-terminus of the protein (Fig. 11.). Its central region (60-90 aa) cannot be identified as ordered or disordered unambiguously.
Fig. 11. PONDR VL-XT analysis of SYN. Disorder prediction values (PONDR scores) for a given residue are plotted against the residue number. The significance threshold is shown (0.5), above which residues are considered to be disordered.

The disordered nature of SYN has been extensively characterized experimentally as well, with numerous methods including CD, NMR, SDS-PAGE and analytical gel filtration (Eliezer et al. 2001, Fauvet et al. 2012).

The protein is composed of three functionally defined regions (Fig. 12). The N-terminal region (1-60 aa) contains a unique, highly conserved apolipoprotein lipid-binding motif of 4x11 imperfect tandem repeats (KTKEGV). The central hydrophobic region (61-95) confers the β-sheet potential (Uéda et al. 1993) and contains the sequence (71-82) necessary and sufficient for its self-assembly into amyloid fibrils (Giasson et al. 2001). The highly disordered C-terminus (96-140) is rich in negatively charged amino acids (10 Glu, 5 Asp). The presence of the five structure disruptor Pro on the C-terminus indicates its disordered state (George et al. 1995, Ulmer et al. 2005). SYN can be phosphorylated on Ser residues 87 and 129, with the latter being the predominant site, by casein kinases 1 and 2 (Okochi et al. 2000), as well as by G-protein-coupled receptor kinases; phosphorylation inhibits its interaction with phospholipids (Pronin et al. 2000).
Fig. 12. Schematic representation of the 140 aa SYN. Several pathogenic mutations as well as the phosphorylation sites are indicated (modified from Bellucci et al. 2012).

Although SYN is predominantly unfolded at physiological conditions, in response to changes in its environment (low pH, high temperature, organic solvents, membranes, agrochemicals, or metal ions), it is capable of adopting structurally unrelated conformations, ranging from intrinsically disordered form to various partially folded conformations with different contents of secondary structural elements (Uversky 2003, Silva et al. 2013). The structure of the membrane bound vesicular SYN contains two α-helices with a short linker region (3-37 and 45-92) in a roughly antiparallel arrangement (Ulmer et al. 2005, Davidson et al. 1998) and it was proposed that during this transition a multimeric SYN is formed (Burré et al. 2014, Luth et al. 2015). The native oligomeric state of the protein is still a matter of debate due to conflicting reports considering the protein either monomeric or tetrameric (Bartels et al. 2011, Dettmer et al. 2013, Fauvet et al. 2012).

3.3.3. Physiological aspects

SYN was first co-purified with synaptic vesicles from the Torpedo electric ray (Maroteaux et al. 1988), and later it was found to be localized in the presynaptic terminals of neurons in the mammalian CNS (Iwai et al. 1995). These results indicated that its functions are related to neurotransmission and the modulation of vesicular synaptic release (Murphy et al. 2000). Direct evidence of interaction was reported by co-immunoprecipitation of SYN with the vesicle fusion mediator SNARE (soluble N-ethylmaleimide-sensitive-factor attachment protein receptor,
Ungar et al. 2003) proteins involved in intracellular trafficking (Burré et al. 2010). SYN was found to dose-dependently facilitate the SNARE complex assembly, binding specifically to the vesicular SNARE protein synaptobrevin-2 (Diao et al. 2013), which emphasizes its role in vesicular membrane transport. Despite extensive research, the physiological properties and functions of SYN are still poorly understood.

Interestingly, a small amount of SYN is secreted from the cells and present in certain body fluids, such as the cerebrospinal fluid (El-Agnaf et al. 2006, Tokuda et al. 2006). The aggregates are also internalized via endocytosis and could be degraded by the lysosomal pathway, while the monomeric form is proposed to translocate across the membranes freely (Lee et al. 2008). Further experiments have shown that both the free and the aggregated SYN can be secreted and taken up by neurons, and cell-to-cell transmission can occur among neurons and multiple glial cell types, including OLG (Bates et al. 2014).

### 3.3.4. Pathological relevance

The etiology of the conformational diseases is a multistep process, initiated by unfolded and/or misfolded proteins forming aberrant protein-protein interactions and insoluble polymeric structures and aggregates, which finally leads to the formation of inclusions then cell death (Dobson 1999). The aggregation of IDPs are characteristic (Uversky 2014, Uversky 2015) for tauopathies (β-amyloid and/or tau) such as Alzheimer’s and Pick’s disease (Lee et al. 2012); prion diseases (prion protein, Safar 2012); Huntington’s disease (mutant huntingtin, Arrasate et al. 2012) and synucleinopathies such as PD, MSA and dementia with Lewy bodies in which SYN is the main constituent of Lewy bodies and glial cytoplasmic inclusions (Stephanis 2012, Vieira et al. 2015, Kim et al. 2014).

PD (Parkinson 1817, reprinted in 2002, Beitz 2014) is the second most common neurodegenerative disorder after Alzheimer's disease, with a prevalence of approximately 0.5 to 1 percent in the age of 65 to 69, rising to 1 to 4 percent among persons 80 years of age and older (de Lau et al. 2006); which presents a growing socio-economic problem with the aging of society. It is not considered as a fatal disease: PD is slowly progressive, coupled with motor symptoms (tremor, bradykinesia, rigidity and postural instability); its development irreversibly decreases the
quality of the patient’s life. The disease is characterized by a relatively selective loss of dopaminergic neuronal cells (Chinta et al. 2005, Surmeier et al. 2010) of the substantia nigra pars compacta and the appearance of intracellular, fibrillar inclusions of SYN, TPPP/p25 and additional proteins in the affected surviving neurons (Wakabayashi et al. 2013).

MSA also belongs to the group of synucleinopathies. It is characterized by oligodendroglial cytoplasmic inclusions (Jellinger et al. 2016), where SYN was found to be co-localized with TPPP/p25 (Kovács et al. 2004). The relocation of TPPP/p25 from myelin to OLG and its aggregation preceded the accumulation of SYN (Song et al. 2007, Ota et al. 2014) and was followed by the hetero-association of the two proteins, which resulted in aggregation and the formation of inclusion bodies mainly consisting of SYN (Arima et al. 1998, Ota et al. 2014, Jellinger et al. 2016).

SYN plays pathological roles in mixed type Alzheimer’s disease as well (Hansen et al. 1990, Hamilton 2000); these observations make the protein a target of excessive research.

Current research states that the small, soluble oligomeric forms with beta-sheet structure are considered the most toxic species (Dehay et al. 2015, Roberts et al. 2015, Winner et al. 2011). Although knowledge on various mechanisms underlying PD and other synucleinopathies has been greatly expanded over the last decades, the pathomechanism is still not well-understood (Dehay et al. 2015, Ozansoy et al. 2013, Roberts et al. 2015).
4. MATERIALS AND METHODS

4.1. DNA manipulation

The TPPP/p25 (N-, C-, double truncated and fluorescently labeled) forms and the SYN (SYN\textsuperscript{1-120} and SYN\textsuperscript{95-140}) mutants were produced by N. Tőkési and T. Szénási as described (Tőkési et al. 2014, Szunyogh et al. 2015, Szénási et al. 2017) with the following sequences: Δ3-43 TPPP/p25 (N-truncated, ΔN TPPP/p25), Δ175-219 TPPP/p25 (C-truncated, ΔC TPPP/p25) and Δ3-43/Δ175-219 TPPP/p25 (double truncated, CORE) form. Both the EGFP-TPPP/p25 and the EGFP-CORE constructs contain the EGFP on the N-terminal region of the proteins, as well as the Venus constructs the Venus fragments (Scheme 1). The sequences of all constructs were verified by restriction mapping and DNA sequencing.

Scheme 1. The Venus constructs of the FL and the CORE TPPP/p25 forms.

4.2. Recombinant protein preparation

The proteins were expressed in \textit{E. coli} BL21 (DE3). 25 μL stock solution of transformed \textit{E. coli} expressing a TPPP/p25- or SYN-variant (30%glycerol in Luria Bertani Broth (LB, 20 g/l) containing the \textit{E. coli}) were inoculated into 5 ml sterile LB with 0.1 mg/ml ampicillin and 0.1 mg/ml chloramphenicol then grown overnight at 37°C with a shake rate of 220 rpm. The starter culture was added to 300 ml LB with 0.1 mg/ml ampicillin and was grown until it reached the optical density of 0.4-0.6 at 600 nm, when they were induced with 1 mM isopropyl β-D-1-
thiogalactopyranoside (IPTG, Sigma-Aldrich) for 3 h at 37°C, then harvested by centrifugation for 15 min at 4°C at 2500 g.

The TPPP/p25 forms and SYN^{95-140} contain a 6xHis tag. In these cases, the pellet was suspended in a lysis buffer containing 50 mM Na₂HPO₄ and 300 mM NaCl pH 7.4 supplemented with protease inhibitor mix (1 mM benzamidine, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride (PMSF)) and 1 mg/ml lysozyme. The sample was sonicated for 6x15 s with 30 s intervals on ice, and centrifuged for 40 min at 4°C at 100,000 g. The supernatant was loaded to a HIS-Select® Nickel Affinity Gel column (Sigma-Aldrich), which was previously equilibrated with lysis buffer. The Ni²⁺ ions selectively bound the 6xHis tag on the proteins. The column was washed extensively, then the proteins were eluted with 50 mM sodium acetate, 300 mM NaCl pH 4.5. The drastic change in the pH caused the Ni²⁺ to release the 6xHis tag. The eluted proteins were concentrated in an Amicon (Millipore) stirred cell with ultrafiltration membranes with nominal molecular weight limit (NMWL) of 10,000 Da for the FL, the ΔC and ΔN TPPP/p25 forms and of 3000 Da for the CORE TPPP/p25 and SYN^{95-140} to a concentration close to 1 mg/ml.

The pellet of SYN and SYN^{1-120} were resuspended with lysis buffer containing 20 mM Tris-HCl, 50 mM NaCl (10 mM NaCl in the case of SYN^{1-120}), 1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM ME pH 7.5 (pH 8.0 in the case of SYN^{1-120}) supplemented with protease inhibitor mix and 1 mg/ml lysozyme. The sample was sonicated for 6x15 s with 30 s intervals on ice, and centrifuged for 25 min at 4°C at 39,000 g. The supernatant was kept at 85°C for 10 min, since SYN is heat stable (Park et al. 2002), then centrifuged for 25 min at 4°C at 39,000 g. The soluble part is mainly consisted of SYN. It was loaded to a Whatman® anion exchange cellulose DE52 column, which was previously equilibrated with lysis buffer. The column was washed extensively, then the proteins were eluted with lysis buffer containing 150 mM NaCl. The eluted proteins were concentrated in an Amicon (Millipore) stirred cell with ultrafiltration membranes with NMWL of 3000 Da. The differences in the salt concentration and pH of the buffer in the case of the two SYN forms are related to the sequence alteration: the last highly acidic amino acids are missing from SYN^{1-120} resulting in a weaker binding to the used anion exchange column.

The concentrated proteins were dialyzed overnight in 50 mM ammonium acetate then lyophilized and stored at -80°C. The purity of the preparation was determined by SDS-PAGE.

Protein concentration was determined from the absorbance at 280 nm using an extinction coefficient of 10955 M⁻¹ cm⁻¹, 10955 M⁻¹ cm⁻¹, 5625 M⁻¹ cm⁻¹, 5625 M⁻¹ cm⁻¹, 5960 M⁻¹ cm⁻¹.
*cm⁻¹, 2980 M⁻¹ *cm⁻¹ and 4470 M⁻¹ *cm⁻¹ for TPPP/p25, ΔN TPPP/p25, ΔC TPPP/p25, CORE TPPP/p25, SYN, SYN¹⁻¹²⁰ and the C-terminal SYN⁹⁵⁻¹⁴⁰, respectively. Extinction coefficients were calculated using ProtParam (http://web.expasy.org/cgi-bin/protparam/protparam).

4.3. Tubulin preparation from bovine brain

The MAP free tubulin was purified from bovine brain according to Na and Timasheff (Na et al. 1986). All preparation steps were conducted in a cold room at 4°C. The cleaned brain was homogenized in lysis buffer (10 mM Na₂HPO₄, 0.24 M sucrose, 0.1 mM GTP, 1 mM dithioerythritol (DTE), 1 mM PMSF, pH 7.0) and centrifuged for 30 min at 4°C at 11,000 g. The supernatant was precipitated with 187 g/L, 32 % (NH₄)₂SO₄ and centrifuged for 30 min at 4°C at 15,000 g. Again, the supernatant was precipitated with (NH₄)₂SO₄ (+71 g/L, 43%). This is the step where tubulin is precipitated along with its MAPs. The solution was centrifuged for 30 min at 4°C with 15,000 g, then the precipitate was suspended in washing buffer (10 mM Na₂HPO₄, 0.1 mM GTP, 1 mM PMSF, 1 mM DTE, pH 7.0). A DEAE Sephadex A-50 anion exchanger column was equilibrated with the washing buffer, then the tubulin containing solution was loaded onto it. The MAPs were removed with a washing buffer containing 0.4 M KCl. The elution was performed with washing buffer supplemented with 0.8 M KCl. After this, another precipitation was carried out with 24.8 g/L (NH₄)₂SO₄. The sample was centrifuged for 30 min at 4°C at 33,000 g. The pellet was resuspended in minimal buffer volume and was loaded into a Sephadex G-50 column to remove the excess salt, then dialyzed into a buffer of 10 mM Na₂HPO₄, 1 M sucrose, 0.5 mM MgCl₂, 0.1 mM GTP, pH 7.0 for storage at -80°C. Concentration was measured in 0.1 M NaOH at 280 nm, purity was verified with SDS-PAGE and the functional properties of the tubulin was measured by turbidimetry.

4.4. SDS-PAGE

The gel electrophoresis was carried out by the Laemmli method (Laemmli et al. 1970), which separates the denatured proteins by their molecular weight. The standard stacking gels
contained 13.5% acrylamide/bis-acrylamide (37.5:1 ratio); the sample contained 20% loading buffer (1.2% SDS, 0.025% bromophenol blue, 40% glycerol and 10% ME if reducing circumstances were needed). The molecular weight marker PageRuler Prestained Protein Ladder was obtained from Thermo Scientific. BioRad Mini-Protean II electrophoresis cell was used for 3 h at 100 V, room temperature (RT). The gels were fixed with a buffer of 10% acetic acid and 25% isopropanol for 30 min then washed for 3x10 min, followed by overnight staining with PageBlue Protein Staining Solution (Thermo Scientific).

The Tricine-containing two-layer-gel was made according to the method of Schägger and von Jagow (Schägger et al. 1987) for low molecular weight proteins. The separating gel was 7%.

4.5. Antibodies, peptides

The following antibodies were used: monoclonal mouse TPPP/p25 antibody (Höftberger et al. 2010), and its biotinylated form produced by T. Berki (Höftberger et al. 2010); rat polyclonal TPPP/p25 antibody (Kovács et al. 2004); mouse monoclonal SYN antibody against the epitope of 121-125 aa (Sigma Aldrich, S5566, clone Syn211); rabbit polyclonal SYN antibody developed against the 111-132 peptide sequence in the C-terminus of the protein (Sigma Aldrich, S3062); rabbit monoclonal SYN antibody developed against the N-terminus of SYN (Merck, 04-1053, clone EP1646Y) and mouse monoclonal tubulin antibody (Sigma Aldrich, T6199, clone DM1A).

The peptides BF180-183 and P1-P4 were synthesized in collaboration with A. Magyar in the Research Group of Peptide Chemistry, ELTE. In the case of BF183 and BF180, a Lys was built into the C-terminus of the peptides to conjugate the biotin derivative to its side chain (ε-amino group). The biotinylated forms were prepared using biotinyl-6-amino-hexanoic acid (long chain biotin). The N-terminus of the peptides was acetylated, while the C-terminus was in amide form. For the P1-P4 peptides, a Cys was built into the N-terminus. The N-terminus of the peptides was acetylated, while the C-terminus was in amide form. The SYN{126-140} peptide (EMPSEEGYQDYEPEA) and its fluorescein-labeled form (FITC-EMPSEEGYQDYEPEA) were purchased from ChinaPeptides (Shanghai, China).
4.6. Turbidity measurements

The assembly of 7 µM tubulin was assessed in polymerization buffer (50 mM MES buffer pH 6.6 containing 100 mM KCl, 1 mM DTE, 1 mM MgCl₂ and 1 mM EDTA) at 37°C in a 500 µl cuvette. The polymerization of tubulin was induced by addition of 3 µM wild type TPPP/p25 or its truncated forms with the addition of 100 µM ZnCl₂ where indicated. When TPPP/p25 was diluted into the cuvette with or without ZnCl₂, it was incubated for 10 minutes and the polymerization was initiated by a stock solution of tubulin, otherwise the polymerization was induced by the addition of TPPP/p25 or its forms from 10 mg/ml stock.

The MT bundling experiments were conducted in polymerization buffer at 37°C, where 10 µM tubulin was polymerized into MTs by the induction of 20 µM paclitaxel, then subsequently 3 µM TPPP/p25 or one of its forms was added to the solution to examine the bundling activities.

At least three independent experiments were performed. A representative polymerization is shown; error of determinations (SEM) is ± 10%. The turbidity was monitored at 350 nm by a Cary 100 spectrophotometer (Varian, Walnut Creek, Australia).

4.7. Pelleting experiment

5 µM tubulin was incubated with 10 µM of the different TPPP/p25 forms for 10 min at 37°C in polymerization buffer, then centrifuged for 15 min at 37°C at 17,000 g and the pellet and supernatant fractions were separated. The pellet fraction was washed and resuspended in polymerization buffer. The pellet and the supernatant fractions were analyzed by Tricine SDS-PAGE with loading buffer containing ME. The distributions of 2 µg TPPP/p25 forms and 4 µg tubulin were analyzed in the fractions.

In the case of MT pelleting, 20 µM tubulin was polymerized with 20 µM paclitaxel for 30 min at 37°C in polymerization buffer, then centrifuged for 15 min at 37°C at 17,000 g and the pellet and supernatant fractions were separated. The pellet fraction was washed and resuspended in 20 µM paclitaxel containing polymerization buffer. 1 mg/ml MT (10 µM tubulin) were incubated with 10 µM TPPP/p25 forms for 15 min at 37°C, then centrifuged for 15 min at 37°C at 17,000 g. The pellet and the supernatant fractions were analyzed by SDS-PAGE with loading
buffer containing ME. The distributions of 3.75 µg TPPP/p25 forms and 15 µg tubulin were analyzed in the fractions.

4.8. Enzyme linked immunosorbent assay (ELISA) and cellular ELISA (cELISA)

The 96-well plate was coated with 5 µg/ml (100 µl/well) TPPP/p25 or SYN forms in phosphate buffered saline (PBS) or in case of the biotinylated peptides with 5 µg/ml (100 µl/well) streptavidin solution in PBS overnight at 4°C. The wells were blocked with 1 mg/ml bovine serum albumin (BSA) in PBS for 1 hour at RT. The next step was the serial dilution or constant amount of the partner(s) as indicated in each experiment for 60 min with or without a preincubation of partners for 30 min. It was followed by the addition of either tubulin, SYN or TPPP/p25 antibody, and the addition of the peroxidase conjugated secondary IgG (1:5000, Sigma-Aldrich), both in PBS buffer containing 1 mg/ml BSA for 1 h. Dilutions were usually 1:5000, for the monoclonal mouse TPPP/p25 antibody it was 1:1000, in the cases of the mouse monoclonal and the rabbit monoclonal SYN antibodies it was 1:2000. Between each incubation step, the wells were washed thrice with PBS containing 0.05% Tween 20 (Sigma Aldrich). The bound antibodies were detected using ortho-phenylenediamine as substrate in the concentration of 3.7 mM with 0.03% H₂O₂ as substrate solution. The reaction was stopped after 10 min with 1 M H₂SO₄ and the absorbance was read at 490 nm with a Wallace Victor 2 multiplate reader (Perkin Elmer) or an EnSpire Multimode Reader (Perkin Elmer). The apparent binding constants (Kd) were evaluated from the saturation curves, using non-linear curve fitting, assuming a single binding site hyperbola model using the Origin v8.0 software. Error bars represent the standard error of the determinations (SEM) (n= 3). * Significant difference, according to Student's t-test, p<0.05.

Differences from this protocol: In the case of P1-P4 peptides: Sulphhydryl-BIND™ (Maleimide) Modified Surface plate (Corning Incorporated) was used according to the manufacturer’s instructions. The plate was coated with 5 µg/ml (100 µl/well) peptide or TPPP/p25 solution in PBS buffer containing 1 mM EDTA pH 6.5 for 1 hour at RT. The next step was the blocking with 0.2% non-fat dry milk in PBS for 30 min at RT. Then the plate was incubated with tubulin as described above.
Sandwich ELISA: The plate was coated with 1 μg/mL (50 μL/well) mouse monoclonal TPPP/p25 antibody in 200 mM Na₂CO₃ buffer pH 9.6 overnight at 4°C. The wells were blocked with 1 mg/mL BSA in PBS for 1 hour at RT. Then the plate was incubated with serial dilutions of 5 μM TPPP/p25 for 1 hour at RT. Then the plate was sequentially incubated with biotinylated monoclonal TPPP/p25 antibody (1 μg/mL) and peroxidase conjugated avidin (Calbiochem) (2.5 μg/mL).

cELISA: After manipulation, the cells in the 96-well tissue culture plate were fixed by ice cold methanol for 10 min. The fixed cells were rehydrated by PBS, and blocked with 1 mg/mL BSA in PBS containing 0.1% Triton-X-100 (Sigma Aldrich) for 1 hour at RT. Then the plate was sequentially incubated with mouse monoclonal TPPP/p25 antibody (1.5 μg/mL ~1:750) and with an anti-mouse IgG-peroxidase conjugate (1:2500, Sigma Aldrich) in PBS containing 1 mg/mL BSA and 0.1% Triton-X-100 for 1 hour at RT. Following each incubation step, the wells were washed thrice with PBS for 5 min. The relative TPPP/p25 concentration was quantified by using o-phenylenediamine in the concentration of 3.7 mM with 0.03% H₂O₂ as substrate. The reaction was stopped after 15 min with 1 M H₂SO₄; absorbance was read at 490 nm with an EnSpire Multimode Reader (Perkin Elmer).

4.9. Circular dichroism spectroscopy

The CD measurements were performed on Jasco J-720 spectropolarimeter at 20 nm/min scan rate, 8 s time constant and 0.5 nm step size in 10 mM phosphate buffer, pH 7.2 at RT, the cuvette path length was 0.1 cm. The protein concentrations were 4 μM for TPPP/p25 forms and 1 μM for tubulin. The mixtures were incubated for 10 min before recording the spectra. Scanning was repeated three times, and the spectra were averaged. Difference of ellipticity (in millidegrees) was calculated by subtracting the ellipticities of TPPP/p25 or its forms and tubulin from that measured with their mixture. Mean molar ellipticity per residue (Θ) in degree square centimeter per decimole was calculated according to the following equation: Θ = Θm/(10*n*c*l), where Θm is the measured ellipticity in millidegrees, n is the number of amino acid residues, c is the concentration in moles and l is the path length of the cuvette in centimeters. The standard error of the determinations (SEM) was ± 10% (n = 3-5).
4.10. Affinity chromatography

SYN was immobilized to CNBr-activated Sepharose 4B (Amersham) according to the manufacturer’s instructions. SYN bound to the resin beads was packed into columns. The binding capacity of a column was ~1.5 mg SYN per 1 ml resin (column volume ~ 2 ml). The affinity column was equilibrated with phosphate buffer (10 mM phosphate pH 7.4 containing 10 mM NaCl). 500 µg TPPP/p25 with or without 500 µg SYN or SYN^{1-120} was loaded to the column in the volume of 500 µl, then the column was washed with phosphate buffer (10 ml, 1 ml fractions). The bound proteins were eluted with phosphate buffer containing 100 mM NaCl (5 ml, 1 ml fractions). After each experiment the column was regenerated using 3 cycles of 0.1 M Na-acetate pH 4.0 buffer containing 0.5 M NaCl and 0.1 M tris(hydroxymethyl)aminomethane (Tris) pH 8.0 buffer containing 0.5 M NaCl. The flow-through (unbound) and the eluted (bound) fractions were analyzed by SDS-PAGE.

4.11. Cell culture and manipulation

The following cell lines were used: CHO10, which is a tetracycline-inducible TPPP/p25 expressing CHO-K1 Tet-On cell line variant generated in the Cell Architecture research group (Tőkési et al. 2010) and the human cervical adenocarcinoma cell line HeLa (ATCC, CCL2).

Hela cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS), 100 unit/ml streptomycin and 100 µg/ml penicillin antibiotics (all cell culture reagents from Sigma Aldrich); CHO10 cells were cultured in DMEM Nutrient Mixture F-12 (DMEM/F12) supplemented with 10% tetracycline free FBS, 100 unit/ml streptomycin and 100 µg/ml penicillin antibiotics. The cells were propagated in a humidified incubator at 37°C with 5% CO₂. Cells were routinely checked for mycoplasma contamination by fluorescence microscopy using 4,6-diamidino-2-phenylindole (DAPI) staining.

The cells were grown on 12 mm diameter coverslips for microscopic analysis (1-2 x 10⁴ cells per coverslip), on 24-well plates for immunoblotting (2.5-5 x 10⁴ cells per well) and on 96-well plates for cELISA (0.5-1 x 10⁴ cells per well). After the passage of cells to the plates/coverslips, they were incubated overnight in the incubator.
The CHO10 cells were treated as follows. For induction, the cells were cultured overnight with 100 ng/ml doxycycline (tetracyclin analogue, sub-maximal induction). Where it is indicated, 10 µM MG132 (Sigma Aldrich) or 10 µM ZnCl₂ (Sigma Aldrich) was added for 3 h.

For microscopic analysis, the uptake of TPPP/p25 and its CORE form was examined by the addition of 2.5 µg TPPP/p25 forms or 3 µg TPPP/p25 and 12 µg SYN forms, when the ability of these proteins to aggregate was examined. The proteins were freshly dissolved in PBS. After a short preincubation of the SYN and TPPP/p25 forms in 1 mg/ml concentrations, the proteins were added to 500 µl medium to be taken up by the cells during the 3 h incubation. The final concentrations of TPPP/p25 and SYN species were 0.006 mg/ml (0.24 µM) and 0.024 mg/ml (1.68) µM (1/7 ratio), respectively. In the case of the FITC labeled SYN peptide, 1 µl of a 10 µg/µl (440 µM, final concentration is 0.02 mg/ml) stock solution was used with the TPPP/p25 forms.

The quantification of microscopic image intensities was performed with ImageJ v1.49 software using the original grayscale pictures. The whole territory of each cell was outlined by the Freehand Line tool and integrated pixel densities were calculated by multiplying the area of each cell with the corresponding average pixel intensity subtracting the background.

For the transfection of HeLa cells with the EGFP-TPPP/p25 and EGFP-CORE TPPP/p25 plasmids and the Venus constructs, the cells were transfected overnight with 0.3 µg DNA of each plasmid using Turbofect (Thermo Scientific) transfection reagent according to the manufacture’s protocol.

4.12. Immunocytochemistry

The HeLa and CHO10 cells on glass coverslips were fixed with ice-cold methanol for 10 min followed by postfixation with 4% formaldehyde (Sigma Aldrich) for 2 min. After washes with PBS (3x10 min), samples were blocked for 30 min in antibody dilution buffer (5% FBS and 0.1% Triton X-100 in PBS). For SYN-staining, the buffer contained 30 µM digitonin instead of Triton X-100.

The samples were incubated with primary antibodies against tubulin, TPPP/p25 and SYN as indicated (dilutions: mouse monoclonal TPPP antibody 1:1000, rat polyclonal TPPP/p25 antibody 1:1500, mouse monoclonal SYN antibody 1:1000, rabbit monoclonal SYN antibody
1:1000, rabbit polyclonal SYN antibody 1:500). Alexa 488 and Alexa546 conjugated antibodies (Invitrogen, 1:1000) were used as dictated by the primary antibody. To detect the FITC-labeled peptide, as well as the EGFP or Venus constructs, intrinsic fluorescence was used. The samples were washed thrice with PBS for 3×10 min after antibody incubation. Nuclei were counterstained with 0.5 μg/ml DAPI. Images of fixed samples were acquired on a Leica DM IL 500 microscope equipped with Leica DFC 395 FX camera and HBO 100w lamp. The equipment software was Leica Application Suite 4.4.0. Chroma UV filter set (No. C40888) was used for DAPI; Chroma 41028 HQ NB GFP filter set (No. C21116) for EGPF, FITC and Alexa488; Chroma 41028 Y GFP filter set (No. C2117) for Venus and Leica filter N2.1 (No. 513832) for Alexa 546 signal acquisition, using a HCX FL Fluotar 40x/0.75 (dry) objective, except Fig.24, which was imaged on a Zeiss LSM710 confocal microscope.

Generally, the images presented on the figures were taken under constant exposure parameters in a given experiment, except panel D on Fig. 35, where lower exposure time was used due to the extreme signal strength of the protein aggregates.
5. RESULTS AND DISCUSSION

5.1. PHYSIOLOGICAL INTERACTIONS OF TPPP/p25

TPPP/p25 is primarily expressed in OLGs, where the tubulin polymerization promoting and MT bundling activities of the protein are crucial in the course of differentiation of the progenitor cells for the development of projections leading to myelination (Lehotzky et al. 2010), the ensheathment of the neuronal axons which facilitates the conduction of electrical impulses between neurons.

5.1.1. Characterization of the interaction of TPPP/p25 with tubulin/microtubules

Tubulin is the main interacting partner of TPPP/p25 (Hlavanda et al. 2002); its binding site was predicted based on sequence homology of the TPPP/p25 and the tau protein (Hlavanda et al. 2007). One of the objectives of my thesis was to identify the TPPP/25 segment(s) involved in the binding of tubulin and MT as well as to establish the functional consequence of their hetero-associations.

![Scheme 2](image)

Scheme 2. Various truncated forms of TPPP/p25 shown schematically.

TPPP/p25 is a disordered protein with unstructured N- and C-terminal segments straddling a flexible, core region as suggested by PONDR predictor (Fig. 2) as well as multinuclear NMR studies (Zotter et al. 2011a). To characterize the role of these segments in its physiological
interactions, different truncated TPPP/p25 variants were produced by recombinant techniques in the Cell Architecture research group (Tőkési et al. 2014, Szénasi et al. 2017). The N- or C-terminal free as well as the double truncated (CORE) TPPP/p25 forms (shown in Scheme 2) were expressed in E. coli, and isolated as described in the Materials and Methods.

The influence of the truncations of the terminal segments of TPPP/p25 on the secondary structure of this disordered protein was established by CD spectroscopy (Fig. 13A). The lack of the N- or the C-terminus resulted in minor spectral alteration, if at all; however, the double truncation reduced the ellipticity, characteristic to the random coil structure, indicating a less disordered state of the protein.

![Normalized far-UV CD spectra and dimerization on SDS-PAGE](image)

Fig. 13. Normalized far-UV CD spectra (A) and the dimerization of the different TPPP/p25 forms (5 µg/lane) on non-reducing SDS-PAGE (B). FL TPPP/p25 (bold line), ΔN TPPP/p25 (solid line), ΔC TPPP/p25 (dashed line), CORE TPPP/p25 (dotted line). B. Molecular weight marker (MM).

The SDS-PAGE images of the wild type and truncated forms of TPPP/p25 used for CD spectroscopy are shown in Fig. 13B that illustrate the presence of both monomeric and dimeric forms in the case of all TPPP/p25 variants, the nature of the truncations apparently do not disturb the monomer-dimer equilibrium of the protein. The dimers are stabilized by covalent intermolecular disulfide bridges by cysteine residues located in the core region of the protein (Fig. 4).
5.1.1.1. TPPP/p25 segments involved in the interaction

The interaction of TPPP/p25 with tubulin results in significant secondary structural alteration as indicated by the difference ellipticity spectrum (Hlavanda et al. 2002). Therefore, CD spectroscopy was used to study the effect of the distinct segments of TPPP/p25 in its interaction with tubulin. As shown in Fig. 14, the difference spectrum of the tubulin-TPPP/p25 complex and proteins alone is reduced by removing the N-terminus that becomes more pronounced in the case of the ΔC TPPP/p25 form; however, the lack of the two unstructured termini diminished the difference ellipticity spectrum. These data indicate that the CORE segment does not have a dominant role in the formation of the TPPP/p25-tubulin complex, consequently the unstructured termini seem to be involved in the formation of the physiological hetero-complex.

Fig. 14. Difference ellipticity spectra of TPPP/p25 forms with tubulin. The concentration of tubulin was 1 μM, that of the TPPP/p25 forms were 4 μM. Bold line: tubulin-TPPP/p25, solid line: tubulin-ΔN TPPP/p25, dashed line: tubulin-ΔC TPPP/p25, dotted line: tubulin-CORE TPPP/p25.

The physiologically relevant function of TPPP/p25, namely its potency to promote the polymerization of tubulin into MTs, was tested by turbidity measurements. The polymerization was induced by the addition of the different TPPP/p25 forms. The results shown in Fig. 15 indicate
that the truncations depending on their nature display significant effects on the tubulin polymerization promoting activity: the lack of the N- or the C-termini reduces the turbidity signals in a less and in a more extent, respectively, while the CORE form lost its ability to produce tubulin polymers. These results provide evidence for the active participation of the unstructured termini not only in the binding of TPPP/p25 to tubulin but in its polymerization promoting potency as well. In fact, electron microscopic studies in the Cell Architecture group in collaboration with the Department of Anatomy, Cell and Developmental Biology of ELTE revealed intact-like MT formation by the wild type TPPP/p25 coupled with extensive bundling activity (Hlavanda et al. 2002); the phenomenon that the formation of the TPPP/p25-induced tubulin polymers is coupled with their cross-linking explains the extremely fast increase of the turbidity.

Fig. 15. The tubulin polymerization promoting potency of the TPPP/p25 forms. The polymerizations were induced by stock TPPP/p25 solutions, the final concentrations were 7 μM for tubulin and 3 μM for the TPPP forms, followed by turbidimetry.

The role of the truncation of TPPP/p25 in its interaction with tubulin/MT was further investigated by pelleting experiments. In one set of the experiments, the different TPPP/p25 forms were incubated with tubulin allowing them to display their potency to produce tubulin assembly, which was followed by separation of the polymerized and soluble tubulin by centrifugation and
analysis of the fractions by SDS-PAGE. As shown in Fig. 16, the FL and the ΔN TPPP/p25 forms were distributed similarly in the pellet (P) and the supernatant (S) fractions, while the ΔC and CORE TPPP/p25 forms appeared mostly in the supernatant indicating their poor, ability to display binding-induced tubulin polymerization.

![Diagram of fraction analysis](image)

**Fig. 16.** The pelleting potency of the various TPPP/p25 forms. 5 μM tubulin was incubated with 10 μM TPPP/p25 forms in polymerization buffer, detected on tricine containing reducing SDS-PAGE.

In another set of pelleting experiments, the effect of truncations on the bundling activity of TPPP/p25 was studied using paclitaxel stabilized MTs instead of tubulin. The stabilized MTs were incubated with the different TPPP/p25 forms and their distribution was analyzed by SDS-PAGE. As illustrated in Fig. 17A, stabilized MTs were pelleted as expected, however, the partition of the TPPP/p25 forms between the pellet and supernatant fractions was dependent on the nature of the truncation: a part of the ΔC and CORE TPPP/p25 remained in the soluble fraction, did not pelleted with the MTs, indicating their lower binding activity. In turbidity measurements, where tubulin was already polymerized by paclitaxel (Fig. 17B), all terminal-free forms lost their ability to bundle MTs (to enhance turbidity) while in similar conditions, the wild type protein produced fast cross-linking effect manifesting in the extensive increase of turbidity.
Fig. 17. The MT binding (A) and bundling (B) potency of the TPPP/p25 forms. A. The polymerization of 10 μM tubulin was induced by 20 μM paclitaxel, then the MTs were bundled by 10 μM TPPP forms, detected on SDS-PAGE. B. The polymerization of 10 μM tubulin was induced by 20 μM paclitaxel, then as indicated by an arrow, 3 μM of the TPPP forms were added, followed by turbidimetry. Bold line: TPPP/p25, solid line: ΔN TPPP/p25, dashed line: ΔC TPPP/p25, dotted line: CORE TPPP/p25.

The results of the binding, polymerization and pelleting experiments performed with wild type and truncated human recombinant TPPP/p25 variants are summarized in Table 1., which illustrates that the C-terminus of TPPP/p25 plays a more dominant role in the tubulin/MT binding and its bundling activity than its N-terminal segment.

<table>
<thead>
<tr>
<th>Characteristics of the interactions of TPPP/p25 forms with tubulin/MT. Qualitative orders from left to right.</th>
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<tbody>
<tr>
<td>Difference in the secondary structure</td>
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<tr>
<td>Tubulin polymerization promoting potency</td>
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<tr>
<td>Binding potency of TPPP/p25 forms to tubulin</td>
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<tr>
<td>MT binding potency of TPPP/p25 forms</td>
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<tr>
<td>MT bundling potency of TPPP/p25 forms</td>
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These *in vitro* data have been supported by experiments performed at cellular level with HeLa cells expressing TPPP/p25 ectopically. As shown in Fig. 18, the FL EGFP-TPPP/p25 co-localizes with the MT network (Lehotzky et al. 2004), modifying the stability of the network by its bundling activity; these cells become more resistant against anti-mitotic agents (Lehotzky et al. 2004). On the contrary, the EGFP-CORE TPPP/p25 does not align along the MTs, but distributed homogeneously within the cytosol as visualized by immunofluorescent microscopy (Tőkési et al. 2014).

![Fig. 18. Localization of TPPP/p25 forms in HeLa cells. The expressed EGFP-TPPP/p25 (A-C, green on merge), EGFP-CORE TPPP/p25 (D-F, green on merge) and the MT network (A-F, red on merge) were visualized. Nuclei were counterstained with DAPI. Scale bar: 10 µm.](image)

Similar results were obtained in living HeLa cells by bimolecular fluorescence complementation (BiFC) technology. In these experiments, two non-fluorescent fragments of the
fluorescent YFP variant mVenus (Kerppola 2008) were fused to the FL and CORE TPPP/p25 forms. If the protein pairs attached to different mVenus fragments interact, the non-fluorescent fragments are brought in proximity to each other complexing into the fluorescent mVenus protein. Both the N- and the C-terminal Venus fragments were fused either to the FL or to the CORE TPPP/p25; BiFC signals were generated by co-transfection of the cells with the TPPP/p25 or CORE forms fused to the different mVenus BiFC constructs. As illustrated in Fig. 19, the mVenus BiFC signal in the case of the FL TPPP/p25 protein (A, D-F), which reflects the TPPP/p25 dimers, is co-localized with the MTs, FL TPPP/p25 stabilizes and bundles them; in the case of the CORE TPPP/p25 (B, G-I), a diffuse signal could be detected in the cytosol, independent of the MT network. The fact that the terminal-free form of TPPP/p25 is unable to associate with the MTs underlines the role of the unstructured termini in the binding to MTs in cellular environment also.
Fig. 19. Intracellular dimerization and localization of the TPPP/p25 and CORE pairs in HeLa cells as detected by BiFC assay. BiFC signals of the N- and C-segments of the Venus constructs coupled with TPPP/p25 (A, D-F) or CORE (CORE) TPPP/p25 pairs (B, G-I) or without them (empty Venus vectors) (C) as detected by immunofluorescence microscopy. BiFC signals (A-D, G) are shown as green, tubulin signals (E, H) are as red on merge. Nuclei were counterstained with DAPI. Scale bar: 10 μm.
5.1.1.2. Determination of the contact surface on TPPP/p25

TPPP/p25 has been reported to be a new MAP protein concerning its MT-stabilizing effect similarly to the tau protein (Ovádi et al. 2005). A main characteristic of these proteins is their unstructured feature (Goedert et al. 1991, Hlavanda et al. 2002). In addition, sequence homology between the tubulin binding motif of the tau protein (Kar et al. 2003) and TPPP/p25 has been suggested (Hlavanda et al. 2007); this segment is localized at the C-terminus of TPPP/p25 (Fig. 20).

![Fig. 20. Sequence homology between the relevant MT binding regions of tau and TPPP/p25](http://www.ebi.ac.uk/Tools/psa/emboss_needle)

To underline the predicted binding segment of TPPP/p25 involved in the tubulin binding, this segment was investigated experimentally using a special ELISA assay. Overlapping TPPP/p25 decapeptides were synthetized as dictated by the sequence homology analysis in collaboration with Dr. Anna Magyar of the Organic Chemistry Department of ELTE. The peptides were tagged on their N-termini with a cysteine residue, which made possible their direct immobilization to an SH-binding ELISA plate. The sequences of the decapeptides were the following:

\[
P1= C^{163}VSRLTDGTKF^{172} \\
P2= C^{168}DTTKFTGSHK^{177} \\
P3= C^{173}TGSNKERFDPSGK^{182} \\
P4= C^{178}ERFDPSGK^{187}
\]

The immobilized peptides were incubated with a serial dilution of tubulin, then the bound tubulin was quantified with a specific monoclonal tubulin antibody.
As Fig. 21 shows, the P4 peptide (178-187 aa) displays comparable affinity with that of the wild type TPPP/p25 in tubulin binding: $K_d = 0.14 \pm 0.04 \, \mu M$ for TPPP/p25 and $0.18 \pm 0.03 \, \mu M$ for the P4 peptide; the P1, P2 and P3 peptides show significantly lower binding potency. Accordingly, the $^{178}{\text{ERFDPSGKGK}}^{187}$ fragment within the C-terminal tail was proposed to be a key segment of TPPP/p25 in its tubulin binding (Tőkési et al 2014).

5.1.2. Characterization of the Zn$^{2+}$-induced effects on TPPP/p25

The classical zinc finger motifs are considered to be consist of 2 cysteine and 2 histidine ($C_2H_2$) or 1 cysteine and 3 histidine ($C_1H_3$) residues centralized around a zinc ion with a short $\beta$-hairpin and a $\alpha$-helix structure (Gower-Winter et al. 2012). In the case of TPPP/p25, His$^{61}(X)_{10}$His$^{72}(X)_{7}$Cys$^{80}(X)_{2}$Cys$^{83}$ segment as a H$_2$C$_2$ zinc finger motif was identified within its flexible CORE region (Zotter et al. 2011b).
5.1.2.1. The effect of Zn$^{2+}$ on the structure

Previously, the Cell Architecture research group reported that the Zn$^{2+}$ binding causes changes in the structure of TPPP/p25 as well as in its tubulin polymerization promoting activity (Zotter et al. 2011b). The mechanism responsible for the Zn$^{2+}$-induced effects was established by sandwich ELISA. This specific ELISA assay renders it possible to detect only the presence of dimeric/oligomeric forms of a given protein (El-Agnaf et al. 2006).

Accordingly, in this set of experiments, a monoclonal TPPP/p25 antibody was immobilized on the plate, TPPP/p25 with or without ZnCl$_2$ was added in dilution series, and then the biotinylated form of the same monoclonal TPPP/p25 antibody was used and detected through peroxidase conjugated avidin reaction. This arrangement does not allow the detection of the monomeric TPPP/p25 species, since they have only a single epitope, thus they are unable to bind simultaneously to the immobilized monoclonal antibody and to the soluble one detecting the bound TPPP/p25. In this experimental setup, TPPP/p25 of different concentrations without or with 2 or 10 µM ZnCl$_2$ was added to the immobilized monoclonal TPPP/p25 antibody; as a further control, a sample contained 10 µM MnCl$_2$ instead of the ZnCl$_2$. In the stock solution, a significant fraction of TPPP/p25 is present in dimeric form (Fig. 5) which dissociates into monomers due to dilution if it is not stabilized by disulfide bonds (Oláh et al. 2012). The data of the sandwich ELISA experiment presented in Fig. 22 provided evidence for the Zn$^{2+}$-enhanced dimerization.
Fig. 22. Effects of the bivalent Zn$^{2+}$ and Mn$^{2+}$ ions on the monomer-dimer equilibrium of TPPP/p25 at 1.25 μM (black column) or 0.3125 μM (white column) protein concentration. * Significant difference, according to the Student's t-test, p < 0.05.

The presence of Zn$^{2+}$ inhibits the dissociation of the dimeric forms in the course of the dilution of TPPP/p25 stock solution, increasing the number of dimers present. Manganase(II) ions, added as MnCl$_2$ from stock solution, has no effect on dimerization (Fig. 22). The Zn$^{2+}$-mediated effect at a relatively high TPPP/p25 concentration is less pronounced since the major part of the disordered protein is in dimeric form even in the absence of Zn$^{2+}$.

The functional consequence of the Zn$^{2+}$-induced structural arrangement was analyzed by means of turbidity assay monitoring the tubulin assembly as illustrated on Fig. 23. Two types of this assay were carried out to get information on the role of Zn$^{2+}$ in the dimerization of TPPP/p25: in one case TPPP/p25 from a stock solution was added to the tubulin diluted in the cuvette with or without Zn$^{2+}$; in the other case the reaction was initiated by the addition of tubulin from a stock solution to the diluted TPPP/p25 with or without Zn$^{2+}$ in the cuvette. The concentrations of tubulin, TPPP/p25 and ZnCl$_2$ were identical in all turbidity assays.
Fig. 23. The effect of Zn\textsuperscript{2+} on tubulin polymerization. The polymerization was induced by addition of TPPP/p25 from a stock solution (400 \(\mu\)M) into the diluted tubulin solution in the absence (bold line) or presence (dashed line) of 100 \(\mu\)M ZnCl\textsubscript{2}; or by the addition of tubulin from the stock solution (280 \(\mu\)M) into the diluted TPPP/p25 solution in the absence (solid line) and presence (dotted line) of ZnCl\textsubscript{2}.

As shown in Fig. 23, the tubulin polymerization promoting potency of TPPP/p25 is significantly higher when the polymerization was initiated by adding a small volume from the stock TPPP/p25 solution as compared to that when the diluted TPPP/25 was in the cuvette and the reaction was started by tubulin (Oláh et al. 2012). The presence of Zn\textsuperscript{2+} apparently counteracts the dilution-mediated reduction of TPPP/p25 activity in both experimental setups, showing that the tubulin assembly mediated by the TPPP/p25 species is favored by the addition of Zn\textsuperscript{2+}. The control experiments (without Zn\textsuperscript{2+}) showed that the dimer-enriched TPPP/p25 sample (stock solution) displays higher polymerization activity, which can be elevated by the addition of Zn\textsuperscript{2+} in both cases. It should be added that without TPPP/p25, Zn\textsuperscript{2+} neither induces tubulin assembly nor influences the paclitaxel-induced tubulin polymerization (Zotter et al. 2011b). In summary, the sandwich ELISA and the turbidity measurements proved that there is a direct relationship between the Zn\textsuperscript{2+}-induced dimerization of TPPP/p25 and its tubulin polymerization enhancing activity.
The effect of $\text{Zn}^{2+}$ on the intracellular TPPP/p25 level

The effect of $\text{Zn}^{2+}$ binding to TPPP/p25 was studied at cellular level by analyzing the intracellular TPPP/p25 concentration in a transgenic Tet-On CHO cell line named CHO10. Since CHO cells do not express TPPP/p25 endogenously, the expression of this brain-specific protein was initiated by doxycycline (a tetracycline analogue) induction in the transgenic line. Induced cells were grown overnight, and then were untreated and treated with 10 $\mu$M $\text{ZnCl}_2$ for 3 hours (Lehotzky et al. 2015). The $\text{Zn}^{2+}$ ion concentration added to the medium appears to be physiologically relevant, similar to that in the plasma and cerebrospinal fluid (10-20 $\mu$M range in plasma, 2-4 $\mu$M in CSF; Mollah et al. 2008). The short time schedule of the experiment minimalizes the potential general protein synthesis enhancing effect of $\text{Zn}^{2+}$ on transcription (Jackson 2008).

The level of TPPP/p25 expression was visualized by immunofluorescence microscopy using mouse monoclonal TPPP/p25 antibody (Fig. 24A), and the immunopositivity was quantified by image densitometry of individual cells (Fig. 24B). These data revealed that $\text{Zn}^{2+}$ significantly increased the intracellular TPPP/p25 level.

Fig. 24. The effect of $\text{Zn}^{2+}$ (10 $\mu$M) on the intracellular TPPP/p25 level (red) in CHO10 cell line represented by immunofluorescence microscopy (A) and quantified by densitometry (B). Nuclei were counterstained with DAPI. Scale bar: 2.5 $\mu$m. * Significant difference, according to the Student's t-test, $p < 0.05$. 
Earlier data provided evidence for the elevation of TPPP/p25 level due to the inhibition of the proteasomal machinery by MG132 in HeLa cells, surmising that the intrinsically unstructured TPPP/p25 is degraded by the proteasome system (Lehotzky et al. 2004). It was an open question whether the Zn\(^{2+}\)-induced structural changes, (the formation of the more compact dimers) could counteract the proteasomal degradation of the protein. To answer this, the TPPP/p25 level was quantified by cELISA in CHO10 cells expressing TPPP/p25 treated with MG132 in the absence and presence of ZnCl\(_2\) (Fig. 25). The control experiment revealed that MG132 elevated the TPPP/p25 level due to the reduced degradation of the protein. The Zn\(^{2+}\) treatment produced similar results. The combination of the addition of MG132 and Zn\(^{2+}\) did not cause further significant elevation of the TPPP/p25 level.

![Bar chart showing relative TPPP/p25 levels](image)

Fig. 25. Quantification of the effect of Zn\(^{2+}\) (10 μM) on the intracellular TPPP/p25 level in CHO10 cells at expression levels modified with MG132 (10 μM). * Significant difference, according to the Student's t-test, p < 0.05.

The possibility that the addition of Zn\(^{2+}\) increases the synthesis of TPPP/p25, which could increase the TPPP/p25 level, was excluded by a similar set of experiments carried out in the presence of cycloheximide, a non-specific inhibitor of the protein synthesis. The treatment of the
cells with cycloheximide reduced the TPPP/p25 level in the control and Zn$^{2+}$ supplemented samples; however, the addition of Zn$^{2+}$ counteracted the reducing effect of cycloheximide on TPPP/p25 (Lehotzky et al. 2015).

These data suggest that the Zn$^{2+}$-induced structural alteration, likely the dimerization, protects this disordered protein against the proteolytic degradation by the proteasomal machinery. The proteasome system could be the main, if not exclusive, degrading system responsible for the elimination of the unbound TPPP/p25 (Lehotzky et al. 2004, Goldbaum et al. 2008), as described for many IDPs (Melo et al. 2011, Suskiewicz et al. 2011, Tsvetkov et al. 2012).

5.2. Pathological interaction of TPPP/p25 with α-synuclein

TPPP/p25, as a NMP protein, displays not only physiological but pathological functions as well (Kovács et al. 2004). TPPP/p25 binds to SYN with high affinity (Oláh et al. 2011), and induces SYN fibrillation in vitro at substoichimetric TPPP/p25 concentration (Lindersson et al. 2005).

5.2.1. Characterization of the pathological interaction

As an innovative strategy, the Cell Architecture research group aimed to identify the contact surface of the pathological TPPP/p25-SYN complex as a potential drug target. Targeting the proteins would inhibit/interfere with their physiological functions, however, targeting only their interface, composed of the segments of the two hallmark proteins would allow the specific inhibition/destruction of the TPPP/p25-SYN complex without affecting the physiological TPPP/p25-tubulin complex. To fulfill this task, the pathological contact segment(s) of TPPP/p25 must be distinct from the unstructured termini that are involved in the physiological interaction with tubulin.

To study the role of the unstructured N- and C-termini of TPPP/p25 in the formation of the TPPP/p25-SYN pathological complex, ELISA experiments were performed with FL and CORE TPPP/p25; TPPP/p25 forms were immobilized on the plate, SYN was added at different concentrations and the bound SYN was detected using a polyclonal rabbit SYN antibody.
As shown in Fig. 26, SYN binds to both TPPP/p25 forms with comparable affinity, in agreement with the data obtained in previous Pepscan experiments (Oláh et al. 2011), suggesting that SYN is associated with the CORE segment of TPPP/p25. The apparent binding affinities of the FL and the CORE TPPP/p25 assuming simple hyperbolic saturation were comparable: $0.59 \pm 0.07$ and $0.81 \pm 0.09 \mu M$, respectively.

5.2.1.1. Determination of the $\alpha$-synuclein binding site on TPPP/p25

To identify the region within the CORE segment of TPPP/p25 involved in the SYN binding, competitive ELISA experiments with synthetized peptides were carried out. The peptides were designed based upon the Pepscan data (Oláh et al. 2011) that suggested the $^{147}$KAPIISGVTAKISSPTVSRL$^{166}$ segment of TPPP/p25 as a potential SYN binding domain. The following overlapping decapeptides with a biotin tag were synthesized by Prof. Magyar lab:
BF180: C-biotinylated: Ac-^{142}RLIEGKAPI^{151}K-NH₂
BF181: N-biotinylated: H-^{142}RLIEGKAPI^{151}N₂
BF182: C-biotinylated: Ac-^{147}KAPIISGVTK^{156}N₂
BF183: C-biotinylated: Ac-^{157}AISSPTVSRL^{166}K-NH₂

The biotinylated peptides were immobilized onto streptavidin covered plates, then they were incubated with a serial dilution of SYN and the bound SYN was quantified with a rabbit polyclonal SYN antibody (Fig. 27).

![Graph](image)

**Fig. 27.** The interaction of TPPP/p25 fragments with SYN. BF180 (●), BF181 (○), BF182 (▲) and BF183 (Δ).

No significant difference was found in the binding affinity of SYN to the fragments immobilized on the plate (direct binding assay), in addition, the binding of SYN is apparently independent of the nature of the decapeptides as well as the position of the biotinylation. The apparent affinity constants (Kₐ) evaluated by fitting the saturation curves with a simple hyperbole were 2-4 µM for the BF180, BF181, BF182 and BF183 fragments. These were significantly higher than that determined in the case of the FL (0.59 ± 0.07 µM) and the CORE (0.81 ± 0.09 µM) TPPP/p25 meaning that the fragments display reduced affinity to SYN (Fig. 26).
To further analyze the interaction of these peptides with SYN, a competitive ELISA experiment was carried out (Fig. 28). In this setup, 10 and 20 μM of the biotinylated peptides were premixed with SYN, then added to the plate coated with FL or CORE TPPP/p25. Therefore, the potential of these peptides to act as competitors of the interaction of SYN and TPPP/p25 can be quantified. The bound SYN was detected by polyclonal rabbit antibody. The concentration of SYN was constant, close to the half saturation value determined with TPPP/p25 (Fig. 26). This experimental setup is appropriate for screening potential drugs modifying the interaction of the two pathologically important proteins.

![Graph showing the competition of BF peptides with SYN (0.5 μM) in the interaction with the TPPP forms. The data presented as the white column corresponds to the FL, the black one to the CORE TPPP/p25. * Significant difference to the control, according to the Student's t-test, p < 0.05.]

The fact that the binding of the FL and the CORE TPPP/p25 to SYN were similarly inhibited by the BF182 fragment shows its inhibition potency in the association of the two proteins and it is in concern with that presented above, namely, that the middle CORE region of TPPP/p25 involves the SYN binding domain. However, the competitive ELISA data seems to contradict the direct binding ELISA experiments, where SYN displayed similar affinity to the biotinylated TPPP/p25 peptides (Kd ~ 2-4 μM). A plausible explanation for this inconsistency is that the peptide biotinylation may play distinct roles in the direct and indirect binding experiments in the
interaction of TPPP/p25 with SYN. Nevertheless, ELISA data suggest that the $^{147}$KAPIISGVTK$^{156}$ segment of the CORE region is involved in the formation of the TPPP/p25-SYN complex.

5.2.1.2. Determination of the TPPP/p25 binding site on $\alpha$-synuclein

Following the interface characterization of the pathological TPPP/p25-SYN complex, I aimed to identify the SYN segment(s) involved in its association with TPPP/p25. Limited proteolytic experiments of SYN with trypsin indicated its unstructured negatively charged C-terminal tail as a potential TPPP/p25 binding region (Szunyogh et al. 2015), therefore, recombinant truncated forms of SYN (Fig. 29A) were produced and used for interaction studies.

![Schematic representation of the prepared SYN forms. B. SDS-PAGE of the SYN forms (2 $\mu$g/lane). C. Selective recognition of the different antibodies against the N-(black column) and C-terminal ($^{12}$DNEAY$^{125}$, white column) of SYN. * Significant difference, according to the Student's t-test, p < 0.05.](image)
The truncated SYN forms used for in vitro studies were as follows: SYN^{95-140}, corresponding to the complete C-terminus; SYN^{1-120}, a truncated form without the last 20 aa of the protein and a peptide corresponding to the last 15 aa of the C-terminus (ordered from the ChinaPeptides Co.). Fig. 29B shows the SDS-PAGE image of the SYN forms. The anomalously slow gel mobility of the relatively small SYN^{95-140} fragment is probably due to its low hydropathy (Rath et al. 2009) and its disordered state (Iakoucheva et al. 2001). Fig. 29C illustrates the immunopositivity of the two different antibodies with epitopes at the N- or the C-terminal SYN that can recognize the C- or N-truncated SYN forms, respectively.

The interaction of the different SYN forms with TPPP/p25 was examined in two sets of experiments. In one set, the binding of TPPP/p25 to SYN was studied by affinity chromatography. SYN was bound covalently onto a CNBr-activated Sepharose 4B beads, TPPP/p25 was loaded to the column in the absence and presence of SYN or its truncated form, and the collected fractions were analysed by SDS-PAGE. The control experiment shows that the SYN affinity column is capable of binding TPPP/p25 according to its capacity (Fig. 30A).
Fig. 30. Competitive binding of TPPP/p25 to the SYN forms and the SYN affinity column. The binding of TPPP/p25 in the absence of further SYN forms (A), in the presence of the FL SYN (B) or SYN$^{1-120}$ (C).

When TPPP/p25 with excess SYN was premixed and loaded into the SYN affinity column (Fig. 30B), a significant fraction of the TPPP/p25 was complexed with the soluble SYN and flowed
through the column; thus the amount of TPPP/p25 retained by the column was significantly reduced compared to that when TPPP/p25 was loaded without soluble SYN (Fig. 30A). When the experiment was carried out with SYN\textsuperscript{1-120} (Fig. 30C), TPPP/p25 showed an elution profile identical to that without SYN (Fig. 30A) indicating that this long segment of SYN cannot associate with TPPP/p25, suggesting that the binding domain is localized on its C-terminal segment.

The binding potencies of the FL SYN, SYN\textsuperscript{1-120} and SYN\textsuperscript{95-140} forms to TPPP/p25 were quantified with ELISA (Fig. 31). TPPP/p25 was added in a serial dilution to the different SYN forms immobilized on the plate, and the bound TPPP/p25 was detected by rat polyclonal TPPP/p25 antibody.

![Graph showing the binding of TPPP/p25 to different SYN forms](image)

**Fig. 31.** The binding of TPPP/p25 to different SYN forms. SYN (●, solid line), SYN\textsuperscript{95-140} (◇, dashed line), SYN\textsuperscript{1-120} (Δ, dotted line).

As illustrated, TPPP/p25 showed poor binding to SYN\textsuperscript{1-120}, while displayed comparable affinity to SYN\textsuperscript{95-140} as SYN. This finding confirms the dominant role of the 121-140 aa segment in the pathological complex formation.

The contact surface of SYN involved in the TPPP/p25 binding was further investigated with competitive ELISA experiments. TPPP/p25 was preincubated with SYN in the presence of
the various SYN mutants and synthetized peptide SYN\textsuperscript{126-140}, then was added to the immobilized SYN. The bound TPPP/p25 was detected with a rat polyclonal TPPP/p25 antibody.

![Graph showing binding values relative to the control (no SYN)](image)

Fig. 32. The competition of the SYN forms with immobilized SYN in the binding to 125 nM TPPP/p25. Binding values relative to the control (no SYN) are shown. The colors indicate the presence of the different SYN forms: SYN (1 µM) is black, SYN\textsuperscript{1-120} (1 µM) is light grey, SYN\textsuperscript{95-140} (1 µM) is gray and SYN\textsuperscript{126-140} (50 µM) is dark gray; the control experiment without SYN is shown as white. * Significant difference, according to the Student's t-test, p < 0.05.

As shown in Fig. 32, the SYN forms containing the C-terminus or its last 15 aa segment, but not the SYN\textsuperscript{1-120}, reduced the binding of TPPP/p25 to the immobilized SYN. Therefore, it further reinforces that the 121-140 aa segment of SYN is involved in the formation of the pathological TPPP/p25-SYN complex.

### 5.2.2. Intracellular visualization of the pathological complex

TPPP/p25 is predominantly expressed in OLGs (Skjoerringe et al. 2006), while SYN is a neuronal protein (Iwai et al. 1995); although they are co-enriched and co-localized in both OLGs
and neurons of human brain in the cases of MSA and PD, respectively (Kovács et al. 2004). The transcellular spreading and cell-to-cell transmission of SYN has been reported (Lee et al. 2008, Bates et al. 2014).

We also provided evidence for the uptake of TPPP/p25 from the medium. Its intracellular appearance in HeLa and CHO10 cells was detected by immunofluorescence microscopy using specific TPPP/p25 antibody. Both the FL and the CORE TPPP/p25 appears intracellularly, although the uptake of the CORE TPPP/p25 resulted in the formation of aggregates in a few cases (Fig. 33).

![Fig. 33. Cellular uptake of the FL (B) and the CORE TPPP/p25 (C) by immunofluorescent microscopy in non-induced CHO10 cells using rat polyclonal TPPP/p25 antibody. Nuclei were counterstained with DAPI. Scale bar: 2.5 μm.](image)

The intracellular associations of SYN with TPPP/p25 or its CORE (SYN binding) form were characterized in the CHO10 cell line. After premixing SYN with either the FL or the CORE TPPP/p25 forms, the proteins were added to the medium to be taken up by the cells. Following incubation, the cells were fixed and the localization and enrichment of both TPPP/p25 forms and SYN were detected by immunocytochemistry.
Fig. 34. Intracellular aggregation of the FL or CORE TPPP/p25 (2.5 μM) with or without SYN (10 μM), the proteins were premixed and added to the medium of CHO10 cells as indicated. Nuclei were counterstained with DAPI. Scale bar: 2.5 μm.

As shown in Fig. 34, the CORE form resulted in more extensive aggregation than the recombinant FL TPPP/p25 due to the fact that the termini-free CORE TPPP/p25 is unable to bind to the MT network (Fig. 9Fig. 18). These findings not only provide evidence that TPPP/p25 and SYN associates intracellularly, but also highlight, in agreement with the in vitro data, that the CORE segment of TPPP/p25 is involved in the formation of their pathological aggregation.

To validate the role of the SYN\textsuperscript{126-140} segment of the C-terminus in the co-aggregation of TPPP/p25 and SYN, a similar set of experiments was carried out with CHO10 cells in the presence of the SYN\textsuperscript{126-140} fragment as a potential binding partner of TPPP/p25. In this type of experiment, concentrations of CORE TPPP/p25 and SYN were slightly higher in order to promote the aggregation of the proteins, to visualize the effectivity of the SYN fragment.
Fig. 35. Intracellular aggregation as a result of the association of the different TPPP/p25 (3 μM) and SYN forms (12 μM). The FL (A, C, E, globally the left side) or CORE (D, F) TPPP/p25 and/or SYN (B, C, D), and FITC-SYN$^{126-140}$ (E, F) were premixed then added to the medium of the cells. The exposure times were 1 s, except in panel D, where it had been reduced to 0.1 s, due to the high fluorescent intensity of the co-aggregates of the CORE TPPP/p25 and SYN (merge). Nuclei were counterstained with DAPI. Scale bar: 5 μm.

The results obtained with TPPP/p25 and SYN forms expected to be involved in the formation of their pathological complex can be visualized in Fig. 35. As shown in Fig. 35A and B, the cells treated with FL TPPP/p25 or SYN showed virtually no aggregations. The premixing of TPPP/p25 with the FL or the FITC labelled SYN$^{126-140}$ (Fig. 35C and E) resulted in small
intracellular co-aggregates. In the case of SYN<sup>1-120</sup> premixed with TPPP/p25, no aggregation was observed (detected with the rabbit monoclonal SYN antibody specific for the N-terminus of SYN, data not shown). The CORE TPPP/p25 premixed with the FL SYN (Fig. 35D) produced intensive aggregation indicating their massive aggregation-prone potency. The co-enrichment of the CORE TPPP/p25 with the SYN<sup>126-140</sup> (Fig. 35F) peptide underlines their role in the constitution of the contact surface of the pathological complex, as well as validates the segments of the CORE TPPP/p25 and the very last aa of the C-terminus of SYN as contact surface of the pathological complex.

**5.2.3. Impact of the presented data in Parkinson research**

Currently there is no cure for PD, but medications can help to control its symptoms, which are caused by the death of dopaminergic neurons, thus the lack of dopamine in the brain (Chinta et al. 2005, Surmeier et al. 2010). Many anti-Parkinson drugs aim either temporarily replenish dopamine or mimic the action of dopamine (levodopa, dopamine agonists; Jankovic et al. 2008). However, current therapies are only symptomatic treatments. The mechanisms which lead to inclusion formation and later the loss of dopaminergic neurons are still under intense investigation.

The results presented in my PhD dissertation could open a door to a new, innovative strategy in Parkinson research: the validation of a novel drug target for PD, which is a key issue. Nowadays, it is clear that SYN and its interacting partner, TPPP/p25 are hallmarks of PD and other synucleinopathies, yet targeting these multifunctional hallmarks themselves cannot provide appropriate therapeutic solution probably since both proteins are involved in physiological and pathological events. In order to destruct the pathological complex without affecting the physiological one, the interface of the pathological TPPP/p25-SYN complex was considered to be a potential drug target. This task could be successful if the contact surface of the pathological complex is distinct from that of the physiological one. The presented data suggest that the segment(s) of TPPP/p25 involved in SYN binding is localized within the flexible, middle CORE region, while the segment associates with tubulin can be found dominantly in its unstructured C-terminus. Therefore, there is an opportunity to develop powerful specific anti-Parkinson agents with limited toxic side effects.
The Cell Architecture research group have developed a specific competitive ELISA assay for the in vitro screening of compounds that inhibit/destroy the TPPP/p25-SYN complex but not the TPPP/p25-tubulin interaction. With this method, the effectivity of the inhibitors could be quantified as well. This test could be a first step in the validation of a potential drug; it renders the evaluation of peptides or peptide derivatives possible leading to the construction of peptidomimetic foldamers. Furthermore, we have established a CHO10 cell model suitable to detect TPPP/p25-SYN aggregates, which gives rise to the possibility to test any promising compounds/fragments also in cell cultures for their potential activity to inhibit or reduce the formation of these pathological aggregates.

The recognition that both SYN and TPPP/p25 can be transferred from the medium into the cells suggests their potency to be transported from extracellular space. This issue largely contributes to our understanding how these proteins can co-enrich and co-localize in inclusion bodies characteristic not only in PD but also in MSA, despite the fact that they are expressed in distinct cell types in normal brain. The occurrence of SYN and TPPP/p25 was detected in cerebrospinal fluid samples of human patients previously (Borghi et al. 2000, Vincze et al. 2011, Marques et al. 2012), which supports this assumption as well.
6. SUMMARY

The brain-specific Tubulin Polymerization Promoting Protein (TPPP/p25) as a Microtubule Associated Protein is an intrinsically disordered protein that primarily is expressed in oligodendrocytes and plays a crucial role in the differentiation of progenitor cells at physiological circumstances; however, its co-enrichment and co-localization with α-synuclein (SYN) occur at pathological condition leading to the etiology of synucleinopathies. Within the frame of my PhD, I aimed to characterize the homo-and hetero-associations of TPPP/p25 and their functional consequences. Experiments with the human recombinant wild type and the truncated variants of TPPP/p25 provided evidence for the ability and significance of dimerization of the unfolded monomeric TPPP/p25 that has a determining role in the microtubule assembly, as well as in the interaction with tubulin and microtubules as physiological partners. The Zn$^{2+}$, as a specific bivalent cation, causes dimerization with a more compact structure. The relevance of the dimeric form was demonstrated in HeLa cells with bimolecular fluorescent microscopy. The Zn$^{2+}$ bound TPPP/p25 is more resistant to proteolytic digestion, therefore Zn$^{2+}$ seems to be involved in the fine-tuning of the physiological TPPP/p25 level. The protein is a prototype of the Neomorphic Moonlighting Proteins, possesses both physiological and pathological functions. The pathological partner is the disordered SYN, hallmark of the Parkinson’s disease and other synucleinopathies. Since both proteins play important physiological roles, they cannot be specific drug targets by themselves. The innovative strategy of the Cell Architecture research group is to target the interface of the pathological TPPP/p25-SYN complex without affecting the physiological interactions. The interfaces of the pathological TPPP/p25-SYN and the physiological TPPP/p25-tubulin complex were identified with truncated protein forms and peptides by circular dichroism, ELISA, turbidimetry and affinity chromatography. Distinct segments of TPPP/p25 are involved in the different complexes: tubulin interacts primarily with the unstructured C-terminus of TPPP/p25; while the C-terminus of the SYN associates with the flexible CORE segment of TPPP/p25. This situation renders it possible to design selective anti-Parkinson drugs. In addition, the results presented in this thesis introduce an in vitro method (competitive ELISA), which is appropriate for screening potential drugs, and a cell model, which is capable of TPPP/p25 and SYN uptake and the two hallmark proteins and their relevant fragments form intracellular aggregates, thus this cell model provides the opportunity to visualize the inhibitory effect of the drugs, selected on the basis of in vitro data, by fluorescence microscopy and also to quantify it by Western blot.
7. ÖSSZEFOGLALÁS

8. LIST OF PUBLICATIONS

Publications in connection with the Ph. D. thesis:


Other publications:

9. REFERENCES


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