

Expression of a noncoding RNA is elevated in Alzheimer's disease and drives rapid feed-forward regulation of β -secretase

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Recent efforts have revealed that numerous protein-coding messenger RNAs have natural antisense transcript partners, most of which seem to be noncoding RNAs. Here we identify a conserved noncoding antisense transcript for β -secretase-1 (*BACE1*), a crucial enzyme in Alzheimer's disease pathophysiology. The *BACE1*-antisense transcript (*BACE1*-AS) regulates *BACE1* mRNA and subsequently *BACE1* protein expression *in vitro* and *in vivo*. Upon exposure to various cell stressors including amyloid- β 1–42 (A β 1–42), expression of *BACE1*-AS becomes elevated, increasing *BACE1* mRNA stability and generating additional A β 1–42 through a post-transcriptional feed-forward mechanism. *BACE1*-AS concentrations were elevated in subjects with Alzheimer's disease and in amyloid precursor protein transgenic mice. These data show that *BACE1* mRNA expression is under the control of a regulatory noncoding RNA that may drive Alzheimer's disease-associated pathophysiology. In summary, we report that a long noncoding RNA is directly implicated in the increased abundance of A β 1–42 in Alzheimer's disease.

Sequential cleavage of amyloid precursor protein (APP) by *BACE1*, the β -site cleaving enzyme essential for A β 1–42 and amyloid- β 1–40 (A β 1–40) biosynthesis¹, and γ -secretase initiates the 'amyloid cascade' that is central to Alzheimer's disease pathophysiology^{2,3}. Oligomers of A β 1–42 produced by *BACE1* influence key aspects of Alzheimer's disease^{4–10}. Studies have revealed elevated brain *BACE1* concentrations in subjects with Alzheimer's disease compared with normal controls^{11–16}. However, controversy exists concerning the extent of *BACE1* upregulation and whether this upregulation involves *BACE1* mRNA or protein^{17–19}.

Loss of *BACE1* results in numerous behavioral and physiological deficits, including memory loss²⁰, emotional deficits²¹, myelination defects in peripheral nerves^{22,23} and loss of synaptic plasticity²¹. Thus, the subtle but crucial boundaries between *BACE1* physiology and pathology indicate that *BACE1* expression must be tightly regulated, allowing the enzyme to perform its physiological functions while avoiding the serious consequences of over- or underexpression.

Here we report that *BACE1*-AS, a natural antisense transcript, plays a part in determining *BACE1* expression. *BACE1*-AS rapidly and reversibly upregulates *BACE1* levels in response to a variety of stresses, including A β 1–42 exposure. Furthermore, we show elevated

BACE1-AS in several brain regions of individuals with Alzheimer's disease. These data suggest that this previously unexamined noncoding RNA has a role in regulating *BACE1* and in driving Alzheimer's disease pathology.

RESULTS

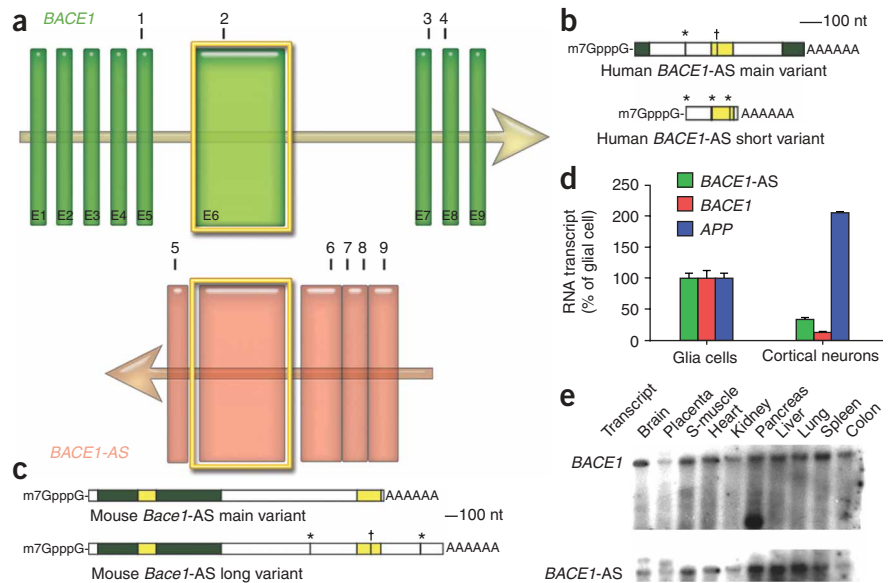
Identification of *BACE1* natural antisense transcript

BACE1-AS was originally identified through the FANTOM large-scale transcriptomics efforts as one member of some 1,000 sense-antisense pairs conserved between human and mouse²⁴. *BACE1*-AS is a conserved ~2-kb RNA transcribed from the positive strand of chromosome 11, on the opposite strand of the *BACE1* locus (11q 23.3), including 104 nucleotides of full complementarity to exon 6 of human *BACE1* mRNA (Fig. 1a). We performed rapid amplification of cDNA ends (RACE) for directional sequencing of 5' and 3' ends and identified two splice variants for human and mouse *BACE1*-AS that overlap the *BACE1* sense transcript (Fig. 1b,c). We found a poly-A tail and cap structure in both human and mouse sequences, indicating that *BACE1*-AS is a fully processed transcript of RNA polymerase II. However, there is no apparent open reading frame. Sequencing data for human and mouse *BACE1*-AS are illustrated in **Supplementary Data 1** and **2** online, and the positions of primers, probes and small

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Figure 1 Genomic organization and expression analysis of *BACE1* and *BACE1-AS*. **(a)** Genomic sequences of *BACE1* and *BACE1-AS*; arrows show the direction of transcription. *BACE1* exons are depicted as vertical bars and marked E1–E9. Human *BACE1-AS* is transcribed from the same region in chromosome 11, but on the opposite strand. Yellow highlighted exons are the overlapping region (104 base pairs) of *BACE1* and *BACE1-AS*, which are conserved across species. Sites numbered 1, 3 and 4 are *BACE1* siRNAs target sites, and site 2 is the northern blot probe site. Sites 5, 6 and 7 are the target sites of the *BACE1-AS* siRNAs and site 8 is the RT-PCR probe target region, which are all in the non-overlapping part of the *BACE1-AS* transcript. Sites 5 and 9 represent the primers for 3' and 5' RACE, respectively. **(b,c)** RACE sequencing data revealed that *BACE1-AS* contains cap structure and a poly-A tail and that this transcript undergoes differential splicing in both human and mouse. The yellow highlighted segments are the overlap region to the *BACE1* sense transcript and the green highlighted segments are additional nucleotides observed from our sequencing data. Point mismatches to the genomic sequence are indicated by stars (*) for A to G and crosses (†) for C to T changes. Nt, nucleotides. **(d)** Expression of *BACE1-AS*, *BACE1* and *APP* mRNA in human cortical neurons (HCN1A) compared to glial cells (M059K). **(e)** Northern blot expression analysis of *BACE1* (top) and *BACE1-AS* (bottom) in ten human tissues. S-muscle, skeletal muscle.



interfering RNA (siRNA) sequences used in these experiments are shown in **Figure 1a** and **Supplementary Table 1** online.

Expression analysis of *BACE1* and *BACE1-AS*

BACE1 mRNA expression levels were 25–75% greater than *BACE1-AS* transcript levels across all samples examined from various tissues and cell lines, in contrast to the relative concentrations in Alzheimer's disease brain (described below). We also observed *Bace1* and *Bace1-AS* transcripts in various regions of the mouse brain. (**Supplementary Figs. 1** and **2** online).

In glia and cortical neurons of human origin, *APP* mRNA was about twice as abundant in neuronal cultures compared to glial cultures, whereas *BACE1* and *BACE1-AS* transcripts were two to three times more abundant in the glial cells (**Fig. 1d**). Northern blot analysis with *in vitro*-transcribed strand-specific RNA probes confirmed that various human tissues express both *BACE1* and *BACE1-AS* (**Fig. 1e**). This observation suggests that *BACE1* and *BACE1-AS* expression may be regulated concordantly, as was recently shown for other sense-antisense pairs^{25,26}.

BACE1-AS regulates *BACE1* RNA and protein *in vitro*

We next investigated whether the *BACE1-AS* transcript regulates expression of *BACE1* mRNA. Unexpectedly, transfection of human SH-SY5Y cells with any one of three distinct siRNA sequences targeting nonoverlapping regions of the *BACE1-AS* transcript resulted in a statistically significant knockdown of not only the targeted *BACE1-AS* transcript, but also *BACE1* mRNA (**Fig. 2a**). There are two types of regulation of sense and antisense transcripts²⁶. In concordant regulation, as in the case of *BACE1-AS*, the antisense transcripts change the level of the sense RNA or the corresponding protein abundance in a positive way. In contrast, in discordant regulation, the antisense transcripts have negative (opposing) effects on sense transcripts. We also observed this concordant pattern of regulation in human HEK293T and HEK-SW cells, in which siRNA-mediated knockdown of *BACE1-AS* resulted in a similar reduction in *BACE1* mRNA (data not shown). In a control experiment in HEK-SW

cells, the level of *BACE2* did not change with *BACE1-AS* siRNA treatment, lending further support to the specificity of the observed *BACE1* regulation by *BACE1-AS* (**Supplementary Fig. 3** online). Furthermore, because three distinct siRNA molecules that exclusively target the *BACE1-AS* transcript resulted in concomitant reduction of sense *BACE1* transcript, it is highly unlikely that the siRNAs acted to knock down *BACE1* transcript through a nonspecific (or 'off-target') mechanism.

To investigate the effects of long-term siRNAs directed against *BACE1-AS* on *BACE1* expression, we generated stable HEK293T cell lines expressing four distinct short hairpin RNAs (shRNAs) to *BACE1* or *BACE1-AS* transcripts. Three of the *BACE1* shRNAs and two of the *BACE1-AS* shRNAs were functional and induced sustained reduction of *BACE1* and *BACE1-AS*, respectively (**Fig. 2b**). Cells expressing *BACE1-AS* shRNA showed reduced *BACE1* levels, and vice versa (**Fig. 2b**).

To examine the concentration-response relationship between *BACE1* mRNA and *BACE1-AS* siRNA, we measured the reduction in *BACE1* mRNA expression across a range of concentrations of *BACE1-AS* siRNA (100 pM–20 nM) in HEK-SW cells. The resulting data confirmed that siRNA targeting *BACE1-AS* reduces the expression of *BACE1* mRNA in a concentration-dependent manner (**Fig. 2c**).

Next, we assessed *BACE1* protein abundance after transfection of HEK293T cells with *BACE1-AS* siRNA or shRNA. Western blotting showed that siRNA against *BACE1-AS* as well as siRNA against *BACE1*, (but not control siRNAs), reduced the expression of *BACE1* protein (**Fig. 2d,e**). Thus, *BACE1-AS* seems to control the expression of *BACE1* at both the mRNA and the protein levels. Additionally, for accurate quantification of the effects of siRNA treatments on *BACE1* protein expression, we established a method for protein quantification by enzyme complementation assay (ECA, **Supplementary Fig. 4** online). We then measured the changes in *BACE1* protein concentration after treatment with siRNAs and shRNAs and observed that siRNAs against either *BACE1* or *BACE1-AS* reduce *BACE1* protein abundance by 40–60% (**Supplementary Fig. 5** online).

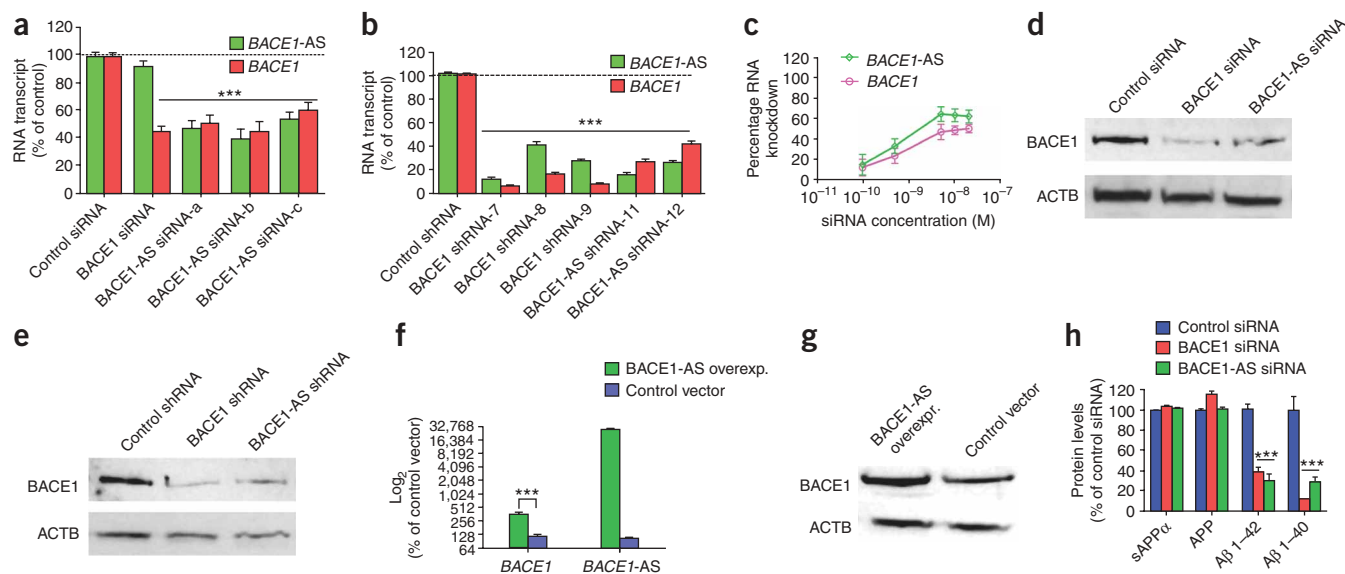


Figure 2 *BACE1-AS* regulates *BACE1* mRNA and protein expression *in vitro*. (a) Targeting *BACE1-AS* transcript with three different siRNAs caused decreases ($P < 0.0001$) in both *BACE1* and *BACE1-AS* transcripts in neuroblastoma cells (SH-SY5Y). (b) Stable transfection of HEK293T cells with shRNA for *BACE1*, *BACE1-AS* and a control shRNA shows that knockdown of *BACE1* for an extended period of time leads to reduction of *BACE1-AS* and knockdown of *BACE1-AS* also leads to reduction of *BACE1* mRNA levels ($P < 0.001$). (c) HEK-SW cells were transfected with 100 pM, 500 pM, 5 nM, 10 nM or 20 nM *BACE1-AS* siRNA. *BACE1-AS* knockdown ranged from 10–60%, and *BACE1* downregulation ranged between 10–50% with increasing concentrations of siRNA. (d,e) Western blot showing that knockdown of either *BACE1* or *BACE1-AS* with siRNA or shRNA leads to reduction of the *BACE1* protein. (f,g) Overexpression of *BACE1-AS* but not an empty control vector leads to increased *BACE1* mRNA (f) ($P < 0.001$) and protein (g) concentrations. (h) HEK-SW cells were transfected with siRNA for *BACE1*, *BACE1-AS* or a control siRNA and analyzed for A β 1–40, A β 1–42, sAPP α and total APP concentrations by ELISA. A β 1–40 and A β 1–42 abundance was reduced ($P < 0.0001$) after transfection of siRNA targeting of either *BACE1* or *BACE1-AS*. Total APP or sAPP α , an enzymatic product of α -secretase, were not changed.

Moreover, overexpression of *BACE1-AS* led to a fourfold increase in *BACE1* mRNA (Fig. 2f). When measured by western blotting, the overexpression of *BACE1-AS* resulted in increased *BACE1* protein abundance (Fig. 2g). These observations further confirm the regulation of *BACE1* expression by *BACE1-AS*, not only at the mRNA but also at the protein level.

Knockdown of *BACE1-AS* reduces A β 1–40 and A β 1–42

We measured by ELISA the amount of A β 1–40 and A β 1–42 after depletion of *BACE1-AS* in the HEK-SW cell line that contains mutated APP, swAPP₇₅₁, the so-called Swedish mutation²⁷. We found a reduced concentration of both A β 1–40 and A β 1–42 in cells treated with *BACE1-AS* siRNA (Fig. 2h). To rule out possible nonspecific effects of siRNA treatment on APP concentration that may account for the observed decreases in A β 1–40 and A β 1–42, we assayed the amount of soluble APP α (sAPP α , soluble product of α -secretase in the supernatants of the HEK-SW cells) and total APP abundance by ELISA. Neither sAPP α nor total APP abundance was altered upon *BACE1* siRNA or *BACE1-AS* siRNA treatment (Fig. 2h). These results suggest that *BACE1-AS* siRNA treatment results in reduced *BACE1* protein function without affecting APP or α -secretase products.

Knockdown of *BACE1-AS* reduces *BACE1* levels *in vivo*

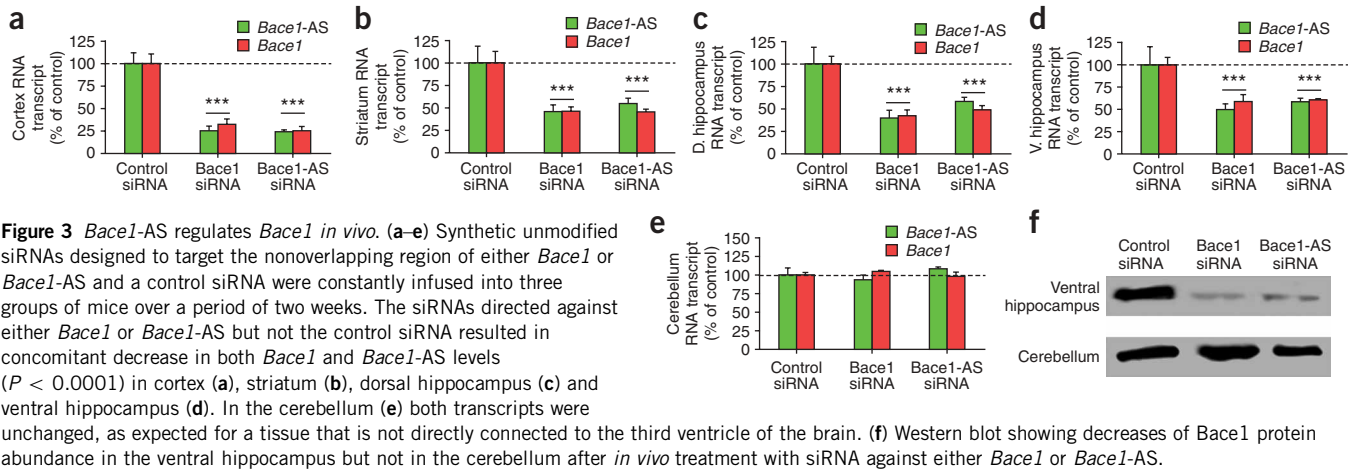
The above data support a role for *BACE1-AS* in regulating *BACE1* function *in vitro* in human cells. Next, we assessed whether orthologous *Bace1-AS* also regulates *Bace1* mRNA and protein *in vivo* in mouse brain. After 14 d of continuous siRNA infusion, *Bace1* mRNA levels were reduced across forebrain regions located adjacent to the third ventricle in mice treated with either *Bace1* siRNA or *Bace1-AS* siRNA, compared to levels unaltered by control siRNA (Fig. 3a–d).

Bace1 and *Bace1-AS* transcripts were unaltered in the cerebellum, a structure that is spatially restricted from the third ventricle, of siRNA-treated mice, consistent with previous work that indicates limited penetration of pump-mediated infusion of siRNA into the brain²⁸ (Fig. 3e). We also measured the amount of *Bace1* protein in the ventral hippocampus and the cerebellum by western blotting after siRNA treatment. We found that both *Bace1* siRNA and *Bace1-AS* siRNA treatment resulted in reduced *Bace1* protein abundance (Fig. 3f). Our *in vivo* findings agree with the *in vitro* data described above and indicate that reduced *Bace1-AS* expression results in reduction of *Bace1* mRNA and protein expression *in vivo*.

Cell stressors increase *BACE1-AS* and *BACE1* protein

Different cell stressors have long been implicated in the pathogenesis of Alzheimer's disease^{13,29}. We exposed HEK-SW cells to hyperthermia, serum starvation, staurosporine, A β 1–42, A β 1–40, hydrogen peroxide (H₂O₂) or high glucose for 12 h. We found that exposure of the cells to high temperature, serum starvation, A β 1–42, H₂O₂ or high glucose resulted in a ~30–130% increase in *BACE1-AS* levels and a ~20–60% increase in *BACE1* mRNA levels (Fig. 4a). Serum starvation generated the strongest response, whereas A β 1–40 and staurosporine exposure did not significantly alter *BACE1-AS* expression levels (Fig. 4a). These results suggest that many, but not all, cell stressors can contribute to the pathogenesis of Alzheimer's disease by altering *BACE1-AS* expression and subsequently *BACE1* enzyme activity.

Accumulating evidence describes A β 1–42 itself as a potent cell stressor^{11,30–33}. To test the hypothesis that A β 1–42 increases *BACE1* expression by a *BACE1-AS* dependent mechanism, we exposed SH-SY5Y cells for 2 h to conditioned media from CHO-7PA2 cells,



which overexpress APP and generate A β 1–42 dimers and oligomers³⁴. Exposure of the SH-SY5Y cells to conditioned media from CHO-7PA2 cells, but not conditioned media from control parental CHO cells resulted in an increase in cytoplasmic concentrations of *BACE1*-AS transcript (Fig. 4b). We obtained similar results when incubating SH-SY5Y cells with synthetic A β 1–42 (1 μ M for 2 h; Fig. 4c). Removal of the cell stressors normalized *BACE1*-AS expression patterns. Using an ECA, we found that synthetic A β 1–42 (1 μ M for 12 h) elicited an increase in *BACE1* protein abundance as well (Fig. 4d). Taken together, the above data indicate that cell stress increases *BACE1*-AS levels, which in turn increases *BACE1* levels; this may result in an increase in APP processing and A β 1–42 production. Subsequently, increased A β 1–42 levels can further increase *BACE1*-AS expression, driving the APP processing cascade in a feed-forward manner.

BACE1-AS forms RNA duplex and increases stability of *BACE1*

We used an RNase protection assay (RPA) on RNA from SH-SY5Y cells to test the possibility of RNA duplex formation (Supplementary Methods online). RT-PCR data showed that the overlapping part of both transcripts was protected from degradation, indicating that *BACE1* and *BACE1*-AS indeed form a RNA duplex (Fig. 5a). We

also validated the RPA data on a 10% Tris-borate-EDTA–urea gel using radiolabeled *BACE1*-AS probes (data not shown).

RNA duplex formation may act to alter the secondary or tertiary structure of *BACE1* and thereby increase its stability. We assessed the stability of *BACE1* and *BACE1*-AS transcripts by blocking new RNA synthesis with α -amanitin and measuring the loss of *BACE1*, *BACE1*-AS, β -actin (*ACTB*) and 18s RNA over a 24-h period. We found that *BACE1*-AS had a shorter half-life than *BACE1* mRNA (8.5 h versus 17.5 h; Fig. 5b). 18s ribosomal RNA, which is a product of RNA polymerase I, was not affected by α -amanitin treatment (Fig. 5b). In a cell line that constitutively expresses *BACE1*-AS shRNA and thereby has depleted *BACE1*-AS levels, we found decreased stability of *BACE1* mRNA compared with cells transfected with a control shRNA (Fig. 5c). Conversely, cells that overexpress *BACE1*-AS showed increased stability of *BACE1* (Fig. 5d). Collectively, our data demonstrate that *BACE1*-AS increases the stability of *BACE1* mRNA.

BACE1-AS is elevated in subjects with Alzheimer's disease

Elevated *BACE1*-AS concentrations may facilitate increased *BACE1* activity and disease progression in the brains of human subjects with Alzheimer's disease. To examine this question, we assessed *BACE1*-AS and *BACE1* mRNA abundance in RNA samples prepared from parietal

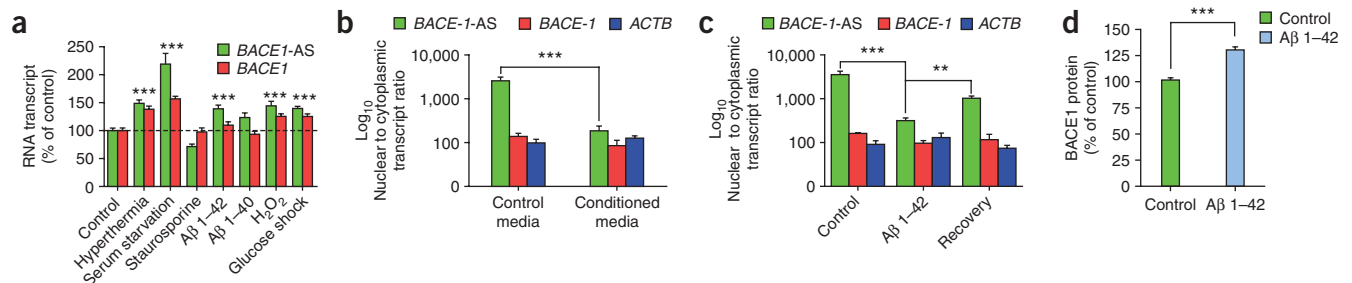


Figure 4 Effect of cell stressors on *BACE1* and *BACE1*-AS. (a) HEK-SW cells were exposed to cell stressors. *BACE1*-AS and *BACE1* transcripts were measured by RT-PCR and normalized to *ACTB* as an endogenous control. Hyperthermia, serum starvation, A β 1–42, hydrogen peroxide or glucose shock caused elevation of *BACE1*-AS and, to a lesser degree, elevation of *BACE1* transcripts. Staurosporine or A β 1–40 did not increase either transcript level. (b) The 7PA2-CHO cells were previously shown to overproduce A β 1–42 dimers and oligomers. Conditioned media from these cells or control parental CHO cells were collected and added to SH-SY5Y cells for 2 h after removal of the regular media. Conditioned media from 7PA2-CHO cells, but not control media, caused the *BACE1*-AS transcript to relocate to the cytoplasm ($P < 0.0001$). (c) Exposure of SH-SY5Y cells to 1 μ M A β 1–42 for 2 h caused an increase in cytoplasmic *BACE1*-AS ($P < 0.001$). The nuclear-cytoplasmic ratio was recovered upon removal of the peptides and maintenance of the cells in regular media for 1 h. (d) Exposure to 1 μ M A β 1–42 peptide caused an elevation in *BACE1* protein abundance ($P < 0.001$). Protein amounts were measured with a β -galactosidase ECA.

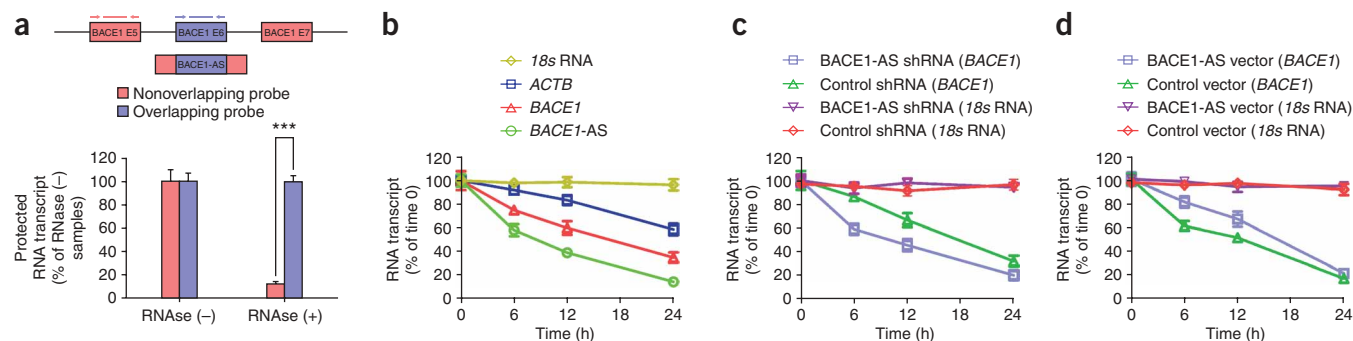


Figure 5 *BACE1-AS* increases the stability of *BACE1* mRNA. **(a)** RPA performed on RNA samples from SH-SY5Y cells. Depicted here are RT-PCR results from two sets of primers and probes covering overlapping and nonoverlapping regions of *BACE1* mRNA. The overlapping region of *BACE1* transcript is protected from degradation by RNase A+T, suggesting RNA duplex formation. **(b)** Stability of *BACE1* and *BACE1-AS* transcripts over time was measured by RT-PCR relative to time 0 after blocking new RNA synthesis with α -amanitin (50 μ M) in HEK293T cells. *BACE1-AS* showed a shorter half-life than *BACE1* and *ACTB*. *18s* RNA, which is a product of RNA polymerase I, was unchanged. **(c)** The stability of *BACE1* mRNA was measured in stably transfected HEK293T cells expressing a *BACE1-AS* shRNA and a second cell line expressing a negative control shRNA. The stability of *BACE1* mRNA was decreased in cells expressing *BACE1-AS* shRNA relative to the control cell line ($P < 0.01$). **(d)** The stability of *BACE1* mRNA increased in HEK293T cells overexpressing *BACE1-AS* in comparison to a cell line transfected with an empty vector.

lobes and cerebellum from five postmortem brains of human subjects with Alzheimer's disease and from five age- and sex-matched control brains. In the Alzheimer's disease samples, the relative quantity of *BACE1-AS* transcript was increased by two to three times, along with a smaller increase in *BACE1* transcript (Fig. 6a and Supplementary Fig. 6a online). In a separate group of 35 subjects with Alzheimer's disease and 35 age- and sex-matched controls³⁵, we examined RNA samples derived from cerebellum (25 Alzheimer's disease samples and 21 control samples), hippocampus (13 Alzheimer's disease samples and 11 control samples), entorhinal cortex (13 Alzheimer's disease samples and 11 control samples) and superior frontal gyrus

(16 Alzheimer's disease samples and 17 control samples). The *BACE1-AS* transcript concentrations were elevated in subjects with Alzheimer's disease by up to sixfold, with an average elevation of about twofold across all brain regions (Fig. 6b–d and Supplementary Fig. 6b,c). We detected a smaller (~30%) increase in *BACE1* mRNA concentrations in these subjects compared to their matched controls (Fig. 6a,b). Taken together, these results support our hypothesis that increases in *BACE1-AS* expression, probably related to cell stressors, drives upregulation of *BACE1* mRNA and protein level, thereby facilitating A β 1–42 biosynthesis in human Alzheimer's disease brain.

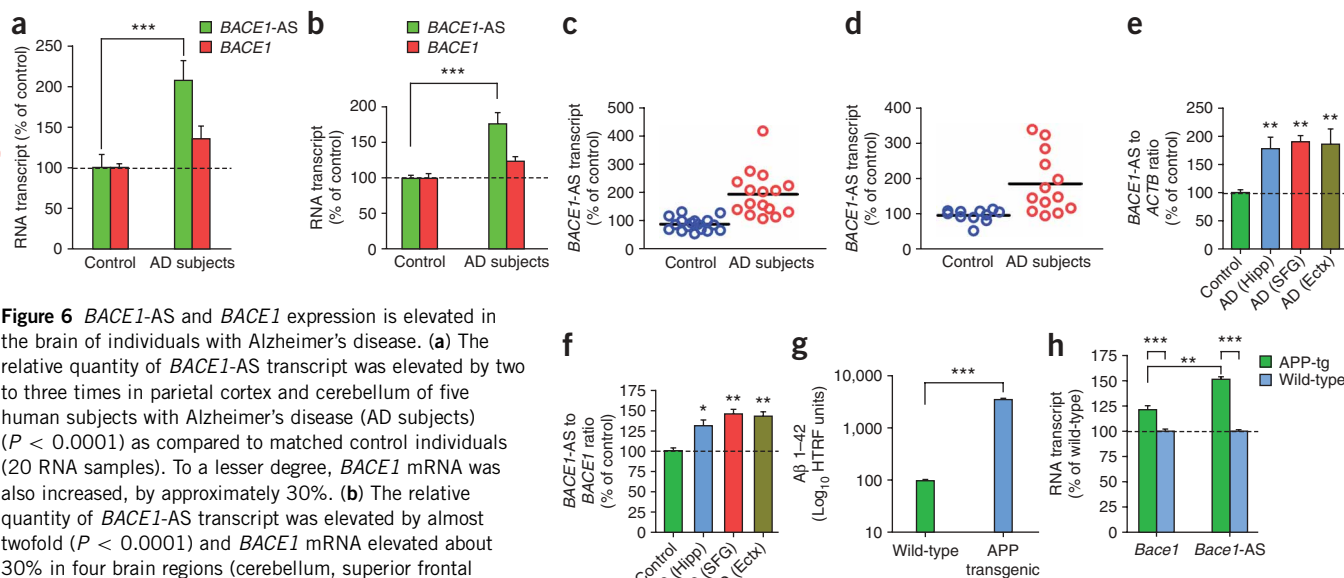


Figure 6 *BACE1-AS* and *BACE1* expression is elevated in the brain of individuals with Alzheimer's disease. **(a)** The relative quantity of *BACE1-AS* transcript was elevated by two to three times in parietal cortex and cerebellum of five human subjects with Alzheimer's disease (AD subjects) ($P < 0.0001$) as compared to matched control individuals (20 RNA samples). To a lesser degree, *BACE1* mRNA was also increased, by approximately 30%. **(b)** The relative quantity of *BACE1-AS* transcript was elevated by almost twofold ($P < 0.0001$) and *BACE1* mRNA elevated about 30% in four brain regions (cerebellum, superior frontal gyrus, entorhinal cortex and hippocampus) of 35 individuals with Alzheimer's disease compared to the average of 35 control individuals (128 RNA samples in total). **(c)** Scatter plot of *BACE1-AS* transcript expression in superior frontal gyrus of 17 control subjects and 16 subjects with Alzheimer's disease. Upregulation ($P < 0.0001$) of *BACE1-AS* was observed in subjects with Alzheimer's disease. **(d)** Scatter plot of *BACE1-AS* transcript expression in hippocampus of 11 controls and 13 individuals with Alzheimer's disease. Upregulation ($P < 0.001$) of *BACE1-AS* was observed in the subjects with Alzheimer's disease. **(e,f)** *BACE1-AS* to *ACTB* ratio **(e)** and *BACE1-AS* to *BACE1* ratio **(f)** in hippocampus (Hipp), superior frontal gyrus (SFG), entorhinal cortex (Ectx) of subjects with Alzheimer's disease compared to control individuals. Both ratios are higher ($P < 0.001$) in subjects with Alzheimer's disease. **(g)** The concentrations of human A β 1–42 peptide are elevated in the brains of APP-tg19599 mice ($P < 0.0001$). **(h)** *Bace1-AS* is elevated by 50% ($P < 0.0001$) in whole brains of APP-tg19599 (APP-tg) mice.

BACE1-AS may have utility as a new biomarker of Alzheimer's disease³⁶. To this end, we calculated the ratio of *BACE1-AS* relative to *BACE1* and *ACTB* mRNA in different brain regions of control subjects and subjects with Alzheimer's disease. We found that the *BACE1-AS* to *ACTB* ratio was increased in various brain regions in subjects with Alzheimer's disease as compared to control individuals (Fig. 6e). A smaller increase in the *BACE1-AS* to *BACE1* ratio was also observed in the brains of individuals with Alzheimer's disease (Fig. 6f). These data demonstrate that the ratio between *BACE1-AS* and other RNA transcripts, including *BACE1*, could potentially be used as a biomarker of Alzheimer's disease.

APP transgenic mice have increased levels of *Bace1-AS*

Tg19959 mice, considered a mouse model of Alzheimer's disease, overexpress a doubly mutated human APP (APP-tg19959)³⁷ and consequently have increased levels of A β 1–42 peptide (ref. 38). Samples from whole brains excised from four six-week-old male mice had increased (~300-fold) levels of A β 1–42 compared with samples from matched wild-type controls, as measured by homogeneous time resolved fluorescence (HTRF) assay (Fig. 6g and Supplementary Methods). Expression of the *Bace1-AS* transcript was increased by about 45%, and *Bace1* mRNA expression was increased by about 25% in the brains of the APP-tg19959 mice compared with wild-type control mice (Fig. 6h), similar to the measurements in the human samples.

DISCUSSION

The contrast between *BACE1*'s essential role in cognitive, emotional and synaptic functions^{20,21} and its pathophysiological dysregulation in Alzheimer's disease^{39,40} highlights the regulatory complexity of this protein. Owing to the consequences of its dysregulation, *BACE1* gene expression must normally maintain tight robust regulatory control.

In this study, we have characterized a conserved noncoding antisense transcript for *BACE1*, called *BACE1-AS*, which functions as a regulator of *BACE1* gene expression. We present data showing that *BACE1-AS* is widely co-expressed with *BACE1* in cell lines, tissues and Alzheimer's disease-sensitive brain regions and that it regulates *BACE1* expression *in vitro* and *in vivo*. We found that selective siRNA targeting of the nonoverlapping regions of the *BACE1-AS* resulted in reduction of *BACE1* mRNA and protein abundance *in vitro*. Administration of siRNA that selectively targeted either *Bace1* or *Bace1-AS* into mouse brains reduced the levels of both transcripts, indicating that this concordant regulation also occurs *in vivo*.

In addition, we have shown that alterations in *BACE1-AS* RNA concentrations can alter A β 1–40 and A β 1–42 production. Considering the narrow window between essential levels and excessive levels (as in Alzheimer's disease) of *BACE1* protein, we believe that neuronal cells must maintain precise physiological regulation of *BACE1* expression by using both pre- and post-transcriptional regulatory mechanisms. The RNA transcript, *BACE1-AS*, seems to function as a regulatory component of this machinery.

Because *BACE1-AS* regulates *BACE1* expression *in vivo*, we propose that the elevation of *BACE1-AS*, resulting from the actions of Alzheimer's disease-related cell stressors, forms a basis for a deleterious feed-forward cycle of Alzheimer's disease progression. Even small changes in *BACE1* activity may lead to a long-lasting and chronic process of A β 1–42 accumulation in the Alzheimer's disease brain^{39,41}. Our current findings provide further evidence for a feed-forward mechanism of stress-dependent and activity-dependent⁴² A β 1–42 production. Recent studies have shown that

amyloid plaques induce elevation of *BACE1* protein expression in adjacent neurons by a post-transcriptional mechanism¹¹. This finding is consistent with the present data in which A β 1–42 was shown to induce increased levels of *BACE1-AS*, thereby driving *BACE1*-mediated APP processing and further accumulation of A β 1–42. In support of the above interpretation, we found that two independent sets of human Alzheimer's disease brain samples as well as an animal model of Alzheimer's disease express elevated levels of both *BACE1-AS* transcript and, to a lesser degree, *BACE1* sense transcript. In contrast to the downregulation of most transcripts reported to date in Alzheimer's disease brains^{43,44}, *BACE1-AS* upregulation may be the driving force behind Alzheimer's disease-related *BACE1* dysregulation^{16,45,46}. Thus, our results implicate a noncoding RNA in the control of gene expression central to the delicate balance between healthy stress response and the pathophysiological β -amyloid cascade.

A β 1–42 induces synaptic depression by triggering endocytosis of glutamatergic *N*-methyl *D*-aspartate receptors from the post-synaptic membrane⁷. Synaptic activity-dependent production of A β 1–42 achieves this depressive effect of *N*-methyl *D*-aspartate receptor endocytosis by a series of common mechanisms that implicate A β 1–42 in the establishment of some forms of long-term depression⁴⁷. Although they contribute to the depth and richness of mammalian memory, if not precisely controlled, these mechanisms may lead to chronic neuronal stress and the onset of Alzheimer's disease. We have previously speculated that noncoding RNAs may be required for some forms of long-term depression⁴⁸, and *BACE1-AS* could well be involved in such a function.

Treatment with *BACE1-AS* siRNA may achieve a preferential reduction of stress-induced increases in *BACE1* expression without disturbing physiologically essential basal expression levels. Thus, we propose that *BACE1-AS* could potentially constitute a drug target candidate well suited to mediate the transition between the essential physiological functions of *BACE1* and its pathological dysregulation in the chronically stressed setting of early Alzheimer's disease⁴⁹. Our *in vivo* experiments using infusion of unmodified synthetic siRNA over an extended period of time in experimental mice support the validity of an siRNA approach to decrease *BACE1* expression, perhaps in humans as well. A recent technological breakthrough suggests that systemic administration of modified siRNA may cross the blood-brain barrier and thereby target RNA transcripts in the brain⁵⁰. Alternatively, proteins involved in *BACE1-AS* localization or turnover could serve as potential targets for therapeutic interventions.

METHODS

Enzyme complementation assay. ECA is a technology developed by DiscoverRx that allows for the measurement of changes in protein abundance (Supplementary Fig. 2). We cloned the cDNA of *BACE1* into a pCMV-ProLabel vector upstream of the ProLabel. We transfected vector into HEK293T cells to produce a fusion protein (*BACE1* and the enzyme donor fragment of β -galactosidase) and made a stable cell line, which we called C3. In our experiments, we treated HEK293T cells with A β 1–42 peptides for 12 h and then added the lysis buffer (DiscoverRx), which includes the enzyme acceptor fragment of β -galactosidase. When the two fragments of the β -galactosidase combine in solution, the enzyme becomes active and hydrolyzes a substrate that produces a chemiluminescent signal. The strength of this signal is proportional to the protein being produced (in this case, *BACE1*). In a separate experiment, we transfected the stable cell line C3 overexpressing *BACE1* with siRNA or shRNA against *BACE1*, *BACE1-AS* or control siRNA and measured protein expression 72 h later with this methodology. We plotted data as a percentage of control siRNA.

Human samples. The first set of human brain samples was prepared at the USC Alzheimer's Disease Research Center. The USC Alzheimer's Disease

Research Center obtained informed consent from all subjects and the USC Institutional Review Board approved the use of the human tissue. RNA was extracted from parietal lobes and cerebellum of postmortem brains of five subjects with Alzheimer's disease and five matched controls. The average age of subjects with Alzheimer's disease was 85 years (range 75–92 years) and 91.8 years (90–95 years) for controls. The postmortem interval ranged from 3.75–10.1 h with a mean of 5.87 h. We treated RNA samples with DNase and purified them with RNeasy mini columns (QIAGEN). We prepared cDNA from 400 ng of RNA samples and used RT-PCR for relative quantification of different transcripts. The second set of human brain samples was prepared from rapid autopsy brain tissue that had been obtained from J. Rogers (Sun Health Research Institute); all enrolled subjects or legal representatives had signed a Sun Health Research Institutional Review Board–approved informed consent form allowing both clinical assessments during life and several options for brain and bodily organ donation after death. These cases included 35 autopsy-confirmed cases of Alzheimer's disease with an average age of 81.8 years (range 64–92 years) and 35 controls with an average age of 72.3 years (range 53–91 years). The postmortem interval ranged from 1.25–5 h with a mean of 2.5 h. The average duration of disease in the subjects with Alzheimer's disease was 9.2 years. Total RNA was isolated via CsCl purification from tissue dissected from specific regions of brain. Although not all regions were available from all cases, we examined a total of 128 RNA samples from superior frontal gyrus, entorhinal cortex, hippocampus and cerebellum for *BACE1* and *BACE1-AS* expression by RT-PCR.

Mouse studies. We obtained approval for mouse studies from the Institutional Animal Care and Use Committee at The Scripps Research Institute.

We used 18 six-month-old male mice for *in vivo* siRNA infusion experiments. We prepared mice with chronic indwelling cannulae in the dorsal third ventricle implanted subcutaneously with osmotic minipumps that delivered continuous infusions (0.25 μ l/h) of synthetic unmodified siRNA directed against *Bace1*, *Bace1-AS* or control siRNA (previously shown to have no effects on the expression of human and mouse genes) at a dose of 0.4 mg/d for 2 weeks. We connected tubing to the exit port of the osmotic minipump and tunneled it subcutaneously to the indwelling cannula, such that siRNAs were delivered directly into the brain.

Pump-mediated infusion of siRNA was previously shown to significantly and specifically knock down expression of targeted mRNAs in the brain, but with a limited tissue penetration²⁸. Indeed, RNA knockdown upon ventricular infusion of siRNAs for 14 consecutive d was usually obtained in brain regions immediately adjacent to the ventricle, with diminishing effects of the siRNA as the distance from the ventricle increased.

We excised five tissues from each mouse for RNA quantitative measurement—the dorsal hippocampus, ventral hippocampus, cortex, dorsal striatum and cerebellum. RNA extraction is described in **Supplementary Methods**.

Tg19959 mice were produced by pronuclear microinjection of (FVB \times 129S6F1) embryos with a cosmid insert containing human APP with two familial Alzheimer's disease mutations (KM670/671NL and V717F) under the control of the hamster PrP promoter. We killed four Tg19959 mice and four control male littermates at 6 weeks old. We used brain tissues for RNA measurements and A β 1–42 detection by HTRE. In a separate experiment, we killed three wild-type male mice and excised their tissues for expression profiling of *Bace1* and *Bace1-AS* by RT-PCR.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

C.W., M.A.F., P.J.K. and G.S.L. conceived and designed the experiments and analyzed the data. M.A.F., F.M., P.J.K. and A.M.K. performed the experiments. D.E.W., B.G.S., T.E.M. and C.E.F. provided human Alzheimer's disease RNA samples. M.A.F., C.W., P.J.K. and G.S.L. wrote the paper.

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