The role of Aβ circRNA in Alzheimer's disease: alternative mechanism of Aβ biogenesis from Aβ circRNA translation

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Abstract:
CircRNAs enrich in neurons and accumulate during brain ageing, indicating possible roles in age-associated neurodegenerative diseases.
Alzheimer's disease (AD) is the most common detrimental dementia. Studies show amyloid-β (Aβ) peptide plays key role in AD. Aβ could accumulate from the full-length APP proteolytic processing, especially in familial AD, but the mechanism of Aβ biogenesis in sporadic AD remains largely unknown.

Here, we demonstrate that a couple of circRNAs are expressed from APP gene, encompassing the Aβ sequence. They are named as Aβ circRNAs. Using circRNA expression strategy based on intron mediated enhancement (IME), we found Aβ circRNA encoded Aβ related peptide. Importantly, this peptide is further processed to produce Aβ, thus representing an alternative mechanism of Aβ biogenesis.
Furthermore, Aβ circRNA up-regulates GSK3β levels and tau phosphorylation, both hallmarks of AD. Thus, Aβ circRNA and translated peptides may not only play a causative role in AD but represent promising therapeutic targets in the AD medicine.

Key words: Neurodegeneration, Alzheimer's disease (AD), Circular RNA (circRNA), intron-mediated enhancement (IME), amyloid beta (Aβ), amyloid beta (Aβ) circRNAs, Aβ circRNA derived peptides (Aβ-circRNA-DP), GSK3β, tau phosphorylation.

Introduction:

Alzheimer's disease (AD) is the most common form of dementia associated with progressive loss of memory, thinking and behaviour capabilities[1-4]. As an ageing-associated neurodegenerative disorder, the greatest known risk factor for AD is increasing age itself[3,5]. Both the leading hypothesis and extensive studies have focused on the causative roles of amyloid-β (Aβ) peptide accumulation and tau hyperphosphorylation[1-4,6-9]. It has been shown that mutations in genes (APP, Presenilin) participating in APP protein proteolytic processing accelerate the accumulation of Aβ peptides (Aβ 36-43). These peptides polymerize into toxic forms, aggregate into insoluble amyloid plaques, and lead to tau protein hyperphosphorylation through GSK3β activation, and subsequently neurofibrillary tangles forming inside neurons, triggering a cascade of events that ultimately cause neuron death[1,2,10-14]. This type of Alzheimer's disease is normally called familial Alzheimer's disease (FAD). Since it develops at an earlier age, it is also described as early-onset familial Alzheimer's disease (eFAD).

Despite well-demonstrated roles of genetic mutations in FAD, this type of AD accounts for less than 1-5% of all AD patients[15,16]. The most common form of AD is late-onset Alzheimer's (LOAD), which normally arises in people aged 65 and older. This type of AD is sporadic, correspondingly it is as also called sporadic AD (SAD); the direct cause or genes responsible for LOAD/SAD are largely unknown.
Although with different age of onset and genetic causes, both FAD and SAD are believed to share a similar overall sequence of symptoms and gradually increasing impairments. The

pathologic hallmarks of both FAD and SAD are plaques and tangles arising from Aβ accumulation and tau hyperphosphorylation. The sporadic incidence of LOAD poses the question of what the exact mechanism of AD is[17,18]. Moreover, how Aβ accumulates in LOAD/SAD is still largely unknown[2,18-20].

Circular RNAs (circRNAs) are a form of evolutionary conserved transcripts with a covalently jointed 5' and 3' structure derived from pre-mRNA back-splicing[21]. Previous studies have found that they are especially enriched in neurons[22]. Interestingly, brain circRNAs are regulated during ageing in C. elegans, fly and mouse, highlighting potential roles of circRNA in brain ageing and ageing-associated neurodegenerative diseases[23-25].

In this study, we found that a couple circRNAs from the human APP gene are well expressed in the human brain. Since this circRNAs contains the Aβ coding sequence, here we call it Aβ circRNAs. Using our previously established method of intron-mediated enhancement (IME) of circRNA expression and translation (BioRxiv: https://doi.org/10.1101/257105), we shown that one of Aβ circRNA copies clearly expressed Aβ related peptide, which could be further proceeded to generate Aβ, thus indicating a new direction in the mechanism of Alzheimer's disease.

Material and methods:

Plasmid construction

The genetic information about circRNAs from the human APP gene were reported by several groups[22,26-28]. One isoform of circRNA from the APP gene, hsa_circ_0007556 (circBase), was called human Aβ circRNA-a for the purpose of this study. The cDNA of human APP circRNA-a (GRCh37/hg19, chr21:27264033-27284274) was inserted into pCircRNA-BE or pCircRNA-DMo vectors to generate pCircRNA-BE-Aβ-a or pCircRNA-DMo-Aβ-a as described previously (BioRxiv: https://doi.org/10.1101/257105). Plasmid DNAs were purified with EndoFree Plasmid Maxi Kit (QIAGEN). Details of oligonucleotides are provided in Supplementary Table 1.

Cell culture and plasmid DNA transfection

Human HEK293 and mouse neuro 2a (N2a) cell line cultures and plasmid DNA transfections were performed as previously described (BioRxiv: https://doi.org/10.1101/257105). Briefly, 2.5 μg of plasmid DNA was transfected into 0.5 million cells for three to six days.

Total RNA isolation and qRT-PCR

Total RNA from HEK293 or N2a cells was isolated with TRIzol reagent (Ambion). Human brain frontal lobe and hippocampus total RNAs were purchased from BioCat GmbH. cDNA synthesis and qRT-PCR were performed as previously described (BioRxiv: https://doi.org/10.1101/257105). Details of qRT-PCR oligonucleotides are provided in Supplementary Table 1.

Aβ circRNAs identification by RT-PCR and sequencing

All Aβ containing circRNA from APP gene were RT-PCR amplified by specific divergent primers targeted at the exon 17 of APP gene from human brain frontal lobe and hippocampus total RNAs. For RNase R treatment, 15 μg total RNAs were digested with RNase R for 1 hour at 37 °C and purified by phenol-chloroform purification. PCR products were digested by BamHI and XhoI, then ligated to pCMV-MIR vector.

PCR primers: AB-cRNAvBF2, atataggatccGTGATCGTCATCACCTTGGTGATGC

AB-cRNAvXR2, tatatctcgagCACCATGAGTCCAATGATTGCACC

Northern blot analysis

Northern blot analysis was performed similarly as previous described[29]. In brief, total RNAs from HEK293 cells were separated on 5% polyacrylamide gel (Bio-Rad) and transferred to positively charged nylon membrane, then blot was hybridized with 5' p^{32} labeled DNA oligo overnight (Aβ-NBR1: CCCACCATGAGTCCAATGATTGCACCTTTGTTTGAACCCAC ATCTTCTGCAAAGAACACC). For RNase R treatment, 15 μg total RNAs were digested with 10 units of RNase R (RNR07250, Epicentre) for 1 hour at 37 °C and then the treated RNAs were directedly separated on gel and analysed by northern blot.

Western blot assays

Total proteins were prepared in RIPA buffer as previously described (BioRxiv: https://doi.org/10.1101/257105). For detecting Aβ circRNA-a-derived peptide in N2a cells, the PIPA buffer-insoluble proteins were extracted by 70% formic acid as described previously[30]. Protein extractions were separated on 18% or 4-20% Criterion™ TGX Stain-Free™ Protein Gel (Bio-Rad) and transferred to 0.2 μm nitrocellulose membranes, then immunoblotted against β-amyloid (β-Amyloid (D54D2) XP® Rabbit mAb #8243, Cell signalling Technology, which also recognizes APP full-length protein), GSK3β (#12456, Cell signalling Technology), tau (#MN1000, Thermo Scientific), phospho-tau (AT8, #MN1020; AT100, #MN1060; ThermoFisher Scientific) and β-Actin (#A5441, Sigma). Quantitative analyses were performed with ImageJ (NIH). Aβ42 (A9810, Sigma) was prepared in DMSO.

Immunoprecipitation/western blotting of Aβ and Aβ related peptides (IP-WB, IP-MS)

Aβ and Aβ related peptides detection was performed through immunoprecipitation of conditioned medium (CM) and HEK293 cell extract in RIPA buffer, followed with western blot analysis as previous described[31]. In brief, HEK293 cells transfected with, pCircRNA-BE-Aβ-a, pCircRNA-DMo-Aβ-a or empty vector control (pCircRNA-DMo) were cultured in serum free medium overnight. Then CM/cell extract was prepared with protease and phosphatase inhibitor (Roche) and precleaned with Protein A/G. Immunoprecipitation was performed with mixture of Aβ antibodies (6E10, 6G8, BioLegend Inc.). Precipitated peptides were resolved in SDS loading buffer and analysed by western blot with antibody against Aβ (D54D2).

Results

Aβ circRNA isoforms expression from the APP gene in human brain

Previous deep-sequencing studies have discovered several candidate circRNAs from human APP gene, and one candidate circRNA (hsa_circ_0007556, circBase) contains the Aβ sequence[22,26-28]. To identify all possible circRNAs encoding Aβ peptide sequence from APP gene, we performed RT-PCR with specifically designed divergent primers to detect Aβ sequence containing circRNA copies in the total RNA of human brain samples (Fig. 1). The amplified RT-PCR products were ligated to pCMV-MIR vector and the resulted constructs were sequenced. As shown in Fig. 1, 14 circRNAs isoforms were identified and hsa_circ_0007556 was confirmed in this list (the junction sites were shown in Supplementary Fig. 1). Since these circRNAs include the sequence of the Aβ peptide, in this study they are called Aβ circRNA (Fig. 1B). Individually, hsa_circ_0007556 is named as Aβ circRNA-a (Fig. 1B).

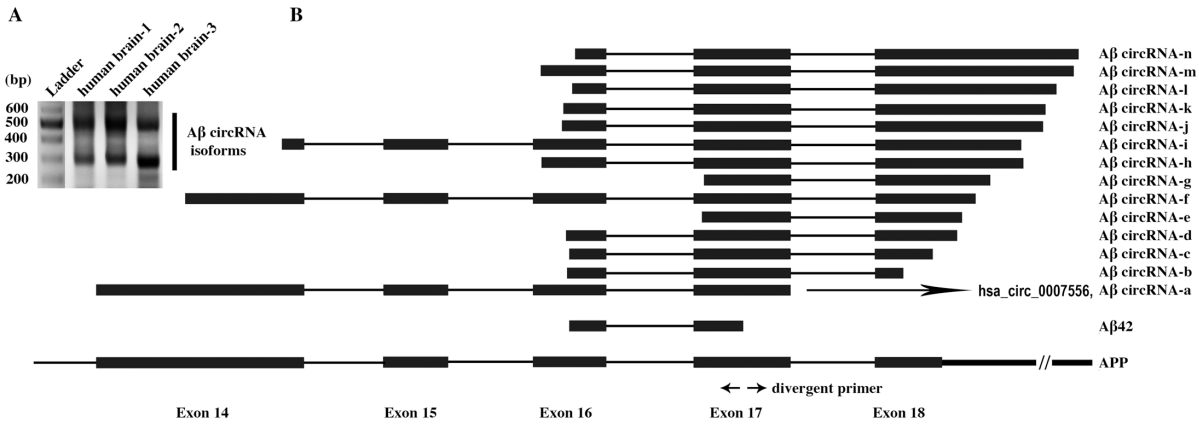

Fig. 1: Aβ circRNA isoforms identification from the APP gene in human brain

A) agarose gel electrophoresis of Aβ circRNA RT-PCR products with divergent primers located in the exon 17 of human APP gene in human brain samples. Three human brain samples were used. The amplified products were ligated to vector and the resulted constructs were sequenced and aligned (results were shown in B).

B) the localizations of Aβ circRNA isoforms in APP gene. Aβ42 sequence was used as location reference. Aβ circRNA isoforms were named as Aβ circRNA-a, b, c, d, e, f, g, h, i, j, k, l, m, n. The short arrow indicates the location of divergent primer. The long arrow indicates has_circ_0007556 circRNA, which is named as Aβ circRNA-a in this study. The original sequencing results were provided in Supplementary sequencing data.

IME promotes Aβ circRNA-a overexpression in cell lines

To further confirm Aβ circRNA-a's existence in human brain, we performed RT-PCR on human frontal lobe and hippocampus total RNA using oligonucleotides specifically designed to detect Aβ circRNA-a (Fig. 2A, C). Indeed, Aβ circRNA-a was clearly well expressed in human frontal lobe and hippocampus (Fig. 2A, C).

To facilitate circRNA functional studies, we recently developed an intron-mediated enhancement (IME) strategy for robust circRNA expression BioRxiv: https://doi.org/10.1101/257105). Here, we applied this method to boost Aβ circRNA-a expression from vectors constructed in a similar manner to the described strategy (Fig. 2A, B) (BioRxiv: https://doi.org/10.1101/257105). Transient expression in HEK293 cells showed that pCircRNA-BE-Aβ-a and pCircRNA-DMo-Aβ-a generated 2185- and 3268-fold more Aβ circRNA-a than was endogenously produced in HEK293 cells transfected with empty vector (Fig. 2C, D). RT-PCR confirmed that the overexpressed Aβ circRNA-a was spliced exactly as the wildtype Aβ circRNA-a (Supplementary Fig. 2). Sequencing the RT-PCR products further confirmed that their sequences were identical to wild type Aβ circRNA-a found in the human brain (Supplementary Fig. 2C, D). Moreover, we used northern blot to detect Aβ circRNA-a expression. As shown in Supplementary Fig. 2E, the detected Aβ circRNA-a migrated faster than the linear counterpart in native agarose gel. RNase R treatment abolished the linear counterpart but the suggested Aβ circRNA-a bands were not affected, demonstrating the circularity of the expressed Aβ circRNA-a in HEK293 cell (Supplementary Fig. 2E).

Since HEK293 is a well-established cell line for Alzheimer's disease research[32,33], the robust IME strategy for transient expression of Aβ circRNA-a in HEK293 cells provides a good model for studying Aβ circRNA-a function. In the endogenous control, qRT-PCR showed that the level of human full-length APP mRNA was not changed (Supplementary Fig. 4), indicating that endogenous APP gene expression was not affected.

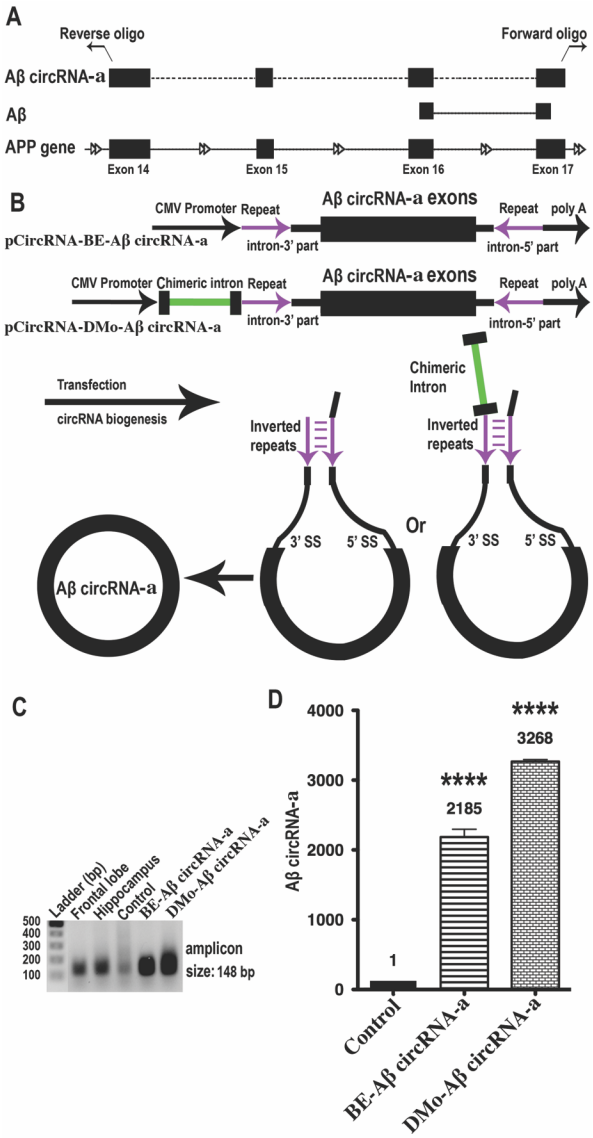

Fig. 2: Aβ circRNA-a overexpressing HEK293 cells.
A) Aβ circRNA-a is encoded by exons 14-17 of the human APP gene, without the introns. The amyloid-β (Aβ) sequence is located in exons 16 and 17. Reverse and forward oligonucleotides were used to amplify Aβ circRNA-a by RT-PCR. B) Aβ circRNA-a over-expression constructs. C) RT-PCR verification of Aβ circRNA-a expression in human brain samples (frontal lobe and hippocampus) and HEK293 cells overexpressing Aβ circRNA-a; Control, pCircRNA-DMo empty vector transfection into HEK293; BE-Aβ circRNA-a, pCircRNA-BE-Aβ-a transfection into HEK293; DMo-Aβ circRNA-a, pCircRNA-DMo-Aβ-a transfection into HEK293; RT-PCR verification of Aβ circRNA-a by another set of oligonucleotides is shown in Supplementary Fig. 2. A, B, C, D) Aβ circRNA-a expression in HEK293 cells mediated by pCircRNA-BE circRNA-a and pCircRNA-DMo circRNA-a vectors. Control, empty vector (pCircRNA-DMo); BE-Aβ circRNA-a, pCircRNA-BE-Aβ-a; DMo-Aβ circRNA-a, pCircRNA-DMo-Aβ-a. All statistical T tests were performed in comparison to the control sample, ****, $P \leq 0.0001$, $n = 4$.

Aβ circRNA-a can be translated into an Aβ related peptide
Previous studies have shown that certain circRNAs are translatable[34-37](BioRxiv: https://doi.org/10.1101/257105). Examination of the Aβ circRNA-a revealed that it contained an open reading frame (ORF) (Fig. 3A) that would translate into a putative protein with a calculated molecular weight of 19.2 kDa (Supplementary Fig. 5). Western blots to detect

whether an Aβ related peptide was translated in HEK293 cells overexpressing Aβ circRNA-a used an antibody (β-Amyloid (D54D2) XP® Rabbit mAb) that detected all Aβ containing peptides. Specifically, we detected an obvious Aβ-related peptide signal with a size around 15 to 20 kDa, confirming the translation of Aβ circRNA-a (Fig. 3B). Products of Aβ circRNA-a translation were called Aβ circRNA-a-derived peptide (Aβ circRNA-a-DP).

Interestingly, control HEK293 cells transfected with empty vector contained detectable amounts of Aβ circRNA-a-DP. As we showed above (Fig. 2), HEK293 cells expressed low levels of endogenous Aβ circRNA-a, thus detectable Aβ circRNA-a-DP in control HEK293 cells represents its endogenous translation. Importantly, such detectable Aβ circRNA-a-DP in HEK293 cells confirmed its authenticity, highlighting its biological relevance.

Clearly, as in our previous study (BioRxiv: https://doi.org/10.1101/257105), intron-mediated enhancement (IME) increased Aβ circRNA-a translation. pCircRNA-DMo-Aβ-a expressed about 3.3-fold more Aβ circRNA-a-DP than the endogenous expression in HEK293 cells, while pCircRNA-BE-Aβ-a only moderately enhanced Aβ circRNA-a-DP levels (1.4-fold; Fig 3B, C). Interestingly, the enrichment of protein product (Aβ circRNA-a-DP) was much less significant than the elevated expression of Aβ circRNA-a, indicating that the translation of Aβ circRNA-a was regulated by other unknown mechanisms.

Next, we used a mouse neuroblastoma cell line to further validate the transcription and translation of Aβ circRNA-a. These N2a cells apparently do not express any detectable Aβ circRNA-a or translation product (Fig 3D, F). However, pCircRNA-DMo-Aβ expressed 4.6-fold more Aβ circRNA-a than pCircRNA-BE-Aβ, whereas the internal control, App mRNA, showed no change (Fig 3D, F). In both Aβ circRNA-a overexpressing N2a cells, Aβ circRNA-a-DP was well expressed, and consistent with HEK293 cells, IME dramatically enhanced Aβ circRNA-a-DP expression (Fig 3D, F). Since N2a cells do not express Aβ circRNA-a-DP or related peptides endogenously, the detection of Aβ related peptide at 15-20 kDa further clearly confirmed that Aβ circRNA-a was the template for translating Aβ circRNA-a-DP. As internal control, the endogenous App mRNA in N2a cell was not changed (Fig 3E).

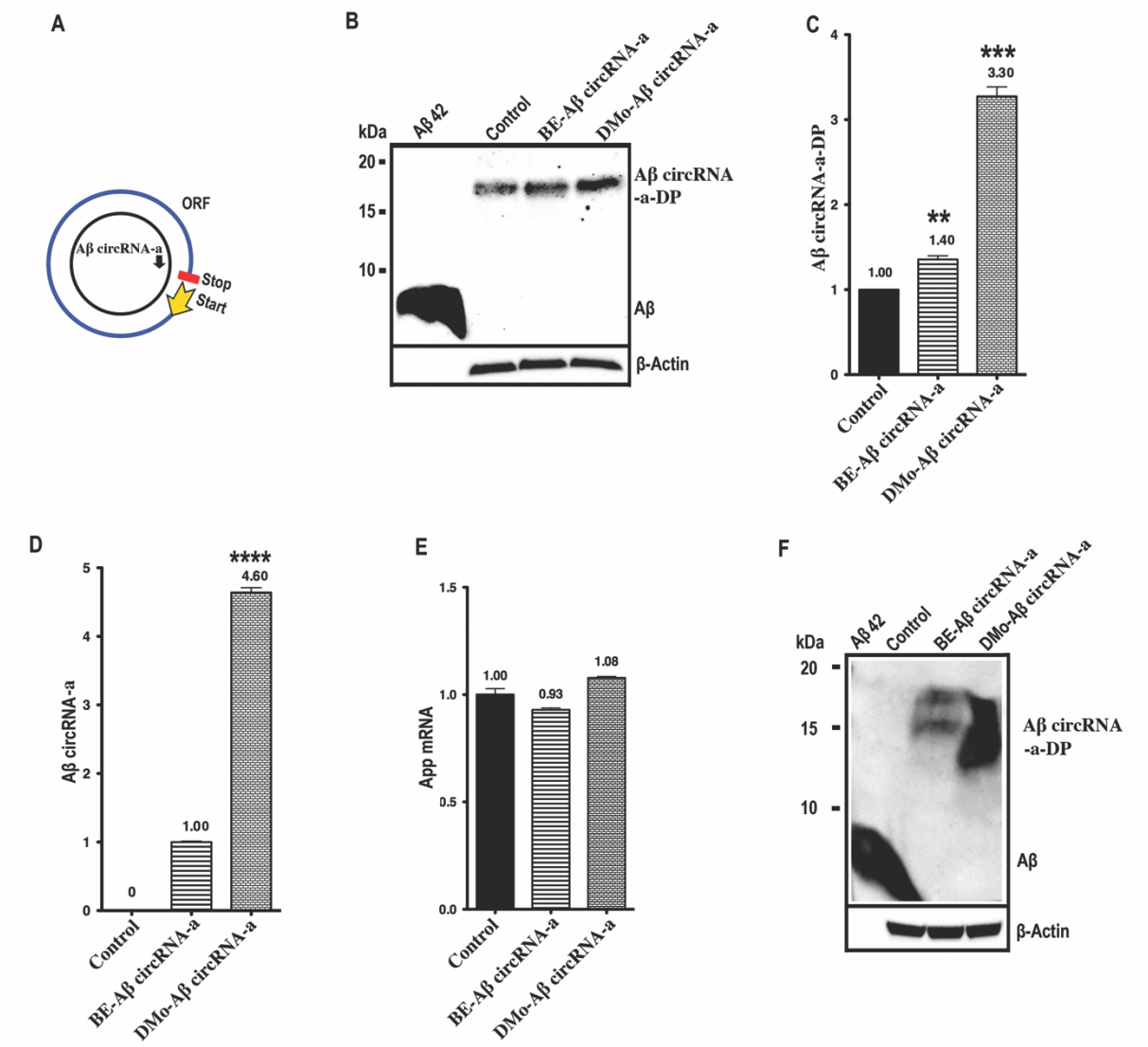

Fig. 3: Expression of Aβ related peptide from Aβ circRNA-a translation in HEK293 and N2a cells.

A) The open reading frame (ORF) of Aβ circRNA-a is represented by a blue circle; yellow arrow, translation start codon; red square, stop codon; the inner black arrow shows the start of the Aβ circRNA-a. B) Western blot of Aβ-related peptides in HEK293 cells. 5 ng of *in vitro* synthesized Aβ42 was used as a positive control peptide and migration indicator. Control, empty vector (pCircRNA-DMo); BE-Aβ circRNA-a, pCircRNA-BE-Aβ circRNA-a; DMo-Aβ circRNA-a, pCircRNA-DMo-Aβ circRNA-a. The detected peptides indicated on the right: Aβ circRNA-a-DP, Aβ circRNA-a-derived protein; Aβ, synthetic peptide Aβ42; β-Actin used as a loading control. C) quantification of Aβ circRNA-a-DP levels.)

D) Aβ circRNA-a expression in N2a cells. Control, empty vector (pCircRNA-DMo); BE-Aβ circRNA-a, pCircRNA-BE-Aβ circRNA-a; DMo-Aβ circRNA-a, pCircRNA-DMo-Aβ circRNA-a. E) Mouse App mRNA expression in Aβ circRNA-a overexpressing N2a cells. F) Western blot of Aβ related peptides in Aβ circRNA-a overexpressing N2a cells. The synthetic Aβ42 peptide (Fig. 3) was used as a positive detection and migration control; Control, empty vector (pCircRNA-DMo); BE-Aβ circRNA-a, pCircRNA-BE-Aβ circRNA-a; DMo-Aβ circRNA-a, pCircRNA-DMo-Aβ circRNA-a. Panels C, D, all statistical T tests were performed with respect to the control sample; **, $P \leq 0.01$; ***, $P \leq 0.001$; $n \geq 3$. Panel D, E, all statistical T tests were performed in comparison to the control sample (in panel E this was BE-Aβ circRNA-a set at 1); ****, $P \leq 0.0001$; $n = 3$.

Aβ circRNA-a derived peptide is further processed to form Aβ.

To further investigate the protein sequence of Aβ circRNA-a derived peptides, we use two specific Aβ antibodies (6E10, 4G8) to immunoprecipitate these peptides from RIPA buffer extract of HEK 293 cell with Aβ circRNA-a overexpression. As shown in Fig. 4A, western blot analysis of these isolated peptides with another Aβ antibody (D54D2) confirmed that they could be specifically immunoprecipitated, further demonstrating the expressed peptides contain Aβ peptide sequence. Importantly, the expression level of Aβ related peptides in the IP output of the three samples (control, pCircRNA-BE-Aβ-a, pCircRNA-DMo-Aβ-a) is consistent with their input levels, demonstrating that the IP manipulation does not changed the relative quantities of the Aβ related peptides in these different samples.

The production of Aβ related peptide from Aβ circRNA-a translation highlights the potential of Aβ biogenesis. Moreover, the predicted protein sequence of Aβ circRNA-a-DP contain β and γ-secretase cleavage site, indicating that Aβ can be produced from Aβ circRNA-a-DP by β and γ-secretase proteolytic processing (Supplementary Fig. 5). So, we used immunoprecipitation/western blotting (IP-WB) by specific Aβ antibodies (6E10, 4G8) to analyse the possible Aβ from the conditioned medium (CM) of Aβ circRNA-a overexpressed HKE293 cells. Strikingly, we detected a peptide band corresponding to Aβ in western blot with another Aβ antibody (D54D2), thus confirming the biogenesis of Aβ from Aβ circRNA-a translation (Fig. 4A, B). Compared to empty vector transfection, pCircRNA-BE-Aβ-a caused 2.6-fold increase of Aβ peptide expression in the condition medium (Fig. 4A, B). Strikingly, pCircRNA-DMo-Aβ-a caused as more as 6-fold increase of Aβ peptide expression (Fig. 4A, B). Of note, the difference trend of Aβ expression level between three samples (control, pCircRNA-BE-Aβ-a, pCircRNA-DMo-Aβ-a) was consistent with the difference trend of Aβ circRNA-a-DP, representing the upregulated Aβ was processed from circRNA-a-DP.

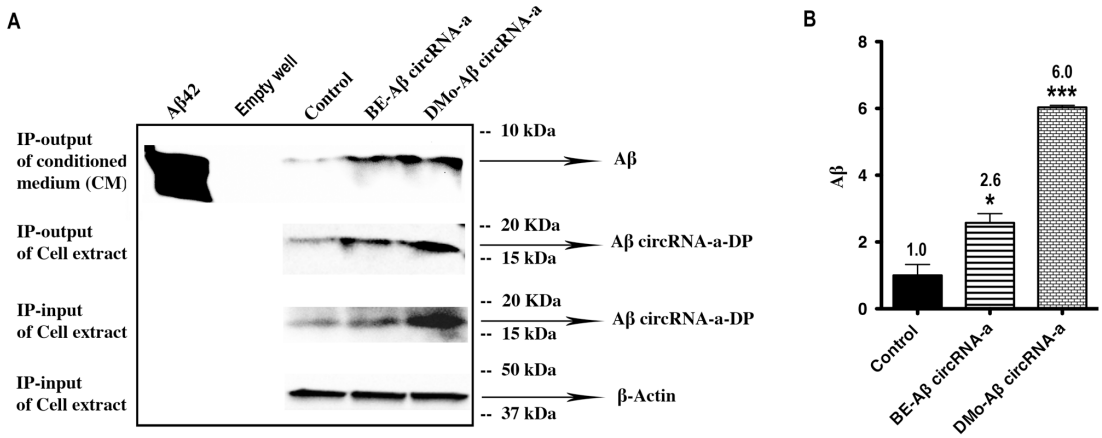

Fig. 4: IP-WB of Aβ peptides from in the conditioned medium of Aβ circRNA-a overexpressed cells.

The conditioned medium of HKE293 with transfection of Aβ circRNA-a overexpression vector was immunoprecipitated with antibody against Aβ (6E10, 4G8). Control, pCircRNA-DMo; BE-Aβ circRNA-a, pCircRNA-BE-Aβ-a; DMo-Aβ circRNA-a, pCircRNA-DMo-Aβ-a; transfected HEK 293 cells were lysed in RIPA buffer and Aβ antibodies (6E10, 4G8) were used in immunoprecipitation; Aβ antibody (D54D2) was used in western blot; β-Actin was used as loading control. 5 ng in vitro synthesised Aβ42 was used as Aβ migration maker in western blot. All statistical T tests were performed in comparison to the control sample; *, $P \leq 0.05$; ***, $P \leq 0.001$; $n = 3$.

Aβ circRNA-a overexpression significantly up-regulates GSK3β and tau phosphorylation

Previously studies have shown that Aβ peptides are toxic to neural cells and cause GSK3β activation, which promotes the phosphorylation of tau proteins. To evaluate the potential roles of Aβ circRNA-a translated peptides, especially the generated Aβ peptides, we analysed GSK3β levels and the phosphorylation of tau proteins in HEK293 cells overexpressing Aβ circRNA-a using western blots. Indeed, Aβ circRNA-a overexpression significantly increased GSK3β levels, by 2.3-fold following pCircRNA-BE-Aβ transfection and 3.5-fold after pCircRNA-DMo-Aβ circRNA-a transfection (Fig. 5A, B). Interestingly, tau protein levels were also upregulated when Aβ circRNA-a was overexpressed (HT7 antibody; Fig. 5A, C). Using AT8 (Ser202, Thr205) and AT100 (Thr212, Ser214) antibodies to analyse tau phosphorylation, pCircRNA-BE-Aβ circRNA-a transfection resulted in a 2.6-fold increase in phosphorylation detected by AT8 and a 2.9-fold increase detected by AT100. Correlating with the IME enhanced higher expression of Aβ circRNA-a-DP, pCircRNA-DMo-Aβ circRNA-a transfected cells contained 3.7-fold or 5.0-fold more phosphorylated tau detected with AT8 or AT100 antibodies (Fig. 5A, C, D, E).

In summary, Aβ circRNA-a significantly up-regulate GSK3β and consequently promote tau phosphorylation.

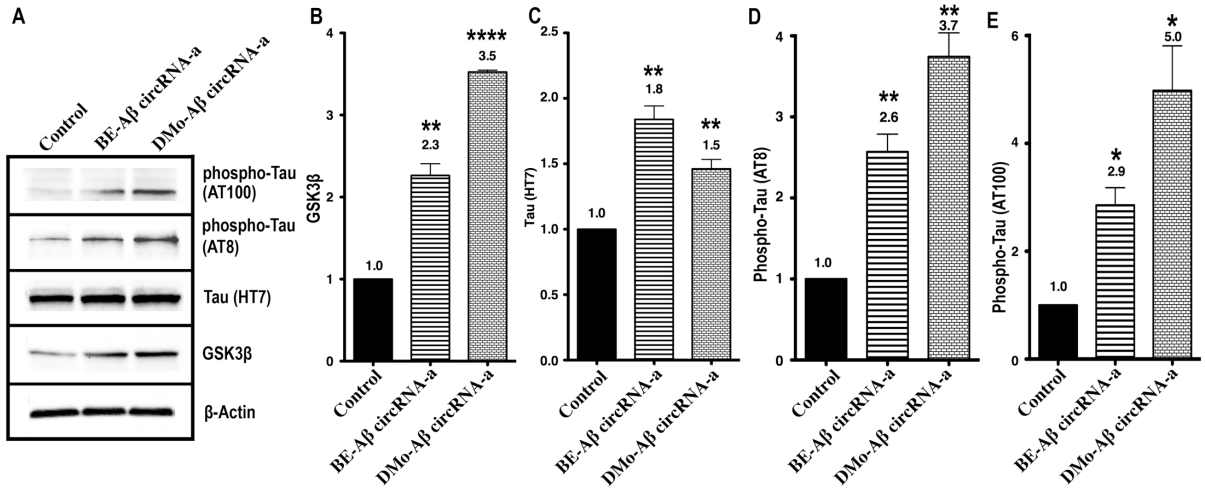

Fig. 5: Aβ circRNA-a up-regulates GSK3β expression and promotes tau protein phosphorylation.
A) GSK3β and tau protein (HT7 antibody) levels, and tau phosphorylation detected in HEK293 cells overexpressing Aβ circRNA-a. β-actin was used as a loading control. Control, pCircRNA-DMo empty vector; BE-Aβ circRNA-a, pCircRNA-BE-Aβ-a; DMo-Aβ circRNA-a, pCircRNA-DMo-Aβ-a. B) Quantification of GSK3β levels; C) quantification of tau levels; D) quantification of tau phosphorylation levels detected by antibody AT8; E) quantification of tau phosphorylation detected by antibody AT100. All statistical T tests were performed with respect to the control sample; **, $P \leq 0.01$; ****, $P \leq 0.0001$; $n \geq 3$.

Discussion

Although extensive studies have illustrated the biogenesis mechanism of Aβ peptide in FAD, the pathways of Aβ peptide production in SAD remain elusive.

In this study, we discovered a couple of Aβ circRNAs from APP gene. With the help of intron-mediated enhancement of circRNA expression, we could verify that Aβ circRNA-a can be translated into Aβ related peptide and subsequently processed to form Aβ, correspondingly inducing GSK3β up-regulation and tau phosphorylation, thus indicating a new direction in the search for molecular mechanisms of Alzheimer' disease (Fig. 6).

Such a mechanism is dramatically different from Aβ accumulation through dysregulation of full length APP prototypic processing. Thus, it provides an alternative pathway of Aβ biogenesis. Unlike factor mutations in familial Alzheimer's disease, no genetic mutation is required for Aβ circRNAs biogenesis. Correspondingly, the entire human population may express Aβ circRNA and Aβ circRNA-DPs, which may play a key role in sporadic Alzheimer's disease.

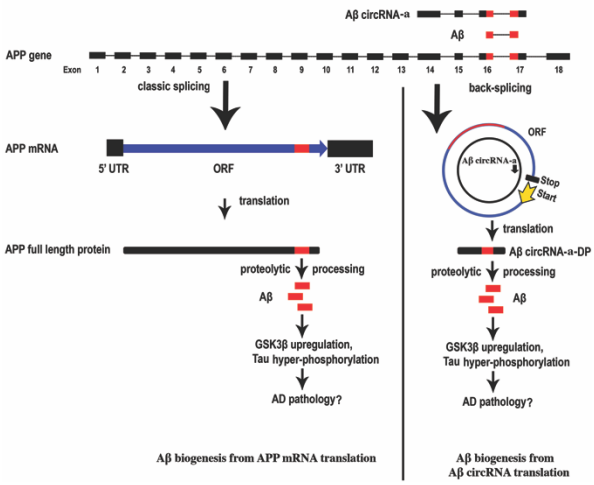

Fig. 6: Two Aβ biogenesis pathways in Alzheimer's disease.
At the top, exon sequences contained in Aβ circRNA-a and Aβ peptides (in red) are aligned with the full-length APP gene. On the left, liner APP mRNA transcribed from the APP gene undergoes classic splicing before being translated into full-length APP protein. Proteolytic processing of APP protein generates Aβ peptides (Aβ40, Aβ42, in red), which play causative roles in AD pathology through GSK3β activation and tau protein hyperphosphorylation. Mutations in factors involved in APP processing cause familial Alzheimer's disease. On the right, Aβ circRNA-a is synthesised by back-splicing of the APP gene. The open reading frame (ORF) is in blue, with the Aβ sequence in red, the start codon in yellow arrow and the stop codon in black. Translation of Aβ circRNA-a produces Aβ-related peptide (Aβ circRNA-a-DP), which is further processed to form Aβ. The accumulation of Aβ could cause the sequential scenario of GSK3β activation and tau protein hyperphosphorylation, leading to AD pathology. Since no mutations are required for Aβ circRNA biogenesis, this process may depend on intracellular environments rather than genetics. Changes in Aβ circRNA expression and translation may cause sporadic Alzheimer's disease.

So far, it is not known that how many percent of Aβ and Aβ related peptides are derived from Aβ circRNAs in aged people and AD patients. The significance of alternative Aβ biogenesis from Aβ circRNA translation in AD pathology remains to be determined.
As an analogy, although Aβ is endogenous expressed in rodent brain, but it is surprising that wildtype mouse and rat neither appears Aβ deposit in old brain nor develops Alzheimer's disease[38-40]. Despite there are three amino acids variations in the N-terminal from human Aβ, endogenous murine Aβ peptides are neurotoxic and have quite similar ability to produce Aβ deposit provided that the murine APP gene harbours FAD mutations[41]. With the discovery of alternative Aβ biogenesis pathway from Aβ circRNA in human, it will be interesting to find out whether mouse express Aβ circRNA or not. Using RT-PCR with divergent primers located at Aβ sequence region, we failed to detect any mouse Aβ circRNA copy (Supplementary Fig. 6). As positive control, a circRNA spanning exon 9 to exon 13 of mouse App gene was detected (Supplementary Fig. 6). Apparently, mouse does not express Aβ circRNA, thus lacking the alternative Aβ biogenesis pathway existed in human, possibly explaining the difference of Aβ

deposition in elderly brain between human and mouse. Moreover, it indicates that the Aβ biogenesis from Aβ circRNA translation may play essential role in human AD development.

In this study, the proteolytic processing of Aβ circRNA-a derived peptide is not studied. It will be interesting to investigate how α, β and γ-secretase participate in the Aβ biogenesis mechanism from Aβ circRNA-a-DP.
Furthermore, other Aβ circRNA isoforms also trigger further investigations. For example, whether they can also produce Aβ related peptides which can be eventually processed to form Aβ.

In AD pathology, ageing itself is the most causative factor. But the mechanism of how Aβ exceptionally accumulate in old people's brain is still largely unknown. Recent studies have implicated circRNA as a regulator of cellular stress[42]. For example, the translation of Circ-ZNF609 circRNA could be induced by heat shock stress[34]. In brain ageing, neurons are particularly vulnerable to various stresses[43]. Presumably, expression of Aβ circRNAs and its translated peptide could be activated by various (age-related) stresses, and cause a detrimental downstream cascade, leading to neurodegeneration.

The rapidly emerging role of circRNA is apparently serving as a template for protein synthesis. However, it is unknown whether peptides produced from circRNA have any function in organisms. Here, we show that the protein produced from Aβ circRNAs translation may play a critical role in Alzheimer's disease, thus endorsing further function exploration of circRNA, and indicating that proteins translated from other circRNAs may also play essential, biologically significant roles.

The discovery of biological roles for Aβ circRNAs and their translated peptides may not only represent a milestone for the function exploration of circRNA, but also reveals a potential ground-breaking mechanism for causing Alzheimer's disease and opens up new strategies for diagnosis, prevention and treatment of Alzheimer's disease.

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Author Contributions
DM designed and conceived the study. DM performed the experiments, analysed the results, prepared the figures and wrote the manuscript. DC and XL participated in some experiments.

Conflict of interest.
None declared.

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The role of Aβ circRNA in Alzheimer's disease: alternative mechanism of Aβ biogenesis from Aβ circRNA translation

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Supplementary table 1

Aβ circRNA expression plasmid construction oligos
Abeta-circF gtttgtttttcagATGAGCTGCTTCAGAAAGAGCAAAACT
ABeta-circR gcatggattattacCTCCACCACACCATGATGAATGG
circDMO-LF GTCGACTGGATCcaacgttaaccc
DMo-Ab-LR CTGAAGCAGCTCATctgaaaaacaaacagaatacaacctcagc
DMo-Ab-RF GGTGTGGTGGAGgtaataatccatgcaccgtctcacc
circDMO-RR cactttgCTCGAGctcatcaacatg

Aβ circRNA verification oligos
AB-cRNAvF2 GTGATCGTCATCACCTTGGTGATGC
AB-cRNAvR2 CACCATGAGTCCAATGATTGCACC

qPCR oligos
human Aβ circRNA
Abeta-cF GTCATAGCGACAGTGATCGTC
Abeta-cR CTTGGTTCACTAATCATGTTGGC

Human APP mRNA
hAPP-mF TTTGTGATTCCCTACCGCTG
hAPP-mR TGCCAGTGAAGATGAGTTTCG

mouse App mRNA qRT-PCR, only amplify mRNA, not circRNA
App-mF ATCTTCACTGGCACACCG
App-mR GCATACAAACTCTACCCCTCG

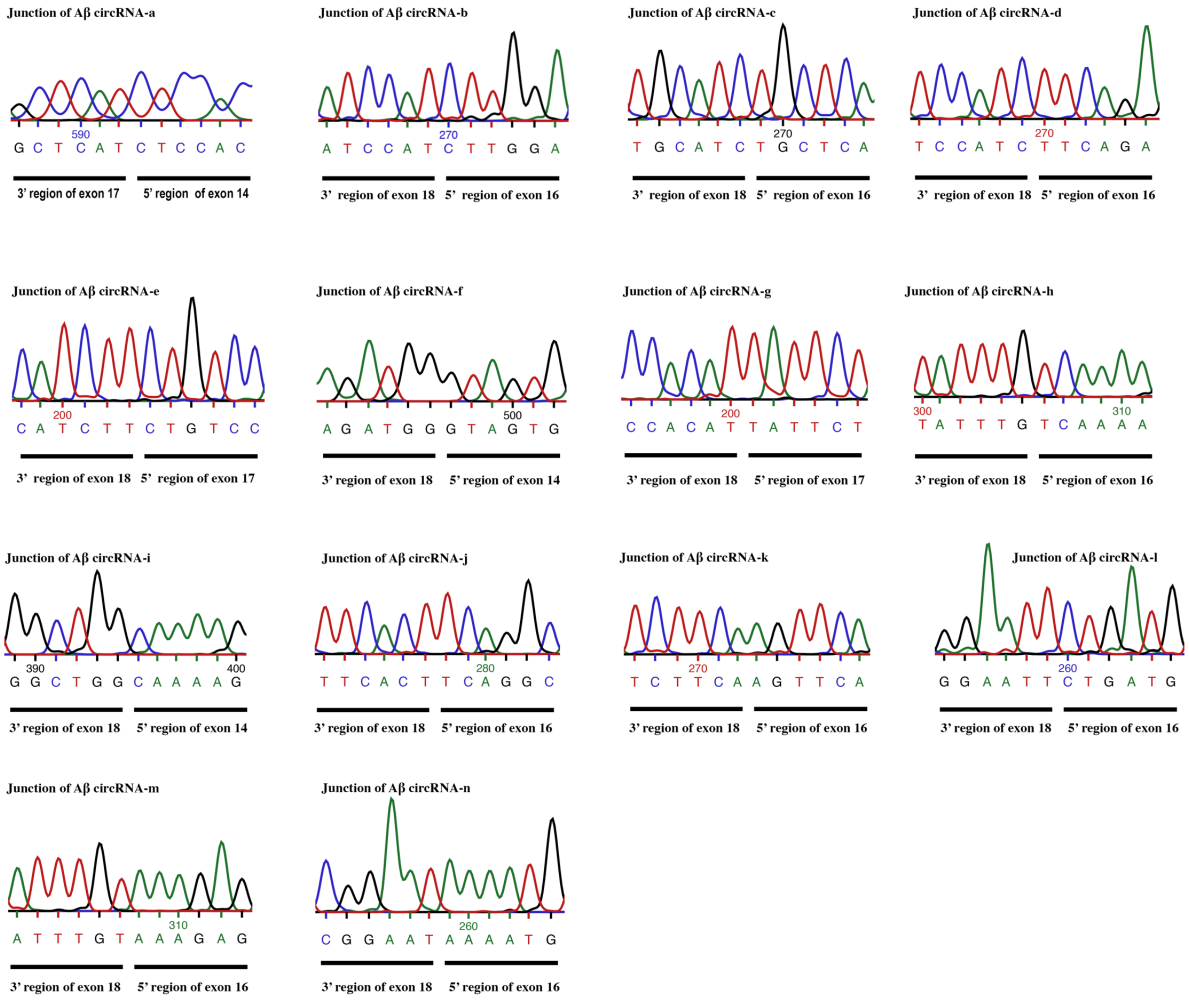
mouse putative Aβ circRNA amplification:
F3 Gctgtctctcattggctgcttcc
R3 agcttgtagagacacacatggccag

mouse App mRNA RT-PCR at Aβ peptide region
F1 GGACATGATTCAGGATTTGAAGTCC
R1 CACCAGGGTGATGACAATCACG

mouse putative Aβ circRNA amplification, same sequence region as mAB-mF, mAB-mR
R2 GGACTTCAAATCCTGAATCATGTCC
F2 CGTGATTGTCATCACCCTGGTG

Mouse Actb mRNA:
mActbF1 ACCTTCTACAATGAGCTGCG,
mActbR1 CTGGATGGCTACGTACATGG

Human ACTB mRNA:
hACTB-F ACCTTCTACAATGAGCTGCG
hACTB-R CCTGGATAGCAACGTACATGG



Supplementary Fig. 1.
the junction region sequencing of Aβ circRNA isoforms.
junction site sequencing of Aβ circRNA-a, b, c, d, e, f, g, h, i, j, k, l, m, n.

>Aβ circRNA-a (524 nt)
ATGAGCTGCTTCAGAAAGAGCAAAACTATTCAGATGACGTCTTGGCCAACATGATTAGTGAACCAAG
GATCAGTTACGGAAACGATGCTCTCATGCCATCTTTGACCGAAACGAAAACCACCGTGGAGCTCCTT
CCCGTGAATGGAGAGTTCAGCCTGGACGATCTCCAGCCGTGGCATTCTTTTGGGGCTGACTCTGTGC
CAGCCAACACAGAAAACGAAGTTGAGCCTGTTGATGCCCGCCCTGCTGCCGACCGAGGACTGACCA
CTCGACCAGGTTCTGGGTTGACAAATATCAAGACGGAGGAGATCTCTGAAGTGAAGATGGATGCAG
AATTCCGACATGACTCAGGATATGAAGTTCATCATCAAAAATTGGTGTTCTTTGCAGAAGATGTGGG
TTCAAACAAAGGTGCAATCATTGGACTCATGGTGGGCGGTGTTGTCATAGCGACAGTGATCGTCATC
ACCTTGGTGATGCTGAAGAAGAAACAGTACACATCCATTCATCATGGTGTGGTGGAG

>Aβ circRNA-b (243 nt)
ATGGATGCAGAATTCCGACATGACTCAGGATATGAAGTTCATCATCAAAAATTGGTGTTCTTTGCAG
AAGATGTGGGTTCAAACAAAGGTGCAATCATTGGACTCATGGTGGGCGGTGTTGTCATAGCGACAG
TGATCGTCATCACCTTGGTGATGCTGAAGAAGAAACAGTACACATCCATTCATCATGGTGTGGTGGA
GGTTGACGCCGCTGTCACCCCAGAGGAGCGCCACCTGTCCAAG

>Aβ circRNA-c (287 nt)
GATGCAGAATTCCGACATGACTCAGGATATGAAGTTCATCATCAAAAATTGGTGT
TCTTTGCAGAAGATGTGGGTTCAAACAAAGGTGCAATCATTGGACTCATGGTGGG
CGGTGTTGTCATAGCGACAGTGATCGTCATCACCTTGGTGATGCTGAAGAAGAAA
CAGTACACATCCATTCATCATGGTGTGGTGGAGGTTGACGCCGCTGTCACCCCAG
AGGAGCGCCACCTGTCCAAGATGCAGCAGAACGGCTACGAAAATCCAACCTACA
AGTTCTTTGAGCA

>Aβ circRNA-d (328 nt)
GATGGATGCAGAATTCCGACATGACTCAGGATATGAAGTTCATCATCAAAAATTG
GTGTTCTTTGCAGAAGATGTGGGTTCAAACAAAGGTGCAATCATTGGACTCATGG
TGGGCGGTGTTGTCATAGCGACAGTGATCGTCATCACCTTGGTGATGCTGAAGAA
GAAACAGTACACATCCATTCATCATGGTGTGGTGGAGGTTGACGCCGCTGTCACC
CCAGAGGAGCGCCACCTGTCCAAGATGCAGCAGAACGGCTACGAAAATCCAACC
TACAAGTTCTTTGAGCAGATGCAGAACTAGACCCCCGCCACAGCAGCCTCTGAA

>Aβ circRNA-e (269 nt)
AAGATGTGGGTTCAAACAAAGGTGCAATCATTGGACTCATGGTGGGCGGTGTTGT
CATAGCGACAGTGATCGTCATCACCTTGGTGATGCTGAAGAAGAAACAGTACAC
ATCCATTCATCATGGTGTGGTGGAGGTTGACGCCGCTGTCACCCCAGAGGAGCGC
CACCTGTCCAAGATGCAGCAGAACGGCTACGAAAATCCAACCTACAAGTTCTTTG
AGCAGATGCAGAACTAGACCCCCGCCACAGCAGCCTCTGAAGTTGGACAG

>Aβ circRNA-f (584 nt)
CCATCTTTGACCGAAACGAAAACCACCGTGGAGCTCCTTCCCGTGAATGGAGAGT
TCAGCCTGGACGATCTCCAGCCGTGGCATTCTTTTGGGGCTGACTCTGTGCCAGC
CAACACAGAAAACGAAGTTGAGCCTGTTGATGCCCGCCCTGCTGCCGACCGAGG
ACTGACCACTCGACCAGGTTCTGGGTTGACAAATATCAAGACGGAGGAGATCTCT
GAAGTGAAGATGGATGCAGAATTCCGACATGACTCAGGATATGAAGTTCATCAT
CAAAAATTGGTGTTCTTTGCAGAAGATGTGGGTTCAAACAAAGGTGCAATCATTG
GACTCATGGTGGGCGGTGTTGTCATAGCGACAGTGATCGTCATCACCTTGGTGAT
GCTGAAGAAGAAACAGTACACATCCATTCATCATGGTGTGGTGGAGGTTGACGC
CGCTGTCACCCCAGAGGAGCGCCACCTGTCCAAGATGCAGCAGAACGGCTACGA
AAATCCAACCTACAAGTTCTTTGAGCAGATGCAGAACTAGACCCCCGCCACAGCA
GCCTCTGAAGTTGGACAGCAAAACCATTGCTTCACTAC

>Aβ circRNA-g (310 nt)
ATGTGGGTTCAAACAAAGGTGCAATCATTGGACTCATGGTGGGCGGTGTTGTCAT
AGCGACAGTGATCGTCATCACCTTGGTGATGCTGAAGAAGAAACAGTACACATC
CATTCATCATGGTGTGGTGGAGGTTGACGCCGCTGTCACCCCAGAGGAGCGCCAC
CTGTCCAAGATGCAGCAGAACGGCTACGAAAATCCAACCTACAAGTTCTTTGAGC
AGATGCAGAACTAGACCCCCGCCACAGCAGCCTCTGAAGTTGGACAGCAAAACC
ATTGCTTCACTACCCATCGGTGTCCATTTATAGAATA

>Aβ circRNA-h (466 nt)
CAAATATCAAGACGGAGGAGATCTCTGAAGTGAAGATGGATGCAGAATTCCGAC
ATGACTCAGGATATGAAGTTCATCATCAAAAATTGGTGTTCTTTGCAGAAGATGT
GGGTTCAAACAAAGGTGCAATCATTGGACTCATGGTGGGCGGTGTTGTCATAGCG
ACAGTGATCGTCATCACCTTGGTGATGCTGAAGAAGAAACAGTACACATCCATTC
ATCATGGTGTGGTGGAGGTTGACGCCGCTGTCACCCCAGAGGAGCGCCACCTGTC
CAAGATGCAGCAGAACGGCTACGAAAATCCAACCTACAAGTTCTTTGAGCAGAT

GCAGAACTAGACCCCCGCCACAGCAGCCTCTGAAGTTGGACAGCAAAACCATTG
CTTCACTACCCATCGGTGTCCATTTATAGAATAATGTGGGAAGAAACAAACCCGT
TTTATGATTTACTCATTATCGCCTTTTGA

>Aβ circRNA-i (553 nt)
CCAGCCAACACAGAAAACGAAGTTGAGCCTGTTGATGCCCGCCCTGCTGCCGACC
GAGGACTGACCACTCGACCAGGTTCTGGGTTGACAAATATCAAGACGGAGGAGA
TCTCTGAAGTGAAGATGGATGCAGAATTCCGACATGACTCAGGATATGAAGTTCA
TCATCAAAAATTGGTGTTCTTTGCAGAAGATGTGGGTTCAAACAAAGGTGCAATC
ATTGGACTCATGGTGGGCGGTGTTGTCATAGCGACAGTGATCGTCATCACCTTGG
TGATGCTGAAGAAGAAACAGTACACATCCATTCATCATGGTGTGGTGGAGGTTGA
CGCCGCTGTCACCCCAGAGGAGCGCCACCTGTCCAAGATGCAGCAGAACGGCTA
CGAAAATCCAACCTACAAGTTCTTTGAGCAGATGCAGAACTAGACCCCCGCCACA
GCAGCCTCTGAAGTTGGACAGCAAAACCATTGCTTCACTACCCATCGGTGTCCAT
TTATAGAATAATGTGGGAAGAAACAAACCCGTTTTATGATTTACTCATTATCGCC
TTTTG

>Aβ circRNA-j (469 nt)
AGTGAAGATGGATGCAGAATTCCGACATGACTCAGGATATGAAGTTCATCATCA
AAAATTGGTGTTCTTTGCAGAAGATGTGGGTTCAAACAAAGGTGCAATCATTGGA
CTCATGGTGGGCGGTGTTGTCATAGCGACAGTGATCGTCATCACCTTGGTGATGC
TGAAGAAGAAACAGTACACATCCATTCATCATGGTGTGGTGGAGGTTGACGCCG
CTGTCACCCCAGAGGAGCGCCACCTGTCCAAGATGCAGCAGAACGGCTACGAAA
ATCCAACCTACAAGTTCTTTGAGCAGATGCAGAACTAGACCCCCGCCACAGCAGC
CTCTGAAGTTGGACAGCAAAACCATTGCTTCACTACCCATCGGTGTCCATTTATA
GAATAATGTGGGAAGAAACAAACCCGTTTTATGATTTACTCATTATCGCCTTTTG
ACAGCTGTGCTGTAACACAAGTAGATGCCTGA

>Aβ circRNA-k (470 nt)
TGAAGATGGATGCAGAATTCCGACATGACTCAGGATATGAAGTTCATCATCAAA
AATTGGTGTTCTTTGCAGAAGATGTGGGTTCAAACAAAGGTGCAATCATTGGACT
CATGGTGGGCGGTGTTGTCATAGCGACAGTGATCGTCATCACCTTGGTGATGCTG
AAGAAGAAACAGTACACATCCATTCATCATGGTGTGGTGGAGGTTGACGCCGCT
GTCACCCCAGAGGAGCGCCACCTGTCCAAGATGCAGCAGAACGGCTACGAAAAT
CCAACCTACAAGTTCTTTGAGCAGATGCAGAACTAGACCCCCGCCACAGCAGCCT
CTGAAGTTGGACAGCAAAACCATTGCTTCACTACCCATCGGTGTCCATTTATAGA
ATAATGTGGGAAGAAACAAACCCGTTTTATGATTTACTCATTATCGCCTTTTGACA
GCTGTGCTGTAACACAAGTAGATGCCTGAACT

>Aβ circRNA-l (475 nt)
AATTCCGACATGACTCAGGATATGAAGTTCATCATCAAAAATTGGTGTTCTTTGC
AGAAGATGTGGGTTCAAACAAAGGTGCAATCATTGGACTCATGGTGGGCGGTGTT
GTCATAGCGACAGTGATCGTCATCACCTTGGTGATGCTGAAGAAGAAACAGTAC
ACATCCATTCATCATGGTGTGGTGGAGGTTGACGCCGCTGTCACCCCAGAGGAGC
GCCACCTGTCCAAGATGCAGCAGAACGGCTACGAAAATCCAACCTACAAGTTCTT
TGAGCAGATGCAGAACTAGACCCCCGCCACAGCAGCCTCTGAAGTTGGACAGCA
AAACCATTGCTTCACTACCCATCGGTGTCCATTTATAGAATAATGTGGGAAGAAA
CAAACCCGTTTTATGATTTACTCATTATCGCCTTTTGACAGCTGTGCTGTAACACA
AGTAGATGCCTGAACTTGAATTAATCCACACATCAG

>Aβ circRNA-m (542 nt)

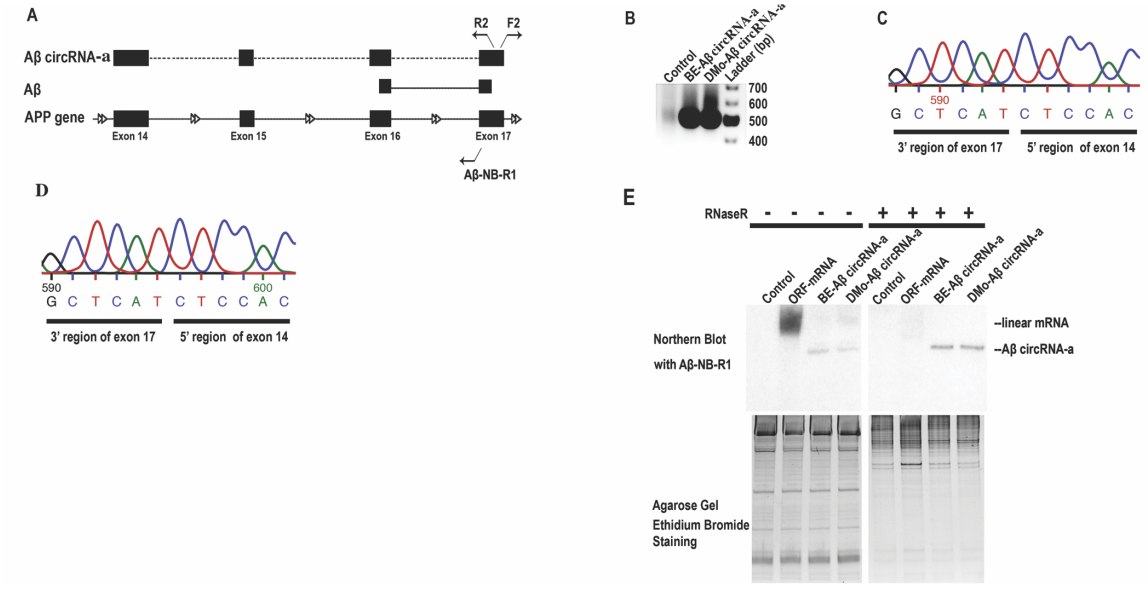
ACAAATATCAAGACGGAGGAGATCTCTGAAGTGAAGATGGATGCAGAATTCCGA
CATGACTCAGGATATGAAGTTCATCATCAAAAATTGGTGTTCTTTGCAGAAGATG
TGGGTTCAAACAAAGGTGCAATCATTGGACTCATGGTGGGCGGTGTTGTCATAGC
GACAGTGATCGTCATCACCTTGGTGATGCTGAAGAAGAAACAGTACACATCCATT
CATCATGGTGTGGTGGAGGTTGACGCCGCTGTCACCCCAGAGGAGCGCCACCTGT
CCAAGATGCAGCAGAACGGCTACGAAAATCCAACCTACAAGTTCTTTGAGCAGA
TGCAGAACTAGACCCCCGCCACAGCAGCCTCTGAAGTTGGACAGCAAAACCATT
GCTTCACTACCCATCGGTGTCCATTTATAGAATAATGTGGGAAGAAACAAACCCG
TTTTATGATTTACTCATTATCGCCTTTTGACAGCTGTGCTGTAACACAAGTAGATG
CCTGAACTTGAATTAATCCACACATCAGTAATGTATTCTATCTCTCTTT

>Aβ circRNA-n (502 nt)
ATTCCGACATGACTCAGGATATGAAGTTCATCATCAAAAATTGGTGTTCTTTGCA
GAAGATGTGGGTTCAAACAAAGGTGCAATCATTGGACTCATGGTGGGCGGTGTTG
TCATAGCGACAGTGATCGTCATCACCTTGGTGATGCTGAAGAAGAAACAGTACAC
ATCCATTCATCATGGTGTGGTGGAGGTTGACGCCGCTGTCACCCCAGAGGAGCGC
CACCTGTCCAAGATGCAGCAGAACGGCTACGAAAATCCAACCTACAAGTTCTTTG
AGCAGATGCAGAACTAGACCCCCGCCACAGCAGCCTCTGAAGTTGGACAGCAAA
ACCATTGCTTCACTACCCATCGGTGTCCATTTATAGAATAATGTGGGAAGAAACA
AACCCGTTTTATGATTTACTCATTATCGCCTTTTGACAGCTGTGCTGTAACACAAG
TAGATGCCTGAACTTGAATTAATCCACACATCAGTAATGTATTCTATCTCTCTTTA
CATTTT

>Aβ42
GATGCAGAATTCCGACATGACTCAGGATATGAAGTTCATCATCAAAAATTGGTGT
TCTTTGCAGAAGATGTGGGTTCAAACAAAGGTGCAATCATTGGACTCATGGTGGG
CGGTGTTGTCATAGCG

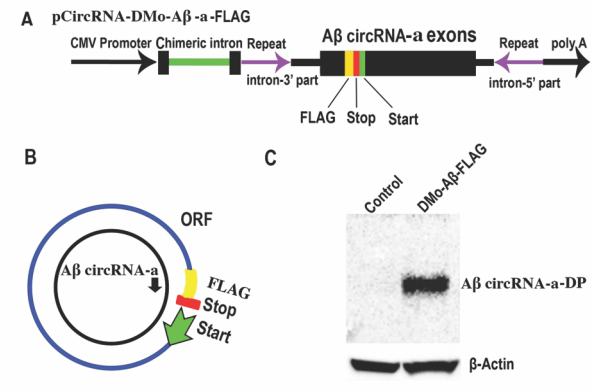
Supplementary sequencing data

The sequences of Aβ circRNA a, b, c, d, e, f, g, h, i, j, k, l, m, n



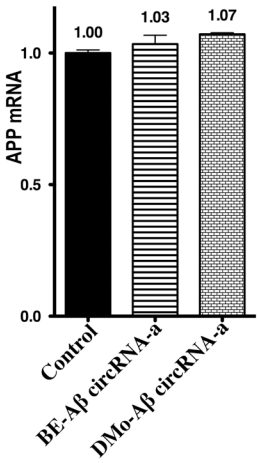
Supplementary Fig. 2. RT-PCR and northern blot verification of Aβ circRNA-a expression in HEK293 cells.

A) RT-PCR primers sites in Aβ circRNA. B) Agarose gel electrophoresis of RT-PCR products (503 bp) using primers Aβ-VR2 and Aβ-VF2; BE-Aβ circRNA-a, pCircRNA-BE-Aβ-a transfected HEK293 cells; DMo-Aβ circRNA-a, pCircRNA-DMo-Aβ-a transfected HEK293 cells. RT-PCR products were cloned, sequenced and aligned, confirming that Aβ circRNA-a were identical and comprised exons 14, 15, 16 and 17 of the APP gene without introns (data not shown); C, D) the sequencing of Aβ circRNA-a junction region of back-splicing from pCircRNA-BE-Aβ-a and pCircRNA-DMo-Aβ-a; E) Northern blot analysis of Aβ circRNA-a expression in HEK293 cells; ORF-mRNA, the linear cognate ORF mRNA of Aβ circRNA-a. For RNase R treatment, 15 μg total RNA was digested with 10 unites of RNase R for 1 hour at 37 °C; -, no treatment; +, with treatment; Oligo Aβ-NB-R1 (CCCACCATGAGTCCAATGATTGCACCTTTGTTTGAACCCACATCTTCTGCAAAGA ACACC) was used in northern blot; ethidium bromide staining of the agarose gel was used as loading control.



Supplementary Fig. 3. Fig. FLAG tagged Aβ circRNA-a derived peptide expression in HEK293 cells.

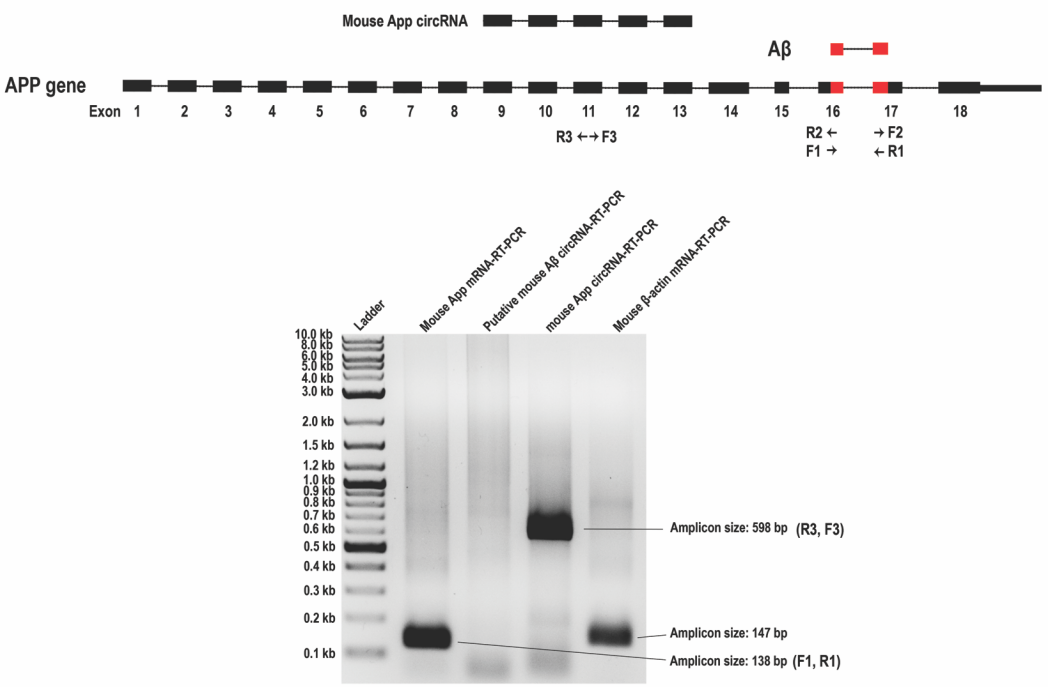
A) schematic of FLAG tagged Aβ circRNA-a expression plasmid. B) schematic of FLAG tagged Aβ circRNA-a. C) Control, pCircRNA-DMo empty vector; DMo-Aβ-FLAG, pCircRNA-DMo-Aβ-a-FLAG. β-Actin was used as loading control.



Supplementary Fig. 4. Endogenous APP mRNA expression in HEK293 cells overexpressing Aβ circRNA-a.

qRT-PCR analysis of endogenous APP mRNA expression in Aβ circRNA-a overexpressing HEK293 cells. Primers targeted exons 3 and 4, which are not contained in Aβ circRNA-a: hAPP-m, TTTGTGATTCCCTACCGCTG and hAPP-mR, TGCCAGTGAAGATGAGTTTCG. Control, pCircRNA-DMo empty vector; BE-Aβ CircRNA-a, pCircRNA-BE-Aβ-CircRNA-a; DMo-Aβ CircRNA-a, pCircRNA-DMo-Aβ CircRNA-a. No significant differences between different groups were observed. n = 4.

Supplementary Fig. 5. The putative amino acid sequence of Aβ circRNA-a–DP. Predicted from the identical sequences of all circRNA RT-PCR products: red, sequence of Aβ42; blue, the unique sequence of Aβ circRNA-a-DP resulting from the circular translation of Aβ circRNA, which is not present in the APP full-length protein. The calculated molecular weight of Aβ circRNA-a-DP is 19.2 kDa; the arrow indicates the possible secretase sites.



Supplementary Fig. 6. Mouse brain does not express Aβ circRNA copies. Primers F1 and R1 were used to amplify App mRNA; the divergent primers R2 and F2 located at the same position as F1 and R1were used to amplify putative Aβ circRNA copies; R3 and F3 were used to amplify the App circRNA (exon 9 to exon 13) as positive control circRNA RT-PCR; amplicon from mouse β-actin mRNA was also used as RT-PCR positive control.