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# Autosomal Recessive Hypercholesterolemia Protein Interacts with and Regulates the Cell Surface Level of Alzheimer's Amyloid β Precursor Protein*

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**The familial Alzheimer's disease gene product amyloid β protein precursor (AβPP) is sequentially processed by β- and γ-secretases to generate the Aβ peptide. Although much is known about the biochemical pathway leading to Aβ formation, because extracellular aggregates of Aβ peptides are considered the cause of Alzheimer's disease, the biological role of AβPP processing is only recently being investigated. Cleavage of AβPP by γ-secretase releases, together with Aβ, a COOH-terminal AβPP intracellular domain, termed AID. Hoping to gain clues about proteins that regulates AβPP processing and function, we used the yeast two-hybrid system to identify proteins that interact with the AID region of AβPP. One of the interactors isolated is the autosomal recessive hypercholesterolemia (ARH) adapter protein. This molecular interaction is confirmed *in vitro* and *in vivo* by fluorescence resonance energy transfer and in cell lysates. Moreover, we show that reduction of ARH expression by RNA interference results in increased levels of cell membrane AβPP. These data assert a physiological role for ARH in AβPP internalization, transport, and/or processing.**

AβPP[1] is a ubiquitous type I transmembrane protein that undergoes extensive proteolytic processing along two major pathways (1–5). In the amyloidogenic pathway, AβPP is cleaved in the ectodomain by the β-secretase forming a C99 membrane-bound intermediate. C99 can be cleaved by the γ-secretase to release Aβ and the APP intracellular domain (AID). Alternatively, AβPP can be cleaved within the Aβ sequence by the α-secretase creating a C83 membrane-bound intermediate which produces P3 and AID after γ-cleavage. AβPP processing is firmly associated with the pathogenesis of Alzheimer's disease (AD) because mutations associated with familial forms of AD are found in AβPP itself and in the highly homologous genes *PS1* and *PS2*, which are key components of a multimolecular complex with γ-secretase activity (6–15).

Although the role of Aβ peptides in the pathogenesis of AD has been extensively studied, reports as to the role of AID are very recent. AID-like peptides have been identified in human brains from cases of sporadic AD (16) and play a role in apoptosis (16), transcription (17–20), and $Ca^{2+}$ release from the endoplasmic reticulum (21).

AβPP processing and AID signaling can be regulated by AβPP-interacting proteins (17–22). Thus, to find AβPP-binding proteins we employed the yeast two-hybrid selection system. This screening resulted in the identification of several proteins that bind the intracellular domain of AβPP. Here we report the novel AβPP interactor ARH, an adapter protein that has been shown to regulate cholesterol uptake by genetic studies (22). These data suggest that ARH may be a mediator of the well described effect of cholesterol metabolism on AβPP processing.

## MATERIALS AND METHODS

*Yeast Two-hybrid System*—The two-hybrid screening was conducted using the Matchmaker system from Clontech according to the manufacturer's instruction. For library screening, Yeast190 expressing GAL4BD-AID fusion proteins were transformed with a human fetal brain cDNA library cloned in the pACT2 vector (Clontech). $2 \times 10^6$ clones were analyzed. Transformed yeast were selected in synthetic drop-out plates lacking tryptophan, leucine, and histidine in the presence of 50 mM 3-aminotriazole (Sigma) and grown for 5 days at 30 °C. Colonies growing on selective media were scored as positive. Assays were done for eight independent transformants.

*cDNA Cloning and Constructs*—The GAL4BD-AβPP bait was constructed using the pAS2 vector (Clontech) and consisted of the COOH-terminal 58 amino acids of AβPP fused to the DNA binding domain of GAL4, respectively. AβPP, AβPPNcas, and AID were made as described previously (16). GST fusion proteins were made in pGEX vectors (Amersham Biosciences). Mutations were introduced by using the transformer site-directed mutagenesis kit (Clontech).

ARH, ARHth (clone AT60), ARHPTB (amino acid 48–175), and ARHΔPTB (amino acids 170–308) were cloned into pECFP-N1 (Clontech), pcDNA3.1, and FLAG-tagged pcDNA3.1 (Invitrogen) for expression in mammalian cells or *in vitro*. Fe65, Fe65N-PTB, and Fe65C-PTB were cloned in FLAG-pcDNA3. All constructs were confirmed by sequencing.

*Cell Lines and Transfections*—Human embryonic kidney (HEK) 293T cells were grown in RPMI 1640 media (Invitrogen) supplemented with glutamine and with 10% heat-inactivated fetal calf serum (Biofluids; Rockville, MD). Transfections were performed in 6-well plates either using Metafectene (Biontex Laboratories Gmbh) with 3 μl per 1 μg of DNA.

*Northern Blot Analysis*—A multitissue blot was purchased from Clontech, and it was hybridized with a $^{32}$P-labeled ARHPTB probe following the manufacturer's instruction. After washing, the blot was developed by autoradiography.

*GST Pull-down, Immunoprecipitation, and Immunoblot Analysis*—

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[1] The abbreviations used are: AβPP, amyloid β protein precursor; AD, Alzheimer's disease; ARH, autosomal recessive hypercholesterolemia; AID, APP intracellular domain; FRET, fluorescence resonance energy transfer; GST, glutathione *S*-transferase; DTT, dithiothreitol; YFP, yellow fluorescent protein; CFP, cyan fluorescent protein; HEK, Human embryonic kidney; siRNA, small interfering RNA; RNAi, RNA interference; FACS, fluorescence-activated cell sorter; PTB, phosphotyrosine-binding domain.

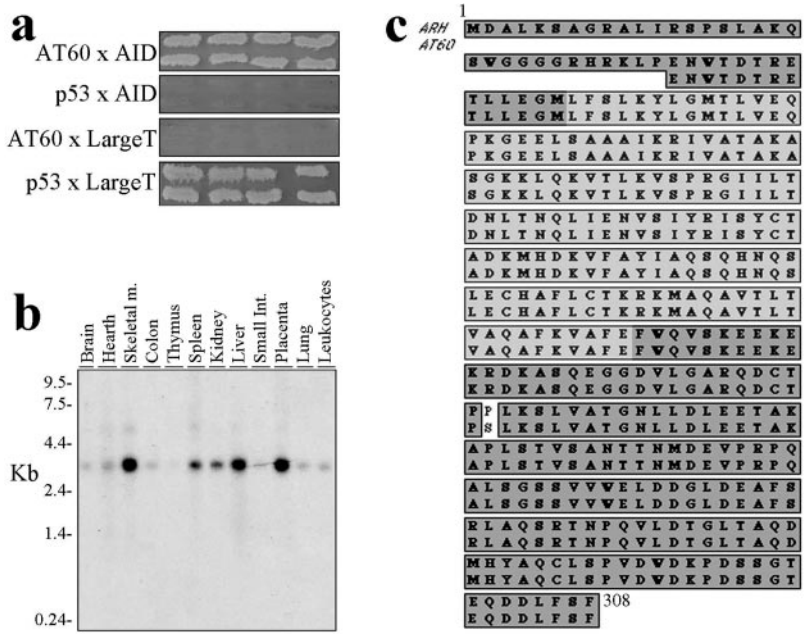


FIG. 1. **ARH interacts with the AβPP cytoplasmic region in yeast.** *a*, the interaction between AT60 and AID in yeast is specific because AT60 does not interact with SV40 large T and AID does not bind p53. The interaction between p53 and large T is shown as a positive control. *b*, Northern blot analysis showing that AT60/ARH is ubiquitously expressed in adult human tissues. *c,* sequence alignment showing that AT60 is identical to ARH. The AT60 clone is missing the $NH_2$-terminal 32 amino acids of ARH. The PTB domain is indicated by the *lighter shading.* The Pro to Ser mutation may represent a polymorphism of ARH.

Recombinant GST fusion proteins were expressed in *Escherichia coli* strain BL21 (Invitrogen) to make non-phosphorylated proteins and strain TKB1 (Stratagene) to make tyrosine-phosphorylated proteins using the pGEX system (Amersham Biosciences) (23). Proteins were purified using glutathione-Sepharose beads.

For GST pull down of proteins produced *in vitro* (Fig. 2*a*), [$^3$H]leucine-labeled proteins were made using the TNT-coupled *in vitro* transcription/translation system (Promega). After synthesis of the radiolabeled protein for 1.5 h, aliquots of the protein were incubated with GST fusion proteins bound to glutathione-Sepharose beads for 2 h at room temperature. The beads were then washed three times with lysis buffer T (1% Triton X-100, 50 mM Tris-HCl, pH 7.4, 45 mM NaCl) and boiled with SDS loading buffer with DTT. The proteins were separated by SDS-PAGE, and the gel were fixed with 50% methanol, 40% $H_2O$, 10% acetic acid. The gel was incubated in Amplify (Amersham Biosciences) for 20 min and dried, and signals were detected using autoradiography.

For GST-pull down of proteins produced *in vivo* (Fig. 2*b*), HEK293 cells were lysed in lysis buffer T containing a protease inhibitor tablet (Roche Applied Science) 24–48 h following transfection. Lysis was allowed to continue for 10 min on ice and was then spun down at full speed at 4 °C for 10 min. Some lysate representing the total lysate was removed and boiled with SDS loading buffer with DTT, whereas the rest was pulled-down with GST fusion proteins. The beads were washed five times with lysis buffer and boiled with SDS loading buffer with DTT. The proteins were separated by SDS-PAGE and blotted onto nitrocellulose (Bio-Rad). Membranes were probed with either αFLAG or rabbit polyclonal αARH (Zymed Laboratories Inc., new product catalog number 36-0400) followed by horseradish peroxidase-conjugated secondary antibodies (Southern Biotech). Proteins were detected using the Supersignal West Pico chemiluminescent system (Pierce).

For immunoprecipitation from transfected cells, lysates were immunoprecipitated for 2 h at room temperature with the αFLAG monoclonal antibody bound to agarose beads (Sigma). After washing and SDS-PAGE, membranes were probed with either the 22C11 monoclonal antibody directed against the ectodomain of AβPP (Calbiochem) or rabbit polyclonal αARH.

For co-precipitation of endogenous proteins, untransfected HEK293 cells were lysed as above. One mg of protein was used for immunoprecipitation with rabbit polyclonal αAPP COOH-terminal (Zymed Laboratories Inc., new product), rabbit polyclonal αARH, or rabbit anti-mouse IgG antibodies (ICN, Aurora, OH). Immunoprecipitations were performed overnight at 4 °C, followed by incubation of immunoprecipitates with protein A-agarose beads, washing, and immunoblot as described above.

*FRET Analysis*—HEK293T cells were plated in 24-well plates and co-transfected with the CFP and YFP fusion proteins using Metafectene. All transfections contained ratios between the two cDNAs determined empirically to yield the best co-expression as follows. Cells were harvested between 18 and 24 h after transfection in their conditioned media. The analysis was performed as described previously (24).

*RNA Interference*—HEK293T cells were plated in 24-well plates and transfected with the following small interfering (si) RNA duplex using TransIT-TKO (Mirus): Scramble (CAGUCGCGUUUGCGACUGGdTdT and CCAGUCGCAAACGCGACUGdTdT); ARH (GCCUUGAGUGGCAGCAGUGdTdT and CACUGCUGCCACUCAAGGCdTdT); and AβPP (CUUGCAUGACUACGGCAUGdTdT and CAUGCCGUAGUCAUGCAAGdTdT).

*FACS Analysis*—For FACS analysis HEK293 cells were collected with phosphate-buffered saline, 0.5 mM EDTA, washed once with FACS media (phosphate-buffered saline, 2% fetal bovine serum), and stained for 1 h at 4 °C in rotation with 2.5 μg/ml P2-1 antibody (BIOSOURCE 44-100) or with the IgG1 isotype control antibody P3 (gift of Dr. Porcelli). Cells were rinsed twice with FACS media and stained with Red-phycoerythrin-conjugated secondary antibody (Southern Biotech 1030-09). Cells were analyzed by FACSCalibur (BD Biosciences).

## RESULTS AND DISCUSSION

To identify proteins interacting with the cytoplasmic domain of AβPP, we have used the yeast two-hybrid selection system. This screening resulted in the identification of two independent clones coding for a novel protein (the interaction in yeast between AID and the representative AT60 clone is shown in Fig. 1*a*). Northern blot analysis showed that AT60 is expressed, albeit at different levels, in all human tissues analyzed (Fig. 1*b*). A recent blast search showed that AT60 is identical to the recently identified ARH adapter protein (22) (Fig. 1*c*). Homozygous ARH mutations cause familial forms of autosomal recessive hypercholesterolemia (ARH). ARH contains a phosphotyrosine-binding domain (PTB), which is known to interact with the PTB-binding motif present in the cytoplasmic tail of AβPP ($^{682}$YENPTY$^{687}$) (19, 23, 24, 28–32). Tyrosine phosphorylation of Tyr$^{682}$ of AβPP is important for its interaction with ShcA and ShcC (23). On the contrary, other binding partners interact with AβPP regardless of Tyr$^{682}$ phosphorylation. Therefore, we sought to determined whether the PTB domain of ARH mediated binding to the $^{682}$YENPTY$^{687}$ of AβPP and whether phosphorylation of AβPP was necessary for binding of ARHs. First, $^3$H-labeled ARH (ARHPTB), Fe65 COOH-terminal (Fe65C-PTB, which binds AβPP), and $NH_2$-terminal (Fe65N-PTB, which does not bind AβPP, Refs. 28 and 30) PTB domains were produced and incubated with GST-AID or GST alone (all bound to glutathione-Sepharose beads). The protein-protein com-

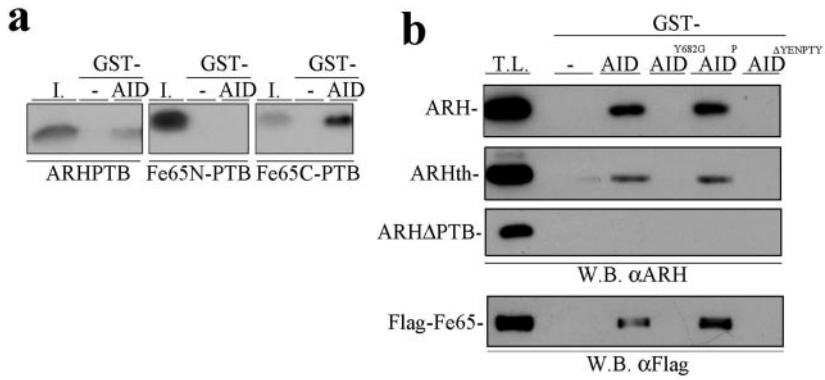


FIG. 2. **ARH interacts with AβPP directly, and the interaction is mediated by the PTB domain of ARH and the PTB-binding motif of AβPP.** *a*, *in vitro* transcribed and translated (i) ARHPTB, Fe65C-PTB (positive control protein), and Fe65N-PTB (negative control protein) were incubated with either GST (−) or GST-AID (AID) fusion proteins. *b*, HEK293T cells were transfected with constructs coding for ARH, ARHth, ARHΔPTB, and FLAG-Fe65. Protein lysates were incubated with the indicated recombinant proteins. After precipitation and gel separation, samples were transferred onto nitrocellulose membranes and probed with either an anti-ARH (Western blot (*W.B.*) *αARH*) or an anti-FLAG (*W.B. αFLAG*) antibody.

plexes were purified, resolved by gel electrophoresis, and visualized by autoradiography. Fig. 2*a* shows that GST-AID specifically and directly binds both ARHPTB and Fe65C-PTB but not Fe65N-PTB. Next, HEK293 cells were transfected with constructs expressing either ARH or ARHΔPTB (a mutant lacking the PTB domain) of FLAG-tagged Fe65. Cell lysates were incubated with GST-AID, GST-AID phosphorylated on $Tyr^{682}$ ($GST\text{-}AID^{P}$), $GST\text{-}AID^{Y682G}$ (in which $tyrosine^{682}$ was mutated to glycine), or GST, and pull downs were resolved by gel electrophoresis. Western blot analysis (Fig. 2*b*) using either αARH or αFLAG antibodies revealed that ARH, like Fe65, interacted with AID, whereas ARHΔPTB did not. We also found that, similarly to Fe65, AβPP binds ARH independent of $Tyr^{682}$ phosphorylation, but this binding is abolished by the Y682G mutation.

In further experiments, HEK293 cells were co-transfected with FLAG-ARH and the following AβPP constructs: wild type AβPP, AβPPNcas (an AβPP mutant lacking the COOH-terminal 31 amino acids that include the YENPTY motif), $AβPP^{Y682F}$ (in which $Tyr^{682}$ was mutated to phenylalanine), $AβPP^{Y687A}$ (in which $Tyr^{687}$ was mutated to alanine), or $AβPP^{T653F}$ (in which $Thr^{653}$ was mutated to phenylalanine). Cell lysates were immunoprecipitated with αFLAG monoclonal antibody and analyzed by Western blot. Fig. 3*a* shows that although FLAG-ARH immunoprecipitates AβPP, it does not immunoprecipitate AβPPNcas which lacks the YENPTY motif. Additionally, $AβPP^{Y682F}$ mutation significantly affects the interaction with ARH.

To detect interaction between ARH and AβPP in living cells, we used fluorescence resonance energy transfer (FRET) (25–27). HEK293T cells were co-transfected with yellow fluorescent protein-AID (Y-AID) and either cyan fluorescent protein-ARH (C-ARH) or C-ARHΔPTB fusion proteins. In FRET on living cells, if the proteins are in close proximity, on the order of 10 nm or less, the energy from the excitation of CFP will be transferred to YFP, and emission at the wavelength of YFP will be detected. If the proteins are not within this proximity, excitation of CFP is not transferred, and only emission at the wavelength of CFP is detected. Importantly, FRET was detected when Y-AID was co-transfected with C-ARH but not with C-ARHΔPTB. Altogether, these data indicate that ARH interacts with the YENPTY motif of AβPP through its PTB domain. Furthermore, this interaction is independent of $Tyr^{682}$ phosphorylation but requires the presence of $Tyr^{682}$.

To determine whether endogenous AβPP and ARH interact, we immunoprecipitated HEK293 lysates either with an αAβPP or an αARH polyclonal antibody. As shown in Fig. 4, which is

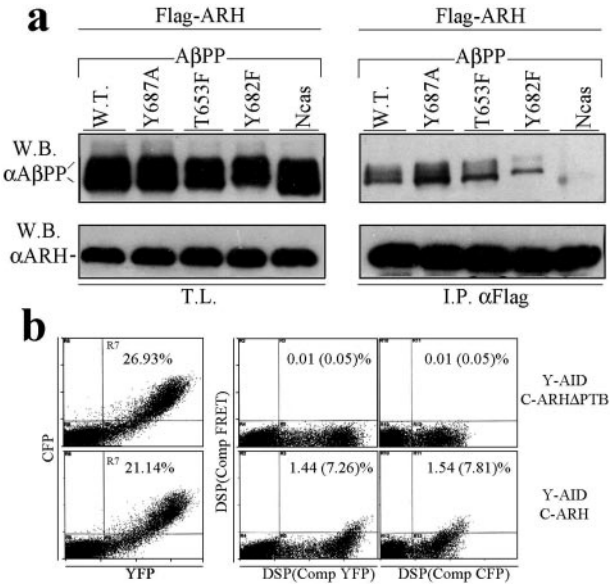


FIG. 3. **ARH interacts with AβPP *in vivo*.** *a*, immunoblot analysis of AβPP and ARH proteins. Equal amounts of cell lysate (*T.L.*) from HEK293T cells transfected with the FLAG-tagged ARH and the indicated AβPP constructs. Expression of AβPP constructs was monitored by Western blot (*W.B.*) analysis with the anti-AβPP antibody 22C11 (*W.B. αAβPP*), whereas expression of ARH was assessed by hybridizing immunoblots with an anti-ARH antibody (*W.B. αARH*). Lysates from transfected cells were immunoprecipitated with the anti-FLAG antibody (*I.P. αFLAG*). After SDS-PAGE, immunoprecipitated samples were analyzed by Western blot using either an anti-ARH or anti-AβPP antibody. *b*, representative plot of CFP *versus* YFP to check for co-transfection efficiency (*left panels*). The R7 gate of each sample was used to calculate the percentage of co-transfected cells exhibiting FRET. Dot plots of representative samples show the presence or lack of FRET. Each plot represents a single co-transfection of the fusion proteins YFP-AID (*Y-AID*) with either CFP-ARHΔPTB (*C-ARHΔPTB*), or CFP-ARH (*C-ARH*). Each *dot*, representing a single cell, is plotted based on the intensity of the FRET signal's intensity *versus* its CFP emission (*right column*), or FRET *versus* YFP emission (*middle panels*). Cells falling in the *upper right quadrant* were scored as FRET-positive cells. Percentages are calculated as the number of FRET-positive cells divided by the total number of cells or, in *parentheses*, as the number of FRET positive cells divided by the number of co-transfected cells (*R7 gate*).

representative of data from two independent experiments, AβPP was immunoprecipitated with both αAβPP and αARH antibodies, whereas AβPP was not immunoprecipitated with a control rabbit anti-mouse IgG antibody (RαM). Similar findings were obtained with the reverse experiment, that is ARH was

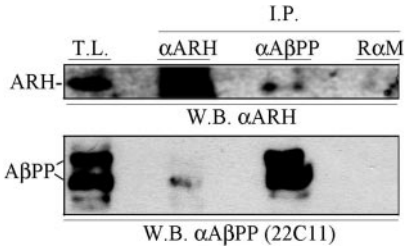


FIG. 4. **Endogenous AβPP and ARH interact in HEK293 cells.** Immunoprecipitations (*I.P.*) were done with rabbit anti-mouse IgG (*RαM*), αAβPP (Zymed Laboratories Inc. anti-COOH-terminal AβPP domain), and αARH antibodies. Western blotting (*W.B.*) was done with either αARH or αAβPP ectodomain antibody 22C11.

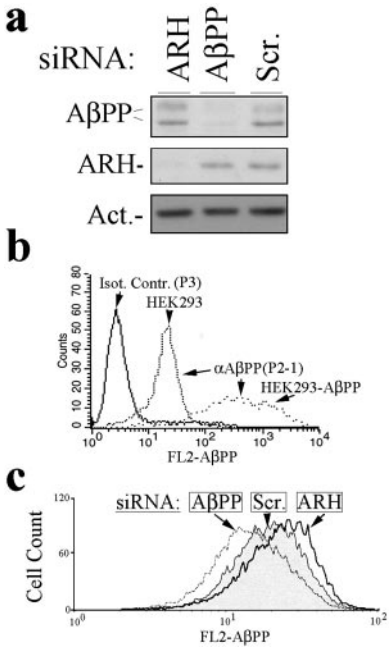


FIG. 5. **ARH depletion results in increased cell membrane AβPP levels.** *a*, HEK293 cells were transfected with the indicated siRNAs and analyzed by Western blot with the indicated antibodies 48 h later. *b*, endogenous AβPP expressed on the cell surface of HEK293 cells can be detected and quantitated using the anti-AβPP monoclonal antibody P2-1 in FACS analysis. The isotype control P3 does not stain these cells, and overexpression of AβPP (HEK293-AβPP) increases P2-1 staining. *c*, the levels of cell membrane AβPP are decreased in cells depleted of AβPP by RNAi. Conversely, reduction of ARH protein levels results in increase of cell surface AβPP. This experiment is representative of three independent experiments. *Scr.,* scrambled sequence.

immunoprecipitated by the αAβPP and the αARH antibodies but not by the RαM IgG control. Altogether, these experiments indicate that endogenous AβPP and ARH associate.

To determine whether ARH physiologically regulates AβPP biology, we have repressed ARH protein expression in HEK293 cell using RNA interference (RNAi), which involves the transfection of small interfering RNA (siRNA) duplexes in cells. We have designed siRNAs duplex specific for human ARH, AβPP, and a duplex of unrelated scrambled sequence. As shown in Fig. 5*a*, ARH and AβPP siRNAs specifically reduce expression of ARH and AβPP proteins, respectively. Because ARH is involved in of low density lipoprotein receptors endocytosis, we next assessed whether ARH may regulate the cell surface levels of AβPP. To this end, we have performed FACS analysis experiments. The monoclonal antibody P2-1, which is specific for the ectodomain of human AβPP, was used in this experiment. The isotype-matched and unrelated monoclonal antibody P3 was used as a negative control. Viable cells were initially stained with either P2-1 or P3. After washing out the unbound antibody, cells were incubated with a phycoerythrin-labeled anti-mouse IgG secondary antibody and analyzed by FACS. P2-1 specifically binds to the cell surface AβPP of HEK293 cells as determined by three complementary facts: (i) the isotype-matched P3 antibody does not bind HEK293 cells (Fig. 5*b*); (ii) overexpression of AβPP in HEK293 cells increases P2-1 binding (Fig 5*b*); (iii) conversely, reduction of AβPP protein levels by RNAi results in decreased P2-1 binding (Fig. 5*c*). Of interest, reduction of ARH protein levels by RNAi results in increased amounts of AβPP on the cell membrane of HEK293 cells (Fig. 5*c*). These data indicate that ARH physiologically regulates the cell surface level of AβPP.

In this study we have demonstrated *in vitro*, *in vivo*, in living cells, and for endogenous proteins the interaction between AβPP and ARH. Moreover, we have shown that ARH regulates AβPP cell membrane levels. These changes in AβPP cell surface levels in ARH-low cells reflect a physiological role for ARH in either AβPP internalization, transport to the cell membrane, and/or shedding of the ectodomain by secretases (α and/or β). Although further work will be required to discriminate among these possibilities, our data nevertheless prove a function for ARH in AβPP biology.

Genetic defects in ARH impair endocytosis of low density lipoprotein receptors family members (22, 33, 34), thereby reducing cholesterol uptake and increasing plasma concentration of cholesterol. Of interest, biochemical, epidemiological, and genetic evidence have involved cholesterol metabolism in the regulation of AβPP processing and the pathogenesis of AD (35–38). The finding that ARH interacts with AβPP and regulates the cell membrane levels of AβPP is provocative and entices us to speculate that ARH may be a molecular link between AβPP processing and cholesterol.

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# Autosomal Recessive Hypercholesterolemia Protein Interacts with and Regulates the Cell Surface Level of Alzheimer's Amyloid β Precursor Protein

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