

SYNERGISM OF TPPP/p25 WITH CHEMICAL INHIBITION AND PROTEOLYSIS OF SIRT2 ON THE MICROTUBULE NETWORK

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ABSTRACT

The cytoskeletal microtubule (MT) network exerts multifarious functions by its decoration with proteins and posttranslational modifications. The disordered Tubulin Polymerization Promoting Protein (TPPP/p25) plays key roles in the regulation of the dynamics and stability of the MT network during the differentiation of oligodendrocytes, the major constituent of the myelin sheath. The NAD⁺-dependent tubulin deacetylase sirtuin-2 (SIRT2) modifies the acetyl-tubulin level of the projections by acting as a component of the myelin proteome. Here we showed that SIRT2 reduces the TPPP/p25-stimulated tubulin polymerization while TPPP/p25 counteracts the SIRT2-derived tubulin deacetylation. Our mathematical model based on in vitro binding data allows ones to quantify the amount of binary and ternary complexes at different protein concentrations and to predict the functional consequences. The co-localization of the SIRT2-TPPP/p25 complex on the MT network was visualized by immunofluorescence microscopy coupled with Bimolecular Fluorescence Complementation technique. In addition, synergistic acetylation effect of TPPP/p25 with both chemical SIRT2 inhibition and chemically induced proteolysis by a thalidomide/sirtuin inhibitor chimera (PROTAC) was established providing a direction for innovative strategy in cancer research.

KEYWORDS

SIRT2 / SIRT2 inhibitors / synergism / TPPP/p25 / tubulin acetylation

INTRODUCTION

Microtubules (MTs) have important functions ranging from maintenance of cell morphology to subcellular transport, cellular signaling, cell migration, and formation of cell polarity (Conde & Caceres, 2009; de Forges et al, 2012; Li & Yang, 2015). At organismal level, MTs are crucial for various biological processes, such as viral entry, inflammation, immunity, learning and memory in mammals (Li & Yang, 2015). The dynamic balance between MT extension and actin contraction regulates mammalian cell shape, division and motility, thus the cytoskeleton is an attractive and successful target for cancer drugs (Chakrabarti et al, 2015; Florian & Mitchison, 2016; Penna et al, 2017). A number of anti-MT drugs have been reported that reduce tumor growth by their depolymerization (e.g. vinca alkaloids) or hyper-stabilization potencies (e.g. taxanes) (Chakrabarti et al, 2015; Florian & Mitchison, 2016; Penna et al, 2017). Regarding cancer therapeutic aspects, these drugs are not specific for exclusively targeting cancer cells thus cause dose-limiting toxicities. Up to now, several data have been reported that posttranslational modifications of tubulin (such as acetylation) and Microtubule Associated Proteins (MAPs) such as tau could be more specific targets of MT subsets by avoiding the disruption of all MT assemblies (Chakrabarti et al, 2015).

Tubulin Polymerization Promoting Protein (TPPP/p25), a recently discovered MAP (Hlavanda et al, 2002; Ovádi, 2008; Oláh et al, 2013), is expressed predominantly in oligodendrocytes (OLGs) (Skjoerringe et al, 2006; Lehotzky et al, 2010). It is a disordered protein with extended unstructured terminal segments straddling a flexible region, which includes important binding domains such as a zinc finger and GTP consensus sequence (Zotter et al, 2011a and b). A number of *in vitro* studies with wild type and mutant recombinant proteins have revealed that TPPP/p25 induces tubulin assembly coupled with MT bundling and aggregation (Oláh et al, 2013 and references therein). These features of TPPP/p25 are related to its physiological functions, namely, the modulation and coordination of the dynamics and stability of the MT network including projection growth in the course of OLG differentiation (Lehotzky et al, 2010). TPPP/p25 has been identified as an interacting partner of tubulin deacetylases, histone deacetylase 6 (HDAC6) and the nicotinamide adenine dinucleotide (NAD⁺)-dependent sirtuin-2 (SIRT2) (Tőkési et al, 2010; Mangas-Sanjuan et al, 2015).

The tubulin subunits are subjected to a number of post-translational modifications, such as acetylation, phosphorylation, tyrosination, polyglycylation and polyglutamylation (Perdiz et al, 2011; Li & Yang, 2015). The acetylation level of tubulin/MTs is controlled by opposing enzymatic activities of tubulin deacetylases and acetyltransferases. The silent information regulator (SIR) proteins belong to the Class III HDACs displaying NAD⁺-dependent deacetylase activity; SIRT2 deacetylates the Lys-40 of α -tubulin both *in vitro* and *in vivo*, interacts with HDAC6, and co-localizes with MTs (North et al, 2003). The inhibition of either HDAC6 or SIRT2 alone by siRNA is sufficient to induce hyperacetylation of tubulin suggesting that both enzymes function interdependently in tubulin deacetylation (North et al, 2003). Abnormal level of this modification is linked not exclusively to cancer but to neurological and heart diseases as well, thereby yielding important therapeutic implications (Li & Yang, 2015). Uniquely among the HDACs, HDAC6 and SIRT2 have cytoplasmic localization and their inhibition by specific inhibitors such as Trichostatin A and AGK2, respectively, increases the intracellular level of acetylated tubulin and alters cell motility (Tőkési et al, 2010 and references therein; Harting & Knöll, 2010).

The human SIRT2 plays a role in mitosis, during which its abundance is increased dramatically (Dryden et al, 2003); Saos2 cells stably overexpressing SIRT2 show a marked prolongation of the mitotic phase of the cell cycle; addition of 26S proteasome inhibitors to these human cells increases the abundance of the SIRT2 protein, indicating that SIRT2 is degraded by the proteasome machinery. SIRT2 is a component of the myelin proteome and is severely reduced in the proteolipid protein (PLP) knockout mouse brain (Werner et al, 2007). In addition, SIRT2 as a tubulin deacetylase is an abundant neuronal protein, its endogenous level correlates with reduced α -tubulin

acetylation in primary mouse cortical neurons, and functions as a predominant MT deacetylase in mature neurons (Maxwell et al, 2011). Nevertheless, the mechanism that regulates SIRT2 expression and activity has not been clarified in details.

High-resolution structures of SIRT2 in complex with highly selective drug-like inhibitors have been evaluated and a unique inhibitory mechanism for the SirReal class of inhibitors has been established (Rumpf et al, 2015). Their potency and unprecedented selectivity of the inhibition is based on ligand-induced structural rearrangement of the active site, which unveiled a yet-unexploited binding pocket. Application of potent Sirtuin-rearranging ligands, MZ25 and MZ242, lead to tubulin hyperacetylation in HeLa cells and induced the destabilization of the checkpoint protein BubR1, consistent with *in vivo* SIRT2 inhibition (Rumpf et al, 2015; Schiedel et al, 2016a and b).

In this paper the mutual effect of the TPPP/p25- and Sirt2-derived tubulin deacetylation on the structural and functional features of the MT system is established at different organization levels. In addition, the interplay of TPPP/p25 and the powerful chemical deacetylase inhibitors is presented in relation to their mutual effects on the tubulin acetylation level.

RESULTS

Characterization of the interaction of SIRT2 with TPPP/p25 and/or tubulin

The circular dichroism (CD) spectrum of the human recombinant SIRT2, similarly to that of tubulin, shows typical features characteristic for α -helical proteins with two prominent minimum at 208 and 222 nm (Greenfield, 2006); in contrast to this, the spectrum of TPPP/p25 corresponds to that of disordered proteins displaying a minimum at 205 nm (Hlavanda et al, 2002) (Fig. 1A). By investigating the pair-wise associations of these proteins, we found changes in their secondary structures indicating complex formation in the case of TPPP/p25, which generated differential ellipticity spectra with a maximum at nearly 207 nm (Fig. 1B). The structural change in the case of the disordered TPPP/p25 was more pronounced when associated with tubulin as compared to that obtained with SIRT2; while there was little difference in the spectra of tubulin and SIRT2.

The interactions of SIRT2 with TPPP/p25 and tubulin were quantified by ELISA as shown in Fig. 2A. SIRT2 was immobilized on the plate, then tubulin or TPPP/p25 was added at various concentrations. The binding of the proteins to the coated SIRT2 was detected by specific antibodies. The binding constants were evaluated by nonlinear fitting of the hyperbolic saturation curves; accordingly, the apparent dissociation constants (K_d) for the interaction of SIRT2-TPPP/p25 and SIRT2-tubulin are 32.4 ± 4.6 nM and > 2 μ M, respectively. For comparative purpose, the association of tubulin with TPPP/p25 was also measured at similar conditions, the binding constant ($K_d = 16.4 \pm 1.4$ nM) corresponds to that published earlier (Mangas-Sanjuan et al, 2015). These data indicate the preferential association of SIRT2 to TPPP/p25 as compared to tubulin.

To establish the nature of the interaction of SIRT2 with its partners, tubulin and TPPP/p25, competitive ELISA experiments were performed (Fig. 2B and C). SIRT2 was immobilized on the plate, then incubated with tubulin at various concentrations as presented in Fig. 2A; however, the tubulin aliquots were pre-incubated with TPPP/p25 of constant concentration. The SIRT2-bound protein was quantified by a specific TPPP/p25 antibody as described in Materials and Methods. As shown in Fig. 2B, the immuno-positivity of SIRT2-bound TPPP/p25 decreases as a function of increasing tubulin concentration suggesting competition between TPPP/p25 and tubulin for SIRT2 binding. However, we realized that this observation apparently contradicted our previous ELISA data (cf. Fig. 2C), namely, the binding of tubulin to immobilized SIRT2 is promoted by TPPP/p25 when it was detected with tubulin antibody (Mangas-Sanjuan et al, 2015). To verify this finding, an ELISA experiment was performed with immobilized SIRT2 by adding 1 μ M tubulin or 0.25 μ M TPPP/p25 or their pre-incubated mixture, and then both the bound tubulin and TPPP/p25 were

detected by specific antibodies. As shown in Fig. 2C, the presence of TPPP/p25 increased the amount of the bound tubulin while the addition of tubulin decreased the TPPP/p25 binding to SIRT2. This observation seems to exclude the competitive binding of tubulin and TPPP/p25 to the immobilized SIRT2; rather, it suggested their simultaneous association with SIRT2.

Modelling of the heteroassociation of tubulin, SIRT2 and TPPP/p25

We evaluated a mathematical model for the heteroassociation of the MT-binding proteins involved in the control of the acetylation level of the MT network. The model includes the pair-wise dissociation constants (K_d) of the complexes of tubulin, SIRT2 and TPPP/p25 and the total (complexed and uncomplexed) concentrations of the individual proteins (For details see Supplementary materials). In the binary model, no ternary complex was assumed, while in the ternary model, the binding of TPPP/p25-SIRT2 to tubulin was allowed.

The constants are established from relevant parameters determined experimentally (cg. Fig. 2A) which allow the quantification of the interactions at macroscopic level. The evaluated model renders the mechanistic description of the mutual interactions possible, including the assembly of the three proteins forming binary and ternary complexes. The parameters presented in Table S1 were used to evaluate the uncomplexed and complexed (binary plus ternary) forms of the proteins as a function of tubulin concentration as shown in Fig. 3A. Thus, the model determines the quality and the quantity of the complexes bound to SIRT2 at different concentrations of tubulin. Fig. 3B illustrates the predicted curve that appropriately simulates the ELISA experimental data, the binding of tubulin to the immobilized SIRT2 in the absence and presence of TPPP/p25. Therefore, the model renders the prediction of the complexes possible at different concentrations of the partner proteins occurring at various conditions related to the potency of TPPP/p25 for the acetylation level of the MT system.

Effect of SIRT2 on the TPPP/p25-promoted tubulin assembly

According to the predicted binding data, the functional consequences of the heterologous associations were characterized: on one hand, the role of SIRT2 in the TPPP/p25-promoted tubulin assembly; on the other hand, the role of TPPP/p25 in the SIRT2-derived tubulin deacetylation. Accordingly, the following types of experiments were performed i) TPPP/p25-induced tubulin polymerization detected by turbidimetry; ii) determination of the partition of SIRT2 in the supernatant and pellet fractions of the samples obtained by the tubulin polymerization assay; iii) quantification of the acetylation degree; iv) visualization of the assembled tubulin structures by electron microscopy.

First of all, the acetylation level of the isolated tubulin and its assemblies were determined by ELISA as well as electron microscopy. These data proved that both the tubulin subunits and the polymerized forms are appropriate substrates of the SIRT2 (Supplementary Fig. 1). Previously we reported that TPPP/p25 promotes tubulin polymerization *in vitro* resulting in intact-like MTs, many of them bundled, as well as amorphous protein aggregate (Hlavanda et al, 2002; Tókési et al, 2010; Oláh et al, 2013). The addition of SIRT2 to the polymerization assay altered the TPPP/p25-induced turbidity of the tubulin (Fig. 4A); the effect appears to be independent on the presence of NAD^+ , i.e. whether the SIRT2 is active or not (cf. Fig. 4C). The samples of the polymerization assay (Fig. 4A) were pelleted and analyzed by SDS/PAGE. According to the SDS/PAGE and Western blot images (Fig. 4B and C), it can be concluded that the presence of SIRT2, independently of the presence of NAD^+ , decreased the tubulin and TPPP/p25 amounts in the pellets (Fig. 4B). The electron microscopic analysis unambiguously shows that SIRT2 counteracts the TPPP/p25-induced tubulin assembly into intact-like MTs and aggregates (Fig. 4D). These data reveal that SIRT2 displays an

anti-MT effect as a “structure” protein affecting the binding of TPPP/p25 to tubulin, but not as a deacetylase.

Effect of TPPP/p25 on the deacetylase efficacy of SIRT2

To study the nature of the effect of TPPP/p25 on the SIRT2-derived tubulin deacetylation, a SIRT2 activity experiment was performed. Fig. 5 shows that SIRT2 decreases the acetylation level of tubulin in a concentration-dependent manner due to its deacetylase activity. The SIRT2 activity was calculated from the decrease of tubulin acetylation level quantified by Western blot using acetyl-tubulin antibody. However, increasing concentrations of TPPP/p25 at a SIRT2 concentration corresponding to its 80% normalized activity, resulted in only a modest increase in the tubulin acetylation level even at 20 μ M TPPP/p25. Under this condition, SIRT2 is expected to be complexed with TPPP/p25 (cf. Fig. 2), yet the deacetylation of tubulin is largely maintained excluding the realization of a competitive binding mechanism. This finding is in accordance with the model based upon the binding data.

In order to obtain information on the SIRT2-derived deacetylation of tubulin as compared to MT, the deacetylase potency of SIRT2 as well as the inhibitory potency of TPPP/p25 were quantified using tubulin heterodimers and paclitaxel-stabilized MTs. The acetylation levels were determined by Western blot using acetyl-tubulin antibody at 0.6 μ M SIRT2 concentration in the absence and presence of 20 μ M TPPP/p25. In this comparative study, the concentrations of tubulin, (subunits of) MT and SIRT2 were similar in both sets of experiments as shown by SDS/PAGE (Fig. 6A); the acetylation levels were quantified by Western blot (Fig. 6B). According to the summarized data presented on Fig. 6C, SIRT2 displays a tendency to be more active on tubulin as compared to MT, while the inhibitory activity of TPPP/p25 seems to be more effective on MT. It can be concluded, that both the assembled and disassembled acetyl-tubulins function similarly as substrates for SIRT2; therefore, the data obtained by *in vitro* experiments with tubulin seems to correspond for MTs, even for MT network as well.

Effect of TPPP/p25 on the SIRT2 activity affected by chemical SIRT2 inhibitors

Dysregulation of human SIRT2 activity has been associated with the pathogenesis of cancer, inflammation, and neurodegeneration, which makes the modulation of its activity a promising strategy for pharmaceutical intervention (Li & Yang, 2015; Rumpf et al, 2015; Schiedel et al, 2017a). A number of SIRT2 modulators have been discovered such as nicotinamide derivatives. However, recently highly potent and isotype-selective deacetylase inhibitors have been discovered and denoted Sirtuin Rearranging Ligands (SirReals); structure-function relationship studies rationalized and optimized their unique features (Rumpf et al, 2015; Schiedel et al, 2016a and b). Recently, a proteolysis targeting chimera (PROTAC) based on the combination of SirReals with thalidomide, a *bona fide* cereblon ligand, has been developed that chemically induces isotype specific degradation of SIRT2 by the proteasome machinery (Schiedel et al, 2017b). The inhibitory potency of these highly potent SIRT2 inhibitors as drug-like compounds were compared with the effectivity of TPPP/p25 as well as their mutual inhibitory potencies were quantified using synthetic substrates and/or tubulin.

The inhibitory potencies of SirReal derivatives and TPPP/p25 on SIRT2 activity were determined with synthetic (the commercial assay) and tubulin heterodimer substrates (Western blot) as described in Materials and Methods. As shown in Fig. 7A, the efficacy of the different compounds to inhibit SIRT2 is comparable with that of TPPP/p25; however, the inhibitory potencies are distinct concerning their effectivity on the nature of the substrate, tubulin or the fluorescence substrate.

TPPP/p25 does not inhibit the deacetylation by SIRT2 acting on the synthetic substrate, suggesting that it may bind to a different domain of the SIRT2.

The effect of chemical inhibitors on the association of 0.25 μ M SIRT2 to the immobilized TPPP/p25 was tested by ELISA. Fig. 7B shows that the compounds MZ242, MZ25 and SH1 (PROTAC) up to 30 μ M concentration do not influence significantly the interaction of SIRT2 and TPPP/p25 indicating that the binding segment/domain of the SIRT2 involved in the heteroassociation with TPPP/p25 is distinct from the inhibitor binding domain. The synergistic inhibitory potency of MZ242 inhibitor and TPPP/p25 is shown in Fig. 7C. Remarkably, TPPP/p25 and MZ242 display significantly higher inhibitory potency than the sum of their individual ones.

Dynamic association of TPPP/p25 and SIRT2 in living HeLa cells

Previously we have revealed the co-localization of TPPP/p25 with the MT network in HeLa cells transiently transfected with TPPP/p25 (Lehotzky et al, 2004). The cancerous HeLa cells express TPPP/p25 endogenously at a very low level, if at all; the MT network is virtually deacetylated, however, ectopic expression of TPPP/p25 induces acetylation of the MT network (Tökési et al, 2010). In order to validate the *in vitro* data obtained with isolated proteins and chemical compounds, we performed experiments with HeLa cells using BiFC technique: mVenus vectors of both SIRT2 and TPPP/p25 (V^N -SIRT2 and V^C -TPPP/p25) (cf. Fig. 8A) were prepared as described in the Materials and Methods.

For BiFC signal, the N-terminal and the C-terminal domains of a fluorescent protein (Venus) are fused separately to two partner proteins expected to interact intracellularly. In the case of the interaction, the two domains of the split fluorescent protein will come into close proximity and fold into the native structure, which results in fluorescence emission upon excitation.

Fig. 8B shows that the application of BiFC technique coupled with immunofluorescence microscopy rendered the visualization of the BiFC complex of SIRT2 and TPPP/p25 possible as well as its co-localization with the MT network. Previously we provided evidence that the “empty” Venus pair does not produce a significant BiFC signal (Szénási et al, 2017). As additional negative control, unlabeled *pTPPP/p25* was co-transfected with the BiFC plasmids, which reduced the BiFC signal significantly as demonstrated by quantification of the fluorescence signal indicating the competition of the unlabeled TPPP/p25 with the Venus-labelled one (Fig. 8C). The merged image in Fig. 8B is similar to that reported for the association of TPPP/p25 to MTs in transfected HeLa cells (Lehotzky et al, 2004; Tökési et al, 2010). Therefore, our finding provides evidence for the formation of a multiprotein complex in agreement with the *in vitro* data.

This cellular system was also used to validate the effect of the chemical inhibitor, MZ25, on the assembly of SIRT2 and TPPP/p25 at the cellular level. The BiFC signal intensity is virtually not influenced by the addition of MZ25 (Fig. 8C) which is in accordance with the *in vitro* ELISA data (Fig. 7B). All these results suggest that the MZ25 does not influence the interaction of SIRT2 with TPPP/p25.

The inhibitory potency of the MZ242 and SH1 (PROTAC) compounds was tested in HeLa cells expressing TPPP/p25 by immunofluorescence microscopy. The conditions were adjusted that they rendered the visualization of the TPPP/p25-, MZ242- or SH1-induced MT acetylation possible, in order to exclude their counteracting and confirm their mutual positive effect on the MT acetylation. As shown in Fig. 9, both TPPP/p25 and the investigated chemical compounds alone increased the acetylation level of MTs, and this effect, according to the *in vitro* data, was more pronounced when cells were transfected with TPPP/p25 in the presence of MZ242 or SH1. In the case of the SH1-treated cells the expression of TPPP/p25 seems to be more pronounced as with MZ242. This could result from the targeted recruitment of SIRT2 to the proteasome induced by the PROTAC which could impede TPPP/p25 degradation.

DISCUSSION

The expression of both SIRT2 and TPPP/p25 are important factors for the differentiation of the progenitor OLGs: while the expression of TPPP/p25 is crucial for the growth of projections, in fact, the reduction of TPPP/p25 expression by siRNA or microRNA counteracts the differentiation of OLGs (Lehotzky et al, 2010); in this process, the structural and functional effects of SIRT2 are largely unknown (Thangaraj et al, 2017). SIRT2 is not neuronally enriched, but is found in the OLGs and myelin sheaths (Tang & Chua, 2008). The knockdown of SIRT2 increases tubulin acetylation and the complexity of cellular arborization (Ji et al, 2011). Our present studies are focused on the connection of SIRT2 and TPPP/p25, both are important interacting partners of the tubulin/MTs, and their mutual influences on the acetylation level of the tubulin/MTs.

Despite the fact that the ubiquitously expressed HDAC6 is regarded the main tubulin deacetylase, it has been evidenced that SIRT2 is also a *bone fide* tubulin deacetylase (North et al, 2003). This issue is supported by the following facts: it is not inhibited by Trichostatin A, an inhibitor of HDAC6 that is specific for Zn²⁺ dependent HDACs and does not act on sirtuins; the deacetylation is a NAD⁺-dependent process; tubulin acetylation is enhanced by SIRT2 depletion with specific siRNA. The co-localization of HDAC6 and SIRT2 has been described when they were co-expressed in cells suggesting that these enzymes are assembled into a multienzyme complex (North et al, 2003). The interaction of TPPP/p25 with HDAC6 has been characterized also in *in vitro* experiments, which revealed its inhibitory potency against the HDAC6-catalyzed tubulin deacetylation (Tókési et al, 2010).

In this work, the interactions of tubulin and its physiological partners, SIRT2 and TPPP/p25 are characterized and the formation of their binary and ternary complex coupled to their tubulin deacetylation function has been established. Indeed, a significant feature of the tubulin acetylation-promoting system has been identified as illustrated by the scheme (Fig. 10). TPPP/p25, as an interacting partner of both tubulin and SIRT2, does not act as a competitor, but it promotes the binding of SIRT2 to tubulin ensuring fine control for the tubulin deacetylation. It is worth noting that we were not able to detect the co-localization of SIRT2 with the MT network in HeLa cells, its homogeneous distribution was detected in the cytosol by immunofluorescence microscopy (Supplementary Fig. 2); however, we were able to visualize the SIRT2 complexed with TPPP/p25 on the MT network. These data are indicative for the formation of a ternary complex supported by the *in vitro* data as well; in addition, a key role of TPPP/p25 is suggested in the formation of a piggy-back complex, i.e. SIRT2 associates indirectly to the MT or it can directly bind to tubulin due to the TPPP/p25-induced conformational change as detected by CD spectroscopy (cf. Fig. 1). The finding that the associated SIRT2 and TPPP/p25 are aligned along the MT network in living HeLa cells underlines that TPPP/p25 as an OLG-specific protein could control the acetylation level of the MT network by its structural and functional integration into the cellular machinery denoted *myelin proteome*. The control of the acetylation level of the MT network is an important issue since both the non-physiological deacetylation and the hyperacetylation have been reported in the etiology of distinct human diseases (Oláh et al, 2017a and references therein).

Tubulin acetylation is an important factor for the regulation of MT architecture and maintenance of MT integrity (Li & Yang, 2015). Consequently, the destruction of the cytoskeletal MT system or its over-stabilization leads to cell death. A number of anti-MT drugs have been reported that display such features (Eira et al, 2016 and references therein). The basic problem in cancer therapy is not to destroy cytoskeletal MT network but to target it specifically in tumor cells. One way to fulfill this task is to develop drugs which can control the acetylation level without the complete inactivation of deacetylases and only to adjust the system that could gently control the dynamics of the MT network via tubulin acetylation. Such promising mechanism could be fulfilled by anti-SIRT2 agents developed recently as SirReal-modified compound, MZ242, MZ25 and SH1

(Rumpf et al, 2015; Schiedel et al, 2016a and b; Schiedel et al, 2017b). These drug-like molecules display their anti-SIRT2 activity with unique mechanisms such as allosteric modification of the active site or degradation of the deacetylase by the proteasome machinery. These compounds, along with TPPP/p25 or its SIRT2-binding fragment, could provide fine control for the acetylation level of the MT network providing a clue for innovative strategy in cancer research.

MATERIALS AND METHODS

Inhibitors

The inhibitors were synthesized according to established procedures: MZ25 (SirReal2) (Rumpf et al, 2015) and MZ242 (cpd 10) (Schiedel et al, 2016b) are inhibitors of the enzymatic activity of SIRT2. SH1 is a proteolysis targeting chimera (PROTAC) linking a partial structure of MZ242 and thalidomide via a spacer (Schiedel et al, 2017b).

Bimolecular fluorescence complementation (BiFC) plasmids

The BiFC plasmids (pBiFC-VN1-173, pBiFC-VC155-238) were the gift of Prof. Péter Várnai (Semmelweis University, Budapest). The V^C-TPPP/p25 plasmid containing the insert for human full length TPPP/p25 was prepared as described previously (Szénási et al, 2017; Oláh et al, 2017b). Plasmid V^N-SIRT2 was produced by inserting the SIRT2 coding region in frame (North et al, 2003) into pBiFC-VN1-173 by XhoI and BamHI restriction enzymes. The sequences of all construct were verified by restriction mapping and sequencing.

Cloning of SIRT2

Human recombinant hexaHis-tagged SIRT2 containing residues 50-356 was cloned and expressed in Escherichia coli (E.coli). BL21 (DE3) cells. The constructs of 50-356 SIRT2 were amplified with 5' primer containing a NdeI site with sequence: 5'-GCACCATATGGACAGCCTGGGCAGCCAGAAGG - 3' and 3' primer containing a XhoI site with sequence 5'-GCAATCTCGAGCGACTGGGCATCTATGC - 3'. The resulting PCR fragments were inserted into the NdeI and XhoI sites of the pET21c vector and were confirmed by sequencing.

Expression and purification of human recombinant wild type TPPP/p25 and SIRT2

The C-terminal hexaHis fusion proteins were expressed in E. coli BL21 (DE3). TPPP/p25 was isolated on HIS-Select™ Cartridge (Sigma-Aldrich) as described previously (Szénási et al, 2017).

SIRT2 was prepared as described by Sun and co-workers (Sun et al, 2006). Briefly, the ampicillin-resistant cells were grown in LB containing 100 mg/l ampicillin, 1 mM MgCl₂ and 5 μM ZnCl₂ at 37°C, then harvested by centrifugation (20 min, 4 °C, 2000 g). The pellet fraction was resuspended in phosphate buffer (50 mM Na₂HPO₄, 300 mM NaCl, pH 8.0 containing 10 μM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 1 mM benzamidine, 1 μg/ml pepstatin and 1 μg/ml leupeptin) and lysed by sonication. Following centrifugation (25 min, 4 °C, 39,000 g), the supernatant containing the soluble proteins was affinity purified by Ni²⁺ affinity gel. The bound protein was eluted by 50 mM Na₂HPO₄, 300 mM NaCl, pH 8.0 containing 50 mM imidazole. Then the protein was concentrated and stored in 50 mM tris(hydroxymethyl)aminomethane (Tris), 200 mM NaCl (pH 8.0), 10% glycerol and 1 mM dithioerythritol.

Concentration of TPPP/p25 and SIRT2 was determined at 280 nm by the extinction coefficients evaluated by ProtParam (<http://web.expasy.org/protparam/>): 10,095 M⁻¹*cm⁻¹ for TPPP/p25 and 33203 M⁻¹*cm⁻¹ for SIRT2, respectively.

Tubulin preparation

Tubulin was prepared from bovine brain according to the method of Na and Timasheff (Na & Timasheff, 1986). It is important to note that the preparation was carried out without adding zinc cation or NAD⁺ to the buffer compositions used for the preparation of cell extracts or to the ammonium sulfate precipitations. In contrast, SIRT2 preparation was performed in the presence of zinc cation (cf. see below).

Circular dichroism (CD)

CD measurements were performed on a Jasco J-720 spectropolarimeter at 20 nm/min scan rate, 8 s time constant and 1 nm step size in 10 mM phosphate buffer, pH 7.4 at room temperature. The path length was 0.1 cm. Protein concentrations were 4 μ M for TPPP/p25, 1 μ M for tubulin and 16 μ M for SIRT2. The difference spectrum was obtained by subtracting the spectra of the individual proteins from that of the mixture of two proteins. Mean molar ellipticity per residue (MRE) in degrees square centimeter per decimole was calculated according to the following equation: $MRE = \Theta_m / (10 * n * c * l)$, where Θ_m is the measured ellipticity (millidegrees), n is the number of amino acid residues in a protein, c (mol) is the concentration and l (cm) is the path length of the cell. Data from three independent measurements were averaged.

Enzyme-linked immunosorbent assay (ELISA)

ELISA experiments were performed as described previously (Tőkési et al, 2014). Briefly, the plate was coated with 5 μ g·mL⁻¹ SIRT2 (or other protein as indicated in the legends of the figures) in phosphate buffered saline (PBS), then the interacting protein at a serial dilution (TPPP/p25 or tubulin) was added. The binding of the protein to the immobilized one was quantified by specific monoclonal TPPP/p25 (Höftberger et al, 2010) or tubulin (Sigma T9026) antibodies followed by addition of the secondary anti-mouse IgG conjugated to HRP (Sigma A2554), and the quantification of the immunocomplex was detected using o-phenylenediamine with hydrogen peroxide as substrate solution; absorbance was read at 490 nm with an EnSpire Multimode Reader (Perkin Elmer). The binding constants (K_d) were evaluated from the saturation curves by non-linear curve fitting assuming single binding site hyperbola model using the Origin 8.0 software.

In the competitive ELISA experiments, the tubulin or TPPP/p25 used in concentration series were pre-incubated with 125 or 250 nM TPPP/p25 or 1 μ M tubulin, respectively.

Turbidimetry and pelleting experiments

The assembly of tubulin (6 μ M) was assessed in polymerization buffer (50 mM 2-(N-morpholino)ethanesulfonic acid buffer, pH 6.6, containing 100 mM KCl, 1 mM dithioerythritol, 1 mM MgCl₂, and 1 mM ethylene glycol tetraacetic acid) at 37°C in the presence of TPPP/p25 (3 μ M) with/without SIRT2 (25 μ M) and NAD⁺ (500 μ M). The tubulin polymerization was induced by the addition of TPPP/p25 pre-incubated with or without SIRT2. The optical density was monitored at 350 nm by a Cary 100 spectrophotometer (Varian, Walnut Creek, Australia).

The polymerized samples (100 μ l aliquot) were centrifuged (15 min, 37°C, 17000 g), and the pellet (P) and the supernatant (S) fractions were separated. The pellet fractions were resuspended in 100 μ l polymerization buffer. The fractions were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE), Western blot using acetyl-tubulin antibody (Sigma T6793), and electron microscopy.

SIRT2 activity assays.

Fluorogenic substrate: SIRT2 activity was measured with the fluorogenic SIRT2 assay kit (BPS Bioscience, 50087) according to the manufacturer's protocol. The normalized effect of TPPP/p25 (20 μ M) or the various drugs (20 μ M) on SIRT2 activity was calculated; background (no

SIRT2) corresponded to 0% activity (100% relative acetylation), while SIRT2 alone corresponded to 100% activity (0% relative acetylation).

Tubulin or MTs: SIRT2 was incubated with 2.64 μ M tubulin, 0.5 mM NAD⁺, 0.1 mg/ml bovine serum albumin (BSA) without or with TPPP/p25 and/or drugs in assay buffer (BPS Bioscience, 50031) for 1 h at 37°C. In the case of MTs, 26.4 μ M tubulin was polymerized by the addition of 20 μ M paclitaxel in polymerization buffer for 30 min at 37°C, then diluted 10-fold with the assay buffer (BPS Bioscience, 50031) and used for SIRT2 activity measurement. The samples were analyzed by 13.5% SDS/PAGE, stained with Coomassie Brilliant Blue R-250 containing 2-mercaptoethanol. The same samples were also electrotransferred onto Immobilon-P^{SQ} transfer membranes and subjected to Western blot. The blot was developed using a monoclonal mouse antibody against acetylated α -tubulin at Lys-40 (1:5000, clone 6-11B-1, Sigma T6793), antibody binding was revealed by anti-mouse IgG-peroxidase conjugate (Fc-specific), (1:5000, Sigma A2554). Peroxidase reaction was detected using Immobilon Western substrate (Millipore) by a Bio-Rad ChemiDoc MP Imaging system and its ImageLab 4.1 software. Intensity of spots was analyzed by ImageJ 1.49 using Measure command and subtracting background values. Relative acetyl-tubulin level corresponds to the difference measured in the absence and presence of 0.6 μ M SIRT2, while the inverse of this value is the relative SIRT2 activity.

Electron microscopy and immunocytochemistry

For electron microscopy, MT-containing samples were pelleted by centrifugation (15 min, 37°C, 17000 g), and the pellets were fixed in a mixture of 2% glutaraldehyde, 0.2% tannic acid in 0.1 M sodium cacodylate, pH 7.4, for 1 h at room temperature and for 24 h at 4°C, and then embedded in Durcupan (Fluka, Switzerland). Ultrathin sections were contrasted with Reynold's lead citrate and examined and photographed in a Jeol JEM-1011 electron microscope, operating at 60kV, equipped with Olympus Morada CCD camera using Olympus iTEM (TEM imaging platform) software.

For immunocytochemical analysis, ultrathin sections (90 nm) were collected on nickel grids. Sections were blocked with 1% BSA and 3% non-fat dry milk in PBS (pH 7.2) for 1 h at room temperature. The samples were then treated with the primary antibody (monoclonal anti-acetylated tubulin (mouse IgG2b isotype) (Sigma T6793, Saint Louis, USA) diluted 1:30 in PBS with 1% BSA and 1.5% non-fat dry milk overnight at 4°C. After short rinses in 1% BSA in PBS (2 times 5 min) the buffer was changed to 1% BSA in Tris-buffered saline (pH 7.6; 2 times 5 min) then to 1% BSA in Tris-buffered saline pH 8.0 with 0.25% Tween 20. The samples were incubated with a 6 nm gold-conjugated secondary antibody (6 nm gold-goat anti-mouse IgG; Sigma, Saint Louis, USA) diluted 1:50 in 1% BSA in TTBS at 4°C for 5 h. After short washes in TBS (3 times 5 min) and nanopure water (2 times 5 min), labeled grids were contrasted with Reynold's lead citrate and examined and photographed as it was described above.

Cell culture and manipulation

HeLa cells (ATCC® CCL-2™, American Type Culture Collection) were grown in Dulbecco's modified eagle medium supplemented with 10% fetal calf serum and 100 μ g/ml kanamycin in a humidified incubator at 37°C with 5% CO₂. Cells were grown on 12-mm-diameter glass coverslips for microscopic analysis. For the evaluation of the BiFC signal, HeLa cells were transfected with mVenus constructs of TPPP/p25 and SIRT2 (0.3 μ g of each plasmid), in the case of competition with an additional 0.1 μ g TPPP/p25 construct (Lehotzky et al, 2004) using Turbofect (Invitrogen) transfection reagent according to the manufacture's protocol. Mouse monoclonal acetyl-tubulin (Sigma T6793), rat polyclonal TPPP/p25 (Kovacs et al, 2004), Alexa 546 conjugated mouse and rat secondary antibodies (Thermo Fisher Scientific A11030 and A11081) were used for the detection of the acetyl-tubulin and TPPP/p25 signal. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). In another experiment, HeLa cells were transfected with *pTPPP/p25* for 24 hs

using the same protocol, then inhibitors (MZ242 and SH1, 10-10 μ M, respectively from DMSO stocks) were added to the samples for the last 3 hs of the experiment. Samples were fixed and processed as above. TPPP/p25-transfected cells were detected with a polyclonal anti-TPPP/p25 serum developed in rat (Kovacs et al, 2004), and labeled by goat anti-rat Alexa 488 (Thermo Fisher Scientific A11006, cross-absorbed against mouse serum). Acetyl-tubulin was detected as above. Images of the mounted 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), Chroma 41028 HQ NB GFP filter set (No. C21116), Chroma 41028 Y GFP filter set (No. C2117) and Leica filter N2.1 (No. 513832) was used for DAPI, Alexa 488 (and EGFP), BiFC and Alexa 546 signal acquisition, respectively, using a HCX FL Fluotar 40x/0.75 (dry) objective.

For image analysis, the pictures were taken under constant exposure parameters. The determination of the BiFC signal (green fluorescence) was performed by using the Analyze, Measure option of the National Institutes of Health ImageJ 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.

Mathematical model.

All the numerical simulations were performed with Mathematica for Students (version 10.0.1.0) software package (Wolfram Research; <http://www.wolfram.com>). For the modeling, the experimentally determined pair-wise dissociation constants were used, all reactions were considered reversible. In the case of the ternary complex, it was assumed that the binding of tubulin to the TPPP/p25-SIRT2 complex is weaker than to TPPP/p25 alone but stronger than to SIRT2 alone. The set of differential equations (see Supplementary data) were solved numerically, which allowed the determination of the time-dependent complex formation and the concentrations characteristic for the equilibrium.

Statistics.

The error bars represent the standard deviation (SD). Comparisons were performed using the two-sided, unpaired Student's t-test and values were considered to be significant if the calculated p value was <0.05 (*).

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AUTHOR CONTRIBUTIONS

Adél Szabó: expression and isolation of the recombinant proteins, performing CD, ELISA experiments, SIRT2 activity measurements, tubulin polymerization, data collection and evaluation; Judit Oláh: performing and supervising in vitro experiments, data collection and evaluations, responsibility for the research activity including mentorship to MSc and PhD fellows; Sándor Szunyogh: transfections of mVenus BiFC and other constructs, immunocytochemistry with HeLa cells; Attila Lehotzky: multiply effects of SIRT2 inhibitors at cellular level: cell manipulations,

immunocytochemistry, fluorescence microscopy including mentorship to the PhD fellows; Tibor Szénási: plan and production of constructs, cloning, mVenus constructs for the BiFC technology; Marianna Csaplár: expression and isolation of the recombinant proteins, performing CD and ELISA experiments; Péter Lőw: design and evaluation of electron microscopic studies; Matthias Schiedel and Manfred Jung: contributed to planning the inhibitor experiments, the data evaluation and the writing of the manuscript; Judit Ovádi: ideas, evolution and coordination of the research aims, planning and execution, acquisition of the financial support for the in vitro and cellular studies.

ABBREVIATIONS

Bimolecular fluorescence complementation, BiFC; bovine serum albumin, BSA; Circular dichroism, CD; 4',6-diamidino-2-phenylindole, DAPI; Enzyme-linked immunosorbent assay, ELISA; Histone deacetylase 6, HDAC6; Microtubule, MT; Microtubule Associated Protein, MAP; nicotinamide adenine dinucleotide, NAD⁺; oligodendrocyte, OLG; phosphate buffered saline, PBS; sirtuin-2, SIRT2; sodium dodecyl sulfate polyacrylamide gel electrophoresis, SDS/PAGE; tris(hydroxymethyl)aminomethane, Tris; Tubulin Polymerization Promoting Protein, TPPP/p25.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

REFERENCES

- Chakrabarti KR, Hessler L, Bhandary L, Martin SS. (2015) Molecular Pathways: New Signaling Considerations When Targeting Cytoskeletal Balance to Reduce Tumor Growth. *Clin Cancer Res* 21: 5209-14
- Conde C, Cáceres A. (2009) Microtubule assembly, organization and dynamics in axons and dendrites. *Nat Rev Neurosci* 10: 319-32
- Dryden SC, Nahhas FA, Nowak JE, Goustin AS, Tainsky MA. (2003) Role for human SIRT2 NAD-dependent deacetylase activity in control of mitotic exit in the cell cycle. *Mol Cell Biol* 23: 3173-85
- Eira J, Silva CS, Sousa MM, Liz MA. (2016) The cytoskeleton as a novel therapeutic target for old neurodegenerative disorders. *Prog Neurobiol* 141: 61-82
- Florian S, Mitchison TJ. (2016) Anti-Microtubule Drugs. *Methods Mol Biol* 1413: 403-21
- de Forges H, Bouissou A, Perez F. (2012) Interplay between microtubule dynamics and intracellular organization. *Int J Biochem Cell Biol* 44: 266-74
- Greenfield NJ. (2006) Using circular dichroism spectra to estimate protein secondary structure. *Nat Protoc* 1: 2876-90
- Harting K, Knöll B. (2010) SIRT2-mediated protein deacetylation: An emerging key regulator in brain physiology and pathology. *Eur J Cell Biol* 89: 262-9
- Hlavanda E, Kovács J, Oláh J, Orosz F, Medzihradszky KF, Ovádi J. (2002) Brain-specific p25 protein binds to tubulin and microtubules and induces aberrant microtubule assemblies at substoichiometric concentrations. *Biochemistry* 41: 8657-64
- Höftberger R, Fink S, Aboul-Enein F, Botond G, Oláh J, Berki T, Ovádi J, Lassmann H, Budka H, Kovacs GG. (2010) Tubulin polymerization promoting protein (TPPP/p25) as a marker for oligodendroglial changes in multiple sclerosis. *Glia* 58: 1847-57
- Ji S, Doucette JR, Nazarali AJ. (2011) Sirt2 is a novel in vivo downstream target of Nkx2.2 and enhances oligodendroglial cell differentiation. *J Mol Cell Biol* 3: 351-9

- Kovács GG, László L, Kovács J, Jensen PH, Lindersson E, Botond G, Molnár T, Perczel A, Hudecz F, Mezo G, Erdei A, Tirián L, Lehotzky A, Gelpi E, Budka H, Ovádi J. (2004) Natively unfolded tubulin polymerization promoting protein TPPP/p25 is a common marker of alpha-synucleinopathies. *Neurobiol Dis* 17: 155-62
- Lehotzky A, Tirián L, Tőkési N, Lénárt P, Szabó B, Kovács J, Ovádi J. (2004) Dynamic targeting of microtubules by TPPP/p25 affects cell survival. *J Cell Sci* 117: 6249-59
- Lehotzky A, Lau P, Tőkési N, Muja N, Hudson LD, Ovádi J. (2010) Tubulin polymerization-promoting protein (TPPP/p25) is critical for oligodendrocyte differentiation. *Glia* 58: 157-68
- Li L, Yang XJ. (2015) Tubulin acetylation: responsible enzymes, biological functions and human diseases. *Cell Mol Life Sci* 72: 4237-55
- Mangas-Sanjuan V, Oláh J, Gonzalez-Alvarez I, Lehotzky A, Tőkési N, Bermejo M, Ovádi J. (2015) Tubulin acetylation promoting potency and absorption efficacy of deacetylase inhibitors. *Br J Pharmacol* 172: 829-40
- Maxwell MM, Tomkinson EM, Nobles J, Wizeman JW, Amore AM, Quinti L, Chopra V, Hersch SM, Kazantsev AG. (2011) The Sirtuin 2 microtubule deacetylase is an abundant neuronal protein that accumulates in the aging CNS. *Hum Mol Genet* 20: 3986-96
- Na GC, Timasheff SN. (1986) Interaction of vinblastine with calf brain tubulin: multiple equilibria. *Biochemistry* 25: 6214-22
- North BJ, Marshall BL, Borra MT, Denu JM, Verdin E. (2003) The human Sir2 ortholog, SIRT2, is an NAD⁺-dependent tubulin deacetylase. *Mol Cell* 11: 437-44
- Oláh J, Tőkési N, Lehotzky A, Orosz F, Ovádi J. (2013) Moonlighting microtubule-associated proteins: regulatory functions by day and pathological functions at night. *Cytoskeleton (Hoboken)* 70: 677-85
- Oláh J, Bertrand P, Ovádi J. (2017a) Role of the microtubule-associated TPPP/p25 in Parkinson's and related diseases and its therapeutic potential. *Expert Rev Proteomics* 14: 301-309
- Oláh J, Szénási T, Szunyogh S, Szabó A, Lehotzky A, Ovádi J. (2017b) Further evidence for microtubule-independent dimerization of TPPP/p25. *Sci Rep* 7: 40594
- Ovádi J. (2008) The tubulin polymerization promoting protein, TPPP/p25. *IUBMB Life* 60: 637-42
- Penna LS, Henriques JA, Bonatto D. (2017) Anti-mitotic agents: Are they emerging molecules for cancer treatment? *Pharmacol Ther* pii: S0163-7258(17)30021-9. doi: 10.1016/j.pharmthera.2017.02.007. [Epub ahead of print] Review.
- Perdiz D, Mackeh R, Poüs C, Baillet A. (2011) The ins and outs of tubulin acetylation: more than just a post-translational modification? *Cell Signal* 23: 763-71
- Rumpf T, Schiedel M, Karaman B, Roessler C, North BJ, Lehotzky A, Oláh J, Ladwein KI, Schmidtkunz K, Gajer M, Pannek M, Steegborn C, Sinclair DA, Gerhardt S, Ovádi J, Schutkowski M, Sippl W, Einsle O, Jung M. (2015) Selective Sirt2 inhibition by ligand-induced rearrangement of the active site. *Nat Commun* 6: 6263
- Schiedel M, Rumpf T, Karaman B, Lehotzky A, Oláh J, Gerhardt S, Ovádi J, Sippl W, Einsle O, Jung M. (2016a) Aminothiazoles as Potent and Selective Sirt2 Inhibitors: A Structure-Activity Relationship Study. *J Med Chem* 59: 1599-612
- Schiedel M, Rumpf T, Karaman B, Lehotzky A, Gerhardt S, Ovádi J, Sippl W, Einsle O, Jung M. (2016b) Structure-Based Development of an Affinity Probe for Sirtuin 2. *Angew Chem Int Ed Engl* 55: 2252-6
- Schiedel M, Robaa D, Rumpf T, Sippl W, Jung M. (2017a) The Current State of NAD⁺ - Dependent Histone Deacetylases (Sirtuins) as Novel Therapeutic Targets. *Med Res Rev* 2017 Jan 17. doi: 10.1002/med.21436. [Epub ahead of print] Review.
- Schiedel M, Herp D, Hammelmann S, Swyter S, Lehotzky A, Robaa D, Olah J, Ovádi J, Sippl W, Jung M. (2017b) Chemically induced degradation of sirtuin 2 (Sirt2) by a proteolysis targeting

chimera (PROTAC) based on sirtuin rearranging ligands (SirReals). *J Med Chem* doi: 10.1021/acs.jmedchem.6b01872. [Epub ahead of print]

Skjoerringe T, Lundvig DM, Jensen PH, Moos T. (2006) P25 α /Tubulin polymerization promoting protein expression by myelinating oligodendrocytes of the developing rat brain. *J Neurochem* 99: 333-42

Sun C, Song D, Marcotte PA, Richardson PL, Hajduk PJ. (2006) High-level bacterial expression and purification of human SirT2 protein for NMR studies. *Protein Expr Purif* 48: 56-60

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

Tang BL, Chua CE. (2008) SIRT2, tubulin deacetylation, and oligodendroglia differentiation. *Cell Motil Cytoskeleton* 65: 179-82

Thangaraj MP, Furber KL, Gan JK, Ji S, Sobchishin L, Doucette JR, Nazarali AJ. (2017) RNA-binding Protein Quaking Stabilizes Sirt2 mRNA during Oligodendroglial Differentiation. *J Biol Chem* 292: 5166-5182

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

Tőkési N, Oláh J, Hlavanda E, Szunyogh S, Szabó A, Babos F, Magyar A, Lehotzky A, Vass E, Ovádi J. (2014) Identification of motives mediating alternative functions of the neomorphic moonlighting TPPP/p25. *Biochim Biophys Acta* 1842: 547-57

Werner HB, Kuhlmann K, Shen S, Uecker M, Schardt A, Dimova K, Orfaniotou F, Dhaunchak A, Brinkmann BG, Möbius W, Guarente L, Casaccia-Bonnel P, Jahn O, Nave KA. (2007) Proteolipid protein is required for transport of sirtuin 2 into CNS myelin. *J Neurosci* 27: 7717-30

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

Zotter Á, Oláh J, Hlavanda E, Bodor A, Perczel A, Szigeti K, Fidy J, Ovádi J. (2011b) Zn²⁺-induced rearrangement of the disordered TPPP/p25 affects its microtubule assembly and GTPase activity. *Biochemistry* 50: 9568-78

FIGURE LEGENDS

Figure 1 - Secondary structural characteristics of SIRT2 and its complexes with tubulin and TPPP/p25 as detected by far-UV CD spectroscopy.

A Normalized CD spectrum of SIRT2 (bold line), TPPP/p25 (solid line) and tubulin (dotted line). 3 independent experiments.

B Difference spectrum of SIRT2-tubulin (bold line), SIRT2-TPPP/p25 (solid line) and tubulin-TPPP/p25 (dotted line). Difference ellipticities were calculated by subtracting the ellipticities of the individual protein from that of their complexes. Concentrations of the proteins: 16 μ M SIRT2, 4 μ M TPPP/p25 and 1 μ M tubulin. 3 independent experiments.

Figure 2 - Quantification of the pair-wise interactions of SIRT2, TPPP/p25 and tubulin by ELISA.

A Saturation curves of TPPP/p25 (●) and tubulin (○), when the plate was coated by SIRT2; and that of tubulin (▲), when the plate was coated with TPPP/p25. Concentrations of the coated

proteins were 5 µg/ml. The data are presented as mean ± SD, n=3 for TPPP/p25-SIRT2, n=3 for tubulin-SIRT2, and n=11 for TPPP/p25-tubulin.

B Displacement of 125 nM TPPP/p25 from the coated SIRT2 by tubulin added at various concentration detected by TPPP/p25 antibody. The data are presented as mean ± SD, n=2.

C Binding of TPPP/p25 (at 250 nM) and tubulin (at 1 µM) to the immobilized SIRT2 as detected by TPPP/p25 and tubulin antibodies, respectively. The data are presented as mean ± SD, n=4. *p=2.64E-4 when TPPP/p25 with tubulin compared to TPPP/p25 alone, and *p=1.76E-2 when tubulin with TPPP/p25 compared to tubulin alone (two-sided, unpaired Student's t-test).

Figure 3 - Mathematical model of the binary and ternary complexes and comparison with the ELISA experiment.

A Concentration of the different complexes according to the binary (dashed lines) and ternary (solid lines) models.

B SIRT2 was immobilized on the plate, then tubulin without (○) or with 200 nM TPPP/p25 (●) was added and the bound tubulin was detected by tubulin antibody. The data are presented as mean ± SD, n=3 for tubulin, n=2 for tubulin with TPPP/p25. SIRT2-tubulin at 0 µM TPPP/p25: relative concentration of SIRT2-tubulin complex without added TPPP/p25. Relative concentrations of SIRT2-tubulin, TPPP/p25-SIRT2-tubulin and the sum of SIRT2-tubulin and TPPP/p25-SIRT2-tubulin according to the ternary model, respectively, are shown. Relative concentration of SIRT2-tubulin according to the binary model is also displayed.

Figure 4 - Effect of SIRT2 on the TPPP/p25-induced tubulin polymerization.

A Tubulin polymerization (6 µM) promoted by TPPP/p25 (3 µM) in the absence and presence of inactive or active (with 500 µM NAD⁺) SIRT2 (25 µM). A representative experiment is shown, n=3 for TPPP/p25, n=3 for TPPP/p25 and SIRT2, and n=1 for TPPP/p25 and SIRT2 with NAD⁺.

B, C Images of SDS/PAGE (supernatant (S) and pellet (P) fractions) and Western blot of the samples of A using acetyl-tubulin antibody.

D Electron microscopic images of the pellet fractions of B.

Figure 5 - Modest inhibitory potency of TPPP/p25 on the deacetylation of tubulin by SIRT2.

A Concentration-dependent tubulin deacetylation and the inhibitory effect of TPPP/p25. The acetylation was determined by Western blot using acetyl-tubulin antibody at 2.64 µM tubulin concentration.

B Quantification of the Western blot data as described in the Materials and Methods. The data are presented as mean ± SD, n=2.

Figure 6 - Acetylation levels of tubulin versus MTs affected by SIRT2 with or without TPPP/p25.

- A SDS/PAGE images of tubulin and MT with and/or without SIRT2 and TPPP/p25.
- B The acetylation levels of the same samples as detected by Western blot using acetyl-tubulin antibody.
- C Summary of the quantified data. For details see Materials and Methods. The data are presented as mean $\pm$ SD, n=4-6. *p=8.07E-4 when SIRT2 with tubulin compared to tubulin alone, and *p=3.08E-3 when SIRT2 with MTs compared to MTs alone (two-sided, unpaired Student's t-test).

Figure 7 - Inhibitory effect of TPPP/p25 and new SIRT2 inhibitors on the deacetylation of tubulin or a synthetic substrate (commercial assay) by SIRT2.

- A Inhibition of deacetylation of 2.64 μ M tubulin (black column) or 20 μ M synthetic substrate (commercial assay) (white column) by 20 μ M TPPP/p25 or chemical compounds; 0.6 and 1 μ M SIRT2 were added to the acetyl-tubulin and synthetic substrates, respectively. The data are presented as mean $\pm$ SD, n=3-11. *p=5.56E-6 for TPPP/p25, *p=1.30E-2 for SH1 and *p=1.38E-3 for MZ242 when the values with and without inhibitor were compared in the case of tubulin as a substrate; *p=2.60E-2 for SH1, *p=9.20E-7 for MZ25, *p=7.76E-11 for MZ242 when the values with and without inhibitor were compared in the case of the fluorescence substrate; #p=1.56E-2 for TPPP/p25 and #p=1.48E-3 for MZ242 when the inhibitory potency obtained with tubulin substrate was compared with that of measured with the fluorescence substrate (two-sided, unpaired Student's t-test).
- B Effect of the inhibitors on the binding of 125 nM TPPP/p25 to the immobilized SIRT2. The data are presented as mean $\pm$ SD, n=3.
- C Mutual effect of the TPPP/p25 and inhibitors on the SIRT2 activity (Western blot data with tubulin). The data are presented as mean $\pm$ SD, n=2. *p=5.14E-3 for TPPP/p25 and *p=4.03E-3 for MZ242 when the values with and without inhibitor were compared (two-sided, unpaired Student's t-test).

Figure 8 - Localization and interaction of TPPP/p25 and SIRT2 in living HeLa cells as detected by immunofluorescence microscopy using BiFC technology.

- A Scheme of the applied BiFC constructs.
- B Co-localization of the TPPP/p25-SIRT2 complex (green) on the MT network (red). MT network was stained with Alexa546, nuclei was counterstained with DAPI (blue). Scale bar: 10 μ m.
- C BiFC signal (green) of the assembly of Venus^N-TPPP/p25 and Venus^C-SIRT2 and its reduction by unlabeled TPPP/p25, quantitative evaluation of the BiFC signals of the images as well as that of obtained with an anti-SIRT2 drug (MZ242). For details see Materials and Methods. The data are presented as mean $\pm$ SD, in each case at least 30 cells were analyzed. *p=4.15E-6 when control (BiFC) cells were compared with those co-transfected with unlabeled pTPPP/p25 (two-sided, unpaired Student's t-test).

Figure 9 - Visualization of the acetylation of the MT network by TPPP/p25 and/or MZ242 and SH1 by widefield immunofluorescence microscopy in HeLa cells.

Acetylated tubulin and transiently transfected TPPP/p25 are labeled by red and green, respectively. Note that double treated samples show enhanced signal of red due to both of inhibitors and TPPP/p25 effects. To clarify red signal and ensure comparability of treatments, corresponding green signal of microscopic areas are shown in the last line. Nuclei was counterstained with DAPI (blue). Scale bar: 5 μ m.

Figure 10 - Heteroassociations of tubulin, TPPP/p25 and SIRT2 and the synergistic effect of the chemical inhibitor MZ242 and TPPP/p25.