APP intracellular domain–WAVE1 pathway reduces amyloid-β production

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An increase in amyloid-β (Aβ) production is a major pathogenic mechanism associated with Alzheimer’s disease (AD)1,2, but little is known about possible homeostatic control of the amyloidogenic pathway. Here we report that the amyloid precursor protein (APP) intracellular domain (AICD) downregulates Wiskott-Aldrich syndrome protein (WASP)-family verprolin homologous protein 1 (WAVE1 or WASF1) as part of a negative feedback mechanism to limit Aβ production. The AICD binds to the Wasp1 promoter, negatively regulates its transcription and downregulates Wasp1 mRNA and protein expression in Neuro 2a (N2a) cells. WAVE1 interacts and colocalizes with APP in the Golgi apparatus. Experimentally reducing WAVE1 in N2a cells decreased the budding of APP-containing vesicles and reduced cell-surface APP, thereby reducing the production of Aβ. WAVE1 downregulation was observed in mouse models of AD. Reduction of Wasp1 gene expression dramatically reduced Aβ levels and restored memory deficits in a mouse model of AD. A decrease in amounts of WASF1 mRNA was also observed in human AD brains, suggesting clinical relevance of the negative feedback circuit involved in homeostatic regulation of Aβ production.

WAVE1, a member of the WASP-WAVE family of proteins, activates the actin-related protein 2/3 (Arp2/3) complex and initiates actin polymerization3. WAVE1 expression is high in the brain4, where it exists as a heteropentameric complex with PIR121, Nap125, Abi2 and HSPC3000 (refs. 5,6). Previously, expression of NCKAP1 (the human gene encoding Nap125) was found to be markedly decreased in AD-affected brains7. Because WAVE1 is a core component of the complex required for actin polymerization, we measured the level of WAVE1 protein in mouse models of AD. We used immunohistochemistry to examine WAVE1 expression in the hippocampus of a triple-transgenic (3×Tg) mouse model harboring transgenes for presenilin 1 (PS1 M146V), Swedish mutant of APP (APPswe) and tau (P301L)8. Compared with brains of wild-type mice, brains of 3×Tg mice showed decreased WAVE1 immunoreactivity (WAVE1-IR) in the CA1 region, where there was high APPswe expression, but comparable WAVE1-IR in the CA2–3 region and dentate gyrus, where there was little expression of APPswe (Fig. 1a). In separate experiments, a lower level of WAVE1 in the entire hippocampus in 3×Tg mice compared with wild-type mice was confirmed by immunoblotting (Fig. 1b). WAVE1 downregulation was also observed in the hippocampus of a transgenic AD mouse model overexpressing only APPswe9 (Tg-APPswe) (Fig. 1c), indicating a critical role for APP in the downregulation of WAVE1. WAVE1 was downregulated by transient overexpression of the APPswe protein, which strongly facilitates β-secretase–mediated processing of APP10, but not by overexpression of wild-type APP695 (APPwt), in neuroblastoma N2a cells (Fig. 1d and Supplementary Fig. 1a). Overexpression of APPwt or APPswe did not affect the level of actin (Supplementary Fig. 1b) and was not associated with cell toxicity (Supplementary Fig. 1c).

To further study the regulation of WAVE1, we used a stable N2a cell line in which all cells express APPwt (N2a-APPwt), unlike the fractional expression of APPwt in transiently transfected cells. WAVE1 was downregulated in N2a-APPwt cells compared with non-transfected N2a cells (Fig. 1e). Treatment with a specific γ-secretase inhibitor led to a marked increase in the level of WAVE1 (Fig. 1f). APP processing by γ-secretase is known to not only generates Aβ but also produces AICD11,12. Higher amounts of AICD were generated in cells expressing APPswe than in cells expressing APPwt (Supplementary Fig. 1d). Notably, the changes in WAVE1 regulation induced by APP overexpression were also observed with overexpression of AICD (Fig. 1g and Supplementary Fig. 1c). Downregulation of WAVE1 by overexpression of APP or AICD takes place at the level of mRNA (Fig. 1e and Supplementary Fig. 1c). AICD is known to be a transcription factor13, and transcriptionally active AICD is preferentially generated via the amyloidogenic pathway14–16. Previously, direct interactions of AICD with several target genes, including a gene encoding an Aβ-degrading enzyme, neprilysin, were characterized by chromatin immunoprecipitation (ChIP) assay17,18. We carried out ChIP assays to examine possible interactions of AICD with the Wasp1 promoter. We transiently expressed 3×Flag-tagged AICD in N2a cells. Immunoprecipitation with

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anti–RNA polymerase (a positive control) or anti-Flag, but not with preimmune immunoglobulin G (IgG), led to coprecipitation of the Wasf1 promoter region (Fig. 1h).

A WASF1 promoter fused-luciferase assay showed suppression of WASF1 promoter activity with overexpression of APPswe or AICD (Fig. 1i). As a positive control, AICD overexpression increased the expression of luciferase in an MME promoter fused-luciferase reporter assay (Supplementary Fig. 1f) as expected18. The potency of WASF1 suppression by overexpression of APPswe or AICD correlated well with the protein levels of AICD in the transfected cells (Supplementary Fig. 1g). WASF1 expression was also regulated by the intracellular domains (ICDs) of APP-like proteins (APLPs) 1 and 2, but not by Notch intracellular domain (NICD) (Supplementary Fig. 2a–e). Overexpression of APLP1-ICD reduced Wasf1 mRNA expression and WASF1 promoter–luciferase activity (Supplementary Fig. 2f,d) but did not significantly alter amounts of WAVE1 protein (Supplementary Fig. 2e). This might have been due to the long half-life of WAVE1 protein (~24 h) (Supplementary Fig. 2f,g) and the relatively weaker inhibitory activity of APLP1-ICD compared to that of AICD and APLP2-ICD in the regulation of the WASF1 promoter (Supplementary Fig. 2d). Together these data suggest a critical role for AICD and ICDs of APLPs in the regulation of WAVE1 expression.

We next investigated the possibility that WAVE1 regulates the amyloidogenic pathway. Reducing WAVE1 expression with a synthetic duplex of small interfering RNA (siRNA) (to 34% of the WAVE1 level in controls; Fig. 2a) decreased the levels of Aβ40 (70% of control) and Aβ42 (53% of control) in a double-transgenic N2a cell line overexpressing APPswe and familial AD presenilin 1 mutant ΔE9 (N2a-APPswe.FS1ΔE9) (Fig. 2b,c). We also observed that reduced WAVE1 expression was associated with a lower level of surface APP (Fig. 2d), a lower level of the soluble ectodomain of APP produced by β-secretase (sAPPβ) (Fig. 2e), a higher level of total APP (Fig. 2f) and an unchanged level of the soluble ectodomain of APP produced by α-secretase (sAPPα) (Fig. 2g). Restoration of WAVE1 levels through expression of siRNA-resistant WAVE1 in conjunction with siRNA knockdown (Fig. 2a) reversed these effects (Fig. 2b–g). To address
the physiological relevance of the regulation of Aβ formation by WAVE1, we crossed double-transgenic AD mice (2×Tg mice) with Wasf1-knockout mice. We chose 2×Tg mice harboring APPsw and PS1ΔE9 (ref. 19) because the pathological phenotype appears earlier in these mice than in Tg-APPsw mouse but is not influenced by tau as it is in 3×Tg mice. We generated constitutive Wasf1-knockout mice by crossing floxed Wasf1 with Cre-deleter mice (Supplementary Fig. 3). The brains of 2×Tg mice with reduced Wasf1 expression compared with that in 2×Tg mice with Wasf1+/+ showed dramatically reduced levels of Aβ40 (Wasf1−/−, 32% and Wasf1−/-, 14% of the level of Aβ40 in Wasf1+/+ mice) and Aβ42 (Wasf1−/−, 57% and Wasf1−/-, 31% of the level of Aβ42 in Wasf1+/+ mice) (Fig. 2h,i). APP is trafficked to and processed in various cellular organelles. After being transported in the endoplasmic reticulum (ER), APP is transported to the plasma membrane via the Golgi apparatus and trans-Golgi network12,20. In this constitutive secretory pathway, immature APP molecules mature by means of post-translational modifications such as glycosylation, phosphorylation and tyrosine sulfation12,20. Surface APP is then endocytosed to endosomes, and a fraction in the endosomes is recycled to the trans-Golgi network or the cell surface. During this trafficking cycle, β-secretase cleaves APP mainly in endosomes, and γ-secretase cleaves APP at the cytoplasmic terminal fragments mainly at the plasma membrane and in endosomes or lysosomes12,20. Immunocytochemical analysis of N2a-APPsw, PS1ΔE9 cells and N2a-APPwt cells showed that WAVE1 and APP were enriched and colocalized in the Golgi apparatus (Fig. 3a,b and Supplementary Fig. 4a). High magnification of the Golgi area showed that Aβ40 and Aβ42 (c, n = 6), total APP (f, n = 4) and actin were measured in cell lysates. Surface APP was measured by biotinylation assay (d, n = 6), sAPPβ (e, n = 4) and sAPPα (g, n = 4) were measured in the medium. Data represent mean and s.e.m. *P < 0.05, **P < 0.01 and ***P < 0.001, two-tailed t-test. (h,i) Amounts of Aβ40 (h) and Aβ42 (i) were measured in mouse brains of 4–5-month-old 2×Tg AD male mice (tg/w+) harboring Wasf1+/+ (n = 13), Wasf1−/− (n = 13) or Wasf1−/- (n = 6). Data are presented as mean and s.e.m. and are representative of one (h,i), two (d–g) or three (a–c) independent experiments. *P < 0.05 and **P < 0.01, one-way ANOVA, Dunnett’s post hoc test.

Figure 2 A reduction in WAVE1 expression leads to reduced levels of Aβ. (a–g) N2a-APPsw, PS1ΔE9 cells were transfected with control siRNA plus control plasmid (Cont), Wasf1 siRNA plus control plasmid (Wasf1 siRNA) or Wasf1 siRNA plus siRNA-resistant plasmid for WAVE1 (WAVE1 add-back). WAVE1, Aβ40 (a, n = 9), Aβ42 (b, n = 6), total APP (c, n = 6), total APP (f, n = 4) and actin were measured in cell lysates. Surface APP was measured by biotinylation assay (d, n = 6), sAPPβ (e, n = 4) and sAPPα (g, n = 4) were measured in the medium. Data represent mean and s.e.m. *P < 0.05, **P < 0.01 and ***P < 0.001, two-tailed t-test. (h,i) Amounts of Aβ40 (h) and Aβ42 (i) were measured in mouse brains of 4–5-month-old 2×Tg AD male mice (tg/w+) harboring Wasf1+/+ (n = 13), Wasf1−/− (n = 13) or Wasf1−/- (n = 6). Data are presented as mean and s.e.m. and are representative of one (h,i), two (d–g) or three (a–c) independent experiments. *P < 0.05 and **P < 0.01, one-way ANOVA, Dunnett’s post hoc test.

In cell lines overexpressing APP, the majority of APP is known to localize to the Golgi apparatus and trans-Golgi network, and only a small fraction of nascent APP molecules (~10%) reach the plasma membrane12. We also observed enrichment of APP in the Golgi apparatus in N2a-APPswt cells (ratio of Golgi to cytoplasm for APP mean density: 12), but we did not observe further accumulation of APP in the Golgi as a result of reduced WAVE1 expression by siRNA knockdown (Supplementary Fig. 7a). Decreasing WAVE1 expression with siRNA also did not alter the turnover rate of Aβ peptides (Supplementary Fig. 7b,c) or protein levels of the γ-secretase complex, BACE1 or NICD (as a readout of γ-secretase activity) (Supplementary Fig. 7d–i). Together these results suggest that down-regulation of WAVE1 results in decreased surface amounts of APP and reduced production of Aβ by the amyloidogenic pathway by reducing vesicular trafficking of APP-containing vesicles from the Golgi. This conclusion is consistent with a growing appreciation for the role of actin polymerization mediated by WASP- and WAVE-family proteins in the facilitation of transport carrier biogenesis21,22.

To assess the behavioral consequences of reduced WAVE1 expression, we tested wild-type mice and 2×Tg mice harboring Wasf1+/+ or Wasf1−/- using the Morris water maze task. We did not test Wasf1−/- mice with this task because of a previously characterized sensorimotor retardation phenotype4. There were no significant differences between the groups in swim time (Supplementary Fig. 8a) or speed (Supplementary Fig. 8b) during the spatial acquisition sessions (sessions 1–4; hidden platform), which indicated that there was no difference.
Figure 3  WAVE1 facilitates budding of APP-containing vesicles from the Golgi apparatus. (a–c) Immunocytochemistry of WAVE1 and APP in N2a-APPsw.1Δ9 cells (a, low magnification; c, high magnification; a and c show different cells) and in N2a-APPwt cells (b). Cells were infected with a viral vector expressing a Golgi-targeting sequence fused to GFP (GFP-Golgi). DAPI counterstaining was used to show nuclei. Line scan (white line in “Merge2” in c) showed coinciding fluorescence signals (blue arrows) for WAVE1, APP and GFP-Golgi (rightmost panel in c). Scale bars, 10 µm (a,b) and 2 µm (c). White arrows (a,b) and arrowheads (c) indicate colocalization of WAVE1 and APP in the Golgi apparatus. A.U., arbitrary units. Images are representative of three independent experiments. (d) A detergent-soluble membrane fraction from N2a-APPwt cells was used for immunoprecipitation with preimmune IgG or anti-WAVE1. WAVE1 pull-downs were probed with anti-WAVE1 or anti-APP antibodies (fold). All levels of APP were reduced by WAVE1 siRNA in vitro budding assay. Reconstituted Golgi membrane and cytosol were incubated at 37 °C for the indicated times, and released vesicles were separated from Golgi membrane by centrifugation. Images (representative of six independent experiments) show the time course of APP-containing vesicle formation (e) and the time-dependent fold increase in APP-containing vesicle formation (f, n = 6, six independent experiments). Data represent mean ± s.e.m. P < 0.0001 for all comparisons of control versus WAVE1 siRNA in f (two-way ANOVA).

in learning. There were also no notable differences between the groups during the cued session when the platform was visible (Supplementary Fig. 8a,b). During the probe trial, we removed the platform and measured the number of platform crossings made where the platform had been located (Fig. 4a) and the amount of time spent swimming in the target quadrant (Fig. 4b). The 2×Tg mice expressing Wasp1−/+ made significantly fewer platform crossings and spent significantly less time in the target quadrant than their non-2×Tg (wild-type) littermates did, indicating a memory deficit. These deficits were not observed in the 2×Tg mice heterozygous for WAVE1 deficiency, which suggested an ameliorated memory deficit. These deficits were not observed in the 2×Tg mice heterozygous for WAVE1 deficiency, which suggested an ameliorated memory deficit.

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nucleus and negatively regulates transcription of WASF1. Decreased WAVE1 expression then retards APP trafficking from the Golgi to the plasma membrane and endosomes, resulting in reduced Aβ production. Thus, the lower expression of WASF1 observed in AD patients compared with controls might reflect negative feedback and homeostatic control for Aβ during disease progression. Previously we characterized WAVE1 as an activity-regulated phosphoprotein in neurons. Recent studies have shown that APP processing is regulated by neuronal activity. Whether WAVE1 signaling pathways are involved in neuronal activity–regulated APP processing remains to be addressed. The role of AICD in AD has remained obscure, probably because of the complex regulation of various downstream targets. Our current study elucidated a signaling mechanism for homeostatic regulation of the amyloidogenic pathway by AICD, and our findings suggest a new pathway with potential for the development of therapeutic agents. In particular, WAVE1 interaction with the cytoplasmic domain of APP could be targeted to achieve specific inhibition of APP vesicular trafficking.

METHODS

Methods and any associated references are available in the online version of the paper.
promoter–luciferase assays. V.B. and I.C. analyzed AICD and Aβ expression. Y.K and I.C. performed metabolic pulse labeling and chase experiments and in vitro budding assays. C.R. analyzed WAVE1 expression in human samples. X.Z. prepared mice. J.G. performed behavioral experiments. G.M., M.B., S.M.S. and Y.K. performed immunocytochemical experiments and data analysis.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Animals. All procedures involving animals were approved by The Rockefeller University Institutional Animal Care and Use Committee and were performed in accordance with the National Institutes of Health guidelines. 3×Tg (jax MMRRC, 034830) and 2×Tg (jax MMRRC, 034832) AD mice were purchased from the Jackson Laboratory. Tg-APPsw AD mice were purchased from Taconic (Model 2789). The constitutive Wasf1-knockout mice were generated by Orgene Pty. Ltd. (Australia) and maintained at The Rockefeller University. We produced the progeny using in vitro fertilization and embryo-transfer techniques to generate the number of animals needed for the biochemical and behavioral tests. Mice were assigned to experimental groups according to genotype. Animal samples were selected from different experimental groups for biochemical analyses randomly and in a blinded fashion. The entire water maze test was run by an experimenter who was blinded to genotype-group allocation. No blinding was done for measurements of WAVE1 expression.

Cell culture and transfection. Wild-type mouse N2a neuroblastoma cells, N2a-APPwt cells, and N2a-APPsw.PS1ΔE9 cells were maintained in medium containing 50% DMEM and 50% Opti-MEM, supplemented with 5% FBS, antibiotics and 200 µg/ml G418 (for the established cell lines only; Invitrogen). We confirmed that all cell lines used in this study were free of mycoplasma contamination (LookOut Mycoplasma PCR Detection Kit, Sigma-Aldrich). For transient transfections of plasmids encoding APP695, APP695sw, AICD, AICD-3×Flag, APP1P-ICD–3×Flag, APP2P-ICD–3×Flag, NIDC-3×Flag, mNotchAE, MME promoter–luciferase and WASF1 promoter–luciferase, we used Fugene 6 (Roche) or Lipofectamine 2000 (Invitrogen). The On-TARGET sequence was AACGATGAGAAAGGGTCTGCG. The cDNA sequence of human APLP1-ICD was amplified from pCAX-APLP1 (Addgene, 30141). The cDNA sequence of human APLP2-ICD was amplified from p3×Flag–CMV7-NICD (Addgene, 20183). The nucleotides of AICD50 were synthesized (Genewiz) and subcloned into the HindIII–BamHI site of 3×Flag–CMV-14 pCMV-Tag4A (Stratagene). 3×Flag-tagged (at C terminus) AICD50 was generated using a 3×Flag-fusion mammalian expression vector system. The cDNA of AICD50 was subcloned into the HindIII–BamHI site of 3×Flag–CMV-14 (Sigma-Aldrich) for 3×Flag-tagging of the C terminus. The cDNA sequence of human Nicd was amplified from p3×Flag–CMV7-NICD (Addgene, 20183). The cDNA sequence of human APLP1-ICD was amplified from pCAX-APLP1 (Addgene, 30141). The cDNA sequence of human APLP2-ICD was amplified from pcDNA-TO-myc-His A-APLP1 (Abgent, D00166). We generated 3×Flag-tagged (at C terminus) NICD, APP1P-ICD and APP2P-ICD by subcloning into the EcoRI–BamHI site of 3×Flag–CMV-14.

Plasmids. pCB6-APP695wt and pCB6-APP695sw were reported previously10. The nucleotides of AICD48 were synthesized (Genewiz) and subcloned into pcMV-Script. The cDNA sequence of human AICD50 was amplified from pCB6-APP695. The full-length sequence of AICD50 was cloned into pGEM-T Easy vector (Promega). Successful clones were confirmed by sequencing. Full-length human AICD50 was also subcloned into the Not I–EcoRI 1 site of pcMV-Tag4A (Strategene). 3×Flag-tagged (at C terminus) AICD50 was generated using a 3×Flag-fusion mammalian expression vector system. The cDNA of AICD50 was subcloned into the HindIII–BamHI 1 site of 3×Flag–CMV-14 (Sigma-Aldrich) for 3×Flag-tagging of the C terminus. The cDNA sequence of mouse Nicd was amplified from p3×Flag–CMV7-NICD (Addgene, 20183). The cDNA sequence of human APLP1-ICD was amplified from pCAX-APLP1 (Addgene, 30141). The cDNA sequence of human APLP2-ICD was amplified from pcDNA-TO-myc-His A-APLP1 (Abgent, D00166). We generated 3×Flag-tagged (at C terminus) NICD, APP1P-ICD and APP2P-ICD by subcloning into the EcoRI–BamHI 1 site of 3×Flag–CMV-14.

SDS-PAGE and immunoblotting. Cultured cells or mouse hippocampal tissues were lysed in buffer A (50 mM Tris-HCl, pH 7.5, 150 mM NaCl and 2 mM MgCl2) supplemented with 1% Triton X-100, a protease-inhibitor cocktail (Complete, EDTA-free, Roche) and phosphatase inhibitors (30 mM NaF, 1 mM orthovanadate and 30 mM pyrophosphate). Cell and tissue lysates were sonicated twice with a probe-type sonicator (Branson) for 10–20 s, and the protein level in the lysates was measured via the BCA or the Bradford method. For sAPPβ and sAPPα, conditioned cell culture media were analyzed. The samples were boiled in standard protein sample buffer and subjected to SDS-PAGE with 4–20% Tris-glycine gels (Life Technologies) followed by protein transfer onto a nitrocellulose membrane. For detection of the N-terminal fragment of presenilin 1, the samples were not boiled, and Tricine gels (Life Technologies) were used. Pre-stained protein-size markers were used (Amersham full-range rainbow molecular weight markers, GE Healthcare; SeeBlue Plus2 Pre-stained Standard, Invitrogen). Immunoblotting was done according to a standard protocol with the following antibodies: anti–C-terminal WAVE1 (rabbit polyclonal, 1:5000); anti-WAVE1 (84-79, mouse monoclonal, 1:1,000, EMD Millipore, 05-618), and anti-APP (6E10, mouse monoclonal, 1:1,000, Covance, SIG-393320) for total APP and sAPPα; and anti-Giantin (rabbit polyclonal, 1:5,000, Abcam, ab24586), anti-GM130 (35/GM130, mouse monoclonal, 1:1,000, BD Transduction Laboratories, 610823), anti-BIP (40/BIP, mouse monoclonal, 1:1,000, BD Transduction Laboratories, 610978), anti-actin (rabbit polyclonal, 1:1,000, Cytoskeleton, AAN-01-A), anti-sAPPβ (a generous gift from Dr. William J. Netzer, The Rockefeller University), anti-N–terminus (PS1 Ab14, rabbit polyclonal, 1:100); anti-Pen2 (EP9200, rabbit monoclonal, 1:1,000, Abcam, ab154830), anti-Notch (Cleaved Notch1 (Val 1744), rabbit polyclonal, 1:1,000, Cell Signaling, 2421), anti-Aph1 (anti-Aph1a, rabbit polyclonal, 1:100, Invitrogen, 38-3600); anti-Nicstrin (35/Nicstrin, mouse monoclonal, 1:1,000, BD Transduction Lab, 612290), and anti-BACE1 (B690 C-terminal, rabbit polyclonal, 1:500, BioLegend, 840101). Flag-tagged ICDs were detected by immunoblotting with anti-Flag (rabbit polyclonal, 1:1,000, Sigma-Aldrich, F7425) after immunoprecipitation with anti-Flag (M2, mouse monoclonal, Sigma-Aldrich, F3165).

Immunoprecipitation. Cultured cells were lysed by sonication in buffer A (plus supplements of inhibitors, but without Triton X-100). After centrifugation at 100,000g for 10 min, the supernatant was discarded and the pellet (membrane fraction) was solubilized with buffer A (plus supplements of inhibitors and 1% Triton X-100). The supernatant obtained after 10 min of centrifugation at 100,000g was used as the detergent-soluble fraction. The detergent-soluble fraction was incubated at 4 °C overnight with anti–C-terminal WAVE1 or control antibody–coupled A/G agarose beads (Pierce) with constant rotation. After three washes with buffer A containing 1% Triton X-100, bound proteins were eluted by being boiled in the SDS sample buffer for 3 min. Samples were subjected to SDS-PAGE with 4–20% Tris-glycine gels and immunoblot analysis with anti-WAVE1 and anti-APP.

RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR). Total RNA was extracted with the RNeasy Plus Mini Kit (Qiagen) according to the manufacturer’s protocol. One microgram of total RNA was converted to complementary DNA with Superscript II reverse transcriptase (Invitrogen) and oligo-(dT)12–18 primers (Bioline) according to the manufacturer’s instructions. qRT-PCR was performed in a 20-µl reaction mixture containing 1 µl complementary DNA, 10 µl Taqman primers for each gene. The Applied Biosystems). Comparative quantitation of each target gene was done on the basis of the cycle threshold (CT), which was normalized to Gapdh.

Enzyme-linked immunosorbent assay (ELISA) for Aβ. Analysis of Aβ40 and Aβ42 was done with an ELISA kit according to the manufacturer’s instructions (Life Technologies). Briefly, supernatants of conditioned media from N2a-APPsw.PS1ΔE9 cells were diluted in a buffer provided with the kit and incubated for 3 h at room temperature in a 96-well plate with antibody to Aβ40 or Aβ42. After carrying out washes to remove unbound material, we added a secondary antibody conjugated to horseradish peroxidase (HRP) for 30 min. Unbound secondary antibody was washed away and subsequently samples were incubated with a developing reagent for 30 min. A stop solution was added to block further reaction between HRP and the colorimetric substrate. An absorbance multiplate reader was used to quantify the colorimetric reaction at 450 nm. For the analysis of mouse brains, 4–5-month-old male mice were euthanized by CO2 followed by decapitation. Brains were removed, frozen...
on dry ice and stored at −80 °C until use. Half-brains were homogenized in 5 volumes of Tris-buffered saline (Sigma-Aldrich) containing protease inhibitors (Roche Diagnostics, Mannheim, Germany) and then centrifuged at 50,000g at 4 °C for 20 min. The resulting pellet was resuspended in 70% formic acid and sonicated for 30 s. The samples were neutralized in 10 volumes of neutralization buffer (1 M Tris-base, 0.5 M Na₂HPO₄, 0.05% NaN₃ pH 9.0), and the amount of Aβ40 or Aβ42 was measured by ELISA. A preliminary study showed that a water-soluble fraction yielded negligible amounts of Aβ40 and Aβ42 from brains of mice 4–5 months of age. Although we analyzed a formic acid–soluble fraction in this study, the size of plaques in mice that age was not big enough to be detectable by immunohistochemistry.

Chromatin immunoprecipitation. ChIP assay was done with the EZ ChIP Kit (Millipore) according to the manufacturer’s instructions. DNA was crosslinked with formaldehyde (1% final concentration for 10 min). N2a cells transfected with 3×FLAG-tagged AICD were lysed in the presence of protease inhibitors. Lysates were sonicated with a Bioruptor (Diagenode) to shear DNA (average size, 200–600 bp). Immunoprecipitation was done with 1 µg of mouse anti-FLAG M2 (Sigma-Aldrich), 1 µg of anti–RNA polymerase II as a positive control or 1 µg of normal mouse IgG as a negative control. Immune complexes collected with protein A/G agarose were decrosslinked by the addition of 200 mM NaCl followed by incubation at 65 °C for 4–5 h. Uncrosslinked samples were treated with RNase and Proteinase K and loaded onto polypropylene spin columns. After columns had been washed, DNA was eluted in a low-salt buffer (the columns, washing and eluting buffers were included in the kit). Conventional PCR was done with primers 5′-GGGCGACAGCTGGTATATGT-3′ and 5′-CTTTCACCAACGCAGCATCTT-3′. The primers were designed with the software Primer3 WWW primer tool, available at http://bioinformatics.umd.edu/bioapps/primer3_www.cgi. Real-time PCR data are represented as a percentage of input.

Cell-surface biotinylation. N2a–APPSwe.PS1ΔE9 cells were cultured on a 60-mm dish and transfected with siRNA targeting Wasf1 or control siRNA. 48 h after transfection, cells were washed three times with ice-cold 1× phosphate-buffered saline (PBS) containing 2.5 mM CaCl₂ and 1 mM MgCl₂ (pH 8.0). Cells were then incubated with either ice-cold PBS (negative control) or ice-cold PBS plus 10 mM Sulfo-NHS-SS-biotin at 4 °C for 30 min. Cells were then washed one time with ice-cold 50 mM Tris (pH 8.0) twice with ice-cold PBS (pH 8.0) to quench the reaction before being lysed in 1× RIPA buffer containing protease inhibitors (EMD Millipore). Cell lysate from each condition was incubated with 50 µl streptavidin-coupled magnetic beads (Invitrogen) while rotating on a tumbler wheel for 1 h at 4 °C. The streptavidin–coupled beads were then washed four times with ice-cold 1× PBS (pH 7.0) containing 0.01% Tween-20 and protease inhibitors. Samples were centrifuged at 5,000 r.p.m. at 4 °C for 5 min and resuspended in Laemmli buffer. Finally, samples were incubated at 55 °C for 15 min and beads were removed with a magnet before analysis by SDS-PAGE and immunodetection with RU-369 antibody (rabbit polyclonal, 1:1,000), which recognizes the C terminus of APP695 (ref. 36).

Wasf1 promoter– and MME promoter–luciferase assays. N2a cells were cotransfected with a plasmid vector expressing luciferase under the human Wasf1 promoter, the MME promoter or a control vector with an empty promoter (a plasmid expressing only luciferase without any gene promoter) (SWITCHGEAR Genomics) with a plasmid expressing APPswe, AICD or a control vector with an empty plasmid. E9 cells were cultured on a 60-mm dish and transfected with siRNA targeting Wasf1 or control siRNA. 48 h after transfection, cells were washed three times with 1× Dulbecco’s phosphate-buffered saline (PBS) (1× Dulbecco’s phosphate-buffered saline, Invitrogen). They were then transferred into 24-well dishes. Triton X-100 (3% in DPBS) was added with DPBS (1× Dulbecco’s phosphate-buffered saline, Invitrogen). They were incubated at 5,000 r.p.m. at 4 °C for 5 min and resuspended in Laemmli buffer. Finally, samples were incubated at 55 °C for 15 min. The resulting pellet was resuspended in 70% formic acid and sonicated for 30 s. The samples were neutralized in 10 volumes of neutralization buffer (1 M Tris-base, 0.5 M Na₂HPO₄, 0.05% NaN₃ pH 9.0), and the amount of Aβ40 or Aβ42 was measured by ELISA. A preliminary study showed that a water-soluble fraction yielded negligible amounts of Aβ40 and Aβ42 from brains of mice 4–5 months of age. Although we analyzed a formic acid–soluble fraction in this study, the size of plaques in mice that age was not big enough to be detectable by immunohistochemistry.

Immunocytochemistry. Cells were grown on poly-1-lysine–coated coverslips (12 mm) in well dishes and were infected with viral vector expressing a fluorescent protein–fused Golgi-targeting sequence from a marker (human Golgi-Golgi-resident enzyme N-acetylgalactosaminyltransferase 2) (GFP Golgi) (Invitrogen) 16 h before fixation. Cells were fixed with 1 ml of 4% parafomaldehyde for 20 min. After fixation, coverslips were washed three times with DPBS (1x Dulbecco’s phosphate-buffered saline, Invitrogen). They were then transferred into 24-well dishes. Triton X-100 (3% in DPBS) was added to each well for 15 min to permeabilize cell membranes. For blocking, 3 ml of 10% normal goat serum (NGS) (Thermo Scientific) or 10% bovine serum albumin (BSA) was added to each well for at least 1 h. After blocking, coverslips underwent a single wash with DPBS. Primary antibodies were diluted in 1% NGS or BSA and added to each well for overnight incubation at 4 °C. The primary antibodies used were anti-WAVE1 (rabbit polyclonal, 1:1,000, V0101 (ref. 37)), anti-APP (6E10, mouse monoclonal, 1:500, Covance, SIG-39320), anti-GM130 (35/GM130, mouse monoclonal, 1:500, BD Transduction Laboratories, 610823) and anti-Giantin (rabbit polyclonal, 1:1,000, Abcam, ab24586). The next day, the coverslips were washed three times with PBS. Secondary antibodies (conjugated to Alexa Fluor 488, 568 and 635/647 dyes, 1:500, Invitrogen) were diluted in 1% NGS or BSA and added to each well for 1 h at room temperature. Coverslips were then washed three times with PBS, removed from their wells, and mounted on microscope slides using ProLong Gold Antifade reagent with or without DAPI mounting medium. After drying overnight, each coverslip was secured to its slide with nail polish.
Brain perfusion and immunohistochemistry. 12-month-old 3×Tg and age-matched control male mice were deeply anesthetized with Nembutal and transcardially perfused with 10 ml of PBS followed by 40 ml of 4% paraformaldehyde (PFA) in PBS. Brains were dissected, post-fixed for 1 h in PFA and cryoprotected in different percentages of sucrose in PBS (wt/vol) at 4 °C (5% for 1 h, 15% overnight, and 30% overnight). Brains were placed in an embedding mold filled with Neg-50 embedding medium (Richard Allan Scientific, Kalamazoo, MI) for 1 h at room temperature and were subsequently incubated on dry ice for 1 h to freeze the embedding medium. Brains were then stored at −80 °C until being sectioned. Sagittal sections (12 µm) were cut, mounted on glass slides, kept at −20 °C overnight, and then transferred to −80 °C until used for immunohistochemistry. Slides were thawed and dried at room temperature for 20 min and washed with PBS. Then, sections were blocked in 2% normal serum (vol/vol) at room temperature for 1 h and incubated overnight at 4 °C with primary antibody to APP (6E10, mouse monoclonal, 1:1,000, Covance, SIG-39320) or WAVE1 (rabbit polyclonal, 1:1,000, V0101 ref. 37)). The specificity of anti-WAVE1 was previously demonstrated by 1:1,000, Covance, SIG-39320) or WAVE1 (rabbit polyclonal, 1:1,000, V0101 incubated on dry ice for 1 h to freeze the embedding medium. Brains were until used for immunohistochemistry. Slides were thawed and dried at room temperature for 20 min and washed with PBS. Then, sections were blocked in 2% normal serum (vol/vol) at room temperature for 1 h and incubated overnight at 4 °C with primary antibody to APP (6E10, mouse monoclonal, 1:1,000, Covance, SIG-39320) or WAVE1 (rabbit polyclonal, 1:1,000, V0101 (ref. 37)). The specificity of anti-WAVE1 was previously demonstrated by immunohistochemistry with the Wasf11−/− brain3 and was also confirmed by us. The next day, sections were washed and incubated with Alexa Fluor− conjugated secondary antibodies (1:500, Invitrogen) at room temperature for 1 h. Slides were mounted with Prolong Gold Antifade containing DAPI (Life Technologies) and dried overnight, and fluorescence was visualized on a Zeiss confocal microscope.

Image acquisition and quantification. Fluorescent images were taken with a confocal microscope (Zeiss LSM 710) with an oil-immersion lens (Plan-Apochromat 100×/1.40 N.A. oil-immersion differential interference contrast) and a 2× digital zoom. DAPI was excited with a 405-nm-emitting diode laser; Alexa Fluor 488 or organelle markers labeled with GFP (Cell Light fluorescent proteins, Invitrogen) were excited with an argon laser (488 nm); Alexa Fluor 568 dye was excited with a 561-nm diode-pumped solid-state laser; and Alexa Fluor 633 and 647 dyes were excited with an HeNe laser (633 nm). To obtain line scans to test for colocalization, we plotted the fluorescence-intensity signals for WAVE1, APP and Golgi-GFP against the distance (µm) for all three colors (Fig. 3c). An average of five pixels was used to obtain line scans for the data shown in Supplementary Figure 4. For the quantification of APP levels in the Golgi apparatus, the z slice with the strongest Golgi signal was chosen. The images were analyzed with MetaMorph software (version 7.7.8). The immunostaining of a Golgi marker protein, Giantin, was used to define the Golgi membrane. Membranes were exposed to autoradiography film (LabScientific) and dried overnight, and fluorescence was visualized on a Zeiss confocal microscope.

Measurement of AICD expression. N2a cells transiently transfected with plasmids were gently scraped in cold PBS and collected by centrifugation at 12,000 r.p.m. for 1 min at 4 °C. Cell pellets were lysed in RIPA buffer (150 mM NaCl, 1.0% IGEPA, 60.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0, Sigma-Aldrich) containing protease inhibitors (Complete Mini, EDTA-free, Roche) and centrifuged at 12,000 r.p.m. for 20 min at 4 °C. The protein concentration of the supernatant was measured via the BCA method (Pierce). The supernatant (vesicles) was concentrated with a 30 kDa centrifugal filter (EMD Millipore) and subsequently stored at −80 °C.

Reconstitution. Golgi membranes (10 µg) were reconstituted with 15 µg of cystosol in a final volume of 150 µl of reaction buffer (12 mM Hepes-KOH, pH 7.2, 110 mM KCl, 2.5 mM MgCl2, 0.1 mM CaCl2) containing an energy-regenerating system consisting of 1 mM ATP, 0.2 mM GTP, 10 mM creatine phosphate and 80 µg/ml creatine phosphokinase. The reaction mixture was incubated at 37 °C for the times indicated in figures. Golgi membranes and vesicles were separated by centrifugation at 11,000g and 4 °C for 1 min. The pellet (Golgi membranes) was resuspended with 45 µl of Laemmli buffer and boiled for 3 min. The supernatant (vesicles) was concentrated with a Microcon 30-kDa centrifugal filter (EMD Millipore) and subsequently dried. The protein that remained on the filter membrane was recovered in 45 µl of Laemmli buffer with vigorous vortexing (the yield of APP protein recovery was more than 95%) and boiled for 3 min. Golgi membranes (5 µl) and vesicles (5 µl) were subjected to 8% Tris-Glycine SDS-PAGE followed by protein transfer onto nitrocellulose membrane and immunoblotting with anti-APP (6E10, mouse monoclonal, 1:1,000, Covance). The number of APP-containing vesicles (inferred from the amount of APP in the vesicle fraction) was quantified as the percentage of total APP (APP in Golgi membranes and vesicles), and the time-dependent fold increase in APP-containing vesicles was calculated.
Analysis of WASF1 expression in human brain tissue. All procedures involving human brain tissue were approved by the institutional review board of the Columbia University Medical Center. Expression profiling was done separately for amygdala (as the affected brain region), cerebellum and parietal-occipital neocortex (as unaffected brain regions) from 19 AD brains and 10 control brains from the New York Brain Bank (http://www.nybb.hs.columbia.edu). All the brains were from individuals with AD or healthy control subjects without clinical or pathological comorbidity, and high-quality RNA samples were available in the brain bank. This three-region analysis allowed us to determine whether the changes in expression patterns of candidate genes such as WASF1 are specific for late-onset AD and consistent with the distribution of AD pathology. For instance, WASF1 downregulation in AD brains was observed only in the amygdala, and not in the cerebellum or parietal-occipital neocortex. This selection of clean brain samples with internal-brain control tissues largely increased the a priori power. Based on power analysis, our sample size was adequate to detect small differences in mean expression level.

For expression profiling of AD and control brains, we used Affymetrix GeneChip Human Exon 1.0 ST Arrays. Frozen brain tissue was ground over liquid nitrogen and stored at −80 °C until use. Total RNA was extracted and purified using the Trizol Plus RNA purification kit (Invitrogen). Quantification and qualification of all RNA preparations was done with an Agilent Bioanalyzer (RNA 6000 nano-kit), and only samples with RNA integrity numbers (RINs) of ≥8 were used in the subsequent RNA amplification and hybridization steps. The GeneChip Expression two-cycle target-labeling kit (Affymetrix) was used for all samples according to Affymetrix protocols. Briefly, the procedure consists of an initial ribosomal RNA (rRNA) reduction step and two cycles of reverse transcription followed by in vitro transcription. For each sample, 1 µg of total RNA was initially subjected to RNA removal using the RibonMinus Transcripnome Isolation Kit (Invitrogen) and spiked with Eukaryotic PolyA RNA controls (Affymetrix). The rRNA-depleted fraction was used for complementary DNA (cDNA) synthesis by reverse transcription primed with T7-random hexamer primers followed by second-strand synthesis. This cDNA served as a template for in vitro transcription to obtain amplified antisense complementary RNA (cRNA). Subsequently, cRNA from the first round was reverse-transcribed using random primers to obtain single-stranded sense DNA. In this second reverse transcription, dUTP was incorporated into the DNA to allow for subsequent enzymatic fragmentation using a combination of UDG and APE1. All reverse and in vitro transcription steps were performed using the GeneChip WT cDNA synthesis and amplification kit (Affymetrix). The resulting fragmented DNA was labeled with Affymetrix DNA Labeling Reagent. Labeled fragmented DNA was hybridized to Affymetrix Human Exon 1.0 ST arrays, washed, and stained using the GeneChip Hybridization, Wash and Stain Kit. Fluorescent images were recorded on a GeneChip scanner 3000 and analyzed with the GeneChip operating software.

Morris water maze. The Morris water maze procedure was adapted from previous reports.40,41 The entire water maze test was run by an experimenter who was blinded to the genotype-group allocation. Testing was done in a white circular escape platform (covered in red tape; 10-cm diameter) for 15 s. A transparent acrylic escape platform (10 cm) was submerged 0.8 cm below the surface of the water in the northeast quadrant of the tank, where it remained for all trials during the acquisition sessions. One session was conducted on each of four consecutive days, and each session consisted of five trials. Each mouse's start position was varied for each trial, and each mouse was given 60 s to locate the escape platform, which it was allowed to sit upon for 15 s before being removed. If the mouse did not locate the platform within 60 s, then the experimenter led the mouse to the platform, where it sat for 15 s. The mouse was then dried and returned to the home cage for an intertrial interval of approximately 20 min. Swim time (seconds) and swim speed (centimeters per second) were recorded during each trial.

Spatial memory was evaluated in a probe trial, which occurred 1 d after the fourth session. During the probe trial (60 s), the platform was removed from the tank. The number of times that the mouse crossed the platform location (i.e., platform crossings) and the amount of time that the mouse spent swimming in the target quadrant (i.e., the quadrant that contained the platform during sessions 1–4) were measured. Higher numbers during the probe trial indicated better performance.

A nonspatial cued water maze test was conducted to evaluate potential sources of non-mnemonic contributions (e.g., swimming ability, visual acuity and motivation) to task performance. Cued testing occurred 1 d after the probe trial and consisted of five trials in one session. During the cued trials, we made the platform visible by raising it above the surface of the water (0.5 cm), covering it with red tape, and attaching a white circular flag to the side of the platform. The extra-maze cues surrounding the tank were removed. The platform was moved to a different location in the tank for each trial. Mice were given 60 s to locate the platform, and the intertrial interval was approximately 20 min. Swim time (seconds) and swim speed (centimeters per second) were recorded during each trial.

Mice included in the data analysis were in good health throughout behavioral testing. Spatial acquisition and cued water maze measurements were averaged within each group for each session. To evaluate the rate of task acquisition, we used ANOVA to analyze a data set from multiple sessions. We used three-way ANOVA with factors of 2×Tg mouse genotype (+/+, +/−, Wasf1−/−) and mouse age group (0–5, 6–10, 11–15 d old). The data passed normality tests. For the probe trial, we analyzed the number of platform crossings and the time spent in the target quadrant during the probe using an unpaired t-test (two-tailed) to compare behavioral performance between two groups, namely, wild-type (non-transgenic) mice and 2×Tg mice harboring Wasf1−/− or Wasf1+/− (GraphPad Prism). Outliers were defined as scores 2 s.d. above or below the group mean in the spatial acquisition task, cued task, or probe task and were excluded before statistical data analysis. Data from one wild-type mouse with Wasf1−/−, two wild-type mice with Wasf1+/−, and three 2×Tg mice with Wasf1+/− were excluded on the basis of this criterion.

Statistics. For all graphic data, n indicates the number of biological replicates. All quantitative data for N2a cells are representative of two or three independent experiments and were replicated at least two times. To determine the number of animals to use per group (for biochemical and behavioral experiments), we based our calculations on accumulated empirical data and on power analysis. The number of animals for each experiment was appropriate to detect biochemical and behavioral differences. Differences between groups were assessed using unpaired two-tailed t-test or ANOVA as indicated in figure legends. The data met the assumptions of the tests. When the variances in two groups were significantly different, we performed an unequal variance t-test (Welch's correction).

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