ARTICLE

WWOX dysfunction induces sequential aggregation of TRAPPC6ΔΔ, TIAF1, tau and amyloid β, and causes apoptosis

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Aggregated vesicle-trafficking protein isoform TRAPPC6ΔΔ (TPC6ΔΔ) has a critical role in causing caspase activation, tau aggregation and Aβ generation in the brains of nondemented middle-aged humans, patients with Alzheimer’s disease (AD) and 3-week-old Wwox gene knockout mice. WWOX blocks neurodegeneration via interactions with tau and tau-phosphorylating enzymes. WWOX deficiency leads to epilepsy, mental retardation and early death. Here, we demonstrated that TGF-β1 induces TPC6ΔΔ in between nucleoli and mitochondria (~40–60 min per round trip), and WWOX reduces the shuttling time by 50%. TGF-β1 initially maximizes the binding of TPC6ΔΔ to the C-terminal tail of WWOX, followed by dissociation. TPC6ΔΔ then undergoes aggregation, together with TIAF1 (TGF-β-induced antiapoptotic factor), in the mitochondria to induce apoptosis. An additional rescue scenario is that TGF-β1 induces Tyr33 phosphorylation and unfolding of WWOX and its the N-terminal WW domain slowly binds TPC6ΔΔ to block aggregation and apoptosis. Similarly, loss of WWOX induces TPC6ΔΔ polymerization first, then aggregation of TIAF1, amyloid β and tau, and subsequent cell death, suggesting that a cascade of protein aggregation leads to neurodegeneration.

INTRODUCTION

In a recent study, we reported the isolation of a truncated vesicle-trafficking protein TRAPPC6ΔΔ (TPC6ΔΔ).1 Wild-type TRAPPC6A (TPC6A) is one of the components in the transport protein particle (TRAPP) complex.2–4 TRAPPC6A gene has been implicated in the neurodegenerative disease.3 TPC6A has an internal deletion of 14 amino acids at the N terminus. Unlike the wild-type protein, TPC6ΔΔ readily undergoes aggregation in the extracellular matrix.1 TPC6ΔΔ aggregates or plaques have been found in the brain cortex and hippocampus in nondemented middle-aged humans and in patients with Alzheimer’s disease (AD), suggesting that TPC6ΔΔ is a marker for early onset of AD.1 Also, in 3-week-old Wwox gene knockout mice,1 aggregates of TPC6ΔΔ, TIAF1 (TGF-β-induced antiapoptotic factor),6,7 tau and amyloid beta (Aβ) can be found in the brain cortex. We have determined that downregulation of WWOX leads to the aggregation of TPC6ΔΔ, TIAF1, tau and Aβ in vitro and in vivo.1,7,9 Sequentially, upon WWOX downregulation, TPC6ΔΔ becomes aggregated first, followed by TIAF1 aggregation, caspase activation, APP degradation for Aβ generation, as well as tau tangles formation.1,7,9 The observations suggest a critical role of WWOX in regulating protein aggregation and AD progression.

Human and mouse WWOX/Wwox gene, which encodes tumor suppressor WW domain-containing oxidoreductase (known as WWOX, FOR or WOX1), is known to have a critical role in blocking neurodegeneration.9–13 WWOX possesses two N-terminal WW domains, a C-terminal short chain alcohol dehydrogenase/reductase domain (SDR) and a nuclear localization signal in between the domains.10,11,14–18 WWOX is essential for embryonic neural development.9,10 However, under stress conditions (e.g., axotomy and constant light), there is an increased expression of WWOX, along with Tyr33 phosphorylation, that allows the activated protein to undergo nuclear accumulation and cause neuronal injury and damage.20–22 When activated WWOX is localized in the nucleus, its N-terminal WW domain may induce the transcription activation of NF-κB-dependent promoter.22 Whether this contributes, in part, to axotomy-induced acute and chronic brain inflammation is unknown and remains to be established.22

We have determined that WWOX is significantly downregulated in the AD hippocampus,18 indicating that loss of WWOX promotes AD progression. Most recently, important studies showed that homozygous nonsense mutations or other alterations of WWOX gene result in protein loss and cause patients to suffer from severe anomalies, including short stature and growth retardation, microcephaly with seizure, retinal degeneration and early death at 16 months of age.13,23–27 We have determined that WWOX blocks neurodegeneration via binding tau and tau-phosphorylating enzymes, including ERK, JNK and GSK-3β.8,28,29 Also, WWOX stimulates neuronal differentiation.29 Together, WWOX possesses dual functions in controlling cell death or survival. Under physiological conditions, WWOX exerts its homeostatic role by using WW domain for signaling and the SDR domain for metabolism. Without WWOX, cells tend to undergo rapid proliferation and apoptosis. Under stress conditions, increased levels of pY33-WWOX are accumulated in the mitochondria and nuclei for causing cell death.

Here, we show that in response to transforming growth factor beta (TGF-β), both wild-type TPC6A and TPC6ΔΔ shuttle from nucleus, nucleolus and then to mitochondrion, and again travel back to the nucleus. The nucleolus–mitochondrion shuttling is novel. However, when TPC6ΔΔ undergoes excessive aggregation

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in the mitochondria, activation of caspases occurs that leads to apoptosis.\textsuperscript{1,7,30} TGF-\(\beta\)/Smad signaling has a critical role in the pathogenesis of AD.\textsuperscript{7,31,32} TGF-\(\beta\)1 expression is upregulated in a transgenic mouse model of familial Alzheimer’s disease, and this leads to neuronal apoptosis.\textsuperscript{32} How WWOX interacts with TPC6A under the effect of TGF-\(\beta\)1 was examined by co-immunoprecipitation (co-IP) and time-lapse microscopy with Förster (fluorescence) resonance energy transfer (FRET) analysis.\textsuperscript{22,28,33,34}

RESULTS
Polymerization of endogenous TPC6A in the nuclei and mitochondria

We have recently demonstrated that downregulation of WWOX leads to aggregation of wild-type TPC6A and TPC6A\(\Delta\) both in Wwox knockout mice and in knockdown experiments using cell lines.\textsuperscript{1} Compared with the wild type, TPC6A\(\Delta\) can readily undergo aggregation in the extracellular matrix of the brain.\textsuperscript{1} To further validate whether polymerization or aggregation of TPC6A\(\Delta\) is universal, different types of mammalian cell lines were used. Transient overexpression of EGFP-TPC6A or -TPC6A\(\Delta\) in COS7 fibroblasts by electroporation resulted in localization of both the proteins mainly in the nucleus (Figure 1a). Cytosolic aggregates of EGFP-TPC6A and -TPC6A\(\Delta\) proteins are also shown (Figure 1a; see white arrows). Aggregate formation was also observed by overexpressing TPC6A\(\Delta\) in cutaneous squamous cell carcinoma SCC-9 cells (Figure 1a) and many cell types (data not shown).

In cutaneous basal cell carcinoma BCC cells, endogenous TPC6A is a 20-kDa monomer in the cytoplasm, and becomes a trimer or larger sizes in the nuclei (~70 kDa and larger), as determined by western blotting (Figure 1b). Exposure of BCC cells to UV irradiation rapidly induced the formation of a trimeric form in 10 min and aggregates of > 200 kDa in 60–120 min in the cytoplasm (Figure 1b). TPC6A appeared to relocate to the nucleus and became aggregated in 20 min post UV exposure, followed by disappearance from the nucleus (Figure 1b). The aggregates appear to be metabolically degradable, whereas no ubiquitination was shown with these proteins (data not shown). Similarly, transforming growth factor beta 1 (TGF-\(\beta\)1) induced self-aggregation of TPC6A and Ser35-phosphorylated TPC6A, and the aggregation exhibited in a ladder-like pattern in melanoma B16F10 cells (Figure 1c). In agreement with our previous observations,\textsuperscript{7} TGF-\(\beta\)1 caused TIAF1 aggregation (Figure 1c).

The endogenous TPC6A is abundant in the nuclei and nucleoli of COS7 cells (Figure 1d). Confocal microscopy analysis revealed that TPC6A was accumulated in the nucleoli and nuclei, and stimulation of these cells with TGF-\(\beta\)1 resulted in the relocation of TPC6A to the mitochondria, as determined by co-localization analysis (Figures 1d and e). We reported that phorbol 12-myristate 13-acetate (PMA) induces the relocation of WWOX to the
mitochondria in Jurkat T cells. When Jurkat T cells were stimulated with PMA (5 μM) for 90 min, endogenous TPC6A and WWOX, along with their phosphorylated forms, were shown in the mitochondria (Figure 1f). TPC6A was present mainly as a monomer in the cytoplasm (data not shown), whereas it became a dimer in the mitochondria (Figure 1f). In other cell types (e.g., monocytic THP-1 and U937 cells), mitochondrial TPC6A may exist as a trimer (data not shown). In addition, in response to TGF-β1, we have demonstrated the relocation of WWOX, along with p53, to the mitochondria and nuclei.15,28,35 TGF-β1 induces endogenous TPC6A to shuttle in between nucleoli and mitochondria We have established Wwox−/− MEF cells. These cells were exposed to TGF-β1 (5 ng/ml), and then stained with specific antibodies for TPC6A and TIAF1. Mitochondria were stained with MitoTracker Red. Wild-type TPC6A shuttles in between nucleoli to mitochondria. Each round trip takes ~60 min. Endogenous TIAF1 is mainly retained in the mitochondria. Approximately 100–120 cells were examined. (b) Similar results were observed by staining Ser35-phosphorylated TPC6A with a specific antibody. (c) TIAF1 co-localizes with TPC6A with Ser35 phosphorylation in the extracellular matrix as aggregates in the human AD hippocampus (also see Supplementary Figure S1).

TGF-β1 induces endogenous TPC6A to shuttle in between nucleoli and mitochondria

We have established Wwox−/− MEF cells. These cells were exposed to TGF-β1, followed by determining the localization of TPC6A. TGF-β1 induced translocation of the wild-type TPC6A from the nucleoli to the mitochondria in 20–30 min. TPC6A then traveled back to the nucleoli in about the same duration (Figures 2a and b). Together, a round trip of TPC6A shuttling in between mitochondria and nucleoli is around 40–60 min. In the wild-type Wwox+/+ MEF cells, the round trip of shuttling time for TPC6A drops down to 20 min, suggesting that WWOX increases the speed of TPC6A shuttling. Indeed, similar results were observed in WWOX-expressing COS7 cells. The round trip of shuttling time for TPC6A is ~10 min in COS7 cells. Endogenous TIAF1 was mainly retained in the mitochondria in Wwox−/− MEF cells (Figure 2a). In the AD hippocampus, TIAF1 co-localizes with Ser35-phosphorylated TPC6A in the extracellular matrix as aggregates (Figure 2c and Supplementary Figure S1). We have determined that TIAF1 participates in the TGF-β1 signaling via binding with Smad4, and that Smad4 prevents self-aggregation of TIAF1.6,7,36 Transiently overexpressed TIAF1 acts together with p53 and WWOX in inducing apoptosis.30

TPC6A physically binds to the C-terminal D3 tail of WWOX

Next, we investigated whether WWOX physically binds TPC6A and affects each other’s function. We have determined that downregulation of WWOX leads to TPC6A aggregation both
in vitro and in vivo.1 Transiently overexpressed EGFP-WWOX exhibited perinuclear distribution in the cytoplasm and the mitochondria of COS7 cells. Co-expression of WWOX and TPC6Δ results in increased nuclear accumulation of WWOX compared with WWOX expression alone (~100 cells counted, mean ± S.D., n = 3; **P < 0.05, Student’s t-test). HEK293 cells were cultured in 10% FBS/medium and grown in a 10-cm dish to 100% confluence. Cell lysates were prepared and processed for co-immunoprecipitation using specific antibodies, respectively, against TPC6A and WWOX. Western blotting analysis showed TPC6A physically interacted with WWOX in resting cells. PreIP = ~40 μg of protein in the input loading. (e) COS7 cells were co-transfected with EYFP-TPC6AΔ and ECFP-WWOX or indicated domains of WWOX. FRET microscopy revealed that TPC6AΔ binds WWOX to its C-terminal D3 tail (see increased FRETc). The relative binding strength is shown in a color scale, where the highest binding energy is indicated in red. (f) Designated WWOX domains for interacting with TPC6AΔ are determined by FRET microscopy. Ten cells from each experimental set were analyzed by Image Pro Plus 6.1 (mean ± S.D.; **P < 0.05, Student’s t-test).

Figure 3. TPC6A physically binds the C-terminal D3 tail of WWOX. (a and b) Transiently overexpressed EGFP-WWOX localized mainly in the perinuclear area in COS7 cells. Co-expression of WWOX and TPC6AΔ results in increased nuclear accumulation of WWOX compared with WWOX expression alone (~100 cells counted, mean ± S.D., n = 3; **P < 0.05, Student’s t-test). (c and d) HEK293 cells were cultured in 10% FBS/medium and grown in a 10-cm dish to 100% confluence. Cell lysates were prepared and processed for co-immunoprecipitation using specific antibodies, respectively, against TPC6A and WWOX. Western blotting analysis showed TPC6A physically interacted with WWOX in resting cells. PreIP = ~40 μg of protein in the input loading. (e) COS7 cells were co-transfected with EYFP-TPC6AΔ and ECFP-WWOX or indicated domains of WWOX. FRET microscopy revealed that TPC6AΔ binds WWOX to its C-terminal D3 tail (see increased FRETc).
Transiently overexpressed TPC6AΔ induces apoptosis and counteracts the function of WWOX in activating promoter governed by NF-κB.

We examined the functional relationship between WWOX and TPC6AΔ. Transiently overexpressed TPC6A and TPC6AΔ induced apoptosis of neuroblastoma SK-N-SH cell and HEK293 fibroblasts (Figure 4a), as well as many types of cells (data not shown). There were no significant differences regarding the efficacy of TPC6A and TPC6AΔ in inducing apoptosis. Transiently overexpressed WWOX induces apoptosis via the mitochondrial pathway.10,15,35,38,39 Interestingly, TPC6A and WWOX nullify each other’s function in causing apoptosis. For example, when transiently overexpressed, both WWOX and TPC6A induced apoptosis (Figures 4b and c). However, in combination, there were no additive effects (Figures 4b and c), suggesting that both proteins counteract each other’s function. To further test this notion, transiently overexpressed TPC6AΔ abolished the function of WWOX in activating NF-κB promoter (Figure 4d). We have previously determined that the WW domain of WWOX binds TPC6AΔ and this binding blocks the aggregation of TPC6AΔ.1 WWOX had no apparent effect on the aggregation of wild-type TPC6A (data not shown). Together, these observations suggest that the D3 tail of WWOX binds TPC6AΔ, and this binding blocks the aggregation of TPC6AΔ.1

TGF-β1 induces dissociation of the WWOX/TPC6AΔ complex for leading to TPC6AΔ aggregation and cell death

When COS7 cells were transiently overexpressed with ECFP-TPC6AΔ and EYFP-WWOX (or ECFP and EYFP) and exposed to TGF-β1, there was an initial increase in the binding of TPC6AΔ with WWOX in the nucleus, followed by reduction, as determined by time-lapse FRET microscopy (Figures 5c and d and Supplementary Videos 1–3). That is, the binding strength of TPC6AΔ and WWOX reached a maximal extent in 6 h in the nucleus. Later, a portion of both proteins relocated to the cytoplasm and underwent aggregation, as revealed by the positive FRET signal in the cytoplasm (see arrows in Figure 5d and Supplementary Videos 1–3). In most cases, the cells underwent apoptosis during a prolonged
WWOX controls TRAPPC6AΔ aggregation

Figure 5. WWOX binds and blocks TPC6AΔ aggregation, and TGF-β1 dissociates TPC6AΔ from WWOX for leading to aggregation. (a) COS7 cells were co-transfected with expression constructs for EYFP, EYFP-TPC6AΔ, EYFP-TPC6AΔ(S35G) and/or EYFP-TPC6AΔ(Y112F), followed by culturing for 24 h and then treating with or without TGF-β1 (5 ng/ml) for another 24 h. The extent of protein aggregation was measured. TGF-β1-induced protein aggregation was statistically analyzed (control versus treated samples; ~ 100 cells analyzed; n = 3). (b) COS7 cells were transfected with ECFP-WWOX and/or EGFP-TPC6AΔ by electroporation. Forty-eight hours later, ectopic TPC6AΔ underwent aggregation, and WWOX suppressed the aggregation by ~ 50%. Dominant negative WWOX (dn-WWOX) failed to block the aggregation of TPC6AΔ. Approximately 300 green fluorescent cells in total were counted, and the average is shown. (c and d) COS7 cells were transiently expressed with EYFP-WWOX and ECFP-TPC6AΔ. After 24 h in culture, cells were treated with TGF-β1 (5 ng/ml) and time-lapse FRET microscopy was performed. TGF-β1 increased the binding of WWOX with TPC6AΔ initially in the nucleus for 6 h, followed by reduction in the binding and increased TPC6AΔ aggregation in the cytoplasm. Also, see Supplementary Videos 1–3. (e and f) COS7 cells were transiently expressed with ECFP-WWOX and EYFP-TPC6AΔ or EYFP-TPC6AΔ(S35G). Upon exposure to TGF-β1 (5 ng/ml), ectopic WWOX and TPC6AΔ became aggregated in the nucleolus, whereas no aggregation was shown for WWOX and TPC6AΔ(S35G). The cell started undergoing apoptosis at hour 15 (see Supplementary Video 6). (g) Similarly, aggregation of EYFP-TPC6AΔ in the nucleolus (top panel) or cytoplasm (middle panel) was observed upon exposure of COS7 cells to TGF-β1 (5 ng/ml). Alteration of Tyr112 to Phe112 resulted in the failure of relocation of the mutant protein to the cytoplasm. (h) Under similar conditions, TGF-β2 (5 ng/ml) induced aggregation of EYFP-TPC6AΔ in the nucleus in 4 h. (i) In summary, two-way endogenous TPC6A shuttling is illustrated. In response to TGF-β1, nuclear TPC6A undergoes Ser35 phosphorylation for entering the nucleoli and then relocates out to the mitochondria as a dimer, which probably requires phosphorylation at Tyr112. Again, TPC6A migrates back to the nucleoli. Ectopic TPC6AΔ, tagged with EGFP or EYFP, undergoes one-way trafficking from the nuclei to the mitochondria only. This is due to TPC6AΔ protein aggregation in the mitochondria.
period of treatment with TGF-β1 for 24–48 h. No protein aggregation was observed in control cells overexpressing ECFP and EYFP (data not shown). Collectively, during a prolonged stimulation, TGF-β1 induces dissociation of the WWOX/TPC6AΔ complex that leads to TPC6AΔ aggregation and eventual cell death.

Ser35 phosphorylation is essential for relocation of TPC6AΔ to the nucleolus and pTyr112 needed for relocation to the cytoplasm. Alternatively, WWOX was tagged with ECFP and TPC6AΔ with EYFP. EYFP-TPC6AΔ relocated to the nucleolus and became self-aggregated in 2 h upon treatment of COS7 cells with TGF-β1 (Figure 5e and Supplementary Video 4). This result is in agreement with the observation that TGF-β1 induced shuttling of endogenous TPC6A to the nucleolus (Figure 2). Without exposure to TGF-β1, there was no translocation of EYFP-TPC6AΔ to the nucleolus (Supplementary Video 5). Alteration of Ser35 to Gly35 abolished TGF-β1-induced relocation of EYFP-TPC6AΔ(S35G) to the nucleolus (Figures 5a and f and Supplementary Video 6). EYFP-TPC6AΔ(S35G) did not undergo aggregation when transiently overexpressed (Figure 5f). Notably, when the strength of binding of ECFP-WWOX with EYFP-TPC6AΔ(S35G) reached maximally at hour 15, the cell underwent apoptosis probably due to the aggregation of WWOX with TPC6AΔ(S35G) (Supplementary Video 6). The EYFP-TPC6AΔ(Y112F) mutant failed to relocate to the cytoplasm (bottom in Figure 5g).

Without ectopic WWOX, aggregation of TPC6AΔ occurred effectively. When COS7 cells were overexpressed with EYFP-TPC6AΔ...
only, TGF-β1 induced relocation of TPC6 Δ to the nucleolus, followed by migrating out to the cytoplasm in less than 6 h (two representative data at top and middle rows from the same video; Figure 5g and Supplementary Video 7). Again, by time-lapse microscopy, we showed TGF-β1 induced nuclear EYFP-TPC6 Δ relocation to the mitochondria (Supplementary Video 8). Mitochondria were stained with MitoTracker Red. We also obtained similar results by treating cells with TGF-β2, which showed the induction of EYFP-TPC6 Δ aggregation in the nuclei and cytoplasm in 2 h (Figure 5h and Supplementary Video 9). Taken together, there is a two-way shuttling for endogenous TPC6A and TPC6 Δ (Figures 2 and 5i). In response to TGF-β1, nuclear TPC6 Δ undergoes Ser35 phosphorylation for entering the nucleoli, and then relocates out to the mitochondria as a dimer with a likely phosphorylation at Tyr112 (Figure 5i). Again, TPC6Δ travels back to the nucleoli. However, transiently overexpressed
TPC6Δ did not relocate back to the nuclei and became accumulated in the mitochondria.

Induction of mitochondrial apoptosis causes nuclear TPC6Δ to relocate to the cytoplasm.

In comparison, CCCP (carbonyl cyanide m-chlorophenyl hydrazone), an inducer of mitochondrial apoptosis, caused relocation of TPC6Δ from the nuclei to the mitochondria. When COS7 cells were transiently overexpressed with TPC6Δ and then treated with CCCP, CCCP rapidly induced loss of mitochondrial membrane permeability in less than 1 h (loss of red fluorescence), as determined by time-lapse microscopy. TPC6ΔA started to relocate to the cytoplasm at hour 3 and became aggregated at hour 6 (see arrows in Figure 6a and Supplementary Videos 8 and 9). Post aggregation, the cells underwent apoptosis (Supplementary Videos 8 and 9). In negative controls, EGFP alone did not relocate to the mitochondria (data not shown). Similar results were observed when cells were exposed to UV irradiation. Relocation of TPC6ΔA from the nuclei to the mitochondria was observed (data not shown).

TGF-β1 regulates the complex formation of TPC6ΔA with WWOX, and apoptosis occurs when the complex dissociates.

We continued to examine how WWOX regulates TPC6ΔA aggregation and apoptosis. COS7 cells were transiently overexpressed with TPC6ΔA and SDR/D3. Upon exposure to TGF-β1, there was a rapid increased binding between TPC6ΔA and SDR/D3 in 30 min in COS7 cells, and the binding lasted for 4 h followed by dissociation. When TPC6ΔA dissociated from SDR/D3, apoptosis occurred (see apoptotic bodies in Figures 6b and c and Supplementary Videos 10–13). During cell death, increased secretion of exosome-like particles to the extracellular space is shown (Supplementary Video 12). In an appropriate control, TGF-β1 did not induce the binding of TPC6ΔA with SDR only (Figure 6d), and no cell death occurred. We observed the release of exosome-like particles to the extracellular matrix during phorbol ester-induced cell death, as well as in UV/cold shock-induced bubbling cell death from the nucleus. However, the functional properties of these exosome-like particles remain to be established.

Without stimulation, TPC6ΔA did not bind the WW domain (Figures 3e and f). When COS7 cells were transiently overexpressed with TPC6ΔA and WW domain only, TGF-β1 did not cause cell death (Figures 6e and f). Notably, upon stimulation with TGF-β1 for 6–8 h, there was an increased binding of TPC6ΔA with WW (Figures 6e and f). This binding and then dissociation did not render cell death. The observations imply that the WW domain of WWOX may provide a rescue mission to block apoptosis.

TPC6A plaques interact with TIAF1 aggregates in vivo

By fluorescent immunostaining, we showed the presence of TPC6A plaques with Ser35 phosphorylation (red) and TIAF1 aggregates (green) in the brain cortex of Wwox−/− mice (Figures 7a–c). The observation suggests that formation of TPC6A plaques occurs first, followed by TIAF1 aggregation. This assumption has been approved by using siRNA to knock down WWOX, TPC6A and TIAF1, respectively.1 Presence of TPC6A and TIAF1 aggregates or plaques was also shown in the extracellular matrix in postmortem human AD hippocampi (Supplementary Figure 52). By antibody-FRET analysis, we showed the binding of TPC6A with TIAF1 (Figures 7a and c). In negative controls, there was no binding (Figure 7b). In addition, we showed the p-TPC6A plaques in the brain cortex of Wwox−/− mice by immunohistochemistry (Figure 7d).

TIAF1 reciprocally increases TPC6A expression

Intriguingly, transient overexpression of EGFPTIAF1 in SH-SY5Y cells significantly raised the expression of endogenous TPC6A (Figure 7e). Binding of EGFPTIAF1 with endogenous wild-type TPC6A occurred mainly in the cytoplasm, but not in the nucleus (Figure 7f). To further determine the relationship between WWOX, TPC6ΔA and TIAF1, we showed that ectopic EYFP-TIAF1 bound strongly with ECFP-TPC6ΔA, as determined by FRET microscopy (Figure 7g). However, binding of ECFP-TPC6ΔA with EYFP-WWOX or EYFP-TPC6ΔA was relatively weak (Figure 7g). The results in vitro are in agreement with the observations in vivo (Figures 7a and c).

DISCUSSION

In this study, we have discovered for the first time that TGF-β1 induces two-way shuttling of TPC6A and TPC6ΔA in between nuclei and mitochondria. The nucleolus–mitochondrion shuttling appears to be a rare event, although many proteins undergo one-way nucelocyttoplasmic shuttling.47 One study reported that SenP5 translocates from the nucleoli to the mitochondria to modulate DRP1-dependent fission during mitosis.48 In yeast, TPC6A participates in tethering of the trafficking protein particle complex to the cis-Golgi membrane.2 In contrast, mammalian TPC6A is mainly localized in the nuclei and the perinuclear regions in mammalian cells. Although endogenous TPC6A and TPC6ΔA may polymerize in the cell compartments, the polymerized proteins do not induce cell death. Presumably, a novel cellular system depolymerizes the aggregated proteins before relocation. Site-directed mutagenesis showed that phosphorylation of Ser35 and Tyr216 is needed for TPC6A to relocate to the nucleolus and mitochondria, respectively. Endogenous WWOX binds TPC6A and reduces the time of round-trip shuttling by 50%. The underlying mechanism of this regard is unknown. Nonetheless, TPC6A nullifies WWOX-mediated activation of the responsive element governed by NF-κB. The observations suggest that endogenous activated WWOX provides a pro-survival signal by activating NF-κB under the stress of neurodegeneration, and its action can be overridden by TPC6A and other transcription factors.22

TPC6ΔA binds the D3 tail of WWOX. TGF-β1 dissociates the binding, which leads to the aggregation of TPC6ΔA in the mitochondria for causing apoptosis. Indeed, WWOX has an intramolecular folding via the binding of the N-terminal WW domain with the C-terminal SDR domain (Chen et al., unpublished). TGF-β1 activates WWOX to phosphorylate at Tyr33.34 The activated form probably has an unfolded WW-SDR binding, thus governing the complex formation of TPC6ΔA with WWOX, and apoptosis occurs when the complex dissociates.22

Functional TGF-β1/Smad signaling is believed to protect against the progression of AD.43 However, aberrant TGF-β signaling caused by interaction of Smads with protein tangles may facilitate AD progression.44 TGF-β1 expression is significantly increased in a transgenic mouse model of familial Alzheimer’s disease and causes neuronal apoptosis.32 In stark contrast, blocking TGF-β1 dissociates the binding protein particle system depolymerizes the aggregated proteins before relocation. Hence, WWOX may be a key player in neuronal survival via NF-κB signaling.

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A6 in AD patients.\textsuperscript{1} The aggregated TPC6A\textsuperscript{Δ} induces TIAF1 aggregation and activates caspases for causing APP degradation and Aβ\textsuperscript{Δ} generation. Caspase activation has been shown in the AD brains.\textsuperscript{46}

Upon stimulation with TGF-β, endogenous TPC6A relocalizes to the nucleus and then nucleolus, and finally migrates out of the nucleus to the cytoplasm or mitochondria. The role of TPC6A in the nuclei is unknown. A likely scenario is that TPC6A may carry RNAs or proteins out of the nuclei, which are needed for mitochondria. A leucine zipper motif is predicted near the C-terminal of TPC6A, suggesting that TPC6A might be a DNA-binding protein. We also predicted two possible phosphorylation sites in TPC6A\textsuperscript{Δ}, Ser35 and Tyr112. Alteration of Ser35 to Gly abolishes TGF-β-mediated aggregation of TPC6A\textsuperscript{Δ}. The Ser35 phosphorylation has been verified by our produced phospho-antibody.\textsuperscript{1} However, alteration of Tyr112 to Phe does not reduce TPC6A\textsuperscript{Δ} aggregation in the presence or absence of TGF-β. Importantly, overexpressed wild-type TPC6A may undergo aggregation, but fails to activates caspases. The binding affinity of TPC6A\textsuperscript{Δ} with wild-type TPC6A is weak, and the binding does not induce caspase activation (data not shown).

Finally, the relationship among WWOX, TIAF1 and TPC6A is being determined. Knockdown of WWOX causes aggregation of TIAF1 and TPC6A.\textsuperscript{3} Interestingly, TPC6A knockdown induces TIAF1 aggregation, whereas TIAF1 knockdown does not induce TPC6A aggregation effectively. That is, the sequential cascade of aggregation is related to WWOX downregulation, which leads to the aggregation of TPC6A followed by TIAF1. The TPC6A and TIAF1 aggregates can be found in the mitochondria of degenerative neurons.\textsuperscript{1} Most recently, we determined that overexpressed TIAF1 exhibits as aggregates together with Smad4 and Aβ in the cancer stroma and peritumor capsules of solid tumors.\textsuperscript{30} Also, TIAF1/Aβ aggregates are shown on the interface between brain neural cells and the metastatic cancer cell mass. TIAF1 is upregulated in developing metastatic tumor, but may disappear in established metastatic tumors. Growing neuroblastoma cells on the extra-cellular matrices from other cancer cell types induces the formation of TPC6A\textsuperscript{Δ}-pEYFP-C1, TPC6A\textsuperscript{Δ}-pEYFP-C1 and/or WWOX-pEYFP-C1. After culturing for 24–48 h, cells were harvested by centrifugation at 2000 r.p.m., gently washed once with PBS and finally fixed with 75% ethanol. Fixed cells were precipitated by centrifugation, washed once by PBS and then stained with propidium iodide (PI) solution (2 μg/ml PI, 10 μg/ml RNase A in PBS) for 30 min at room temperature. Flow cytometry/FACS (Becton Dickinson, BD, Franklin Lakes, NJ, USA) analysis was carried out to determine DNA contents. Three independent experiments were performed, and Student’s t-test was used to analyze data among the controls and experiments.

ABSTRACT

AD, Alzheimer’s disease; Aβ, β-amyloid; APP, amyloid precursor protein; BACE, APP-β-secretase; FRET, Förster (fluorescence) resonance energy transfer; FTD, frontotemporal dementia; HD, Huntington disease; PMA, phorbol 12-myristate 13-acetate; NFT, neurofibrillary tangles; TGF-β, transforming growth factor beta; TIAF1, TIAF1-β-related protein complex 6A delta (TRAPPC6A\textsuperscript{Δ}), an N-terminal internal deletion isoform of wtTPC6A; WWOX, WWOX domain-containing oxidoreductase.

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COMPETING INTERESTS

The authors declare no conflict of interest.
11. Supplementary Information accompanies the paper on the Cell Death and Differentiation website (http://www.nature.com/cddiscovery)

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