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Expression of AHI1 Rescues Amyloidogenic Pathology in Alzheimer's Disease Model Cells

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Abstract

A hallmark of Alzheimer's disease (AD) pathogenesis is the accumulation of extracellular plaques mainly composed of amyloid-β (Aβ) derived from amyloid precursor protein (APP) cleavage. Recent reports suggest that transport of APP in vesicles with huntingtin-associated protein-1 (HAP1) negatively regulates Aβ production. In neurons, HAP1 forms a stable complex with Abelson helper integration site-1 (AHI1), in which mutations cause neurodevelopmental and psychiatric disorders. HAP1 and AHI1 interact with tropomyosin receptor kinases (Trks), which are also associated with APP and mediate neurotrophic signaling. In this study, we hypothesize that AHI1 participates in APP trafficking and processing to rescue AD pathology. Indeed, AHI1 was significantly reduced in mouse neuroblastoma N2a cells expressing human Swedish and Indiana APP (designed as AD model cells) and in 3xTg-AD mouse brain. The AD model cells as well as *Ahi1*-knockdown cells expressing wild-type APP-695 exhibited a significant reduction in viability. In addition, the AD model cells were reduced in neurite outgrowth. APP C-terminal fragment-β (CTFβ) and Aβ42 were increased in the AD cell lysates and the culture media, respectively. To investigate the mechanism how AHI1 alters APP activities, we overexpressed human AHI1 in the AD model cells. The results showed that AHI1 interacted with APP physically in mouse brain and transfected N2a cells despite APP genotypes. AHI1 expression facilitated intracellular translocation of APP and inhibited APP amyloidogenic process to reduce the level of APP-CTFβ in the total lysates of AD model cells as well as Aβ in the culture media. Consequently, AHI1–APP interactions enhanced neurotrophic signaling through Erk activation and led to restored cell survival and differentiation.

Keywords Alzheimer's disease (AD) · Abelson helper integration site-1 (AHI1) · Amyloid precursor protein (APP) · Amyloid-β (Aβ) · Huntingtin-associated protein-1 (HAP1) · Extracellular signal-regulated protein kinase (Erk)

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Abbreviations

Aβ	Amyloid-β
AD	Alzheimer's disease
AHI1	Abelson helper integration site-1
APP	Amyloid precursor protein
APP-Swe/Ind	Swedish and Indiana APP
CTF	C-Terminal fragment
ELISA	Enzyme-linked immunosorbent assay
Erk	Extracellular signal-regulated protein kinase
GFP	Green fluorescent protein
HAP1	Huntingtin-associated protein-1
PSEN	Presenilin
sAPPα	Soluble APPα
Trk	Tropomyosin receptor kinase

Introduction

Neurodegenerative disorders accompanying dementia are characterized by progressive neuropsychiatric dysfunction and loss of specific subtypes of neuron. Alzheimer's disease (AD) is the most common form of dementia worldwide. As the disease progresses, AD patients suffer long-term memory impairment, psychiatric disturbance, and gradual loss of physical function, eventually leading to death [1]. There are still no disease-specific treatments approved for AD, and there remains a need to identify optimal assessment tools or biomarkers to maximize the information acquired from clinical trials [2].

Progressive neuronal loss found in AD can result from the accumulation of extracellular amyloid plaques and intracellular neurofibrillary tangles. Additionally, advanced glycation end products can contribute to oxidative stress and autoimmunity in AD [3]. The extracellular plaques comprise amyloid-β (Aβ) generated through sequential proteolytic cleavage of amyloid precursor protein (APP) by secretases [4]. The α-secretase controls the non-amyloidogenic production of APP fragments, including the soluble APPα (sAPPα) and the 83-residue C-terminal fragment (CTFα or C83) within the plasma membrane. Mutation at certain sites of APP or other amyloidogenic activities may increase its cleavage by β-secretase (or BACE1), producing a 99-residue C-terminal fragment (CTFβ or C99) within the membrane. Sequential cleavage by γ-secretase, having presenilins (PSENs) in the core, generates Aβ peptides of 39–43 amino acids that can misfold and form extracellular plaques, as well as intracellular oligomers [5, 6]. APP is a type I transmembrane receptor-like protein. It plays a role in promoting neurite outgrowth, which is a critical aspect of neuronal maturation, plasticity, and regeneration [7]. Three major variants of APP with 695, 751, and 770 residues are generated as a result of alternative mRNA splicing [8]. In the brain, APP-695 is expressed at a relatively higher level than the other variants. Most missense mutations of APP are pathogenic, resulting in early-onset AD [9]. Examples were reported as Swedish double mutation K670N/M671L (K596N/M597L on APP-695) and Indiana mutation V717F (V642F on APP-695) [6, 10]. Swedish mutation is near the β-secretase cleavage site on APP and causes enhanced Aβ formation. Indiana mutation is near the γ-secretase cleavage site, causing increased production and secretion of toxic Aβ42 [11]. These mutations have been developed as cellular AD models [6, 12, 13]. Following endocytosis, APP is delivered in endosomes and recycled to the cell surface or Golgi apparatus [14]. Abnormal transport of misfolded proteins found in the plaques is apparent in the pathogenesis.

Neurons are highly polarized cells and rely on microtubule-dependent transport machinery for survival and development. Mounting evidence shows that mutations of proteins involved in microtubule-dependent transport machinery induce neurodegeneration [15]. An early pathological sign of AD is the appearance of neurite swellings containing the microtubule-binding motor proteins kinesin and dynein/dynactin [16]. In addition, microtubule-dependent transport of endosomes and vesicles containing APP and tropomyosin receptor kinases (Trks) is inhibited in AD mouse models [17]. Recent study suggests that APP interacts with NGF receptor TrkA and mediates neuronal survival and differentiation through neurotrophic signaling including the activation of extracellular signal-regulated protein kinase (Erk) [18]. Activation of Erk has been related to sAPPα secretion and decreased Aβ production [19]. The transport of APP and Trks involves huntingtin-associated protein-1 (HAP1) which downregulates Aβ production in neurons [20–22]. Found predominantly in neurons, HAP1 was the first identified huntingtin-interacting protein, implying its role in neurodegenerative pathology [23]. HAP1 regulates neurogenesis through Trk sorting [24]. In mice, deletion of *Hap1* leads to early postnatal death; however, postnatal loss of *Hap1* causes depression [25, 26]. In neurons, HAP1 forms a stable complex with Abelson helper integration site-1 (AHI1 or Jouberin), in which mutations are associated with abnormal neural development and psychiatric disorders [27–31]. The AHI1–HAP1 complex is required for vesicle transport and signaling of tropomyosin receptor kinase B (TrkB), as elimination of either AHI1 or HAP1 decreases levels of TrkB, p-TrkB, and p-Erk in neurons and causes neurodevelopmental defects and psychiatric phenotypes in mice [29, 31].

In the present study, we investigated the levels of AHI1 and HAP1 in AD model cells expressing Swedish and Indiana APP (APP-Swe/Ind) in comparison with control cells expressing APP-695. We hypothesize that AHI1 plays a key role participating in APP transport and processing.

Materials and Methods

AD Model Cells The AD model cell line was established as described previously [13]. N2a mouse neuroblastoma cells

(ATCC, CCL-131) were transfected with the plasmid pCAX vector, pCAX-APP-695 bearing human wild-type APP variant 695, or pCAX-APP-Swe/Ind bearing the mutated Swedish and Indiana APP on the variant 695 (K595N/M596L/V642F) (Addgene) [32] and pEGFP-C3 or pEG-HA-AHI1 bearing the HA-tagged full-length human AHI1 using Lipofectamine 2000 (Invitrogen). Cells were maintained in minimum essential medium (Eagle) with 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1.5 g/L sodium bicarbonate, and 1.0 mM sodium pyruvate (Life Technologies) and supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies), 100 units/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL amphotericin B (Life Technologies). Cultures were incubated at 37 °C with 5% CO_2 in a humidified incubator.

Mice Wild-type (C57BL/6) and 3xTg-AD mice (B6; 129-Psen1tm1Mpm Tg (APPSwe, TauP301L) 1Lfa/Mmjax)) expressing three mutant genes, Psen1-M146V, APP-K670N-M671L (APP-Swe), and Tau-P301L, were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). All animal procedures were approved by the Institutional Animal Care and Use Committee in Taipei Medical University (LAC-2015-0191 and LAC-2017-0379).

Western Blot Analysis Total protein concentration was determined using Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Inc.). Protein samples at 50 μg were diluted in loading buffer with protease inhibitor cocktail in PBS buffer. Proteins were separated by 10% or 10–18% SDS-PAGE and transferred onto nitrocellulose or PVDF membrane. After blocking in 5% nonfat milk PBS, the membrane was incubated with an antibody against HAP1 (Santa Cruz), AHI1 (Abnova), C-terminal APP (A8717, Sigma-Aldrich), Erk1/2 (Cell Signaling), p-Erk1/2 (Thr202/Tyr204) (Cell Signaling), α-tubulin (Cell Signaling), or β-actin (GeneTex) in 3% BSA/PBS at 4 °C overnight. After sufficient washing, the membrane was incubated with an HRP-conjugated secondary antibody at 25 °C for 1 h. The membrane was then washed and developed with chemiluminescent substrate (Thermo Scientific) in a luminescence/fluorescence imaging system (ImageQuant Las-4000). Quantitative analysis was done using Un-Scan-It 6.1 (Silk Scientific Crop.).

Cell Viability Assay The viability of N2a cells was determined using cell counting kit-8 (Life sciences) or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded in 96-well plates at a density of 5×10^4 cells/ml and allowed to grow for 24 h. Afterward, cells were transfected with plasmids and treated with 50 μM U0126 (Sigma), an Erk kinase inhibitor, as indicated, and cultured for another 24 h, 48 h, or 72 h. Cells were incubated with the kit reagent for 1–4 h. The absorbance at 450 or 570 nm was measured using the plate reading spectrophotometer Synerge HT (Biotech).

Immunofluorescent Microscopy After 48 h of transfection, N2A cells were fixed with 4% paraformaldehyde. After sufficient washing, the cells were blocked with 5% bovine serum albumin in PBST (0.1% Triton X-100 in PBS) at 4 °C for 1 h. Cells were then labeled with APP or HA antibody at 4 °C overnight and Dylight 594- or 488-conjugated secondary antibody at 4 °C for 1 h. The cells transfected with the vector pEGFP-C3 expressed green fluorescent protein (GFP) and were used to be the control for HA-tagged AHI1. To calculate the percentage of cells with neurite outgrowth, images were processed using Nikon eclipse te200-U microscopy system. The percentage of differentiated cells with neurites longer than two folds of their cell bodies was calculated. High-quality microscopic images were processed using GE Healthcare DeltaVision Personal Deconvolution Microscope in the core facility center at Taipei Medical University. Images of co-localized area were generated using Image J 1.48v (Wayne Rasband, NIH, USA), and fluorescence densities were quantified using Un-Scan-It 6.1. In order to minimize the variation of fluorescent densities among different cell images, APP density in neurite was normalized with total density per cell.

Co-immunoprecipitation A wild-type mouse (C57BL/6) whole brain was homogenized in 20 mM HEPES containing 100 μg/ml PMSF and 1× protease inhibitors (Merck Millipore). N2a cells were harvested 24 h after transfection and homogenized in 1X PBS, 0.1% Triton X100, 100 μg/ml PMSF, and 1× protease inhibitors (Merck Millipore). The homogenate was centrifuged at 15,000 rpm for 15 min at 4 °C. Antibody against APP or AHI1 was added to protein-G or protein-A magnetic beads (Recenttec) and incubated for 2 h at 4 °C to immobilize the antibody. The centrifuged supernatant was incubated with immobilized antibody with continuous mixing at 4 °C overnight. The immunoprecipitates were washed five times with the same buffer and then eluted with 100 mM glycine-HCl, pH 2. The proteins were resolved in SDS-PAGE and transferred onto PVDF membrane (BioRad) for Western blotting.

ELISA The concentration of Aβ1-42 in cell culture media after 48 h of transfection was measured using commercial ELISA kit (Elabscience). Standards or samples were added into the antibody-precoated wells on a microtiter plate and incubated with conjugate at 37 °C for 1 h. After washed and dried completely, each well was incubated with substrates at 25 °C for 15 min. Absorbance was measured through the microplate reader Synerge HT. The concentrations were determined with standard reduction.

Statistical Analysis Results were expressed as mean ± standard error and analyzed through Student's *t* test and one-way ANOVA using SigmaPlot 12.5 (Systat Software Inc.). Values of $p < 0.05$ (*), $p < 0.01$ (**), or $p < 0.001$ (***) were considered statistically significant.

Results

Reduction of AHI1 in AD Models

To study AD pathology in cells, we transfected N2a cells with APP plasmids. In these cells, APP can be cut by α- and β-secretases to generate 83- and 99-residue membrane-associated APP-CTFα and APP-CTFβ, respectively [4]. Further cleavage of APP-CTFβ by γ-secretase releases a ~4-kDa amyloidogenic Aβ product. Through Western blotting, we found that overexpression of APP-695 increased the production of APP-CTFs especially CTFα, while overexpression of APP-Swe/Ind significantly increased APP-CTFβ, supporting a pathologic process of APP-Swe/Ind in the cells (Fig. 1a). Along with full-length APP (APP-FL) expression, endogenous mouse AHI1 and HAP1 expression also increased within the cells. Strikingly AHI1 and HAP1 proteins decreased in APP-Swe/Ind-overexpressed cells with statistical significance for AHI1. This reduction was not a result of gene regulation, since *Ahi1* mRNA level was not changed in the AD model cells (Fig. S1). The findings in 3xTg-AD mice expressing three mutant genes, PSEN1-M146V, APP-K670N-M671L (APP-Swe), and Tau-P301L (Fig. S2) were consistent. AHI1 was reduced in the brain of 3xTg-AD mice compared to wild-type mice with a significant difference in cerebral cortex, hippocampus, and other brain areas (Fig. 1b). Knocking down *Ahi1* increased APP-CTFs in APP-overexpressing N2a cells (Fig. S3A) and reduced cell viability with a significant difference in cells expressing APP-695 (Fig. S3B). These results support a notion that AHI1 has roles in APP activities within established AD models.

Neuronal Protection by AHI1 Expression

To test the effects of AHI1 in AD model cells, we expressed human AHI1 along with either human APP-695 or APP-Swe/Ind in N2a cells and monitored their viabilities and neurite outgrowth. The viability of cells expressing either APP-695 or APP-Swe/Ind was slightly reduced after 24 and 48 h (Fig. 2a). After 72 h, the viability of cells expressing APP-695 remained similar to that of 24 and 48 h. In comparison, after 72 h, the viability of AD model cells expressing APP-Swe/Ind was significantly reduced. When co-expressing AHI1, the AD model cell viability was restored significantly at 72 h, suggesting a protective role of AHI1 on AD pathology.

The capacity of AHI1 and APP in promoting neurite outgrowth was examined in immunofluorescent microscopy. As expected, the cells expressing AHI1, APP-695, or both displayed a higher percentage of neurite outgrowth than control cells expressing GFP and AD model cells expressing APP-Swe/Ind (Fig. 2b). Strikingly, when co-expressing AHI1, the AD model cells restored the capacity to neurite outgrowth. Note that the control cells transfected with vectors (expressing GFP) had some activities of neurite outgrowth, which may be partially due to the basal levels of endogenous APP and AHI1 (Fig. 1a). In addition, a portion of the cells was not transfected so that the effects of the expressed proteins could be greater than those shown here. These results demonstrate that AHI1 expression may overcome AD pathology in the cells.

Inhibition of Amyloidogenic Processing by AHI1 Expression

Amyloidogenic APP processing and Aβ generation are key events in AD pathogenesis. N2a cells co-expressing AHI1 with APP-695 or APP-Swe/Ind were analyzed in Western blotting and ELISA to determine the effects of AHI1 expression on the production of APP-CTFβ in the cell lysates, as well as the production of Aβ in the culture media. APP-CTFα was present abundantly in the cells expressing either APP-695 or APP-Swe/Ind despite AHI1 expression (Fig. 3a). In contrast, APP-CTFβ was present at high levels in the cells expressing APP-Swe/Ind, and it was noticeably reduced in AHI1-expressing cells. These results suggest an inhibitory effect of AHI1 on the β-secretase cleavage of APP. Measurement of Aβ in the culture media confirmed the inhibition of amyloidogenic APP processing by AHI1 (Fig. 3b).

Interactions of APP with AHI1

To obtain the insights of interactions between AHI1 and APP, we performed co-immunoprecipitation and immunofluorescent microscopy. Using the antibody against either AHI1 or APP, we found that these two proteins precipitated each other from wild-type mouse brain lysates (Fig. 4a). Their interactions did not seem affected by Swedish and Indianan mutations in APP as both APP-695 and APP-Swe/Ind precipitated similar amount of AHI1 protein in N2a cells co-expressing AHI1 with either APP-695 or APP-Swe/Ind (Fig. 4b).

APP and AHI1 were both labeled in N2a cells to monitor their intracellular locations. In agreement with the results in Fig. 2b, APP-695 promoted neurite outgrowth of the cells while APP-Swe/Ind did not (Fig. 5a). As expected, cells co-expressing AHI1 and APP-Swe/Ind were restored in neurite

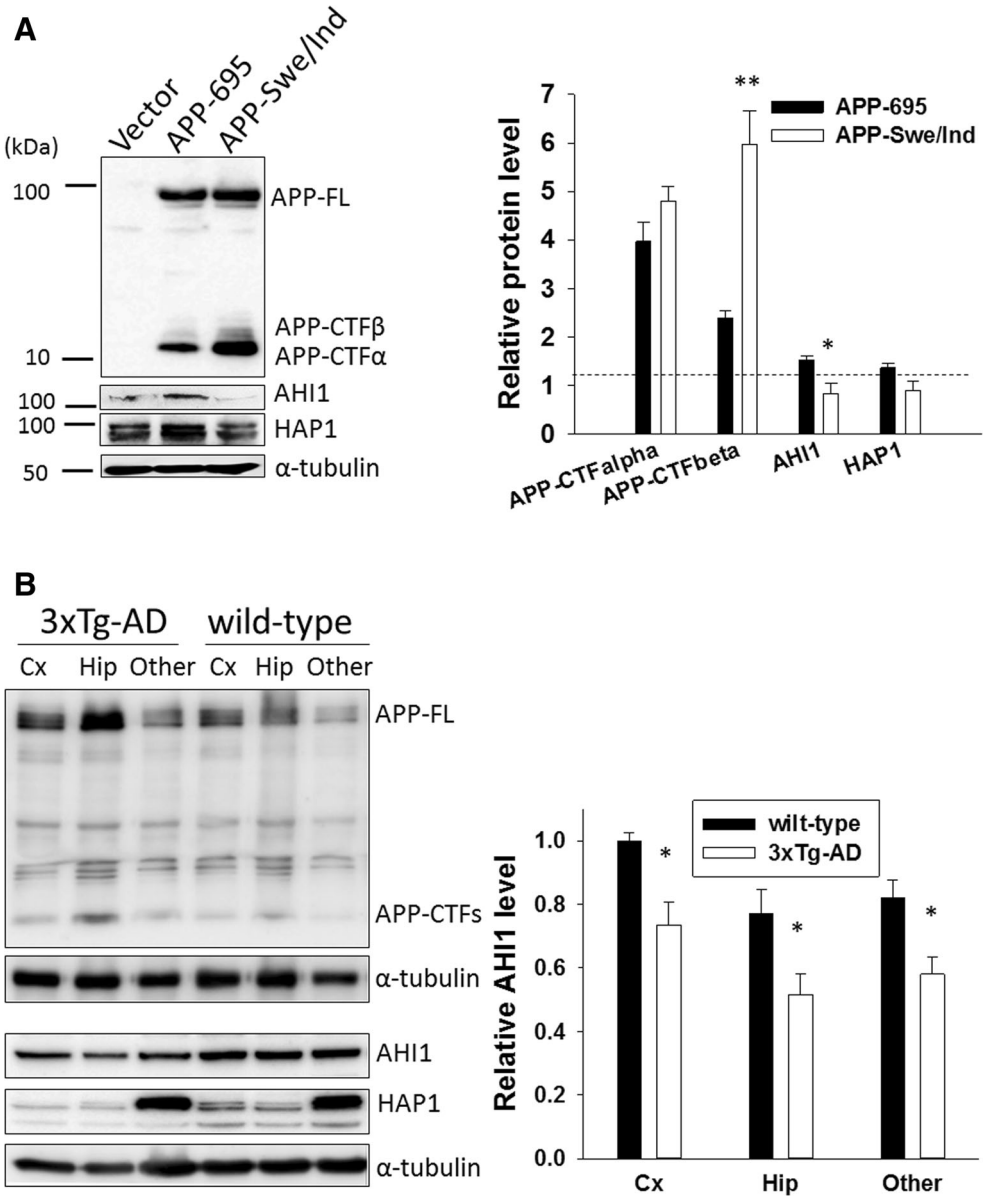

Fig. 1 AHI1 and related protein levels in AD models. The lysates from cells or brain tissues were analyzed using Western blotting. The full-length APP (APP-FL) and APP C-terminal fragments (APP-CTFα and APP-CTFβ), as well as mouse AHI1, HAP1, and α-tubulin were labeled. **a** N2a cells transfected with pCAX vector, pCAX-APP-695, or pCAX-APP-Swe/Ind. The band densities were quantified and normalized with those from the cells transfected with the vector. Three independent experiments were performed. **b** Brain tissues from age-matched 3xTg-AD and wild-type mice (6 months old or older). AHI1 densities were normalized with α-tubulin, and the value of wild-type Cx was set as one. Four sets of mouse brain were analyzed. Cx cerebral cortex, Hip hippocampus. $^{*}p < 0.05$, $^{**}p < 0.01$

outgrowth capacity comparing to the cells co-expressing GFP and APP-Swe/Ind. In the merged images, AHI1 was co-localized with both APP-695 and APP-Swe/Ind in the soma and neurites, suggesting that AHI1 may participate in intracellular APP trafficking. The fluorescent densities of APP, AHI1 and their co-localization area were quantified. The co-localization ratio of AHI1 with APP-695 was similar to that with APP-Swe/Ind (Fig. 5b), which is consistent with the results in co-immunoprecipitation (Fig. 4b). Strikingly, AHI1 expression enhanced average APP density in neurites, suggesting that AHI1 may facilitate intracellular APP translocation (Fig. 5c). The cells co-expressing APP-Swe/Ind and GFP developed very few neurites and were not analyzed for APP density in neurites. These results support our hypothesis that AHI1 participates in APP translocation physically, and enhancing AHI1 expression may improve both familial and sporadic AD conditions.

Activation of the Neurotrophic Pathway by AHI1

Neurotrophic activities of TrkA and TrkB are associated with APP and AHI1, respectively [31, 33]. It is very possible that changes to APP processing by AHI1 will affect neurotrophic signaling through Erk activation. As expected, expression of AHI1 or APP-695 in N2a cells enhanced Erk1/2 phosphorylation with statistical significance (Fig. 6a). Co-expression of AHI1 and APP-695 seemed to reduce Erk1/2 activation probably due to low expression of both proteins. AD model cells expressing APP-Swe/Ind were reduced in Erk1/2 phosphorylation compared to those expressing APP-695. Strikingly, in the AD model cells, AHI1 expression restored Erk1/2 phosphorylation significantly. Note that the endogenous level of AHI1 in N2a cells was very low so that a slight

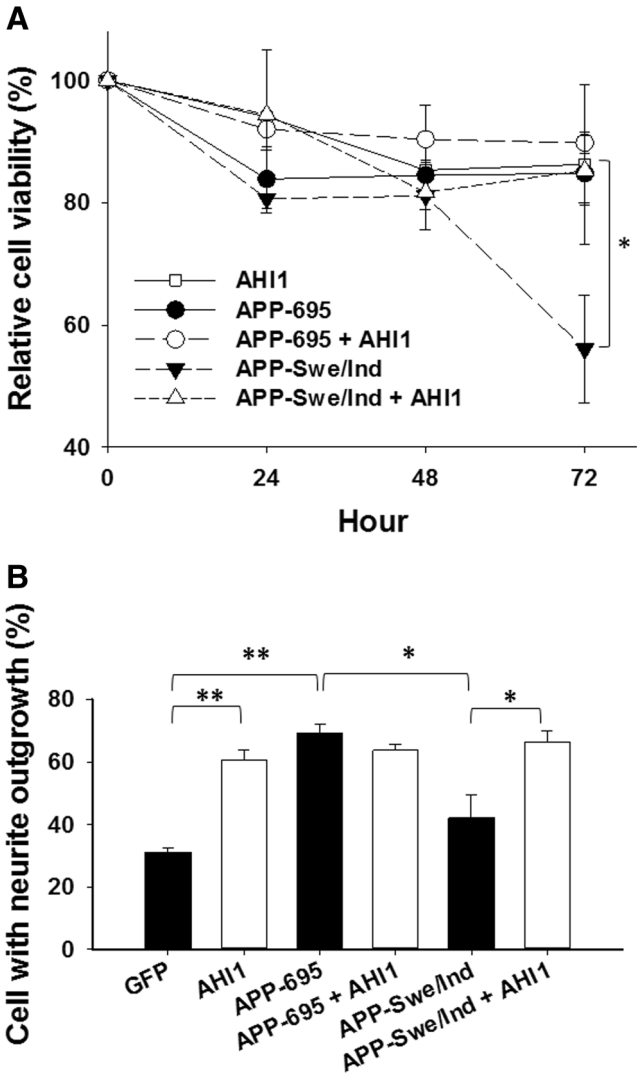

Fig. 2 Effects of AHI1 expression on AD model cell viability and differentiation. N2a cells were co-transfected with pCAX vector, pCAX-APP-695 or pCAX-APP-Swe/Ind, and pEGFP-C3 or pEG-HA-AHI1 for 24, 48, or 72 h. **a** The cell viability was determined and normalized with that of cells transfected with the vectors. **b** Images from fluorescent microscopy were used to calculate the percentage of differentiated cells with neurite outgrowth. At least, three independent experiments were performed. $*p < 0.05$; $**p < 0.01$

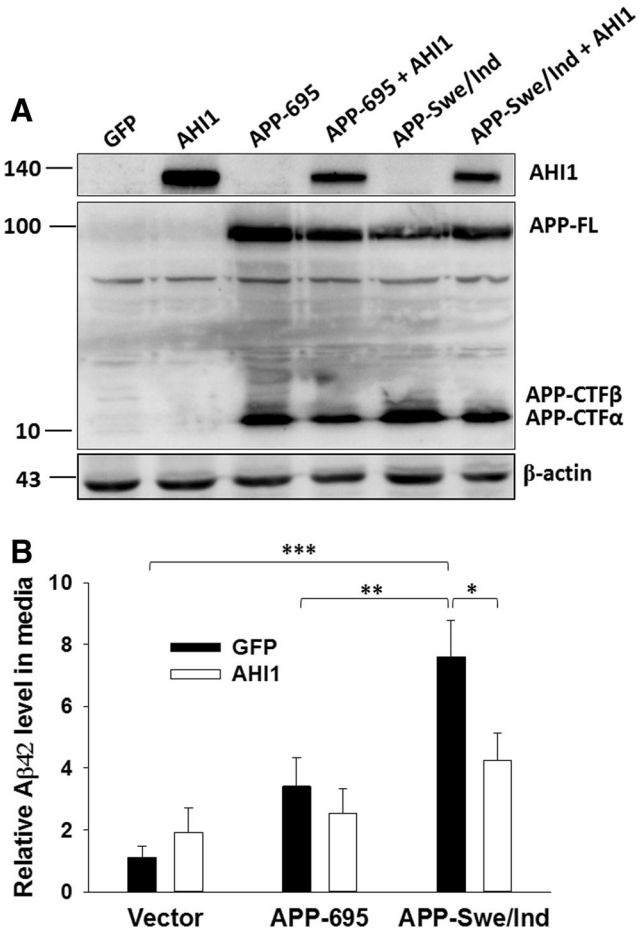

Fig. 3 Effect of AHI1 expression on APP processing. N2a cells were co-transfected with pCAX vector, pCAX-APP-695, or pCAX-APP-Swe/Ind and pEG-HA-AHI1 or pEGFP-C3 for 48 h. **a** APP-CTFs in cell lysates. The total lysates from cells were analyzed by Western blotting with antibodies against AHI1, C-terminal APP, or β-actin. **b** Aβ production. The culture media were collected. The concentrations of Aβ42 were measured and normalized with that produced from the cells transfected with the vectors. Three independent experiments were performed. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$

variation in sample loading or unevenly developed blots can mask the results. However, overexpression overcame this issue.

To verify the involvement of Erk pathway in AHI1 function, AHI1-expressing cells were treated with the Erk kinase inhibitor U0126 in MTT assay. Indeed, those treated cells were reduced in viability at 72 h with statistical significance (Fig. 6b). The protective effect of AHI1 expression on AD model cell viability was interrupted when Erk pathway was inhibited. These results suggest that APP-Swe/Ind may intervene its interaction with and activation of Trks to reduce phosphorylation of Erk. A high level of AHI1 may recover APP-Swe/Ind and rescues neurotrophic activities including neuronal survival and differentiation through Erk activation.

Discussion

AHI1 protein level was significantly reduced in the AD model cells and mice (Fig. 1), suggesting that AHI1 may be abnormally degraded in AD cells. To investigate the mechanisms how AHI1 could potentially alter the APP activities, we overexpressed AHI1 in the AD model cells. The results indicate that AHI1 interact with APP physically despite APP genotypes (Fig. 4). Increasing AHI1 expression enhanced intracellular density of APP in the neurites (Fig. 5) and decreased the level of APP-CTFβ in the total lysates of AD model cells and Aβ in the culture media (Fig. 3). It enhanced the AD cell survival signaling through Erk activation (Fig. 6) and led to restored cell viability and differentiation (Fig. 2). These results support the role of AHI1 in inhibiting AD pathogenesis.

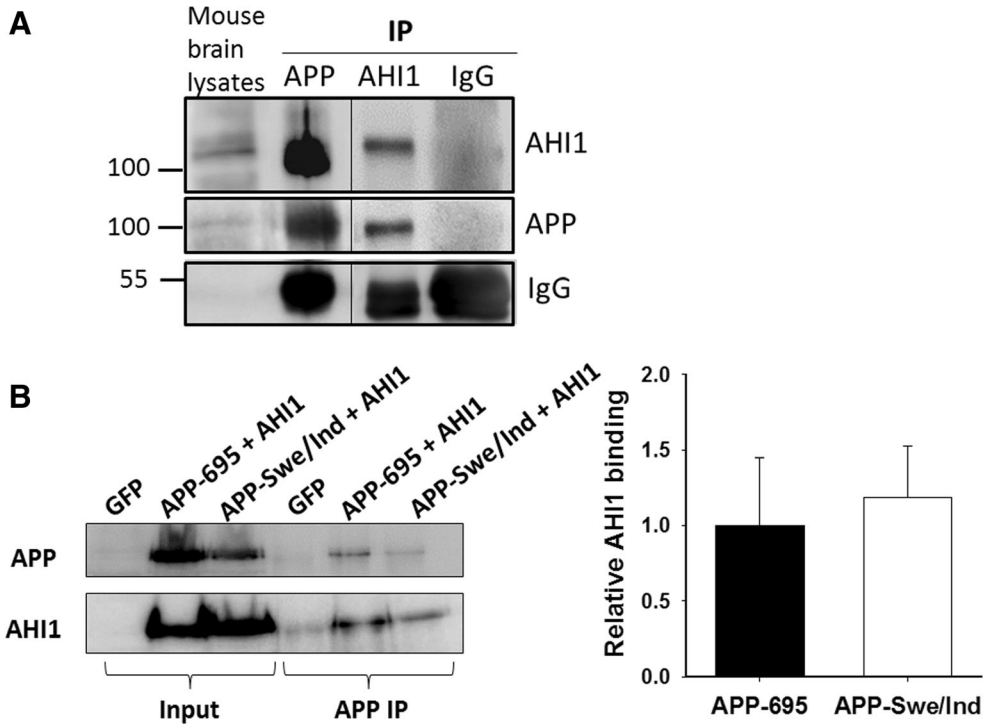

Fig. 4 Physical interactions of AHI1 and APP. **a** Co-immunoprecipitation from mouse brain. Rabbit antibody against mouse APP or AHI1 was used in immunoprecipitation. Normal rabbit IgG was used as a negative control. The co-precipitated proteins were then analyzed by Western blotting using the same antibodies. **b** Co-immunoprecipitation from transfected N2a cells. N2a cells were co-transfected with pEG-HA-AHI1 and pCAX-APP-695 or pCAX-APP-Swe/Ind. Cells transfected with pCAX vector were used as a control. After 48 h of transfection, the cells were homogenized. The lysates were incubated with APP antibody for immunoprecipitation. The co-precipitated proteins were then analyzed by Western blotting using AHI1 or APP antibody. The band densities were quantified and normalized. The ratio of precipitated AHI1 to APP-695 was set as one. Three independent experiments were performed

Most AD patients can develop neuropsychiatric symptoms, such as depression, at any stage of the disease. These symptoms become more prevalent throughout disease progression, primarily as a result of the increasing severity of neurodegeneration, dementia, and neuroinflammation [34]. Therefore, depressive symptoms are an important focus for treatment of AD. A better understanding of the etiopathogenesis of these symptoms in AD will improve development of reliable treatment strategies. Recent studies suggest that the AHI1 gene is associated with susceptibility to schizophrenia, a major neuropsychiatric disorder associated with depression [35]. Supporting evidence also demonstrates that neuronal AHI1 deficiency reduces TrkB level and neurotransmitter release in the brain, and AHI1 deficiency leads to development of depressive phenotypes in mice [31, 36, 37]. Our present study further suggests that AD pathology reduces AHI1 level in the model cells and mouse brain areas including cerebral cortex and hippocampus (Fig. 1). Strikingly when we increased AHI1 level in the AD model cells, amyloidogenic processing of APP was inhibited, and the cells were survived and differentiated like normal cells (Figs. 2, 3, and 5).

We propose that APP works with AHI1 in the neurotrophic vesicle transporting complex. APP and HAP1 form a complex in neurons, and HAP1 knockdown increases Aβ levels [20]. The researchers noted that APP-HAP1 interactions may involve other adaptor proteins. In this study, we showed that APP was co-localized and co-precipitated with AHI1 (Figs. 4 and 5), suggesting that AHI1 is a major player in this APP interactome. Elimination of either AHI1 or HAP1 destabilizes many other proteins and causes previously mentioned neuropsychiatric phenotypes. HAP1 and AHI1 are close partners of neurotrophic receptors such as TrkA and TrkB, as well as motor proteins such as kinesin subunits and dynactin [38, 39]. Furthermore, HAP1 and AHI1 closely interact with vesicle transport adaptor proteins required in neuronal development and functions. In AD pathology, on one hand, amyloidogenic activity is increased due to an enhanced physical approximation of APP and β-secretase [40]. Any events that increase amyloidogenic activity may also disturb the interaction between APP and its partners within the complex, as well as accelerate their degradation as shown in this study. AHI1 and HAP1 levels were decreased when amyloidogenic genes were expressed in AD model cells or mice (Fig. 1). On the other hand, defective transcytosis of APP is also a major problem in the pathology [41]. APP-CTFβ disrupts endocytic transport so that APP was decreased in axons in a familial AD

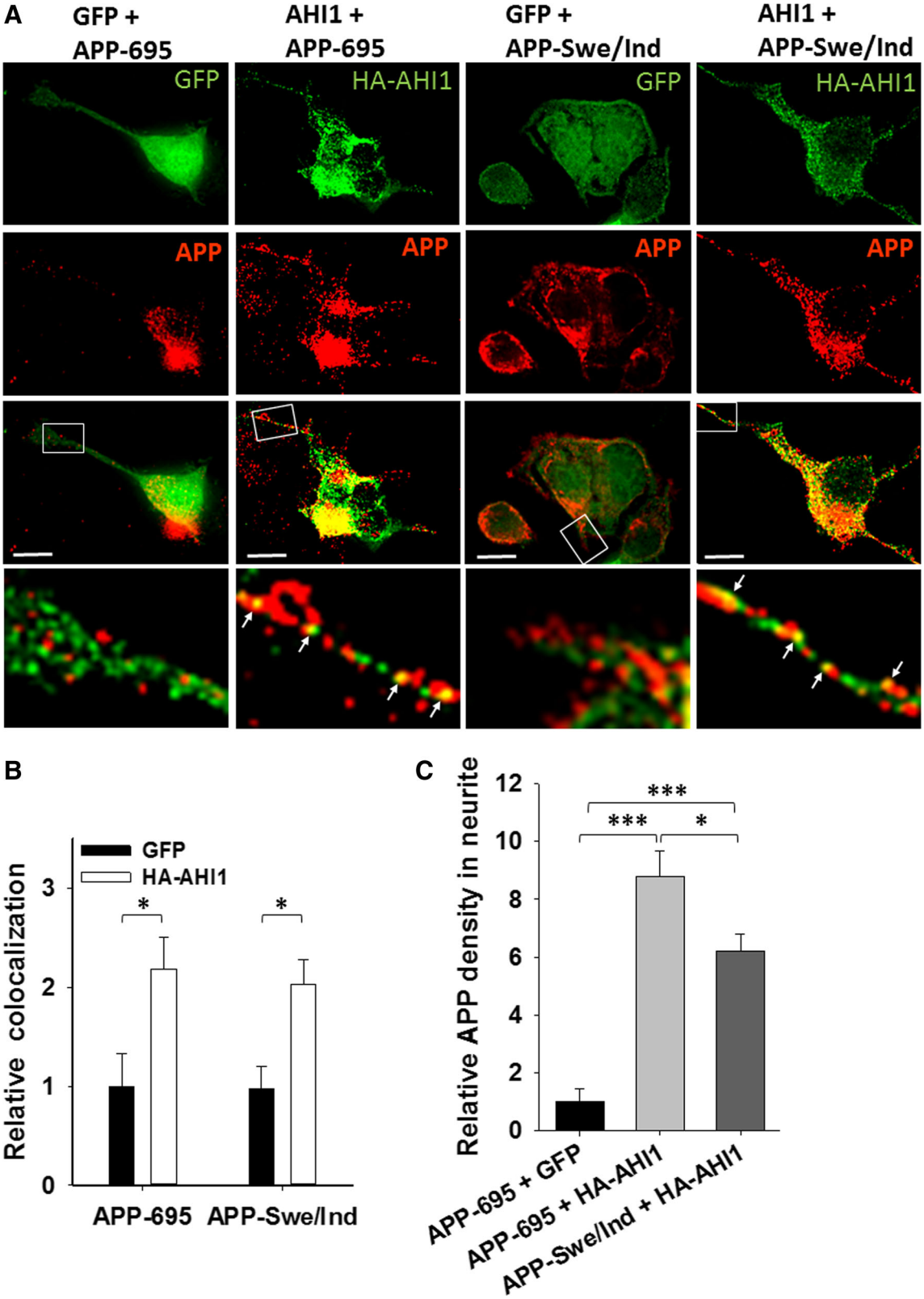

Fig. 5 Localization of APP and AHI1. **a** Deconvolution imaging of N2a cells co-expressing HA-AHI1 (green) or green fluorescent protein (GFP) with APP-695 (red) or APP-Swe/Ind (red). Scale bar, 10 μm. The neurite images framed in rectangle were magnified in the bottom panel. Arrows indicated co-localization of AHI1 and APP. Fluorescence densities were quantified using Un-Scan-It 6.1. **b** Statistical analysis showing the relative co-localization of APP and AHI1, or GFP for control, in the cells. **c** Statistical analysis showing the relative APP density in neurites per unit length per cell. All values were normalized with that of cells expressing APP-695 and GFP. At least three independent sets of image were analyzed. *$p < 0.05$; ***$p < 0.001$

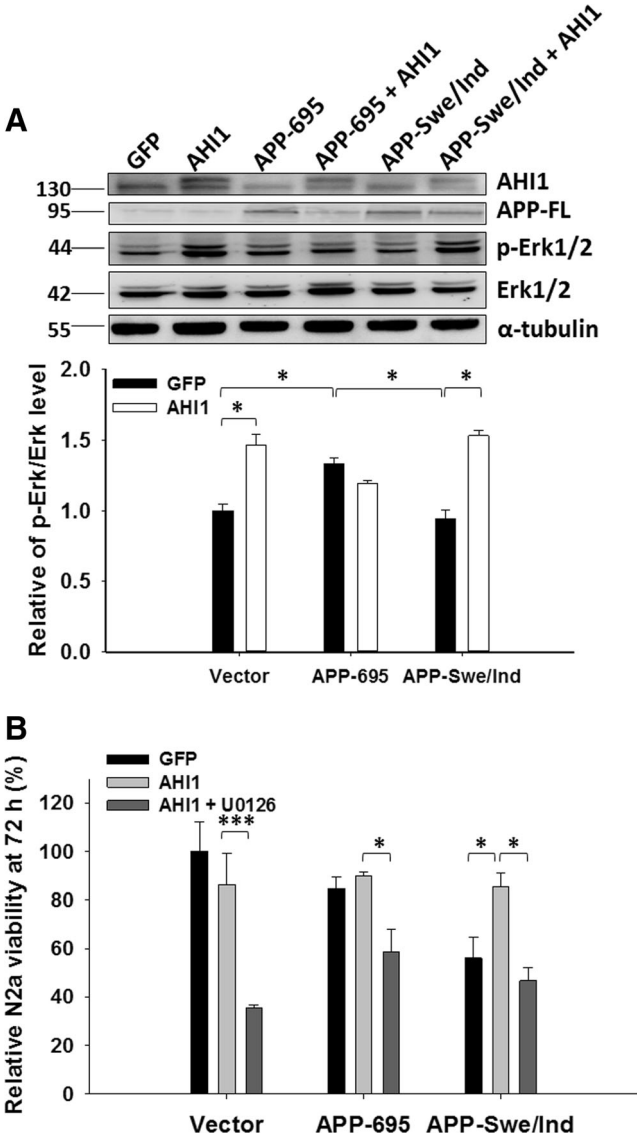

Fig. 6 Activation of Erk with AHI1 expression in AD model cells. N2a cells were co-transfected with pCAX vector, pCAX-APP-695, or pCAX-APP-Swe/Ind and pEGFP-C3 or pEG-HA-AHI1. **a** Western blotting. After 48 h of transfection, the cell lysates were analyzed by Western blotting with antibodies against p-Erk1/2, total Erk1/2, and α-tubulin. Tubulin was used as an internal control. The band densities were quantified. The levels of p-Erk1/2 were divided by total Erk1/2 and normalized with the values of cells transfected with vectors. **b** Cell viability with Erk inhibition. Transfected cells were treated with 50 μM U0126. MTT assay was performed, and the relative cell viabilities at 72 h were shown. Three independent experiments were performed. *$p < 0.05$; ***$p < 0.001$

mouse model expressing mutant PSEN1. In our study, the AD model cells expressing APP-Swe/Ind produced high levels of CTFβ (Fig. 3) that may contribute to the reduction of intracellular level of APP-Swe/Ind and its transport to the neurites. Although the AD model cells developed very few axons and may not be ideal for APP quantification analysis in neurites, overexpression of AHI1 dramatically increased neurite localization of both APP-695 and APP-Swe/Ind, suggesting an important role of AHI1 in APP transport.

Interactions of APP and AHI1 contribute not only in neurite outgrowth but also in other neurotrophic activities such as survival. In our cell viability study, cells expressing APP-695 survived well, while those expressing APP-Swe/Ind were dramatically suffered at 72 h of transfection (Fig. 2a). It is known that APP is cleaved by α-secretase to produce sAPPα in the non-amyloidogenic pathway. This product promotes cell proliferation and protection which can be related to activation of Erk and is implicated as a link to cancer [19]. We also showed that AHI1 enhanced the survival of cells co-expressing APP-Swe/Ind significantly through Erk activation (Fig. 2a and 6). Interestingly, AHI1 is also identified as an oncogene acting through cellular endocytosis in leukemia cells [42]. Finding a way to fine tuning AHI1 expression and APP-related transport machinery is a potential strategy to treat AD. Looking forward, further experiments may be done in order to fill in the gaps to support this hypothesis.

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Study conception and design: LLT, YFL.

Providing critical materials: LLT, HTL, SL, XJL, YMK.

Acquisition of the data: SFY, THN, IST, FYT, YHT, FYC, YFL.

Analysis and interpretation of the data: HTL, SFY, IST, CKL, SHK, YFL.

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Compliance with Ethical Standards

All animal procedures were approved by the Institutional Animal Care and Use Committee in Taipei Medical University (LAC-2015-0191 and LAC-2017-0379).

Conflict of Interest The authors declare that they have no conflict of interest.

References

1. Brookmeyer R, Johnson E, Ziegler-Graham K, Arrighi HM (2007) Forecasting the global burden of Alzheimer's disease. Alzheimers Dement 3(3):186–191. https://doi.org/10.1016/j.jalz.2007.04.381

2. Aisen P, Touchon J, Amariglio R, Andrieu S, Bateman R, Breitner J, Donohue M, Dunn B et al (2017) EU/US/CTAD task force: lessons learned from recent and current Alzheimer's prevention trials. J Prev Alzheimers Dis 4(2):116–124. https://doi.org/10.14283/jpad.2017.13
3. Lin CY, Sheu JJ, Tsai IS, Wang ST, Yang LY, Hsu IU, Chang HW, Lee HM et al (2018) Elevated IgM against nepsilon-(carboxyethyl)lysine-modified apolipoprotein A1 peptide 141-147 in Taiwanese with Alzheimer's disease. Clin Biochem 56:75–82. https://doi.org/10.1016/j.clinbiochem.2018.04.009
4. Wolfe MS (2003) The secretases of Alzheimer's disease. Curr Top Dev Biol 54:233–261
5. Alonso Vilatela ME, Lopez-Lopez M, Yescas-Gomez P (2012) Genetics of Alzheimer's disease. Arch Med Res 43(8):622–631. https://doi.org/10.1016/j.arcmed.2012.10.017
6. Citron M, Oltersdorf T, Haass C, McConlogue L, Hung AY, Seubert P, Vigo-Pelfrey C, Lieberburg I et al (1992) Mutation of the beta-amyloid precursor protein in familial Alzheimer's disease increases beta-protein production. Nature 360(6405):672–674. https://doi.org/10.1038/360672a0
7. Sosa LJ, Caceres A, Dupraz S, Oksdath M, Quiroga S, Lorenzo A (2017) The physiological role of the amyloid precursor protein as an adhesion molecule in the developing nervous system. J Neurochem 143(1):11–29. https://doi.org/10.1111/jnc.14122
8. Nalivaeva NN, Turner AJ (2013) The amyloid precursor protein: a biochemical enigma in brain development, function and disease. FEBS Lett 587(13):2046–2054. https://doi.org/10.1016/j.febslet.2013.05.010
9. Tcw J, Goate AM (2017) Genetics of beta-amyloid precursor protein in Alzheimer's disease. Cold Spring Harb Perspect Med 7(6). https://doi.org/10.1101/cshperspect.a024539
10. Zeldenrust SR, Murrell J, Farlow M, Ghetti B, Roses AD, Benson MD (1993) RFLP analysis for APP 717 mutations associated with Alzheimer's disease. J Med Genet 30(6):476–478
11. Sinha S, Lieberburg I (1999) Cellular mechanisms of beta-amyloid production and secretion. Proc Natl Acad Sci U S A 96(20):11049–11053
12. Cai XD, Golde TE, Younkin SG (1993) Release of excess amyloid beta protein from a mutant amyloid beta protein precursor. Science 259(5094):514–516
13. Tsai YF, Yang DJ, Ngo TH, Shih CH, Wu YF, Lee CK, Phraekanjanavichid V, Yen SF et al (2019) Ganglioside Hp-s1 analogue inhibits amyloidogenic toxicity in Alzheimer's disease model cells. ACS Chem Neurosci 10:528–536. https://doi.org/10.1021/acschemneuro.8b00406
14. Wang X, Huang T, Bu G, Xu H (2014) Dysregulation of protein trafficking in neurodegeneration. Mol Neurodegener 9:31. https://doi.org/10.1186/1750-1326-9-31
15. Salinas S, Bilsland LG, Schiavo G (2008) Molecular landmarks along the axonal route: axonal transport in health and disease. Curr Opin Cell Biol 20(4):445–453. https://doi.org/10.1016/j.ceb.2008.04.002
16. Stokin GB, Lillo C, Falzone TL, Brusch RG, Rockenstein E, Mount SL, Raman R, Davies P et al (2005) Axonopathy and transport deficits early in the pathogenesis of Alzheimer's disease. Science 307(5713):1282–1288. https://doi.org/10.1126/science.1105681
17. Lazarov O, Morfini GA, Pigino G, Gadadhar A, Chen X, Robinson J, Ho H, Brady ST et al (2007) Impairments in fast axonal transport and motor neuron deficits in transgenic mice expressing familial Alzheimer's disease-linked mutant presenilin 1. J Neurosci 27(26):7011–7020. https://doi.org/10.1523/JNEUROSCI.4272-06.2007
18. Zhang YW, Chen Y, Liu Y, Zhao Y, Liao FF, Xu H (2013) APP regulates NGF receptor trafficking and NGF-mediated neuronal differentiation and survival. PLoS One 8(11):e80571. https://doi.org/10.1371/journal.pone.0080571
19. Galvao F Jr, Grokoski KC, da Silva BB, Lamers ML, Siqueira IR (2019) The amyloid precursor protein (APP) processing as a biological link between Alzheimer's disease and cancer. Ageing Res Rev 49:83–91. https://doi.org/10.1016/j.arr.2018.11.007
20. Yang GZ, Yang M, Lim Y, Lu JJ, Wang TH, Qi JG, Zhong JH, Zhou XF (2012) Huntingtin associated protein 1 regulates trafficking of the amyloid precursor protein and modulates amyloid beta levels in neurons. J Neurochem 122(5):1010–1022. https://doi.org/10.1111/j.1471-4159.2012.07845.x
21. Rong J, McGuire JR, Fang ZH, Sheng G, Shin JY, Li SH, Li XJ (2006) Regulation of intracellular trafficking of huntingtin-associated protein-1 is critical for TrkA protein levels and neurite outgrowth. J Neurosci 26(22):6019–6030. https://doi.org/10.1523/JNEUROSCI.1251-06.2006
22. Lim Y, Wu LL, Chen S, Sun Y, Vijayaraj SL, Yang M, Bobrovskaya L, Keating D et al (2018) HAP1 is required for endocytosis and signalling of BDNF and its receptors in neurons. Mol Neurobiol 55(3):1815–1830. https://doi.org/10.1007/s12035-016-0379-0
23. Li XJ, Li SH, Sharp AH, Nucifora FC Jr, Schilling G, Lanahan A, Worley P, Snyder SH et al (1995) A huntingtin-associated protein enriched in brain with implications for pathology. Nature 378(6555):398–402. https://doi.org/10.1038/378398a0
24. Xiang J, Yang H, Zhao T, Sun M, Xu X, Zhou XF, Li SH, Li XJ (2014) Huntingtin-associated protein 1 regulates postnatal neurogenesis and neurotrophin receptor sorting. J Clin Invest 124(1):85–98. https://doi.org/10.1172/JCI69206
25. Xiang J, Yan S, Li SH, Li XJ (2015) Postnatal loss of hap1 reduces hippocampal neurogenesis and causes adult depressive-like behavior in mice. PLoS Genet 11(4):e1005175. https://doi.org/10.1371/journal.pgen.1005175
26. Li SH, Yu ZX, Li CL, Nguyen HP, Zhou YX, Deng C, Li XJ (2003) Lack of huntingtin-associated protein-1 causes neuronal death resembling hypothalamic degeneration in Huntington's disease. J Neurosci 23(17):6956–6964
27. Ferland RJ, Eyaid W, Collura RV, Tully LD, Hill RS, Al-Nouri D, Al-Rumayyan A, Topcu M et al (2004) Abnormal cerebellar development and axonal decussation due to mutations in AHI1 in Joubert syndrome. Nat Genet 36(9):1008–1013. https://doi.org/10.1038/ng1419
28. Lotan A, Lifschytz T, Mernick B, Lory O, Levi E, Ben-Shimol E, Goelman G, Lerer B (2017) Alterations in the expression of a neurodevelopmental gene exert long-lasting effects on cognitive-emotional phenotypes and functional brain networks: translational evidence from the stress-resilient Ahi1 knockout mouse. Mol Psychiatry 22(6):884–899. https://doi.org/10.1038/mp.2016.29
29. Sheng G, Xu X, Lin YF, Wang CE, Rong J, Cheng D, Peng J, Jiang X et al (2008) Huntingtin-associated protein 1 interacts with Ahi1 to regulate cerebellar and brainstem development in mice. J Clin Invest 118(8):2785–2795. https://doi.org/10.1172/JCI35339
30. Weng L, Lin YF, Li AL, Wang CE, Yan S, Sun M, Gaertig MA, Mitha N et al (2013) Loss of Ahi1 affects early development by impairing BM88/Cend1-mediated neuronal differentiation. J Neurosci 33(19):8172–8184. https://doi.org/10.1523/JNEUROSCI.0119-13.2013
31. Xu X, Yang H, Lin YF, Li X, Cape A, Ressler KJ, Li S, Li XJ (2010) Neuronal Abelson helper integration site-1 (Ahi1) deficiency in mice alters TrkB signaling with a depressive phenotype. Proc Natl Acad Sci U S A 107(44):19126–19131. https://doi.org/10.1073/pnas.1013032107
32. Young-Pearse TL, Bai J, Chang R, Zheng JB, LoTurco JJ, Selkoe DJ (2007) A critical function for beta-amyloid precursor protein in neuronal migration revealed by in utero RNA interference. J Neurosci 27(52):14459–14469. https://doi.org/10.1523/JNEUROSCI.4701-07.2007
33. Canu N, Pagano I, La Rosa LR, Pellegrino M, Ciotti MT, Mercanti D, Moretti F, Sposato V et al (2017) Association of TrkA and APP is

promoted by NGF and reduced by cell death-promoting agents. Front Mol Neurosci 10:15. https://doi.org/10.3389/fnmol.2017.00015

34. Rosenberg PB, Nowrangi MA, Lyketsos CG (2015) Neuropsychiatric symptoms in Alzheimer's disease: what might be associated brain circuits? Mol Asp Med 43-44:25–37. https://doi.org/10.1016/j.mam.2015.05.005
35. Amann-Zalcenstein D, Avidan N, Kanyas K, Ebstein RP, Kohn Y, Hamdan A, Ben-Asher E, Karni O et al (2006) AHI1, a pivotal neurodevelopmental gene, and C6orf217 are associated with susceptibility to schizophrenia. Eur J Hum Genet 14(10):1111–1119. https://doi.org/10.1038/sj.ejhg.5201675
36. Ren L, Qian X, Zhai L, Sun M, Miao Z, Li J, Xu X (2014) Loss of Ahi1 impairs neurotransmitter release and causes depressive behaviors in mice. PLoS One 9(4):e93640. https://doi.org/10.1371/journal.pone.0093640
37. Xu Z, Guo X, Yang Y, Tucker D, Lu Y, Xin N, Zhang G, Yang L et al (2017) Low-level laser irradiation improves depression-like behaviors in mice. Mol Neurobiol 54(6):4551–4559. https://doi.org/10.1007/s12035-016-9983-2
38. Wu LL, Zhou XF (2009) Huntingtin associated protein 1 and its functions. Cell Adhes Migr 3(1):71–76
39. Huang PT, Chen CH, Hsu IU, Salim SA, Kao SH, Cheng CW, Lai CH, Lee CF et al (2015) Huntingtin-associated protein 1 interacts with breakpoint cluster region protein to regulate neuronal differentiation. PLoS One 10(2):e0116372. https://doi.org/10.1371/journal.pone.0116372
40. Sun J, Roy S (2018) The physical approximation of APP and BACE-1: a key event in Alzheimer's disease pathogenesis. Dev Neurobiol 78(3):340–347. https://doi.org/10.1002/dneu.22556
41. Woodruff G, Reyna SM, Dunlap M, Van Der Kant R, Callender JA, Young JE, Roberts EA, Goldstein LS (2016) Defective transcytosis of APP and lipoproteins in human iPSC-derived neurons with familial Alzheimer's disease mutations. Cell Rep 17(3):759–773. https://doi.org/10.1016/j.celrep.2016.09.034
42. Liu X, Rothe K, Yen R, Fruhstorfer C, Maetzig T, Chen M, Forrest DL, Humphries RK et al (2017) A novel AHI-1-BCR-ABL-DNM2 complex regulates leukemic properties of primitive CML cells through enhanced cellular endocytosis and ROS-mediated autophagy. Leukemia, **UK** 31(11):2376–2387. https://doi.org/10.1038/leu.2017.108