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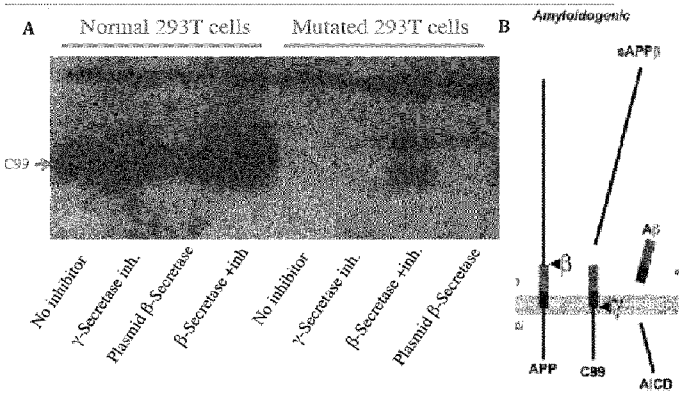

(57) Abstract: Methods and products related to genome editing using the CRISPR/Cas9 system to introduce an A673T substitution into an APP gene, such as guide RNAs and recombinant proteins, are described for decreasing APP levels produced by a cell, and use for the treatment of Alzheimer's disease and/or age-related cognitive decline in a cell from a subject in need thereof.

WO 2015/168800 A1

TITLE OF THE INVENTION

Prevention and Treatment of Alzheimer's Disease by Genome Editing Using the CRISPR/Cas System

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] The present application claims the benefit of U.S. Provisional Application Serial No. 61/991,054 filed on May 9, 2014, which is incorporated herein by reference in their entirety.

SEQUENCE LISTING

[0002] This application contains a Sequence Listing in computer readable form entitled "11229_339_SeqList.txt", created May 8, 2015 and having a size of about 132 KB. The computer readable form is incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates to the introduction of modifications in the Amyloid β Precursor Protein (APP) gene and uses thereof to reduce the production of toxic Aβ peptides. Such reduction may be used for example to prevent or treat Alzheimer's disease and/or age-related cognitive decline.

BACKGROUND OF THE INVENTION

[0003] More than 5% of the population of the Western world above the age of 60 is affected by dementia. Two thirds of these cases are due to Alzheimer's disease (AD) (1-3). The prevalence of AD doubles every 5 years after age 65. Thus in persons older than 90 years of age there is a prevalence of more than 25% (3). The diagnosis of AD is confirmed by 2 major histopathologic hallmarks: senile plaques, which are extracellular deposits of amyloid β (Aβ) peptide, and neurofibrillary tangles, which are somatic inclusions of the microtubule-associated protein tau.

[0004] The amyloid cascade hypothesis: structure and metabolic processing of the Amyloid β Precursor Protein (APP)

[0005] A role of Amyloid β (Aβ) peptide aggregation and deposition in AD pathogenesis is widely accepted. Toxic peptides are produced by the metabolic processing of Amyloid β Precursor Protein (APP). APP is an integral membrane protein expressed in many tissues and concentrated in the synapses of neurons. APP proteolysis generates beta amyloid (Aβ), a 37 to 49 amino acid peptide whose amyloid fibrillar form is the primary component of amyloid plaques found in the brains of Alzheimer's disease patients.

[0006] Genetic alterations in APP suffice to cause early-onset Familial Alzheimer's disease. Abnormalities

induced by aggregated Aβ have been linked to synaptic and neuronal degeneration. This is consistent with the "dying-back" pattern of degeneration that characterizes neurons affected in AD.

[0007] Amyloid plaque formation is a central pathological feature of AD. These plaques largely consist of Aβ peptides (4). The Aβ peptides are formed through sequential proteolytic processing of APP made by the β- and γ-secretases (5) (Figure 1). The γ-secretase is part of the presenilin complex. The aspartyl protease β-site APP cleaving enzyme 1 (BACE1) was originally identified over a decade ago (6, 7). It cleaves APP mostly at a unique site. However, the γ-secretase complex cleaves the resulting carboxy-terminal fragment at several places, especially at positions 40 and 42. The cleavage by this enzyme leads to the formation of amyloid-β1–40 (Aβ1–40) and Aβ1–42 peptides (5). Alternative processing of APP at the α-site prevents the formation of amyloid-β, because the α-site is located within amyloid-β. The neurotoxic potential of the Aβ peptides results from their biochemical properties that favor aggregation into insoluble oligomers and protofibrils. These oligomers and protofibrils accumulate into senile and neuritic plaques. These plaques, along with a reduction of Aβ clearance from the brain, leads to the extracellular accumulation of Aβ. This leads to activation of neurotoxic cascades and ultimately to cytoskeletal changes, neuronal dysfunction and cellular death.

[0008] The amyloid cascade hypothesis is based mostly on findings from *in vitro* and *in vivo* studies, and is further strengthened by the discovery of genetic mutations associated with early-onset, familial AD. Familial AD is a severe form of the disease, in which massive intra-cerebral amyloidogenesis occurs prematurely as a consequence of mutations all affecting APP metabolism (i.e., mutations in the APP gene in chromosome 21, and in presenilin 1 and 2 (PS-1 and PS-2) genes in chromosomes 14 and 1 respectively). Currently, two proteins are deemed as intimately involved in the clearance of Aβ peptides from the brain: apolipoprotein E (APOE) and the insulin-degrading enzyme (IDE). Disadvantageous genetic polymorphisms (such as the ε4 allele of APOE) and pathological conditions related to abnormal IDE homeostasis (e.g., diabetes mellitus) also favor the amyloidogenic cleavage of APP and/or decrease the Aβ clearance from the brain. This facilitates the accumulation of Aβ in the neural tissues and promote downstream effects of the amyloid cascade (8).

[0009] Mutations in APP that are responsible for early onset familial Alzheimer's disease

[0010] Over thirty coding mutations in the APP gene have been identified (6). Twenty five of these mutations are pathogenic, usually resulting in early onset autosomal dominant Alzheimer's disease (Figure 2A). Substitutions at or near the β- and γ-proteolytic sites result in over-production of either total amyloid-β or a shift in the $A\beta_{1-40}$:$A\beta_{1-42}$ ratio towards formation of the more toxic $A\beta_{1-42}$ peptide. On the other hand, substitutions within the amyloid-β peptide result in formation of Aβ, which aggregates more easily (9). Mutations in APP are also responsible for the common, late-onset form of Alzheimer's disease.

[0011] Mutation that prevents Alzheimer's disease

[0012] **Jonsson** et al. (10) searched for low-frequency variants in the APP gene, which significantly reduces the risk of Alzheimer's disease. They studied coding variants in APP in whole-genome sequence data obtained from 1,795 Icelanders. They reported a coding mutation, i.e., an alanine to threonine substitution at position 673 in the APP gene (A673T), which protects against Alzheimer's disease. This mutation is adjacent to the aspartyl protease β-site in APP and is located at position 2 in the amyloid-β peptide. The proximity of A673T mutation to the proteolytic site of BACE1 suggests that this variant might result in impaired BACE1 cleavage of APP.

[0013] The A673T mutation reduces by about 40% the formation of amyloidogenic peptides *in vitro* (10). The strong protective effect of the A673T mutation against Alzheimer's disease provided a proof of principle that reducing the β-cleavage of APP may protect against the disease. Moreover, the A673T mutation also protects against cognitive decline in the elderly without Alzheimer's disease. The carriers of this A673T mutation have a 1.47 times greater chance of reaching the age of 85 without developing AD than non-carriers. Jonsson et al. (10) concluded that the A673T mutation confers a strong protection against Alzheimer's disease. Kero et al. (11) found the A673T variant in one person who died at the age of 104.8 years with little beta-amyloid pathology. This observation supports the concept that this variant protects the brain against β-amyloid pathology and Alzheimer disease.

[0014] The present description refers to a number of documents and sequence database entries, the content of which is herein incorporated by reference in their entirety.

SUMMARY OF THE INVENTION

[0015] The present invention relates to introducing one or more modifications into a target gene involved in Aβ peptide production by gene editing using one or more site-specific endonucleases and one or more donor or patch nucleic acids. The modifications introduced are specifically designed to decrease the production of Aβ peptide (e.g., by reducing APP expression and/or APP processing). In an aspect, a guide RNA (gRNA) is designed and used in combination with a Cas9 nuclease or nickase to specifically introduce a cut in the targeted gene DNA. This cut, allows to specifically modify the target gene by introducing in the target gene (e.g., by homologous recombination) a donor (patch) nucleic acid comprising one or more modifications of interest. The target gene thus modified, will ultimately reduce the level of Aβ peptide produced by the cell. The present invention further relates to uses of such gRNAs, site-specific endonucleases and donor/patch nucleic acids for decreasing Aβ-peptide expression/levels in a cell. The present invention also concerns uses of such gRNAs, site-specific endonucleases and donor/patch nucleic acid for the prevention or treatment of Alzheimer's disease and/or age related cognitive decline.

[0016] Accordingly, in an aspect, the present invention provides a method for decreasing Amyloid Precursor Protein (APP) processing into Aβ peptide by a cell comprising introducing at least one modification within an

endogenous polynucleotide target gene sequence of the cell, wherein the modification decreases the amount of Aβ peptide produced by the cell and wherein the endogenous polynucleotide target gene encodes a protein that regulates Aβ-peptide production.

[0017] In an embodiment, the target gene encodes an α-secretase, a β-secretase or a γ-secretase. In an embodiment, the target gene encodes APP.

[0018] In an embodiment, the above-mentioned methods comprise providing the cell with:

i) a site-specific endonuclease specifically targeting a nucleic acid sequence in the endogenous APP polynucleotide gene sequence of the cell; and

ii) a donor nucleic acid comprising an APP polynucleotide gene sequence or fragment thereof, which comprises at least one modification with respect to the endogenous APP polynucleotide gene sequence present in the cell.

[0019] In an embodiment, the site-specific endonuclease is a meganuclease, a zinc finger nuclease, a transcription activator-like effector nuclease (TALEN), a Cas9 nuclease (including a a dCas-FokI nuclease), or a Cas9 nickase. .

[0020] In an embodiment, the donor nucleic acid further comprises on each side of the APP polynucleotide gene sequence or fragment thereof, a target sequence recognized by a gRNA for cutting the donor nucleic acid in the presence of the gRNA and a Cas9 nuclease, or a Cas9 nickase or a dCas9-FokI.

[0021] In an embodiment, the donor nucleic acid comprises a modification, which introduces an additional endonuclease restriction site when the donor nucleic acid is integrated in the endogenous APP polynucleotide gene sequence in the cell.

[0022] In an embodiment, the donor nucleic acid comprises a modified APP polynucleotide gene fragment encoding exon 16 or exon 17.

[0023] In an embodiment, the at least one modification in the donor nucleic acid results in an APP polynucleotide gene sequence encoding an APP protein in which the alanine at position 673 has been substituted with another amino acid when the donor nucleic acid is integrated in the endogenous APP polynucleotide gene sequence of the cell. In an embodiment, the at least one modification changes the alanine at position 673 of the APP protein into a threonine. In another embodiment, the at least one modification in the donor nucleic acid results in a modification of one or more amino acids recognized by an α, β, or γ secretase in the APP protein encoded by the APP polynucleotide gene sequence. In an embodiment, the at least one modification corrects a mutation associated with an increased risk of developing Alzheimer's disease present in the endogenous APP polynucleotide gene

sequence of the cell.

[0024] In an embodiment, the above-mentioned methods comprise providing the cell with:

(i) at least one guide RNA (gRNA) comprising:
  (a) a gRNA guide sequence comprising a seed region of at least 10 consecutive nucleotides of a target sequence in an endogenous APP gene polynucleotide sequence present in said cell;
  (b) a Cas9 recognition sequence,

  wherein the target sequence of the gRNA guide sequence is contiguous to a protospacer adjacent motif (PAM) in the endogenous APP gene polynucleotide sequence and wherein said PAM is recognized by a ribonucleoprotein complex comprising a Cas9 nuclease or nickase;

(ii) a Cas9 nuclease or nickase or a nucleic acid encoding a Cas9 nuclease or nickase; and

(iii) a donor nucleic acid comprising an APP polynucleotide gene sequence or fragment thereof, which comprises at least one modification with respect to the endogenous APP polynucleotide gene sequence present in said cell,

wherein the donor nucleic acid is integrated in said APP gene polynucleotide sequence of said cell and wherein said modification decreases the amount of Aβ produced by said cell.

[0025] In an embodiment, the site-specific endonuclease is a Cas9 nuclease or nickase; and the method further comprises providing the cell with at least on one guide RNA (gRNA) comprising a nucleotide seed region perfectly complementary to a target sequence (minus (-) strand) in the endogenous APP polynucleotide gene sequence of the cell.

[0026] In an embodiment, the Cas9 nuclease or nickase is a hSpCas9 nuclease, a hSaCas9, a hSpCas9 nickase, a hSaCas9 nickase or a dCas9-FokI nuclease.

[0027] In an embodiment, the guide RNA (gRNA) comprises a nucleotide seed region of at least 8 nucleotides perfectly complementary to the target nucleic acid sequence (minus (-) strand) in the endogenous APP polynucleotide gene, wherein the target nucleic acid sequence is immediately adjacent to a protospacer adjacent motif (PAM) recognized by a Cas ribonucleoprotein complex comprising the Cas9 nuclease or nickase.

[0028] In an embodiment, the PAM comprises a NGG trinucleotide-sequence or an NNGRR nucleotide sequence.

[0029] In an embodiment, the donor nucleic acid is a single stranded oligodeoxynucleotide (ssODN) or a PCR amplicon. In an embodiment, the donor nucleic acid is comprised in a vector. In an embodiment, the donor nucleic acid further comprises on each side of the APP polynucleotide gene sequence or fragment thereof, a target sequence recognized by a gRNA for cutting said donor nucleic acid in the presence of said gRNA and said Cas9 nuclease or nickase. In an embodiment, the donor nucleic acid comprises a modification which introduces an additional endonuclease restriction site when the donor nucleic acid is integrated in the endogenous APP polynucleotide gene sequence in said cell. In an embodiment, the donor nucleic acid comprises a modified APP polynucleotide gene fragment encoding exon 16, or a fragment thereof. In an embodiment, the donor nucleic acid comprises a modified APP polynucleotide gene fragment comprising at least part of intron 15, exon 16, and at least part of intron 16 of the endogenous APP polynucleotide sequence.

[0030] In an embodiment, the least one modification is located in Exon 16 of the APP polynucleotide gene sequence. In an embodiment, the at least one modification in the donor nucleic acid results in an APP polynucleotide gene sequence encoding an APP protein comprising at least one amino acid substitution between amino acid positions 656 and 688, wherein said amino acid positions are with respect to the APP protein sequence as set forth in SEQ ID NO: 30 (Figure 3). In an embodiment, the at least one amino acid substitution corresponds to at least one substitution at amino acid position 670, 671, 673, 678, 682, 692, 603, 694, 704, 711, 712, 713, 714, and/or 715 of the APP protein. In an embodiment, the at least one amino acid substitution introduces a threonine at position 673. In an embodiment, the at least one modification in said donor nucleic acid results in a modification of one or more amino acids recognized by an α, β, or γ secretase in the APP protein encoded by said APP polynucleotide gene sequence present in said cell. In an embodiment, the at least one modification corrects a mutation associated with an increased risk of developing Alzheimer's disease present in said endogenous APP polynucleotide gene sequence present in said cell.

[0031] In an embodiment of the above-mentioned method, the cell is from a subject having at least one family member which has been diagnosed with Alzheimer's disease. In a related embodiment, the cell is from a subject having at least one mutation associated with early onset Alzheimer's disease.

[0032] In a preferred embodiment of the above methods, the guide RNA (gRNA) sequence of the present invention consists of at least 19 or 20 contiguous nucleotides of the target sequence in the endogenous APP polynucleotide gene.

[0033] In an embodiment, the target nucleic acid sequence of the above-mentioned gRNA is located in an exon of the APP polynucleotide gene sequence encoding the APP protein. In an embodiment, the target sequence of said gRNA is located in intron 15, exon 16 or intron 16 of the endogenous APP polynucleotide gene sequence present in said cell.

[0034] In an embodiment, the above-mentioned target sequence is located between (i) nucleotide 277141 and nucleotide 279540; (ii) nucleotide 277567 and nucleotide 27845; (iii) nucleotide 277141 and nucleotide 278147; (iv) nucleotide 278149 and nucleotide 278250; (v) nucleotide 278251 and nucleotide 279540; (vi) nucleotide 277277 and nucleotide 278147; (vii) nucleotide 277559 and nucleotide 278147; (vii) nucleotide 277567 and nucleotide 278147; (viii) nucleotide 278149 and nucleotide 278220; (ix) nucleotide 278202 and nucleotide 278250; (x) nucleotide 278721 and nucleotide 279540; (xi) nucleotide 278781 and nucleotide 279540; (xii) nucleotide 279245 and nucleotide 279540 of the APP polynucleotide gene sequence set forth in Figure 5, or the complement thereof, or in a corresponding location in the endogenous APP polynucleotide gene sequence present in said cell.

[0035] In an embodiment, the above-mentioned target sequence of said gRNA comprises the following nucleic acid sequence:

(i) 5'-ATTTATGAGTAAAACTAAT-3 (SEQ ID NO: 1)';
(ii) 5'-TTTAATTATGATGTAATAC-3'(SEQ ID NO: 3);
(iii) 5'-TATGATGTAATACAGGTTC-3'(SEQ ID NO: 5);
(iv) 5'-ATGATGTAATACAGGTTCT-3'(SEQ ID NO: 7);
(v) 5'-GGGTTGACAAATATCAAGA-3'(SEQ ID NO: 9);
(vi) 5'-TTGACAAATATCAAGACGG-3'(SEQ ID NO: 11);
(vii) 5'-GAGATCTCTGAAGTGAAGA-3'(SEQ ID NO: 13);
(viii) 5'-CAGAATTCCGACATGACTCA-3'(SEQ ID NO: 15);
(ix) 5'-GAAGTTCATCATCAAAAAT-3'(SEQ ID NO: 17);
(x) 5'-CCAAATGACCTATTAACTC-3'(SEQ ID NO: 19)

of the human APP polynucleotide gene sequence having reference number NCBI NG_007376.1.

[0036] In an embodiment, the above-mentioned target sequence of the gRNA of the present invention comprises the following nucleic acid sequence:

(i) 5'- CTACCCAAAACTTCTTTCT-3' (SEQ ID NO: 21);
(ii) 5'- CATCATAATTAAAGTATGC-3' (SEQ ID NO: 23);
(iii) 5'- TTCATATCCTGAGTCATGT-3' (SEQ ID NO: 25); or
(iv) 5'- GACAAACAGTAGTGGAAAG-3' (SEQ ID NO: 27)

of the complementary strand (minus strand) of human APP polynucleotide gene sequence having reference number NCBI NG_007376.1.

[0037] In an embodiment, the above-mentioned methods comprises providing the cell with a gRNA targeting

the nucleic acid sequence CAGAATTCCGACATGACTC corresponding to nucleotide positions 278202 to 278220 of the human APP polynucleotide gene sequence having reference number NCBI NG_007376.1.

[0038] In an embodiment, of the above-mentioned methods the at least one modification within said endogenous APP polynucleotide gene sequence of said cell is introduced by Non-Homologous End Joining. In an embodiment, the at least one modification within said endogenous APP polynucleotide gene sequence of said cell is introduced by homologous recombination between said donor nucleic acid and said endogenous APP polynucleotide gene sequence.

[0001] In a specific embodiment, the method comprises providing the cell with a gRNA targeting the nucleic acid sequence CAGAATTCCGACATGACTC (SEQ ID NO:16) corresponding to nucleotide positions 278202 to 278220 of the human APP polynucleotide gene sequence having reference number NCBI NG_007376.1.In another aspect, the present invention provides a gRNA and a donor nucleic acid as defined in the above-mentioned methods. The present invention also provides a vector comprising the above-mentioned gRNAs and/or donor nucleic acids and host cells expressing such gRNA, site-specific nucleases (e.g., Cas9 nucleases or nickases) and donor nucleic acids. In an embodiment, the vector is a viral vector. In an embodiment, the viral vector is a lentiviral vector, Adeno-Associated viral vector, adenovirus viral vector or herpes virus viral vector.

[0002] In a particular embodiment, the above-mentioned vectors further comprise a nucleic acid encoding a site-specific endonuclease or nickase. In an embodiment, the vector further comprises a nucleic acid encoding a Cas9 nuclease (e.g., hSpCas9, hSaCas 9, dCas9-FokI) or a Cas 9 nickase (e.g., hSpCas9, hSaCas 9). In an embodiment, the Cas9 nuclease is a dCas9-FokI nuclease.

[0003] In a further aspect, the present invention concerns a composition comprising: i) one or more of the above-mentioned gRNAs, ii) one or more of the above-mentioned donor nucleic acids; and/or iii) one or more of the above-mentioned vectors, and a pharmaceutically acceptable carrier. The present invention also concerns a kit comprising: i) one or more of the above-mentioned gRNAs, ii) one or more of the above-mentioned donor nucleic acids; and/or iii) one or more of the above-mentioned vectors, and instructions to use the kit in accordance with the present invention.

[0004] In an embodiment, the composition or kit further comprises a site-specific nuclease. In an embodiment, the site-specific nuclease is a Cas9 nuclease (e.g., hSpCas9, hSaCas 9, dCas9-FokI) or a Cas9 nickase (e.g., hSpCas9, hSaCas 9, dCas9-FokI).

[0005] In an embodiment, the present invention concerns the above-mentioned gRNAs, donor nucleic acids, vectors, host cells, compositions and/or kits for use in decreasing Amyloid Precursor Protein (APP) processing into Aβ peptide by a cell.

[0006] In another embodiment, the present invention concerns the above-mentioned gRNAs, donor nucleic acids, vectors, host cells, compositions and/or kits for use in treating or preventing Alzheimer's disease or age-related cognitive decline in a subject in need thereof.

[0007] In a related aspect, the present invention concerns the above-mentioned gRNAs, donor nucleic acids, vectors, host cells,compositions and/or kits for the preparation of a medicament for decreasing Amyloid Precursor Protein (APP) processing into Aβ peptide by a cell.

[0008] The present invention also concerns the above-mentioned gRNAs, donor nucleic acids, vectors, host cells, compositions and/or kits for the preparation of a medicament for treating or preventing Alzheimer's disease or age-related cognitive decline in a subject in need thereof.

[0009] The present invention further concerns the use of the above-mentioned gRNAs, donor nucleic acids, vectors, host cells, compositions and/or kits for decreasing Amyloid Precursor Protein (APP) processing into Aβ peptide by a cell.

[0010] In another embodiment, the present invention relates to the use of the above-mentioned gRNAs, donor nucleic acids, vectors, host cells, compositions and/or kits for preventing or treating Alzheimer's disease or age-related cognitive decline in a subject in need thereof.

[0011] In another embodiment, the present invention relates to a method of preventing or treating Alzheimer's disease or age-related cognitive decline in a subject in need thereof, comprising introducing into or contacting a cell of the subject with the above-mentioned gRNAs, donor nucleic acids, vectors, host cells and/or compositions.

[0012] In an embodiment of the above-mentioned methods, the cell is from subject in need thereof. In a particular embodiment, the subject in need thereof is a subject at risk of developing Alzheimer's disease. In another embodiment, the subject in need thereof is a subject diagnosed with Alzheimer 's disease or age-related cognitive decline.

[0013] Other objects, advantages and features of the present invention will become more apparent upon reading of the following non-restrictive description of specific embodiments thereof, given by way of example only with reference to the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014] Figure 1 shows pathways for metabolic processing of Amyloid β Precursor Protein (APP). The figure illustrates the two most prominent metabolic routes and the APP proteolytic derivatives. APP is the substrate for either α- or β-secretases. Thus, the two pathways (nonamyloidogenic and the amyloidogenic) are mutually exclusive.

β-site APP-cleaving enzyme 1 (BACE1) is the only known aspartylprotease with β-secretase activity. The fragments C83 and C99 (also known as α- and β-carboxy-terminal fragments) are substrates for the γ-secretase, a protein complex that includes at least 4 different proteins: anterior pharynx defective 1, nicastrin, presenilin 1 (PS-1) or presenilin 2 (PS-2), and presenilin enhancer-2. AICD is the intracytoplasmic domain of APP. The figure was adapted from E.A. Bignante et al. Neurobiology of Aging 34 (2013) 2525-2537;

[0015] Figure 2 is a schematic representation of the domain structure of amyloid β precursor protein (APP). The E1 domain consisting of the Growth Factor Like Domain (GFLD) and the Copper-Binding Domain (CuBD). The acidic domain (AcD) and the Kunitz-type inhibitory domain (Kunitz protease inhibitor [KPI], not present in neurons) bridge the E1 and E2 domains. The E2 domain consists of 2 coiled-coil substructures connected through a continuous helix. The amino acid sequence of amyloid β (Aβ)/transmembrane/intracellular (intracytoplasmic domain of APP [AICD]) domains is shown in the lower part of the figure (SEQ ID NO: 29). Asterisks denote amino acids substituted in familiar forms of AD and APP variants. Arrows indicate cleavage sites for both secretases and caspases. Binding motifs for heterotrimeric Go/s proteins (Go/s-BM) and scaffolding proteins (SP-BM; e.g., Fe65, Mint/X11-family proteins, Dab1, c-Jun N-terminal kinase) are underlined. The Aβ sequence is also underlined. Figure is from E.A. Bignante et al. Supra;

[0016] Figure 3 shows the complete amino acid sequence of the human Amyloid β Precursor Protein (APP) gene (NCBI Reference Sequence: NG_007376.1, (SEQ ID NO: 30), wild-type sequence). The position of mutation A673T is located in exon 16 of the human APP gene and is shown in bold and is underlined;

[0017] Figure 4 (A) shows the nucleotide (SEQ ID NO: 31) and amino acid (SEQ ID NO: 32) sequences of exon 16 from APP (NCBI Reference sequence NG_007376.1). (B) shows the nucleotide (SEQ ID NO: 33) and amino acid (SEQ ID NO: 34) sequences of mutated APP exon 16 after Homology Directed Repair with pMiniT-Patch. The amino acid alanine (A) at position 673 (in bold) in Panel A is modified to a threonine (T) to protect against Alzheimer's disease (see panel B). The highlighted sequence (CAGAATTCCGACATGACTC, SEQ ID NO: 15) in panel A is the sequence targeted by the gRNA#8 (Tables 3 and 4). The sequence in italic (*AGG*) located after the highlighted sequence in panels A and B is the PAM recognized by the Cas9 complex;

[0018] Figure 5 shows a partial sequence of the wild type APP gene. (A) is a schematic representation of a partial sequence of the wild type APP gene extending before and after exon 16. (B) presents the partial genomic nucleotide sequence of the human APP gene shown in (A) (nucleotides 277141 to 279540 of NCBI NG_007376.1/GI: 166795291, SEQ ID NO: 35) which includes a partial sequence of intron 15 (SEQ ID NO:36), followed by the complete sequence of exon 16 (nucleotides 278148-278248, underlined and bold, (SEQ ID NO: 31)) and a partial sequence of intron 16 (SEQ ID NO: 37). Primer sequences (Cfw (SEQ ID NO: 38), Afw (SEQ ID NO: 39), Brev (SEQ ID NO: 40), Arev (SEQ ID NO: 41) and Crev (SEQ ID NO: 42)) and the gRNA #8 target sequence + PAM (SEQ ID

NO: 15, positions 278202 to 278223) are shown in *italics*. Other PAM sequences used to design alternative gRNAs (gRNAs 1-7 and 9 to 14) are in bold and shaded (see Table 4, in Example 2). The sequence of primer Bfw (SEQ ID NO: 43) is underlined;

[0019] Figure 6 shows that gRNAs of the present invention efficiently target the human APP gene in HEK 293T cells. (A) Schematic representation of the pX330™ plasmid encoding the humanized Cas9 enzyme (hSpCas9) and gRNAs transfected in HEK 293T cells to induce mutations in the endogenous APP gene. Figure 6A is from the information pamphlet regarding the pX330™ plasmid. It can be viewed at the Addgene website at: http://www.addgene.org/crispr/zhang/. The guide sequence insertion site of plasmid pX330™ is shown (SEQ ID NO: 44). For proper sequence insertion into the pX330™ plasmid at the *Bbs*I sites, the guide sequences should have CACC and CAAA overhangs (in 5'). The CACC overhang should preferably be followed by a G for increased expression under the U6 promoter (it can either be present in the gRNA target sequence or be added before the target sequence in the gRNA insert). The Cas9 recognition sequence is also shown (SEQ ID NO:134 (DNA) and SEQ ID NO: 135 (RNA)) (B) Schematic representation of the APP gene fragment of HEK 293T cells amplified by PCR and used in the Surveyor™ Enzyme Test to detect micro-insertions or micro-deletion (INDELs) induced by the expression of gRNAs and Cas9 enzyme. Primers Bfw (SEQ ID NO: 43) and Brev (SEQ ID NO: 40) (see Table 3 for specific sequence) were used. (C) Results of Surveyor™ Enzyme Test for fourteen (14) different gRNAs (see Table 4 for specific sequences). Cells were either not-transfected (C) or transfected with a eGFP plasmid, or with the pX330™ plasmid coding for a humanized Cas9 derived from S. pyogenes and one of the fourteen 14 different gRNAs (1 to 14). The DNA was collected from the cells 72 hours post transfection and part of the APP gene (including exon 16) was amplified by PCR with primers Bfw and Brev. The PCR amplicon was then digested with the Surveyor™ enzyme, which cuts miss-matched DNA strands indicating that mutations were introduced in the APP gene;

[0020] Figure 7 shows the pMiniT-Patch plasmid construct encoding a donor DNA used to modify the APP gene by homologous recombination. (A) Schematic representation of the pMiniT-Patch plasmid. (B) Nucleic acid sequence of the donor plasmid MiniT-Patch (SEQ ID NO: 45). The donor DNA patch is (SEQ ID NO: 46), located between the two gRNA#8 sequences. The nucleotide sequence of exon 16 is shown in italics. Primer sequences (MiniTfw (SEQ ID NO: 51); Bfw (SEQ ID NO: 43); IRMUfw (SEQ ID NO: 49); ILMUrev (SEQ ID NO: 50); MiniT rev (SEQ ID NO: 52)) and gRNA#8 target sequence +PAM (SEQ ID NO: 16) are also identified. Mutated nucleotides in Exon 16 are in lowercase. Two of these mutations change the alanine at position 673 to a threonine, which has been previously shown to reduce the production of toxic Aβ peptides and to protect against Alzheimer's disease. Other nucleotides have been changed (i.e., mutations introduced) but without changing the resulting amino acid (i.e., silent mutation). These changes have been made to prevent recognition of the corrected APP gene by the gRNA and thus, recuting of the modified APP gene;

[0021] Figure 8 shows how the APP gene is corrected by the pMini-Patch plasmid using Homologous Directed

Repair (HDR). (A) Schematic representation of the correction of the APP gene by Homologous Directed Repair. (B) Schematic representation of the primers used to amplify the mutated APP gene. (C) Partial sequence (SEQ ID NO: 47) of the mutated (corrected) APP gene comprising a A673T substitution. The nucleic acid sequence includes a partial sequence of intron 15, followed by the complete sequence of exon 16 (nucleotides 278148-278248 in italic, SEQ ID NO: 48, corrected Exon 16) and a partial sequence of intron 16. Various primer positions are shown. The mutated oligonucleotides are in bold. A new Spe1 site was introduced at position 278275-278280. The sequences of forward primers Cfw (SEQ ID NO: 38), Bfw (SEQ ID NO: 43) and IRMUfw (SEQ ID NO: 49) are underlined. The sequences of primers Afw (SEQ ID NO: 39), Brev (SEQ ID NO: 40), Arev (SEQ ID NO: 41) and Crev (SEQ ID NO: 42) are highlighted and bold;

[0022] Figure 9 shows that the pMiniT-Patch plasmid when expressed with the gRNA#8 and Cas9 nuclease can produce either micro-insertions or micro-deletions (INDELs) or Homologous Directed Repair (HDR) in the APP gene. (A) Schematic representation of PCR amplification used to verify insertion of the pMiniT-Patch sequence. (B) Surveyor™ enzyme test of the PCR product obtained by amplification of the APP gene with primers Afw and Arev in human cells (293T) transfected with the following plasmids. Lane 1: 5µg of pMiniT-patch plasmid alone in cells grown in a 6 well plate. There is no additional band in this negative control because no Cas9 nuclease was introduced in the cells and thus no mutations in the APP gene were present. In lanes 2-5, cells were transfected with decreasing amounts of pX330™ (coding for gRNA#8 and Cas9). Lane 2: 5 µg of pX330™, Lane 3: 4 µg of pX330™ and 1 µg of mock/carrier DNA (pEGFP), Lane 4: 2.5 µg of pX330™ and 2.5 µg of carrier/mock DNA and Lane 5: 1 µg of pX330 and 4 µg of carrier/mock DNA. As seen in lanes 2-5, the Surveyor™ enzyme produced additional bands because the gRNA#8 and the Cas9 induced cuts in the APP gene that were repaired by Non Homologous End Joining (NHEJ). In lanes 6, 7, 8 various amount of pMiniT-Patch and pX330™ plasmids were transfected in human cells. Lane 6: Four (4) µg of pMiniT-Patch and 1 µg of pX330™. Lane 7: 2.5 µg pMiniT-Patch and 2.5 µg of pX330™. Lane 8: 1 µg of pMiniT-Patch and 4 µg of pX330™. The presence of additional bands on the gel is due to the cut introduced in the APP gene by the expressed gRNA#8 and Cas9 nuclease. This cut is repaired endogenously either by NHEJ or by Homologous Directed Repair (HDR) with the pMiniT-patch. Lane 9: negative control corresponding to untransfected cells;

[0023] Figure 10 shows that the pMiniT-Patch sequence is cut by the gRNA#8 and Cas9. (A) Schematic representation of the pMiniT-Patch plasmid showing the position of the primers along the pMiniT-Patch nucleic acid sequence. (B) Human cells (HEK 293T) were transfected with various amounts of pX330™ alone or in combination with the pMiniT-Patch plasmid. Lane 1: Lane 1: 5 µg of pMiniT-patch plasmid alone. Lane 2: 5 µg of pX330™. Lane 6: 4 µg of pMiniT-Patch and 1 µg of pX330™. Lane 7: 2.5 µg pMiniT-Patch and 2.5 µg of pX330™. Lane 8: 1 µg of pMiniT-Patch and 4 µg of pX330™. The left side of panel B corresponds to Surveyor™ enzyme test following amplification of the pMiniT-Patch plasmid present in the transfected cells using one primer (MiniTfw) hybridizing with

a backbone of the pMiniT-Patch plasmid and one primer (Brev) hybridizing with a sequence in the APP sequence present in the pMiniT-Patch plasmid. The right side of panel B shows the Surveyor™ enzyme test following amplification of the pMiniT-Patch plasmid present in the transfected cells using one primer (MiniTrev) hybridizing with a backbone of the pMiniT-Patch plasmid and one primer (Bfw) hybridizing with a sequence in the APP patch present in the pMiniT-Patch plasmid. The cuts induced by the Surveyor™ enzyme are due to double stranded breaks produced by the presence of gRNA#8 and Cas9 in the gRNA#8 target sequences, which is present at each end of the APP patch in the pMini-Patch plasmid. These cuts were repaired by NHEJ resulting in a mutated plasmid and thus the amplicons contained mutations, which were cut by the Surveyor™ enzyme. These results confirm that the pMiniT-Patch plasmid was cut by the CRISPR system (gRNA and Cas9) inside the cells and thus that the APP patch was linearized and liberated from the pMiniT plasmid backbone;

[0024] Figure 11 shows the PCR amplification with primers IRMUfw (SEQ ID NO: 49) and Arev (SEQ ID NO: 41) of the mutated APP gene in human cells. The mutation was introduced by Homologous Recombination between the pMiniT-Patch plasmid and the endogenous APP gene following cell transfection with the pMiniT-Patch and pX330™ plasmids (encoding gRNA#8 and Cas9 nuclease) as described in Example 3. (A) Schematic representation of the primer positions on the targeted mutated APP gene. Primer IRMUfw (SEQ ID NO: 49) binds specifically to the mutated sequence of APP exon 16 present in the donor plasmid (pMiniT-Patch) and in the mutated genomic DNA. Primer Arev (SEQ ID NO: 41) hybridizes with a sequence in the APP gene in 5' of the sequence that is present in the pMiniT-Patch. Amplification is possible only if the APP gene has been modified by Homology Directed Repair with the patch present in the pMiniT-Patch plasmid. (B) RedSafe™ agarose gel showing amplified product (608 bp). Lane 1: negative control: cells in a 6 well plate transfected only with the pMiniT-patch plasmid. Lane 6: cells transfected with 4 μg of pMiniT-Patch and 1 μg of pX330™. Lane 7: cells transfected with 2.5 μg pMiniT-Patch and 2.5 μg of pX330™. Lane 8: cells transfected with 1 μg of pMiniT-Patch and 4 μg of pX330™;

[0025] Figure 12 shows the PCR amplification with primers Cfw (SEQ ID NO: 38) and ILMUrev (SEQ ID NO: 50) of the mutated APP gene in human cells. The mutation was introduced by Homologous Recombination between the patch sequence (including the A673T APP sequence) and the endogenous APP gene following cell transfection with the pMiniT-Patch and pX330™ plasmids (encoding gRNA#8 and Cas9 nuclease) as described in Example 3. A. Schematic representation of the primer positions on the targeted mutated APP gene. The Cfw primer targets a sequence, which is present in the endogenous APP gene but not in the repair pMiniT-Patch plasmid. The ILMUrev primer hybridizes with a sequence, which is present only when the APP gene has been repaired by homologous recombination and the patch sequence introduced in the APP gene. The sequence targeted by the ILMUrev sequence includes a portion in the patch/repair sequence (from the MiniT-Patch plasmid) and a sequence in the APP gene, which is present only if the gene has been repaired by HR. (B) RedSafe™ agarose gel showing PCR- amplified product (947 bp). Lane 1: negative control: cells in a 6 well plate transfected only with 5μg of the pMiniT-patch

plasmid. Lane 2: 293T cells in a 6 well plate transfected with 4 µg of pX330™ (coding for gRNA#8 and Cas9) and with 1 µg of pMiniT-Patch;

[0026] Figure 13 shows the PCR amplification with primers IRMUfw and Crev of the mutated APP gene in human cells. The mutation was introduced by Homologous Recombination between the pMiniT-Patch plasmid and the endogenous APP gene following cell transfection with the pMiniT-Patch and pX330™ plasmids (encoding gRNA#8 and Cas9 nuclease) as described in Example 3. (A) Schematic representation of the primer positions on the targeted mutated APP gene. Primer Crev (SEQ ID NO: 42) targets a sequence, which is present only in the endogenous APP gene but not in the pMiniT-Patch repair plasmid. The IRMUfw primer (SEQ ID NO: 49) hybridizes with a sequence in the repair plasmid and with a sequence in the APP gene only if the gene has been repaired by homologous recombination. (B) RedSafe™ agarose gel showing PCR amplified product (1073 bp). Lane 1: negative control: cells in a 6 well plate transfected only with 5 µg of the pMiniT-patch plasmid shows a non-specific PCR fragment (~450 pb). Lane 2: 293T cells transfected with 4 µg of pX330™ (coding for gRNA#8 and Cas9) and with 1 µg of pMiniT-Patch;

[0027] Figure 14 shows a nucleic acid sequence comparison between the amplicon in lane #8 (L8, (SEQ ID NO: 53)) of Figure 11 and the wild type (WT) APP gene sequence (SEQ ID NO: 54). The IRMUfw (5' forward, (SEQ ID NO: 49)) and the Arev (3' reverse, (SEQ ID NO: 41)) primer sequences are underlined. The additional Spe1 site introduced in the APP gene with the pMiniT-Patch plasmid is in bold and underlined and is indicated at position 161 of the amplicon;

[0028] Figure 15 shows a nucleic acid sequence comparison between a portion of WT APP (SEQ ID NO: 55) and an amplicon containing the mutated APP gene following the modification with gRNA#8 and the MiniT-Patch nucleic acid (SEQ ID NO: 56). The sequences of the Cfw (5' forward, (SEQ ID NO: 42)) and ILMUrev (3' reverse, (SEQ ID NO: 50)) primers are underlined. Since the Cfw primer hybridizes with a sequence, which is present in the genome but not in the pMiniT-Patch, and the ILMUrev primer hybridizes with a sequence, which is present in the pMiniT-Patch but not in the wild type genome, DNA amplification with these primers demonstrates that the genomic DNA has been modified by Homology Directed Repair with the pMiniT-Patch;

[0029] Figure 16 shows a nucleic acid sequence comparison between the wild type (WT) APP nucleic acid sequence (SEQ ID NO: 57) and that of a bacterial clone (D, (SEQ ID NO: 58)) containing the mutated APP gene following modification with gRNA#8/Cas9 and the MiniT-Patch sequence. The sequences of the IRMUfw (5' forward, (SEQ ID NO: 49)) and Crev (3' reverse, (SEQ ID NO: 42)) primers are underlined. Since the Crev primer anneals with a sequence, which is present in the genome but not in the pMiniT-Patch, and the ILMUfw anneals with a sequence, which is in the pMiniT-Patch but not in the wild type genome, these primers can be used to demonstrate that the genomic DNA has been modified by Homology Directed Repair with the pMiniT-Patch sequence. Homologous

recombination between the APP gene and the pMiniT-Patch introduces an additional Spe1 endonuclease restriction site (ACTAGT, in bold and underlined) at position 85 of the amplicon obtained with clone D. This site is used to confirm the presence of the mutation and proper integration;

[0030] Figure 17 shows that the endogenous APP gene can be modified by various donor/Patch nucleic acid sequences. (A) Nucleic acid sequence of the single stranded oligodeoxynucleotide (ssODN) used to modify the APP gene as described in Example 5 (SEQ ID NO: 59). Underlined nucleotides correspond to those, which are modified in the wild-type APP gene to introduce the A673T mutation, to prevent the binding of the gRNA#8 to the mutated gene and to insert a new Spe1 site in intron 16. The portion of the sequence in lowercase corresponds to the coding sequence of exon 16 in the APP gene. (B) Correction of the APP gene with the single stranded oligonucleotide described in (A) or with a PCR product (amplicon synthesized with primers Bfw and Brev (SEQ ID NO: 60), see Example 5), with a FLAG or FLAG-STOP patch/donor sequence. The cell DNA was amplified 72 hours post transfection with primers Cfw (SEQ ID NO: 38) and ILMUrev (SEQ ID NO: 42). The presence of a PCR product confirmed that the APP gene had been modified by homologous recombination. The 293T cells in a 24 well plate were transfected with the following plasmids/nucleic acids. Lane 1: 400 ng of pLenti-eGFP and 400 ng of pX330™ (i.e., gRNA#8 and Cas9); Lane 2: APP amplicon (150 ng) of part of pMiniT-Patch and pX330™; Lane 3: 2.5 µg of single stranded oligonucleotide and 400 ng of pX330™; Lane 4: 5 µg of single stranded oligonucleotide and 400 ng of pX330™; Lane 5: 400 ng of pMiniT-Patch-Flag and 400 ng of pX330™; Lane 6: 400 ng of pMiniT-Patch-Flag-Stop and 400 ng of pX330; Lane 7: no transfection (negative control); Lane 8: 400 ng of pMiniT-Patch-Flag (no pX330™);

[0031] Figure 18 shows the nucleic acid sequence of the pMiniT-Patch-FLAG-STOP donor sequence (SEQ ID NO: 61). The portion of the nucleic acid sequence coding for exon 16 of the APP gene is shown in lower case and the portion of the nucleic acid sequence coding for the FLAG tag (SEQ ID NO: 62) (DYKDHDGDYKDHDIDYKDDDDK, SEQ ID NO: 63) is shown in bold. There are two stop codons after the FLAG (sequence underlined) in this version of the patch sequence;

[0032] Figure 19 shows the nucleic acid sequence of the pMiniT-Patch-FLAG donor sequence (SEQ ID NO: 64). The portion of the nucleic acid sequence coding for exon 16 of the APP gene is shown in lower case and the portion of the nucleic acid sequence coding for the FLAG tag (SEQ ID NO: 62) (DYKDHDGDYKDHDIDYKDDDDK, SEQ ID NO: 63) is shown in bold;

[0033] Figure 20 shows that the APP gene in human cells has been modified to introduce a FLAG or FLAG Stop sequence in exon 16 of the endogenous APP gene. 293T cells were transfected as follows: Lane 1: 400 ng of pX330™ (containing the gRNA#8 and the Cas9 gene) and 400 ng of pMiniT-Patch-FLAG, Lane 2: 400 ng of pX330™ and 400 ng of pMiniT-Patch-FLAG-STOP, Lane 3: only 400 ng of pX330™ and Lane 4: only 400 ng of pMiniT-Patch-FLAG. The genomic DNA was extracted 72 hours post transfection and the APP nucleic acid sequence was amplified

with primers Cfw and Crev. The 1993 bp PCR product was digested with Spe1 and migrated on a 2% agarose gel. Two additional bands (1002 and 991 bp) were detected in Lanes 1 and 2 confirming that the APP gene had been mutated by HDR resulting in the insertion of the new Spe1 site, which is present in the donor DNA (pMiniT-Patch-FLAG or pMiniT-Patch-FLAG-STOP) and is thus introduced in intron 16 of the endogenous APP nucleotide sequence following HDR;

[0034] Figure 21 shows the nucleic acid sequence of pMiniT-Patch plasmid with wild type APP gene insert and gBlock with APP gene mutations. (A) Wild-type APP insert (SEQ ID NO: 65, 1180 bp APP intron 15/exon 16/ intron 16) in the pMiniT plasmid. The mutations to be introduced are shown in lowercases. Primer sequences are also identified (underlined: gBlockfw (SEQ ID NO 69); gBlockrev (SEQ ID NO: 70), Bfw, and Brev are also shown) **(**B) gBlock of 441 bp (SEQ ID NO: 66) including 9 mutations in APP gene. The mutations are in lowercase, bold and underlined. The sequence shown in lowercase corresponds to the sequence coding for exon 16 of the APP gene (C) Patch repair final sequence of 1224 bp in the pMiniT plasmid (SEQ ID NO: 67) including two gRNAs target sites (in italics). The sequence of Exon 16, as corrected, (SEQ ID NO: 68) is shown in lower case. Primer sequences are underlined;

[0035] Figure 22 shows the nucleic acid sequence of the gBlock for 3XFLAG and 3XFLAG-STOP donor/patch sequence. (A) gBlock 3XFLAG: 507 pb (SEQ ID NO: 71). (B) gBlock 3XFLAG-STOP: 513 pb (SEQ ID NO: 72). (C) Sequencing result of insert of plasmid pMiniT-Patch-3XFLAG (SEQ ID NO: 73), gRNAs sequences are in bold and primer targeting sequences (Bfw and Brev) are underlined; and (D) Sequencing result of insert of plasmid pMiniT-Patch-3XFLAG-STOP(SEQ ID NO: 74), gRNAs sequences are in bold and primer targeting sequences are underlined;

[0036] Figure 23 shows the nucleic acid sequence of the pX330-U6-Chimeric_BB-CBh-hSpCas9 vector sequence used in accordance with an embodiment of the present invention (gift from Feng Zhang, plasmid #42230 from Addgene). (A) Vector sequence of pX330™ (SEQ ID NO: 75). The BbsI cloning sites for cloning the gRNA target sequence are shown (GTCTTCgaGAAGAC, SEQ ID NO: 76). Expression of the gRNA is under the control of the U6 promoter, therefore a "G" is preferably present in 5' of the target sequence (or it can be added to the insert) for optimal expression of the gRNA. The humanized Cas9 (hSpCas9) nuclease expressed by this vector comprises a 3X Flag tag (in N-terminal, for purification or for verifying expression by western blot) and a nuclear localization signal (SV40 NLS) for proper nuclear targeting of the nuclease. The humanized Cas9 is derived from *S. pyogenes* and is under the control of the CBh promoter. The sequence coding for the 3X FLAG TAG is shaded and in bold (SEQ ID NO: 77). The NLS (CCAAAGAAGAAGCGGAAGGTC, SEQ ID NO: 78) is shaded and underlined and the portion coding for the hSpCas9 is underlined (SEQ ID NO: 79). (B) Amino acid sequence of the hSpCas9 nuclease expressed by the pX330™ vector (SEQ ID NO: 80; SEQ ID NO:114 (haspCas9 without NLS and TAG), the NLS is in bold and the TAG is underlined and bold;

[0037] Figure 24 shows sequences from the pX458™ plasmid (SEQ ID NO: 81) used in an embodiment of the present invention. The pX458™ plasmid (gift from Feng Zhang, Addgene plasmid # 48138) expresses a humanized form of *S pyogenes* Cas9 nuclease (SEQ ID NOs: 79 and 80) fused to GFP (SpCas9-2A-GFP) under the Cbh promoter. The hSpCas9 is fused to a 3X FLAG (SEQ ID NO: 77) at its N-terminal end and comprises a nuclear localization signal (SV40 NLS, SEQ ID NO: 78) for proper nuclear targeting of the nuclease. The vector includes a cloning backbone for gRNA expression under the U6 promoter (https://www.addgene.org/48138/) as in the pX330™ plasmid described in Figure 23;

[0038] Figure 25 shows a sequence Comparison between the wild type APP (SEQ ID NO: 82) sequence and the sequence of the mutated clone (SEQ ID NO:83) described in Example 7;

[0039] Figure 26 shows that the APP protein of cells (in which both alleles have been modified to incorporate the A673T protective mutation by the combination of CRISPR and homologous recombination) is less frequently cut by the β-Secretase enzyme than the wild type APP protein. (A) Normal 293T cells and a clone of 293T cells having both APP alleles containing the A673T mutation were used for the experiment. The C99 fragment was abundantly present in all conditions in the normal cells (lanes 1 to 4 of the figure). However, the C99 fragment was not detected in the mutated cells with (lane 6) or without (lane 5) the γ-Secretase inhibitor. Even in the presence of the β-Secretase plasmid, the C99 fragment was not detected (lane 8). However the C99 fragment was detected in the mutated cells in presence of both the β-Secretase plasmid and of the γ-Secretase inhibitor, which permitted an accumulation of the C99 fragment (lane 7). (B) Schematic representation of the processing pathway of APP by the beta and gamma secretases.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

[0040] The present invention shows that it is possible to introduce one or more modifications into a target gene involved in Aβ peptide production by gene editing using one or more site-specific endonucleases and one or more donor or patch nucleic acids. The modifications introduced are specifically designed to decrease the production of Aβ peptide (e.g., by reducing APP expression and/or APP processing).

[0041] Applicants show herein that the endogenous APP polynucleotide gene sequence within a cell may be efficiently genetically modified to introduce one or more modifications designed to decrease Aβ-peptide production. The modifications are introduced by providing the cell with a donor nucleic acid (patch sequence) and a site-specific endonuclease (e.g., Cas 9) specifically targeting and cutting the endogenous APP polynucleotide gene sequence. The donor nucleic acid has a nucleic acid sequence, which is highly similar to the endogenous APP polynucleotide gene sequence but which comprises one or more modifications, which, once introduced into the endogenous APP polynucleotide gene sequence in the cell will reduce Aβ peptide production.

[0042] Following the cut(s) (e.g., a double stranded break (DSB) or two single strand breaks (SSB)) introduced by the site-specific endonuclease/nickase into the APP DNA, the donor or patch sequence is integrated (e.g., it replaces the corresponding endogenous APP polynucleotide sequence by homologous recombination) in the endogenous APP polynucleotide gene sequence, thereby modifying it.

[0043] Using such methods, modifications into the target gene can be made to correct an endogenous mutation associated with increased risk of Aβ-peptide production or accumulation or to introduce one or more modifications, which decrease APP expression or processing into Aβ-peptide.

[0044] For example, mutations in the APP gene associated with familial forms of Alzheimer's disease may be corrected in accordance with the present invention by targeting the endogenous APP gene with a donor or patch sequence encoding the wild-type APP sequence or a portion thereof. In another aspect of the present invention, the APP gene may be modified to introduce a protective mutation into the APP gene associated with decreased levels of Aβ-peptide production or accumulation (a A673T substitution in the APP protein sequence).

[0045] As used herein, the terms "APP gene", "APP nucleic acid" and "APP polynucleotide sequence" are used interchangeably and refer to the nucleic acid sequence encoding the Amyloid Precursor Protein (Entrez 351; Ensembl ENSG00000142192; UniProt P05067; RefSeq mRNA NM_000484; RefSeq (protein) NP_000475). A wild-type APP nucleic acid is a nucleic acid, which has the nucleotide sequence of the APP gene naturally found in subjects and which does not comprise mutations (in the coding region of the APP protein or elsewhere in the APP gene), which are associated with an increased risk of developing Alzheimer's disease. A wild type APP nucleic acid thus includes allelic variants not associated with familial forms of Alzheimer's disease and encodes the wild type APP protein (e.g., NM_000484.3; Uniprot P05067, and Figure 3 (SEQ ID NO: 30)). In a particular embodiment, the wild type APP nucleic acid sequence has the sequence of NCBI Reference Sequence: NG_007376.1. An endogenous APP polynucleotide gene sequence in a cell refers to the unmodified APP nucleic acid sequence found in such cell.

[0046] Many mutations causing Alzheimer's disease have been reported in the APP gene and these may be corrected in accordance with the present invention. In a particular aspect of the present invention, the correction involves the replacement of the mutated nucleotide(s) with nucleotide(s) normally found in the APP gene. In another aspect, the correction involves the replacement of the mutated nucleotides with nucleotides encoding the wild type amino acids of the APP protein. In such a case, the replacement nucleotides may be the same as those found in the wild type APP nucleic acid sequence or may be different (e.g., due to codon degeneracy), as long as the corrected sequence encodes the wild type APP protein. Mutations in the exons encoding the APP protein, in the promoter or in any other regulatory sequence in the APP gene modulating the expression of the APP protein may be targeted in accordance with the present invention. For example mutations in any of the 18 exons of the APP gene may be targeted and corrected in accordance with the present invention (i.e., mutations in exon 1, exon 2, exon 3, exon 4,

exon 5, exon 6, exon 7, exon 8, exon 9, exon 10, exon 11, exon 12, exon 13, exon 14, exon 15, exon 16, exon 17 and exon 18). Modifications introduced in the portion of the polynucleotide gene sequence encoding the APP protein (e.g., exon sequences) will be reflected in the APP protein expressed by the cell (i.e., the cell will normally express a modified/corrected APP protein).

[0047] Non-limiting examples of mutations in the APP protein that may be corrected include: KM670/671NL (12), Ala673Val (13), His677Arg (14), Asp678Asn (15), Asp678His (16), Glu682Lys (17), Ala692Gly (18), Glu693Gln (19), Glu693Gly (20), Glu693del (21), Asp694Asn (22), Leu705Val (23), Ala713Thr (24), Ala713Thr (25), Ala713Val (26), Thr714Ala (27), Thr714Ile (28), Val715Met (29), Ile716Val (30), Ile716Phe (31), Val717Ile (32), Val717Leu (33), Val717Phe (34), Val717Gly (35), Leu723Pro (36), Lys724Asn (37) and His733Pro (38). Thus, possible modifications include amino acid at position 670, 671, 673, 677, 678, 682, 692, 693, 694, 705, 713, 714, 715, 716, 717, 723, 724, and/or 733. The amino acid numbering is with respect to the APP amino acid sequence shown in Figure 3 (SEQ ID NO: 30). Other modifications also exist. All mutations located between amino acids 656 and 688 are located in exon 16 (SEQ ID NOs: 31 and 34) of the APP gene. In an embodiment, mutations at amino acid positions 670 (lysine in WT APP), 671 (methionine in WT APP), 673 (alanine in WT APP), 677 (histidine in WT APP), 678 (aspartic acid in WT APP) and/or 682 (glutamic acid in WT APP) may be corrected in accordance with the present invention by replacing exon 16 or a part thereof in the endogenous APP polynucleotide gene sequence. Exon 16 has been shown to be efficiently targeted and replaced using an embodiment of the method of the present invention.

[0048] Other mutations associated with Alzheimer's disease, such as those found in the Presenilin 1 (PS1) and Presenilin 2 (PS2) genes may also be corrected using the method of the present invention.

[0049] In addition to correcting mutations associated with increased risk of developing Alzheimer's disease (e.g., mutations associated with familial forms of AD), it is also possible to introduce modifications in the APP gene, which are known to protect against Alzheimer's disease. One such modification is the replacement of the alanine at position 673 of the APP protein sequence with a threonine (A673T substitution in exon 16). The presence of the A673T variant in subjects has been found to reduce the risk of developing Alzheimer's disease and to protect against age-related cognitive decline. The A673T substitution is adjacent to the beta secretase cleavage site in APP and results in a 40% reduction in the formation of toxic amyloid beta peptides.

[0050] Introduction of the A673T substitution in accordance with the present invention may be made alone in the APP gene, or in combination with one or more other modifications aiming, for example, at correcting endogenous mutations associated with an increased risk of developing AD or age-related cognitive decline. For example, mutations associated with increased risk of developing AD present in exon 16 may advantageously be corrected at the same time as the A673T protective substitution is introduced in the APP gene since all targeted modifications are present in the same region of the APP gene. Of course mutations located elsewhere (i.e., in exons other than exon

16) may also be concurrently corrected but the correction will involve the replacement of longer stretch of sequences or of multiple smaller targeted regions in the APP gene.

[0051] Modifications in the APP gene or other genes associated with AD may be made in cells (neurons or glial cells) of a subject in need thereof. As used herein, "a subject in need thereof" is a subject, which may benefit from a decreased production of Aβ peptides. Non-limiting examples of a subject in need thereof include a subject having cells showing an increased level of Aβ peptide production (APP expression and/or APP maturation) or activity as compared to cells from a normal subject. In an embodiment, the subject in need thereof is a healthy subject (e.g., a subject at risk of developing AD or age-related cognitive decline) or a subject already diagnosed with AD or age-related cognitive decline. As used herein, a subject at risk of developing AD or age-related cognitive decline, is a subject that has not yet been diagnosed with the disease or condition but which, due to certain factors (age, familial history, heredity) is likely to develop de disease or condition later on in his/her life. In an embodiment, the subject at risk is a subject having a mutation in the APP, PS-1 and/or PS-2 gene(s). In another embodiment, the subject at risk is a subject having a mutation in the APOE-e4 gene. In another embodiment, the subject at risk is a subject having at least one family member (e.g., a mother, father, brother, sister or child) diagnosed with Alzheimer's disease or age-related cognitive decline. In an embodiment, the subject at risk is a subject having a mutation associated with early onset Alzheimer's disease or familial Alzheimer's disease (FAD). In an embodiment, the subject is a mammal, preferably, a human.

[0052] The correction or modification may be made in a single allele of a targeted gene within a cell but is preferably made in both alleles of the gene, when necessary.

[0053] Modifications in genes associated with AD (e.g., APP, PS-1 and PS-2) in accordance with the present invention can be used to prevent or treat Alzheimer's disease or age-related cognitive decline. As used herein, the term "prevention/preventing/prevent" means that the modification(s) avoid(s) or delay(s) the onset of the disease. As used herein, the term "treat/treating/treatment" includes instances where the genetic modification(s) reduce(s) partially or completely the progression of the disease and instances where symptoms associated with the disease are reduced partially or completely (i.e., one or more symptoms associated with Aβ peptide neurotoxicity).

[0054] Preferably, the one or more modifications in a targeted gene (e.g., APP) in cells (e.g., neurons) of a subject are introduced as early as possible after the identification of a risk of developing AD or soon after AD diagnosis. In a particular embodiment, the one or more genetic modifications in cells are made after the detection by Magnetic Resonnance Imaging (MRI) of plaques comprising extracellular deposits of amyloid β (Aβ) peptides in the subject's brain.

[0055] Methods of introducing one or more genetic modifications in a targeted gene in accordance with the

present invention preferably involve Homologous Recombination (HR). The proposed treatment requires the introduction of a double strand break (DSB) in the targeted gene (e.g., APP) using specifically designed endonucleases or nickases. The introduction of a DSB is then used together with a donor (patch) sequence to modify the targeted gene to, for example, reduce the formation of toxic Aβ peptides (e.g., by correcting one or more endogenous mutations in a targeted gene, which is/are associated with increased risk to develop AD and/or by introducing a protective modification in the targeted gene, which reduces Aβ plaque formation on neurons).

[0056] As used herein, "donor" or "patch" nucleic acid are used interchangeably and refers to a nucleic acid that corresponds to a fragment of the endogenous targeted gene of a cell (in some embodiments the entire targeted gene), but which includes the desired modifications at specific nucleotides (e.g., the wild type sequence of the targeted gene (e.g., wild type APP gene) in the case of the correction of an endogenous mutation associated with AD or a modified sequence including a protective mutation to be introduced in the targeted gene). The donor (patch) nucleic acid must be of sufficient size and similarity to permit homologous recombination with the targeted gene. Preferably, the donor/patch nucleic acid is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% and 99% identical to the endogenous targeted polynucleotide gene sequence. The patch nucleic acid may be provided for example as a ssODN, as a PCR product (amplicon) or within a vector. Preferably, the patch/donor nucleic acid will include modifications with respect to the endogenous gene which i) precludes it from being cut by a gRNA once integrated in the genome of a cell and/or which facilitate the detection of the introduction of the patch nucleic acid by homologous recombination.

[0057] As used herein, a "targeted gene" or "targeted polynucleotide gene sequence" corresponds to the polynucleotide within a cell that will be modified by the introduction of the patch nucleic acid. It corresponds to an endogenous gene naturally present within a cell. The targeted gene may comprise one or more mutations associated with a risk of developing AD or age-related cognitive decline which will be corrected by the introduction of the patch nucleic acid (i.e., will be modified to correspond to the WT gene or to a form which is no longer associated with increased risk of developing AD or age-related cognitive decline). One or both alleles of a targeted gene may be corrected within a cell in accordance with the present invention.

[0058] Although genetic modifications in a targeted gene are preferably introduced by HR, DSBs in cell DNA may also be spontaneously repaired by Non Homologous End Joining (NHEJ) leading to the presence micro-insertions or micro-deletions (INDELs) in the targeted gene. Although these forms of genetic modifications are not preferred they can nevertheless be useful to prevent the formation of the Aβ peptide (e.g., by for example reducing completely or partially the level of APP synthesized in cells or by preventing a cut of the APP protein by the β-secretase).

[0059] Various types of endonucleases or nickases may be used to induce a DSB at selected site(s) in a

targeted gene (e.g., APP, PS-1, PS-2, etc.). Non-limiting examples of useful endonucleases and nickases include meganucleases, Zinc Finger Nucleases (ZFNs), Transcription Activator-Like Effector nucleases (TALENs) or the Cas nucleases or Cas nickases used in combination with at least one (e.g., one or two) guide RNA(s) (gRNA) in the Clustered regularly interspaced short palindrome repeat (CRISPR) system (39-41). Each of these technologies can be used to modify a targeted gene in accordance with the present invention.

[0060] Preferably, the present invention uses the CRISPR system (i.e., combination of gRNA and Cas nuclease or nickase) together with a donor (patch) sequence to introduce one or more genetic modifications in a targeted gene (e.g., APP). Applicants demonstrate herein that specific gRNAs can be produced and used with a Cas9 nuclease and patch sequence to efficiently modify the endogenous APP gene in human cells.

[0061] THE CRISPR SYSTEM

[0062] Recent discoveries in the field of bacterial immunity have led to the development of a new system for controlling gene expression in cells. Bacterial and archaea have developed adaptive immune defenses termed clustered regularly interspaced short palindromic repeats (CRISPR) systems, which use crRNAs and Cas proteins to degrade complementary sequences present in invading viral and plasmid DNA (42). Jinek et al. (43) and Mali et al. (42) have engineered a type II bacterial CRISPR system using custom guide RNA (gRNA) to induce double strand break(s) in DNA. Because the original Cas9 nuclease combined with a gRNA may produce off-target mutagenesis, one may alternatively use in accordance with the present invention a pair of specifically designed gRNAs in combination with a Cas9 nickase (44) or in combination with a dCas9-FokI nuclease (45) to cut both strands of DNA.

[0063] Applicant shows herein for the first time that the CRISPR system can be used in combination with a donor (patch) nucleic acid to efficiently modify APP in cells. The introduction of modifications preferably occurs by homologous recombination although Non Homologous End Joining repair may also occur in the presence of a gRNA and Cas9 nuclease or nickase. Various gRNAs targeting the human APP gene were designed and shown to induce Cas9-dependent DSBs in human cell DNA and to enable the efficient targeted modification of the APP gene. Accordingly, a protective modification in the APP gene (i.e., the A673T mutation in Exon 16 of the APP gene) was introduced. This modification has been shown to significantly reduce Aβ peptide formation in cells.

[0064] Accordingly, in an aspect, methods of the present invention involve the design of one or more gRNAs for inducing a DSB (or two SSB in the case of a nickase) in a targeted gene involved in toxic Aβ peptide formation and deposition. The gRNA(s), targeting a region of interest in the targeted gene, and a Cas nuclease or nickase (e.g., Cas9) are then used in combination with a donor/patch nucleic acid to introduce the desired modification(s) in the endogenous polynucleotide gene sequence within the cell by homologous recombination. The present invention further relates to uses of such targeted genetic modification(s), such as for reducing Aβ peptide formation in cells from

a subject in need thereof, such as for the treatment of Alzheimer's disease and/or age-related cognitive decline.

[0065] In order to cut DNA at a specific site, Cas9 proteins require the presence of a gRNA and a protospacer adjacent motif (PAM), which immediately follows the gRNA target sequence in the targeted polynucleotide gene sequence (46). The PAM is located at the 3' end of the gRNA target sequence but is not part of the gRNA. Different Cas proteins require a different PAM. Accordingly, selection of a specific polynucleotide gRNA target sequence (e.g., on the APP nucleic acid sequence) by a gRNA is generally based on the recombinant Cas protein used. The PAM for the *S. pyogenes* Cas9 CRISPR system is 5'-NRG-3', where R is either A or G, and characterizes the specificity of this system in human cells. The PAM of S. aureus is NNGRR (65). The *Streptococcus pyogenes* Type II system naturally prefers to use an "NGG" sequence, where "N" can be any nucleotide, but also accepts other PAM sequences, such as "NAG" in engineered systems. Similarly, the Cas9 derived from *Neisseria meningitidis* (NmCas9) normally has a native PAM of NNNNGATT, but has activity across a variety of PAMs, including a highly degenerate NNNNGNNN PAM. In a preferred embodiment, the PAM for a Cas9 protein used in accordance with the present invention is a NGG trinucleotide-sequence.

gRNAs

[0066] As used herein, the expression "gRNA" refers to a guide RNA which is a fusion between the gRNA guide sequence (crRNA) and the Cas9 recognition sequence (tracrRNA). It provides both targeting specificity and scaffolding/binding ability for Cas9 nuclease or nickase. gRNAs of the present invention do not exist in nature, i.e., is a non naturally-occuring nucleic acid.

[0067] The gRNAs of the present invention generally comprises (or consists of) a "gRNA guide sequence" and a Cas (e.g., Cas9) recognition sequence, which is necessary for Cas (e.g., Cas9) binding to the targeted APP gene.

[0068] As used herein, the expression "gRNA guide sequence" refers to the nucleotides that precede the PAM (i.e., in 5' of the PAM) in the genomic DNA. It corresponds to the protospacer on the target polynucleotide gene sequence. It is what gets put into a gRNA expression plasmid, it does not include the PAM sequence. It is the sequence that confers target specificity. It requires a Cas9 recognition sequence (tracrRNA) to bind to Cas9. The "gRNA guide sequence" is between 15-22 nucleotides, preferably between 18-22 nucleotides and even more preferably 19 nucleotides or 20 nucleotides long. The gRNA guide sequence recognizes and binds to the targeted gene of interest. It hybridizes with (i.e., is complementary to) the opposite strand of a target gene sequence, which comprises the PAM (i.e., it hybridizes with the DNA strand opposite to the PAM).

[0069] A Cas recognition sequence (e.g., Cas9 recognition sequence) refers to the portion of the gRNA that links the gRNA guide sequence (crRNA) to the Cas nuclease. It acts as a guide for the endonuclease or nickase, which will cleave the nucleic acid. In an embodiment, Cas recognition sequence is a Cas9 recognition sequence

having at least 65 nucleotides. In a particular embodiment, the Cas9 recognition sequence comprises the sequence as set forth in SEQ ID NO: 129, 131, 133 or 135 (corresponding DNA sequences for RNA SEQ ID NOs: 131, 133 and 135 SEQ ID NOs are SEQ ID NO:s130, 132 and 134). In an embodiment, Cas recognition sequence is a Cas9 recognition sequence having at least 65 nucleotides In an embodiment, Cas recognition sequence is a Cas9 recognition sequence having at least 85 nucleotides.

[0070] A "target region", "target sequence" or "protospacer" in the context of gRNAs and CRISPR system are used herein interchangeably and refers to the region of the target gene, which is targeted by the CRISPR/dCas9-based system, without the PAM. The CRISPR/Cas9-based system may include at least one gRNA, wherein the gRNAs target different DNA sequences on the target gene. The target DNA sequences may be overlapping. The target sequence or protospacer is followed by a PAM sequence at the 3 ' end of the protospacer. In an embodiment, the target sequence is immediately adjacent to the PAM sequence and is located on the 5' end of the PAM.

[0071] The gRNA comprises a “gRNA guide sequence” or “gRNA target sequence” which corresponds to the target sequence on the target polynucleotide gene sequence that is followed by a PAM sequence. The gRNA may comprise a "G" at the 5' end of the polynucleotide sequence. The presence of a “G” in 5' is preferred when the gRNA is expressed under the control of the U6 promoter. The CRISPR/Cas9 system of the present invention may use gRNA of varying lengths. The gRNA may comprise at least a 10 nts, at least 11 nts, at least a 12 nts, at least a 13 nts, at least a 14 nts, at least a 15 nts, at least a 16 nts, at least a 17 nts, at least a 18 nts, at least a 19 nts, at least a 20 nts, at least a 21 nts, at least a 22 nts, at least a 23 nts, at least a 24 nts, at least a 25 nts, at least a 30 nts, or at least a 35 nts of the target APP DNA sequence which is followed by a PAM sequence. The “gRNA guide sequence” or “gRNA target sequence” may be least 17 nucleotides (17, 18, 19, 20, 21, 22, 23), preferably between 17 and 30 nts long, more preferably between 18-22 nucleotides long. In an embodiment, gRNA guide sequence is between 10-40, 10-30, 12-30, 15-30, 18-30, or 10-22 nucleotides long. The PAM sequence may be "NGG", where "N" can be any nucleotide. gRNA may target any region of the a target gene (e.g., APP) which is immediately upstream (contiguous, adjoining, in 5') to a PAM (e.g., NGG) sequence. In an embodiment, the gRNA may target any region which is followed by a PAM identified on the APP polynucleotide gene sequence set forth in Figure 5 or in Entrez 351; Ensembl ENSG00000142192; or NG_007376.1.

[0072] Although a perfect match between the gRNA guide sequence and the DNA sequence on the targeted gene is preferred, a mismatch between a gRNA guide sequence and target sequence on the gene sequence of interest is also permitted as along as it still allows hybridization of the gRNA with the complementary strand of the gRNA target polynucleotide sequence on the targeted gene. A seed sequence of between 8-12 consecutive nucleotides in the gRNA, which perfectly matches a corresponding portion of the gRNA target sequence is preferred for proper recognition of the target sequence. The remainder of the guide sequence may comprise one or more mismatches. In general, gRNA activity is inversely correlated with the number of mismatches. Preferably, the gRNA

of the present invention comprises 7 mismatches, 6 mismatches, 5 mismatches, 4 mismatches, 3 mismatches, more preferably 2 mismatches, or less, and even more preferably no mismatch, with the corresponding gRNA target gene sequence (less the PAM). Preferably, the gRNA nucleic acid sequence is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% and 99% identical to the gRNA target polynucleotide sequence in the gene of interest (e.g., APP). Of course, the smaller the number of nucleotides in the gRNA guide sequence the smaller the number of mismatches tolerated. The binding affinity is thought to depend on the sum of matching gRNA-DNA combinations.

[0073] Non-limiting examples of gRNAs guide sequences are presented in Table 4 below. Any gRNA guide sequence can be selected in the target gene, as long as it allows introducing at the proper location, the patch/donor sequence of the present invention. Accordingly, the gRNA guide sequence or target sequence of the present invention may be in coding or non-coding regions of the APP gene (i.e., introns or exons). In an embodiment, the gRNA guide sequence is selected in intron 15, exon 16 and/or intron 16 of the APP gene. In an embodiment, the gRNA guide sequence, in combination with the donor/patch sequence allows to replace at least a portion (preferably entirely) exon 16 of an endogenous APP polynucleotide gene sequence within a cell. Figure 5 presents a fragment of the APP polynucleotide gene sequence comprising part of intron 15, exon 16 and part of intron 16 from which gRNA guide sequence may easily be selected in accordance with the present invention. Of course the complementary strand of the sequence shown in Figure 5 may alternatively and equally be used to identify proper PAM and gRNA guide sequence. As shown below, gRNA guide sequence may also be selected within a vector nucleotide sequence to allow the removal of the patch sequence, when such patch/donor sequence is provided in a vector.

[0074] In an embodiment, the target nucleic acid sequence (gRNA guide sequence) of the gRNA of the present invention is located between (i) nucleotide 277141 and nucleotide 279540; (ii) nucleotide 277567 and nucleotide 27845; (iii) nucleotide 277141 and nucleotide 278147; (iv) nucleotide 278149 and nucleotide 278250; (v) nucleotide 278251 and nucleotide 279540; (vi) nucleotide 277277 and nucleotide 278147; (vii) nucleotide 277559 and nucleotide 278147; (vii) nucleotide 277567 and nucleotide 278147; (viii) nucleotide 278149 and nucleotide 278220; (ix) nucleotide 278202 and nucleotide 278250; (x) nucleotide 278721 and nucleotide 279540; (xi) nucleotide 278781 and nucleotide 279540; (xii) nucleotide 279245 and nucleotide 279540 of the APP polynucleotide gene sequence set forth in Figure 5, or the complement thereof, or in a corresponding location in the endogenous APP polynucleotide gene sequence present in said cell.

[0075] In an embodiment, the gRNA guide sequence on the APP polynucleotide gene sequence is not rich in polyG or polyC. In an embodiment, the gRNA guide sequence on on the APP polynucleotide gene sequence does not comprise more than one PAM (e.g., NGG sequence) within its sequence. In an embodiment, the gRNA target sequence on the on the APP polynucleotide gene sequence does not include an NGG (although it is adjacent to a PAM).

[0076] The number of gRNAs administered to or expressed in a cell (or subject) or subject in accordance with the methods of the present invention may be at least 1 gRNA, at least 2 gRNAs, at least 3 gRNAs at least 4 gRNAs, at least 5 gRNAs, at least 6 gRNAs, at least 7 gRNAs, at least 8 gRNAs, at least 9 gRNAs, at least 10 gRNAs, at least 11 gRNAs, at least 12 gRNAs, at least 13 gRNAs, at least 14 gRNAs, at least 15 gRNAs, at least 16 gRNAs, at least 17 gRNAs, or at least 18 gRNAs. The number of gRNAs administered to or expressed in a cell may be between at least 1 gRNA and at least 15 gRNAs, at least 1 gRNA to and least 10 gRNAs, at least 1 gRNA and at least 8 gRNAs, at least 1 gRNA and at least 6 gRNAs, at least 1 gRNA and at least 4 gRNAs, at least 1 gRNA to and least 3 gRNAs, at least 2 gRNA and at least 5 gRNAs, at least 2 gRNA and at least 3 gRNAs. lDifferent or identical gRNAs may be used to cut the endogenous target gene of interest and liberate the donor/patch nucleic acid, when provided in a vector.

Nucleases and nickases

[0077] Recently, Q Tsai et al. (45) have designed recombinant dCas9-FoKI dimeric nucleases (RFNs) that can recognize extended sequences and edit endogenous genes with high efficiency in human cells. These nucleases comprise a dimerization-dependent wild type FokI nuclease domain fused to a catalytically inactive Cas9 (dCas9) protein. Dimers of the fusion proteins mediate sequence specific DNA cleavage when bound to target sites composed of two half-sites (each bound to a dCas9 (i.e., a Cas9 nuclease devoid of nuclease activity) monomer domain) with a spacer sequence between them. The dCas9-FoKI dimeric nucleases require dimerization for efficient genome editing activity and thus, use two gRNAs for introducing a cut into DNA.

[0078] The recombinant Cas protein that may be used in accordance with the present invention is i) derived from a naturally occurring Cas; and ii) has a nuclease (or nickase) activity to introduce a DSB (or two SSBs in the case of a nickase) in cellular DNA when in the presence of appropriate gRNA(s). Thus, as used herein, the term "Cas9 nuclease" refers to a recombinant protein which is derived from a naturally occurring Cas9 which has nuclease activity and which function with the gRNAs of the present invention to introduce DSBs in the targeted DNA. In an embodiment, the Cas9 nuclease is a dCas9 protein (i.e., a mutated Cas9 protein devoid of nuclease activity) fused with a dimerization-dependent FokI nuclease domain [45]. In another embodiment, the Cas protein is a Cas9 protein having a nickase activity [39]. As used herein, the term "Cas9 nickase" refers to a recombinant protein which is derived from a naturally occurring Cas9 and which has one of the two nuclease domains inactivated such that it introduces single stranded breaks (SSB) into the DNA. It can be either the RuvC or HNH domain. In a further embodiment, the Cas protein is a Cas9 nuclease. In accordance with the present invention, the Cas9 protein can be derived from any naturally occurring source.

[0079] For example, Cas9 proteins are natural effector proteins produced by numerous species of bacteria including *Streptococcus pyogene* (47), *Streptococcus thermophiles* (48), *Staphylococcus aureus* (65), and *Neisseria*

meningitides (46). Accordingly, in an embodiment, the Cas protein of the present invention is a Cas9 nuclease/nickase derived from *Streptococcus pyogene*, *Streptococcus thermophiles, Staphylococcus aureus* or *Neisseria meningitides.* In an embodiment, the Cas9 recombinant protein of the present invention is a human-codon optimized Cas9 derived from *S. pyogenes* (hSpCas9). In an embodiment, the Cas9 recombinant protein of the present invention is a human-codon optimized Cas9 derived from S. aureus (hSaCas9). In an embodiment, the hSpCas9 consists essentially of the amino acid sequence set forth in Figure 23B (SEQ ID NO: 80). In an embodiment, the amino acid sequence of the Cas9 nuclease protein of the present invention comprises an amino acid sequence at least 90%, at least 95% (in embodiments at least 96%, 97%, 98% or 99%) identical to the Cas9 sequence set forth in Figure 23B (SEQ ID NO: 80).

[0080] The Cas9 cuts 3-4bp upstream of the PAM sequence. There can be some off-target DSBs using wildtype Cas9. The degree of off-target effects depends on a number of factors, including: how closely homologous the off-target sites are compared to the on-target site, the specific site sequence, and the concentration of Cas9 and guide RNA (gRNA). These considerations only matter if the PAM sequence is immediately adjacent to the nearly-homologous target sites. The mere presence of additional PAM sequences should not be sufficient to generate off-target DSBs; there needs to be extensive homology of the protospacer followed by PAM.

[0081] In addition to Cas9 derived nucleases or nickases, other nucleases may be used in accordance with the present invention to introduce site specific cuts into DNA, thereby allowing modifications to be introduced in the targeted polynucleotide gene sequence by homologous recombination or non-homologous end joining repair. Such nucleases may be used with a donor (patch) sequence of the present invention to introduce specific modifications into the targeted endogenous polynucleotide gene sequence. Such nucleases/nickases include but are not limited to meganucleases, Zinc finger nucleases and transcription activator-like effector nucleases (TALENs).

[0082] The Cas or other nuclease/nickase recombinant protein of the present invention preferably comprises at least one Nuclear Localization Signal (NLS) to target the protein into the cell nucleus. Accordingly, as used herein the expression “nuclear localization signal” or “NLS” refers to an amino acid sequence, which 'tags' a protein for import into the cell nucleus by nuclear transport. Typically, this signal consists of one or more short sequences of positively charged lysines or arginines exposed on the protein surface. Different nuclear localized proteins may share the same NLS. An NLS has the opposite function of a nuclear export signal, which targets proteins out of the nucleus. Classical NLSs can be further classified as either monopartite or bipartite. The first NLS to be discovered was the sequence PKKKRKV (SEQ ID NO: 84) in the SV40 Large T-antigen (a monopartite NLS). The NLS of nucleoplasmin, KR[PAATKKAGQA]KKKK (SEQ ID NO: 85) , is the prototype of the ubiquitous bipartite signal: two clusters of basic amino acids, separated by a spacer of about 10 amino acids. The Cas9 protein exemplified herein is a mutated Cas9 nuclease comprising a NLS sequence.

[0083] There are many other types of NLS, which are qualified as "non-classical", such as the acidic M9 domain of hnRNP A1, the sequence KIPIK in yeast transcription repressor Matα2, the complex signals of U snRNPs as well as a recently identified class of NLSs known as PY-NLSs. Thus, any type of NLS (classical or non-classical) may be used in accordance with the present invention as long as it targets the protein of interest into the nucleus of a target cell. In an embodiment, the NLS is derived from the simian virus 40 large T antigen. In an embodiment, the NLS of the recombinant protein of the present invention comprises the following amino acid sequence: SPKKKRKVEAS (SEQ ID NO: 86). In an embodiment the NLS comprises the sequence KKKRKV (SEQ ID NO: 87). In an embodiment, the NLS comprises the sequence SPKKKRKVEASPKKKRKV (SEQ ID NO: 88). In another embodiment, the NLS comprises the sequence KKKRK (SEQ ID NO: 89).

[0084] The nuclease/nickase recombinant protein of the present invention may optionally advantageously be coupled to a protein transduction domain to ensure entry of the protein into the target cells. Alternatively the nucleic acid coding for the gRNA and for the nuclease or nickase (e.g., Cas9 nuclease/nickase) and the donor/patch nucleic acid sequence may be delivered in targeted cells using various viral vectors, virus like particles (VLP) or exosomes.

[0085] Protein transduction domains (PTD) may be of various origins and allow intracellular delivery of a given therapeutic by facilitating the translocation of the protein/polypeptide into a cell membrane, organelle membrane, or vesicle membrane. PTD refers to a polypeptide, polynucleotide, carbohydrate, or organic or inorganic compound that facilitates traversing a lipid bilayer, micelle, cell membrane, organelle membrane, or vesicle membrane. A PTD attached to another molecule facilitates the molecule traversing a membrane, for example going from extracellular space to intracellular space, or cytosol to within an organelle including the mitochondria.

[0086] In an embodiment, a PTD is covalently linked to the amino terminus of a recombinant protein of the present invention. In another embodiment, a PTD is covalently linked to the carboxyl terminus of a recombinant protein of the present invention. Exemplary protein transduction domains include but are not limited to a minimal undecapeptide protein transduction domain (corresponding to residues 47-57 of HIV-1 TAT comprising YGRKKRRQRRR (SEQ ID NO: 90); a polyarginine sequence comprising a number of arginines sufficient to direct entry into a cell (e.g., 3, 4, 5, 6, 7, 8, 9, 10, or 10-50 arginines); a VP22 domain (49); an Drosophila Antennapedia protein transduction domain (50); a truncated human calcitonin peptide (51); RRQRRTSKLMKR (SEQ ID NO: 91); Transportan GWTLNSAGYLLGKINLKALAALAKKIL (SEQ ID NO: 92); KALAWEAKLAKALAKALAKHLAKALAKALKCEA (SEQ ID NO:93); and RQIKIWFQNRRMKWKK (SEQ ID NO:94). Further exemplary PTDs include but are not limited to, KKRRQRRR (SEQ ID NO: 95), RKKRRQRRR (SEQ ID NO: 96); or an arginine homopolymer of from 3 arginine residues to 50 arginine residues.

[0087] Other non-limiting examples of PTD include an endosomal escape peptide. Non-limiting examples of such endosomal escape peptides are listed in the Table 1 below.

[0088] Table 1: Endosomal escape peptides

Peptide	Primary sequence	Mechanism	References
DT	VGSSLSCINLDWDVIRDKTKTKIE SLKEHGPIKNKMSESPNKTVSEE KAKQYLEEFHQTALEHPELSELKT VTGTNPVFAGANYAAWAVNVAQ VIDSETADNLEKTTAALSILPGIGS VMGIADGAVHHNTEEIVAQSIALS SLMVAQAIPLVGELVDIGFAAYNF VESIINLFQVVHNSYNRPAYSPG	Fusion	(52), (SEQ ID NO: 99)
GALA	WEAALAEALAEALAEHLAEALAE ALEALAA	Membrane destabilization, pore formation and flip-flop of membrane lipids	(53), (SEQ ID NO: 100)
PEA	VLAGNPAKHDLDIKPTVISHRLHF PEGGSLAALTAHQACHLPLETFT RHRQPRGWEQLEQCGYPVQRLV ALYLAARLSWNQVDQVIRNALAS PGSGGDLGEAIREQPEQARLALT	Pore formation	(54), (SEQ ID NO: 101)
INF-7	GLFEAIEGFIENGWEGMIDGWYG C	Membrane fusion and destabilization	(55), (SEQ ID NO: 102)
LAH4	KKALLALALHHLAHLALHLALALK KA	Membrane destabilization	(56), (SEQ ID NO: 103)
CM18	KWKLFKKIGAVLKVLTTG	Membrane destabilization	(57), (SEQ ID NO: 104)
HGP	LLGRRGWEVLKYWWNLLQYWS QEL	Pore formation and fusion	(58), (SEQ ID NO: 105)
H5WYG	GLFHAIAHFIHGGWH GLIHGWYG	Membrane destabilization	(59), (SEQ ID NO: 106)
HA2	GLFGAIAGFIENGWEGMIDGWYG	Membrane fusion and destabilization	(60), (SEQ ID NO: 107)
EB1	LIRLWSHLIHIWFQNRRLKWKKK	Membrane destabilization	(61), (SEQ ID NO: 108)
DT	VGSSLSCINLDWDVIRDKTKTKIE SLKEHGPIKNKMSESPNKTVSEE KAKQYLEEFHQTALEHPELSELKT VTGTNPVFAGANYAAWAVNVAQ VIDSETADNLEKTTAALSILPGIGS VMGIADGAVHHNTEEIVAQSIALS SLMVAQAIPLVGELVDIGFAAYNF VESIINLFQVVHNSYNRPAYSPG	Fusion	(52), (SEQ ID NO: 109)

[0089] In an embodiment, the protein transduction domain is TAT or Pep-1. In an embodiment, the protein transduction domain is TAT and comprises the sequence SGYGRKKRRQRRRC (SEQ ID NO:97). In another embodiment, the protein transduction domain is TAT and comprises the sequence YGRKKRRQRRR (SEQ ID NO:

90). In another embodiment, the protein transduction domain is TAT and comprises the sequence KKRRQRRR (SEQ ID NO: 95). In another embodiment, the protein transduction domain is Pep-1 and comprises the sequence KETWWETWWTEWSQPKKKRKV(SEQ ID NO: 98).

[0090] In addition or alternatively to the above-mentioned protein transduction domains, the nuclease/nickase (e.g., Cas9 nuclease) recombinant protein or nucleic acid, patch nucleic acid sequence and gRNA(s) of the present invention may be coupled to liposomes to further facilitate their delivery into the cells.

[0091] Genetic constructs encoding a Cas 9 protein (nuclease or nickase) in accordance with the present invention can be made using either conventional gene synthesis or modular assembly. A humanized Cas9 construct is publicly available for example at the repository Addgene (for example Addgene plasmids pX330™, pX335™ (nickase), pX458™, pX459™, pX460™, pX461™, pX462™, pX165™ pX260™, pX334™ (nickase)).

[0092] In an aspect, the gRNAs, recombinant nuclease/nickase (e.g., Cas9) protein and donor/patch nucleic acid of the present invention may be used to decrease toxic Aβ peptides production by decreasing APP mRNA and APP protein in cells. In another aspect, the gRNAs, Cas9 recombinant nuclease/nickase protein and donor/patch nucleic acid of the present invention may be used to decrease APP cleavage at the β-secretase site (e.g., between amino acid 671 and 672 of APP) thereby inhibiting completely or partially the production of toxic Aβ peptides. As used herein, the expression “decreasing” in “decreasing the expression of toxic Aβ peptides in a cell” is meant to include circumstances where, in the absence of a gRNA, of a recombinant nuclease/nickase (e.g., Cas9) protein and of a patch nucleic acid sequence of the present invention, the toxic Aβ peptides are expressed at certain amount (baseline amount), which is decreased in their presence. It comprises decreasing/reducing/inhibiting the expression of APP and/or its maturation (cleavage) into toxic Aβ peptides in cells completely or partially. The cell may be a cell expressing a normal level of APP or Aβ peptides or an abnormal/higher level of Aβ peptides (as compared to normal conditions).

[0093] In an embodiment, the gRNA and Cas9 recombinant protein and patch nucleic acid of the present invention may be used to decrease transcription of the APP promoter and expression of the APP protein in cells from a subject in need thereof.

[0094] In an embodiment, the present invention relates to a method of decreasing Aβ peptide expression, production or accumulation in a subject in need thereof comprising administering to the subject an effective amount of a gRNA, a Cas recombinant nuclease or nickase protein and a donor/patch nucleic acid of the present invention in order to introduce one or more genetic modifications in a target gene (e.g., APP, PS-1 or PS-2). In an embodiment, the recombinant protein, gRNA and/or donor/patch nucleic acid are specifically formulated for crossing the plasma membrane and reaching the nucleus. In an embodiment, the present invention provides a composition comprising a

Cas9, based recombinant protein donor/patch nucleic acid and/or gRNA of the present invention together with a pharmaceutically acceptable carrier. In an embodiment, the method of the present invention corrects a mutation present in a target gene, which is associated with increased risk of developing Alzheimer's disease. In an embodiment, the mutation increases the expression or maturation of APP into toxic Aβ peptides. In another embodiment, the method of the present invention introduces a modification in a target gene, which protects against Alzheimer's disease (i.e., reduces the risk of developing Alzheimer's disease). In an embodiment, the modification decreases the production of toxic Aβ peptide by modifying one or more of the beta and gamma secretases cleavage sites in the APP protein, thereby reducing toxic Aβ peptides secretion. In an embodiment the modification is a modification at amino acid 673 or other amino acids close to that position of the APP protein, which reduces maturation by the beta secretase. In an embodiment, the modification replaces an alanine at position 673 with a threonine.

[0095] Optimization of codon degeneracy

[0096] Because several site-specific nuclease proteins, such as Cas9, are normally expressed in bacteria, it may be advantageous to modify their nucleic acid sequences for optimal expression in eukaryotic cells (e.g., mammalian cells). This has been done for the embodiment of the Cas9 nuclease protein of the present invention described herein.

[0097] Codon degeneracy may also be used to distinguish two nucleic acids encoding for the same protein. For example, the donor/patch nucleic acid sequence of the present invention may comprise one or more modifications with respect to the wild type targeted gene sequence, which do not translate into modifications at the amino acid level. These one or more modifications allow distinguishing between the wild type endogenous sequence, the patch sequence and the integrated patch sequence into the targeted endogenous gene. In an embodiment of the present invention the wild type, donor/patch nucleic acid and modified targeted gene cane be distinguished using the appropriate combination of oligonucleotide primers and/or probes. In an embodiment, the oligonucleotide primer(s) or probe(s) overlap the integration site. In another embodiment, the modifications introduced at the nucleic acid level add one or more restriction sites normally not present in the wild-type targeted gene sequence and/or in the donor/patch sequence. The one or more restriction sites may allow identifying a properly integrated patch/donor sequence into the targeted endogenous gene sequence in accordance with the method of the present invention.

[0098] Accordingly, the following codon chart (Table 2) may be used, in a site-directed mutagenic scheme, to produce nucleic acids encoding the same or slightly different amino acid sequences of a given nucleic acid:

Table 2: Codons encoding the same amino acid

Amino Acids			Codons					
Alanine	Ala	A	GCA	GCC	GCG	GCU		
Cysteine	Cys	C	UGC	UGU				
Aspartic acid	Asp	D	GAC	GAU				
Glutamic acid	Glu	E	GAA	GAG				
Phenylalanine	Phe	F	UUC	UUU				
Glycine	Gly	G	GGA	GGC	GGG	GGU		
Histidine	His	H	CAC	CAU				
Isoleucine	Ile	I	AUA	AUC	AUU			
Lysine	Lys	K	AAA	AAG				
Leucine	Leu	L	UUA	UUG	CUA	CUC	CUG	CUU
Methionine	Met	M	AUG					
Asparagine	Asn	N	AAC	AAU				
Proline	Pro	P	CCA	CCC	CCG	CCU		
Glutamine	Gln	Q	CAA	CAG				
Arginine	Arg	R	AGA	AGG	CGA	CGC	CGG	CGU
Serine	Ser	S	AGC	AGU	UCA	UCC	UCG	UCU
Threonine	Thr	T	ACA	ACC	ACG	ACU		
Valine	Val	V	GUA	GUC	GUG	GUU		
Tryptophan	Trp	W	UGG					
Tyrosine	Tyr	Y	UAC	UAU				

[0099] Sequence similarity

[00100] "Homology" and “homologous” refers to sequence similarity between two peptides or two nucleic acid molecules. Homology can be determined by comparing each position in the aligned sequences. A degree of homology between nucleic acid or between amino acid sequences is a function of the number of identical or matching nucleotides or amino acids at positions shared by the sequences. As the term is used herein, a nucleic acid sequence is "homologous" to another sequence if the two sequences are substantially identical and the functional activity of the sequences is conserved (as used herein, the term “homologous” does not infer evolutionary relatedness, but rather refers to substantial sequence identity). Two nucleic acid sequences are considered substantially identical if, when optimally aligned (with gaps permitted), they share at least about 50% sequence similarity or identity, or if the sequences share defined functional motifs. In alternative embodiments, sequence similarity in optimally aligned substantially identical sequences may be at least 60%, 70%, 75%, 80%, 85%, 90% or 95%. For the sake of brevity,

the units (e.g., 66, 67…81, 82,…91, 92%….) have not systematically been recited but are considered, nevertheless, within the scope of the present invention.

[00101] Substantially complementary nucleic acids are nucleic acids in which the complement of one molecule is substantially identical to the other molecule. Two nucleic acid or protein sequences are considered substantially identical if, when optimally aligned, they share at least about 70% sequence identity. In alternative embodiments, sequence identity may for example be at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 98% or at least 99%. Optimal alignment of sequences for comparisons of identity may be conducted using a variety of algorithms, such as the local homology algorithm of Smith and Waterman, 1981, Adv. Appl. Math 2: 482, the homology alignment algorithm of Needleman and Wunsch, 1970, J. Mol. Biol. 48:443, the search for similarity method of Pearson and Lipman (62), and the computerized implementations of these algorithms (such as GAP, BESTFIT, FASTA and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, Madison, WI, U.S.A.). Sequence identity may also be determined using the BLAST algorithm, described in Altschul et al. (63) 1990 (using the published default settings). Software for performing BLAST analysis may be available through the National Center for Biotechnology Information (through the internet at http://www.ncbi.nlm.nih.gov/). The BLAST algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence that either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold. Initial neighborhood word hits act as seeds for initiating searches to find longer HSPs. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Extension of the word hits in each direction is halted when the following parameters are met: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T and X determine the sensitivity and speed of the alignment. One measure of the statistical similarity between two sequences using the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. In alternative embodiments of the invention, nucleotide or amino acid sequences are considered substantially identical if the smallest sum probability in a comparison of the test sequences is less than about 1, preferably less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

[00102] An alternative indication that two nucleic acid sequences are substantially complementary is that the two sequences hybridize to each other under moderately stringent, or preferably stringent, conditions. Hybridization to filter-bound sequences under moderately stringent conditions may, for example, be performed in 0.5 M NaHPO4, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65°C, and washing in 0.2 x SSC/0.1% SDS at 42°C (64). Alternatively, hybridization to filter-bound sequences under stringent conditions may, for example, be performed in 0.5 M NaHPO4,

7% SDS, 1 mM EDTA at 65°C, and washing in 0.1 x SSC/0.1% SDS at 68°C (64). Hybridization conditions may be modified in accordance with known methods depending on the sequence of interest. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point for the specific sequence at a defined ionic strength and pH.

[00103] In another aspect, the invention further provides one or more nucleic acids encoding the above-mentioned Cas nuclease/nickase recombinant protein, gRNA and/or patch/donor sequences. The invention also provides one or more vector(s) comprising one or more of the above-mentioned nucleic acids. In an embodiment, the vector further comprises a transcriptional regulatory element operably-linked to the above-mentioned nucleic acid. A first nucleic acid sequence is "operably-linked" with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably-linked to a coding sequence if the promoter affects the transcription or expression of the coding sequences. Generally, “operably-linked” DNA sequences are contiguous and, where necessary to join two protein coding regions, in reading frame. However, since, for example, enhancers generally function when separated from the promoters by several kilobases and intronic sequences may be of variable lengths, some polynucleotide elements may be operably-linked but not contiguous. "Transcriptional regulatory element" is a generic term that refers to DNA sequences, such as initiation and termination signals, enhancers, and promoters, splicing signals, polyadenylation signals, which induce or control transcription of protein coding sequences with which they are operably-linked.

[00104] As indicated above, gRNAs, patch/donor and Cas recombinant nucleic acids of the present invention may be delivered into cells using various viral vectors. Accordingly, preferably, the above-mentioned vector is a viral vector for introducing the gRNA and/or Cas nucleic acid encoding the Cas9 nuclease/nickase and/or the donor patch sequence of the present invention in a target cell (preferably a neurone). Non-limiting examples of viral vectors include retrovirus, lentivirus, Herpes virus, adenovirus or adeno Associated Virus, as well known in the art. Herpesvirus, adenovirus, Adeno-Associated virus and lentivirus derived viral vectors have been shown to efficiently infect neuronal cells. Preferably, the viral vector is episomal and not cytotoxic to cells. In an embodiment, the viral vector is an AAV or a Herpes virus.

[00105] In yet another aspect, the present invention provides a cell (*e.g.*, a host cell) comprising the modified targeted gene. The invention further provides a recombinant expression system, vectors and host cells, such as those described above, for the expression/production of a recombinant protein, using for example culture media, production, isolation and purification methods well known in the art.

[00106] In another aspect, the present invention provides a composition (e.g., a pharmaceutical composition) comprising the above-mentioned gRNA, recombinant nuclease/nickase (e.g., Cas9) nucleic acid or protein and a donor/patch nucleic acid. In an embodiment, the composition comprises the above-mentioned viral vector for

targeting the gRNA, nuclease/nickase (e.g., Cas9) and donor/patch nucleic acids into a cell. In an embodiment, the cell is a neuronal cell. In an embodiment, the composition further comprises one or more pharmaceutically acceptable carriers, excipients, and/or diluents.

[00107] As used herein, "pharmaceutically acceptable" (or "biologically acceptable") refers to materials characterized by the absence of (or limited) toxic or adverse biological effects in vivo. It refers to those compounds, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the biological fluids and/or tissues and/or organs of a subject (e.g., human, animal) without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

[00108] The present invention further provides a kit or package comprising one or more (at least one) of the above-mentioned gRNA, recombinant nuclease/nickase protein and/or patch/donor nucleic acids or compositions, together with instructions for decreasing Aβ peptide production levels (expression or maturation) in a cell or for the treatment of Alzheimer's disease or age-related cognitive decline.

[00109] DEFINITIONS

[00110] The articles "a," "an" and "the" are used herein to refer to one or to more than one (i.e., to at least one) of the grammatical object of the article.

[00111] As used in this specification and claim(s), the words "comprising" (and any form of comprising, such as "comprise" and "comprises"), "having" (and any form of having, such as "have" and "has"), "including" (and any form of including, such as "includes" and "include") or "containing" (and any form of containing, such as "contains" and "contain") are inclusive or open-ended and do not exclude additional, un-recited elements or method steps and are used interchangeably with, the phrases "including but not limited to" and "comprising but not limited to".

[00112] The present invention is illustrated in further details by the following non-limiting examples.

EXAMPLE 1

REAGENTS AND METHODS

[00113] Preparation of donor plasmids and ssODNs for homologous DNA recombination (HDR) to mutate exon 16 of APP gene.

[00114] All oligonucleotides and gBlocks gene fragments used to construct the donor plasmids were synthesized by IDT (Integrated DNA technologies, Iowa, USA). The pMiniT™ plasmid was obtained from the NEB PCR cloning kit (Catalog #E1202S, New England Biolabs, MA USA). Phusion™ High-Fidelity Polymerase (Thermo

Fisher Scientific). Qiaquick™ gel extraction Kit and Qiaquick™ PCR purification kit were from Qiagen.

[00115] A) Construction of the pMiniT-Patch plasmid

[00116] Step 1: A portion of the APP gene containing exon 16 and parts of introns 15 and 16 was initially amplified by PCR from DNA extracted from of 293T cells. The PCR product was sequenced to confirm that the nucleotide sequence was the same as the reference sequence published in NCBI (accession number NG_007376). The procedure for this PCR amplification was as follows: the PCR mix (50 µL) contained 100 ng of each primer Bfw (TTCAGCAGACGAACCAATTACA , in intron 15 (SEQ ID NO: 43)) and Brev (CACGAACTTTGCTGCCTTGTAG, in intron 16, (SEQ ID NO: 40)), 200 mM dNTP, 100 ng genomic DNA (from 293T cells), 1X Phusion™ HF buffer and 0.5 µL Phusion™ DNA Polymerase (2U/ µL).The PCR amplification was done in the Hybaid PCR Express™ apparatus using the following cycling program: 1 cycle of denaturation at 98°C for 1 min; followed by 30 cycles comprising a denaturation step at 98°C for 10 sec, an annealing step at 58°C for 20 sec. and elongation step at 72°C for 40 sec; followed by 1 cycle of final elongation at 72°C for 10 min and hold at 4°C indefinitely.

[00117] The analysis of the PCR fragment was done on a 1.4% agarose gel stained with Red Safe staining (Intron Biotechnology). After electrophoresis, a PCR product of 1180 pb was observed and extracted from the agarose gel to be further purified with a gel extraction kit (QIAquick™ Gel Extraction Kit, Qiagen). The purified PCR fragment was then cloned directly in a pMiniT plasmid included in the cloning kit (NEB) as follows: 150 ng purified PCR fragment (4 µL), 25 ng/µL linearized pMiniT plasmid (1 µL), 5 µL Cloning Master Mix making a final volume of 10 µL. The mix was left at room temperature 5 minutes and then placed on ice at least 2 minutes before the transformation in competent DH5α bacteria and plated on agar containing ampicillin. After overnight incubation at 37°C, six colonies were picked up and the plasmids were extracted (mini-prep method) from these colonies to verify the presence of the insert by plasmid digestion with the EcoR1 restriction enzyme. Three plasmids containing the insert with appropriate size were sequenced using primers MiniTfw (5´- ACCTGCCAACCAAAGCGAGAAC- 3´, (SEQ ID NO: 51)) and MiniTrev (5´- TCAGGGTTATTGTCTCATGAGCG- 3´, (SEQ ID NO: 52)). The sequences of the 3 inserts (1180 pb, Figures 15 and 16) in the pMiniT plasmid all showed 100% homology with the NCBI sequence (accession number NG_007376).

[00118] Step 2: Design of a gBlock permitting to introduce the A673T mutation in the APP gene and additional mutations to prevent a subsequent cut of the mutated APP gene by gRNA#8.

[00119] *Step 2A*: The plasmid pMiniT including the 1180 pb PCR fragment (obtained in step1) corresponding to a portion of the APP gene (portion of intron 15, exon 16 and portion of intron 16) showed two unique restriction enzyme sites (Stu1 and BstX1) near exon 16 (Figure 21A). These two restriction sites served to introduce different DNA fragments in the original plasmid donor to insert various mutations or an optional tracker (FLAG) to evaluate the

efficiency of the correction by HDR. A gBlock was thus designed containing these two restriction enzyme sites and several mutations within the nucleotide sequence targeted in wild exon 16 by sgRNA#8 and including the critical mutation amino acid Alanine (A) in position 673 (coded by gca) to a Threonine (coded by acg) (Figure 21B). The gBlock also included a nucleotide mutation C for G creating a Spe1 restriction site localized in intron 16 to detect more easily the HDR repair produced by the gRNA8 with the Cas9 nuclease (Figure 21B). Thus to make all the desired mutations discussed above, the gBlock gene fragment synthesized by IDT (see Figure 21B) contained a total of 9 mutations (8 nucleotide mutations inside of exon 16 and another one in intron 16 creating a Spe1 site). The gBlock (Figure 21B) was amplified to obtain a PCR fragment able to be cloned between the restriction enzymes sites Stu 1 and BstX1 of the pMiniT plasmid containing the wild type exon 16 described in step 1.

[00120] *Step2B*: The amplification of the gBlock was done as follows: the PCR reaction (50 µL) contained 100 ng of each primer gBlockfw (5'-ATCAATTTTCTTCTAACTTCAGG-3', (SEQ ID NO: 69)) and gBlockrev (5'-CCACCCGCCTTGGCCTCCCAAAG-3', (SEQ ID NO: 70)), 200 mM dNTP, 100 ng genomic DNA (from 293T cells), 1X Phusion™ HF buffer and 0.5 µL Phusion™ DNA Polymerase (2U/ µL). The PCR amplification was done in the PCR apparatus (Hybaid PCR Express™) according to the following cycling program: 1 cycle, denaturation at 98°C for 1 min; 30 cycles, denaturation at 98°C for 10 sec, annealing at 60°C for 10 sec and elongation at 72°C for 10 sec; 1 cycle, elongation at 72°C for 10 min and hold at 4°C indefinitely.

[00121] Step 2C: The PCR product was electrophoresed on a 1.4% agarose gel and the PCR product (441 pb) was then purified with the Qiaquick™ gel extraction kit (Qiagen). The purified PCR fragment was then directly cloned in a pMiniT plasmid as described in the step1, transformed in competent DH5α bacteria and spread on ampicillin agar plate. A plasmid containing the expected 441 pb insert was prepared (mini-prep preparation) from one the resulting clones. This plasmid was digested during 1 hour with restriction enzymes BstX1 followed by Stu1 in 100 µL digestion medium as follows respectively: 10 µL purified plasmid (~5 µg), 10 µL NEBuffer 3.1 (NEB), 90 µL H_20 and 2.5 µL BstX1 (10 000 U/ml). This mixture was incubated during one hour at 37°C followed by purification of the digested product through a Qiaquick™ PCR column and elution with 90 µL of water. 10 µL of CutSmart™ buffer (NEB) and 2.5 µL of Stu 1 (10 000 U/ml) were added to 90 µL of the purified BstX1 digested product and incubated for one hour at 37°C. The double digested product was loaded directly on a 1.6 % agarose gel and electrophoresed to separate the DNA fragment (BstX1/Stu1) (~400 pb). This fragment was purified using the Qiagen gel extraction kit and eluted in 100 µL. This fragment was treated with phenol/Chloroform (24:1) (75 µL) followed by Chloroform only (60 µL), recuperated in 100 µL of water, purified through a Qiaquick™ PCR column and eluted in 30 µL of water.

[00122] Step 2D: The purified PCR fragment (BstX1/Stu1) (~400 pb) described in the step 2C was cloned between the unique sites (Stu1 and BstX1) found in the plasmid pMiniT previously described in Step 1 (figure 20A). The same procedure for the digestion and purification of the plasmid was done as described previously in Step 2C for

the PCR fragment.

[00123] The purified PCR fragment was then cloned in the digested (BstX1/Stu1) plasmid pMiniT containing the wild APP gene sequence (portion of intron 15, exon 16 and portion of intron 16). For the cloning, we proceeded as follows: 100 ng of plasmid vector (1 µL), 300 ng of PCR fragment (BstX1/Stu1) (~400 pb), 5 µL of 2X Quick Ligation Reaction Buffer (NEB) and 0.5 µL of Quick T4 DNA Ligase (provided in the Quick Ligation Kit, NEB) were left at room temperature 5 minutes and then placed on ice before transformation in competent bacteria DH5α as described earlier. The resulting clones were sequenced to confirm that all of the expected 9 nucleotide mutations (Figure 21B) have been successfully introduced in the original wild type gene (Figure 21A) by the modified DNA provided by the gBlock.

[00124] Step2E: The final step was to add the specific DNA sequence targeted by the gRNA#8 at each 5' and 3' ends of the insert introduced in the pMiniT plasmid containing mutations described in Step 2D. To proceed the plasmid (step 2D) was amplified with the following long primers:

[00125] Tgt8bfw: 5'-atagcagaattccgacatgactcaggttcagcagacgaaccaattaca-3' (48 nts, (SEQ ID NO:112))

[00126] Tgt8brev: 5'-cctgagtcatgtcggaattctgcacgaactttgctgccttgtag-3' (49 nts, (SEQ ID NO:113))

[00127] The underlined nucleotides of both primers correspond to the target sequence of gRNA#8 (including in this case a PAM sequence for proper recognition by Cas9), while the rest of the nucleotide sequences correspond to the end of the insert of the plasmid MiniT containing mutations described in step 2D. To produce the final donor/patch, a PCR product (1224 pb) was obtained as follows: the PCR reaction (50 µL) contained 100 ng of each primer Tgt8bfw and Tgt8brev, 200 mM dNTP, 10 ng plasmid DNA (step 2D), 1X Phusion HF buffer and 0.5 µL Phusion DNA Polymerase (2U/ µL). The PCR amplification was done in the Hybaid PCR Express apparatus according to the following cycling program: 1 cycle, denaturation at 98°C for 1 min; 5 cycles, denaturation at 98°C for 10 sec, annealing at 55°C for 20 sec and elongation at 72°C for 40 sec; 25 cycles, denaturation at 98°C for 10 sec, annealing at 60°C for 20 sec and elongation at 72°C for 40 sec; 1 cycle, elongation at 72°C for 10 min and hold at 4°C indefinitely. At the end of the reaction, the PCR product was analyzed on a 1.4% agarose gel and purified with Qiaquick™ gel extraction kit as described earlier. The purified PCR product (1224 pb) was cloned directly in a pMiniT plasmid (NEB PCR cloning kit) as described previously and cloned in competent bacteria DH5α. The clones resulting from the transformation were sequenced to confirm the expected configuration of the pMiniT Patch. The originality of this construct to produce HDR is that the target sites of gRNA#8 (i.e., the same target sequence as in the wild type APP gene) are present at in each side of the plasmid mini-T insert (1224 pb). This feature permits to liberate a linear fragment cassette to facilitate the HDR.

[00128] Construction of plasmid pMiniT-Patch containing a 3XFLAG or a 3XFLAG-STOP

[00129] To construct the plasmid pMiniT-Patch containing the 3XFLAG or the 3XFLAG-STOP, two gBlocks described in Figures 22 A and B were synthesized by IDT. The same procedure described in the above section A steps 2C and 2D for introducing in the plasmid pMiniT-Patch digested with Stu1/BstX1 a DNA fragment obtained by PCR amplification for each gBlock was used. The final cloning result gave a plasmid pMiniT-Patch containing a 3XFLAG and another pMiniT-Patch containing a 3XFLAG-STOP. The inserts in these two plasmids were confirmed by sequencing and the nucleotide sequence insert in the pMiniT-Patch-3X-FLAG (SEQ ID NO:73) and pMiniT-Patch-3XFLAG-STOP (SEQ ID NO: 74) are shown in the Figure 22 C and D respectively.

[00130] Design and synthesis of ssODNs 199 nts

[00131] A long single stranded oligodeoxynucleotides (ssODNs) of 199 nts was designed to correspond to the entire exon 16 of the APP gene extended at each side by 90 nucleotides corresponding respectively to a portion of the 3' end of intron 15 and of the 5' end of intron 16. This ssODN was synthesized by IDT (4 nmoles of Ultramer DNA oligo) and the nucleotide sequence is presented in the Figure 17A. The ssODN 199 nts contained all the nucleotide mutations described for the plasmid pMiniT-Patch.

[00132] Co-Transfection in 293T cells of gRNA#8 with plasmids donors MiniT-Patch, MiniT-Patch-3XFLAG and MiniT-Patch-3XFLAG-STOP, ssODN 199 nts, and PCR fragment of Patch:

[00133] Materials: all the transfection reagents (Lipofectamine 2000TM transfection reagent and Opti-MEM-1 TM culture media) were from Life Technologies.

[00134] The day before the transfection, 100,000 293T cells were seeded per well in a 24 well plates in DMEM medium supplemented with 10% FBS and antibiotics (penicillin/streptomycin 1X). The following morning, the culture medium was changed for 500 µl of DMEM medium supplemented with 10% FBS without antibiotics. The plate was incubated at 37°C in the incubator for the time required to prepare the transfection solution. For the transfection, solutions A and B were first prepared. Solution A contained 48 µl of Opti-MEM™ and 2 µl of Lipofectamine™ 2000 for a final volume of 50 µl. Solution B was prepared as follows: a volume of DNA solution containing 150 ng to 800 ng of DNA was mixed with a volume of Opti-MEM™ to obtain a final volume of 50 µl. For each co-transfection, various amounts of the following plasmids were used: pX330™ (containing gRNA#8 and Cas9, see Figure 23), pMiniT-Patch, pMiniT-Patch-3XFLAG or pMiniT-Patch-3XFLAG-STOP, ssODN 199 or a PCR fragment of pMiniT-Patch (see Figures 17-22 (amplified with Bfw and Brev). Solutions A and B were then mixed by up and down movement and incubated at room temperature for 20 minutes. Then, 100 µl of the mixed solution were added to each well. The plate was let in the CO_2 incubator for a period of 4 to 6 hours. The medium was changed by 1 ml of DMEM supplemented with 10% FBS and antibiotics. The plate was incubated for 48-72 hours in the CO_2 incubator before extraction of genomic DNA.

[00135] DNA Extraction

[00136] 293T cells were detached from wells by performing up and down movements in 1 ml culture medium with a pipette. These cells were transferred in an Eppendorf tube and centrifuge at 8000 RPM for 10 minutes. The supernatant medium was carefully removed without disturbing the cell pellets. These cell pellets were washed once with 1 ml of HBSS solution and centrifuged at 8000 RPM for 10 minutes. The HBSS was then carefully removed without disturbing the cell pellets. The cells were lysed with 100 µl of lysis buffer containing 1% Sarkosyl and 0.5M EDTA pH 8 supplemented with 10 µl of proteinase K solution (20 mg/ml). These tubes were incubated at 50oC for 15 minutes. 400 µl of 50 mM Tris pH 8 were then added to each tube. Next, 500 µl of a mixture of phenol: chloroform: isoamyl alcohol (respectively 25:24:1) was added. The tubes were centrifuged at 16 000 RPM for 2 minutes. The aqueous upper phase was transferred to a new tube. 50 µl of NaCl 5 M were added to each tube and mixed thoroughly. One (1) ml of ice cold ethanol 100% was added to each tube and mixed for genomic DNA precipitation. The tubes were centrifuged at 16000 RPM for 7 minutes and ethanol was carefully removed to avoid disturbing the DNA pellets. These DNA pellets were washed once with 400 µl ethanol 70%. The tubes were centrifuged at 16000 RPM for 5 minutes and ethanol was removed to permit to dry the DNA pellet rapidly by lyophylisation. The DNA was solubilized in 50-100 µl of sterile water and stored at -20°C until quantification was performed. The DNA solutions were dosed at 260 nm with a spectrophotometer.

[00137] Genomic DNA PCR amplification

[00138] For PCR amplification of genomic DNA of 293T cells cotransfected with a patch/donor sequence and a gRNA/Cas9 plasmid the following mix was used: genomic DNA (25 ng/µl, 2 µl), 5X HF Phusion™ buffer (10 µl), dNTP 10 mM (1 µl), forward primer (100 ng/µl, 1 µl), reverse primer (100 ng/µl,1 µl), water (34.75 µl) and Phusion™ enzyme (2U/µl, 0.25 µl). PCR amplification was as follows: denaturation at 98oC for 30 sec. followed by (98oC-10 sec, 60 oC-20 sec. and 72 oC 45 sec. to 1 min) for 35 cycles; and 72 oC for 5 minutes as a final elongation cycle.

[00139] Surveyor™ nuclease assay for confirmation of genome modification

[00140] After transfection, the 293 T cells were incubated at 37°C for 72 hrs and genomic DNA was extracted as described above. The genomic region flanking the gRNA#8 target site in exon 16 of the APP gene was PCR amplified with different forward primers Bfw or Cfw and different reverse primers Brev or Crev using Phusion™ DNA Polymerase (New England Biolabs) as described in genomic DNA PCR amplification section. After the PCR amplification, 20 µl of unpurified PCR products were heated at 95°C for 5 minutes and slowly cooled down (5°C per 30 seconds) to 25°C using a thermocycler. After the formation of heteroduplexes, 1 µL of Surveyor™ enzyme (Transgenomics inc. Omaha, NE, USA) and 1 µL of enhancer in Phusion™ HF Buffer (NEB) were added with to each tube to obtain a 1X final concentration. The mixes were incubated at 42°C for 25 minutes. 5 µl of loading buffer were added to each tube. All Surveyor™ analysis were done on 2% agarose gels containing RedSafe™ Nucleic Acid staining solution (Froggobio inc., Toronto, ON, Canada) for UV visualization.

[00141] CRISPR construction in the pX330™ and pX458™ plasmids

[00142] The pX330™-U6-Chimeric_BB-CBh-hSpCas9 plasmid was purchased from Addgene inc (plasmid #42230 from Addgene). In order to clone the target sequence into the pX330™ (or pX458™ backbone), two oligos having the following sequences were purchased from IDT:

[00143] Oligo 1: 5' – CACCGNNNNNNNNNNNNNNNNNNN – 3' (SEQ ID NO: 110)

[00144] Oligo 2: 3' – CNNNNNNNNNNNNNNNNNNNCAAA – 5' (SEQ ID NO: 111)

[00145] These oligos are complementary, and their sequences correspond to the gRNA target site and will serve, once cloned into the plasmid to express the desired gRNA. The overhangs serve to clone the annealed oligos into the plasmid using BbsI sites. The following mixture was prepared: 1 µg of pX330™ with 1 µl FastDigest™ BbsI (Fermentas), 1 µl FastAPTM (Fermentas), 2 µl 10X FastDigestTM Buffer. The volume was completed to 20 µl with ddH2O. The mixture was incubated for 30 min at 37°C. The same protocol applies to the use of the p260 and p458 plasmids.

[00146] The pX260™, pX330™ or pX458™ plasmids were purified using QIAquick™ Gel Extraction Kit and eluted in the elution buffer.

[00147] To phosphorylate and anneal each pair of complementary oligos, the following procedure was used: 1 µl oligo 1 (1 µg), 1 µl oligo 2 (1 µg), 1 µl 10X T4 Ligation Buffer (NEB), 6.5 µl ddH_2O, 0.5 µl T4 PNK (NEB) for a total 10 µl. The oligos were annealed in a thermocycler using the following parameters: 37°C 30 min, 95°C 5 min and then ramp down to 25°C at 5°C/min.

[00148] The ligation reaction was done as follows: 50 ng of BbsI digested pX260™, pX330™ or pX458™ from step 2, 1 µl phosphorylated and annealed oligo duplex from step 3 (1:200 dilution), 5 µl 2X Quick ligation™ Buffer (NEB), 1 µl Quick Ligase (NEB) and ddH_2O to obtain a total volume of 11 µl. The mixture was incubated at room temperature for 10 min.

Table 3: Primer and gRNA guide sequences targeting the human APP gene

Location/target	Nucleotide position In genome	Nucleotide sequence of primers/gRNA 5' to 3'	SEQ ID NO:
3' end of intron 15	277141-278147		SEQ ID NO : 36
Cfw: in intron 15	277277-277300	CACGGTAGAGAAGATGACTTCAAA	SEQ ID NO: 38
Afw: in intron 15	277559-277577	GCTCTTTATTCAGCAGACG	SEQ ID NO: 39
Bfw: intron 15	277567-277588	TTCAGCAGACGAACCAATTACA	SEQ ID NO: 43
Exon 16	278149-278250		SEQ ID NOs: 31/32
Target of gRNA#8	278202-278220	CAGAATTCCGACATGACTC	SEQ ID NO: 15

5' end of intron 16	278251-279540		SEQ ID NO : 37
Brev: intron 16 (reverse)	278721-278745	CACGAACTTTGCTGCCTTGTAG	SEQ ID NO: 40
Arev: intron 16 (reverse)	278781-278803	GTGGAAGTCAAAGTGGCTGCTAT	SEQ ID NO: 41
Crev: intron 16 (reverse)	279245-279278	CCTCTTACTGCACCTACTGATAAG	SEQ ID NO: 42

[00149] The nucleotide positions indicated in Table 3 above correspond to the nucleotide positions in the human APP genomic sequence of NCBI reference sequence NG_007376.1.

EXAMPLE 2

gRNAs EFFICIENTLY TARGET THE HUMAN APP GENE IN CELLS

[00150] In an to attempt to modify the APP gene to reduce the formation of toxic Aβ peptides, fourteen different gRNAs targeting nucleotide sequences near the sequence coding for the alanine at position 673 in the APP gene were first prepared. The mutation A673T is located in exon 16 of the human APP gene. Figure 4 illustrates the nucleotide and amino acid sequences of exon 16 and Figure 5 illustrates the nucleotide sequence of part of intron 15, exon 16 and part of intron 16 of the human APP gene. Figure 4 also indicates the amino acid alanine (A) in position 673 that is to be changed to a threonine (T). Table 4 below, indicates the nucleotide sequences in intron 15, exon 16 and intron 16 in the human APP gene, which were targeted by these gRNAs. Each targeted sequence includes in 3' an NGG sequence, which is the protospacer adjacent motif (PAM), necessary for the formation of a complex between the gRNA, the DNA and the Cas9 nuclease used in the instant examples. The PAM sequence is not part of the gRNA expressed in cells.

Table 4: gRNAs target sequences in the human APP gene

gRNA #	Targeted sequence + PAM sequence (underlined)	Sense/ Antisense	Starting position	Ending position	SEQ ID NOs:	Location
1	5'ATTTATGAGTAAAACTAATTGG3'	Sense	278092	278113	SEQ ID NOs: 1, 2	Intron 15
2	5'TTTAATTATGATGTAATACAGG3'	Sense	278127	278148	SEQ ID NOs: 3, 4	In 15/Ex 16
3	5'TATGATGTAATACAGGTTCTGG3'	Sense	278133	278154	SEQ ID NOs: 5, 6	In 15/Ex 16
4	5'ATGATGTAATACAGGTTCTGGG3'	Sense	278134	278155	SEQ ID NOs: 7, 8	In 15/Ex 16
5	5'GGGTTGACAAATATCAAGACGG3'	Sense	278153	278174	SEQ ID NOs: 9, 10	Exon 16
6	5'TTGACAAATATCAAGACGGAGG3'	Sense	278156	278177	SEQ ID NOs: 11, 12	Exon 16

7	5'GAGATCTCTGAAGTGAAGATGG3'	Sense	278177	278198	SEQ ID NOs: 13, 14	Exon 16
8	5'CAGAATTCCGACATGACTCAGG3'	Sense	278202	278223	SEQ ID NOs: 15, 16	Exon 16
9	5'GAAGTTCATCATCAAAAATTGG3'	Sense	278228	278249	SEQ ID NOs: 17, 18	Exon 16
10	5'CCAAATGACCTATTAACTCTGG3'	Sense	278291	278312	SEQ ID NOs: 19, 20	Intron 16
11	3'GGATCTTTCTTCAAAACCCATC5'	Antisense	278049	278070	SEQ ID NOs: 21, 22	Intron 15
12	3'GGACGTATGAAATTAATACTAC5'	Antisense	278117	278138	SEQ ID NOs: 23, 24	Intron 15
13	3'GGCTGTACTGAGTCCTATACTT5'	Antisense	278209	278230	SEQ ID NOs: 25, 26	Exon 16
14	3'GGAGAAAGGTGATGACAAACAG5'	Antisense	278266	278287	SEQ ID NOs: 27, 28	Intron 16

Table 5: gRNA sequences corresponding to gRNA target sequences set forth in Table 4.

gRNA #	gRNA sequence	SEQ ID NO:
1	5' AUUUAUGAGUAAAACUAAU 3'	SEQ ID NO: 115
2	5' UUUAAUUAUGAUGUAAUAC 3'	SEQ ID NO: 116
3	5' UAUGAUGUAAUACAGGUUC 3'	SEQ ID NO: 117
4	5' AUGAUGUAAUACAGGUUCU 3'	SEQ ID NO: 118
5	5' GGGUUGACAAAUAUCAAGA 3'	SEQ ID NO: 119
6	5' UUGACAAAUAUCAAGACGG 3'	SEQ ID NO: 120
7	5' GAGAUCUCUGAAGUGAAGA 3'	SEQ ID NO: 121
8	5' CAGAAUUCCGACAUGACUCA 3'	SEQ ID NO: 122
9	5' GAAGUUCAUCAUCAAAAAU 3'	SEQ ID NO: 123
10	5' CCAAAUGACCUAUUAACUC 3'	SEQ ID NO: 124
11	5' CUACCCAAAACUUCUUUCU 3'	SEQ ID NO: 125
12	5' CAUCAUAAUUAAAGUAUGC 3'	SEQ ID NO: 126
13	5' UUCAUAUCCUGAGUCAUGU 3'	SEQ ID NO: 127

14	5' GACAAACAGUAGUGGAAAG 3'	SEQ ID NO: 128

[00151] Each gRNA targeting the APP gene was inserted into plasmid pX330™ (Plasmid 42230: pX330-U6-Chimeric_BB-CBh-hSpCas9 from Addgene) under the control of the U6 promoter and transfected in HEK 293T cells. This plasmid also codes for the humanized Cas9 nuclease (hSpCas9, Figure 6A and Figure 23), which cuts double stranded DNA at a specific site when a gRNA efficiently hybridizes with its target sequence. The insert coding for the gRNA in the plasmid corresponds to the gRNA target sequence on the genomic DNA without the PAM. The expressed gRNA includes the target sequence and a tracrRNA sequence (+85 nts) for efficient targeting efficiency/Cas9 recruitment. Once cut, the double stranded breaks in the DNA introduced by Cas9 are then spontaneously repaired by Non Homologous End Joining (NHEJ) resulting in micro-insertions or micro-deletions (INDELs) in the APP gene.

[00152] To detect INDELs resulting from Cas9/gRNA expression, cellular DNA was extracted and the APP gene amplified by PCR using primers Bfw and Brev (see Table 3). The amplicons were then heated at 95°C to separate the two DNA strands and slowly cooled at room temperature to permit the formation of mismatched DNA strands if INDELs were present. These miss-matched DNA were then cut with the Surveyor™ enzyme, resulting in additional bands in an agarose gel.

[00153] As shown in Figure 6C, incubation of amplified APP fragments with the Surveyor™ enzyme resulted in the presence of an additional band on the agarose gel for all gRNAs tested. Some gRNAs were however more efficient than others in cutting the target sequence. These results indicate that transfection of human cells with the various pX330™ plasmids can efficiently target and induce double strand breaks in the APP gene. These breaks were spontaneously repaired by Non Homologous End Joining (NHEJ) resulting in micro-insertions or micro-deletions (INDELs) in the APP gene.

[00154] The gRNA#8 (Figure 5B, Tables 3 and 4, SEQ ID NO: 15) was selected for further investigation but other gRNAs were shown to be effective and may be used in accordance with the present invention.

EXAMPLE 3

Endogenous APP gene and pMini-Patch plasmid are efficiently targeted by the CRISPR system in human cells

[00155] The next step toward modifying the APP gene to reduce toxic Aβ production is to make a donor DNA that can be used to modify the gene by homologous recombination (Figure 7). The donor DNA (called pMiniT-Patch) prepared contained about 500 nucleotides of the APP gene (homology sequences) on each side of the sequence

targeted by the gRNA#8 (see Figures 7A and B). This donor DNA also contained a mutation in the codon encoding amino acid 673 of the APP gene, which replaced the alanine (A) by a threonine (T). The donor DNA also contained modifications in some of the nucleotides of exon 16 to prevent the APP gene from being cut again by gRNA#8/Cas9 after its modification by Homologous Directed Repair (HDR, see Figures 4B and 7B). One nucleotide of intron 16 of the APP gene was also modified to a G in pMiniT-Patch to modify the APP gene sequence to ACTA**G**T, which can be cut by the restriction enzyme Spe1 (Figure 7B). This is to permit a rapid detection of the corrected APP gene. Finally, the pMiniT-Patch also contained at each end of the homology sequence used for repair of the APP gene, the sequence targeted by the gRNA#8. This is to permit linearization of the pMiniT-Patch plasmid and to separate the correcting oligonucleotide from the backbone of the plasmid (see Example 1 for more details about plasmid constructions). The pMiniT-patch plasmid allows replacing whole Exon 16 in the APP gene, together with portions of intron 15 and intron 16.

[00156] Human cells (HEK 293T) were next transfected with the pMiniT-Patch plasmid described above and with various amounts of the pX330™ plasmid (from 5 µg to 1µg) containing or not the gRNA#8 and containing or not the nucleic acid sequence encoding a Cas9 nuclease. Genomic DNA was extracted from the cells 72 hours post-transfection.

[00157] The APP gene was amplified with primers Afw and Arev (see positions of primers in Figure 8B and C and Table 3) resulting in a 1245 bp amplicon. Next, a Surveyor™ enzyme test on the PCR product obtained by amplification of the APP gene was performed. As expected, transfection of cells with the pMiniT-Patch plasmid alone did not result in the presence of any additional amplification product and thus is unable, alone (i.e., without the presence of Cas9 and gRNA) to introduce a mutation in the APP gene (Figure 9B, lane 1). However, digestion of the PCR product obtained on DNA extracted from cells transfected with decreasing amounts (from 5 µg to 1µg) of pX330™ (coding for gRNA#8 and Cas9) with the Surveyor™ enzyme resulted in the presence of 2 additional bands (602 bp and 643 bp) (Figure 9B). These results demonstrate that the gRNA#8 can produce either micro-insertions or micro-deletions (INDELs) or Homologous Directed Repair (HDR) in the APP gene (Lanes 2-5 of Figure 9). The presence of additional bands reflects the ability of gRNA#8 and Cas9 encoded by the pX330™ plasmid to target and cut the endogenous APP gene. The cuts are later repaired by Non Homologous End Joining (NHEJ) and are detected by the Surveyor™ enzyme.

[00158] Co-transfection of various ratios of pMiniT-Patch and pX330™ plasmids (4:1 µg, 2.5:2.5 µg and 1:4 µg of pMiniT-Patch: pX330™) also resulted in the presence of additional bands due to the cut of the APP gene by the gRNA#8 and the Cas9, repaired either by NHEJ or by Homologous Directed Repair (HDR) with the pMiniT-Patch (Figure 9B, lanes 6-8).

[00159] The DNA of the human cells transfected or not with pMiniT-Patch and pX330™ (coding for gRNA#8 and

Cas9) was also amplified with primers MiniTfw and Brev or with MiniTrev and Bfw (see position and sequence of these primers in Figure 7), which specifically amplify the donor plasmid but not the genomic DNA. The amplicons (respectively 1339 bp and 1317 bp) were incubated with the Surveyor™ enzyme. This generated in each case 2 fragments (respectively 150 pb + 1189pb and 125 pb + 1192pb) (Figure 10). These results demonstrate that the donor plasmid (pMiniT-Patch) was cut by the gRNA#8/Cas9 combination. These cuts were produced because of the presence of the target sequence of the gRNA#8 on both sides of the patch inserted in the pMiniT-Patch donor plasmid. These two gRNA target sequences were inserted in the donor plasmid to permit its linearization by the combined action of the gRNA#8 and Cas9 nuclease. This linear fragment is expected to favor homologous recombination with genomic DNA following a cut in the targeted APP DNA by the CRISPR system (i.e., gRNA#8 and Cas9 nuclease).

EXAMPLE 4

The pMiniT-Patch plasmid/gRNA/Cas9 combination efficiently introduce a protective mutation in the endogenous APP gene of human cells

[00160] The presence of mutations in the APP gene introduced by the pMiniT-Patch plasmid/gRNA#8/Cas9 combination was confirmed by amplifying DNA of human cells transfected or not with pMiniT-Patch and pX330™ (coding for gRNA#8 and Cas9) with 3 different sets of primers: 1) IRMUfw and Arev, 2) Cfw and ILMUrev and 3) IRMUfw and Crev (see position and sequence of these primers in Figure 8B and Table 3). The ILMUrev and the IRMUfw can only hybridize with the mutated sequence of APP exon 16 present in the donor plasmid (i.e., pMiniT-Patch) and in the mutated genomic DNA but not with the wild type APP gene. On the contrary, the Arev, Cfw and Crev primers only hybridize with APP gene sequences, which are not present in the pMiniT-Patch plasmid. Thus, DNA amplification with these pairs can only be obtained if the APP gene has been modified by Homology Directed Repair with the donor DNA.

[00161] Amplification with all 3 pairs of primers on DNA collected 72 hours post-transfection resulted in the presence of amplicons of expected sizes of 608 bp (primers IRMUfw and Arev-Figure 11), 947bp (primers Cfw and ILMUrev Figure 12) and 1073 bp (IRMUfw and Crev Figure 13) suggesting that the protective A673T mutation was efficiently introduced in the APP gene. These amplicons were cloned in the pMiniT plasmid and sequenced. The sequencing results are presented in Figures 14, 15 and 16.

[00162] The mutated sequences amplified with the IRMUfw and Arev primer pair and the IRMUfw and Crev primer pair contained the additional Spe1 site introduced with the pMiniT-Patch plasmid at position 161 and 85 of the amplicon respectively. These sequences confirmed the presence of the mutations (A673T and Spe1 site) in the APP gene. Since the pMiniT-Patch donor plasmid contained the codons for all the wild type amino acids of exon 16 of APP

(with the exception of the A673T mutation), the homologous recombination thus also permits to correct any mutation present in the APP gene and responsible for a familial form of Alzheimer's disease.

[00163] The results of these 3 amplification and sequencing experiments demonstrated that the APP gene can be efficiently modified by Homology Directed Repair using the Patch/CRISPR system of the present invention. The sequencing experiments clearly confirm that the APP gene was appropriately corrected in the cell genome.

Example 5

An ssODN encoding a portion of the APP gene and a PCR amplicon from the pMiniT-Patch plasmid efficiently introduce a protective mutation in the endogenous APP gene of human cells

[00164] The APP gene may also be corrected by transfecting the gRNA#8 and the Cas9 with a donor sequence, which is smaller than the pMiniT-Patch. Two different donor DNAs were used: 1) a single stranded oligodeoxynucleotide (ssODN, 199 nucleotides in length, Figure 17A, SEQ! ID NO: 59) synthetized by IDT (Integrated DNA Technologies inc.) and 2) an amplicon obtained by amplifying a 1179 bp sequence of pMiniT-Patch with primers Bfw and Brev (SEQ ID NO: 60). The 293T cells were transfected with the pX330™ (gRNA#8 and hSpCas9) and with the ssDNA or the amplicon. The presence of mutations in the APP gene induced by both types of donor was confirmed by amplifying the genomic DNA with primers Cfw and ILMUrev (Figure 17B, lane 2 for the amplicon and lanes 3 and 4 for the ssODN). The presence of the corrections in the APP gene was confirmed in both cases by cloning and sequencing of the PCR product as described in Example 4.

Example 6

Modification of the endogenous APP polynucleotide gene using a FLAG donor sequence or FLAG-STOP donor sequence

[00165] The APP gene may also be modified by introducing a FLAG sequence into exon 16. This was achieved by modifying the pMiniT-Patch to introduce only the FLAG in exon 16 (pMiniT-Patch-FLAG, Figure 18) or a FLAG followed by a stop codon (pMiniT-Patch-FLAG-Stop, Figure 19). The 293T cells were transfected with plasmids pX330™ and the pMiniT-Patch-Flag or the pMiniT-Patch-Flag-Stop. The cell DNA was amplified 72 hours post-transfection with primers Cfw and ILMUrev. The presence of a PCR product confirmed that the APP gene had been modified by homologous recombination (Figure 17B lanes 5 and 6 respectively). The presence of the recombination was confirmed by cloning and sequencing the PCR products.

[00166] 293T cells were transfected with various plasmids: well # 1: 400 ng of px330 (gRNA#8 and the Cas9 gene) and 400 ng of pMiniT-Patch-FLAG, well #2 : 400 ng of px330 (gRNA#8 and the Cas9 gene) and 400 ng of pMiniT-Patch-FLAG-STOP, well # 3: only 400 ng of px330 (gRNA#8 and the Cas9 gene) (no donor DNA) and well #

4: only 400 ng of the donor DNA (pMiniT-Patch-FLAG) but no px330 (gRNA#8 and the Cas9 gene). The genomic DNA was extracted 72 hours post-transfection and the APP gene was amplified with Cfw and Crev. The PCR product of 1993 bp was digested with Spe1 and migrated on a 2% agarose gel stained with Redsafe™. Two additional bands (1002 and 991 bp) were detected in lanes 1 and 2 (Figure 20) confirming that the APP gene had been mutated by HDR resulting in the insertion of the new Spe1 site, which was not present when the donor DNA (pMiniT-Patch-FLAG or pMiniT-Patch-FLAG-STOP) or the gRNA#8 and the Cas9 gene were absent.

[00167] The PCR amplification of amplicons of expected sizes and the sequencing results demonstrated that the APP gene has been modified by Homology Directed Repair with the pMiniT-Patch, pMiniT-Patch-FLAG and pMiniT-Patch-FLAG-STOP. All the sequencing results clearly confirm that the APP gene was corrected in the cell's genome.

Example 7

The β-secretase-dependent C99 fragment is not produced by cells harboring the A673T mutation in both APP alleles

[00168] 293T cells were nucleofected with plasmid pX458™ (pSpCas9(BB)-2A-GFP), (gift from Feng Zhang, purchased from Addgene plasmid # 48138, see Figure 24), coding for the gRNA#8 and the Cas9-2A-eGFP, and with the plasmid pMiniT-Patch described above. The pX458™ plasmid was prepared and tested as described in Example 1 for the px330 plasmid. The fluorescent green cells were separated by FACS and individual cells were deposited in each well of a 96 wells plate. The cell clones were expanded. The DNA and the proteins were extracted from individual clones. Part of intron 15 to intron 16 of the APP gene was amplified by PCR using primers Cfw and Brev (see sequence in attachment). The amplicons were first digested with EcoR1 enzyme, which cuts only wild type APP gene, and with the Spe1 enzyme, which cuts only the mutated APP gene. Clones that were cut by both enzymes are clones in which only one of the two APP alleles was mutated. Only clones, which amplicons were not cut by EcoR1 but cut by Spe1, were kept for further investigation because this cutting pattern occurs only when both APP alleles are mutated. The amplicon of such a clone was cloned in the pMiniT plasmid and sequenced. The sequence confirmed the presence of the A673T and of the Spe1 mutation in that clone (see Figure 25). The cells of this mutated clone and a normal cell population were transfected with a plasmid coding for the β-Secretase enzyme. This plasmid was obtained by amplifying a human brain cDNA library and the sequence is identical to the BACE1 beta-site APP-cleaving enzyme 1 (ref NM_0121104.4). The transfected cells were also grown with or without the presence of a γ-Secretase inhibitor (L-685,458 Tocris Bioscience #2627). Control cells (no inhibitor) were not transfected with the β-secretase plasmid and not incubated with the γ-Secretase inhibitor. The proteins were extracted from the cells 48 hours after the transfection. A western blot was made using a mAb (Sigma #A8717) against the C terminal of the APP protein.

[00169] In normal cells, the C99 fragment was detected and increased when the γ-Secretase inhibitor prevented its degradation (Figure 26, lanes 1 and 2). When the β-Secretase plasmid was transfected in normal cells to increase the cutting of the mutated APP protein by this enzyme, the C99 fragment was also detected (Figure 26, lane 3). This C99 fragment was however detected more abundantly when the normal cells transfected with the β-Secretase plasmid were incubated with the γ-Secretase inhibitor to permit the accumulation of the fragment (Figure 26, lane 4). In the cells that contained the A673T mutation, the C99 fragment was not detected, even when the cells were incubated with the γ-Secretase inhibitor (Figure 26, lanes 5 and 6). This indicated that the C99 fragment resulting form the β-Secretase cutting was rare and not detected even when the γ-Secretase inhibitor prevented its degradation. When the β-Secretase plasmid was transfected in these mutated cells to increase the cutting of the mutated APP protein by this enzyme, the C99 fragment was not detected (Figure 26, lane 8). This C99 fragment was however detected when the mutated cells transfected with the β-Secretase plasmid were incubated with γ-Secretase inhibitor to permit the accumulation of the fragment (Figure 26, lane 7). This experiment demonstrated that the APP protein, containing the A673T mutation following by homologous recombination induced the cutting of the APP gene by the CRISPR system, is less frequently cut by the β-Secretase enzyme than the wild type APP protein.

[00170] The scope of the claims should not be limited by the preferred embodiments set forth in the examples, but should be given the broadest interpretation consistent with the description as a whole.

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CLAIMS

1. A method of decreasing Amyloid Precursor Protein (APP) processing into Aβ peptide by a cell comprising providing said cell with:

(iv) at least one guide RNA (gRNA) comprising:

(c) a gRNA guide sequence comprising a seed region of at least 10 consecutive nucleotides of a target sequence in an endogenous APP gene polynucleotide sequence present in said cell;

(d) a Cas9 recognition sequence,

wherein the target sequence of the gRNA guide sequence is contiguous to a protospacer adjacent motif (PAM) in the endogenous APP gene polynucleotide sequence and wherein said PAM is recognized by a ribonucleoprotein complex comprising a Cas9 nuclease or nickase;

(v) a Cas9 nuclease or nickase or a nucleic acid encoding a Cas9 nuclease or nickase; and

(vi) a donor nucleic acid comprising an APP polynucleotide gene sequence or fragment thereof, which comprises at least one modification with respect to the endogenous APP polynucleotide gene sequence present in said cell,

wherein said donor nucleic acid is integrated in said APP gene polynucleotide sequence of said cell and wherein said modification decreases the amount of Aβ produced by said cell.

2. The method of claim 1, wherein said Cas9 nuclease or nickase is a hSpCas9 nuclease, a hSaCas9 nuclease, a hSpCas9 nickase, a hSaCas9 nickase or a dCas9-FokI nuclease.

3. The method of claim 1 or 2, wherein the donor nucleic acid is a single stranded oligodeoxynucleotide (ssODN) or a PCR amplicon.

4. The method of claim 1 or 2, wherein the donor nucleic acid is comprised in a vector.

5. The method of any one of claims 1-4, wherein the donor nucleic acid further comprises on each side of the APP polynucleotide gene sequence or fragment thereof, a target sequence recognized by a gRNA for cutting said donor nucleic acid in the presence of said gRNA and said Cas9 nuclease or nickase.

6. The method of any one of claims 1-4, wherein said donor nucleic acid comprises a modification which introduces an additional endonuclease restriction site when the donor nucleic acid is integrated in the endogenous APP polynucleotide gene sequence in said cell.

7. The method of any one of claims 1-6, wherein the donor nucleic acid comprises a modified APP polynucleotide gene fragment encoding exon 16, or a fragment thereof.

8. The method of 7, wherein the donor nucleic acid comprises a modified APP polynucleotide gene fragment comprising at least part of intron 15, exon 16, and at least part of intron 16 of the endogenous APP polynucleotide sequence.

9. The method of any one of claims 1-8, wherein said at least one modification is located in Exon 16 of the APP polynucleotide gene sequence.

10. The method of claim 9, wherein said at least one modification in said donor nucleic acid results in an APP polynucleotide gene sequence encoding an APP protein comprising at least one amino acid substitution between amino acid positions 656 and 688, wherein said amino acid positions are with respect to the APP protein sequence as set forth in SEQ ID NO: 30 (Figure 3).

11. The method of claim 10, wherein said at least one amino acid substitution corresponds to at least one substitution at amino acid position 670, 671, 673, 678, 682, 692, 603, 694, 704, 711, 712, 713, 714, and/or 715 of the APP protein.

12. The method of claim 10, wherein the at least one amino acid substitution introduces a threonine at position 673.

13. The method of any one of claims 1-6, wherein said at least one modification in said donor nucleic acid results in a modification of one or more amino acids recognized by an α, β, or γ secretase in the APP protein encoded by said APP polynucleotide gene sequence present in said cell.

14. The method of any one of claims 1-13, wherein said at least one modification corrects a mutation associated with an increased risk of developing Alzheimer's disease present in said endogenous APP polynucleotide gene sequence present in said cell.

15. The method of any one of claims 1-14, wherein said cell is from a subject having at least one family member which has been diagnosed with Alzheimer's disease.

16. The method of any one of claims 1-14, wherein said cell is from a subject having at least one mutation associated with early onset Alzheimer's disease.

17. The method of any one of claims 1-16, wherein the guide RNA (gRNA) sequence consists of at least 20 contiguous nucleotides of the target sequence in the endogenous APP polynucleotide gene.

18. The method of claim 17, wherein the PAM is a NGG trinucleotide-sequence or a NNGRR nucleotide sequence..

19. The method of any one of claims 1-18, wherein the target sequence of said gRNA is located in an exon of the APP polynucleotide gene sequence encoding the APP protein.

20. The method of any one of claims 1-18, wherein the target sequence of said gRNA is located in intron 15, exon 16 or intron 16 of the endogenous APP polynucleotide gene sequence present in said cell.

21. The method of any one of claims 1-18, wherein the target sequence is located between (i) nucleotide 277141 and nucleotide 279540; (ii) nucleotide 277567 and nucleotide 27845; (iii) nucleotide 277141 and nucleotide 278147; (iv) nucleotide 278149 and nucleotide 278250; (v) nucleotide 278251 and nucleotide 279540; (vi) nucleotide 277277 and nucleotide 278147; (vii) nucleotide 277559 and nucleotide 278147; (vii) nucleotide 277567 and nucleotide 278147; (viii) nucleotide 278149 and nucleotide 278220; (ix) nucleotide 278202 and nucleotide 278250; (x) nucleotide 278721 and nucleotide 279540; (xi) nucleotide 278781 and nucleotide 279540; (xii) nucleotide 279245 and nucleotide 279540 of the APP polynucleotide gene sequence set forth in Figure 5, or the complement thereof, or in a corresponding location in the endogenous APP polynucleotide gene sequence present in said cell.

22. The method of any one of claims 1-21, wherein the target sequence of said gRNA comprises the following nucleic acid sequence:

(i) 5'-ATTTATGAGTAAAACTAAT-3 (SEQ ID NO: 1)';
(ii) 5'-TTTAATTATGATGTAATAC-3'(SEQ ID NO: 3);
(iii) 5'-TATGATGTAATACAGGTTC-3'(SEQ ID NO: 5);
(iv) 5'-ATGATGTAATACAGGTTCT-3'(SEQ ID NO: 7);
(v) 5'-GGGTTGACAAATATCAAGA-3'(SEQ ID NO: 9);
(vi) 5'-TTGACAAATATCAAGACGG-3'(SEQ ID NO: 11);
(vii) 5'-GAGATCTCTGAAGTGAAGA-3'(SEQ ID NO: 13);
(viii) 5'-CAGAATTCCGACATGACTCA-3'(SEQ ID NO: 15);
(ix) 5'-GAAGTTCATCATCAAAAAT-3'(SEQ ID NO: 17);
(x) 5'-CCAAATGACCTATTAACTC-3'(SEQ ID NO: 19)

of the human APP polynucleotide gene sequence having reference number NCBI NG_007376.1.

23. The method of any one of claims 1-21, wherein said target sequence of said gRNA comprises the following nucleic acid sequence:

(i) 5'- CTACCCAAAACTTCTTTCT-3' (SEQ ID NO: 21);

(ii) 5'- CATCATAATTAAAGTATGC-3' (SEQ ID NO: 23);

(iii) 5'- TTCATATCCTGAGTCATGT-3' (SEQ ID NO: 25); or

(iv) 5'- GACAAACAGTAGTGGAAAG-3' (SEQ ID NO: 27)

of the complementary strand of human APP polynucleotide gene sequence having reference number NCBI NG_007376.1.

24. The method of claim 22, wherein said method comprises providing the cell with a gRNA targeting the nucleic acid sequence CAGAATTCCGACATGACTC corresponding to nucleotide positions 278202 to 278220 of the human APP polynucleotide gene sequence having reference number NCBI NG_007376.1.

25. The method of any one of claims 1-24, wherein said at least one modification within said endogenous APP polynucleotide gene sequence of said cell is introduced by Non-Homologous End Joining.

26. The method of any one of claims 1-24, wherein said at least one modification within said endogenous APP polynucleotide gene sequence of said cell is introduced by homologous recombination between said donor nucleic acid and said endogenous APP polynucleotide gene sequence.

27. A gRNA as defined in any one of claims 1 and 17 to 24.

28. A donor nucleic acid as defined in any one of claims 1 and 3 to 14.

29. A vector comprising a nucleic acid encoding the gRNA of claim 27 and/or the donor nucleic acid of claim 28.

30. The vector of claim 29, further comprising a nucleic acid encoding a Cas9 nuclease or nickase.

31. The vector of claim 29 or 30, wherein said vector is a viral vector.

32. The vector of claim 31, wherein said viral vector is a lentiviral vector, Adeno-Associated viral vector, adenovirus viral vector or herpes virus viral vector.

33. A composition comprising :

i) the gRNA of claim 27,

ii) the donor nucleic acid of claim 28;

iii) the vector of any one of claims 29-32; or

iv) any combination of (i) to (iii); and

and a pharmaceutically acceptable carrier.

34. The composition of claim 33, further comprising a Cas9 nuclease or nickase, or a vector comprising a nucleic acid encoding said Cas9 nuclease or nickase.

35. The composition of claim 33, wherein said Cas9 nuclease or nickase is a hSpCas9, a hSaCas9, a Cas9 nickase or a dCas9-FokI nuclease.

36. The gRNA of claim 27, the donor nucleic acid of claim 28, the vector of any one of claims 29 to 32 and/or the composition of any one of claims 33-35 for decreasing Amyloid Precursor Protein (APP) processing into Aβ peptide by a cell.

37. The gRNA of claim 27, the donor nucleic acid of claim 28, the vector of any one of claims 29 to 32 and/or the composition of any one of claims 33-35 for treating or preventing Alzheimer's disease or age-related cognitive decline in a subject in need thereof .

38. The gRNA of claim 27, the donor nucleic acid of claim 28, the vector of any one of claims 29 to 32 and/or the composition of any one of claims 33-35 for use in the preparation of a medicament for decreasing Amyloid Precursor Protein (APP) processing into Aβ peptide by a cell.

39. The gRNA of claim 27, the donor nucleic acid of claim 28, the vector of any one of claims 29 to 32 and/or the composition of any one of claims 33-35 for use in the preparation of a medicament for treating or preventing Alzheimer's disease or age-related cognitive decline in a subject in need thereof .

40. The gRNA of claim 27, the donor nucleic acid of claim 28, the vector of any one of claims 29 to 32 and/or the composition of any one of claims 33-35 for decreasing Amyloid Precursor Protein (APP) processing into Aβ peptide by a cell.

41. The gRNA of claim 27, the donor nucleic acid of claim 28, the vector of any one of claims 29 to 32 and/or the composition of any one of claims 33-35 for preventing or treating Alzheimer's disease or age-related cognitive decline in a subject in need thereof.

42. Use of the gRNA of claim 27, the donor nucleic acid of claim 28, the vector of any one of claims 29 to 32 and/or the composition of any one of claims 33-35 in the preparation of a medicament for decreasing Amyloid Precursor Protein (APP) processing into Aβ peptide by a cell.

43. Use of the gRNA of claim 27, the donor nucleic acid of claim 28, the vector of any one of claims 29 to 32 and/or the composition of any one of claims 33-35 in the preparation of a medicament for treating or preventing Alzheimer's disease or age-related cognitive decline in a subject in need thereof .

44. The use of claim 41 or 43, wherein said subject in need thereof is a subject having at least one family member which has been diagnosed with Alzheimer's disease.

45. The use of claim 41 or 43, wherein said subject in need thereof is subject having at least one mutation associated with early onset Alzheimer's disease.

46. The use of claim 42, wherein said cell is from a subject having at least one family member which has been diagnosed with Alzheimer's disease.

47. The use of claim 42, wherein said cell is from a subject having at least one mutation associated with early onset Alzheimer's disease.

48. A method of decreasing Amyloid Precursor Protein (APP) processing into Aβ peptide by a cell comprising introducing at least one modification within an endogenous polynucleotide target gene sequence of said cell, wherein said modification decreases the amount of Aβ peptide produced by said cell and wherein said endogenous polynucleotide target gene encodes a protein that regulates Aβ-peptide production.

49. The method of claim 48, wherein said target gene encodes an α-secretase, a β-secretase or a γ-secretase.

50. A method of preventing or treating Alzheimer's disease or age-related cognitive decline in a subject in need thereof, comprising introducing into or contacting a cell of the subject with the gRNA of claim 27, the donor nucleic acid sequence of claim 28, the vector of any one of claims 29 to 32 and/or the composition of any one of claims 33-35.

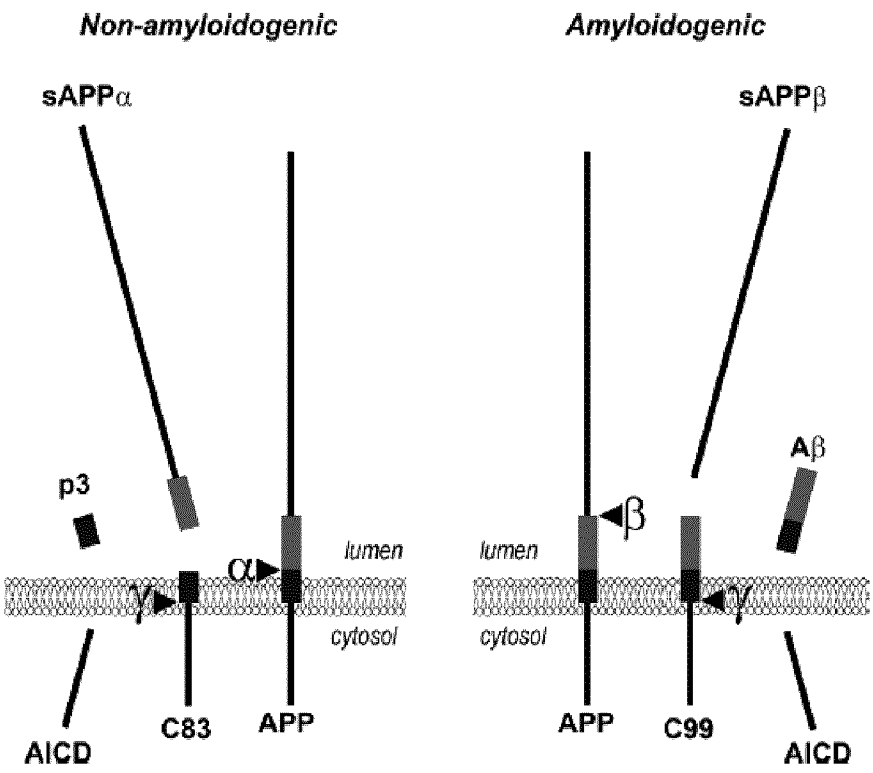
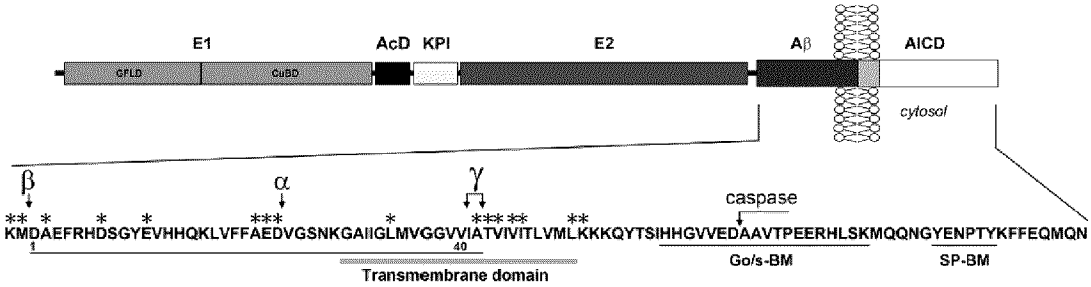

Non-amyloidogenic
Amyloidogenic
sAPPα
sAPPβ
p3
Aβ
α
β
γ
γ
lumen
lumen
cytosol
cytosol
AICD
C83
APP
APP
C99
AICD

Figure 1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1
40
Transmembrane domain
Go/s-BM
SP-BM

Figure 2

MLPGLALLLLAAWTARALEVPTDGNAGLLAEPQIAMFCGRLNMHMNVQNGKWDSDPSGTKTCIDTKEGILQYCQEVYP
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GADSVPANTENEVEPVDARPAADRGLTTRPGSGLTNIKTEEISEVKMD**A**EFRHDSGYEVHHQKLVFFAEDVGSNKGAI
IGLMVGGVVIATVIVITLVMLKKKQYTSIHHGVVEVDAAVTPEERHLSKMQQNGYENPTYKFFEQMQN

Figure 3

A

```
Gt tct ggg ttg aca aat atc aag acg gag gag atc tct gaa gtg aag
    S   G   L   T   N   I   K   T   E   E   I   S   E   V   K
              target of gRNA#8
atg gat gca gaa ttc cga cat gac tca gga tat gaa gtt cat cat caa
 M   D   A   E   F   R   H   D   S   G   Y   E   V   H   H   Q
aaa ttg
 K   L
```

B

```
Gt tct ggg ttg aca aat atc aag acg gag gag atc tct gaa gtg aag
    S   G   L   T   N   I   K   T   E   E   I   S   E   V   K

atg gat AcG gaG ttT AgG caC gaT tca gga tat gaa gtt cat cat caa
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aaa ttg
 K   L
```
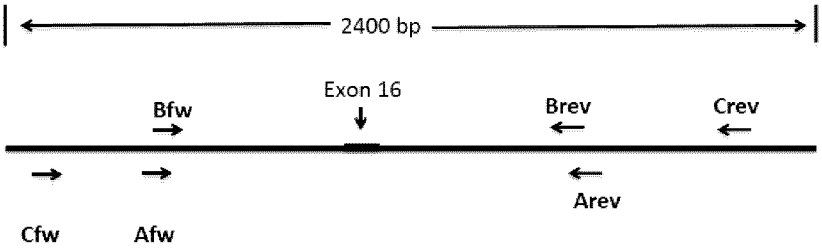

Figure 4

A



B

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                                  Cfw
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277441 tgaaattcat cagaacaatt agtgttaaga atcatatagc aatttataga aaaggaagag
277501 ttcgtaggtt ataaattctg ttagttgcta agaagcattt ttaaaattat gtactatagc
          Afw /Bfw
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278041 acttcaggcc tagaaagaag ttttgggtag gctttgtctt acagtgttat tatttatgag
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                                                          gRNA#8
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                                     Arev
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                    Crev
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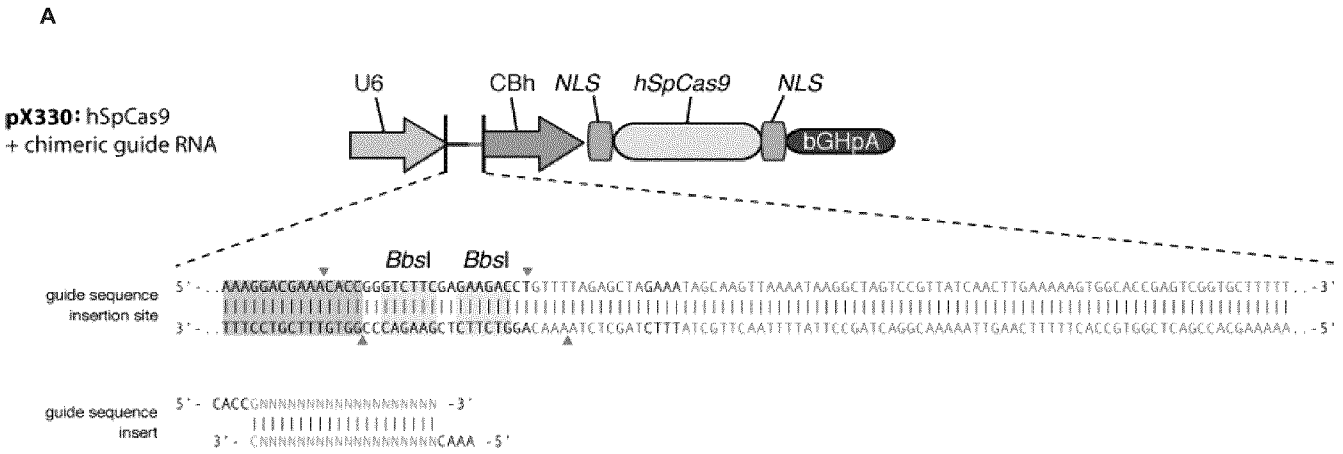
Figure 5


A
pX330: hSpCas9
+ chimeric guide RNA
U6
CBh
NLS
hSpCas9
NLS
bGHpA
BbsI
BbsI
guide sequence
insertion site
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3'-..TTTCCTGCTTTGTGGCCCAGAAGCTCTTCTGGACAAAATCTCGATCTTTATCGTTCAATTTTATTCCGATCAGGCAAAAATTGAACTTTTTCACCGTGGCTCAGCCACGAAAAA..-5'
guide sequence
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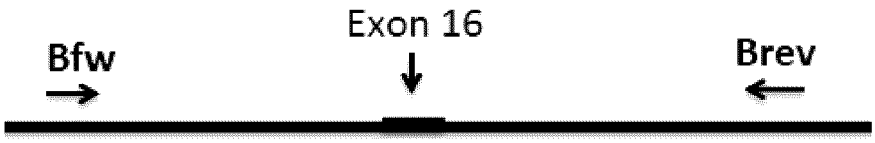
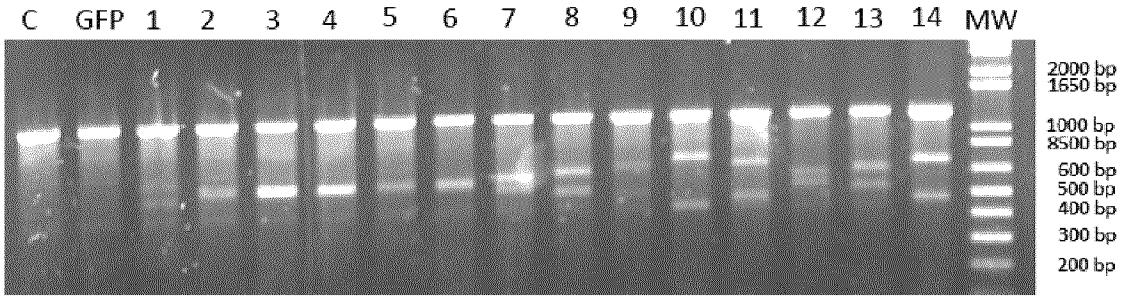
Figure 6A

B



C



Figures 6B and 6C

A

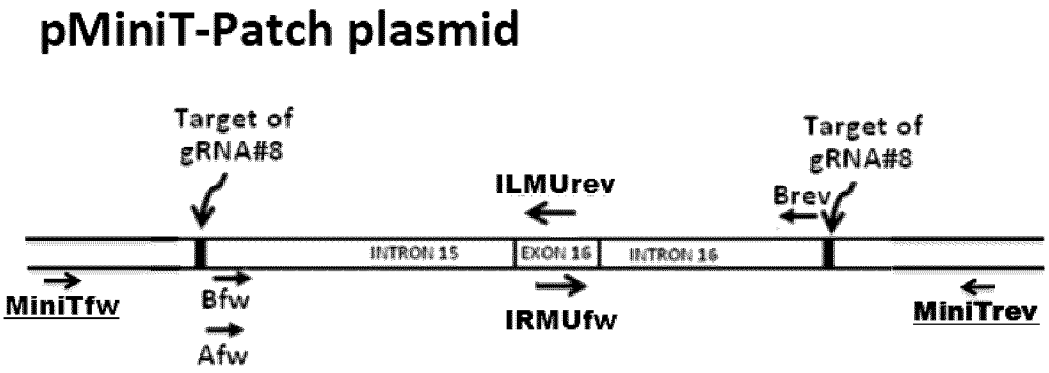

pMiniT-Patch plasmid
Target of gRNA#8
ILMUrev
Target of gRNA#8
Brev
INTRON 15
EXON 16
INTRON 16
MiniTfw
Bfw
Afw
IRMUfw
MiniTrev

Figure 7A

CCGGTTCAGACAGGATAAAGAGGAAAGAATGTTAGACAACACCCGCTTACGCATAGCTATTCAGAAATCAGGCCGTTTAAGC
GATGATTCACGAGAATTGCTGGCCCGCTGCGGCATAAAAATTAATTTACACACTCAGCGCTGATGAATCCCCTAATGATTTT
GGTAAAAATCATTAAGTTAAGGTGGACACACATCTTGTCATATGATTAAATGGTTTCGCGAAAAATCAATAATCAGACAACA
AGATGTGCGAACTCGATATTTTACACGACTCTCTTTACCAATTCTGCCCCGAATTACACTTAA

AACGACTCAACAGCTTAACGTTGGCTTGCCACGCATTACTTGACTGTAAAACTCTCACTCTTACCGAACTTGGCCGTA

MiniTfw

ACCTGCCAACCAAAGCGAGAACAAAACATAACATCAAACGAATCGACCGATTGTTAGGTAATCGTCACCTCCACAAAG

gRNA#8target

AGCGACTCGCTGTATCGCTCGAGGGATCCGAATTCAGGAGGTAAAAACCATGAT**ATAGCAGAATTCCGACATGACTCA**

Bfw

GGTTCAGCAGACGAACCAATTACAATCTGTGTAACTAGAACACTTGACTAAAATTATATAATTTTTACAACGCTTCACTGCA
TAGATACATGAACATAATTTATTTGTAATTGGAACAAAGCCCCAAAGTAGCAGTTTTGTTCTACCAGGTAATTAATGCTCAT
TTTTAAAGCCTTTTATTATTATTTCTGAAGTAATGAGTGCACATGGAAAAAGACACATAATAGGCTAAACAATAAGCCCGTA
AGCCAAGCCAACATATTCCAGGAACAAATCCTTGCCAACCTCTCAACCAGGATTTAACTTCTGCTTTTCCCCCATTTTCAAA
AATTATAGCATGTATTTAAAGGCAGCAGAAGCCTTACTTTCAGGTTTCCCTTACCCTTTCATTTCTTTTTGTTCAAAATAGG
TAGTAATTGAAGTTTTAAATATAGGGTATCATTTTTCTTTAAGAGTCATTTATCAATTTTCTTCTAACTTCAGGCCTAGAAA
GAAGTTTTGGGTAGGCTTTGTCTTACAGTGTTATTATTTATGAGTAAAACTAATTGGTTGTCCTGCATACTTTAATTATGAT
GTAATACAG*GTTCTGGGTTGACAAATATCAAGACGGAGGAGATCTCTGAA*

IRMUfw

*GTGAAGATGGATaCgGAgTTtaGgCAcGAtTCAGGATATGAAGTTCATCATCAAAAATTGG*TACGTAAAATAATTTAC

ILMUrev

New Spe1

CTCTTTCCACTAGTGTTTGTCTTGCCAAATGACCTATTAACTCTGGTTCATCCTGTGCTAGAAATCAAATTAAGGAAAAGAT
AAAAATACAATGCTTGCCTATAGGATTACCATGAAAACATGAAGAAAATAAATAGGCTAGGCTGAGCGCAGTGGCTCAAGCC
TGTAATCCCAGCACTTTGGGAGGCCAAGGCGGGTGGATCACGAGGTCAGAAATTCGAGACCAGCCTGGCCAATATGGTGAAA
CCCCATCTNTACTAAAAATACAAAAAAGATTAGCTGGGTGTGGTGGCAAACNCCTGTAGTCCCAGCTGCTGGGGAGGCTGAC
GCAGGAGACTTGCTTGAACCCAGGAGGTGGAGGTTGCAGTGAGCTGAGATCGTGCCTAGGCGACAGAGCGAGACTCCATCCC
AAAAAAAAAAAAAGAAAAGAAAGAGGCTGTATGTATAGTTCTTTCAGACTACAAGGCA

gRNA#8target

GCAAAGTTCGTGCAGAATTCCGACATGACTCAGGACTGATAATAATGACGTCAGAATTCTCGAGTCGGGGAAATGTGC

MiniTRev

GCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCTTCA
ATAATATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCT
GTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGG
ATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTATG
TGGCGCGGTATTATCCCGTATTGACGCCGGGCAAGAGCAACTCGGTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAG
TACTCACCAGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATA
ACACTGCGGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACAACATGGGGGATCATGT
AACTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACCACGATGCCTGTAGCAATG
GCAACAACGTTGCGCAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGCGG
ATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGG
GTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCA
ACTATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGTAACTGTCAGACCAAGTTTACT
CATATATACTTTAGATTGATTTAAAACTTCATTTTTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGAC
CAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTT
TTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTACCAA
CTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATACTGTTCTTCTAGTGTAGCCGTAGTTAGGCCACCA
CTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCG
TGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTGCACACAGC
CCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAG
AAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTAT
CTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGA
AAAACGCCAGCAATGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCC
TGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACCGAGCGCAGCGAGTCA
GTGAGCGAGGAAGCGGAAGAGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAG

Figure 7B

A

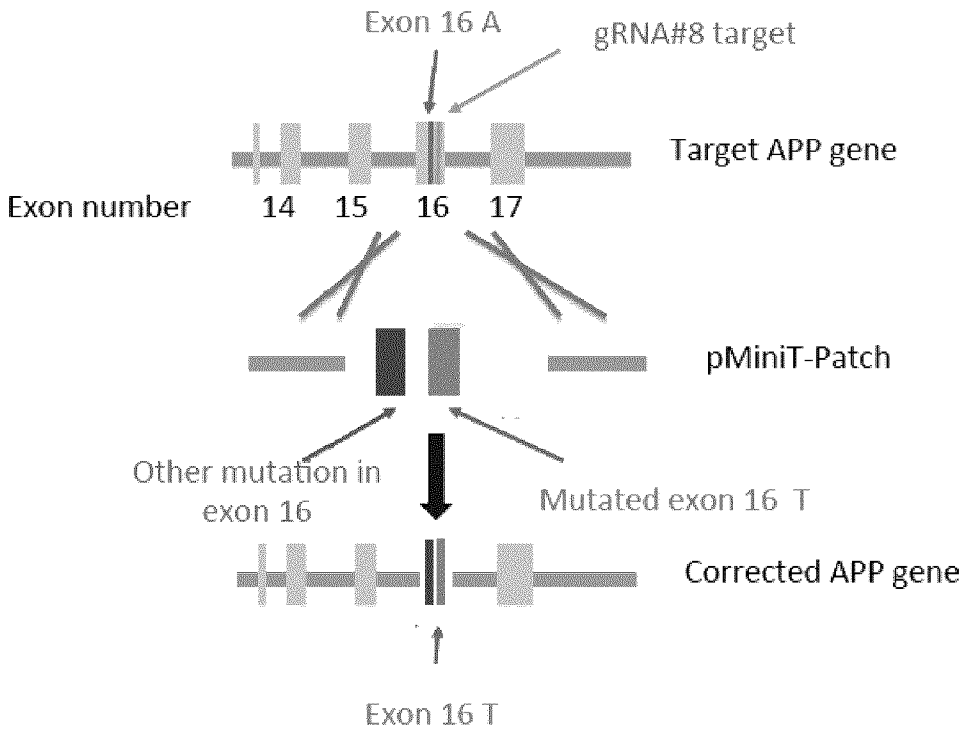

Exon 16 A
gRNA#8 target
Target APP gene
Exon number
14
15
16
17
pMiniT-Patch
Other mutation in exon 16
Mutated exon 16 T
Corrected APP gene
Exon 16 T

Figure 8A

B

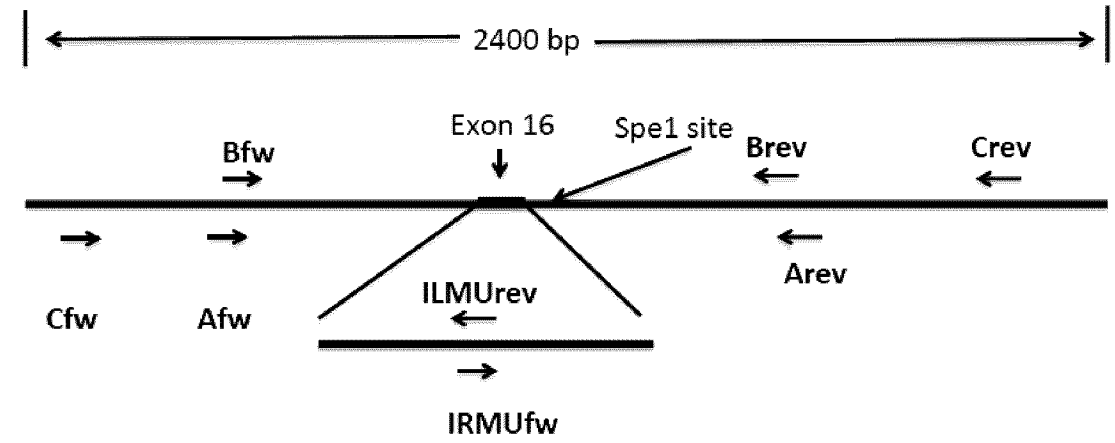
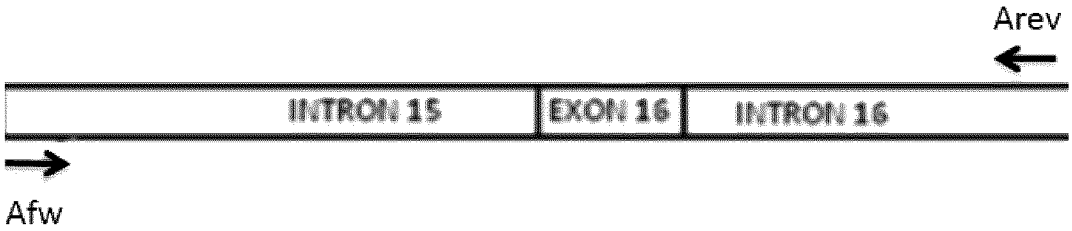

2400 bp
Exon 16
Spe1 site
Bfw
Brev
Crev
Cfw
Afw
ILMUrev
Arev
IRMUfw

Figure 8B

```
277141 aagttacagg gaagctgatt ctggcttcat gtaaaaaaag gacagtttgg gcaggcaaat
277201 ctatcaaaaa atggagggaa attgatacat tcctctatgt tcaaacagga actgacaatc

                                  Cfw
277261 tgcccctggg tgggaacacg gtagagaaga tgacttcaaa agccctttc atcctaaaat
277321 tctgatgttt gataattaaa tgttatagca tggacactga catttacatt ttttacttat
277381 gtttttggtt tttaaatgac tctgcatttt gttttaagct tcaaattatt atttgaataa
277441 tgaaattcat cagaacaatt agtgttaaga atcatatagc aatttataga aaaggaagag
277501 ttcgtaggtt ataaattctg ttagttgcta agaagcattt ttaaaattat gtactatagc

            Afw /Bfw
277561 tctttattca gcagacgaac caattacaat ctgtgtaact agaacacttg actaaaatta
277621 tataattttt acaacgcttc actgcataga tacatgaaca taatttattt gtaattggaa
277681 caaagcccca aagtagcagt tttgttctac caggtaatta atgctcattt ttaaagcctt
277741 ttattattat ttctgaagta atgagtgcac atggaaaaag acacataata ggctaaacaa
277801 taagcccgta agccaagcca acatattcca ggaacaaatc cttgccaacc tctcaaccag
277861 gatttaactt ctgcttttcc cccattttca aaaattatag catgtattta aaggcagcag
277921 aagccttact ttcaggtttc ccttaccctt tcatttcttt ttgttcaaaa taggtagtaa
277981 ttgaagtttt aaatataggg tatcattttt ctttaagagt catttatcaa ttttcttcta
278041 acttcaggcc tagaaagaag ttttgggtag gctttgtctt acagtgttat tatttatgag
278101 taaaactaat tggttgtcct gcatacttta attatgatgt aatacaggtt ctgggttgac

                                                  IRMUfw
278161 aaatatcaag acggaggaga tctctgaagt gaagatggat acggagttta ggcacgattc

                                                            Spe1 site
278221 aggatatgaa gttcatcatc aaaaattggt acgtaaaata atttacctct ttccactagt
278281 gtttgtcttg ccaaatgacc tattaactct ggttcatcct gtgctagaaa tcaaattaag
278341 gaaaagataa aaatacaatg cttgcctata ggattaccat gaaaacatga agaaaataaa
278401 taggctaggc tgagcgcagt ggctcaagcc tgtaatccca gcactttggg aggccaaggc
278461 gggtggatca cgaggtcaga aattcgagac cagcctggcc aatatggtga aaccccatct
278521 ctactaaaaa tacaaaaaag attagctggg tgtggtggca aacacctgta gtcccagctg
278581 ctggggaggc tgacgcagga gacttgcttg aacccaggag gtggaggttg cagtgagctg
278641 agatcgtgcc taggcgacag agcgagactc catcccaaaa aaaaaaaaga aaagaaagag

                                  Brev
278701 gctgtatgta tagttctttc agactacaag gcagcaaagt tcgtgcatga ctcgggactt

                                  Arev
278761 aaagtggaat taatttcaat atagcagcca ctttgacttc cactgtgttt tctgggaaaa
278821 taggtttaca ataggtttat ttgaaggatc aaacacatgc atacactgct tggttttaca
278881 gaacacttta tgtggcttaa attcacatcc ggaactgtct tcctttaccc attcatttct
278941 cccccagctc tttcttttca ttccctcccc tacctcccat gatttaactt ctcttgcaag
279001 agtaagatca tggagtgagc aggaccccat gatgttcccg atagtgttat tcatcaaaag
279061 gtttgtgcaa agaagacagc agcttccttt tcagatgaaa tcacttttcc cccctaatgt
279121 tagaattgga gtaaatcaaa aagccacatc tcctttgtgg tcagctctag tagttatata
279181 aaatccttta ccaaaagctt agaaatggag ataaatcaaa tcgtggatta tgttagggtt

                  Crev
279241 ccatcttatc agtaggtgca gtaagagggt taaattaatg aagacgacaa ttttatcaca
279301 ttcagtggtg gacagaaaaa tggtaagaaa atttccatag caataatact taaagttatc
279361 tcaggcactt cttttgtttt gttttgtgtg tgtgtgtgtg tgagtgttac ttttttccaa
279421 gcagaaaatg tcttttcaat attcataaag ttgataaatc ctagtattaa tctctaaaag
279481 aaacacctcc aaattattat ttatgcctta cttgactcca aataattgta gcaaataaaa
```

Figure 8C

A


Arev
INTRON 15
EXON 16
INTRON 16
Afw

B

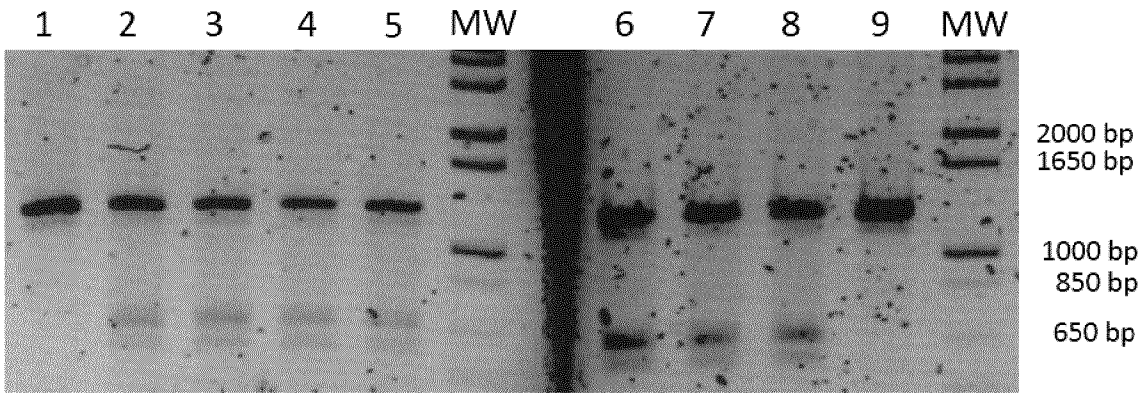
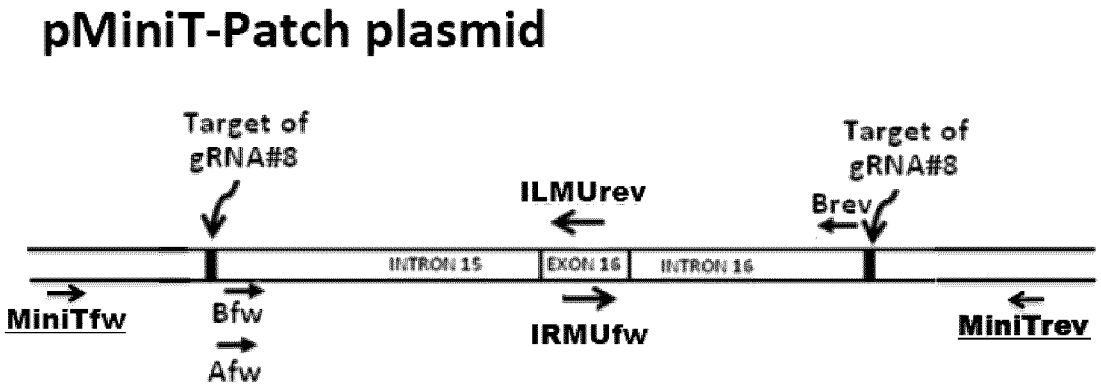

1
2
3
4
5
MW
6
7
8
9
MW
2000 bp
1650 bp
1000 bp
850 bp
650 bp

Figure 9

A


pMiniT-Patch plasmid
Target of gRNA#8
ILMUrev
Target of gRNA#8
Brev
INTRON 15
EXON 16
INTRON 16
MiniTfw
Bfw
Afw
IRMUfw
MiniTrev

B

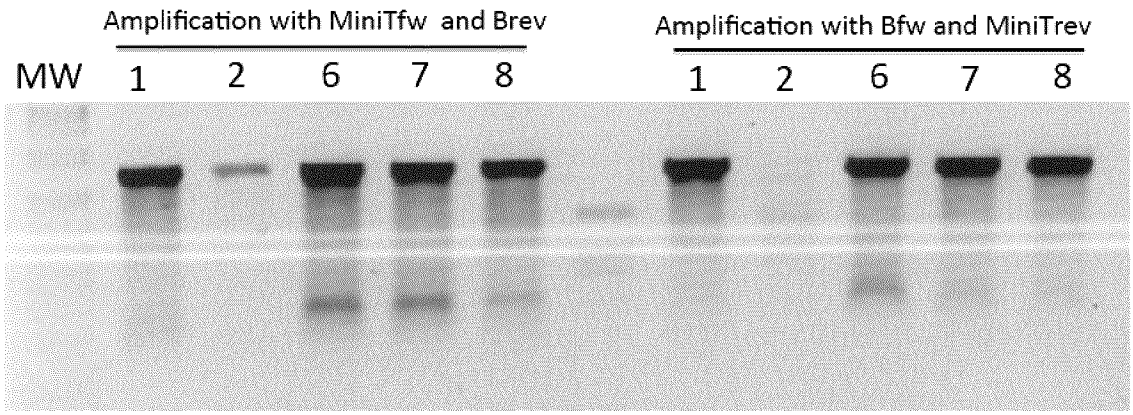
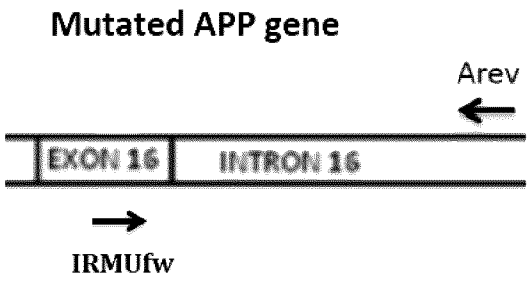

Amplification with MiniTfw and Brev
Amplification with Bfw and MiniTrev
MW
1
2
6
7
8
1
2
6
7
8

Figure 10

A


Mutated APP gene
Arev
EXON 16
INTRON 16
IRMUfw

B

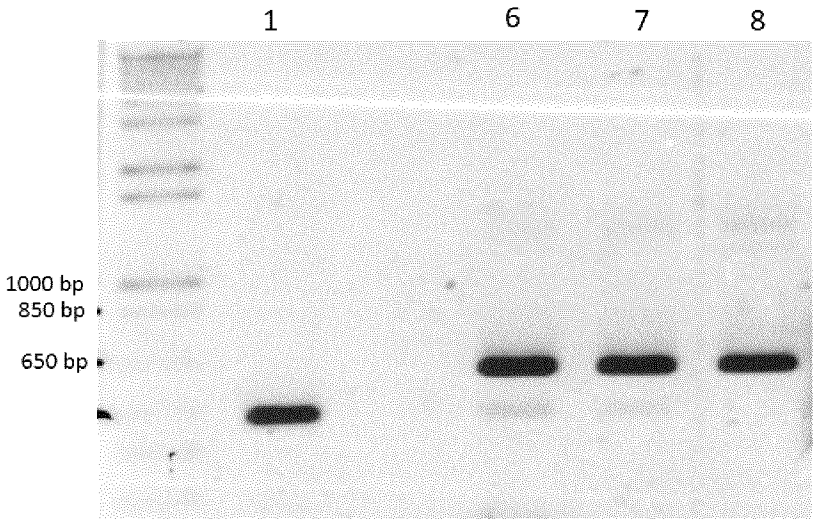
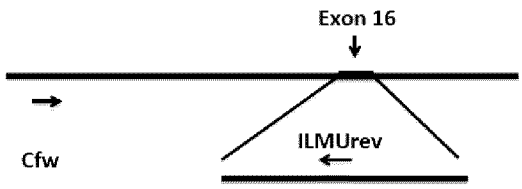

1
6
7
8
1000 bp
850 bp
650 bp

Figure 11

A


Mutated APP gene
Exon 16
Cfw
ILMUrev

B

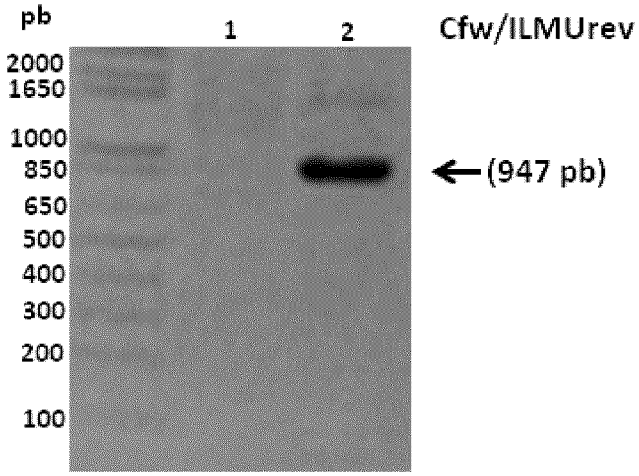
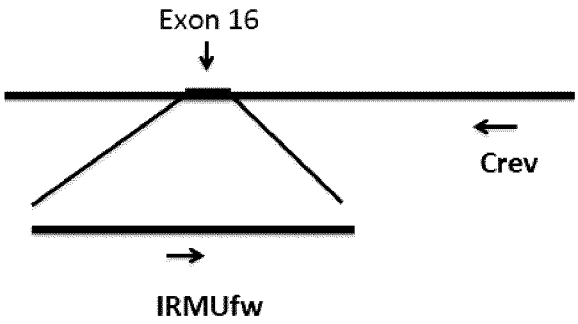

pb
1
2
Cfw/ILMUrev
2000
1650
1000
850
650
500
400
300
200
100
(947 pb)

Figure 12

A


Exon 16
Crev
IRMUfw

B

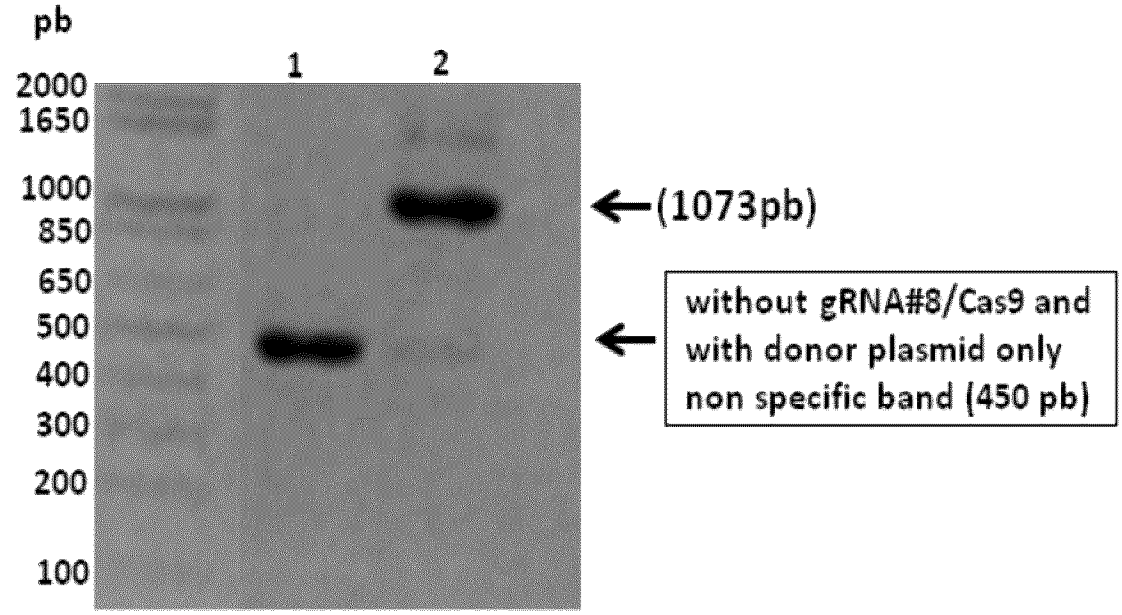

pb
1
2
2000
1650
1000
850
650
500
400
300
200
100
(1073pb)
without gRNA#8/Cas9 and
with donor plasmid only
non specific band (450 pb)

Figure 13

IRMUfw/Arev (PCR 608pb)

```
                              10        20        30        40
WT 608                  TGGATGCAGAATTCCGACATGACTCAGGATATGAAGTTCATCAT
                        ::::: : :: ::  : :: :: ::::::::::::::::::::
L8                      TGGATACGGAGTTTAGGCACGATTCAGGATATGAAGTTCATCAT
               70        80        90        100       110       120

           50        60        70        80        90       100
WT 608 CAAAAATTGGTACGTAAAATAATTTACCTCTTTCCACTACTGTTTGTCTTGCCAAATGAC
       :::::::::::::::::::::::::::::::::::: :::::::::::::::::::::
L8     CAAAAATTGGTACGTAAAATAATTTACCTCTTTCCACTAGTGTTTGTCTTGCCAAATGAC
             130       140       150       160       170       180
                                          SpeI
           110       120       130       140       150       160
WT 608 CTATTAACTCTGGTTCATCCTGTGCTAGAAATCAAATTAAGGAAAAGATAAAAATACAAT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
L8     CTATTAACTCTGGTTCATCCTGTGCTAGAAATCAAATTAAGGAAAAGATAAAAATACAAT
             190       200       210       220       230       240

           170       180       190       200       210       220
WT 608 GCTTGCCTATAGGATTACCATGAAAACATGAAGAAAATAAATAGGCTAGGCTGAGCGCAG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
L8     GCTTGCCTATAGGATTACCATGAAAACATGAAGAAAATAAATAGGCTAGGCTGAGCGCAG
             250       260       270       280       290       300

           230       240       250       260       270       280
WT 608 TGGCTCAAGCCTGTAATCCCAGCACTTTGGGAGGCCAAGGCGGGTGGATCACGAGGTCAG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
L8     TGGCTCAAGCCTGTAATCCCAGCACTTTGGGAGGCCAAGGCGGGTGGATCACGAGGTCAG
             310       320       330       340       350       360

           290       300       310       320       330       340
WT 608 AAATTCGAGACCAGCCTGGCCAATATGGTGAAACCCCATCTCTACTAAAAATACAAAAAA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
L8     AAATTCGAGACCAGCCTGGCCAATATGGTGAAACCCCATCTCTACTAAAAATACAAAAAA
             370       380       390       400       410       420

           350       360       370       380       390       400
WT 608 GATTAGCTGGGTGTGGTGGCAAACACCTGTAGTCCCAGCTGCTGGGGAGGCTGACGCAGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
L8     GATTAGCTGGGTGTGGTGGCAAACACCTGTAGTCCCAGCTGCTGGGGAGGCTGACGCAGG
             430       440       450       460       470       480

           410       420       430       440       450       460
WT 608 AGACTTGCTTGAACCCAGGAGGTGGAGGTTGCAGTGAGCTGAGATCGTGCCTAGGCGACA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
L8     AGACTTGCTTGAACCCAGGAGGTGGAGGTTGCAGTGAGCTGAGATCGTGCCTAGGCGACA
             490       500       510       520       530       540

           470       480       490       500       510       520
WT 608 GAGCGAGACTCCATCCCAAAAAAAAAAAAAAGAAAAGAAAGAGGCTGTATGTATAGTTCTT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
B)     GAGCGAGACTCCATCCCAAAAAAAAAAAAAAGAAAAGAAAGAGGCTGTATGTATAGTTCTT
             550       560       570       580       590       600

           530       540       550       560       570       580
WT 608 TCAGACTACAAGGCAGCAAAGTTCGTGCATGACTCGGGACTTAAAGTGGAATTAATTTCA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
L8     TCAGACTACAAGGCAGCAAAGTTCGTGCATGACTCGGGACTTAAAGTGGAATTAATTTCA
             610       620       630       640       650       660

           590       600
WT 608 ATATAGCAGCCACTTTGACTTCCAC
       :::::::::::::::::::::::::
L8     ATATAGCAGCCACTTTGACTTCCAC
             670       680
```

Figure 14

```
WT 947 CACGGTAGAGAAGATGACTTCAAAAGCCCTTTTCATCCTAAAATTCTGATGTTTGATAAT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
C      CACGGTAGAGAAGATGACTTCAAAAGCCCTTTTCATCCTAAAATTCTGATGTTTGATAAT
              10        20        30        40        50        60

              70        80        90       100       110       120
WT 947 TAAATGTTATAGCATGGACACTGACATTTACATTTTTTACTTATGTTTTTGGTTTTTAAA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
C      TAAATGTTATAGCATGGACACTGACATTTACATTTTTTACTTATGTTTTTGGTTTTTAAA
              70        80        90       100       110       120

             130       140       150       160       170       180
WT 947 TGACTCTGCATTTTGTTTTAAGCTTCAAATTATTATTTGAATAATGAAATTCATCAGAAC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
C      TGACTCTGCATTTTGTTTTAAGCTTCAAATTATTATTTGAATAATGAAATTCATCAGAAC
             130       140       150       160       170       180

             190       200       210       220       230       240
WT 947 AATTAGTGTTAAGAATCATATAGCAATTTATAGAAAAGGAAGAGTTCGTAGGTTATAAAT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
C      AATTAGTGTTAAGAATCATATAGCAATTTATAGAAAAGGAAGAGTTCGTAGGTTATAAAT
             190       200       210       220       230       240

             250       260       270       280       290       300
WT 947 TCTGTTAGTTGCTAAGAAGCATTTTTAAAATTATGTACTATAGCTCTTTATTCAGCAGAC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
C)     TCTGTTAGTTGCTAAGAAGCATTTTTAAAATTATGTACTATAGCTCTTTATTCAGCAGAC
             250       260       270       280       290       300

             310       320       330       340       350       360
WT 947 GAACCAATTACAATCTGTGTAACTAGAACACTTGACTAAAATTATATAATTTTTACAACG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
C      GAACCAATTACAATCTGTGTAACTAGAACACTTGACTAAAATTATATAATTTTTACAACG
             310       320       330       340       350       360

             370       380       390       400       410       420
WT 947 CTTCACTGCATAGATACATGAACATAATTTATTTGTAATTGGAACAAAGCCCCAAAGTAG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
C      CTTCACTGCATAGATACATGAACATAATTTATTTGTAATTGGAACAAAGCCCCAAAGTAG
             370       380       390       400       410       420

             430       440       450       460       470       480
WT 947 CAGTTTTGTTCTACCAGGTAATTAATGCTCATTTTTAAAGCCTTTTATTATTATTTCTGA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
C)     CAGTTTTGTTCTACCAGGTAATTAATGCTCATTTTTAAAGCCTTTTATTATTATTTCTGA
             430       440       450       460       470       480

             490       500       510       520       530       540
WT 947 AGTAATGAGTGCACATGGAAAAAGACACATAATAGGCTAAACAATAAGCCCGTAAGCCAA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
C      AGTAATGAGTGCACATGGAAAAAGACACATAATAGGCTAAACAATAAGCCCGTAAGCCAA
             490       500       510       520       530       540

             550       560       570       580       590       600
WT 947 GCCAACATATTCCAGGAACAAATCCTTGCCAACCTCTCAACCAGGATTTAACTTCTGCTT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
C      GCCAACATATTCCAGGAACAAATCCTTGCCAACCTCTCAACCAGGATTTAACTTCTGCTT
             550       560       570       580       590       600

             610       620       630       640       650       660
WT 947 TTCCCCCATTTTCAAAAATTATAGCATGTATTTAAAGGCAGCAGAAGCCTTACTTTCAGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
C      TTCCCCCATTTTCAAAAATTATAGCATGTATTTAAAGGCAGCAGAAGCCTTACTTTCAGG
             610       620       630       640       650       660

             670       680       690       700       710       720
WT 947 TTTCCCTTACCCTTTCATTTCTTTTTGTTCAAAATAGGTAGTAATTGAAGTTTTAAATAT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
C      TTTCCCTTACCCTTTCATTTCTTTTTGTTCAAAATAGGTAGTAATTGAAGTTTTAAATAT
             670       680       690       700       710       720
```

Figure 15A

```
              730       740       750       760       770       780
WT  947 AGGGTATCATTTTTCTTTAAGAGTCATTTATCAATTTTCTTCTAACTTCAGGCCTAGAAA
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
C       AGGGTATCATTTTTCTTTAAGAGTCATTTATCAATTTTCTTCTAACTTCAGGCCTAGAAA
              730       740       750       760       770       780

              790       800       810       820       830       840
WT  947 GAAGTTTTGGGTAGGCTTTGTCTTACAGTGTTATTATTTATGAGTAAAACTAATTGGTTG
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
C       GAAGTTTTGGGTAGGCTTTGTCTTACAGTGTTATTATTTATGAGTAAAACTAATTGGTTG
              790       800       810       820       830       840

              850       860       870       880       890       900
WT  947 TCCTGCATACTTTAATTATGATGTAATACAGGTTCTGGGTTGACAAATATCAAGACGGAG
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
C       TCCTGCATACTTTAATTATGATGTAATACAGGTTCTGGGTTGACAAATATCAAGACGGAG
              850       860       870       880       890       900

              910       920       930       940
WT  947 GAGATCTCTGAAGTGAAGATGGATGCAGAATTCCGACATGACTCAGG
        ::::::::::::::::::::::: : :: ::  : :: :: :::::
C       GAGATCTCTGAAGTGAAGATGGATACGGAGTTTAGGCACGATTCAGG
```

Figure 15B

IRMUfw/Crev (PCR 1073 pb)

```
                10        20        30        40        50        60
WT 107 TGGATGCAGAATTCCGACATGACTCAGGATATGAAGTTCATCATCAAAAATTGGTACGTA
       ::::: : :: ::  : :: :: :::::::::::::::::::::::::::::::::::::
D      TGGATACGGAGTTTAGGCACGATTCAGGATATGAAGTTCATCATCAAAAATTGGTACGTA
                10        20        30        40        50        60

                70        80        90       100       110       120
WT 107 AAATAATTTACCTCTTTCCACTACTGTTTGTCTTGCCAAATGACCTATTAACTCTGGTTC
       ::::::::::::::::::::::: ::::::::::::::::::::::::::::::::::::
D      AAATAATTTACCTCTTTCCACTAGTGTTTGTCTTGCCAAATGACCTATTAACTCTGGTTC
                70        80        90       100       110       120
                           SpeI
               130       140       150       160       170       180
WT 107 ATCCTGTGCTAGAAATCAAATTAAGGAAAAGATAAAAATACAATGCTTGCCTATAGGATT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
D      ATCCTGTGCTAGAAATCAAATTAAGGAAAAGATAAAAATACAATGCTTGCCTATAGGATT
               130       140       150       160       170       180

               190       200       210       220       230       240
WT 107 ACCATGAAAACATGAAGAAAATAAATAGGCTAGGCTGAGCGCAGTGGCTCAAGCCTGTAA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
D      ACCATGAAAACATGAAGAAAATAAATAGGCTAGGCTGAGCGCAGTGGCTCAAGCCTGTAA
               190       200       210       220       230       240

               250       260       270       280       290       300
WT 107 TCCCAGCACTTTGGGAGGCCAAGGCGGGTGGATCACGAGGTCAGAAATTCGAGACCAGCC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
D      TCCCAGCACTTTGGGAGGCCAAGGCGGGTGGATCACGAGGTCAGAAATTCGAGACCAGCC
               250       260       270       280       290       300

               310       320       330       340       350       360
WT 107 TGGCCAATATGGTGAAACCCCATCTCTACTAAAAATACAAAAAAGATTAGCTGGGTGTGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
D      TGGCCAATATGGTGAAACCCCATCTCTACTAAAAATACAAAAAAGATTAGCTGGGTGTGG
               310       320       330       340       350       360

               370       380       390       400       410       420
WT 107 TGGCAAACACCTGTAGTCCCAGCTGCTGGGGAGGCTGACGCAGGAGACTTGCTTGAACCC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
D      TGGCAAACACCTGTAGTCCCAGCTGCTGGGGAGGCTGACGCAGGAGACTTGCTTGAACCC
               370       380       390       400       410       420

               430       440       450       460       470       480
WT 107 AGGAGGTGGAGGTTGCAGTGAGCTGAGATCGTGCCTAGGCGACAGAGCGAGACTCCATCC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
D      AGGAGGTGGAGGTTGCAGTGAGCTGAGATCGTGCCTAGGCGACAGAGCGAGACTCCATCC
               430       440       450       460       470       480

               490       500       510       520       530       540
WT 107 CAAAAAAAAAAAAGAAAAGAAAGAGGCTGTATGTATAGTTCTTTCAGACTACAAGGCAGC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
D      CAAAAAAAAAAAAGAAAAGAAAGAGGCTGTATGTATAGTTCTTTCAGACTACAAGGCAGC
               490       500       510       520       530       540

               550       560       570       580       590       600
WT 107 AAAGTTCGTGCATGACTCGGGACTTAAAGTGGAATTAATTTCAATATAGCAGCCACTTTG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
D      AAAGTTCGTGCATGACTCGGGACTTAAAGTGGAATTAATTTCAATATAGCAGCCACTTTG
               550       560       570       580       590       600

               610       620       630       640       650       660
WT 107 ACTTCCACTGTGTTTTCTGGGAAAATAGGTTTACAATAGGTTTATTTGAAGGATCAAACA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
D      ACTTCCACTGTGTTTTCTGGGAAAATAGGTTTACAATAGGTTTATTTGAAGGATCAAACA
               610       620       630       640       650       660
```

Figure 16A

```
              670       680       690       700       710       720
WT 107 CATGCATACACTGCTTGGTTTTACAGAACACTTTATGTGGCTTAAATTCACATCCGGAAC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
D      CATGCATACACTGCTTGGTTTTACAGAACACTTTATGTGGCTTAAATTCACATCCGGAAC
              670       680       690       700       710       720

              730       740       750       760       770       780
WT 107 TGTCTTCCTTTACCCATTCATTTCTCCCCCAGCTCTTTCTTTTCATTCCCTCCCCTACCT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
D      TGTCTTCCTTTACCCATTCATTTCTCCCCCAGCTCTTTCTTTTCATTCCCTCCCCTACCT
              730       740       750       760       770       780

              790       800       810       820       830       840
WT 107 CCCATGATTTAACTTCTCTTGCAAGAGTAAGATCATGGAGTGAGCAGGACCCCATGATGT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
D      CCCATGATTTAACTTCTCTTGCAAGAGTAAGATCATGGAGTGAGCAGGACCCCATGATGT
              790       800       810       820       830       840

              850       860       870       880       890       900
WT 107 TCCCGATAGTGTTATTCATCAAAAGGTTTGTGCAAAGAAGACAGCAGCTTCCTTTTCAGA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
D      TCCCGATAGTGTTATTCATCAAAAGGTTTGTGCAAAGAAGACAGCAGCTTCCTTTTCAGA
              850       860       870       880       890       900

              910       920       930       940       950       960
WT 107 TGAAATCACTTTTCCCCCCTAATGTTAGAATTGGAGTAAATCAAAAAGCCACATCTCCTT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
D      TGAAATCACTTTTCCCCCCTAATGTTAGAATTGGAGTAAATCAAAAAGCCACATCTCCTT
              910       920       930       940       950       960

              970       980       990      1000      1010      1020
WT 107 TGTGGTCAGCTCTAGTAGTTATATAAAATCCTTTACCAAAAGCTTAGAAATGGAGATAAA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
D      TGTGGTCAGCTCTAGTAGTTATATAAAATCCTTTACCAAAAGCTTAGAAATGGAGATAAA
              970       980       990      1000      1010      1020

             1030      1040      1050      1060      1070
WT 107 TCAAATCGTGGATTATGTTAGGGTTCCATCTTATCAGTAGGTGCAGTAAGAGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::
D      TCAAATCGTGGATTATGTTAGGGTTCCATCTTATCAGTAGGTGCAGTAAGAGG
             1030      1040      1050      1060      1070
```

Figure 16B

A

AATTGGTTGTCCTGCATACTTTAATTATGATGTAATACAGgttctgggttgacaaatatc
aagacggaggagatctctgaagtgaagatggatacggagtttaggcacgattcaggatat
gaagttcatcatcaaaaattgGTACGTAAAATAATTTACCTCTTTCCACTAGTGTTTGTC
TTGCCAAATGACCTATTAA

B

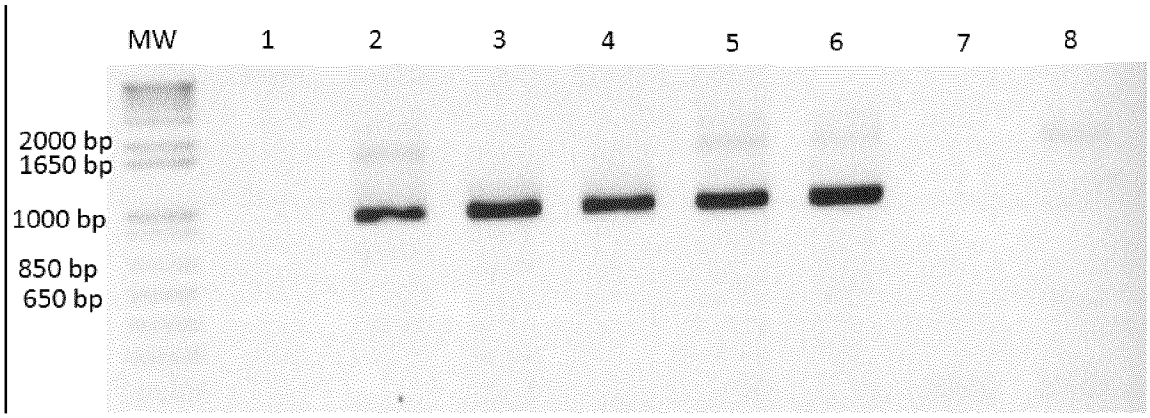


Figure 17

CNCTTGGGCANGNNNNNNNNNCNCTNTCACGAATCGACCGATTGTTAGGTAATCGTCACC
TCCACAAAGAGCGACTCGCTGTATCGCTCGAGGGATCCGAATTCAGGAGGTAAAAACCAT
GATATAGCAGAATTCCGACATGACTCAGGTTCAGCAGACGAACCAATTACAATCTGTGTA
ACTAGAACACTTGACTAAAATTATATAATTTTTACAACGCTTCACTGCATAGATACATGA
ACATAATTTATTTGTAATTGGAACAAAGCCCCAAAGTAGCAGTTTTGTTCTACCAGGTAA
TTAATGCTCATTTTTAAAGCCTTTTATTATTATTTCTGAAGTAATGAGTGCACATGGAAA
AAGACACATAATAGGCTAAACAATAAGCCCGTAAGCCAAGCCAACATATTCCAGGAACAA
ATCCTTGCCAACCTCTCAACCAGGATTTAACTTCTGCTTTTCCCCCATTTTCAAAAATTA
TAGCATGTATTTAAAGGCAGCAGAAGCCTTACTTTCAGGTTTCCTTACCCTTTCATTTCT
TTTGTTCAAATAGGTAGTAATTGAAGTTTAAATATAGGGTATCATTTTCTTTAAGAGTCA
TTTATCAATTTTCTTCTAACTTCAGGCCTAGAAAGAAGTTTTGGGTAGGCTTTGTCTTAC
AGTGTTATTATTTATGAGTAAAACTAATTGGTTGTCCTGCATACTTTAATTATGATGTAA
TACAGgttctgggttgacaaatatcaagacggaggagatctctgaagtgaagatggatac
ggagtttaggcacgattcaggatatgaagttcatcatcaaaaattg**gactacaaagacca**
tgacggtgattataaagatcatgacatcgactacaaggatgacgatgncaagtgataaGT
ACGTAAAATAATTTACCTCTTTCCACTAGTGTTTGTCTTGCCAAATGACCTATTAANTCT
GGTTCATCCTGTGCTAGAAATCAAATTAAGGAAAAGATAAAAATACAATGCTTGCCTATA
GGATTACCATGAAAACATGAAGAAAATAAATAGGCTAGGCTGAGCGCAGTGGCTCAAGCC
TGTAATCCCAGCACTTTGGGAGGCCAAGGCGGGTGGATCACGAGGTCAGAAATTCGAGAC
CAGCCTGGCCAATATGGTGAAACCCCATCTNTACTAAAAATACAAAAAAGATTAGCTGGG
TGTGGTGGCAAACACCTGTAGTCCCAGCTGCTGGGGAGGCTGACGCAGGAGACTTGCTTG
AACCCAGGAGGTGGAGGTTGCAGTGAGCTGAGATCGTGCCTAGGNGACAGAGCGAGACTC
CATCCCAAAAAAAAAAAAAGAAAAGAAAGAGGCTGTATGTATAGTTCTTTCAGACTACAA
GGCAGCAAAGTTCGTGCAGAATTCCGACATGACTCAGGACTGATAATAATGACGTCAGAA
TTCTCGAGTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTCTAAANCATCAANN
GNANNTNNNNTNCCCCTCNNATG

Figure 18

CNCTTGGGCANGNNNNNNNNNCNCTNTCACGAATCGACCGATTGTTAGGTAATCGTCACC
TCCACAAAGAGCGACTCGCTGTATCGCTCGAGGGATCCGAATTCAGGAGGTAAAAACCAT
GATATAGCAGAATTCCGACATGACTCAGGTTCAGCAGACGAACCAATTACAATCTGTGTA
ACTAGAACACTTGACTAAAATTATATAATTTTTACAACGCTTCACTGCATAGATACATGA
ACATAATTTATTTGTAATTGGAACAAAGCCCCAAAGTAGCAGTTTTGTTCTACCAGGTAA
TTAATGCTCATTTTTAAAGCCTTTTATTATTATTTCTGAAGTAATGAGTGCACATGGAAA
AAGACACATAATAGGCTAAACAATAAGCCCGTAAGCCAAGCCAACATATTCCAGGAACAA
ATCCTTGCCAACCTCTCAACCAGGATTTAACTTCTGCTTTTCCCCCATTTTCAAAAATTA
TAGCATGTATTTAAAGGCAGCAGAAGCCTTACTTTCAGGTTTCCTTACCCTTTCATTTCT
TTTGTTCAAATAGGTAGTAATTGAAGTTTAAATATAGGGTATCATTTTCTTTAAGAGTCA
TTTATCAATTTTCTTCTAACTTCAGGCCTAGAAAGAAGTTTTGGGTAGGCTTTGTCTTAC
AGTGTTATTATTTATGAGTAAAACTAATTGGTTGTCCTGCATACTTTAATTATGATGTAA
TACAGgttctgggttgacaaatatcaagacggaggagatctctgaagtgaagatggatac
ggagtttaggcacgattcaggatatgaagttcatcatcaaaaattg**gactacaaagacca**
TgacggtgattataaagatcatgacatcgactacaaggatgacgatgncaagGTACGTAA
AATAATTTACCTCTTTCCACTAGTGTTTGTCTTGCCAAATGACCTATTAANTCTGGTTCA
TCCTGTGCTAGAAATCAAATTAAGGAAAAGATAAAAATACAATGCTTGCCTATAGGATTA
CCATGAAAACATGAAGAAAATAAATAGGCTAGGCTGAGCGCAGTGGCTCAAGCCTGTAAT
CCCAGCACTTTGGGAGGCCAAGGCGGGTGGATCACGAGGTCAGAAATTCGAGACCAGCCT
GGCCAATATGGTGAAACCCCATCTNTACTAAAAATACAAAAAAGATTAGCTGGGTGTGGT
GGCAAACACCTGTAGTCCCAGCTGCTGGGGAGGCTGACGCAGGAGACTTGCTTGAACCCA
GGAGGTGGAGGTTGCAGTGAGCTGAGATCGTGCCTAGGNGACAGAGCGAGACTCCATCCC
AAAAAAAAAAAAAGAAAAGAAAGAGGCTGTATGTATAGTTCTTTCAGACTACAAGGCAGC
AAAGTTCGTGCAGAATTCCGACATGACTCAGGACTGATAATAATGACGTCAGAATTCTCG
AGTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTCTAAANCATCAANNGNANNT
NNNNTNCCCCTCNNATG

Figure 19

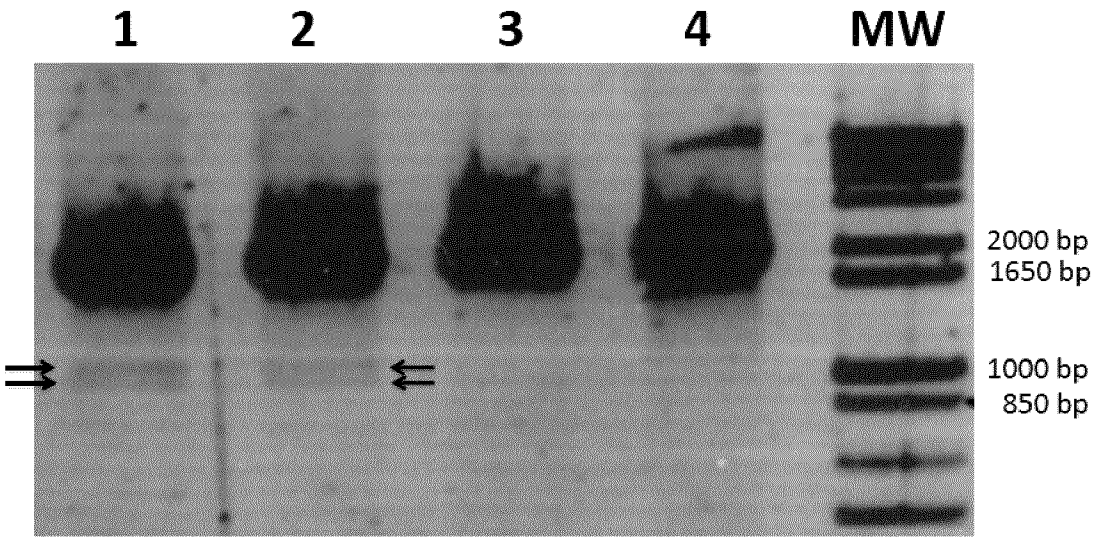


Figure 20

A

TTCAGCAGACGAACCAATTACAATCTGTGTAACTAGAACACTTGACTAAAATTATATAAT
Bfw

TTTTACAACGCTTCACTGCATAGATACATGAACATAATTTATTTGTAATTGGAACAAAGC
CCCAAAGTAGCAGTTTTGTTCTACCAGGTAATTAATGCTCATTTTTAAAGCCTTTTATTA
TTATTTCTGAAGTAATGAGTGCACATGGAAAAAGACACATAATAGGCTAAACAATAAGCC
CGTAAGCCAAGCCAACATATTCCAGGAACAAATCCTTGCCAACCTCTCAACCAGGATTTA
ACTTCTGCTTTTCCCCCATTTTCAAAAATTATAGCATGTATTTAAAGGCAGCAGAAGCCT
TACTTTCAGGTTTCCCTTACCCTTTCATTTCTTTTTGTTCAAAATAGGTAGTAATTGAAG
TTTTAAATATAGGGTATCATTTTTCTTTAAGAGTCATTTATCAATTTTCTTCTAACTTCa
gBlockfw

ggcttAGAAAGAAGTTTTGGGTAGGCTTTGTCTTACAGTGTTATTATTTATGAGTAAAAC
TAATTGGTTGTCCTGCATACTTTAATTATGATGTAATACAGGTTCTGGGTTGACAAATAT
CAAGACGGAGGAGATCTCTGAAGTGAAGATGGATgCaGAaTTccGaCAtGAcTCAGGATA

TGAAGTTCATCATCAAAAATTGGTACGTAAAATAATTTACCTCTTTCCACTAcTGTTTGT
CTTGCCAAATGACCTATTAACTCTGGTTCATCCTGTGCTAGAAATCAAATTAAGGAAAAG
ATAAAAATACAATGCTTGCCTATAGGATTACCATGAAAACATGAAGAAAATAAATAGGCT
AGGCTGAGCGCAGTGGCTCAAGCCTGTAATCccagcactttgggaggcCAAGGCGGGTGG
GBlockrev

ATCACGAGGTCAGAAATTCGAGACCAGCCTGGCCAATATGGTGAAACCCCATCTCTACTA
AAAATACAAAAAAGATTAGCTGGGTGTGGTGGCAAACACCTGTAGTCCCAGCTGCTGGGG
AGGCTGACGCAGGAGACTTGCTTGAACCCAGGAGGTGGAGGTTGCAGTGAGCTGAGATCG
TGCCTAGGCGACAGAGCGAGACTCCATCCCAAAAAAAAAAAAAGAAAAGAAAGAGGCTGT
ATGTATAGTTCTTTCAGACTACAAGGCAGCAAAGTTCGTG
Brev

B

ATCAATTTTCTTCTAACTTCAGGCCTAGAAAGAAGTTTTGGGTAGGCTTTGTCTTACAGT
GTTATTATTTATGAGTAAAACTAATTGGTTGTCCTGCATACTTTAATTATGATGTAATAC
AG**gttctgggttgacaaatatcaagacggaggagatctctgaagtgaagatggatacgga**
gtttaggcacgattcaggatatgaagttcatcatcaaaaattgGTACGTAAAATAATTTA
CCTCTTTCCACTAgTGTTTGTCTTGCCAAATGACCTATTAACTCTGGTTCATCCTGTGCT
AGAAATCAAATTAAGGAAAAGATAAAAATACAATGCTTGCCTATAGGATTACCATGAAAA
CATGAAGAAAATAAATAGGCTAGGCTGAGCGCAGTGGCTCAAGCCTGTAATCCCAGCACT
TTGGGAGGCCAAGGCGGGTGG

Figures 21A and 21B

C

*CAGAATTCCGACATGACTCAGG*TTCAGCAGACGAACCAATTACAATCTGTGTAACTAGAA
gRNA#8 target site Bfw

CACTTGACTAAAATTATATAATTTTTACAACGCTTCACTGCATAGATACATGAACATAAT
TTATTTGTAATTGGAACAAAGCCCCAAAGTAGCAGTTTTGTTCTACCAGGTAATTAATGC
TCATTTTTAAAGCCTTTTATTATTATTTCTGAAGTAATGAGTGCACATGGAAAAAGACAC
ATAATAGGCTAAACAATAAGCCCGTAAGCCAAGCCAACATATTCCAGGAACAAATCCTTG
CCAACCTCTCAACCAGGATTTAACTTCTGCTTTTCCCCCATTTTCAAAAATTATAGCATG
TATTTAAAGGCAGCAGAAGCCTTACTTTCAGGTTTCCCTTACCCTTTCATTTCTTTTTGT
TCAAAATAGGTAGTAATTGAAGTTTTAAATATAGGGTATCATTTTTCTTTAAGAGTCATT
TATCAATTTTCTTCTAACTTCAGGCCTAGAAAGAAGTTTTGGGTAGGCTTTGTCTTACAG
TGTTATTATTTATGAGTAAAACTAATTGGTTGTCCTGCATACTTTAATTATGATGTAATA
CAGgttctgggttgacaaatatcaagacggaggagatctctgaagtgaagatggatacgg
agtttaggcacgattcaggatatgaagttcatcatcaaaaattgGTACGTAAAATAATTT
ACCTCTTTCCACTAgTGTTTGTCTTGCCAAATGACCTATTAACTCTGGTTCATCCTGTGC
TAGAAATCAAATTAAGGAAAAGATAAAAATACAATGCTTGCCTATAGGATTACCATGAAA
ACATGAAGAAAATAAATAGGCTAGGCTGAGCGCAGTGGCTCAAGCCTGTAATCCCAGCAC
TTTGGGAGGCCAAGGCGGGTGGATCACGAGGTCAGAAATTCGAGACCAGCCTGGCCAATA
TGGTGAAACCCCATCTCTACTAAAAATACAAAAAAGATTAGCTGGGTGTGGTGGCAAACA
CCTGTAGTCCCAGCTGCTGGGGAGGCTGACGCAGGAGACTTGCTTGAACCCAGGAGGTGG
AGGTTGCAGTGAGCTGAGATCGTGCCTAGGCGACAGAGCGAGACTCCATCCCAAAAAAAA
AAAAAGAAAAGAAAGAGGCTGTATGTATAGTTCTTTCAGACTACAAGGCAGCAAAGTTCG
Brev

TG*CAGAATTCCGACATGACTCAGG*
gRNA#8 target site

Figure 21C

A

ATCAATTTTCTTCTAACTTCAGGCCTAGAAAGAAGTTTTGGGTAGGCTTTGTCTTACAGT
GTTATTATTTATGAGTAAAACTAATTGGTTGTCCTGCATACTTTAATTATGATGTAATAC
AGgttctgggttgacaaatatcaagacggaggagatctctgaagtgaagatggatacgga
gtttaggcacgattcaggatatgaagttcatcatcaaaaattggactacaaagaccatga
cggtgattataaagatcatgacatcgactacaaggatgacgatgacaagGTACGTAAAAT
AATTTACCTCTTTCCACTAgTGTTTGTCTTGCCAAATGACCTATTAACTCTGGTTCATCC
TGTGCTAGAAATCAAATTAAGGAAAAGATAAAAATACAATGCTTGCCTATAGGATTACCA
TGAAAACATGAAGAAAATAAATAGGCTAGGCTGAGCGCAGTGGCTCAAGCCTGTAATCCC
AGCACTTTGGGAGGCCAAGGCGGGTGG

B

ATCAATTTTCTTCTAACTTCAGGCCTAGAAAGAAGTTTTGGGTAGGCTTTGTCTTACAGT
GTTATTATTTATGAGTAAAACTAATTGGTTGTCCTGCATACTTTAATTATGATGTAATAC
AGgttctgggttgacaaatatcaagacggaggagatctctgaagtgaagatggatacgga
gtttaggcacgattcaggatatgaagttcatcatcaaaaattggactacaaagaccatga
cggtgattataaagatcatgacatcgactacaaggatgacgatgacaagtgataaGTACG
TAAAATAATTTACCTCTTTCCACTAGTGTTTGTCTTGCCAAATGACCTATTAACTCTGGT
TCATCCTGTGCTAGAAATCAAATTAAGGAAAAGATAAAAATACAATGCTTGCCTATAGGA
TTACCATGAAAACATGAAGAAAATAAATAGGCTAGGCTGAGCGCAGTGGCTCAAGCCTGT
AATCCCAGCACTTTGGGAGGCCAAGGCGGGTGG

C

GGGTNGGCCCCGAACNNNCNNTAATAACGATCGACCGATTGTTAGGTAATCGTCACCTCC
ACAAAGAGCGACTCGCTGTATCGCTCGAGGGATCCGAATTCAGGAGGTAAAAACCATGAT
ATAG**CAGAATTCCGACATGACTCAGG**TTCAGCAGACGAACCAATTACAATCTGTGTAACT
AGAACACTTGACTAAAATTATATAATTTTTACAACGCTTCACTGCATAGATACATGAACA
TAATTTATTTGTAATTGGAACAAAGCCCCAAAGTAGCAGTTTTGTTCTACCAGGTAATTA
ATGCTCATTTTTAAAGCCTTTTATTATTATTTCTGAAGTAATGAGTGCACATGGAAAAAG
ACACATAATAGGCTAAACAATAAGCCCGTAAGCCAAGCCAACATATTCCAGGAACAAATC
CTTGCCAACCTCTCAACCAGGATTTAACTTCTGCTTTTCCCCCATTTTCAAAAATTATAG
CATGTATTTAAAGGCAGCAGAAGCCTTACTTTCAGGTTTCCCTTACCCTTTCATTTCTTT
TGTTCAAAATAGGTAGTAATTGAAGTTTTAAATATAGGGTATCATTTTCTTTAAGAGTCA
TTTATCAATTTTCTTCTAACTTCAGGCCTAGAAAGAAGTTTTGGGTAGGCTTTGTCTTAC
AGTGTTATTATTTATGAGTAAAACTAATTGGTTGTCCTGCATACTTTAATTATGATGTAA
TACAGGTTCTGGGTTGACAAATATCAAGACGGAGGAGATCTCTGAAGTGAAGATGGATAC
GGAGTTTAGGCACGATTCAGGATATGAAGTTCATCATCAAAAATTGGACTACAAAGACCA
TGACTGTGATTATAAAGATCATGACATCGACTNCAAGGATGACGATGACAAGTGATAAGT
ACGTAAAATAATTTACCTCTTTCCACTAGTGTTTGTCTTGCCAAATGACCTATTAACTCT
GGTTCATCCTGTGCTAGAAATCAAATTAAGGAAAAGATAAAAATACAATGCTTGCCTATA
GGATTACCATGAAAACATGAAGAAAATAAATAGGCTAGGCTGAGCGCAGTGGCTCAAGCC
TGTAATCCCAGCACTTTGGGAGGCCAAGGCGGGTGGATCACGAGGTCAGAAATTCGAGAC
CAGCCTGGCCAATATGGTGAAACCCCATCTCTACTAAAAATACAAAAAAGATTAGCTGGG
TGTGGTGGCAAACACCTGTAGTCCCAGCTGCTGGGGAGGCTGACGCAGGAGACTTGCTTG
AACCCAGGAGGTGGAGGTTGCAGTGAGCTGAGATCGTGCCTAGGCGACAGAGCGAGACTC
CATCCCAAAAAAAAAAAAAGAAAAGAAAGAGGCTGTATGTATAGTTCTTTCAGACTACAA
GGCAGCAAAGTTCGTG**CAGAATTCCGACATGACTCAGG**ACTGATAATAATGACGTCAGAA
TTCTCGAGTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTCTAAANCATCAANGG

Figures 22A-C

D

GGGTNGGCCCCGAACNNNCNNTAATAACGATCGACCGATTGTTAGGTAATCGTCACCTCCA
CAAAGAGCGACTCGCTGTATCGCTCGAGGGATCCGAATTCAGGAGGTAAAAACCATGATAT
AG**CAGAATTCCGACATGACTCAGG**TTCAGCAGACGAACCAATTACAATCTGTGTAACTAGA
ACACTTGACTAAAATTATATAATTTTTACAACGCTTCACTGCATAGATACATGAACATAATTTA
TTTGTAATTGGAACAAAGCCCCAAAGTAGCAGTTTTGTTCTACCAGGTAATTAATGCTCATTT
TTAAAGCCTTTTATTATTATTTCTGAAGTAATGAGTGCACATGGAAAAAGACACATAATAGGC
TAAACAATAAGCCCGTAAGCCAAGCCAACATATTCCAGGAACAAATCCTTGCCAACCTCTCA
ACCAGGATTTAACTTCTGCTTTTCCCCCATTTTCAAAAATTATAGCATGTATTTAAAGGCAGC
AGAAGCCTTACTTTCAGGTTTCCCTTACCCTTTCATTTCTTTTGTTCAAAATAGGTAGTAATT
GAAGTTTTAAATATAGGGTATCATTTTCTTTAAGAGTCATTTATCAATTTTCTTCTAACTTCAG
GCCTAGAAAGAAGTTTTGGGTAGGCTTTGTCTTACAGTGTTATTATTTATGAGTAAAACTAAT
TGGTTGTCCTGCATACTTTAATTATGATGTAATACAGGTTCTGGGTTGACAAATATCAAGAC
GGAGGAGATCTCTGAAGTGAAGATGGATACGGAGTTTAGGCACGATTCAGGATATGAAGTT
CATCATCAAAAATTGGACTACAAAGACCATGACTGTGATTATAAAGATCATGACATCGACTN
CAAGGATGACGATGACAAGTGATAAGTACGTAAAATAATTTACCTCTTTCCACTAGTGTTTG
TCTTGCCAAATGACCTATTAACTCTGGTTCATCCTGTGCTAGAAATCAAATTAAGGAAAAGAT
AAAAATACAATGCTTGCCTATAGGATTACCATGAAAACATGAAGAAAATAAATAGGCTAGGC
TGAGCGCAGTGGCTCAAGCCTGTAATCCCAGCACTTTGGGAGGCCAAGGCGGGTGGATCA
CGAGGTCAGAAATTCGAGACCAGCCTGGCCAATATGGTGAAACCCCATCTCTACTAAAAAT
ACAAAAAAGATTAGCTGGGTGTGGTGGCAAACACCTGTAGTCCCAGCTGCTGGGGAGGCT
GACGCAGGAGACTTGCTTGAACCCAGGAGGTGGAGGTTGCAGTGAGCTGAGATCGTGCCT
AGGCGACAGAGCGAGACTCCATCCCAAAAAAAAAAAAAGAAAAGAAAGAGGCTGTATGTAA
GTTCTTTCAGA**CTACAACAAAGTTCGTG****CAGAATTCCGACATGACTCAGG**ACTGATAATAAT
GACGTCAGAATTCTCGAGTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTCTAAAN
CATCAANGGGTTAANANNNANNNNNNNNNG

Figure 22D

A

gagggcctatttcccatgattccttcatatttgcatatacgatacaaggctgttagagagataat
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Figure 23A - 1

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Figure 23A-2

AGCCCATCAGAGAGCAGGCCGAGAATATCATCCACCTGTTTACCCTGACCAATCTGGGAGCCCCT
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Figure 23A-3

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B

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KVLSMPQVNIVKKTEVQTGGFSKESILPKRNSDKLIARKKDWDPKKYGGFDSPTVAYSVLVVAKVE
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Figures 23A-4 and 23B

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CAAAG**AAGAAGCGGAAGGTC**GGTATCCACGGAGTCCCAGCAGCCGACAAGAAGTACAGCATCGGCCTGGACATCGGCA
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Figure 24A

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TGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGT
TCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACA
AGCTGGAGTACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCA
AGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCC
CCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACA
TGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGgaattctaaCTAGAGC
TCGCTGATCAGCCTCGACTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGCCCCTCCCCCGTGCCTTCCTTGACCCT
GGAAGGTGCCACTCCCACTGTCCTTTCCTAATAAAATGAGGAAATTGCATCGCATTGTCTGAGTAGGTGTCATTCTAT
TCTGGGGGGTGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGGAAGAgAATAGCAGGCATGCTGGGGAgcggccgca
ggaacccctagtgatggagttggccactccctctctgcgcgctcgctcgctcactgaggccgggcgaccaaaggtcgc
ccgacgcccgggctttgcccgggcggcctcagtgagcgagcgagcgcgcagctgcctgcaggggcgcctgatgcggta
ttttctccttacgcatctgtgcggtatttcacaccgcatacgtcaaagcaaccatagtacgcgccctgtagcggcgca
ttaagcgcggcgggtgtggtggttacgcgcagcgtgaccgctacacttgccagcgccctagcgcccgctcctttcgct
ttcttcccttcctttctcgccacgttcgccggctttccccgtcaagctctaaatcgggggctccctttagggttccga
tttagtgctttacggcacctcgaccccaaaaaacttgatttgggtgatggttcacgtagtgggccatcgccctgatag
acggtttttcgccctttgacgttggagtccacgttctttaatagtggactcttgttccaaactggaacaacactcaac
cctatctcgggctattcttttgatttataagggattttgccgatttcggcctattggttaaaaaatgagctgatttaa
caaaaatttaacgcgaattttaacaaaatattaacgtttacaattttatggtgcactctcagtacaatctgctctgat
gccgcatagttaagccagccccgacacccgccaacacccgctgacgcgccctgacgggcttgtctgctcccggcatcc
gcttacagacaagctgtgaccgtctccgggagctgcatgtgtcagaggttttcaccgtcatcaccgaaacgcgcgaga
cgaaagggcctcgtgatacgcctatttttataggttaatgtcatgataataatggtttcttagacgtcaggtggcact
tttcggggaaatgtgcgcggaacccctatttgtttatttttctaaatacattcaaatatgtatccgctcatgagacaa
taaccctgataaatgcttcaataatattgaaaaaggaagagtatgagtattcaacatttccgtgtcgcccttattccc
ttttttgcggcattttgccttcctgtttttgctcacccagaaacgctggtgaaagtaaaagatgctgaagatcagttg
ggtgcacgagtgggttacatcgaactggatctcaacagcggtaagatccttgagagttttcgccccgaagaacgtttt
ccaatgatgagcacttttaaagttctgctatgtggcgcggtattatcccgtattgacgccgggcaagagcaactcggt
cgccgcatacactattctcagaatgacttggttgagtactcaccagtcacagaaaagcatcttacggatggcatgaca
gtaagagaattatgcagtgctgccataaccatgagtgataacactgcggccaacttacttctgacaacgatcggagga
ccgaaggagctaaccgctttttgcacaacatgggggatcatgtaactcgccttgatcgttgggaaccggagctgaat
gaagccataccaaacgacgagcgtgacaccacgatgcctgtagcaatggcaacaacgttgcgcaaactattaactggc
gaactacttactctagcttcccggcaacaattaatagactggatggaggcggataaagttgcaggaccacttctgcgc
tcggcccttccggctggctggtttattgctgataaatctggagccggtgagcgtggaagccgcggtatcattgcagca
ctggggccagatggtaagccctcccgtatcgtagttatctacacgacggggagtcaggcaactatggatgaacgaaat
agacagatcgctgagataggtgcctcactgattaagcattggtaactgtcagaccaagtttactcatatatactttag
attgatttaaaacttcatttttaatttaaaaggatctaggtgaagatccttttgataatctcatgaccaaaatccct
taacgtgagttttcgttccactgagcgtcagaccccgtagaaaagatcaaaggatcttcttgagatccttttttttctg
cgcgtaatctgctgcttgcaaacaaaaaaaccaccgctaccagcggtggtttgtttgccggatcaagagctaccaact
ctttttccgaaggtaactggcttcagcagagcgcagataccaaatactgtccttctagtgtagccgtagttaggccac
cacttcaagaactctgtagcaccgcctacatacctcgctctgctaatcctgttaccagtggctgctgccagtggcgat
aagtcgtgtcttaccgggttggactcaagacgatagttaccggataaggcgcagcggtcgggctgaacgggggttcg
tgcacacagcccagcttggagcgaacgacctacaccgaactgagatacctacagcgtgagctatgagaaagcgccacg

Figure 24B

cttcccgaagggagaaaggcggacaggtatccggtaagcggcagggtcggaacaggagagcgcacgagggagcttcca
gggggaaacgcctggtatctttatagtcctgtcgggtttcgccacctctgacttgagcgtcgatttttgtgatgctcg
tcaggggggcggagcctatggaaaaacgccagcaacgcggccttttacggttcctggccttttgctggccttttgct
cacatgt

Figure 24C

```
  WT  792    TAGGCTTTGTCTTACAGTGTTATTATTTATGAGTAAAACTAATTGGTTGTCCTGCATACT  851
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
clone  761    TAGGCTTTGTCTTACAGTGTTATTATTTATGAGTAAAACTAATTGGTTGTCCTGCATACT  702

   WT  852    TTAATTATGATGTAATACAGGTTCTGGGTTGACAAATATCAAGACGGAGGAGATCTCTGA  911
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
clone  701    TTAATTATGATGTAATACAGGTTCTGGGTTGACAAATATCAAGACGGAGGAGATCTCTGA  642

   WT  912    AGTGAAGATGGATGCAGAATTCCGACATGACTCAGGATATGAAGTTCATCATCAAAAATT  971
                |||||||||||||  |  ||  ||   |  ||  ||  ||||||||||||||||||||||||||||
clone  641    AGTGAAGATGGATACGGAGTTTAGGCACGATTCAGGATATGAAGTTCATCATCAAAAATT  582

   WT  972    GGTACGTAAAATAATTTACCTCTTTCCACTACTGTTTGTCTTGCCAAATGACCTATTAAC
1031
                |||||||||||||||||||||||||||||  ||||||||||||||||||||||||||||
Clone  581    GGTACGTAAAATAATTTACCTCTTTCCACTAGTGTTTGTCTTGCCAAATGACCTATTAAC  522

   WT  1032   TCTGGTTCATCCTGTGCTAGAAATCAAATTAAGGAAAAGATAAAAATACAATGCTTGCCT
1091
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Clone  521    TCTGGTTCATCCTGTGCTAGAAATCAAATTAAGGAAAAGATAAAAATACAATGCTTGCCT  462

   WT  1092   ATAGGATTACCATGAAAACATGAAGAAAATAAATAGGCTAGGCTGAGCGCAGTGGCTCAA
1151
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Clone  461    ATAGGATTACCATGAAAACATGAAGAAAATAAATAGGCTAGGCTGAGCGCAGTGGCTCAA  402
```

Figure 25

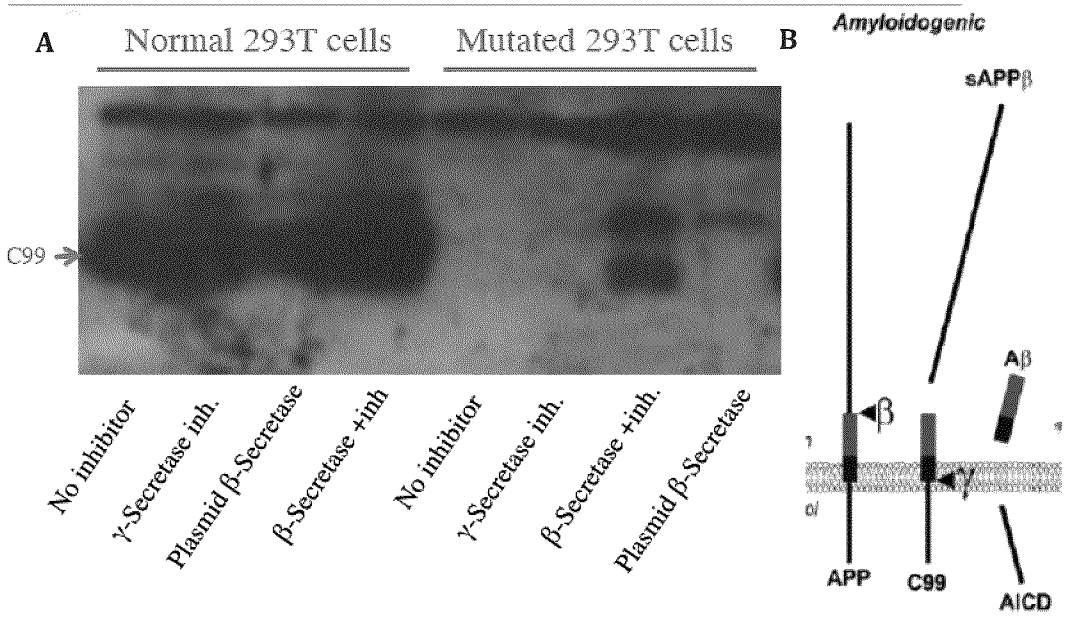
A
Normal 293T cells
Mutated 293T cells
C99
No inhibitor
γ-Secretase inh.
Plasmid β-Secretase
β-Secretase +inh
No inhibitor
γ-Secretase inh.
β-Secretase +inh.
Plasmid β-Secretase
B
Amyloidogenic
sAPPβ
Aβ
β
γ
APP
C99
AICD

Figure 26

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2015/050411

A. CLASSIFICATION OF SUBJECT MATTER
IPC: ***C12N 15/63*** (2006.01), ***A61K 48/00*** (2006.01), ***A61P 25/28*** (2006.01), ***C12N 15/09*** (2006.01), ***C12N 15/113*** (2010.01), ***C12N 15/12*** (2006.01) (more IPCs on the last page)

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC: ***C12N 15/63*** (2006.01), ***A61K 48/00*** (2006.01), ***A61P 25/28*** (2006.01), ***C12N 15/09*** (2006.01), ***C12N 15/113*** (2010.01), ***C12N 15/12*** (2006.01), ***C12N 15/55*** (2006.01), ***C12N 15/85*** (2006.01), ***C12N 15/86*** (2006.01), ***C12N 15/90*** (2006.01)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)
Canadian Patent Database, WIPO database, Questel-Orbit, Medline/Pubmed, Google Search, www.alzforum.org
Keywords: CRISPR, Alzheimer's, A673T, ZFN, TALEN, guide RNA, gRNA, PAM, protospacer adjacent motif, Cas, Cas9, Fok, APP, α-secretase, β-secretase, γ-secretase, mutation, modification

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2014/093595 A1, (Zhang, F., et al.) 19 June 2014 (19-06-2014) *whole document*	1-47, 50
Y	JONSSON, T., et al., "A mutation in *APP* protects against Alzheimer's disease and age-related cognitive decline", *Nature*, Vol. 488, Pages 96-98, 2 August 2012 (02-08-2012), ISSN: 0028-0836 *whole document"	1-47, 50

☑ Further documents are listed in the continuation of Box C. ☑ See patent family annex.

*	Special categories of cited documents:
"A"	document defining the general state of the art which is not considered to be of particular relevance
"E"	earlier application or patent but published on or after the international filing date
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O"	document referring to an oral disclosure, use, exhibition or other means
"P"	document published prior to the international filing date but later than the priority date claimed
"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&"	document member of the same patent family

Date of the actual completion of the international search 13 August 2015 (13-08-2015)	Date of mailing of the international search report 25 August 2015 (25-08-2015)
Name and mailing address of the ISA/CA Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 001-819-953-2476	Authorized officer Debora Fujimoto (819) 997-1855

Box No. I **Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)**

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

a. ☑ forming part of the international application as filed:

☑ in the form of an Annex C/ST.25 text file.

☐ on paper or in the form of an image file.

b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

SEQ ID NO:45: missing <223> at position 1543

Box No. II **Observations where certain claims were found unsearchable (Continuation of item 2 of the first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☑ Claim Nos.: 50
because they relate to subject matter not required to be searched by this Authority, namely:

Claim 50 is directed to a method to treatment of the human or animal body by surgery or therapy, which the International Searching Authority is not required to examine under Rule 67.1(iv) of the PCT.
However, this Authority has carried out a search based on the alleged effect or purpose/use of the product defined in claim 50.

2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III **Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:
The claims are directed to a plurality of inventive concepts, as follows:
Group A – Claims 1-47 are directed to a method of decreasing amyloid precursor protein (APP) processing into Aβ peptide using a CRISPR/Cas9 system to introduce a A673T substitution in the endogenous APP gene sequence in a cell; guide RNA designed to an endogenous APP gene target sequence, donor nucleic acid; vector and compositions for integration into a wild type target APP sequence; and the use to decrease Aβ peptide in a cell from a subject with an association with Alzheimer's disease or age-related cognitive decline; and
(Continued on last page)

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☑ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claim Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant=s protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SHAH, S.A., et al., "Protospacer recognition motifs", *RNA Biology*, Vol. 10, No. 5, Pages 891-899, May 2013 (05-2013), ISSN: 1547-6286 *whole document*	1-47, 50
Y	MENKE, D.B., et al., "Engineering subtle targeted mutations into the mouse genome", *Genesis*, Vol. 51, Pages 605-618, 2013, online ISSN: 1526-968X *whole document, particularly pages 611-613*	1-47, 50
A	TING, S.K.S., et al., "Absence of A673T amyloid-β precursor protein variant in Alzheimer's disease and other neurological diseases", *Neurobiology of Aging*, Vol, 34, Pages 2441.e7-2441.e8, 2013, ISSN: 0197-4580 *whole document*	1-47, 50
X	WOODRUFF, G., et al., "The presenilin-1 ΔE9 mutation results in reduced γ-secretase activity, but not total loss of PS1 function, in isogenic human stem cells", *Cell Rep.*, 27 November 2013 (27-11-2013), Vol. 5, No. 4, Pages 974-985, ISSN: 2211-1247 *whole document*	48, 49
A	JINEK, M., et al., "A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity", *Science*, Vol 337, Pages 816-821, 17 August 2012 (17-08-2012), ISSN: 0036-8075 *whole document*	1-47, 50
A	GAJ, T., et al., "ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering", *Trends in Biotechnology*, Vol. 31, No. 7, Pages 397-405, 2013, ISSN: *whole document*	1-47, 50
A	MALI, P., et al., "RNA-guided human genome engineering via Cas9", *Science*, Vol. 339, Pages 823-826, 15 February 2013 (15-02-2013), ISSN: 0036-8075 *whole document*	1-47, 50

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CA2015/050411

Patent Document Cited in Search Report	Publication Date	Patent Family Member(s)	Publication Date
WO2014093595A1	19 June 2014 (19-06-2014)	WO2014093595A1	19 June 2014 (19-06-2014)
		WO2014093595A9	06 August 2015 (06-08-2015)
		AU2013359123A1	30 July 2015 (30-07-2015)
		AU2013359199A1	30 July 2015 (30-07-2015)
		AU2013359212A1	30 July 2015 (30-07-2015)
		AU2013359238A1	30 July 2015 (30-07-2015)
		AU2013359262A1	30 July 2015 (30-07-2015)
		CA2894668A1	19 June 2014 (19-06-2014)
		CA2894681A1	19 June 2014 (19-06-2014)
		CA2894684A1	19 June 2014 (19-06-2014)
		CA2894688A1	19 June 2014 (19-06-2014)
		CA2894701A1	19 June 2014 (19-06-2014)
		DK2771468T3	26 May 2015 (26-05-2015)
		DK2784162T3	13 July 2015 (13-07-2015)
		EP2764103A2	13 August 2014 (13-08-2014)
		EP2771468A1	03 September 2014 (03-09-2014)
		EP2771468B1	11 February 2015 (11-02-2015)
		EP2784162A1	01 October 2014 (01-10-2014)
		EP2784162B1	08 April 2015 (08-04-2015)
		EP2825654A1	21 January 2015 (21-01-2015)
		EP2840140A1	25 February 2015 (25-02-2015)
		EP2848690A1	18 March 2015 (18-03-2015)
		EP2896697A1	22 July 2015 (22-07-2015)
		EP2898075A1	29 July 2015 (29-07-2015)
		ES2536353T3	22 May 2015 (22-05-2015)

ES2542015T3	29 July 2015 (29-07-2015)
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US8697359B1	15 April 2014 (15-04-2014)
US2014170753A1	19 June 2014 (19-06-2014)
US8771945B1	08 July 2014 (08-07-2014)
US2014179006A1	26 June 2014 (26-06-2014)
US8795965B2	05 August 2014 (05-08-2014)
US2014242700A1	28 August 2014 (28-08-2014)
US8865406B2	21 October 2014 (21-10-2014)
US2014273231A1	18 September 2014 (18-09-2014)
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US8895308B1	25 November 2014 (25-11-2014)
US2014273232A1	18 September 2014 (18-09-2014)
US8906616B2	09 December 2014 (09-12-2014)
US2014248702A1	04 September 2014 (04-09-2014)
US8932814B2	13 January 2015 (13-01-2015)
US2014227787A1	14 August 2014 (14-08-2014)
US8945839B2	03 February 2015 (03-02-2015)
US2014186958A1	03 July 2014 (03-07-2014)
US8993233B2	31 March 2015 (31-03-2015)
US2014256046A1	11 September 2014 (11-09-2014)
US8999641B2	07 April 2015 (07-04-2015)

US2014179770A1	26 June 2014 (26-06-2014)
US2014186843A1	03 July 2014 (03-07-2014)
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US2014357530A1	04 December 2014 (04-12-2014)
US2015020223A1	15 January 2015 (15-01-2015)
US2015031134A1	29 January 2015 (29-01-2015)
US2015079681A1	19 March 2015 (19-03-2015)
US2015184139A1	02 July 2015 (02-07-2015)
US2015203872A1	23 July 2015 (23-07-2015)
WO2014093622A2	19 June 2014 (19-06-2014)
WO2014093622A3	07 August 2014 (07-08-2014)
WO2014093622A9	06 November 2014 (06-11-2014)
WO2014093622A8	04 December 2014 (04-12-2014)
WO2014093635A1	19 June 2014 (19-06-2014)
WO2014093635A9	24 July 2014 (24-07-2014)
WO2014093655A2	19 June 2014 (19-06-2014)
WO2014093655A3	31 July 2014 (31-07-2014)
WO2014093655A9	04 December 2014 (04-12-2014)
WO2014093661A2	19 June 2014 (19-06-2014)
WO2014093661A3	07 August 2014 (07-08-2014)
WO2014093661A9	16 October 2014 (16-10-2014)
WO2014093694A1	19 June 2014 (19-06-2014)
WO2014093694A9	16 October 2014 (16-10-2014)

WO2014093701A1	19 June 2014 (19-06-2014)
WO2014093701A9	06 November 2014 (06-11-2014)
WO2014093709A1	19 June 2014 (19-06-2014)
WO2014093712A1	19 June 2014 (19-06-2014)
WO2014093712A9	21 August 2014 (21-08-2014)
WO2014093718A1	19 June 2014 (19-06-2014)
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WO2014204724A1	24 December 2014 (24-12-2014)
WO2014204725A1	24 December 2014 (24-12-2014)
WO2014204725A8	28 May 2015 (28-05-2015)
WO2014204726A1	24 December 2014 (24-12-2014)
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WO2014204727A1	24 December 2014 (24-12-2014)
WO2014204727A9	12 March 2015 (12-03-2015)
WO2014204728A1	24 December 2014 (24-12-2014)
WO2014204728A8	09 July 2015 (09-07-2015)
WO2014204729A1	24 December 2014 (24-12-2014)

A. CLASSIFICATION OF SUBJECT MATTER (IPCs continued)

C12N 15/55 (2006.01), ***C12N 15/85*** (2006.01), ***C12N 15/86*** (2006.01), ***C12N 15/90*** (2006.01)

Box No. III (continued)

Group B – Claims 48 and 49 are directed to a method of decreasing APP processing into Aβ peptide by a cell comprising introducing at least one modification within an endogenous polynucleotide target gene sequence of said cell, wherein said modification decreases the amount of Aβ peptide and said endogenous polynucleotide target gene encodes an α-secretase, a β-secretase or a γ-secretase.

The claims must be limited to one inventive concept as set out in Rule 13 of the PCT.