

A family of membrane proteins associated with presenilin expression and γ -secretase function

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ABSTRACT Presenilin 1 (PS1) forms the γ -secretase complex with at least three components: nicastrin, APH-1, and PEN-2. This complex mediates intramembrane cleavage of amyloid precursor protein (APP) to generate β -amyloid protein (A β) as well as other type 1 transmembrane proteins. Although PS1 mutations linked to familial Alzheimer's disease influence these cleavages, their biological consequences have not been fully understood. In this study, we used mRNA differential display analysis to identify a gene, denoted *adoplin-1/ORMDL-1*, which displays significantly reduced expression in association with PS1 mutations. *Adoplin-1* and two highly homologous genes (*adoplin-2*, *-3*) constitute a gene family that encodes transmembrane proteins. The mRNA and protein levels of *adoplins* (particularly *adoplin-1*, *-2*) were markedly elevated in PS-deficient fibroblasts, compared to wild-type cells. Moreover, knockdown of the three *adoplins* by RNA interference affected maturation of nicastrin and its association with PS1. *Adoplin* knockdown additionally resulted in elevated levels of APP C-terminal fragments and decreased A β production, suggestive of reduced γ -secretase activity. Our data collectively indicate that *adoplins* are unique molecules with PS-related expression and functions that may play important role(s) in the maturation and activity of the γ -secretase complex.—Araki, W., Takahashi-Sasaki, N., Chui, D.-H., Saito, S., Takeda, K., Shirotani, K., Takahashi, K., Murayama, K. S., Kametani, F., Shiraishi, H., Komano, H., Tabira, T. A family of membrane proteins associated with presenilin expression and γ -secretase function. *FASEB J.* 22, 000–000 (2008)

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ALZHEIMER'S DISEASE (AD) IS THE MOST COMMON neurodegenerative dementia in the elderly population, characterized pathologically by neuronal and synaptic loss and extensive formation of senile plaques and

neurofibrillary tangles. The principal component of senile plaques is β -amyloid protein (A β), of which accumulation appears to play a key role in the pathogenesis of AD. A β is produced by sequential proteolysis of its precursor, amyloid precursor protein (APP), by β - and γ -secretases. β -Secretase cleavage of APP generates the secreted derivative, sAPP- β , and β -C-terminal fragment, β -CTF. The latter is subsequently cleaved by γ -secretase, yielding A β 40 and A β 42. Alternative cleavage of APP by α -secretase generates sAPP- α and α -CTF, precluding A β production (1).

Presenilin 1 (PS1) and PS2 genes are linked to autosomal dominant early onset familial AD (2, 3). PS1 or PS2 comprises the catalytic component of PS- γ -secretase, a novel type of aspartyl protease complex composed of at least four membrane proteins, including PS1 or PS2, nicastrin, APH-1, and PEN-2 (4–6). Recent reports that CD147 and TMP21 are regulatory cofactors of the γ -secretase complex (7, 8) have yet to be confirmed clearly. The PS complex catalyzes γ -cleavage of APP within the membrane to generate A β . Familial AD-associated PS mutations lead to alterations in the γ -secretase processing of APP to increase the generation of highly amyloidogenic A β 42, relative to A β 40 (9). The PS complex is additionally responsible for processing various type 1 membrane proteins, including Notch and cadherins (10), and AD-causing PS mutations influence their cleavage (11–14). Thus, the biological consequences of PS mutations are complicated, and their characterization may aid in improving our understanding of the pathological mechanisms of AD.

In this study, we used mRNA differential display (DD) analysis (15) to identify genes that are differentially expressed in neuronal cells with wild-type PS1 *vs.*

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those with familial AD-associated mutant PS1. We isolated a novel gene (adoplin-1 or ORMDL-1) that is specifically down-regulated in association with PS1 mutations. This gene and two highly homologous genes (adoplin-2 and -3/ORMDL-2 and -3) constitute an evolutionally conserved gene family that encodes integral membrane proteins with unknown functions (16). Our subsequent analyses further indicate that adoplins are unique molecules with PS-related expression and functions.

MATERIALS AND METHODS

Differential display analysis

Total RNA was isolated from wild-type and mutant PS1-transfected SH-SY5Y cells, as described previously (17). To preclude selection bias, total RNA preparations from two different clones were mixed and used for cDNA synthesis with T11VV downstream primers. PCR was performed using multiple primer sets (9 down primers \times 24 up primers; Takara, Tokyo, Japan) and ^{35}S -dATP. Amplified products were separated on a polyacrylamide gel, followed by autoradiography. DNA recovered from differential display bands on the gel was reamplified using modified primer pairs (extra 8–9 bases were added to the 5' ends of primers; 18), subcloned into pUC18, and sequenced.

Northern blot analysis

mRNA was extracted with the Poly(A)PureTM mRNA isolation kit (Ambion, Austin, TX, USA) and further purified with phenol/chloroform. Northern blotting was performed according to a previous report (17). Signals of mRNA were quantified using a BAS5000 image analyzer (Fuji Film Co., Tokyo, Japan). β -actin labeling was used as an internal control to normalize RNA loading.

Cell cultures and transfection

Murine wild-type fibroblasts and PS1-deficient, PS2-deficient, and PS1/PS2-doubly deficient fibroblasts immortalized with a large T antigen were maintained as described previously (19). Wild-type or mutant (I143T or G384A) PS1 cDNA were transfected into SH-SY5Y or H4 cells, and stable transformants were obtained by selection with hygromycin, as described previously (20, 21). Adoplin-1, -2, and -3 cDNA were subcloned into pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA) and transfected into SH-SY5Y cells. Stable transfectants were selected with G418. SH-SY5Y cells stably transfected with APP were described previously (21). HeLa cells were transfected with "Swedish" mutant APP (swAPP) cDNA, and stable transfectants obtained as above. Notch ΔE cDNA with the C-terminal myc epitope subcloned into pcDNA3 vector was provided by Dr. Raphael Kopan (22). Transient transfection was performed using Lipofectamine 2000 (Invitrogen) or FuGENE6 (Roche, Mannheim, Germany), according to the manufacturer's instructions. Rat primary cerebral cortical neurons were cultured as described previously (23).

Antibodies

Polyclonal rabbit antibodies to adoplin-1 (anti-Adp1) were raised against the synthetic peptide corresponding to residues

134–153 of human adoplin-1 and purified by affinity chromatography on a HiTrap NHS-activated HP column (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) coupled with the peptide. Other antibodies employed include polyclonal anti-PS1-loop (anti-CX against residues 346–363 of human PS1), monoclonal anti-PS1N (24), polyclonal anti-nicastrin (Sigma, St. Louis, MO, USA), anti-APH-1aL (Covance, Berkeley, CA, USA), anti-PEN-2 (provided by Dr. Gopal Thinakaran) (25), anti-APP C-terminal (R37) (26), monoclonal anti-APP N-terminal (Chemicon, Temecula, CA, USA), and anti-myc (Invitrogen).

Western blot analysis

Cells were lysed in RIPA buffer containing protease inhibitors and processed according to a previous report (23). For adoplin analysis, samples containing 100–150 μg protein were immunoprecipitated with anti-Adp1 antibodies. Immunoprecipitated proteins were subjected to Western blotting with the same antibodies. Postmortem human brain tissues were homogenized in 10 mM Tris, pH 8; 150 mM NaCl containing protease inhibitors; and centrifuged at 100,000 g for 1 h. The resultant pellet was lysed in RIPA buffer and analyzed as above.

Immunoprecipitation

Cells were lysed in buffer containing 1% CHAPSO and protease inhibitors (27). Lysates were incubated with anti-PS1N covalently attached to protein G-agarose beads. Immunoprecipitated proteins were analyzed by Western blotting with the appropriate antibodies.

Immunohistochemistry

Human brain tissue sections were prepared, and double-immunohistochemical staining was performed following a previously documented method (28). Briefly, sections were incubated with anti-Adp1 and subsequently with FITC-conjugated anti-rabbit IgG. For double-staining, sections were treated with primary antibodies [monoclonal anti-NeuN (Chemicon), anti-glial fibrillary acidic protein (GFAP) (DakoCytomation, Kyoto, Japan), or anti- $\text{A}\beta$ (6F/3d, DakoCytomation)], followed by incubation with Texas-Red conjugated anti-mouse IgG. Next, sections were observed with a confocal laser scanning microscope imaging system (FLUOVIEW, Olympus, Tokyo, Japan). Immunocytochemical staining of cultured neurons was performed according to a previous report (23).

RNA interference

Single-stranded gene-specific sense and antisense RNA oligomers were synthesized by Qiagen (Hilden, Germany). The specific siRNA sequences were directed to the following targets: adoplin-1, 5'-ACACAGCTTCTCTCCTGAG-3'; adoplin-2, 5'-AGGCTCGGCTACTGACACA-3'; adoplin-3, 5'-AAGTACGACCAGATCCATT-3'. Control nonsilencing siRNA was obtained from Qiagen. siRNA duplexes were transfected using a HiPerFect transfection reagent (Qiagen), according to the manufacturer's instructions. We used two consecutive rounds of transfection (29). For measurement of secreted $\text{A}\beta$, culture media were changed one day after the second transfection. Cells and conditioned media were harvested the next day. A mixture of siRNAs to adoplin-1, -2, and -3 was used to achieve downregulation of adoplin expression (10 nM each).

Control nonsilencing siRNA was used at a concentration of 30 nM.

Ab measurement

Ab1–40 in conditioned media was measured using a sandwich enzyme-linked immunosorbent assay (ELISA) kit (IBL, Gunma, Japan), based on the manufacturer's instructions.

RESULTS

Isolation of the adoplín-1 gene by differential display analysis

Human SH-SY5Y neuroblastoma cells stably transfected with wild-type or familial AD-associated mutant PS1 (I143T or G384A; designated SH-WT PS1, SH-I143T PS1, and SH-G384A PS1, respectively; 20, 21) were used for mRNA DD analysis. The expression levels of PS1 proteins in all transfectants were equivalent (data not shown). After repeated DD experiments, we identified two clones that were down-regulated in both SH-I143T PS1 and SH-G384A PS1 cells, compared to SH-WT PS1 cells. Subsequent sequence analyses revealed that the

one clone corresponded to partial ORMDL-1 cDNA (16) and the other to human ribosomal protein L23a cDNA. We isolated full-length cDNA clones of the former gene encoding a protein of 153 amino acid residues (**Fig. 1A**) and redesignated the gene “adoplín-1” (a down-regulated gene in mutant presenilin 1 cells) for convenience.

Decreased adoplín-1 mRNA expression in mutant PS1-transfected cells was verified by Northern blotting (**Fig. 1B**). Specifically, the adoplín-1 mRNA levels in SH-I143T PS1 and SH-G384A PS1 cells were 36 ± 1 and $33 \pm 7\%$ of control SH-WT PS1 cells, respectively. Mutant PS1-associated down-regulation of adoplín-1 mRNA was additionally observed in stably transfected human neuroglioma H4 cells (44 and 59% in H4 cells expressing I143T PS1 and G384A PS1, respectively, compared to H4 cells expressing wild-type PS1). Northern blot analysis of human tissues disclosed adoplín-1 mRNA transcripts in various tissues, including brain, as a major ~1.4 kb band (**Fig. 1C**). Adoplín-1 mRNA was expressed in different subregions of the brain at similar levels (data not shown). Relatively higher mRNA expression was observed in the heart, placenta, and pancreas.

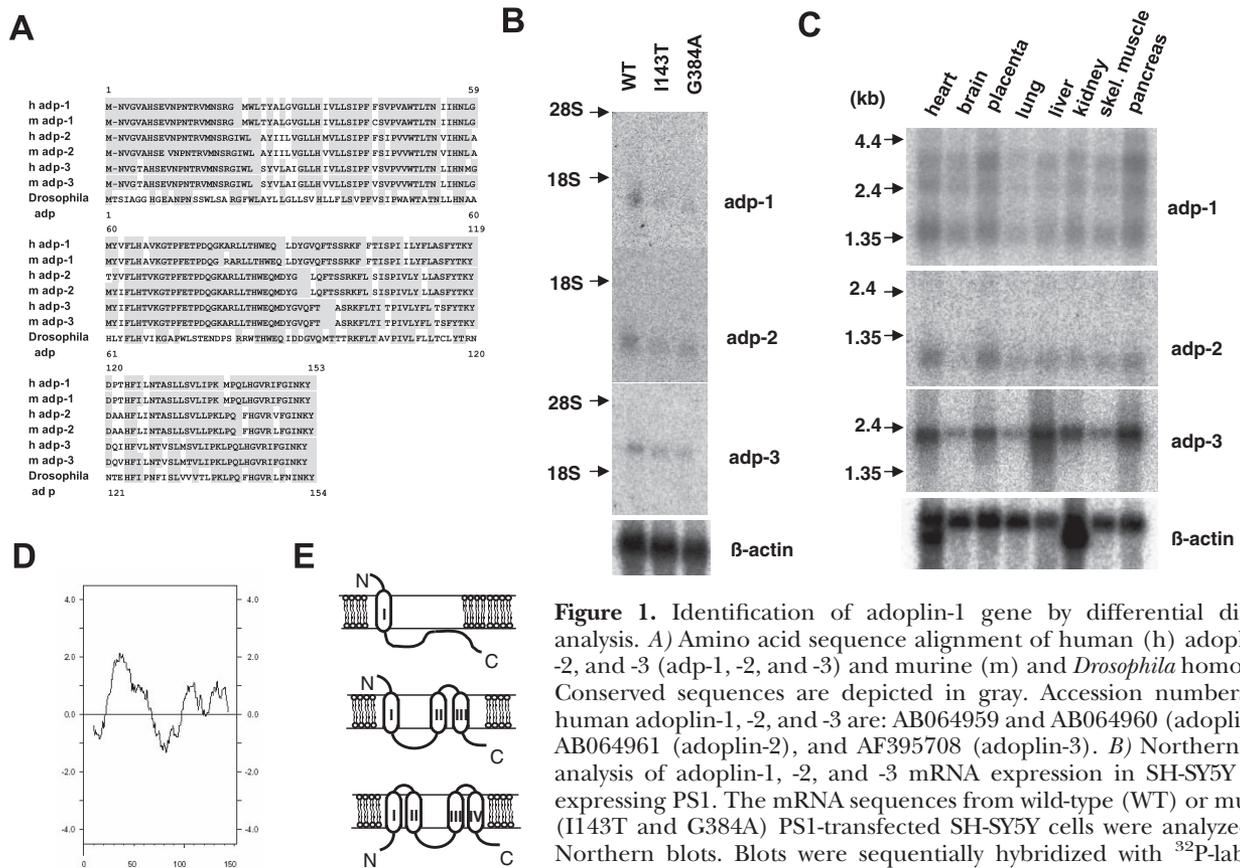


Figure 1. Identification of adoplín-1 gene by differential display analysis. **A**) Amino acid sequence alignment of human (h) adoplín-1, -2, and -3 (adp-1, -2, and -3) and murine (m) and *Drosophila* homologs. Conserved sequences are depicted in gray. Accession numbers for human adoplín-1, -2, and -3 are: AB064959 and AB064960 (adoplín-1), AB064961 (adoplín-2), and AF395708 (adoplín-3). **B**) Northern blot analysis of adoplín-1, -2, and -3 mRNA expression in SH-SY5Y cells expressing PS1. The mRNA sequences from wild-type (WT) or mutant (I143T and G384A) PS1-transfected SH-SY5Y cells were analyzed on Northern blots. Blots were sequentially hybridized with ³²P-labeled adoplín-1, adoplín-2, adoplín-3, and β-actin cDNA. **C**) Adoplín-1, -2, and -3 mRNA expression in human peripheral tissues. Human multiple tissue Northern blots (Clontech, Palo Alto, CA, USA) were

hybridized with ³²P-labeled probes, as in **B**. **D**) The hydropathy profile of adoplín-1 was plotted using the hydrophobicity indices of Kyte and Doolittle (40), with a window size of 19 residues. The hydropathy profiles of adoplín-2 and -3 are essentially similar to those of adoplín-1. **E**) Models of the possible transmembrane topology of adoplín protein. Three possible models with one, three, or four transmembrane segments are depicted.

We further found that two paralogs of human adoplin-1/ORMDL-1 exist in the database (designated adoplin-2 and -3/ORMDL-2 and -3) and cloned their cDNAs (16). Adoplin-2 and -3 mRNA were observed ubiquitously (Fig. 1C). Interestingly, adoplin-2 and -3 mRNA levels in SH-I143T PS1 and SH-G384A PS1 cells were lower than those in SH-WT PS1 cells (Fig. 1B), although their decreases were not as significant as those observed with adoplin-1. Homologous genes to human adoplin-1, -2, and -3 have been identified in mouse, and a single homologue exists in *Drosophila* (Fig. 1A). Northern blot analysis of mouse tissues confirmed that the three adoplin genes are expressed ubiquitously (Fig. S1). Weakly homologous genes have additionally been identified in fungi and plants (16). The estimated hydrophathy profile of the adoplin-1 protein is depicted in Fig. 1D. Hydrophathy analysis and the PSORT program (<http://psort.nibb.ac.jp>) predict that adoplin-1 protein is a type 1b integral membrane protein with one transmembrane region, although its exact transmembrane topology remains unclear at present (Fig. 1E; 16). The THWEQ sequence in the middle part of the adoplin sequences is highly conserved among different species and may represent a functionally important region.

Expression of adoplin in neurons

To determine adoplin-1 protein expression, we generated a specific antibody to the C-terminus of human adoplin-1 (anti-Adp1). Western blotting with this antibody allowed the detection of overexpressed and endogenous proteins (~17 kDa) in adoplin-1-transfected SH-SY5Y cells and nontransfected cells, respectively (Fig. 2A). Since the C-terminal region is highly conserved among the three adoplin homologs, the antibody also recognized adoplin-2 and adoplin-3 proteins in cells transfected with the corresponding constructs (Fig. 2A, not shown). His-tagged adoplin-1 proteins expressed in *E. coli* displayed a similar molecular weight to those in SH-SY5Y cells (data not shown), implying that no extensive modifications of adoplin-1 occur in mammals. Western blot analysis further showed that

endogenous adoplin protein levels in SH-I143T PS1 and SH-G384A PS1 cells were similar to those in SH-WT PS1 cells (data not shown), despite lower adoplin-1 mRNA expression in the former cultures.

We also observed the presence of ~17 kDa adoplin proteins in the membrane but not cytosolic, fractions of primary rat cerebral cortical neurons (data not shown) and human brain tissues (Fig. 2A). Immunocytochemical staining of primary cortical neurons showed that adoplin immunoreactivity was localized in both cell bodies and neurites (Fig. 2B). Adoplin proteins were primarily expressed in neurons, as evident from immunohistochemical staining of human cerebral cortices of control (data not shown) and AD brains (Fig. 2C) with the anti-Adp1 antibody. This finding was further confirmed by double-staining with anti-Adp1 and either anti-NeuN (a marker of neurons) or anti-glial fibrillary acidic protein (GFAP, a marker of astrocytes) (data not shown). Adoplin immunoreactivity did not colocalize with A β in senile plaques (Fig. 2C). No adoplin immunoreactivity was observed when the primary antibody was replaced with nonimmune rabbit IgG (data not shown). These observations suggest that adoplins are chiefly neuronal proteins.

Increased adoplin expression in PS-deficient cells

The relationship between PS and adoplin was further investigated using PS-deficient cells. We compared the adoplin protein levels in fibroblasts derived from wild-type, PS1-deficient, PS2-deficient, and PS1/PS2-doubly deficient mice (19). Interestingly, endogenous adoplin protein expression was increased marginally (~2-fold) in PS1- or PS2-deficient cells and markedly (~6-fold) in PS1/PS2 doubly deficient cells, compared to wild-type cells (Fig. 3A, B). Northern blot analyses disclosed that adoplin-1 and -2 mRNA levels were increased by 1.7-fold and 2.0-fold in PS1-deficient cells, 2.5-fold and 2.8-fold in PS2-deficient cells, and 4.7-fold and 6.5-fold in double PS-deficient cells, respectively, relative to wild-type cells; adoplin-3 mRNA levels were similar among the cells examined (Fig. 3C, D). Immunocytochemical staining with the anti-Adp1 antibody additionally showed that the intensity of adoplin immunoreac-

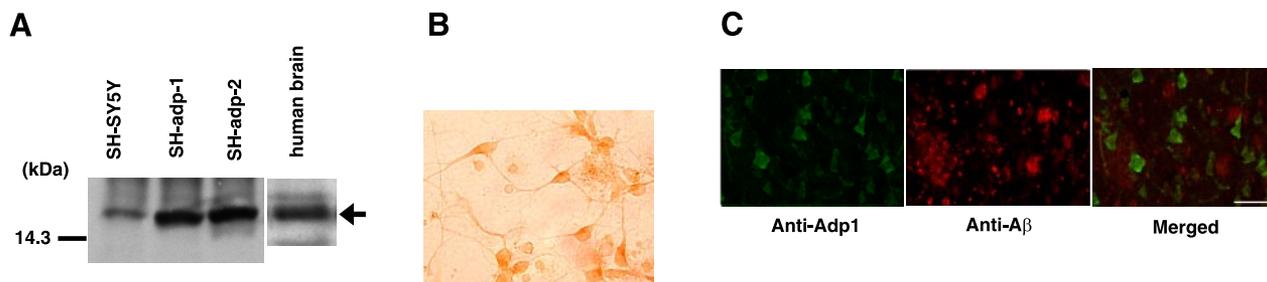


Figure 2. Adoplin protein expression in cultured cells and human brain tissues. *A*) Western blot analysis of adoplin expression in SH-SY5Y cells stably transfected with vector alone, adoplin-1, or adoplin-2, and the membrane fraction of human brain tissues. Anti-Adp1 antibody detected a ~17 kDa protein, as indicated with an arrow. *B*) Immunostaining of primary rat cerebral cortical neurons with anti-Adp1. *C*) Immunohistochemical analysis of adoplin proteins in AD brain tissues. Brain sections were doubly immunostained with anti-Adp1 (green) and anti-A β (red), as described in Materials and Methods. Scale bar = 60 μ m.

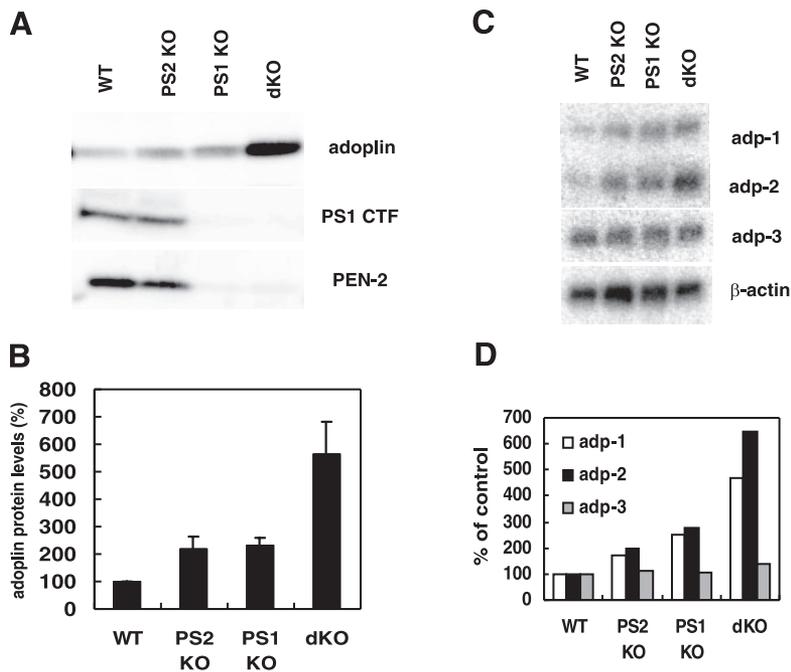


Figure 3. Enhanced expression of adoplins in PS-deficient cells. *A*) Western blot analysis of adoplins expression in wild-type fibroblasts and PS1-deficient (PS1 KO), PS2-deficient (PS2-KO), or PS1/PS2-deficient (dKO) fibroblasts. *B*) Adoplins protein expression levels were quantified, and the relative levels were plotted. Data are presented as means \pm SE from three independent experiments. *C*) Northern blot analysis of adoplins-1, -2, and -3 mRNA in wild-type fibroblasts and PS1-, PS2-, or PS1/PS2-deficient fibroblasts. *D*) mRNA levels of adoplins-1, -2, and -3 were quantitated, and relative levels were plotted. Data are presented as means \pm SE from three independent experiments.

tivity was higher in PS1/PS2-deficient cells than wild-type cells (data not shown). These data clearly indicate that PS deficiency results in enhanced adoplins-1 and -2 mRNA and protein expression.

Adoplins could possibly be a substrate of γ -secretase that accumulates in the absence of PS. To investigate this theory further, we examined the effect of the γ -secretase inhibitor, DAPT (*N*-[*N*-(3,5-difluorophenylacetyl)-L-alanyl]-(*S*)-phenylglycine *t*-butyl ester) (30), on adoplins protein expression. Treatment with DAPT did not significantly alter adoplins levels in adoplins-1-expressing cells but led to a marked increase in APP CTF levels in APP-expressing cells (Fig. 4*A, B*). These findings indicate that augmented levels of adoplins proteins in PS-deficient cells do not result from reduced degradation by γ -secretase but from enhanced transcription of adoplins genes.

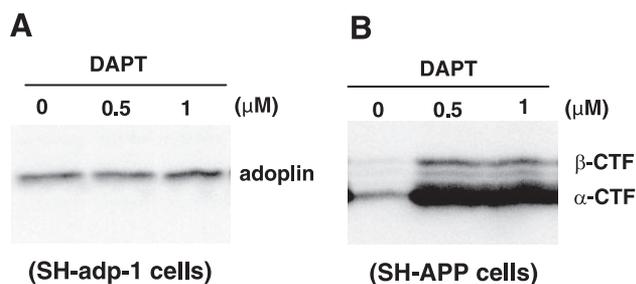


Figure 4. The γ -secretase inhibitor, DAPT, has no effect on adoplins expression. *A*) SH-SY5Y cells stably transfected with adoplins-1 were treated with the indicated concentrations of DAPT for 20 h, followed by Western blot analysis with the anti-Adp antibody. *B*) SH-SY5Y cells stably transfected with APP were treated with DAPT as in (*A*), followed by Western blot analysis with an anti-APP antibody. APP α -CTF and β -CTF levels were markedly elevated in DAPT-treated cells.

Adoplins knockdown affects γ -secretase function

To clarify the functional relationship between adoplins proteins and PS1- γ -secretase, we first examined whether adoplins overexpression has any influence on PS complex proteins. The levels of PS1 complex proteins were unaffected in SH-SY5Y cells overexpressing adoplins-1 (Fig. 5*A*), suggesting that adoplins overexpression does not influence the expression of PS complex proteins.

Next, we performed RNAi experiments using HeLa cells. For downregulation of adoplins protein expression, cells were transfected with three small interfering RNAs (siRNAs) specific for adoplins-1, -2, and -3. As negative controls, cells were transfected with nonsilencing siRNAs. Significant reduction (\sim 80%) in adoplins protein levels was observed following treatment with adoplins siRNAs (Fig. 5*B*). The adoplins siRNAs did not affect cellular viability, although some morphological changes such as thinner cell shape were observed (data not shown). Adoplins knockdown did not affect the expression of endogenous PS1, PS2, APH-1, or PEN-2. However, the levels of the immature nicastrin were significantly augmented (Fig. 5*B*). Densitometric quantification estimated that immature nicastrin levels were increased approximately 2-fold, and mature nicastrin levels were almost unchanged in adoplins siRNA-treated cells compared with control cells (Fig. 5*C*). However, adoplins knockdown did not influence maturation of APP (Fig. 5*B*). Thus, adoplins downregulation appears to specifically impair nicastrin maturation. In these RNAi experiments, nonspecific induction of interferon responses (31) was unlikely because no upregulation of Stat1, one of the interferon-stimulated genes, was observed in siRNA-treated cells on Western blots (data not shown).

We then determined whether siRNA-mediated adoplins suppression affects PS complex formation. CHAPSO

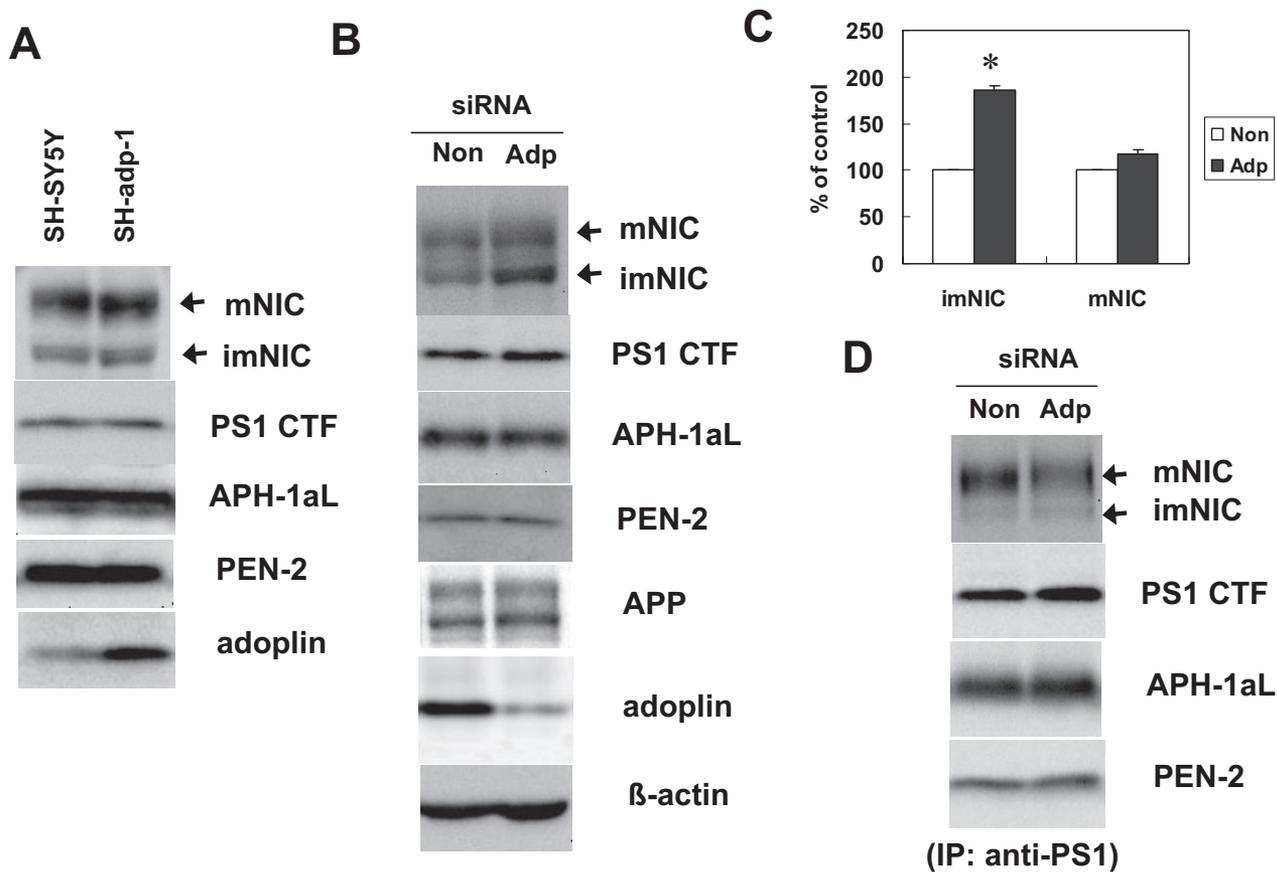


Figure 5. Suppression of adoplins expression causes impaired maturation of nicastrin. *A*) Effects of adoplins overexpression on PS1, nicastrin, APH-1, and PEN-2. Cell lysates of SH-SY5Y and adoplins-1-expressing SH-SY5Y cells were subjected to Western blotting with the relevant antibodies. *B*) Effects of RNAi-mediated suppression of adoplins expression on PS1, PS2, nicastrin, APH-1, and PEN-2. HeLa cells were treated with either nonsilencing (Non) or adoplins (Adp) siRNAs (mixture of siRNAs for three adoplins), as described in Materials and Methods. Cell lysates were subjected to Western blotting with the relevant antibodies. *C*) Immature and mature nicastrin bands from *B* were quantitated, and relative levels were calculated. Data represent means \pm SE from three independent experiments. * $P < 0.01$ differed significantly from control by the paired *t* test. *D*) Effects of adoplins suppression on PS1 complex-associated proteins. SiRNA-treated HeLa cells were lysed in buffer containing 1% CHAPSO. PS1 complexes were immunoprecipitated with anti-PS1N antibodies, and immunoprecipitated proteins were analyzed by Western blotting as in *B*. imNIC, immature NIC; mNIC, mature NIC.

extracts of siRNA-treated HeLa cells were immunoprecipitated with monoclonal anti-PS1 antibodies, and PS1-associated proteins were analyzed by Western blotting. Levels of PS1-associated APH-1aL, PEN-2, and PS1 remained almost unchanged in siRNA-treated cells, compared with control cells (Fig. 5*D*). In contrast, significantly less mature nicastrin ($\sim 75\%$ of control) was associated with PS1 in adoplins siRNA-treated cells (Fig. 5*D*). Immature nicastrin barely associated with PS1 in both cell types, consistent with previous reports (32, 33). Adoplins proteins did not appear to be in close physical association with PS1 or nicastrin because endogenous adoplins proteins did not coimmunoprecipitate with neither PS1 nor nicastrin (data not shown).

Next, we examined whether downregulation of adoplins affects γ -secretase-mediated cleavage of APP and Notch. In HeLa cells transfected with adoplins siRNAs, a significant (~ 3 fold) increase in the APP α -CTF level was observed, compared to those transfected with con-

trol siRNA (Fig. 6*A, B*). Following transient transfection of APP β -CTF, both β - and α -CTF bands were augmented in these cells (Fig. 6*A*). Furthermore, transient transfection of Notch ΔE (22), the direct substrate of γ -secretase, led to a significant increase in its level while that of Notch intracellular domain (NICD) remained unaltered in adoplins siRNA-treated cells (Fig. 6*C*). The data indicate that γ -secretase cleavage of APP and Notch ΔE are inhibited due to loss of adoplins. The effects of adoplins downregulation on $A\beta$ generation were assessed by measuring $A\beta$ levels in conditioned media of HeLa cells stably expressing swAPP. Levels of $A\beta_{1-40}$ in media from adoplins siRNA-treated cells were decreased to $\sim 40\%$ of that from control cells (Fig. 6*E*). Levels of $A\beta_{1-42}$ were below detection limits. Since the cellular level of full-length APP remained unaltered (Fig. 6*D*), it is likely that $A\beta$ secretion was suppressed due to inhibition of γ -secretase cleavage of APP. These results suggest that adoplins knockdown affects not only nicastrin maturation but also γ -secretase activity.

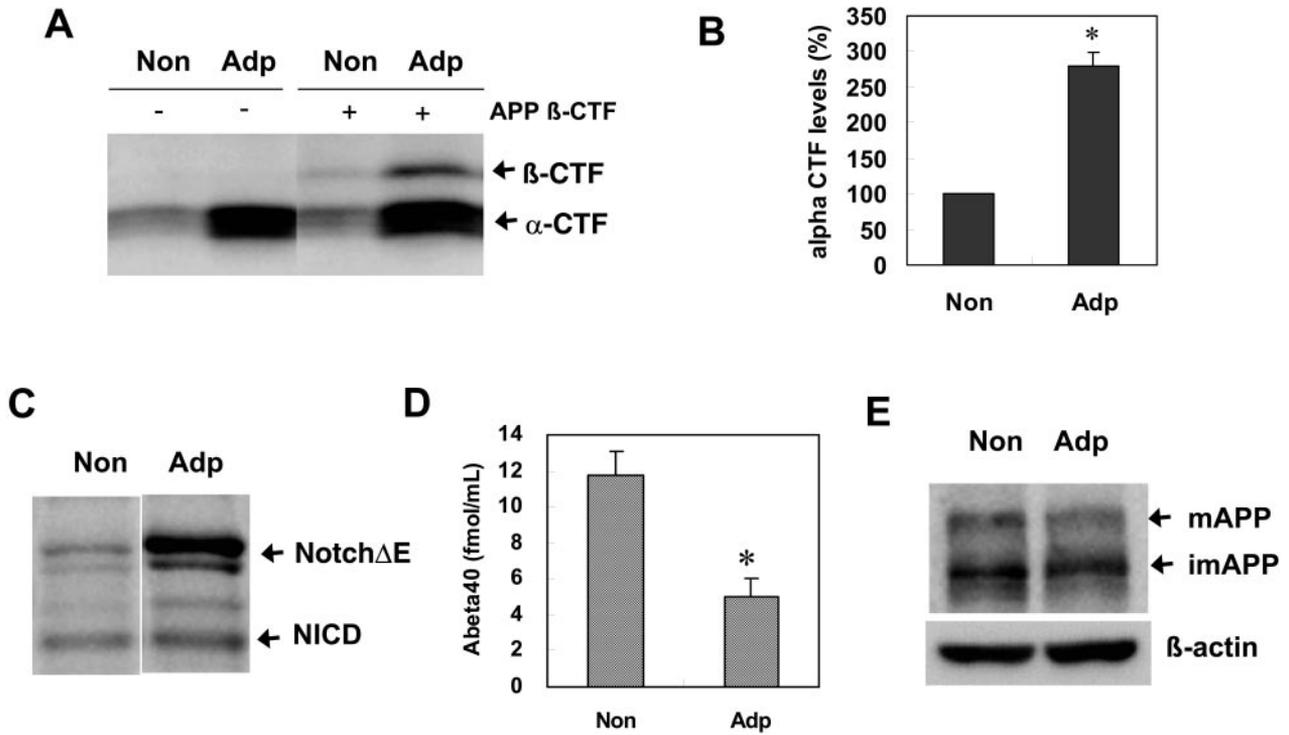


Figure 6. γ -Secretase function is affected by adoplins knockdown. *A*) Effect of adoplins RNAi on cellular levels of APP CTF. HeLa cells were treated with nonsilencing (Non) or adoplins (Adp) siRNAs and were transfected further with APP β -CTF cDNA or vector. APP CTF in cell lysates was analyzed by Western blotting with anti-APP (R37). *B*) α -CTF levels in control and adoplins siRNA-treated cells were quantitated, and relative levels were calculated. Data means \pm SE from three independent experiments. * $P < 0.05$ by the paired *t* test. *C*) Effects of adoplins RNAi on Notch cleavage. HeLa cells treated with siRNA were transfected with Notch Δ E cDNA, and cell lysates analyzed by Western blotting with anti-myc. *D*) Effects of adoplins suppression on A β production. HeLa cells stably expressing swAPP were treated with nonsilencing or adoplins siRNAs, and A β 1–40 levels in 24 h conditioned media were measured by sandwich ELISA. Data are presented as means \pm SE of three independent experiments. * $P < 0.01$ by the paired *t* test. *E*) Cellular APP protein expression in cells used in *C*, as analyzed by Western blotting with anti-APP antibodies. imAPP, immature APP; mAPP, mature APP.

DISCUSSION

Familial AD-associated PS mutations alter the γ -cleavage of APP, increasing the ratio of A β 42 relative to A β 40, which is a pathologically significant event. Notably, AD-causing PS mutations additionally alter proteolytic function toward several different substrates, such as Notch and cadherins. Thus, the molecular effects of PS mutations are complicated, and some find dispute over the underlying AD-inducing mechanisms (34, 35). In this study, we employed mRNA DD analysis to identify a novel gene, adoplins-1/ORMDL-1, which displays reduced expression in neuronal cells containing mutant PS1 relative to those with wild-type PS1. Adoplins-1 (or ORMDL-1) and two highly homologous genes, adoplins-2 and -3 (or ORMDL-2 and -3), constitute an evolutionally conserved family encoding integral membrane proteins of 153 amino acids (Fig. 1A). Due to the lack of characteristic functional domains, the physiological functions of adoplins are currently unknown. Expression patterns of these proteins were characterized by using Northern and Western blot analyses and immunohistochemistry. We further investigated the possible relationship between adoplins, PS expression, and γ -secretase function using PS-null cells

and RNAi techniques. Unexpectedly, expression of adoplins protein and mRNA was markedly up-regulated in PS-deficient cells. Furthermore, our RNAi experiments revealed that siRNA-mediated adoplins suppression affects nicastrin maturation and PS- γ -secretase activity, thus highlighting its relevance in PS- γ -secretase function.

Northern blot analysis showed that the three adoplins genes are expressed ubiquitously, in keeping with a previous report (16). Adoplins-1 and -2 mRNA expression patterns are different in human and mouse tissues. Specifically, mouse but not human adoplins-1 and -2 transcripts are particularly abundant in the liver and kidney. An antibody specific for the C-terminus of adoplins-1 was employed to determine its expression in cultured neuronal cells and human brain tissues. This antibody is sufficiently sensitive to detect a ~17 kDa endogenous adoplins protein in the membrane fraction on Western blots, although it cannot distinguish among the three homologs. Immunohistochemical analyses disclosed that adoplins proteins are primarily expressed in the neurons of cerebral cortices. Moreover, adoplins is localized mainly in the cell bodies and neurites of primary cortical neurons, which shows possible role(s) in nerve cells. Adoplins immunoreactivity is not colocal-

ized in senile plaques, which suggests that the protein does not function in the extracellular deposition of A β .

In contrast to the significant downregulation of adoplin-1 mRNA in mutant PS1-expressing cells, mRNAs of adoplin-1 and -2 are up-regulated moderately in PS1- or PS2-deficient cells and markedly in PS1-/PS2-doubly deficient cells. One possible explanation for this is that adoplin gene expression is under transcriptional control by PS (Fig. 7). We postulate that the expression of adoplin-1 and -2 mRNA is maintained at low levels under normal conditions involving regulated PS-associated signaling. Adoplin expression may be controlled *via* signaling pathway(s) downstream of specific γ -secretase substrate(s). Alternatively, Wnt/ β -catenin signaling may be involved in the upregulation of adoplins, since PS deficiency results in increased β -catenin stability (36). Our experiments with a γ -secretase inhibitor indicate that adoplin is not a substrate of γ -secretase. Thus, it is unlikely that the significant enhancement in adoplin expression in PS-null cells results from decreased degradation by PS- γ -secretase.

The mechanism underlying mutant PS1-associated downregulation of adoplin-1 is yet to be established but may be a result of gain-of-function, as suggested in a previous microarray study (37). A number of genes were identified that exhibit reverse-expression alterations in the brains of PS1 conditional knockout mice and mutant PS1 transgenic mice. The authors suggest that the familial AD-linked PS1 variant produces transcriptome changes primarily by gain of aberrant function (37). We did not observe a clear reduction in adoplin protein expression in mutant PS1-expressing cells despite the significant downregulation of adoplin-1 mRNA. The reason for this discrepancy is unclear. It is possible that the extent of downregulation of adoplin-1 is not sufficiently large to alter total adoplin protein expression.

Our RNAi experiments show that adoplin knock-

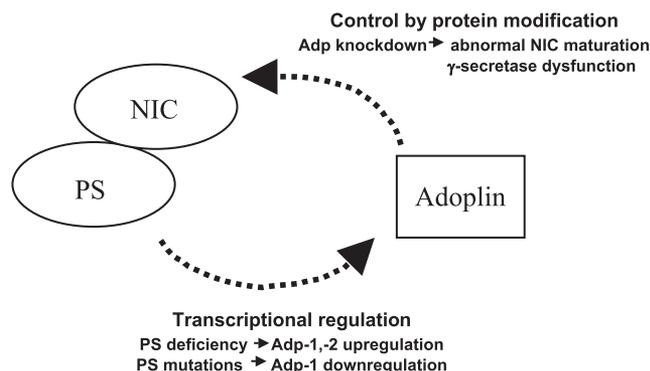


Figure 7. Schema illustrating the relationship between adoplin and PS. Adoplin expression appears to be under transcriptional control by PS. PS deficiency results in the upregulation of adoplin-1 and adoplin-2 mRNA, while familial AD-associated PS mutations lead to down-regulation of adoplin-1 mRNA. However, adoplin knockdown induces abnormal maturation of nicastrin, as well as reduced γ -secretase function. Abnormal nicastrin maturation may be directly associated with the γ -secretase dysfunction, although the precise underlying mechanism remains unclear.

down does not affect the expression of endogenous PS1, APH-1, and PEN-2 but significantly influences nicastrin maturation. The level of immature nicastrin is clearly augmented by adoplin knockdown. This effect appears specific, since maturation of APP remains unaffected. Importantly, significantly less mature nicastrin is associated with PS1 in adoplin knockdown cells than control cells, suggesting disruption of PS complex formation. Furthermore, loss of adoplin results in decreased γ -secretase cleavage of APP and Notch (as indicated by accumulation of APP CTF and Notch ΔE) and reduced A β production, implying significantly reduced γ -secretase activity. Nicastrin plays a critical role in maintaining the PS- γ -secretase complex and serving as a γ -secretase substrate receptor (38, 39). Thus, impaired nicastrin maturation may be directly associated with the apparent reduction in γ -secretase activity following adoplin knockdown (Fig. 7). However, the possibility that adoplin suppression affects γ -secretase function by a different unknown mechanism cannot be eliminated. A previous study demonstrates that adoplin/ORMDL proteins localize in endoplasmic reticulum membranes (16). Consistent with this report, our preliminary data of biochemical subcellular fractionation indicate that adoplin mainly localizes in the endoplasmic reticulum, where it coresides with PS1, PS2, nicastrin, APH-1, and PEN-2 (data not shown). Experiments using yeast knockout strains also suggest that adoplin/ORMDL functions in correct protein folding and/or trafficking in the endoplasmic reticulum (16). Adoplin may participate in nicastrin trafficking in the endoplasmic reticulum. However, since neither PS1 nor nicastrin coprecipitate with endogenous adoplin, close interactions between adoplin and these proteins appear unlikely. Thus, further research is required to elucidate the mechanism of adoplin action in nicastrin maturation.

We have identified adoplin/ORMDL genes whose transcription is possibly regulated by PS. Our data additionally indicate that adoplins are relevant in PS- γ -secretase function. Thus, adoplins are unique molecules that display PS-related expression and function. In view of the reduced γ -secretase activity following adoplin knockdown, we hypothesize that adoplin may be a therapeutic target for AD. Further clarification of the role(s) of adoplins in relation to PS- γ -secretase may aid in the development of novel therapeutic strategies for AD. FJ

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