Title: Adenosine triphosphate Binding Cassette subfamily C member 1 (ABCC1) overexpression reduces APP processing and increases alpha- versus beta-secretase activity, *in vitro*.

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Contributions:

Wayne M. Jepsen designed, carried-out, and analyzed the experiments, as well as wrote the manuscript.

Matthew De Both assisted with the analyses.

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Keri Ramsey contributed to the acquisition of data.

Ignazio S. Piras assisted with the analyses.

Marcus Naymik assisted with the analyses.

Adrienne Henderson contributed to the acquisition of data.

Matthew J. Huentelman is the principal investigator and guarantor of the work.

Abstract:

The organic anion transporter Adenosine triphosphate Binding Cassette subfamily C member 1 (*ABCC1*), also known as *MRP1*, has been demonstrated in murine models of Alzheimer's disease (AD) to export amyloid beta (Abeta) from the endothelial cells of the blood-brain barrier to the periphery, and that pharmaceutical activation of ABCC1 can reduce amyloid plaque deposition in the brain. Here, we show that ABCC1 is not only capable of exporting Abeta from the cytoplasm of human cells, but also that it's overexpression significantly reduces Abeta production and increases the ratio of alpha- versus beta-secretase mediated cleavage of the Amyloid Precursor Protein (APP), likely via indirect modulation of alpha-, beta-, and gamma-secretase activity.

Background:

Alzheimer's disease (AD) is the sixth leading cause of death in the United States, and no current treatment exists that can effectively prevent or slow progression of the disease. For this reason, it is imperative to identify novel drug targets that can dramatically alter the physiological cascades that lead to neuronal cell death resulting in dementia and ultimately loss of life.

The deposition of aggregated amyloid beta (Abeta) in the brain is one of the major pathological hallmarks of AD, and Abeta species result from the differential cleavage of the Amyloid Precursor Protein (APP) (Selkoe and Hardy, 2016). APP is a single-pass transmembrane protein that is highly expressed in the brain and can be cleaved by a variety of secretases to produce unique peptide fragments, the two major pathways of which are known as the alpha- and beta-secretase pathways (Selkoe and Hardy, 2016). Cleavage by an alpha-secretase releases the soluble APP alpha (sAPPalpha) fragment from the membrane into the extracellular space, which has been shown to be neuroprotective and increase neurogenesis, in vitro (Ohsawa *et al.*, 1999), as well as play a positive role in synaptic plasticity (Ring *et al.*, 2007; Hick *et al.*, 2015) and memory formation (Bour *et al.*, 2004). Alpha-secretase cleavage of APP is the by far the most common cleavage of APP in the brain (Haass and Selkoe, 1993). If, instead, the APP molecule is cleaved by a beta-secretase, soluble APP beta (sAPPbeta) is released into the extracellular space, and subsequent cleavage of the remaining membrane-bound fragment by the gamma-secretase complex results in the production of Abeta, the peptide that aggregates to form amyloid plaques (Baranello *et al.*, 2015). Because alpha-secretases cleave

APP within the Abeta domain, and beta-secretases cleave within the sAPPalpha domain, APP cleaved by an alpha-secretase cannot be cleaved by a beta-secretase, and vice versa (Haass and Selkoe, 1993), the two pathways are mutually exclusive and stoichiometrically related. It has been hypothesized that decreasing Abeta production could slow progression of AD, and although direct beta-secretase inhibition has failed in clinical trials (Das and Yan, 2019), control of these pathways via pharmaceutical intervention may still prove to be a viable AD treatment.

Our lab investigated the role of *ABCC1* in AD because we identified rare *ABCC1* single nucleotide polymorphisms (SNPs) in a familial case of late-onset AD, and in an early-onset AD patient with no family history of AD. ABCC1 has been previously shown to export Abeta from the cerebral spinal fluid to the peripheral blood (Krohn *et al.*, 2011), and *Abcc1* knockout mouse models have increased cerebral amyloid plaque deposition and soluble Abeta (Krohn *et al.*, 2011, 2015). Though our final experiments demonstrated no significant difference between the human reference *ABCC1* and either of the mutant alleles, our study revealed that *ABCC1* overexpression results in a significant reduction in extracellular Abeta1-40, Abeta1-42, and sAPPbeta species, while increasing the ratio of alpha- to beta-secretase mediated cleavage of APP, likely via indirect transcriptional modulation of proteins involved in APP metabolism. Our results indicate that ABCC1 is a valid drug target for the treatment of AD because of its multimodal influence on Abeta deposition: via exportation of Abeta species, as well as modulation of APP processing away from the amyloidogenic pathway.

Results and Discussion:

Briefly, for the APP metabolite experiments, ABCC1-overexpressing cells, or empty vector control cells, were plated at 1.4e7 cells per well of a 6-well plate weekly, with daily media changes. On the fourth day, supernatant was harvested and clarified, and cells were lysed for RNA or protein extraction. All samples were stored at -80 °C until 3 weeks of experiments were assayed together. A more complete description of the experimental approach can be found in the Materials and Methods section.

In the first experiment, ABCC1-overexpressing cells had a 34.02% decrease in extracellular Abeta1-40 ($t=11.184$, $df=4$, $p=3.64e-04$) and a 32.85% decrease in extracellular Abeta1-42 ($t=4.26$, $df=4$, $p=0.013$). The second experiment saw a 33.08% decrease in Abeta1-40 ($t=5.26$, $df=4$, $p=6.26e-03$) and a 43.90% decrease in Abeta1-42 ($t=3.37$, $df=4$, $p=0.028$) (see **Figure 1**). These results were surprising because, as previously

stated, ABCC1 has been shown to export Abeta from the cytoplasm to the extracellular space, and if Abeta is, in fact, a substrate for ABCC1, we would expect to see higher extracellular concentrations of Abeta species.

To test whether ABCC1 exports Abeta, both cell lines were incubated with 200nM fluorescent Abeta1-42 (Beta-Amyloid (1-42), HiLyte Fluor 555-labeled, Human, AnaSpec, Fremont, CA, USA) for 18 hours, and then cells were subject to flow cytometry (FACSCanto II, BD Biosciences, Franklin Lakes, NJ) to quantify the percentage of fluorescent cells. 79.7% of the empty vector control cells were fluorescent, while only 68.4% of ABCC1-overexpressing cells displayed intracellular fluorescence. Furthermore, when incubated with fluorescent Abeta1-42 and 25uM thiethylperazine (MilliporeSigma, Burlington, MA, USA), a small molecule previously shown to increase ABCC1-mediated transport of Abeta (Krohn *et al.*, 2011), we observed a 23.6% decrease in population fluorescence in the empty vector control cells, and an even greater 38.0% decrease in the ABCC1-overexpressing cells (see **Figure 2**). This confirms that our model is working as expected because it agrees with previous reports: that ABCC1 does export Abeta, and that thiethylperazine increases ABCC1 transport activity.

Because we demonstrated that ABCC1 does export Abeta from the cytoplasm to the extracellular space, we hypothesized that ABCC1 may alter transcript levels of proteins capable of altering APP metabolism. To this end, we conducted RNA-sequencing of the cell lines. Analysis revealed 2470 differentially expressed genes (DEGs) with adjusted p-values less than or equal to 0.001, of which 2192 were protein coding. We hypothesized that because of the drastic reduction in extracellular Abeta, if a single gene were responsible for the altered APP processing, that it would have a log base two fold change (log2FC) with an absolute value greater than or equal to 1.5, which left 268 genes of interest (GOIs). Each gene was manually researched for their association to Alzheimer's disease and amyloid pathology. This left 55 GOIs, 10 of which have known roles in APP/Abeta metabolism or transport – but whose expression levels are altered in the opposite direction one would expect for the observed ELISA results – and two with expression levels that may account for the lower levels of extracellular Abeta. All GOIs are discussed in **Supplementary Table 1** with a focus on this experiment, and in the context of the proceeding two RNA-seq experiments discussed later.

The genes whose expression levels may account for the reduced extracellular Abeta levels are *CD38* and *TIMP3*. *CD38* encodes the Cluster of Differentiation 38, an enzyme that synthesizes and hydrolyzes cyclic adenosine 5'-diphosphate-ribose, a molecule that regulates intracellular calcium signaling (Chini *et al.*, 2002). It

has been shown that *Cd38* knockout AD mouse models have improved cognitive deficits, decreased cerebral amyloid burden, and that primary neurons cultured from those mice secrete significantly less Abeta species (Blacher *et al.*, 2015). The authors found that knockout of Cd38 alters beta- and gamma-secretase activity, effectively reducing both (Blacher *et al.*, 2015). This aligns with the observations made in our experiment, that when *CD38* expression is reduced (log2FC=-2.98, N=6, n=3, p=7.21e-09, padj=1.78e-07), extracellular Abeta levels are also reduced. Therefore, the reduction of *CD38* expression may contribute to the altered APP processing, though the mechanism by which ABCC1 alters *CD38* expression is not known.

TIMP3, our second candidate gene, encodes the Tissue Inhibitor of Metalloproteinases 3, a protein that can irreversibly inhibit APP-cleaving alpha-secretases like ADAM10 and ADAM17 (Hoe *et al.*, 2007). *TIMP3* expression is also reduced in AD brain tissue (Dunckley *et al.*, 2006), which may play a role in increased Abeta production. In our experiment, we saw TIMP3 expression reduced with a log2FC of -1.95 in the ABCC1-overexpressing cell line compared to the empty vector control (N=6, n=3, p=2.54e-110, padj=7.56e-107). Logically, if an alpha-secretase inhibitor is significantly decrease in expression, alpha-secretase activity would be increased, which would result in the reduction of secreted Abeta species because of the mutual exclusivity of the alpha- versus beta-secretase cleavage of APP previously discussed. It is also possible that the reduction of *CD38* and *TIMP3* works synergistically to reduce extracellular Abeta by decreasing beta- and gamma-, and increasing alpha-secretase activity.

To confirm our results, experiments were repeated with cryogenically preserved cells, as well as freshly transfected cells (to ensure that the transcriptional changes observed are not due to locus-specific integration of the transposable vectors), and APP metabolites were measured using the Meso Scale Discovery (MSD) platform (Meso Scale Diagnostics LLC, Rockville, MD, USA) which allows for the simultaneous, single-well measurement of Abeta1-40 and Abeta1-42, or sAPPalpha and sAPPbeta. Again, ABCC1-overexpressing cells had a 36.96% (t=10.97, df=10, p=6.74e-07) and a 35.21% (t=7.84, df=10, p=1.40e-05) reduction in extracellular Abeta1-40, as well as a 39.66% (t=11.42, df=10, p=4.66e-07) and 35.75% (t=9.73, df=10, p=9.03e-03) reduction in extracellular Abeta1-42. Furthermore, the two experiments saw a 29.45% (t=6.64, df=10, p=5.81e-05) and a 23.55% reduction (t=3.64, df=10, p=4.56e-03) in extracellular sAPPbeta, with no significant effect on sAPPalpha levels in the first experiment, but with a 16.27% reduction (t=3.21, df=10, p=9.30e-03) in the second experiment. Because the MSD platform allows for the simultaneous measurement

of sAPPalpha and sAPPbeta in a single well, we used the ratio of sAPPalpha over sAPPbeta (sAPPalpha/sAPPbeta) to monitor alpha- versus beta-secretase cleavage of APP molecules because it controls for many of the confounding factors that could influence our measurements, and instead offers a mole-to-mole comparison. Indeed, in both experiments, ABCC1-overexpressing cells had a 35.20% (t=-10.89, df=10, p=7.24e-07) and 9.41% increase (t=-2.71, df=10, p=0.022) in sAPPalpha/sAPPbeta, implying a significant increase or reduction of alpha- or beta-secretase activity, respectively. Results are summarized in **Figure 3**.

Cells were again subject to RNA-seq. In both experiments, TIMP3 was significantly downregulated, with a log2FC of -0.64 in cryopreserved cells (N=6, n=3, p=0.015) and -0.82 in newly transfected cells (N=6, n=3, p=5.7e-03). CD38 had an insignificant log2FC of -0.53 in cryopreserved cells (N=6, n=3, p=0.24) and -0.48 (N=6, n=3, p=0.069) in the newly generated cell line; however, we do not believe that this is necessarily a reason to completely disregard the involvement of CD38 in the altered APP metabolism observed, as it is trending towards significance in the newly generated cell line. Furthermore, this confirms that the reduction in extracellular Abeta species is likely not due to integration of the transposable vectors within genes that alter APP processing, but rather that the increase in ABCC1 protein expression is likely altering transcription of genes whose products are capable of altering APP metabolism.

To determine if the transcriptional effects were cell line specific, we co-transfected the vectors (with SB100X) into ReNcell VM cells (MilliporeSigma), a human neural progenitor line, and extracted RNA from differentiated cells (14 days without growth factors). Transcripts were quantified using TaqMan (Applied Biosystems, Foster City, CA, USA) quantitative reverse transcriptase PCR (qRT-PCR), with targeted transcripts normalized *ACTB* expression, using the relative quantification (RQ) method (Livak and Schmittgen, 2001). *TIMP3* and *CD38* mean RQs were 11.10% lower (t=3.236, df=22, p=3.80e-03) and 76.0% lower (t=-12.76, df=22, p=1.21e-11), respectively, in the *ABCC1*-overexpressing cells versus the empty vector control (see **Figure 4**). These results agree with our previous results, that ABCC1 overexpression significantly alters the transcription levels of *TIMP3* and *CD38*, in a direction consistent with the reduced extracellular Abeta, and increased alpha- over beta-secretase cleaved APP molecules, and further demonstrates that altered transcriptional regulation of this gene is due to increased expression of ABCC1, rather than disruption of these genes due to transposable integration of the vectors.

Taken together, our work confirms what previous labs have reported – that Abeta is a substrate for ABCC1-mediated export – but also provides novel insight: that increased ABCC1 expression reduces extracellular Abeta levels, likely via the alteration of alpha-, beta-, and gamma-secretase activity due to transcriptional modification of *TIMP3* and *CD38*. How ABCC1 alters these transcripts is unknown, but we hypothesize it is due to increased export of ABCC1's canonical substrates, though further functional studies will be required to completely delineate the mechanism. Regardless, compounds that can dramatically increase ABCC1 transport activity or those that can increase ABCC1 expression, may prove to be viable drugs for the treatment or prevention of AD by not only increasing clearance of Abeta from the brain, but also by reducing the amount of Abeta that is produced. Many drugs have already been developed to block ABCC1 transport to prevent chemoresistance in cancer (Stefan and Wiese, 2019). Compounds identified in these drug development pipelines that have the opposite effect should be studied in the context of Alzheimer's disease.

Materials and Methods:

Cell line generation:

BE(2)-m17: Human APP and ABCC1 codon-optimized cDNA was cloned into the Sleeping Beauty transposable vectors pSBbi-Hyg and pSBbi-Pur, respectively (gifts of Eric Kowarz, Addgene plasmids #60524 and #60523) by GenScript (Piscataway, NJ, USA). pSBbi-Hyg-APP was cotransfected with the transposase-encoding vector pCMV(CAT)T7-SB100 (a gift of Zsuzsanna Izsvak, Addgene plasmid #34879) into BE(2)-m17 human neuroblastoma cells (ATCC, Manassas, VA, USA), using the Cell Line Nucelofector Kit V and the Amaxa Nucleofector II Device (Lonza Group AG, Basel, CH). Stable cells were selected for with 1mg/mL hygromycin B (Invitrogen, Carlsbad, CA, USA). This APP-overexpressing cell line (now referred to as BE(2)-m17-APP) was then used to create the two experimental cell lines to ensure that APP expression is not variable due to transfection conditions. To this end, pSBbi-Pur-ABCC1 or empty vector was cotransfected with SB100X into BE(2)-m17-APP, and stable cells selected for with 10ug/mL puromycin (Gibco, Thermo Fisher Scientific, Waltham, MA), and maintained with 2ug/mL puromycin and 200ug/mL hygromycin B.

ReNcell VM: pSBbi-Pur-ABCC1 or empty vector were cotransfected with SB100X using the same Amaxa Nucleofector II and kit V (Lonza Group AG). Stably expressing cells were selected for using 10ug/mL

puromycin (Gibco). When maintained with human epidermal growth factor (EGF) and fibroblast growth factor basic (bFGF) proteins (SigmaMillipore), ReNcell VM's remain as human neuronal precursor cells. Upon removal of the growth factors, the cells will terminally differentiate and begin to mature into neurons and astrocytes.

APP metabolite experiments and cellular extracts:

Weekly, 1.4e7 cells per line were plated in a well of a 6-well plate without antibiotics (hygromycin and puromycin) and with daily media changes. On the fourth day, supernatant was harvested and supplemented to a final concentration of 1.0mM of an irreversible serine protease inhibitor, AEBSF (Thermo Fisher Scientific), then clarified at 10,000g for 10minutes at room temperature. Resulting supernatant was transferred to a new tube and stored at -80 °C until analysis. Cells in the plate were lysed for either protein extraction using RIPA buffer (Thermo Fisher Scientific) or RNA extraction using the Quick-RNA miniprep kit (Zymo Research, Irvine, CA, USA), either of which were stored at -80 °C until analysis.

For the first two sets of APP metabolite experiments, after 3 weeks of samples have been stored, supernatants were diluted 4-fold and assayed with the Amyloid beta 40 Human ELISA Kit and either the Amyloid beta 42 Human ELISA Kit or the Amyloid beta 42 Human ELISA Kit Ultrasensitive (Invitrogen), according to the manufacturer's instructions. For the second two sets, supernatants were diluted 4-fold and assayed with the V-PLEX Plus Abeta Peptide Panel 1 (6E10) Kit and the sAPPalpha/sAPPbeta Kit (Meso Scale Discovery), according to the manufacturer's protocol.

Flow cytometry assay:

Both cell lines were incubated with media supplemented with 200nM human Beta-Amyloid (1-42) HiLyte Fluor 555 (AnaSpec, Fremont, CA, USA), with or without thiethylperazine (MilliporeSigma) for approximately 18 hours. Cells were then washed twice with phosphate buffered saline (PBS), trypsinized, and spun-down. Pelleted cells were washed once with ice cold PBS, then resuspended in 1% FBS in ice cold PBS, and kept on ice until assayed. Sorting occurred on the FACSCanto II (BD Biosciences), and initially gated using untreated cells. Values are reported as the percentage of fluorescent cells.

RNA sequencing:

The first RNA-seq experiment utilized the TruSeq RNA Library Prep Kit v2 on the NextSeq500 (Illumina), and results mapped to 37,703 unique Ensembl IDs. The mean total reads per sample was 58.0±15.1 million. The next two RNA-seq experiments utilized the SMARTer Stranded Total RNA-Seq Kit v2 – Pico Input Mammalian (Takara Bio Inc., Kusatsu, Shiga, JP), and were sequenced on the NovaSeq 6000 (Illumina). Results mapped to 54,723 and 55,109 unique Ensembl IDs, respectively. The mean total reads per sample was 73.2±14.6 and 50.6±7.6 million reads, respectively. FASTQs were generated with bcl2fastq v2.18 (Illumina). Reads were aligned with STAR v2.7.3a (Dobin and Gingeras, 2016) to generate BAM files, and differential expression analysis was accomplished using featureCounts from Subread package v2.0.0 (Liao, Smyth and Shi, 2014) and DeSeq2 v1.26.0 (Love, Huber and Anders, 2014).

qRT-PCR:

Reverse transcription (RT) and no-RT reactions were achieved using SuperScript IV VILO Master Mix (Thermo Fisher Scientific), and qPCR was performed using TaqMan Fast Advanced Master Mix (Applied Biosystems) and multiplexed with primer/probe set for ACTB (Hs01060665_g1, VIC-MGB) and either TIMP3 (Hs00165949_m1, FAM-MGB) or CD38 (Hs00120071_m1, FAM-MGB). Reactions were run on the QuantStudio 6 Flex Real-Time PCR System (Applied Biosciences), according to the manufacturer's protocol. Samples were measured in quadruplicate an quantified using the RQ = 2^(-(delta delta CT)) method (Livak and Schmittgen, 2001), and values reported as means of those technical replicates.

FIGURES AND LEGENDS:

Figure 1: ABCC1 overexpression in BE(2)-m17 cells significantly decreases extracellular Abeta1-40 and 1-42 levels. [A] and [B] are experiment 1 and 2, respectively. The empty vector cell line is labeled "Puro" (grey boxes), and ABCC1-overexpressing cells are labeled "ABCC1" (blue boxes). Each point on the plots is the mean of technical quadruplicates, as measured by ELISA. P-values reported on each plot are calculated from Student's two-sample t-test by comparing the two groups in that plot (N=6, n=3 for each plot).

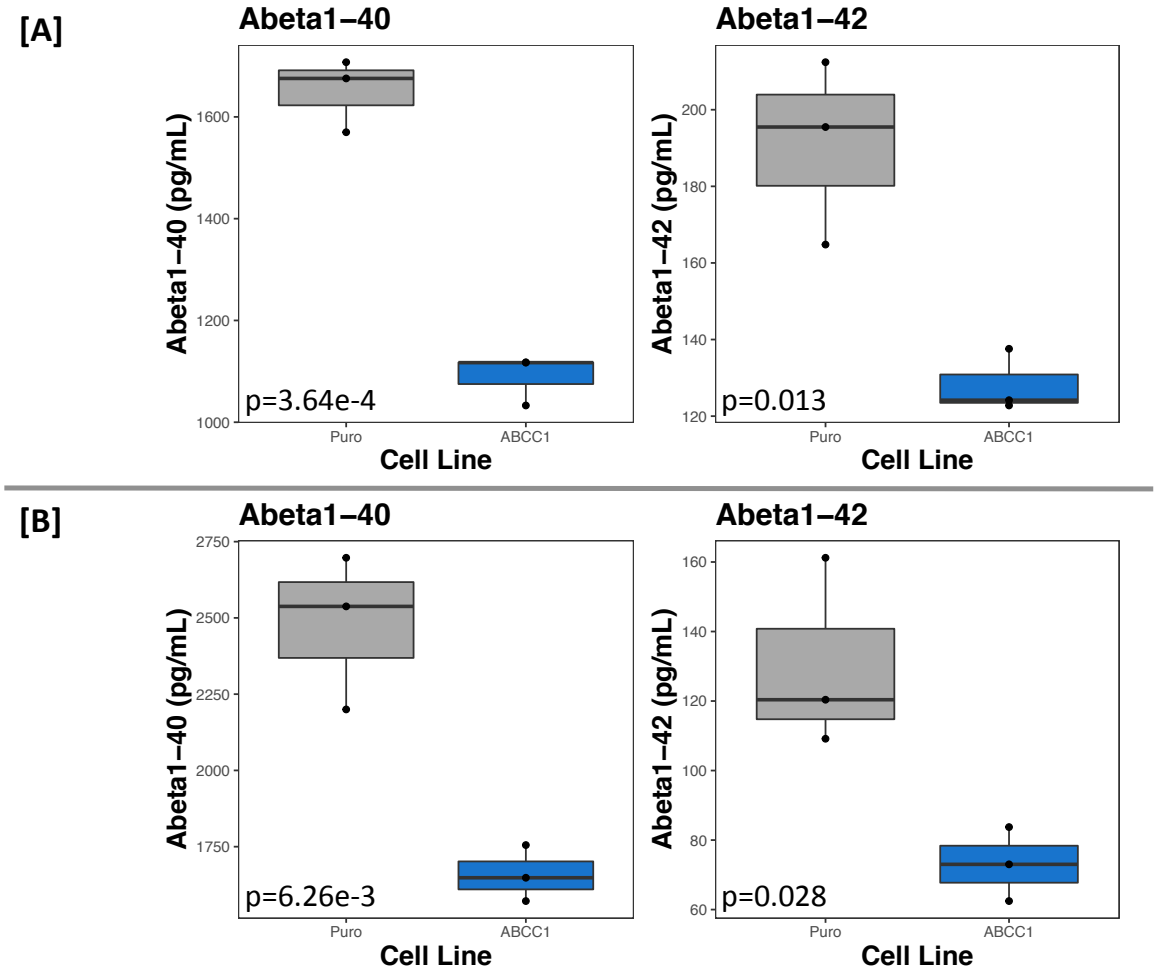


Figure 2: ABCC1 exports Abeta, and that activity is increased by thiethylperazine. [A] shows the original gating using untreated (unstained) cells to identify singlets and set a threshold for fluorescence. [B] shows the results of the cytometry experiment when empty vector cells (Puro, grey bars) or ABCC1 over-expressing cells (ABCC1, blue bars) are treated with fluorescent Abeta1-42 with or without thiethylperazine (TEP). Cells in quadrant 1 (Q1) are considered fluorescent, while those in Q4 are not. Percentage of fluorescent cells is plotted as a bar graph in [C].

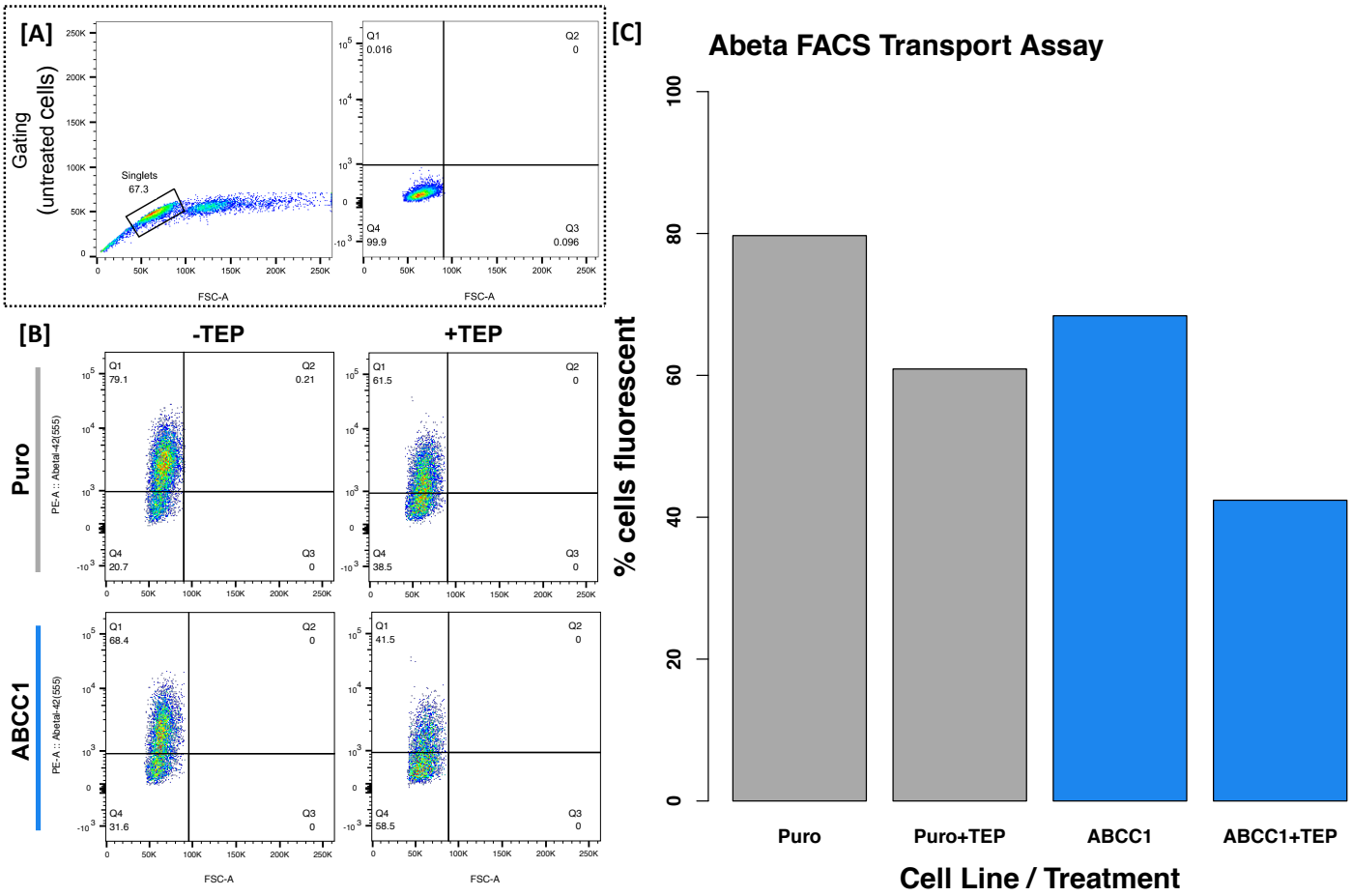


Figure 3: ABCC1 overexpression in BE(2)-m17 cells significantly decreases extracellular Abeta1-40, Abeta1-42, and sAPPbeta levels, and increases the ratio of alpha- to beta-secretase cleaved APP molecules. [A] and [B] show the third (cryopreserved cells) and fourth (newly transfected cells) APP metabolite experiments, respectively, measured using the MSD platform. The empty vector cell line is labeled "Puro" (grey boxes), and ABCC1-overexpressing cells are labeled "ABCC1" (blue boxes). All points on the plot are means of technical quadruplicates. P-values reported on each plot are calculated from Student's two-sample t-test by comparing the two groups in that plot (N=12, n=6 for each plot). The results in [A] demonstrate that the decrease in extracellular Abeta species is not temporal, and [B] demonstrates that the location of integration of the transposable vectors is not the reason for altered APP metabolism.

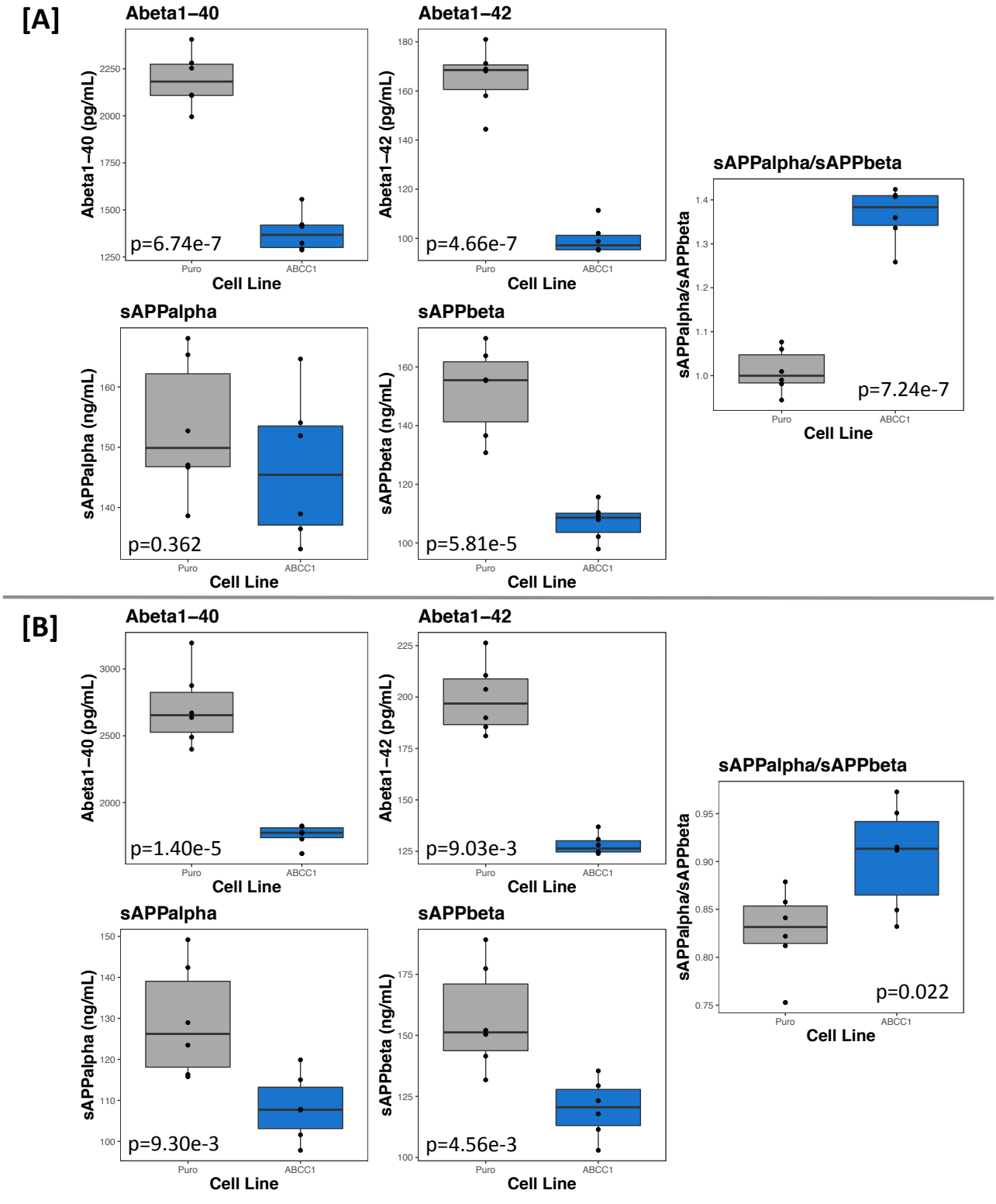
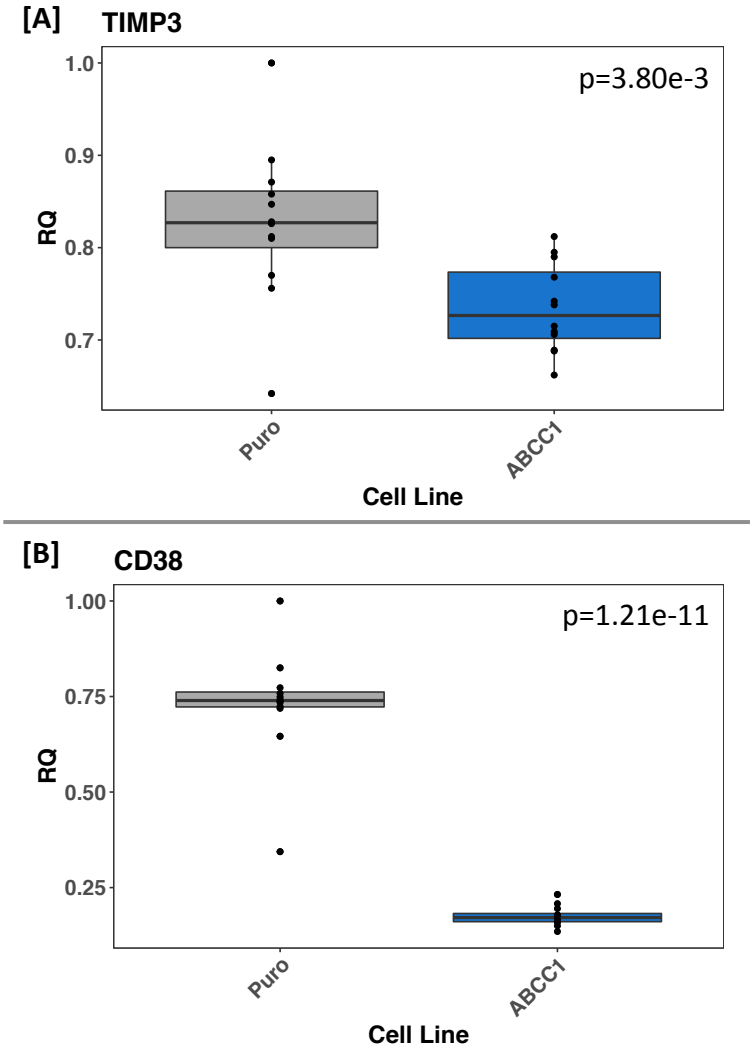


Figure 4: ABCC1 overexpression in ReNcell VM, a human neural progenitor cell line, significantly decreases mRNA levels of *TIMP3* and *CD38*. [A] and [B] show the relative quantification (RQ) of *TIMP3* and *CD38* mRNA in ReNcell VM. The empty vector cell line is labeled "Puro" (grey boxes), and ABCC1-overexpressing cells are labeled "ABCC1" (blue boxes). All points on the plots are means of technical quadruplicates. P-values reported on each plot are calculated from Student's two-sample t-test by comparing the two groups in that plot (N=24, n=12 for each plot). The results confirm that altered expression of these genes is not due to location-specific genomic integration of the vectors, but rather because of increased ABCC1 expression. Furthermore, this demonstrates that decreased expression of these genes due to ABCC1 overexpression is not specific to the BE(2)-m17 human neuroblastoma cell line.



Supplementary Table 1: *TIMP3* and *CD38* are the only two differentially expressed genes with log2FCs that can account for the altered APP processing observed, according to current literature. Of the 55 GOIs identified, 12 are linked to altered APP processing or metabolism, but 10 of those genes have expression level changes in the opposite direction than are known in the literature to be able to account for the altered extracellular levels of APP-derived peptides observed. This may represent a lack of knowledge about these genes' complete functions in APP metabolism, or it may indicate that their known roles have rather subtle effects. GOIs with expression levels consistent with our observations are highlighted in blue, while those that are directly inconsistent are highlighted in red.

Gene Name	Ensembl Gene ID	Chr.	Log2FC	Stat	p-value	p-adjusted	Justification	Final GOI?
GHRH	ENSG00000118702	20	8.00958478	6.5363983	6.30E-11	2.16E-09	A GHRH antagonist, MIA-690, reduced Abeta1-42 and Tau deposition in 5xFAD mouse brains (Jaszberenyi et al., 2012); therefore, if GHRH were responsible for the reduced extracellular Abeta observed in our experiment, we would expect to see it down-, rather than up-regulated.	No
GC	ENSG00000145321	4	6.02179664	4.38997943	1.13E-05	0.000140051	GC, aka DBP, can bind to Abeta to reduce oligomerization (Moon et al., 2013). Therefore, it may be possible, though unlikely, that increased GC is binding to Abeta and making it unrecognizeable by antibodies used in assays. However, GC upregulation is not significant in the second RNA-seq experiment, and is downregulated in the third experiment. Therefore, we do not believe that the differential expression of GC can account for reduced Abeta.	No
CYP1A2	ENSG00000140505	15	5.43876407	4.12430361	3.72E-05	0.000403413	Only publication linking the two terms refers to the enzymatic profile of a drug that blocks Abeta-induced apoptosis (Elkamhawy et al., 2017). There is no known direct link to APP or Abeta metabolism.	No
PLXNA4	ENSG00000221866	7	2.2827125	17.6165379	1.84E-69	1.31E-66	Transfection of 3 isoforms of PLXNA4 did not alter APP processing, but the full-length isoform increased TAU phosphorylation (Jun et al., 2014). Literature does not demonstrate a direct connection between PLXNA4 expression and APP/Abeta metabolism.	No
PTGS1	ENSG00000095303	9	1.96085251	4.05140616	5.09E-05	0.000536458	Expression of COX1, encoded by PTGS1, in CHO cells, resulted in increased Abeta1-42 (Qin et al., 2003); therefore, if PTGS1 expression were responsible for the decrease in extracellular Abeta observed, we would expect to see PTGS1 down-, rather than up-regulated.	No
HCN1	ENSG00000164588	5	1.71441095	5.05079538	4.40E-07	7.73E-06	Link to Abeta processing has to do with neuroexcitability, but authors also found that overexpression of HCN1 in Neuro2a cells decreased Abeta production (Chang et al., 2019). However, HCN1 was not significantly upregulated in the second experiment, and was downregulated in the third experiment. Expression of HCN1 cannot account for reduced Abeta in all experiments.	No
FOXC1	ENSG00000054598	6	1.67804982	9.68683622	3.43E-22	3.32E-20	Transcription factor found to be differentially expressed in AD (Rahman et al., 2020), not a catalytic enzyme. Furthermore, FOXC1 was insignficantly downregulated in the second experiment and insignificantly upregulated in the third experiment. Expression is not consistent to even account for changes in APP metabolizing enzymes.	No
TGFB3	ENSG00000119699	14	1.63851504	9.48843155	2.35E-21	2.11E-19	Oligomeric Abeta can induce TGFB3 production (Tapella et al., 2018), though the gene product is not linked to APP/Abeta metabolism. Furthermore, TGFB3 was insignificantly downregulated in the second experiment, and insignificantly upregulated in the third experiment. TGFB3 cannot logically account for altered APP processing observed.	No
SORCS2	ENSG00000184985	4	-1.5340787	-9.9562092	2.37E-23	2.52E-21	Increased SORCS2 decreased APP processing, and decreased SORCS2 increased gamma-secretase activity (Reitz et al., 2013). SORCS2 was significantly downregulated in all 3 RNA-seq experiments, which is the opposite of what we would expect if SORCS2 expression levels accounted for altered APP processing observed.	No
SEZ6	ENSG00000063015	17	-1.5364914	-14.958268	1.38E-50	5.39E-48	SEZ6 is a substrate for BACE1/2 (Pigoni et al., 2016). SEZ6 overexpression results in reduced Abeta species (Paracchini et al., 2018). SEZ6 expression was significantly downregulated in all 3 RNA-seq experiments, and thus it's expression is in the opposite direction expected to account for reduced extracellular Abeta species.	No
NKX6-1	ENSG00000163623	4	-1.5453016	-4.6731491	2.97E-06	4.25E-05	Association with amyloid is islet amyloid pancreatic polypeptide.	No
NOX5	ENSG00000255346	15	-1.5880465	-15.399964	1.64E-53	7.00E-51	Plays a role in oxidative stress in Alzheimer's disease (Tarafdar and Pula, 2018). No direct link to APP/Abeta metabolism.	No
PTGER4	ENSG00000171522	5	-1.6250699	-4.0742588	4.62E-05	0.000491256	PTGER4 stimulation can result in PSEN1 endocytosis, and activation of the gamma-secretase (Hoshino et al., 2009), thus a decrease in PTGER4, as we observed, may result in reduced gamma-secretase activity. However, PTGER4 downregulation in the second and third RNA-seq experiments was statistically insignificant and carried very minimal log2FC of -0.189 and -0.021, respectively. Therefore, differnetial expression of PTGER4 does not likely account for decreased extracellular Abeta species seen in all experiments.	No
MAFA	ENSG00000182759	8	-1.6918124	-4.564746	5.00E-06	6.81E-05	Association with amyloid is islet amyloid pancreatic polypeptide.	No
SEMA3A	ENSG00000075213	7	-1.6940126	-18.902189	1.09E-79	1.12E-76	sAPPalpha can bind SEMA3A and inhibit it (Magdesian et al., 2011), and Sema3a may induce neuronal collapse in mice (Good et al., 2004), but Sema3a stimulation has been shown to increase Tau phosphorylation with no observed differences in APP processing (Jun et al., 2014). SEMA3A is significantly downregulated in all 3 RNA-seq experiments; however, we cannot find a link between SEMA3A and APP metabolism in the literature.	No
FAS	ENSG00000026103	10	-1.775488	-8.4014521	4.41E-17	2.92E-15	FAS is implicated in AD because of apoptosis (Ethell and Buhler, 2003). No direct link in literature between FAS and altered APP processing. FAS downregulation in the second and third RNA-seq experiments was insignificant.	No
SSTR1	ENSG00000139874	14	-1.7856036	-11.357719	6.79E-30	1.11E-27	SST (somatostatin) has been shown to bind to Abeta (Lau et al., 2017), and is the reason for the link between SSTR1 and Abeta; however, SSTR1 is a G-protein coupled receptor, with no known APP processing abilities, and its downregulation in the second and third RNA-seq experiments was insignificant. There is no reason to believe SSTR1 can be directly responsible for lowering extracellular Abeta.	No
CYP19A1	ENSG00000137869	15	-1.8706075	-14.752983	2.94E-49	1.10E-46	A SNP in CYP19A1 is associated with AD (Huang and Poduslo, 2006), but it has been shown that the SNP identified is not associated with altered APP processing (Han, Schellenberg and Wang, 2010). No direct link between CYP19A1 and APP metabolism found.	No
NAV3	ENSG00000067798	12	-1.9473467	-5.548492	2.88E-08	6.35E-07	Regulates axon guidance and has been shown to be downregulated in young APPswe/PS1deltaE9 mice (Zong et al., 2015). No direct link between APP processing, and downregulation was statistically insignificant in second and third RNA-seq experiments.	No
TIMP3	ENSG00000100234	22	-1.9539687	-22.301951	3.5374E-110	7.5565E-107	TIMP3 encodes the Tissue Inhibitor of Metalloproteinases 3, a protein that can irreversibly inhibit APP-cleaving alpha-secretases like ADAM10 and ADAM17 (Hoe et al., 2007). TIMP3 expression has also been shown to be reduced in AD brain tissue (Dunckley et al., 2006), which may play a role in increased cerebral Abeta. Logically, if TIMP3 expression is downregulated, more APP molecules are more likely to be cleaved by an alpha-secretase. This would result in decreased extracellular Abeta and an increase in alpha- versus beta-secretase cleavage of APP.	**YES**
WNT4	ENSG00000162552	1	-1.9799445	-14.536432	7.12E-48	2.43E-45	No link to APP/Abeta metabolism.	No
NEUROD1	ENSG00000162992	2	-2.0840307	-7.3142458	2.59E-13	1.18E-11	Related to Alzheimer's disease because of it's role in neurogenesis and differentiation (Dard, Dahan and Rampon, 2019), but linked to amyloid because of islet amyloid pancreatic polyprotein, as the gene is strongly associated with diabetes.	No
SNCAIP	ENSG00000064692	5	-2.2299991	-7.2718384	3.55E-13	1.58E-11	siRNA targeting SNCAIP reduced aggresome activity, and the aggresome is capable of storing and degrading aggregated proteins (Zaarur et al., 2015). No literature shows a direct link between SNCAIP and APP processing.	No
PLCG2	ENSG00000197943	16	-2.320263	-8.5616116	1.11E-17	7.67E-16	One study found PLCG2 is significantly upregulated when cells exposed to Abeta (Sierksma et al., 2020), and another found that a variant in PLCG2 protects against Tau phosphorylation, and expression seems to be responsive to Abeta (Kleineidam et al., 2020). No study has demonstrated that altered PLCG2 expression alters APP processing. Furthermore, downregulation of PLCG2 was not significant in the second or third experiments.	No
ACE2	ENSG00000130234	X	-2.3287002	-4.3401745	1.42E-05	0.000171912	ACE2 activation reduces hippocampal soluble Abeta1-42 (Evans et al., 2020), which is in opposition to our results. However, ACE2 can convert Abeta1-43 to Abeta1-42, which can then be converted to Abeta1-40 by ACE (Liu et al., 2014). If, in our system, less Abeta1-43 is being converted to Abeta1-42, and is thus not detectable by our assay, downregulation of ACE2 may account for some of the reduction in extracellular Abeta observed. However, the downregulation of ACE2 was insignificant in the second RNA-seq experiment, and was insignificantly upregulated in the third experiment.	No
FGFR3	ENSG00000068078	4	-2.3335884	-10.261628	1.05E-24	1.20E-22	FGFR3 is released by neurons stimulated by oligomeric Abeta1-42 to recruit microglia to damaged tissue (Noda et al., 2014). No literature linking FGFR3 to APP/Abeta metabolism.	No
MBP	ENSG00000197971	18	-2.3392196	-3.9941838	6.49E-05	0.000662943	It has been shown that MBP can act as a chaperone protein for Abeta, limiting its fibrilation (Hoos et al., 2007), and that MBP has serine proteinase activity that can degrade Abeta (Liao et al., 2009). Therefore, if MBP could account for reduced extracellular Abeta, we would expect to see it up-, not down-regulated, in the ABCC1-overexpressing cells.	No
ABI3	ENSG00000108798	17	-2.5271572	-4.8113809	1.50E-06	2.31E-05	Clusters of ABI3+ microglia are associated with amyloid plaques, which may suggest a role for ABI3+ in microglia motility (Satoh et al., 2017), and ABI3(S209F) has been found to be a risk factor for AD (Sims et al., 2017), but no literature links ABI3 directly to APP processing or metabolism.	No
GRIK1	ENSG00000171189	21	-2.6364395	-4.9042159	9.38E-07	1.52E-05	Stimulation of GRIK1 with kainic acid increased Abeta and oligomeric Abeta, likely because GRIK1 signaling increases phosphorylation and activation of NF-kappa B (Ruan et al., 2019), a transcription factor. Therefore, if the downregulation of GRIK1 observed in our experiment played a role in reducing extracellular Abeta, we would expect that the lack of GRIK1 signalling through NF-kappa B alters transcription of genes capable of altering APP metabolism, rather than GRIK1 directly playing a role.	No
ERBB4	ENSG00000178568	2	-2.6467963	-16.468033	6.23E-61	3.66E-58	ERBB4 is the neuroregulin (NRG1) receptor, and is cleaved by factor-alpha converting enzyme, then subsequently by PSEN-dependent gamma-secretase (Woo et al., 2012), the same gamma-secretase that cleaves the APP molecule. However, no literature directly links ERBB4 to altered APP/Abeta metabolism.	No

CD38	ENSG00000004468	4	-2.9798536	-5.78603	7.20694E-09	1.78449E-07	CD38 encodes the Cluster of Differentiation 38, an enzyme that synthesizes and hydrolyzes cyclic adenosine 5'-diphosphate-ribose, a molecule that regulates intracellular calcium signaling (Chini et al., 2002). It has been shown that Cd38 knockout AD mouse models have improved cognitive deficits, decreased cerebral amyloid burden, and that primary neurons cultured from those mice secrete significantly less Abeta species (Blacher et al., 2015). The authors found that knockout of Cd38 alters beta- and gamma-secretase activity, effectively reducing both (Blacher et al., 2015). Therefore, downregulation of CD38 resulting in decreased extracellular Abeta is consistent with the literature.	**YES**
KCNIP4	ENSG00000185774	4	-2.9838801	-27.415115	1.81E-165	8.51E-162	The longest isoform of KCNIP4 can interact with PSEN1 of the gamma-secretase, and decreasing expression of this longest isoform increases Abeta1-40 and Abeta1-42 production (Massone et al., 2011). In another study, KCNIP4, aka CALP, overexpression did not alter gamma-cleavage of APP (Morohashi et al., 2002). Taken together, a decrease in KCNIP4 long isoform expression would increase extracellular Abeta, and a decrease in the short isoform should have no effect on APP processing. We see KCNIP4 expression decrease while extracellular Abeta species decrease. This is the opposite of what would be expected based on the literature.	No
SORCS3	ENSG00000156395	10	-3.2424713	-5.4616931	4.72E-08	1.00E-06	Expression reduced in frontal cortex after amyloid deposition in APP/PS1 mice (Hermey et al., 2019), and knockdown of SORCS3 increased APP processing (Reitz et al., 2013). Therefore, if SORCS3 could account for reduced extracellular Abeta, we would expect to see it up-, rather than down-regulated.	No
MUSK	ENSG00000030304	9	-3.373102	-4.5068209	6.58E-06	8.67E-05	Link between MUSK and APP is that they are LRP4 coreceptors at the neuromuscular junction (Choi et al., 2013), with no known link to APP processing.	No
TNFRSF1B	ENSG00000028137	1	-3.6063976	-4.4895388	7.14E-06	9.34E-05	A single study found that stimulation of their TNFRSF1B-APP-GFP chimeric receptor increased APP processing (Sim and Heese, 2010). The downregulation of TNFRSF1B is statistically insignificant in the second and third RNA-seq experiments. Downregulation of this receptor (with no known enzymatic function) cannot not directly account for altered APP processing.	No
CTNNA3	ENSG00000183230	10	-3.8116282	-14.065955	6.15E-45	1.78E-42	One study found SNPs in CTNNA3 were associated with increased cerebral Abeta levels (Ertekin-Taner et al., 2003), but two other studies found that the association is not significant (Blomqvist et al., 2004; Busby et al., 2004), and a fourth found the association only in females (Miyashita et al., 2007). However, no direct link between CTNNA3, a cell-to-cell adhesion molecule, and APP/Abeta metabolism has been found in the literature. Interestingly, knockdown of a gene nested within the CTNNA3 locus, LRRTM3, has been shown to decrease secreted Abeta species through its interaction with APP and BACE1 (Lincoln et al., 2013). That study found a relationship between mRNA levels of CTNNA3, LRRTM3, BACE1, and APP, but did not demonstrate that CTNNA3 alters APP metabolism. Furthermore, in the same study, when LRRTM3 is knocked down in SH-SY5Y cells, CTNNA3 mRNA levels increased (Lincoln et al., 2013); therefore, it seems that an increase in CTNNA3 would be associated with decreased secreted Abeta species, if any relationship exists at all.	No
C4BPB	ENSG00000123843	1	-4.1219575	-8.0779878	6.58E-16	3.93E-14	Link between C4BPB and amyloid is that C4BPB, amyloid A, and protein Z serum levels may serve as biomarkers for pulmonary tuberculosis (Jiang et al., 2017).	No
CCK	ENSG00000187094	3	-4.3958699	-5.4313899	5.59E-08	1.17E-06	One study found that CCK expression does not correlate with Abeta1-42 in AD patients (Plagman et al., 2019), and most studies linking CCK to amyloid pertain to islet amyloid pancreatic polypeptide.	No
DCC	ENSG00000187323	18	-4.7379038	-7.2792154	3.36E-13	1.51E-11	APP interacts with DCC and enhances Nestin signalling (Rama et al., 2012), and PSEN1 processes DCC to ensure fidelity of axon guidance (Bai et al., 2011), but no link between DCC and APP processing was found. Downregulation of DCC was statistically insignificant in second and third RNA-seq experiments.	No
CD34	ENSG00000174059	1	-4.882655	-16.260743	1.87E-59	9.79E-57	Decreased counts of circulating CD34+ cells correlates with Abeta1-42 levels in AD patients (Maler et al., 2006), but no link between CD34 and APP/Abeta processing.	No
GLI3	ENSG00000106571	7	-4.8874424	-8.4404417	3.16E-17	2.10E-15	GLI3 is a transcription factor that can repress Pitrm1 (Town et al., 2009), a metalloprotease capable of degrading Abeta when it accumulates in mitochondria (Falkevall et al., 2006). If GLI3 suppression were increasing PITRM1 expression, resulting in decreased Abeta species, PITRM1 would be on this list. There is no reason to believe that suppression of the transcription factor, itself, would decrease extracellular Abeta.	No
HLA-DRB1	ENSG00000196126	6	-4.9849761	-11.456865	2.17E-30	3.59E-28	HLA-DRB1 is part of the major histocompatibility complex, and specific isoforms have shown high immunoreactivity to Abeta species (Zota et al., 2009). A link between HLA-DR1 and amyloid, then, makes sense *in vivo*, not in a monoculture of neuroblastoma cells. Furthermore, if Abeta were binding to HLA-DRB1, thus removing it from the supernatant, and that was the reason for measuring reduced extracellular Abeta, we would expect HLA-DRB1 expression to be up-, rather than down-regulated.	No
BMP6	ENSG00000153162	6	-5.4269376	-4.0259947	5.67E-05	0.000590154	It has been shown that Abeta increases BMP6 levels, in vitro (Crews et al., 2010), and BMP6 is neuroprotective against toxic effects of Abeta25-35 (Sun et al., 2014), but no link found between BMP6 and APP processing.	No
PLA2G4A	ENSG00000116711	1	-5.6292257	-5.0104561	5.43E-07	9.30E-06	PLA2G4A has been shown to contibute to Abeta-induced lysosomal membrane permeabilization (Sarkar et al., 2020), and cytosolic phospholipase A2 activation facilitates oligomeric Abeta endocytosis by microglia (Teng et al., 2019). If PLA2G4A expression were to account for the reduced extracellular Abeta level observed, we would expect to see it up-, rather than down-regulated.	No
TNFSF10	ENSG00000121858	3	-6.0304536	-4.0878419	4.35E-05	0.000465899	Administration of antibodies that neutralize TNFSF10 reduces Abeta1-42 in the hippocampi of 3xTg AD mice (Cantarella et al., 2015); however, TNFSF10 is a cytokine, not an enzyme. Therefore, we would expect that downregulation of this gene would not directly account for altered APP processing, but reduced signalling by TNFSF10 may alter levels of other genes capable of altering APP processing.	No
OTX2	ENSG00000165588	14	-6.0337592	-4.1299129	3.63E-05	0.000394606	OTX2 is a transcription factor involved in the maturation of perineuronal nets (Soleman et al., 2013). Reduced OTX2 may reduce expression of genes that can alter APP processing, but the transcription factor, itself, would not logically perform this task.	No
APLN	ENSG00000171388	X	-6.2095007	-19.586904	2.00E-85	2.47E-82	Administration of exogenous apelin-13, a peptide fragment resulting from cleavage of the APLN gene product, reduced Abeta production by decreasing BACE1 (beta-secretase) activity and increasing neprilysin (an Abeta degrading enzyme) activity (Luo et al., 2019). Therefore, if APLN expression could account for reduced extracellular Abeta observed in our experiment, we would expect APLN expression to be up-, rather than down-regulated.	No
HDC	ENSG00000140287	15	-6.5182389	-6.0764451	1.23E-09	3.40E-08	Converts histidine to histamine (Hocker et al., 1996). May be part of the signalling that results in differential expression of genes capable of altering APP metabolism, but a change in expression in either direction should not directly result in altered APP processing.	No
CFI	ENSG00000205403	4	-6.6038489	-4.8502187	1.23E-06	1.94E-05	CFI activity can be decreased by Abeta, which can activate the complement cascade (Lashkari et al., 2018), but no link found between CFI and APP/Abeta processing.	No
HNF1B	ENSG00000108753	17	-6.9354737	-5.5473661	2.90E-08	6.38E-07	Transcritption factor that controls expression of many genes implicated in glucose-induced insulin resistance (Wang et al., 2002). Again, a trasnciption factor, itself, should not logically have a direct role in altering APP processing unless altering the expression levels of genes capable of this task.	No
INPP5D	ENSG00000168918	2	-6.9920428	-5.6192849	1.92E-08	4.38E-07	INPP5D is believed to be involved in the immune response in the brain, regulating clearance of misfolded proteins by microglia (Efthymiou and Goate, 2017). If INPP5D had this function in our neuroblastoma monoculture, we would expect INPP5D to be up-, rather than down-regulated, if its expression accounted for the reduced extracellular Abeta observed.	No
CD3D	ENSG00000167286	11	-7.0480262	-5.2660668	1.39E-07	2.71E-06	Only article linking CD3D to amyloid discusses reduction of CD3D positive T-lyphocytes in sRAGE-MSC treated rats (Oh et al., 2018). CD3D is part of the T-cell receptor with no known link between CD3D and APP processing.	No
KCNMB2	ENSG00000197584	3	-7.1102768	-18.687604	6.25E-78	6.11E-75	KCNMB2 locus was associated with hippocampal sclerosis in a GWAS (Beecham et al., 2014), but no direct link in the literature between KCNMB2 and APP/Abeta metabolism.	No
DPP4	ENSG00000197635	2	-8.5053795	-6.9526959	3.58E-12	1.41E-10	Inhibition of DPP4 with sitagliptin can delay amyloid deposition by increasing GLP-1 levels in the brain (D'Amico et al., 2010), and GLP-1 receptor stimulation can lower Abeta secretion, in vitro (Li et al., 2010). However, if DPP4 downregulation in our study is resulting in increased GLP-1 levels, since GLP-1 is a hormone, not an enzyme, we would expect that GLP-1 signalling would alter the transcript levels of proteins involved with APP/Abeta processing, rather than directly activate/deactivate available enzymes with mRNA levels that are consistent between the two cell lines.	No
MEGF10	ENSG00000145794	5	-11.550718	-17.774019	1.12E-70	8.52E-68	The MEGF10 protein has been shown to function as a phagocytosis receptor for the uptake of Abeta (Singh et al., 2010). If MEGF10 were responsible for the decrease in extracellular Abeta, we would expect to see higher levels of MEGF10, which would likely result in increased phagocytosis of Abeta, and thus decreased extracellular levels of the peptides; however, the opposite of this was observed.	No

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