The role of a small transforming growth factor beta (TGF-β)-induced TIAF1 (TGF-β1-induced antiapoptotic factor) in the pathogenesis of Alzheimer’s disease (AD) was investigated. TIAF1 physically interacts with mothers against DPP homolog 4 (Smad4), and blocks SMAD-dependent promoter activation when overexpressed. Accordingly, knockdown of TIAF1 by small interfering RNA resulted in spontaneous accumulation of Smad proteins in the nucleus and activation of the promoter governed by the SMAD complex. TGF-β1 and environmental stress (e.g., alterations in pericellular environment) may induce TIAF1 self-aggregation in a type II TGF-β receptor-independent manner in cells, and Smad4 interrupts the aggregation. Aggregated TIAF1 induces apoptosis in a caspase-dependent manner. By filter retardation assay, TIAF1 aggregates were found in the hippocampi of nondemented humans and AD patients. Total TIAF1-positive samples containing amyloid β (Aβ) aggregates are 17 and 48%, respectively, in the nondemented and AD groups, suggesting that TIAF1 aggregation occurs preceding formation of Aβ. To test this hypothesis, in vitro analysis showed that TGF-β-regulated TIAF1 aggregation leads to dephosphorylation of amyloid precursor protein (APP) at Thr668, followed by degradation and generation of APP intracellular domain (AICD), Aβ and amyloid fibrils. Polymerized TIAF1 physically interacts with amyloid fibrils, which would favorably support plaque formation in vivo.

Neurodegenerative diseases are frequently encountered in the aging populations in the human societies. The neuro-pathology of Alzheimer’s disease (AD) is characterized by extracellular accumulation of fibrillar amyloid β (Aβ) peptide and intracellular deposition of neurofibrillary tangles made of cytoskeletal protein tau.1 These protein aggregates invoke neuronal death and block neurogenesis and learning and memory capabilities in AD patients.

Role of transforming growth factor beta (TGF-β) in the pathogenesis of AD is controversial.2-5 Type II receptor for TGF-β (TβRII) is central to the TGF-β/mothers against DPP homolog (Smad) signaling. Reduced levels of TβRII are observed in human AD brains, and this correlates with age-dependent neurodegeneration and AD-like disease in a mouse model.6 However, in a transgenic AD mouse model, TGF-β1 is constitutively upregulated and neuronal apoptosis occurs.7 Increased levels of TGF-β in AD are associated with perivascular deposition of extracellular matrix, which hinders clearance of Aβ and allows the development of cerebral amyloid angiopathy.8 Blockade of TGF-β/Smad innate immune signaling mitigates Alzheimer-like pathology in transgenic TβRII mouse model.9

Here, we identified TGF-β1-induced antiapoptotic factor (TIAF1) protein aggregation, which may cause neuronal death. TIAF1, a 12-kDa TIAF1,6 participates in TGF-β signaling9 and controls p53 activation.8 TIAF1 is implicated in the activation of TH2 helper T lymphocytes in chronic organ rejection9 and development of regulatory T cells,10 as well as...
association with Hirschsprung’s disease. TIAF1 tends to aggregate, and the protein aggregates are found in the hippocampi of postmortem nondemented humans and AD patients. Importantly, TIAF1 self-aggregation leads to generation of Aβ and amyloid fibrils from amyloid precursor protein (APP) in vitro.

Results

TIAF1 binds and blocks Smad4-dependent promoter activation. TIAF1 is an effector of the TGF-β signaling. By yeast two-hybrid analysis, we determined that TIAF1 physically interacted with Smad4 (Figure 1a). Stimulation of COS7 fibroblasts with TGF-β for 30 min resulted in the binding of endogenous TIAF1 with Smad4, and the complex was accumulated in the nuclei (Figure 1b).

COS7 cells were infected with a retroviral small interfering RNA-targeting TIAF1 (TIAF1si) or an empty retrovirus. After 48 h, spontaneous relocation of Smad3 and 4 in the nuclei occurred in TIAF1-knockdown cells, but not in control cells, as determined by immunofluorescence microscopy (Figure 1c). Similarly, COS7 cells were transfected with enhanced green fluorescence protein (EGFP)-Smad4 plasmid and subsequently infected with retroviral TIAF1si or empty retrovirus. By yeast two-hybrid analysis, we determined that when TGF-β-induced TIAF1 self-binding occurs independently of TβRII. In TβRII-expressing cells, TGF-β/1 cross-links with TβRII for recruiting TβRI (type I receptor) and signaling via the Smad2/3/4 complex. Human colon HCT116 cells are deficient in TβRII. TGF-β/1 binds membrane hyaluronidase type 2 (Hyal-2) for recruiting tumor suppressor WW domain-containing oxidoreductase (WOX1) (also named WWOX or FOR) and Smad4 to relocate to the nuclei, which may dramatically increase the transcriptional activation for SMAD and apoptosis. HCT116 cells were transiently overexpressed with TIAF1/TIAF1 and the cells became sensitive to TGF-β/1-induced apoptosis (Figure 2c).

TIAF1 self-binding induces apoptosis. To determine TIAF1 in TGF-β/1-mediated growth suppression, TGF-β/1-sensitive mink lung Mv1Lu epithelial cells were transfected with a mixture of ECFP and enhanced yellow fluorescence protein (EYFP) (yellow), or ECFP-TIAF1 and EYFP-TIAF1 plasmids by liposome-based GeneFector. Protein–protein interactions were measured by FRET ( Förster/resonance energy transfer) microscopy. TGF-β/1 rapidly induced apoptosis of cells overexpressing TIAF1/TIAF1 in 1 h, but had no effect on ECFP/EYFP-expressing cells or non-transfected cells in time-lapse microscopy (Figure 2a). No apoptosis was observed in cells overexpressing a dominant-negative TIAF1 (E222/23A) (TIAF1 with Glu22 and 23 mutated to Ala acting as dominant negative) alone (Figure 2a), and/or wild-type TIAF1 (Supplementary Figure S1). Similarly, human neuroblastoma SK-N-SH cells overexpressing TIAF1/TIAF1 were sensitive to UV light-induced apoptosis, whereas ECFP/EYFP-overexpressing cells were resistant (Figure 2b).

TGF-β/1-induced TIAF1 self-binding occurs independently of TβRII. In TβRII-expressing cells, TGF-β/1 cross-links with TβRII for recruiting TβRI (type I receptor) and signaling via the Smad2/3/4 complex. Human colon HCT116 cells are deficient in TβRII. TGF-β/1 binds membrane hyaluronidase type 2 (Hyal-2) for recruiting tumor suppressor WW domain-containing oxidoreductase (WOX1) (also named WWOX or FOR) and Smad4 to relocate to the nuclei, which may dramatically increase the transcriptional activation for SMAD and apoptosis. HCT116 cells were transiently overexpressed with TIAF1/TIAF1 and the cells became sensitive to TGF-β/1-induced apoptosis (Figure 2c).

TIAF1 self-binding induces apoptosis essentially in every cell dependently of caspases. Transient overexpression of TIAF1 is sufficient to sensitize cells to TGF-β/1-mediated apoptosis. Human lung NCI-H1299 cells were transfected with EYFP-TIAF1 only. TGF-β/1-induced TIAF1 aggregation (see punctates) and apoptosis in cells in a time-dependent manner (Supplementary Figure 3a and Supplementary Video 1). EYFP alone had no effect. Similar results were observed using other types of cells, as well as using EGFP- or ECFP-tagged TIAF1 (data not shown).

TIAF1 self-binding is apparently a universal occurrence. When SK-N-SH, SH-SYS5, Mv1Lu and NCI-H1299 cells were transiently overexpressed with TIAF1/TIAF1, apoptosis occurred significantly in 24 h, compared with cells overexpressing ECFP/EYFP (measured at the SubG0/G1 phase; Figure 3b). Reduction by ~50–75% in the G0/G1, S and G2/M phases of the cell cycle was shown in cells.
Figure 1  TIAF1 physically binds Smad4 and blocks Smad4-regulated promoter activation. (a) By yeast two-hybrid library screening, two Smad4 clones (No. 7 and 17), with an identical DNA sequence, were isolated as a TIAF1-binding protein. Both Smad4/TIAF1 interactions and positive control bindings for WOX1/p53 and MafB self-interaction are shown, as evidenced by the growth of yeast at 37°C using a selective galactose-containing agarose plate (six representative colonies). Empty pSos/pMyr vectors and pS3/TIAF1 plasmid constructs were regarded as negative controls. (b) COS7 cells were cultured in 10% FBS and treated with or without TGF-β1 (5 ng/ml) for 30 min, followed by isolating cytosolic and nuclear fractions for co-immunoprecipitation with anti-TIAF1 IgG. TGF-β1 increased the binding of TIAF1 with Smad4. Non-immune IgG was used as a negative control in co-immunoprecipitation. (c) COS7 cells were infected with retroviral TIAF1si or empty retrovirus and cultured for 48 h, followed by determining protein nuclear localization for Smad3 and 4 by immunofluorescence microscopy. TIAF1si knocked down endogenous TIAF1 protein by ~70%. (d) COS7 cells were transfected with EGFP-Smad4 plasmid (using liposome) and infected with retroviral TIAF1si or empty retrovirus. The cells were grown for 48 h, fixed and immunostained with TIAF1. Nuclei were stained with DAPI. Knockdown of TIAF1 significantly induced nuclear accumulation of EGFP-Smad4. (e and f) Human monocytic U937 cells and mouse L929 fibroblasts were infected with TIAF1si or empty retrovirus and cultured for 48 h, followed by isolating nuclear and cytosolic fractions. Spontaneous accumulation of Smad3 and 4 in the nuclei was shown in cells infected with TIAF1si. Reduction of endogenous TIAF1 protein by siRNA is shown (60–75% reduction; a representative set of data from two experiments). (g) COS7 cells were transfected with ECFP or ECFP-TIAF1 expression constructs, grown for 24 h, and treated with TGF-β1 (5 ng/ml) for 1–8 h. The extent of accumulation of Smad2, 3 or 4 in the nuclei post TGF-β1 treatment for 4 h is shown (n = 3, ~100 cells counted per experiment), as determined by immunofluorescence microscopy. (h) COS7 cells were transfected with a SMAD-responsive element DNA construct (using GFP as a reporter), in the presence or absence of Smad4 and/or TIAF1 expression constructs. After 48 h, positive promoter activation in cells with green fluorescence was counted. Compared with Smad4 alone, TIAF1 significantly blocked the ectopic Smad4-induced activation of the promoter (P < 0.001 for the last two bars at the right, ~200 cells counted in each experiment with three repeats; mean ± S.D., Student’s t-test). SP, promoter driven by SMAD. (i) COS7 cells were transfected with the SMAD promoter reporter construct and simultaneously infected with the TIAF1si or empty retrovirus. Knockdown of TIAF1 resulted in spontaneous activation of the SMAD promoter (~1.5-fold increase). Ectopic Smad4-regulated promoter activation was increased by ~2-fold in the TIAF1-knockdown cells.
overexpressing TIAF1/TIAF1, compared with cells overexpressing ECFP/EYFP or nothing. These observations were reproduced in human breast cancer MCF7 and MDA-MB-231 cells, prostate DU145 cells, Jurkat T lymphocytes and murine L929 fibroblasts. TIAF1 self-binding-mediated cell death was blocked by a pan-caspase inhibitor zVAD-FMK (30 μM; inhibition by 85%).

When TIAF1/TIAF1-expressing NCI-H1299, MCF7 and SK-N-SH cells were exposed to TGF-β1 or -β2 for 24 h, no enhanced cell death (SubG1 phase) and growth suppression (G0/G1 phase) were observed (Supplementary Figures S2–S4). Indeed, during exposure of TIAF1/TIAF1-overexpressing cells to TGF-β1 for <3 h, there was an enhanced TIAF1 self-binding and cell death (Figures 2 and 3b). Beyond the initial time points, occurrence of cell death appears to depend on the extent of TIAF1 self-binding. Without binding interaction between the wildtype and mutant TIAF1, no cell death occurred (Supplementary Figure S1).

Smad4 prevents TIAF1 self-binding and determines cell fate. Whether Smad4 affects TIAF1 self-aggregation was examined. NCI-H1299 cells were transiently overexpressed with EYFP-TIAF1 and ECFP-Smad4. By time-lapse microscopy, when cells expressed a greater amount of Smad4 than TIAF1 (see big blue), they were highly sensitive to TGF-β1-induced apoptosis, as evidenced by rapid cell shrinking and membrane blebbing (Figure 3c; see blue line). That is, TGF-β1 activated the overexpressed Smad4 to cause apoptosis. TGF-β1 rapidly increased the binding of Smad4 with TIAF1 in 20 min (red color line for FRETc), and concurrently cell death occurred (reduction in cell size).

In contrast, cells became refractory to TGF-β1-mediated apoptosis, when the expressed EYFP-TIAF1 level was higher than ECFP-Smad4. Under this condition, the binding affinity of these proteins was reduced.

**Figure 2** TIAF1 self-binding leads to apoptosis. (a) TGF-β1-sensitive mink lung Mv1Lu epithelial cells, overexpressing ECFP/EYFP, ECFP-TIAF1/EYFP-TIAF1 or EYFP-TIAF1(E22/23A), were exposed to TGF-β1 (5 ng/ml). Time-lapse microscopy was carried out (total 8 h; one picture taken per 5 min). TGF-β1 rapidly increased apoptosis of TIAF1/TIAF1-expressing cells, but not ECFP/EYFP-expressing cells, in 1 h. No apoptosis was observed in cells overexpressing a dominant-negative TIAF1(E22/23A) or in non-transfected cells. (b) Human neuroblastoma SK-N-SH cells overexpressing TIAF1/TIAF1 were sensitive to UV light-induced apoptosis, but ECFP/EYFP-overexpressing cells were resistant. (c) Human TβRII-deficient colon HCT116 cells, overexpressing TIAF1/TIAF1, were treated with TGF-β1 (5 ng/ml) and imaged by time-lapse FRET microscopy. TGF-β1 significantly increased the self-binding of TIAF1/TIAF1 (left panel). When the binding reached a maximal strength (measured as FRETc), the cell underwent apoptosis (left panel). When cells were co-transfected with TIAF1 and Smad4, apoptosis was blocked (right panel). The expression of Smad4 was optimized to a low level, so as to prevent apoptosis by the overexpressed Smad4 (see Figure 3).

**Figure 3** Smad4 regulates TIAF1 self-binding and cell fate. (a) TGF-β1 (5 ng/ml) induced self-aggregation of TIAF1 (see green punctate formation), which leads to apoptosis of NCI-H1299 cells overexpressing EYFP-TIAF1, as determined by time-lapse microscopy. Also, see Supplementary Video 1. (b) Cell lines, including neuroblastoma SK-N-SH and SH-SY5Y cells, normal lung epithelial Mv1Lu cells, and lung cancer NCI-H1299 cells, were transfected with ECFP-TIAF1 and EYFP-TIAF1 by electroporation and then cultured for 24 h. These cells underwent apoptosis significantly, compared with cells overexpressing ECFP and EYFP (measured at the SubG0/G1 phase; P < 0.0005, n = 3, Student's t-test). Cont, control (no electroporation). Sham, electroporated with medium only. CY, ECFP and EYFP; T/T, ECFP-TIAF1 and EYFP-TIAF1. (c) When ECFP-Smad4 was expressed at a greater level than ECFP-TIAF1 in NCI-H1299 cells, TGF-β1 (5 ng/ml) rapidly increased the binding of Smad4 with TIAF1 in 20 min (red color line for FRETc), and concurrently cell death occurred (reduction in cell size). (d) In contrast, cells became refractory to TGF-β1-mediated apoptosis, when the expressed EYFP-TIAF1 level was higher than ECFP-Smad4. Under this condition, the binding affinity of these proteins was reduced.
Extracellular stimuli induce TIAF1 self-polymerization. When L929 cells were grown to 80% confluence and then exposed to TGF-β1 for 1 h, TIAF1 expression was increased (12–17 kDa), along with the presence of high molecular size proteins (43 and 46 kDa; Figure 4a). Similarly, TGF-β1 rapidly induced formation of large size TIAF1 (55 and 90 kDa) in SK-N-SH cells (Figure 4a). At high cell densities (100% and higher), multiple molecular sizes of TIAF1 are shown in NCI-H1299 and human teratocarcinoma NT2D1 cells (Figures 4b and c), as well as in COS7 and many other types of cells (data not shown). These observations are in parallel with the results from TGF-β1-induced TIAF1 self-aggregation in real time (Figure 3a). On treatment with estrogen (E2 or 17β-estradiol) for 24 h, TIAF1 aggregation was reduced in NCI-H1299 cells (Figure 4b). Also, UV irradiation rapidly suppressed the aggregation in NT2D1 cells in 90 min (Figure 4c).

Tumor necrosis factor (TNF)-α rapidly induced TIAF1 polymerization in L929 cells in 1 h in a dose-related manner, which correlates with generation of Aβ and amyloid fibrils (Figure 4d). Monoclonal antibody against Aβ (MCA2172), which was used in the entire experiments, did not cross-react with APP (Figure 4d, Supplementary Figures S6–S8). Also, APP antibody (MAB348) did not bind Aβ (Figure 4d, Supplementary Figures S6 and S7). Amyloid fibrils were stained with Chemicon/Millipore’s (Billerica, MA, USA) antibody against A/β oligomers. Treatment of L929 cells with TGF-β1, -β2 and/or TNF-α for 24 h resulted in induction of high molecular sizes of TIAF1 and Aβ (Supplementary Figure S7). Failure of detection of 4.5 kDa Aβ monomers was probably because of their release from the cells to the culture supernatants. By immunoprecipitation using specific antibodies against TIAF1 and Aβ, Aβ monomer was enriched (Figures 6d and f and 7e).

When L929 cells were grown to a high cell density, TIAF1 became a dimer (Figure 4e). Both hyaluronidase PH20 and complement C1q suppressed the expression of TIAF1 (Figure 4e). In contrast, NCI-H1299 cells expressed the dimeric TIAF1, and C1q and PH20 promoted the formation of TIAF1 dimer, tetramer and higher molecular sizes (Figure 4e).

TIAF1 aggregation can be superinduced, especially when cells are cultured on the extracellular matrix derived from other types of cells pretreated with Prima1. p53-negative NCI-H1299 cells were cultured overnight on Petri dishes and pretreated with or without Prima1 for 1 h (to restore mutant p53 function). The cells were then treated with 50 mM EDTA (in phosphate-buffered saline) at 4°C for 5 min, followed by removal using repeat pipetting. HEK293 cells were then seeded on the matrix and grown for 24 h. When cells were grown on Prima1-activated extracellular matrix, at 20 min, and membrane blebbing at 40–70 min.

UV light rapidly induced apoptosis of NCI-H1299 cells overexpressing TIAF1/TIAF1. Reduction in cell size by 50% occurred at 120–180 min, and membrane blebbing at 200–240 min.
Pi 
im is an inhibitor of p53 and blocks the binding of p53 with Bcl-xL.17 Finally, in rat primary glial cells, TIAF1 is expressed as 17- and 24-kDa proteins and high molecular sizes at a high cell density (Supplementary Figure S9). Lactacystin, a proteasome inhibitor, appeared to block TIAF1 expression (Supplementary Figure S9). Nocodazole, an inhibitor of microtubule polymerization, did not cause TIAF1 degradation. Together, lactacystin and nocodazole suppressed the expression of TIAF1 in 3 h, without APP fragmentation and Aβ production (Supplementary Figure S9).

Identification of TIAF1 aggregates in human hippocampus. Postmortem human hippocampal tissue sections were pre-stained with Bielschowski stain (containing silver), followed by staining with specific antibody against TIAF1. Fluoro-Jade C Red was used to identify degenerative neurons. Nuclei were stained with DAPI. Sequential magnification of aggregated TIAF1 (green; from × 40 to × 400) shows its colocalization with degenerative neurons (both in red and blue). (b) TIAF1 aggregation is shown in the extracellular matrix containing no signal for nuclei. A representative negative control. The above sections were stained with a secondary antibody (green), Fluoro-Jade C Red and DAPI. Scale bars a–c, 20 μm for × 400 magnification, and 200 μm for × 40 magnification. (d) A confocal image of a plaque, containing TIAF1 and Aβ, is shown (× 600; also see data in Supplementary Figure S10). Scale bar, 20 μm for × 600 magnification. (e) A confocal image of a TIAF1 plaque, containing de novo synthesized Aβ aggregates in the epicenter, is shown from the hippocampal section of an APP/PS1 transgenic mouse (also see Supplementary Figure S11). TIAF1 aggregates in degenerative neurons in the AD hippocampus. (a) Human hippocampal tissue sections were pre-stained with Bielschowski stain (containing silver), followed by staining with specific antibody against TIAF1. Fluoro-Jade C Red was used to identify degenerative neurons. Nuclei were stained with DAPI. Sequential magnification of aggregated TIAF1 (green; from × 40 to × 400) shows its colocalization with degenerative neurons (both in red and blue). (b) TIAF1 aggregation is shown in the extracellular matrix containing no signal for nuclei. A representative negative control. The above sections were stained with a secondary antibody (green), Fluoro-Jade C Red and DAPI. Scale bars a–c, 20 μm for × 400 magnification, and 200 μm for × 40 magnification. (d) A confocal image of a plaque, containing TIAF1 and Aβ, is shown (× 600; also see data in Supplementary Figure S10). Scale bar, 20 μm for × 600 magnification. (e) A confocal image of a TIAF1 plaque, containing de novo synthesized Aβ aggregates in the epicenter, is shown from the hippocampal section of an APP/PS1 transgenic mouse (also see Supplementary Figure S11).
and that TIAF1 is likely to protect the formation of Aβ aggregates.

**TIAF1 aggregates are abundant in the hippocampi of nondemented humans.** Presence of TIAF1 aggregates is shown in water-soluble protein extracts of postmortem frozen human hippocampi by western blots (Figure 6a). Multiple aggregated TIAF1 forms are >200 kDa in both AD patients and nondemented controls (Figure 6a, Supplementary Figure S13). These aggregates are essentially devoid of ubiquitin, as determined in western blotting using the same blots (Supplementary Figure S13).

Filter retardation assay was carried out to determine the presence of water-insoluble TIAF1 aggregates (>0.2 μm in diameter) in human hippocampi. TIAF1 aggregation was present in 59.0% of nondemented human hippocampi (age 59.0 ± 17.0, n = 41), and 54% in older postmortem AD patients (age 80.0 ± 8.8, n = 97) (Figure 6c). There were 15 and 48% for Aβ aggregates in controls and AD samples, respectively (Figure 6c). Further analysis revealed that there was only 17% of the total TIAF1-positive samples possessing Aβ aggregates in the control group, and 48% for the AD group.

**TIAF1 self-aggregation precedes production of Aβ and amyloid fibrils.** We validated the occurrence of TIAF1 aggregation preceding Aβ in vitro. Although Aβ monomer can be secreted from cells, we enriched the monomer by immunoprecipitation using anti-Aβ antibody (A/H43). Transient overexpression of TIAF1/TIAF1 in L929 cells induced generation of amyloid fibrils (Figure 6d). Ectopic dominant-negative and wild-type TIAF1 did not generate amyloid fibrils (Figure 6e). The dominant negative did not bind wild-type TIAF1, as determined by FRET analysis. Interestingly, TGF-β1-induced generation of pre-fibrillar Aβ oligomers (A/H43) in cell lysates (Figure 6d or see Figure 7). Transient overexpression of TIAF1/TIAF1 in L929 cells induced generation of amyloid fibrils (AO antibody), but not in cells overexpressing ECFP/EYFP (C/Y) or GFP-TIAF1/mutant EYFP-TIAF1 (Tc/Ty). TGF-β1 (5 ng/ml) induced the expression of prefibrillar Aβ oligomers (A11 antibody) in C/Y-expressing L929 cells. Mutant or dominant-negative TIAF1 is with an E22/23A alteration. (g) Similar experiments were performed in Mv1Lu cells, which shows Tc/Ty-induced amyloid fibrils (a single band under reducing SDS-PAGE; ~60 kDa). (h and i) NCI-H1299, B16F10 and SH-SYSY cells were treated with medium C, TGF-β1 (5 ng/ml), -β2 (5 ng/ml), -β3 (5 ng/ml), TNF-α (50 ng/ml) and TNF-β (25 ng/ml) for 24 h. Distinct APP degradation patterns are shown. A representative protein gel is shown from three repeats e-i.
SK-N-SH cells were overexpressed with TIAF1/TIAF1. On exposure to TGF-β1 for 24 h, cell sizes were reduced. These cells had significantly overexpressed APP and Aβ, compared with control cells (Figures 7a–d). FRET analysis showed that TGF-β1 increased the self-binding of TIAF1 during treatment for 24 h (Figures 7a–d). Importantly, phosphorylation of endogenous APP at Thr668 was significantly increased in cells overexpressing TIAF1/TIAF1, and that TGF-β1 suppressed the phosphorylation (Figure 7d). Increased phosphorylation at Thr668 in APP prevents cleavage by γ-secretase and generation of Aβ.

Finally, in western blotting, TGF-β1 was shown to increase the phosphorylation of endogenous APP at Thr668, but suppressed the binding of TIAF1 with phosphorylated APP (≈ 70 kDa) in MCF7 cells (Figure 7e). TGF-β1 did not increase the binding of TIAF1 with Aβ monomer (Figure 7e). Indeed, TIAF1 constantly bound a basal level of Aβ monomer in COS7, L929 and other cells (data not shown). TGF-β2 increased the binding of TIAF1 with amyloid fibrils in L929 cells during treatment for 24 h (Figure 7f). However, exposure of B16F10 cells to TGF-β1 for 1 h reduced the binding of TIAF1 with amyloid fibrils, whereas the effect was barely visible in HCT116 cells in this short-term experiment (Figure 7g).

**Discussion**

We have discovered for the first time that TIAF1 undergoes self-aggregation in response to TGF-β1. TIAF1 participates in the TGF-β signaling. Knockdown of TIAF1 with siRNA induces spontaneous nuclear accumulation of Smad proteins and activation of promoter governed by the SMAD protein complex. TIAF1 aggregates are expressed in degenerating neurons and form plaques in the hippocampus. Most importantly, TIAF1 aggregates possessing Aβ in the hippocampi are significantly increased in postmortem AD patients (age 71–90; n = 97), compared with younger nondemented controls (age 42 to 76; n = 41), suggesting that formation of TIAF1 aggregation occurs before that of Aβ.

Under physiological conditions, TβRII has a central role in the TGF-β signaling, which involves the TGF-β/Smad2/3/4 signal complex (see the schematic summary; Figure 8). Recently, we discovered that TGF-β1 binds membrane Hyal-2, and the TGF-β1/Hyal-2 complex recruits tumor
suppressor WOX1 and Smad4 for signaling.\textsuperscript{14} We determined here that TGF-\(\beta\)1 signals the binding of TIAF1 with Smad4, and the complex relocates to the nuclei to modulate gene transcription (Figure 8a). Smad4 is critical for supporting the generation of membrane APP via regulation at the gene transcription level.\textsuperscript{21,22} Smad4 prevents TIAF1 self-aggregation. The TGF-\(\beta\)/Smad4/TIAF1 signaling is expected to occur in every normal cell. How TIAF1 affects Smad4 in controlling APP production remains to be established.

TGF-\(\beta\) signaling is probably aberrant under pathological conditions (Figure 8b).\textsuperscript{2–5,23,24} TGF-\(\beta\)1 is crucial in promoting neuroprotection and neurodegeneration.\textsuperscript{24} Long-term overexpression of TGF-\(\beta\)1 in mice causes neurodegeneration.\textsuperscript{24} TIAF1 interacts with Thr668-phosphorylated APP, suggesting that TIAF1 binds and stabilizes membrane APP. TGF-\(\beta\)1 induces TIAF1 aggregation and reduces its binding with APP, and causes APP dephosphorylation. The dephosphorylated APP is subjected to enzymatic cleavage and subsequent production of A\(/\) mono-mer,\textsuperscript{20} AICD and amyloid fibrils. Aggregated TIAF1 binds amyloid fibrils, which facilitates gradual formation of TIAF1/A\(/\) plaques. How AICD is generated because of TIAF1 aggregation remains to be established.\textsuperscript{25} AICD is involved in signaling and differentiation of neurons.\textsuperscript{26} Thus, a balanced status of phosphorylation of APP is critical in determining plaque formation.

Neuroblastoma, melanoma and many other cancer cells are refractory to TGF-\(\beta\)-mediated growth suppression and apoptosis.\textsuperscript{27,28} TGF-\(\beta\)1 and -\(\beta\)2 could rapidly induce APP degradation and production of A\(/\) and amyloid fibrils in breast MCF7 and other cancer cells. A\(/\) appears to be cleared up swiftly in MCF7 cells (data not shown), suggesting a novel mechanism for cancer cells to escape A\(/\) cytotoxicity.

Endogenous TIAF1 is prone to aggregate under stress stimuli, which leads to generation of A\(/\) and amyloid fibrils. UV light, estrogen and lactacystin inhibit TIAF1 expression, or probably induce its degradation. In vitro analyses showed that overexpressed TIAF1 undergoes self-binding, and TGF-\(\beta\)1 enhances the effect. The aggregated TIAF1 appears to cause APP phosphorylation at Thr668, which is located at the C-terminal tail of APP presenting in the cytoplasm. By surface plasmon-enhanced TIRF (total internal reflection fluorescence) microscopy,\textsuperscript{29,30} we have recently determined that complement C1q destabilizes cell adherence by generating dynamic ‘turbulence’ of plasma membrane in tumor suppressor WOX1-expressing cells. It would be of interest to examine TGF-\(\beta\)-induced membrane turbulence for de-phosphorylating APP and generating A\(/\) and amyloid fibrils. Thr668-phosphorylated APP affects the cell metabolism regarding its tendency in generation of A\(/\).\textsuperscript{26,31–33}

Smad4 physically interacts with TIAF1, and the binding prevents TIAF1 aggregation. Indeed, the concentration of Smad4 is critical in determining the strength of its binding with TIAF1. The overexpressed Smad4/TIAF1 complexes tend to remain in the perinuclear area, thereby abolishing the transcriptional function of Smad4. Overexpressed Smad4 alone induces apoptosis of cancer and neuronal cells. Smad4 is involved in the TGF-\(\beta\)-induced transcriptional activation of APP gene.\textsuperscript{21,22} Conceivably, TGF-\(\beta\)-induced TIAF1 aggregation and Smad4-mediated APP gene activation occur concurrently (Figure 8), and that aggregated TIAF1 binds Smad4 to prevent nuclear relocation.

In summary, protein aggregation has a critical role in the pathogenesis of neurodegeneration.\textsuperscript{33–35} Apparently, TIAF1 aggregation occurs preceding generation of A\(/\) and amyloid fibrils.
fibrils, and the TIAF1/amyloid fibril aggregates facilitate plaque formation.

Materials and Methods
Cell lines, postmortem human hippocampi, brain tissue sections, antibodies, immunohistochemistry, fluorescence immunostaining and confocal microscopy. Cell lines used were human neuroblastoma SH-SY5Y and SK-N-SH cells, lung non-small cell lung cancer NCI-H1299 cells, breast cancer MCF7 and MDA-MB-231 cells, colon cancer HCT116 cells, prostate DU145 cells, teratocarcinoma NT2D1 cells, and murine L929 fibroblasts (American Type Cell Collections). Recombinant TGF-β1 was from PeproTech (Rocky Hill, NJ, USA). Antibodies against TIAF1 were either homegrown37,38 or from Abcam. Aβ antibodies were from (1) AbD-Serotec (MCA2172; Oxford, UK), (2) Covance (BE10; Princeton, NJ, USA) and (3) Santa Cruz Biotechnologies (H43; Santa Cruz, CA, USA). Prefibrillar oligomer Aβ antibody39 was from Invitrogen (Carlsbad, CA, USA). Amyloid fibril-specific antibody (AO),36 APP antibody (MAb348) and Fluoro-Jade C Red were from Chemicon/Millipore. Antibody against phospho-APP at Thr668 was from Cell Signaling Technology (Danvers, MA, USA), AICD antibody (SIG-39152) was from Covance. Aβ(1–42)40 and Aβ(1–40) were synthesized by AnaSpec (Fremont, CA, USA). Human recombinant TGF-β1 (DNase I buffer (20 mM Tris-HCl, pH 8.0, 15 mM MgCl₂, 1 mg/ml DNase I), and then incubated for 1 h at 37 °C. Protein preparations were quantified by BCA assay kit, Pierce, Waltham, MA, USA). Each sample, containing 10, 30 and 60 μg protein, respectively, was diluted in 100 μl sample buffer (1% SDS, 8% β-mercaptoethanol in PBS), boiled for 10 min at 95 °C, and then filtered through 0.2 μm cellulose acetate membranes using a dot-blot apparatus (Bio-dot apparatus, Bio-Rad, Hercules, CA, USA). Each well was washed by 200 μl 0.1% SDS twice. Then, the membranes were analyzed by standard western blotting to determine the presence of Aβ1-42 and TIAF1.

cDNA expression constructs and Cytotrap yeast two-hybrid analysis. TIAF1 cDNA was tagged in-frame with EGFP, ECFP or EYFP (in cDNA expression constructs and Cytotrap yeast two-hybrid constructs were used for cell experiments as specified. To identify TIAF1-binding TE2000-U; Tokyo, Japan) and confocal microscopy (Olympus (Tokyo, Japan) and appropriate controls, was performed using the CytoTrap system.

Epi-fluorescence, time-lapse, confocal microscopy, FRET and cell cycle analysis. Epi-fluorescence, time-lapse microscopy (Nikon Eclipse TE2000-U; Tokyo, Japan) and confocal microscopy (Olympus (Tokyo, Japan) and Zeiss (Hamburg, Germany)) were performed to test the effect of TGF-β1 on protein aggregation and apoptosis. Time-lapse FRET analysis was performed for TIAF1 self-binding and its interaction with Smad.15,23,30 The FRET images were corrected for background fluorescence from an area free of cells. The spectrally corrected FRETc was calculated using a software program (Image-Pro Plus 6.1) by Youvan’s equation (Image-Pro 6.1, Media Cybernetics, Bethesda, MD, USA).15,23,30 When indicated, cellular transfection with the TIAF1 constructs and cultured for 48 h, followed by determining the extent of apoptosis and growth suppression by cell cycle analysis using a fluorescence-activated cell sorting/flow cytometry machine (BD, Sparks, MD, USA), as described.3,12

Conflict of interest
The authors declare no conflict of interest.

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