1-1-2011

Wild type and Tangier disease ABCA1 mutants modulate cellular amyloid-beta production independent of cholesterol efflux activity

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Abstract

Cerebral amyloid-β (Aβ) deposition is a critical feature of Alzheimer's disease. Aβ is derived from the amyloid-β protein precursor (AβPP) *via* two sequential cleavages that are mediated by β-secretase and the γ-secretase complex. Such amyloidogenic AβPP processing occurs in lipid raft microdomains of cell membranes and it is thought that modulating the distribution of lipids in rafts may regulate AβPP processing and Aβ production. Certain ATP-binding cassette (ABC) transporters regulate lipid transport across cell membranes and, as recent studies reveal, within membrane microdomains. ABCA1 also regulates Aβ metabolism in the brain although its direct impact on AβPP remains an open question. Here we assessed the capacity of three ABCA1 mutants (that do not promote lipid efflux) to modulate AβPP processing. Unexpectedly, these non-functional mutants also reduced Aβ production similar to wild type ABCA1. ABCA1 expression did not alter AβPP localization in lipid rafts, and co-immunoprecipitation experiments indicated ABCA1 and AβPP physically interact. These data suggest that ABCA1 may regulate AβPP processing independent of its impact on membrane lipid homeostasis.

Keywords

independent, cholesterol, efflux, wild, activity, type, tangier, disease, abca1, mutants, modulate, cellular, amyloid, beta, production, CMMB

Disciplines

Life Sciences | Physical Sciences and Mathematics | Social and Behavioral Sciences

Publication Details

Woojin, K. S., Hill, A. F., Fitzgerald, M. L., Freeman, M. W., Evin, G. & Garner, B. (2011). Wild type and Tangier disease ABCA1 mutants modulate cellular amyloid-beta production independent of cholesterol efflux activity. Journal of Alzheimer's Disease, 27 (2), 441-452.

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Wild Type and Tangier Disease ABCA1 Mutants Modulate Cellular Amyloid-Beta Production Independent of Cholesterol Efflux Activity

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Short title: Tangier mutant ABCA1 inhibits Aβ production

Key words: ABCA1, AβPP processing, membrane biology, lipid transport, Tangier disease, Alzheimer's disease,

Abbreviations: ABCA1, ATP-binding cassette transporter A1; AβPP; amyloid-β precursor protein; Aβ, amyloid-beta peptide; CHO-AβPP, Chinese hamster ovary cell line stably expressing human AβPP

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ABSTRACT

Cerebral amyloid-beta (Aβ) deposition is a critical feature of Alzheimer's disease. Aβ is derived from the amyloid precursor protein (AβPP) *via* two sequential cleavages that are mediated by beta-secretase and the gamma-secretase complex. Such amyloidogenic AβPP processing occurs in lipid raft microdomains of cell membranes and it is thought that modulating the distribution of lipids in rafts may regulate AβPP processing and Aβ production. Certain ATP-binding cassette (ABC) transporters regulate lipid transport across cell membranes and, as recent studies reveal, within membrane microdomains. ABCA1 also regulates Aβ metabolism in the brain although its direct impact on AβPP remains an open question. Here we assessed the capacity of three ABCA1 mutants (that do not promote lipid efflux) to modulate AβPP processing. Unexpectedly, these non-functional mutants also reduced Aβ production similar to wild type ABCA1. ABCA1 expression did not alter AβPP localization in lipid rafts, and co-immunoprecipitation experiments indicated ABCA1 and AβPP physically interact. These data suggest that ABCA1 may regulate AβPP processing independent of its impact on membrane lipid homeostasis.

INTRODUCTION

Several members of the ATP-binding cassette (ABC) transporter family regulate lipid transport across cell membranes [1,2]. ABCA1 is the most studied ABC transporter in the lipid homeostasis context; in part this is due to the discovery that loss of function mutations in the ABCA1 gene cause Tangier disease [3-5]. Tangier disease is characterised by severely impeded capacity for cells to efflux cholesterol and concomitant low levels of plasma high-density lipoprotein (HDL). These conditions result in cellular lipid accumulation and premature atherosclerosis.

An important role for ABCA1 in the regulation of cholesterol transport in the brain has also been established. Cholesterol efflux from neurons is accelerated in the presence of lipid-poor apoA-I and apoE, and in vitro studies demonstrated that the function of these cholesterol acceptors is largely dependent on ABCA1 (reviewed in [6]). Astrocyte ABCA1 (and ABCG1) expression also plays an important role in apoE lipidation [7-9]. Studies in mice show that ABCA1-mediated lipidation of apoE in the brain regulates apoE turnover and this has important implications for the apoE-dependent metabolism of amyloid-beta (Aβ) peptide; a neurotoxic and pro-inflammatory peptide that impairs memory, and represents a major constituent of cerebral amyloid plaques in Alzheimer's disease (AD) [10-13]. The direct role that ABCA1 might play in the modulation of neuronal amyloid precursor protein (AβPP) processing to generate Aβ remains an open question.

Previous studies have shown that over-expression of ABCA1 in neuronal and AβPP -expressing non-neuronal cell lines resulted in an apparent inhibition of Aβ production [14,15]. Interestingly, this inhibition of Aβ production was observed in the absence of an extracellular cholesterol acceptor. It is thus unclear if ABCA1 cholesterol efflux activity is a functional prerequisite for its modulation of AβPP processing. Lipid raft microdomains are the major site for amyloidogenic processing of AβPP [16] and previous work has shown that depletion of membrane cholesterol reduces AβPP localisation in rafts [17]. This in turn inhibits AβPP proteolytic processing by β- and γ-secretase thereby suppressing Aβ production [17-19]. Based on the fact that ABCA1 not only stimulates cholesterol efflux across membranes to apolipoprotein acceptors (as mentioned above) but also promotes the lateral redistribution of cholesterol from lipid rafts to non-raft microdomains in membranes [20,21], it seems plausible that ABCA1 may influence AβPP processing via regulation of intra-membrane cholesterol distribution and trafficking of AβPP to rafts. In line with this idea, the effect of ABCA1 on the lateral redistribution of membrane lipids out of rafts was shown to have a significant impact on the localisation of transferrin receptor (TfR) and toll-like receptor 4 (TLR4) in membrane microdomains of HeLa cells and macrophages, respectively [22,23].

In the present study we have assessed the impact that wild type and mutant ABCA1 has on cellular Aβ production. We have also investigated the potential regulation of AβPP localisation in lipid rafts by ABCA1. Unexpectedly, wild type and three different ABCA1 mutants all suppressed cellular Aβ production and this was

independent of AβPP raft localisation. Co-immunoprecipitation experiments did however reveal an interaction between ABCA1 and AβPP that may contribute to suppression of cellular Aβ production.

MATERIALS AND METHODS

Materials

Cell culture media and additives were obtained from Invitrogen (Melbourne, Australia) unless stated otherwise. Human wild type and mutant ABCA1 cDNA and expression plasmids were generated as described previously [24,25]. The CHO cell line stably expressing the human 695 amino acid AβPP (CHO-AβPP) was generated as described previously [26].

Cell Culture

The SK-N-SH and HEK293 cell lines were obtained from the ATCC (Manassas, VA). Cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), 2 mM glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin at 37°C in humidified air containing 5% CO_2. HEK293 cells were grown on poly-D-lysine coated plates to ensure maximal adhesion. The CHO-AβPP cell line stably expressing the human 695 amino acid AβPP was maintained using zeocin (200 μg/ml). Transfected cells were cultured in RPMI 1640 medium containing 10% FCS, 2 mM glutamine and 100 IU/ml penicillin and 100 μg/ml streptomycin.

Transfection

Transient transfection was performed using Lipofectamine 2000 and Opti-MEM I (Invitrogen) following the manufacturer's protocol. Briefly, cells were seeded at approximately 90% confluency in 12 well plates using antibiotic-free medium. cDNA-Lipofectamine complex was added to the cells and after 24 h of incubation samples were collected for gene expression analysis. In the case of cholesterol efflux assays the cells were cultured for up to an additional 16 h.

Cholesterol Efflux Assay

Cellular cholesterol efflux was measured as described previously [27]. In brief, cells were labelled with 2 μCi/ml [3H]-cholesterol (Amersham Biosciences) for 16 h, rinsed with phosphate-buffered saline (PBS) and incubated for 2 h in medium containing 0.1% (w/v) bovine serum albumin (BSA) to allow equilibration of [3H]-cholesterol in intracellular pools. The cells were rinsed once more in PBS and then incubated in serum-free medium containing 0.1% BSA with or without cholesterol acceptors for up to 16 h (i.e. 0.1% BSA is always present). Media samples were collected at the specified time point and cleared of any cellular debris by centrifugation at 1000 *g* for 5 min. The cells were lysed with 0.1 M NaOH and radioactivity in the media samples and cell lysates were measured by scintillation counting. Cholesterol effluxed to the medium was calculated as a percentage of total radioactivity in the cell lysates and medium. Experiments were performed in triplicate and repeated twice. Human apoA-I (15 μg / ml) was used as a cholesterol acceptor and was purified from human high-density lipoprotein (HDL) by ultracentrifugation and anion exchange chromatography as previously described [28].

Western Blotting

Cells expressing wild type or mutant ABCA1 and/or AβPP 695 were cultured in 6 well plates, rinsed with cold PBS and lysed in lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P40, 0.5% deoxycholate, 0.1% SDS, and protease and phosphatase inhibitors). Bicinchoninic acid protein assays was performed on lysates and equal amounts of protein were separated on SDS-PAGE gels (6% acrylamide) and transferred onto 0.45 μm nitrocellulose membranes at 100 volts for 30 min. Membranes were blocked overnight at 4°C in PBS containing 5% non-fat dry milk and probed with the relevant antibodies to reveal the major bands at the appropriate M_r: ABCA1 250 kDa (Novus, rabbit polyclonal 1/1000), AβPP 90 kDa (Sigma, 6E10 monoclonal 1/2000) at 22˚C for 2 h. The membranes were washed three times in PBS containing 0.1% Tween-20 and then incubated with horseradish peroxidase-conjugated goat anti-rabbit (Dako, 1/2000) or rabbit anti-mouse (Dako, 1/1000) secondary antibody for 2 h. Signals were detected using enhanced chemi-luminescence (ECL, Amersham Biosciences) and X-ray films. The signal intensity was quantified using NIH ImageJ software.

Western blotting of secreted Aβ peptides was carried out as previously described [15]. Briefly, Aβ in the culture medium was separated on 10–20% Tris/Tricine gels or 12% SDS-PAGE gels and transferred onto 0.2 μm nitrocellulose membranes at 65 volts for 15 min. Membranes were boiled in milli-Q H_2O for 10 min, probed with WO2 monoclonal antibody followed by rabbit anti-mouse horseradish peroxidase-conjugated secondary antibody and ECL detection applied as described above. AβPP

was also analysed by western blotting under non-reducing conditions. CHO-AβPP cells were homogenized in cold hypotonic buffer (250 mM sucrose, 10 mM HEPES, pH 7.4, 1 mM EDTA containing protease and phosphatase inhibitors) using microfuge tubes and tight pestles. The samples were centrifuged at 800 *g* for 20 min at 4°C, the supernatant mixed with SDS-free loading buffer and electrophoresed on Bis-Tris PAGE gels and analysed by western blotting as described above.

Western blotting for cellular levels of the AβPP C-terminal fragment (CTFβ) was performed as described previously [29] using rabbit polyclonal antibody raised against C-terminal amino acids 676-695 of human AβPP695 (Sigma, Cat# A8717). Western blotting for cellular presenilin 1 (PS1), BACE1 and nicastrin was performed as described previously [30,31] using in-house antibodies 98/1 and 00/5 for PS1 and BACE1, respectively, and a commercial antibody (Sigma, Cat # N-1660) for nicastrin.

Quantitative real-time PCR

RNA was isolated from cells using TRIzol reagent (Invitrogen) following the manufacturer's protocol. All procedures were carried out using RNase-free reagents. Four μg of RNA was reverse transcribed into cDNA as previously described [32]. cDNA was used as a template in the quantitative real-time PCR assay, which was carried out using a Mastercycler ep realplex S (Eppendorf) and the fluorescent dye SYBR Green (Eppendorf), following the manufacturer's protocol. Briefly, each reaction (20 μl) contained 1x RealMasterMix, 1x SYBR green, 5 pmoles of primers and 1 μl of template. Amplification was carried out with 40 cycles of 94°C for 15 sec

and 60°C for 1 min. All gene expression was normalized to β-actin, which served as an internal control for the quality of RNA isolated from each cell sample. Experiments were performed in triplicate and at least three samples were analysed for each cell type. Conventional PCR amplification was also carried out with 30 cycles of denaturation (94°C, 30 s), annealing (60°C, 30 s) and extension (72°C, 30 s), and the PCR products were visualized after electrophoresis in 1% agarose gels. Details of all primers used are provided as Supplementary Data (Supplementary Table 1).

Preparation of cell membrane lipid raft fractions

Lipid raft and non-raft fractions were prepared from CHO-AβPP and SK-N-SH cells as described previously [33]. Briefly, confluent cells grown in 175 cm^2 flasks were rinsed twice with phosphate buffered saline (PBS), resuspended in Buffer M (50 mM HEPES pH 7.4, 10 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA plus protease inhibitor cocktail (Complete Mini, Roche Applied Science), 1 mM Na_3VO_4, 1 mM NaF and 1 mM $Na_4P_2O_7$.10 dH_2O) and homogenized in microfuge tubes using tight pestles (Sigma, Castle Hill, NSW, Australia) while on ice. The cell lysates were centrifuged at 16,000 *g* for 20 min at 4°C. The pellets were resuspended in Buffer A (25 mM 2-(*N*-morpholino)-ethanesulfonic acid, 150 mM NaCl, pH 6.5) and mixed with an equal volume of Buffer A containing 2% Triton X-100 and phosphatase and protease inhibitors. The samples were kept on ice for 60 min, centrifuged at 16,000 *g* for 20 min at 4°C and the supernatants collected as non-raft fraction. The pellets were rinsed briefly with Buffer A and resuspended in Buffer B (10 mM Tris-Cl, pH 7.6, 150 mM NaCl, 60 mM β-octylglucoside and phosphatase and protease inhibitors). The samples

were kept on ice for 30 min, centrifuged at 16,000 *g* for 20 min at 4°C and the supernatants collected as the lipid raft fraction.

Immunoprecipitation

Immunoprecipitation of ABCA1 was carried out as described previously [34]. Briefly, CHO-AβPP cells were rinsed once with cold PBS, resuspended in homogenization buffer (100 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Triton X-100 plus protease inhibitor cocktail), homogenized in microfuge tubes using tight pestles while on ice, and cell lysates collected after centrifugation at 1,000 *g* for 10 min at 4°C. The cell lysates were mixed (in rotation) with the anti-AβPP antibody WO2 and Protein G Sepharose beads (Sigma, cat# P3296) at 4°C overnight, and then washed three times in the homogenization buffer and analysed by western blotting as described above.

Quantification of Aβ1-40 by ELISA

The concentration of Aβ1-40 in cell culture supernatants was determined using ELISA kits (BioSource International) following the manufacturer's instructions. Culture supernatants were diluted 1:10 in 55 mM NaHCO3 (pH 9.0) and all standards and samples were assayed in triplicate.

Statistical Analysis

Experiments were routinely performed in triplicate and repeated three times. Data are presented as means with SE shown by error bars. Differences were considered significant where $p < 0.05$ as determined by the 2-tailed Student's *t*-test for unpaired data.

RESULTS

In previous studies we have shown that in the absence of an extracellular acceptor to stimulate cholesterol efflux, ABCA1 expression significantly reduced cellular Aβ production [15]. This suggested that ABCA1-dependent removal of cholesterol from cell membranes was not a strict requirement for modulation of AβPP processing. In order to investigate the impact that non-functional ABCA1 mutants may have on cellular AβPP processing and Aβ production we first verified the loss of cholesterol efflux function in two ABCA1 mutants (R587W and W590S) associated with Tangier disease and an additional mutant in which the C-terminal 46 amino acids (Δ46) have been deleted (Fig 1A). Transient transfection of HEK293 cells resulted in similar levels of both mRNA and protein for WT and mutant ABCA1 cDNAs (data not shown). Consistent with previous studies [24,25], apoA-I mediated cholesterol efflux was strongly induced by WT ABCA1, weakly induced by $ABCA1_{W590S}$ and not induced by either $ABCA1_{R587W}$ or $ABCA1_{\Delta 46}$ expression as compared to mock transfected cells (Fig 1B). The loss of ABCA1 function in the mutants was previously ascribed to the variations in ABCA1 cell surface expression and apoA-I binding as summarised in Figure 1C.

We next examined the potential for both WT and mutant ABCA1 to modulate AβPP processing in CHO-AβPP cells. Transient transfection of CHO-AβPP cells resulted in similar levels of both mRNA and protein for WT and mutant ABCA1 (Fig 2A-C).

Transfection of CHO-AβPP cells did not alter the expression of the housekeeper gene β-actin at either mRNA or protein levels (Fig 2A-C), nor did it affect the expression of the stably transfected human AβPP695 cDNA at the mRNA level (Fig 2A). Western blot analysis revealed that transfection of CHO-AβPP cells with either WT ABCA1 or the $ABCA1_{R587W}$ and $ABCA1_{W590S}$ mutants increased the level of AβPP detected at the protein level by 11% to 25% (Fig 2D-E), whereas levels of sAβPPα detected in the culture medium were not altered by transfection with any of the ABCA1 constructs as compared to the mock-transfected conditions (Fig 2D-E). These data suggest that the processing of AβPP via the non-amyloidogenic α-secretase pathway is not substantially altered by expression of either WT or mutant ABCA1.

To gain insight into the potential modulation of AβPP processing via the amyloidogenic β- and γ-secretase pathway, we used additional techniques to quantify levels of the cellular C-terminal fragment (CTF) product of AβPP β-secretase processing (CTFβ) as well as extracellular levels of Aβ. The data derived from western blot analysis indicate that transient expression with either WT or mutant ABCA1 resulted in an increase in CTFβ and a decrease in secreted Aβ levels (Fig 3). The magnitude of increased CTFβ varied depending on the ABCA1 construct used; with the WT and $ABCA1_{\Delta 46}$ mutant increasing CTFβ levels by ~2.5 fold, and the $ABCA1_{R587W}$ and $ABCA1_{W509S}$ mutants increasing CTFβ levels by ~2-fold and 25%, respectively (Fig 3B and D). In contrast, levels of extracellular Aβ peptide were significantly decreased by both WT and mutant ABCA1 transfection by a similar degree (36% to 45% on average, Fig 3B and D). This pattern of increased cellular

CTFβ level associated with decreased extracellular Aβ in ABCA1-transfected cells could theoretically be due to a decrease in γ-secretase activity or possibly a change in AβPP trafficking that results in its reduced localisation in lipid rafts. Since we have previously shown that the close relative of ABCA1, ABCA7, inhibits CHO-AβPP cell Aβ production independent of effects on either α-, β- or γ-secretase activities [35], we focused on the impact that ABCA1 expression has on AβPP localisation in lipid rafts.

The methodology for lipid raft isolation requires scaling up of the cell culture system. Our first experiments therefore aimed to confirm that transient expression of ABCA1 in 175 cm^2 flasks resulted in the predicted inhibition of cellular Aβ production. Because the WT and three ABCA1 mutants all inhibited Aβ generation to a similar degree (Fig 3), we also limited this phase of the study to the WT ABCA1. Our data indicated that ABCA1 transfection of CHO-AβPP cells in the scaled-up cell culture system resulted in reduced extracellular Aβ levels to approximately the same extent observed when cluster plates were used for cell culture (Fig 4A cf Fig 3A and C). The decrease in Aβ levels in the cell culture medium was also confirmed by ELISA analysis of Aβ1-40 (mock 1.66 $\pm$ 0.10 ng/ml vs ABCA1 1.02 $\pm$ 0.04 ng/ml, mean $\pm$ SE, n=4, p = 0.02); the predominant Aβ species produced by the CHO-AβPP cell line.

We then isolated lipid raft and non-raft membrane fractions according to an established method [33]. Flotillin-2 and calnexin were used as markers to confirm the purity of the non-raft and raft fractions, respectively. As expected, flotilin-2 was highly enriched in rafts whereas calnexin was exclusively located in the non-raft

fraction (Fig 4B). When CHO-AβPP cells were transfected with ABCA1, we detected the vast majority (85% to 90%) of ABCA1 protein in the non-raft domains of the membranes as assessed after equal total protein loading of each of the fractions onto the gels (Supplementary Fig 1). Interestingly, ABCA1 was detected as a doublet band (which is known to be due to glycosylation heterogeneity [36]), and there was a clear preferential distribution of the higher molecular weight band in lipid rafts (Fig 4C and D, Supplementary Fig 1). A faint ABCA1 band was also detected in the non-raft fraction of mock-transfected cells, presumably as a result of cross-reaction of the antibody with hamster ABCA1 (Fig 4C, Supplementary Fig 1). Similarly, most (~80%) AβPP was located in non-raft fractions of both mock- and ABCA1-transfected CHO-AβPP cells (Supplementary Fig 1). Importantly, AβPP levels in either raft or non-raft fractions were not significantly altered by transfection with ABCA1 (Fig 4C to E), although trends for reduced levels of AβPP and flotilin-2 in rafts were detected (Fig 4E). It should be noted that in order to compare protein expression levels by western blotting, ~8-fold more protein was loaded on gels assessing the lipid raft proteins as compared to non-raft proteins (i.e. panels in Fig 4C and 4D can not be directly compared to each other). These data indicate that despite an ~15-fold up-regulation of ABCA1 protein levels in both lipid raft and non-raft fractions, the relative amount of AβPP localised to lipid rafts was not significantly altered. We also considered the possibility that ABCA1 expression may reduce cellular levels of γ-secretase components or BACE1, however, levels of PS1, nicastrin and BACE1 were unaltered in whole cell lysates transfected with ABCA1 as compared to mock conditions (Suppl Fig 3). In addition, we could find no evidence that ABCA1 altered PS1 levels in lipid rafts. Intriguingly, ABCA1 transfection

increased nicastrin levels in lipid rafts by ~40%, whereas BACE1 levels were not reliably detected in rafts under our experimental conditions (Suppl Fig 3).

Modulation of intramembrane AβPP trafficking therefore does not appear to be the mechanism by which ABCA1 down-regulates cellular Aβ production. One possible caveat in this conclusion is that the CHO-AβPP cells express a large amount of AβPP in a cell type that would not normally express this protein and thus subtle changes in AβPP trafficking may not be detected in this system. We therefore extended the study to assess the impact that ABCA1 has on endogenous AβPP processing and Aβ production in the human SK-N-SH neuroblastoma cell line. The levels of endogenous Aβ secreted into the cell culture medium is at least two orders of magnitude less than observed using the CHO-AβPP cell line. Cell culture medium was therefore concentrated approximately 10-fold before analysis of Aβ by western blotting and ELISA techniques. We used the same scaled-up culture methods that were used for the CHO-AβPP cell experiments to transfect SK-N-SH neurons in 175 cm^2 flasks. The western blot data indicate that ABCA1 transfection reduced Aβ production by 32% ($p < 0.05$). Three higher molecular weight species of Aβ were also detected by western blotting of SK-N-SH culture medium (Fig 5A) that may represent Aβ oligomers (although we cannot the possibility that these bands may represent as yet uncharacterised small AβPP proteolytic fragments). The levels of all these putative oligomeric forms of Aβ were also significantly reduced by ABCA1 transfection (*p*-values for the three arrowed bands all <0.05 for mock versus ABCA1-transfected cells). These results were confirmed by ELISA analysis of Aβ1-40 in the concentrated

media samples (mock 0.182 ± 0.010 ng/ml vs ABCA1 0.132 ± 0.008 ng/ml, mean ± SE, n=4, $p < 0.05$).

It was not possible to quantify sAβPPα in the concentrated SK-N-SH supernatants as the western blot was signal was over-loaded (in order to allow Aβ detection), however, blotting of non-concentrated samples indicated neuronal sAβPPα production was not influenced by ABCA1 transfection (Supplementary Figure 2).

Flotillin-2 was not reliably detected in the SK-N-SH cells so we used another established raft marker, Gαi-2 [33], in these experiments. Gαi-2 was exclusively located in rafts whereas calnexin was exclusively located in the non-raft fraction (Fig 5B). ABCA1 was also exclusively detected in the non-raft domains of SK-N-SH membranes under both mock- and ABCA1-transfected conditions (Fig 5C and 5D). Similar to what was observed with CHO-AβPP cells, most AβPP (~90%) was located in non-raft fractions of both mock- and ABCA1-transfected SK-N-SH neurons (data not shown). Interestingly, AβPP levels were slightly, but significantly, increased in the lipid raft fraction of ABCA1-transfected cells, whereas a trend for a reduced level of the raft marker Gαi-2 was detected (Fig 5D and 5E). These data provide two important pieces of information. Firstly, the results highlight the fact that protein distribution in lipid rafts can vary depending on cell type (which in turn may be related to either the heterogeneity in the total complement of raft proteins in different cell types or due to differences in membrane lipid composition); and secondly, the results indicate that despite a 6.5-fold up-regulation of ABCA1 in the transfected neurons, the relative amount of AβPP localised to lipid rafts was not reduced (in fact

there was a slight increase). Therefore an inhibition of AβPP trafficking to lipid rafts does not appear to be the mechanism by which ABCA1 down-regulates neuronal Aβ production.

Our findings that ABCA1 inhibits Aβ production via mechanisms that are not dependent on cholesterol efflux or modulation of AβPP localisation in lipid rafts led us to consider other pathways by which WT and mutant ABCA1 may have an impact on AβPP metabolism. Recent studies have shown that AβPP dimerisation inhibits its processing by γ-secretase thereby resulting in decreased Aβ production [37]. Using previously established PAGE conditions we analysed AβPP under non-reducing conditions and confirmed the presence of AβPP dimers in CHO-AβPP cell lysates (Fig 6A). However, transfection of CHO-AβPP cells with WT or mutant ABCA1 had no impact on the proportion of AβPP detected as dimers (Fig 6A); indicating that this is unlikely to contribute to the mechanism by which ABCA1 inhibits Aβ production.

A large body of research indicates that AβPP interacts with several membrane and adapter proteins and that this can alter its processing by secretases as well as have an important impact on AβPP trafficking and degradation [38-45]. A previous proteomic analysis of ABCA1 binding partners identified more than 200 interacting proteins, 76 of which specifically interacted with the C-terminal 40 amino acid region of ABCA1 [46]. Intriguingly, co-immunoprecipitation studies utilising human IMR-32 neuroblastoma cells or transfected HEK293 cells suggest a direct interaction between ABCA1 and AβPP695 [34]. In a final series of experiments we therefore used a co-

immunoprecipitation approach to assess whether ABCA1 mutants also interact with AβPP. Our data indicate that ABCA1 co-purifies with immunoprecipitated AβPP in CHO-AβPP cells transfected with either WT or mutant ABCA1 but not in mock-transfected cells (Fig 6B).

DISCUSSION

This study shows for the first time that WT ABCA1 and three non-functional ABCA1 mutants all suppress cellular Aβ production by pathways that are associated with a moderate accumulation of AβPP CTFβ (e.g. compared to the pronounced accumulation of CTFβ that results when γ-secretase inhibitors are used to suppress Aβ production in CHO-AβPP cell lines [29]). It is established that removal of cholesterol from lipid rafts inhibits the amyloidogenic processing of AβPP and this results in decreased production of Aβ [17,47,48]. Other raft lipids, such as glycosphingolipids (GSLs), also modulate AβPP processing [29,49]. The mechanisms by which membrane lipid composition regulates Aβ generation may involve control of AβPP translocation to rafts, modulation of clathrin-dependent AβPP endocytosis, as well as direct regulation of β- and γ-secretase function [48-52]. Based on previous work indicating that ABCA1 inhibits Aβ generation in vitro and also promotes the removal of cholesterol from lipid rafts to non-raft domains, we speculated that ABCA1-mediated changes in membrane lipid composition could account for its anti-amyloidogenic action. Unexpectedly, however, we found that non-functional ABCA1 mutants also suppressed cellular Aβ production and that transient transfection of both

CHO-AβPP cells and SK-N-SH neurons with ABCA1 did not alter the proportion of AβPP localised to lipid rafts. This indicates that the pathway by which ABCA1 influences AβPP processing is not related to modulation of AβPP localisation in lipid rafts, nor is it likely to be dependent on ABCA1 lipid transport function (particularly as assessed in the present study using cholesterol efflux to the amphipathic apoA-I acceptor protein). It is also unlikely that the inhibition of AβPP amyloidogenic processing is simply due to the over-expression of a large multi-transmembrane protein as we have previously shown that robust expression of ABCA2 (46% homologous to ABCA1) in CHO-AβPP cells has no impact on AβPP proteolytic processing or Aβ production [15]. Interestingly, studies by Chen et al. indicated that ABCA2 increased both AβPP expression and Aβ production in 293 EBNA cells stably transfected with AβPP695 whereas a similar sized control ABC transporter (not described in detail) had no impact on either AβPP expression or Aβ production [53]. Clearly, not all ABC transporters / large multi-transmembrane proteins inhibit AβPP amyloidogenic processing.

Independent of effects on membrane lipid transport, several membrane proteins that interact with AβPP either directly or through adaptor proteins can inhibit (e.g. SorLA, X11 proteins) or promote (e.g. LRP, flotillin-2) amyloidogenic processing of AβPP [39,40,42-44,54] and there is increasing evidence that these types of interactions can modulate AβPP localisation to rafts or to subcellular compartments that alter the proteolytic processing of AβPP due factors such as reduced pH and colocalisation with secretase components [55-57]. As noted above, ABCA1 also interacts with a

large number of proteins and a direct interaction between ABCA1 and AβPP695 has been reported [34,46]. The molecular basis for this interaction (which was confirmed by our present study) remains uncertain. Using a human macrophage proteomics approach, 76 proteins were found to bind to the C-terminal 40 amino acids of ABCA1 [46]. Interestingly, ABCA1 the ABCA1 C-terminal region was found to interact with nicastrin, an essential component of the γ-secretase complex that is required for γ-secretase activity and for its function as a regulator of AβPP trafficking [46,58]. Whether nicastrin represents an intermediate binding protein for the ABCA1-AβPP interaction is unclear. The fact that $ABCA1_{\Delta 46}$ interacts with AβPP (albeit at lower levels than for the WT and other ABCA1 mutants, see Fig 6B) suggests that the C-terminal region is not essential for interaction with AβPP. In the case of the SorLA-AβPP interaction, it is the extracellular carbohydrate-linked domain of AβPP that is crucial [42]. Given that both AβPP and ABCA1 bind multiple membrane and adaptor proteins, it seems reasonable to conclude that there may be more than one type of interaction linking these proteins and this may be dependent on the cell type, membrane composition and subcellular location.

The current data should also be considered in terms of possible cell-specific functions that ABCA1 may play related to Aβ homeostasis. There is compelling evidence that astrocyte ABCA1 plays a role in the lipidation of apoE and that this in turn regulates Aβ uptake and metabolism [7,59-61]. In addition to astroglia, neurons also express ABCA1 (reviewed in [6]) and data from postmortem tissue indicate ABCA1 immunostaining in human CA1 hippocampal neurons is significantly up-regulated in AD [62]. We speculate that neuronal ABCA1 may be up-regulated in response to

changes that occur in AD tissue in an attempt to limit further Aβ production. Previous studies have shown that even in the absence of extracellular apoE to promote cholesterol efflux from the cell membrane, ABCA1 transfection still has the capacity to reduce extracellular Aβ levels in vitro [15]. This earlier finding is consistent with our current data indicating that loss-of-function mutations do not prevent this apparent direct anti-amyloidogenic action of ABCA1. We speculate that astroglial and neuronal ABCA1 may act in concert, possibly via different pathways, to reduce the accumulation of Aβ in the brain.

In conclusion, our data indicate that the mechanisms underlying the inhibitory effect that ABCA1 has on amyloidogenc AβPP processing are independent of its lipid transporter function and do not appear to be related to changes in AβPP raft localisation.

ACKNOWLEDGEMENTS

We are grateful to Prof Kerry-Anne Rye for provision of human apoA-I, to Prof Colin Masters and Dr Qiao-Xin Li for providing WO2 antibody, and to Dr Janetta Culvenor for providing 98/1 antibody. This work was supported by the National Health and Medical Research Council of Australia (NHMRC Grant No. 510148). Prof Garner is supported by a Fellowship from the Australian Research Council (FT0991986).

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Supplementary Table 1. PCR primers

Gene	GenBank accession	Primer 5' – 3' Forward & Reverse	Product size (bp)
ABCA1	NM_005502	F AACTCTACATCTCCCTTCCCG R CTCCTGTCGCATGTCACTCC	123
AβPP	NM_000484	F ACATGCACATGAATGTCCAG R CACCAGTTCTGGATGGTCAC	169
β-actin	NM_001101	F GAATTCTGGCCACGGCTGCTTCCAGCT R AAGCTTTTTCGTGGATGCCACAGGACT	162

FIGURE LEGENDS

FIG. 1. **Functional properties of wild type and mutant ABCA1.** A, Schematic representation of ABCA1 protein structure and the position of Tangier disease missense mutations, NBD, nucleotide binding domain. B, Ability of wild type and mutant ABCA1 to stimulate cholesterol efflux to apoA-I. HEK293 cells were transfected with mock empty vector (M), wild type (WT), R587W (R), W590S (W) or Δ (Δ46) ABCA1, labeled with [^{3}H]cholesterol, and then incubated with either bovine serum albumin (BSA) (light bars) or apoA-I (15 μg/ml) (dark bars). Data are mean values derived from triplicates with SE represented by the error bars, *p<0.05, ** p<0.01, *** p<0.001. C, Tabulated summary of the functional properties of wild type and mutant ABCA1. Data used to generate the table in are from previously published studies [24,25].

FIG. 2. **Effect of wild type and mutant ABCA1 on AβPP processing.** CHO-AβPP cells (stably expressing the human AβPP695) were transiently transfected with wild type (WT) and mutant ABCA1. The transfection efficiency of ABCA1 cDNAs was confirmed at the mRNA level by PCR amplification and agarose gel electrophoresis (A) and at the protein level by western blotting using anti-ABCA1 antibody (B). β-actin was used as an internal control in both cases. The expression of the human AβPP695 in these transfected cells was measured at the mRNA level (A) and at the cellular protein level (D). The expression of secreted sAβPPα was also measured by western blotting (D). The signal intensity of the protein bands was quantified using ImageJ software (C, E). Mock empty vector (M), wild type (WT), R587W (R),

W590S (W), Δ46 (Δ). Data are mean values derived from triplicates with SE represented by the error bars, *p<0.05, ** p<0.01, *** p<0.001.

FIG. 3. **Impact of wild type and mutant ABCA1 on Aβ peptide generation.** CHO-AβPP cells (stably expressing the human AβPP695) were transiently transfected with wild type (WT) and mutant ABCA1 and the secreted Aβ and cellular CTFβ were measured by western blotting (A, C). The signal intensity of the protein bands was quantified using ImageJ software (B, D). Mock empty vector (M), wild type (WT), R587W (R), W590S (W), Δ46 (Δ). Data are mean values derived from triplicates with SE represented by the error bars, *p<0.05, ** p<0.01, *** p<0.001.

FIG. 4. **Impact of ABCA1 on AβPP localisation in lipid rafts.** A, CHO-AβPP cells (stably expressing the human AβPP695) grown in 175 cm^2 flasks were transiently transfected with mock empty vector or ABCA1 and the secreted Aβ and sAβPPα were measured by western blotting. B, The transfected cells were harvested and lipid raft (LR) and non-raft (NR) membrane fractions were isolated and probed with flotillin-2 (Flot) and calnexin (Clnx) antibodies to confirm the purity of the fractions. The expression of ABCA1 and AβPP was analysed by western blotting in the non raft (C) and lipid raft (D) fractions. Calnexin (Clnx) and flotillin-2 (Flot), respectively, were used as loading controls. E, The signal intensity of the protein bands was quantified using ImageJ software. Data are mean values derived from triplicates with SE represented by the error bars, ** p<0.01, *** p<0.001.

FIG. 5. **Impact of ABCA1 on endogenous AβPP processing in human SK-N-SH neurons.** A, SK-N-SH neuroblastoma cells grown in 175 cm^2 flasks were transiently transfected with mock empty vector or ABCA1, the culture media concentrated and Aβ and sAβPPα measured by western blotting (see also Suppl Fig 2). B, The transfected cells were harvested and lipid raft (LR) and non-raft (NR) membrane fractions were isolated and probed with Gαi-2 and calnexin (Clnx) antibodies to confirm the purity of the fractions. The expression of ABCA1 and AβPP was analysed by western blotting in the non-raft (C) and lipid raft (D) fractions. Calnexin (Clnx) and Gai-2 respectively were used as loading controls. E, The signal intensity of the protein bands was quantified using ImageJ software. Data are mean values derived from triplicates with SE represented by the error bars, *p<0.05, ** p<0.01.

FIG. 6. **Analysis of AβPP dimerization and interaction with ABCA1.** A, CHO-AβPP cells were transiently transfected with wild type and mutant ABCA1 and protein lysates were subjected to non-reducing gel electrophoresis and western blotting with AβPP antibody. β-actin was used as a loading control. B, In a separate experiment, CHO-AβPP cells were transiently transfected with wild type and mutant ABCA1 and protein lysates were immunoprecipitated with anti-AβPP antibody and then probed with anti-ABCA1 antibody, stripped, and re-probed with anti-AβPP antibody. Mock empty vector (M), wild type (WT), R587W (R), W590S (W), Δ46 (Δ).

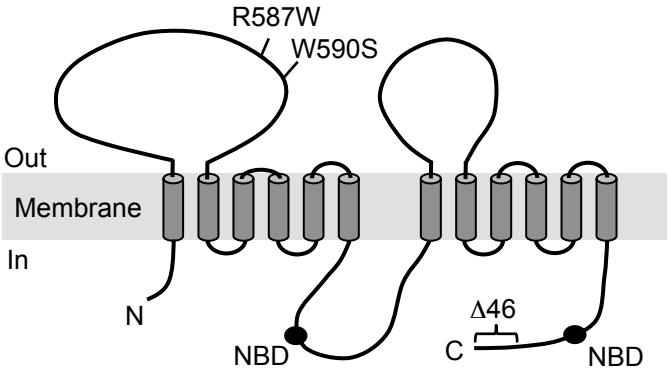
SUPPLEMENTARY FIG. 1. **Analysis of ABCA1 and AβPP proteins in lipid raft and non-raft membrane fractions.** A, CHO-AβPP cells were transiently transfected with mock empty vector or ABCA1 and lipid raft and non-raft membrane fractions were isolated. Equal amounts of protein was loaded onto each well and analysed by western blotting with ABCA1 and AβPP antibodies. B, A longer exposure (3h) of film of the ABCA1 western blot was used to enhance the signal.

SUPPLEMENTARY FIG. 2. **Impact of ABCA1 on endogenous sAβPPα production by human SK-N-SH neurons.** SK-N-SH neuroblastoma cells grown in 175 cm^2 flasks were transiently transfected with mock empty vector or ABCA1 and the culture media sAβPPα measured by western blotting. Western blotting of cell lysates for β-actin was used as a control for cellular protein levels.

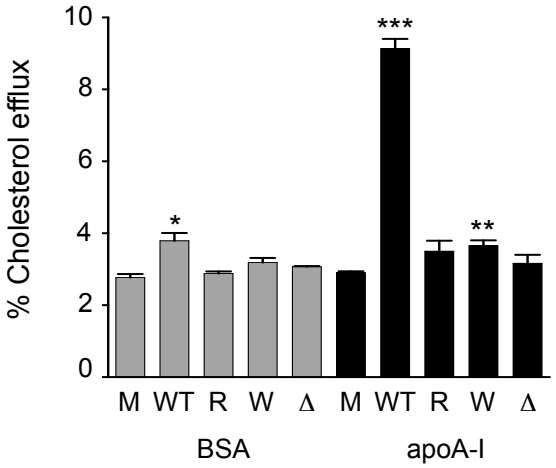
SUPPLEMENTARY FIG. 3. **Impact of ABCA1 on γ-secretase and BACE1 proteins in cell lysates and lipid raft membrane fractions.** A, CHO-AβPP cells were transiently transfected with mock empty vector or ABCA1 and cell lysates (A) and lipid raft membrane fractions (B) were analysed for presenilin 1 (PS1), nicastrin (Nic.), BACE1 (BACE) by Western blotting as indicated. β-Actin and flotilin-2 (Flot.) were used to confirm equal protein loading of the lysates and raft fractions, respectively. Quantification of the signal intensity relative to the relevant control proteins is shown in the histogram (C). Note we did not detect a reliable signal for BACE1 in the lipid raft membrane fractions in these experiments (not shown). Data are representative of two experiments performed in triplicate. **, $p < 0.01$.

Kim et al Fig 1

A



B

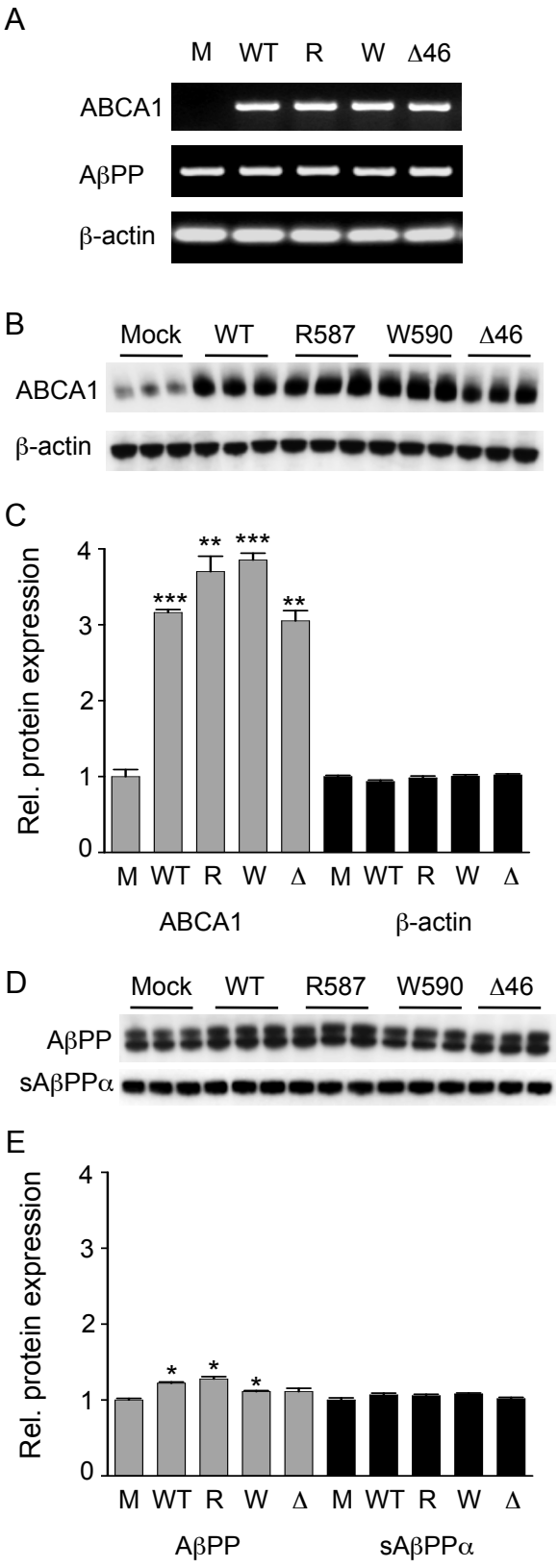


C

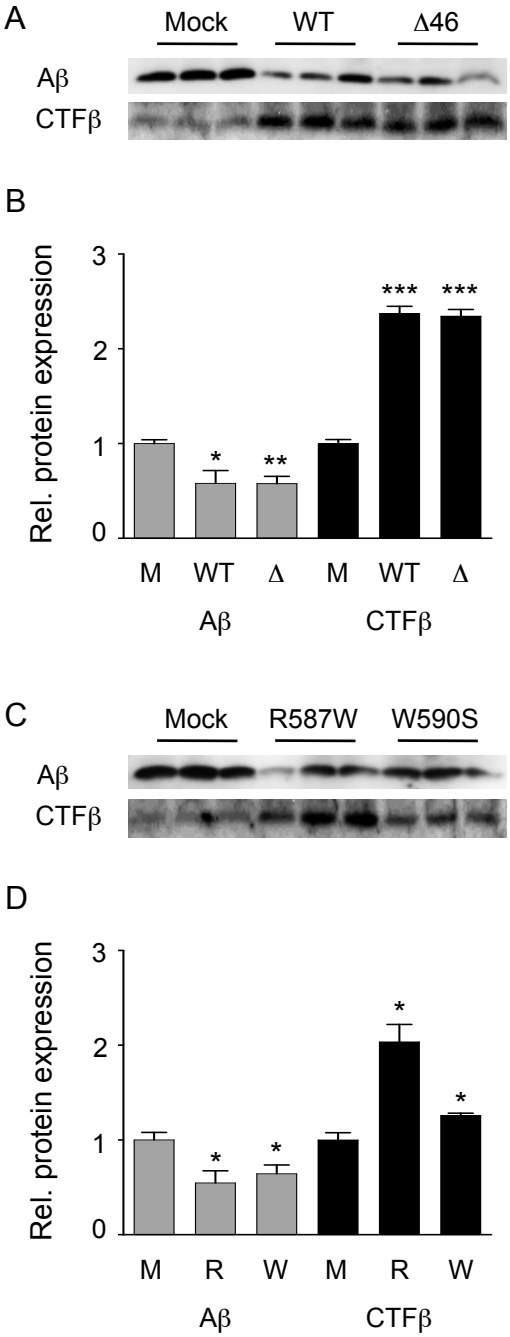
	Cell surface Expression	ApoA-I binding	Cholesterol efflux
WT	high	high	high
R587W	high	low	ND
W590S	high	high	low
Delta46	low	ND	ND

ND, not detectable

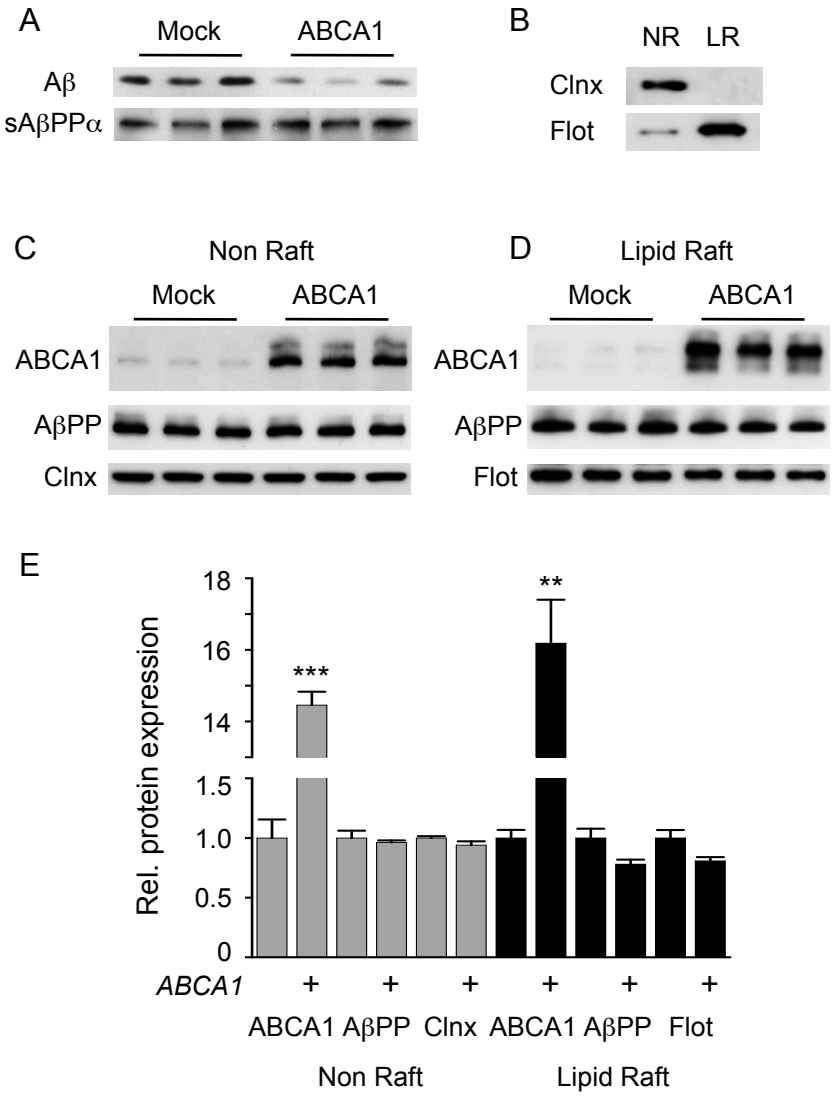
Kim et al Fig 2



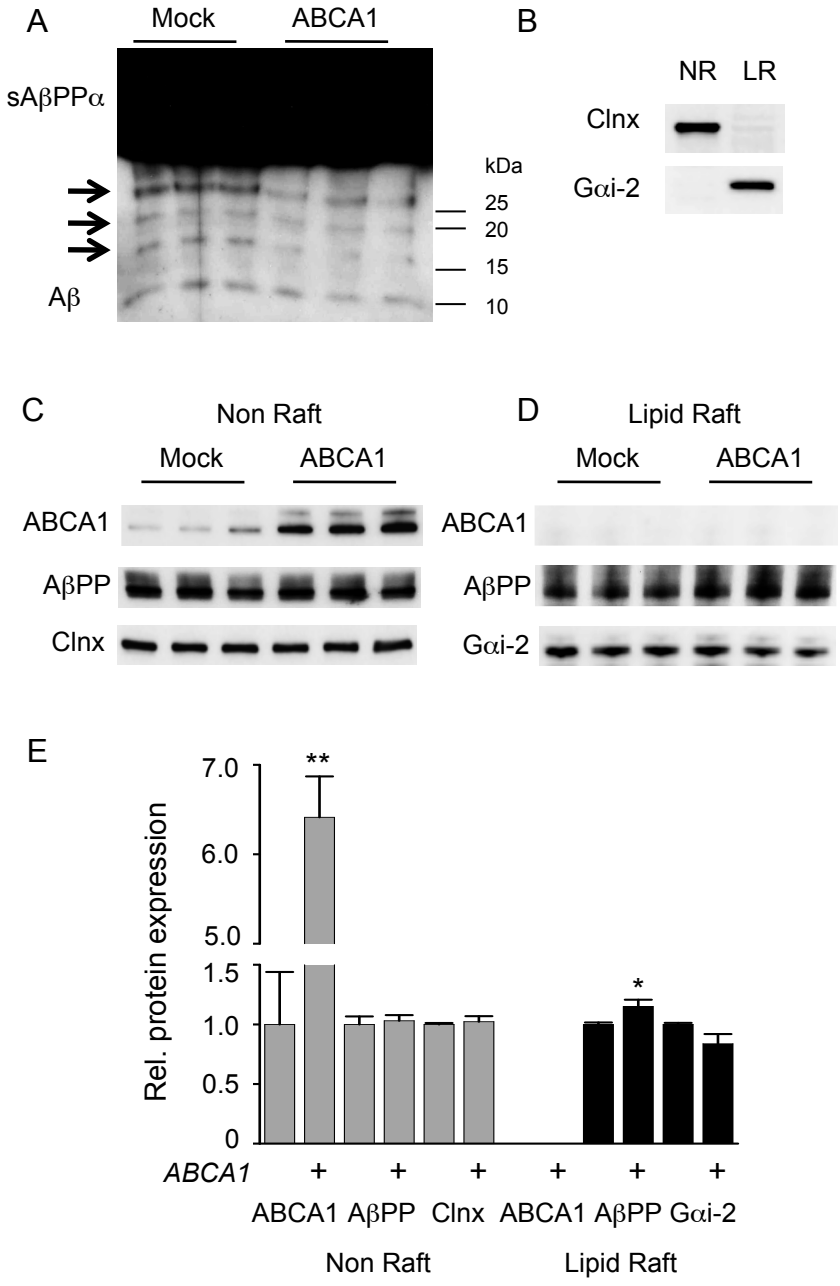
Kim et al Fig 3



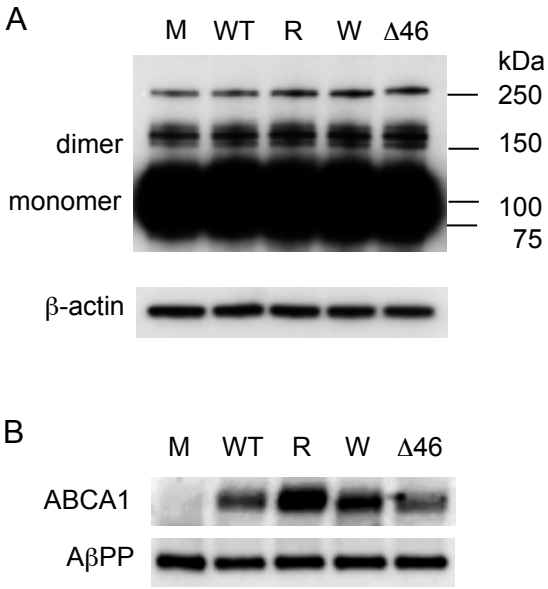
Kim et al Fig 4



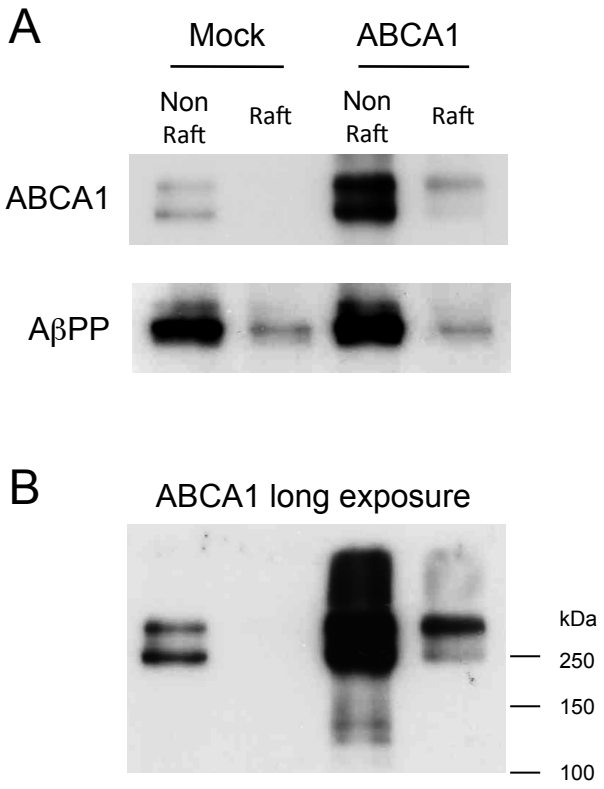
Kim et al Fig 5



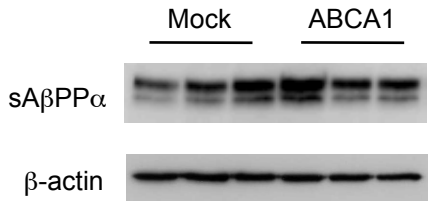
Kim et al Fig 6



Kim et al Suppl Fig 1



Kim et al Suppl Fig 2



Kim et al Suppl Fig 3