(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) **EP 3 697 914 B9**

(12) **CORRECTED EUROPEAN PATENT SPECIFICATION**

(15) Correction information:
Corrected version no 1 (W1 B1)
Corrections, see
Sequence listing
Remarks
Sequence listing replaced or added

(48) Corrigendum issued on:
04.10.2023 Bulletin 2023/40

(45) Date of publication and mention of the grant of the patent:
07.06.2023 Bulletin 2023/23

(21) Application number: **18786778.3**

(22) Date of filing: **17.10.2018**

(51) International Patent Classification (IPC):
C12N 15/86 *(2006.01)*

(52) Cooperative Patent Classification (CPC):
C12N 15/86; C12N 2750/14143; C12N 2830/48

(86) International application number:
PCT/EP2018/078384

(87) International publication number:
WO 2019/076973 (25.04.2019 Gazette 2019/17)

(54) VECTORS FOR THE TREATMENT OF FRIEDREICH'S ATAXIA

VEKTOREN ZUR BEHANDLUNG DER FRIEDREICH-ATAXIE

VECTEURS DESTINÉS AU TRAITEMENT DE L'ATAXIE DE FRIEDREICH

(84) Designated Contracting States:
AL AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO RS SE SI SK SM TR

(30) Priority: **17.10.2017 EP 17382691**

(43) Date of publication of application:
26.08.2020 Bulletin 2020/35

(60) Divisional application:
22170787.0 / 4 089 173

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Processed by Luminess, 75001 PARIS (FR)

(Cont. next page)

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Remarks:

The complete document including Reference Table(s) and the Sequence Listing(s) can be downloaded from the EPO website

Description

Technical field

[0001] The present invention can be included in the field of new therapeutics for the treatment of Friedreich's ataxia. Specifically, the present application relates to new products for gene therapy. The products are capable of treating Friedreich's ataxia.

Background art

[0002] Friedreich's ataxia (FRDA; OMIM #229300) is a rare inherited neurodegenerative disease that causes progressive damage to the nervous system resulting in symptoms ranging from gait disturbances and language problems to heart disease or cardiomyopathy. The disease was named after the physician Nikolaus Friedreich, who was the first to describe it in the 1860s. Ataxia, referred to problems of motor coordination such as awkward movements and instability, occurs in Friedreich's ataxia by degeneration of nervous tissue in the spinal cord and nerves that control the muscular movement of the arms and legs. The spinal cord shrinks with nerve cells losing part of their myelin sheath. Friedreich's ataxia, although uncommon, is the most common hereditary ataxia, ranging from 1 in 30,000-50,000 live births, with a prevalence of 2-4 per 100,000. Both sexes are affected equally. Symptoms usually begin between 5 and 15 years of age but may, on rare occasions, appear as early as 18 months or as late as 50 years of age. The first symptom that appears is usually difficulty walking, or gait ataxia. Ataxia gradually worsens and spreads slowly to the arms and then to the trunk. Foot deformities such as pes cavus, involuntary folding of the toes, or hammer toes may appear as early signs. Over time, the muscles begin to weaken and to be consumed, especially in the feet, legs and hands, and the deformities develop. Other symptoms include loss of tendon reflexes, especially in the knees and ankles. There is often a gradual loss of sensation in the extremities, which can spread to other parts of the body. Dysarthria develops, and the patient gets tired easily. The rapid, rhythmic and involuntary movements of the eyes called nystagmus are common. Most people with Friedreich's ataxia develop scoliosis, which if severe, can affect breathing.

[0003] Other symptoms that can occur are chest pain, shortness of breath and palpitations. These symptoms are the result of various forms of heart disease that often accompany Friedreich's ataxia, such as hypertrophic cardiomyopathy, myocardial fibrosis, or heart failure. Heart rate abnormalities such as tachycardia and heart block are also common. About 20% of people with Friedreich's ataxia develop carbohydrate intolerance and 30% show diabetes mellitus. Some affected people lose hearing or sight. The speed of disease progression varies from person to person. Generally, between 10 and 20 years after the onset of the first symptoms, the person is confined to a wheelchair, and in later stages of the disease individuals become totally disabled. Life expectancy may be affected. Many people with Friedreich's ataxia die in adulthood due to associated heart disease, the most common cause of death. However, some people with less severe symptoms of Friedreich's ataxia sometimes live to 60 or 70 years.

[0004] Neuroimaging such as MRI shows normal appearance in the early stages of the disease, although progressively reveals variable atrophy of the cervical spinal cord and cerebellum. This often shows atrophy of the superior peduncle. Electrophysiological studies show conduction velocities greater than 40 m/s with absence or reduction of sensory action potentials, and absence of reflex H. At the neuropathological level, marked atrophy of the spinal cord, posterior roots, occasionally of the cerebellum, and cardiac hypertrophy are observed. Neurodegeneration is identified in peripheral sensory nerves with progressive loss of large dorsal root ganglion sensory neurons, dorsal column degeneration, transynaptic degeneration of neurons in Clarke's spine and spinocerebellar fibers, pyramidal tracts, and atrophy of the gracile and cuneiform nuclei. Secondary lesions may include atrophy of the dentate nucleus in the cerebellum affecting large glutamatergic neurons, and atrophy of Betz cells and corticospinal tracts.

[0005] In 1996, the cause of Friedreich's ataxia was identified as a molecular defect in the FXN gene located on chromosome 9 (9q21 band). The mutation consists of an abnormal homozygous expansion of the GAA triplet located inside an ALU sequence in the first intron of the FXN gene. About 98% of patients with FRDA have 2 chromosomes with a number between 201 and 1,700 GAA repeats (more frequently between 600 and 900) in each of them. However, 2-5% of patients with FRDA present a GAA expansion and a point mutation in the FXN gene in composite heterozygosis (Table 1). To date, more than 17 different mutations in the FXN gene have been described capable of triggering FRDA. The GAA expansion in FRDA is very unstable during meiosis and interferes with FXN gene transcription causing abnormally low levels of frataxin mRNA. GAA expansion would silence transcription of the FXN gene and thus abolish frataxin expression by forming triple DNA structures or DNA-RNA hybrids, or both. More recently, it has been proposed that the GAA expansion would block the transition from initiation to elongation of transcription due to the formation of heterochromatin-like structures in the vicinity of GAA hyperexpansion. It has also been proposed that GAA expansion would lead to epigenetic methylation of the CpG sites located in the 5' upstream region of the FXN gene causing their silencing. Thus, as a consequence of the GAA mutation in the FXN gene, a deficiency of the frataxin protein occurs in FRDA.

Table 1. Most prevalent allelic variants identified in the FXN gene causing Friedreich's ataxia.

OMIM	DISEASE ALLELIC VARIANT
606829.0001	GAA expansion within intrón 1
606829.0002	Transversion within exon 3: LEU106X
606829.0003	Transition c.385-2A>G affecting splicing
606829.0004	Missense variant: Ile154Phe in exon 4
606829.0005	Missense mutation: Gly130Val
606829.0006	Missense mutation affecting start codon: Met1Ile
606829.0007	Missense mutation: Trp173Gly
606829.0008	Deletion of 1 nt in codon 75 provoking protein truncation
606829.0009	Deletion of 6 nt and insertion of 15 nt (c.371_376del6ins15) in exon 3

[0006] The FXN gene encodes a small conserved mitochondrial protein that contains 210 amino acids (aa) in its precursor form, with a molecular weight of 23,135 Da named frataxin (Q16595). This precursor protein form contains an N-terminal sequence of transit that directs it to the mitochondrial matrix where the mitochondrial peptidase converts it into different smaller isoforms (FXN42-210, FXN56-210, FXN81-210, FXN78-210) of the mature protein frataxin being the FXN81-210 of 130 aa and 14.2 kDa the most abundant. In humans, frataxin is detected in the mitochondrial matrix in association with its inner membrane in a large variety of tissues, and the most abundant levels are identified in cardiac tissue, spinal cord and dividing lymphoblasts, and remarkably the lowest levels in the cerebellum. Frataxin has not been detected in the cerebral cortex to date. Since the gene responsible for the disease was identified and with the generation of several animal models, different functions have been postulated for frataxin such as mitochondrial iron homeostasis, iron storage, response to oxidative stress, biogenesis of Fe-S clusters, modulation of mitochondrial aconitase activity and regulation of oxidative phosphorylation. In FRDA, frataxin deficiency produced by homozygosis expansion of the GAA expansion in the FXN gene leads to insufficient biosynthesis of the iron-sulphur clusters necessary for electron transport in the mitochondria and aconitase assembly leading to dysregulation of mitochondrial function and mitochondrial iron accumulation by alterations of its homeostasis. Frataxin also modulates the DNA binding capacity of the protein aconitase 1 (ACO1), which in addition to participating in the cycle of citric acid in the mitochondrial matrix, regulates the uptake and utilization of cellular iron.

[0007] Several animal and cellular models for Friedreich's ataxia have been generated by genetic manipulation. Generating an AF model in the mouse mimicking as close as possible the human disease has been an arduous task, and currently there are 8 distinct murine models of FRDA. Unfortunately, none of them present with the combination of all the phenotypic symptoms of FA in the same animal such as lesions in the dorsal root ganglia (DRG) and cerebellar dentate nuclei, although they are useful for the study of isolated aspects of the molecular neurodegenerative process in FRDA and for the evaluation of different treatments in specific symptoms. The most used in pre-clinical treatments are the Prp-CreERT and YG8R mice. The Prp-CreERT mice specifically develop cerebellar and progressive sensory ataxia, the most prominent neurological functions of Friedreich's Ataxia. Histological studies in these animals show abnormalities of the spinal cord and dorsal root ganglia with absence of motor neuropathy, a hallmark of human disease, as well as arborisation defects in Purkinje cells of the cerebellum. In contrast, YG8R transgenic mice in the absence of the endogenous murine frataxin protein exhibit slowly progressive FRDA pathology. In contrast to humans, these mice do not show cardiac deficits.

[0008] Among viral and non-viral vectors used in gene therapy for human pathologies, vectors derived from adeno-associated virus (AAV) have shown important clinical benefits and prolonged expressions in animal models of Gaucher disease, Fabry disease, Pompe disease, Metachromatic leukodystrophy, Niemann-Pick A disease and mucopolysaccharidosis I, II, III A, III B, IV and VII among others. Intravenous administration of AAV vectors in animal models of lysosomal storage diseases has led to increases in enzyme activity of up to 16 times normal values in blood, liver, spleen, kidney and muscle, making them very useful for treating this type of pathologies. Over the past 10 years, AAVs have been the vectors of choice in most clinical trials conducted to treat central and peripheral nervous system pathologies (http://www.abedia.com/wiley/index.html). Importantly, no adverse effects have been observed with these vectors to date and the results are very promising. Thus, several factors have made AAVs become the ideal gene delivery vehicle for the central nervous system (CNS). Adeno-associated virus vectors comprising a frataxin sequence have been used to treat FRDA in mice (Gérard et al., 2014. Mol Ther Methods Clin Dev. 1: 14044; Chapdelaine et al., 2016. Gene Ther. 23(7): 606-614; Tremblay et al., 2015. Mol Ther. 23(Suppl. 1): pS153; WO 2016/150964). But, the AAV vectors disclosed

in the prior art either over- or under-express frataxin and do not reach all target cells and neurons affected in FRDA, which make them unsuitable for the efficient treatment of FRDA and in particular its neurological signs. Thus, an AAV vector expressing the frataxin protein at a therapeutically effective amount is needed.

[0009] At present there is no effective therapy for the treatment of FRDA and therefore there is a need for new therapeutics for the treatment of FRDA. It is an objective of the present invention to provide a suitable therapy for treating FRDA.

Figures

[0010]

Figure 1. Evaluation of the SYN (synapsin) and FXN (frataxin; 1,255 bp) human promoters on frataxin (FXN) protein expression. To test the levels of human frataxin (FXN) protein expression under the regulation of a fragment of the endogenous promoter (phFXN), and the synapsin neuronal promoter (phSYN), in comparison to the expression under the high-expressing constitutive promoter CMV, Human Neuroblastoma SH-SY5Y cells were transfected with empty vector (lane 1), or constructs encoding the human synapsin (phSYN) (lane 3), or a 1,255 bp fragment of the frataxin (phFXN) promoters (lane 4). Constructs 1.2 and 1.3 using the phSYN or the phFXN (1,255 bp) to express the human FXN coding sequence (hFXN CDS) did not result in expression of the recombinant frataxin protein (rFXN). HA, hemagglutinin tag.

Figure 2. Evaluation of the SYN (synapsin), NSE (neuron-specific enolase), and FXN (frataxin; 1,255 bp) human promoters together with the WPRE sequence on frataxin (FXN) protein expression. The effect of a 320 bp fragment of the 5'end from FXN, and WPRE sequences on the expression of FXN from the phSYN promoter previously shown in Fig.1 (lane 3, 4 vs 8) and of phNSE (lane 6) was evaluated. The different constructs were transfected into HEK cells and the lysates analysed 48 hrs after transfection. The efficiency of the WPRE sequences in the stabilization of RNA is seen in the construct expressing FXN from the CMV promoter (lane 2). However, the addition and combination of the regulatory elements shown above did not result in the expression of FXN from the phSYN, hpFXN, or phNSE. HA, hemagglutinin tag; iFXN, intermediate form FXN; mFXN, mature form FXN; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element.

Figure 3. Evaluation of the SYN (synapsin), NSE (neuron-specific enolase), and FXN (frataxin; 1,255 bp) human promoters together with the CMV enhancer and WPRE sequences on frataxin (FXN) protein expression. HEK and mouse neuroblastoma cells N2a were transfected with constructs comprising the CMV enhancer together with either SYN, NSE or FXN (1,255 bp) promoters. The combination of the regulatory elements shown above did not result in the expression of FXN from the phSYN, phFXN, or phNSE promoters. HA, hemagglutinin tag; iFXN, intermediate form FXN; mFXN, mature form FXN; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element.

Figure 4. Evaluation of the effect of the human promoters SYN (synapsin), NSE (neuron-specific enolase), and FXN (frataxin; 1,255 bp) human promoters with addition of the KOZAK (5') and WPRE (3') sequences on frataxin (FXN) protein expression. The different constructs were transfected into HEK cells and the lysates analysed 48 hrs after transfection. The efficiency of the WPRE sequences in the stabilization of RNA is seen in the construct expressing FXN from the CMV promoter (lane 1 vs 2). The further FXN expression enhancement by the addition of the kozak sequence and the 105 nt sequences between the promoter and the FXN coding sequence is seen in lanes 2 vs 3. However, the addition and combination of the regulatory elements shown above did not result in the expression of FXN from the phSYN, phFXN, or phNSE. HA, hemagglutinin tag; iFXN, intermediate form FXN; mFXN, mature form; pFXN, precursor form; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element.

Figure 5. Evaluation of the combination of a defined linker between the human promoters PGK1 (phosphoglycerate kinase 1), NSE (neuron-specific enolase), SYN (synapsin) or FXN (frataxin; 1,255 bp) and the coding region (CDS) of FXN in addition to the KOZAK (5') and WPRE (3') sequences on frataxin (FXN) protein expression. The different constructs shown were transfected into either N2a (A, B and E) or HEK (C and D) cells and the lysates were analysed 48 hrs after transfection. Expression of FXN was consistently detected from the phPGK, but not from the phNSE or phFXN (1,255 bp) promoter when using the 105 bp linker between the promoter and the coding region (A,C, and D: Lane 4 ; B: lane 5 and E: lane 8). HA, hemagglutinin tag; iFXN, intermediate form FXN; mFXN, mature form; pFXN, precursor form; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element.

Figure 6. Expression of luciferase (LUC) under the PGK1 (phosphoglycerate kinase 1) human promoter following intrathecal injection of the rAAV9 vector (4.6×10^{12} vg/Kg) in YG8R Tg/- and WT mice. The expression of luciferase

was detected after intraperitoneal injection of 150 μls of D-luciferin at 150 mg/Kg mouse-weight 3.5 months after intrathecal injection of the vector (A). Organs were dissected and luciferase substrate was added fresh before capturing the image (relative scales shown after left and right panels (B)).

Figure 7. In vivo expression of frataxin mRNA in 7 months-old mice intrathecally administered with rAAV9-PGK1-FXN. Frataxin mRNA levels were quantified by qRT-PCR in liver, heart, lumbar dorsal root ganglia (DRG-L), lumbar spinal cord (SC-L), thorasic spinal cord (SC-T), cervical spinal cord (SC-C), cerebellum (Cb), and brain (frontal region C1) tissues from 7-months-old YG8R hemizygous transgenic (Tg/-) or homozygous (Tg/Tg) mice. YG8R hemizygous transgenic mice (Tg/-) were administered with either rAAV-Null (shown in black) or rAAV9-PGK1-FXN (shown in light grey) and the FRDA homozygous transgenic mice (Tg/Tg) (shown in dark grey) were not injected. Unlike the YG8R hemizygous mice (Tg/-) carrying two tandem copies of the human FXN gene with ~82 and ~190 GAA trinucleotide sequence repeats in one of the chromosomes, the homozygous YG8R mice contain the two tandem copies in each of the chromosomes. Neither the homozygous or the hemizygous YG8R mice for the human FXN express endogenous Fxn since they have a mouse Fxn knockout genetic background. The homozygous YG8R mice served as a higher expressing control for the human FXN mRNA since it contains more copies of the human transgene yet exhibiting normal functions. Mice were injected intrathecally at 2.5 months of age and human FXN-specific primers were used to quantify FXN mRNA in the different tissues. Levels of total FXN mRNA were normalized to levels of those detected in the YG8R hemizygous mice (Tg/-) treated with rAAV9-Null for each of the tissues analysed. Five months after intrathecal injection of the rAAV9-PGK1-FXN vector, the levels of the FXN mRNA were higher (around 1.3 and 3.2-fold) in the rAAV9-PGK1-FXN injected hemizygous YG8R mice (Tg/-) with the dosage tested but not higher than levels in the homozygous YG8R mice (Tg/Tg) which in all tissues, compared with the levels in Tg/- mice AAV-Null treated. A) Relative levels of human FXN mRNA in both females and males mice n= 6 (3 females and 3 males). Fold differences between Friedreich ataxia mice model YG8R mice injected with the control AAV-null compared to the rAAV9-PGK1-FXN injected YG8R mice are statistically significant in liver, dorsal root ganglia and spinal cord ($p=0.019$, $p=0.004$, and $p= 0.024$; denoted by asteriks *) with increasing trends in all other tissues shown. More variability was observed in male compared to female mice with the dose used. B) Relative levels of hFXN mRNA in female YG8R mice. Statistically significant fold differences in frataxin levels are detected in all tissues studied as denoted by asteriks and listed in the table below. The fold increases in the rAAV9-PGK1-FXN injected mice were never higher than levels detected in the Tg/Tg YG8R mice. Statistical significance, $p<0.05$*, $p<0.005$**

Figure 8. In *vivo* expression of the recombinant frataxin protein from the rAAV9-hPGK1-FXN vector after intrathecal injection. Lysates from cultured cells N2a transfected with empty vector or the frataxin expressing vector which was used as a positive control (Fig.8A and 8B: lanes 1 and 2). The endogenous FXN protein in the N2a cells and WT mice are detected with the anti-FXN antibody (A and B). The human FXN protein from the transgene in the YG8R mice as well as the human recombinant FXN protein in the injected mice are detected in both the liver and the mice brain (A and B). Incubation of the blots with the anti-FXN antibody for one hour begins to reveal the human FXN protein while overnight (o/n) incubation reveals both the mouse FXN in the WT mice, and the human FXN in the hemizygous (Tg/-) and homozygous mice (Tg/Tg). Levels of expression of the recombinant human FXN protein expressed from the injected rAAV9-FXN are similar to the levels of the endogenous mouse FXN protein in the WT mice. HA, hemagglutinin tag; iFXN, intermediate form FXN; mFXN, mature form FXN; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element.

Figure 9. Restoration of the clasping reflex in the YG8R mice over time following injection with the rAAV9-FXN vector. Clasping of hindlimbs was scored from 0 to 3 (see Methods), denoting less to more impairment, in hemizygous (Tg/-) mice and WT injected with rAAV9-FXN or rAAV9-null vectors. Results for females and males together (n=10, 5 males and 5 females for each group). Alterations in the clasping reflex are detected as early as 4 months of age in the hemizygous YG8R mice. Following intrathecal injection of the rAAV9-FXN vector the clasping reflex appears to normalize, indicating a restoration over time of this neurological pathological phenotype. Asterisks (*) denote significant differences between values for WT and the Tg/- mice injected with rAAV9-null and the pound signs (#) denotes significant differences between values obtained for the Tg/- mice injected with rAAV9-null and Tg/- mice injected with rAAV9-FXN. *, # : $P<0.05$

Figure 10. *In vivo* expression of recombinant frataxin protein in the rAAV9-hPGK1-FXN injected mice restores the electrophysiological properties at the different distances from the stimulus (D1-D4) of the caudal nerve. Asterisks (*) denote significant differences between WT and Tg/- YG8R FRDA mice treated with rAAV9-null, while the pound (#) sign denotes significant differences between the rAAV9-null and the rAAV9-FXN YG8R-treated mice. Intrathecal injection of the mice at 2.5 months of age with the rAAV9-FXN vector prevents or slows the defects in nerve conduction

in the FRDA mouse model for FRDA (YG8R). WT, green (n = 10); Tg/- YG8R FRDA mice treated with the rAAV9-null vector, grey (n= 10); Tg/-YG8R FRDA mice treated with the rAAV9-FXN vector, blue (n = 10). Measurements were obtained at 1 cm, 2 cm, 3 cm and 4 cm from the tail tip denoted as values for sites D1, D2, D3, and D4, respectively * , # : P<0.05.

Figure 11. Preservation of dorsal root ganglia in the rAAV9-FXN treated FRDA mouse model YG8R mice. The caliber of the dorsal root ganglia lumbar appeared preserved as well as the mitochondria morphology in the FRDA mouse model treated with the rAAV9-FXN compared with the same mice treated with the rAAV9-null virus.

Summary of the invention

[0011] The present invention provides an adeno-associated virus (AAV) vector comprising a nucleic acid, wherein the nucleic acid comprises:

(i) a nucleic acid sequence encoding frataxin;
(ii) a phospho-glycerate-kinase (PGK) promoter consisting of SEQ ID NO: 1; and
(iii) a woodchuck hepatitis virus posttranscriptional regulatory element (WPRE);

wherein (ii) and (iii) are operably linked to and regulate the expression of (i), wherein there is an operationally functional linker between (i) and (ii), wherein said linker consists of SEQ ID NO: 6, and wherein the AAV vector is an AAV serotype 9 vector.

[0012] The present invention also provides a transfer vector which comprises the nucleic acid and additional nucleic acid elements for promoting integration or transposition of the transfer vector into an AAV serotype 9 vector. Further described herein is the use of the transfer vector for the production of the AAV vector of the present invention. Also, the present invention provides a pharmaceutical composition and the AAV vector of the invention for use as a medicament, specifically for use in the treatment of FRDA.

Detailed description of the invention

Definitions

[0013] The terms "*treatment*" and "*therapy*", as used in the present application, refer to a set of hygienic, pharmacological, surgical and/or physical means used with the intent to cure and/or alleviate a disease and/or symptoms with the goal of remediating the health problem. The terms "*treatment*" and "*therapy*" include preventive and curative methods, since both are directed to the maintenance and/or reestablishment of the health of an individual or animal. Regardless of the origin of the symptoms, disease and disability, the administration of a suitable medicament to alleviate and/or cure a health problem should be interpreted as a form of treatment or therapy within the context of this application.

[0014] The term "*therapeutically effective amount*" refers to an amount of matter which has a therapeutic effect and which is able to treat FRDA.

[0015] The terms "*individual*", "*patient*" or "*subject*" are used interchangeably in the present application and are not meant to be limiting in any way. The "*individual*", "*patient*" or "*subject*" can be of any age, sex and physical condition.

[0016] As used herein, "*pharmaceutically acceptable carrier*" or "*pharmaceutically acceptable diluent*" means any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed and, without limiting the scope of the present invention, include: additional buffering agents; preservatives; co-solvents; antioxidants, including ascorbic acid and methionine; chelating agents such as EDTA; metal complexes (e.g., Zn-protein complexes); biodegradable polymers, such as polyesters; salt-forming counterions, such as sodium, polyhydric sugar alcohols; amino acids, such as alanine, glycine, glutamine, asparagine, histidine, arginine, lysine, ornithine, leucine, 2-phenylalanine, glutamic acid, and threonine; organic sugars or sugar alcohols, such as lactitol, stachyose, mannose, sorbose, xylose, ribose, ribitol, myoinisitose, myoinisitol, galactose, galactitol, glycerol, cyclitols (e.g., inositol), polyethylene glycol; sulphur containing reducing agents, such as urea, glutathione, thioctic acid, sodium thioglycolate, thioglycerol, [alpha]-monothioglycerol, and sodium thio sulphate; low molecular weight proteins, such as human serum albumin, bovine serum albumin, gelatin, or other immunoglobulins; and hydrophilic polymers, such as polyvinylpyrrolidone.

[0017] The term "*frataxin*" refers to a protein which is encoded by the FXN gene and which is usually localized in the mitochondrion. Details of the protein can be found in the UniProtKB database under accession number Q16595.

[0018] The term "*promoter*" refers to a DNA sequence to which RNA polymerase can bind to in order to initiate

transcription. The sequence may further contain binding sites for various proteins that regulate transcription, such as transcription factors. The promoter sequence may be composed of different promoter fragments (either different or the same fragments) that are localized closely in the DNA sequence and may be separated by linkers or spacers. Such promoters are referred to as chimeric promoters. In a preferred embodiment, the term "*promoter*" refers to a phospho-glycerate-kinase (PGK) promoter.

[0019] The term "*posttranscriptional regulatory element*" refers to a DNA sequence that when transcribed creates a tertiary structure which enhances or inhibits the expression of a protein.

[0020] The term "*operably linked*" refers to two or more nucleic acid sequences that are connected in a way that allows one nucleic acid sequence to influence another. For example, the PGK promoter and the WPRE are operably linked to the nucleic acid sequence encoding frataxin so that the expression levels of frataxin are regulated by the PGK promoter and WPRE.

[0021] The term "*functional variant*" refers to nucleic or amino acids whose nucleic or amino acid sequence differs in one or more positions from the parental nucleic or amino acid sequence, whereby differences might be additions, deletions and/or substitutions of nucleic acids or amino acid residues, and which are still functional and therefore a suitable for treating FRDA. A skilled person may determine functional variants by seeking homologues with a BLAST search or by studying the variability of the protein or gene in a population.

[0022] The term "*Adeno-associated virus*" refers to a small virus which infects humans and some other primate species. A "*vector*" is any vehicle which can be used to artificially carry foreign genetic material into a cell. Thus, an "*AAV vector*" refers to a recombinant AAV which carries a nucleic acid into a cell.

AAV vector

[0023] In a first aspect, the present invention provides an adeno-associated virus (AAV) vector comprising a nucleic acid, wherein the nucleic acid comprises:

(i) a nucleic acid sequence encoding frataxin;
(ii) a phospho-glycerate-kinase (PGK) promoter consisting of SEQ ID NO: 1; and
(iii) a woodchuck hepatitis virus posttranscriptional regulatory element (WPRE);

wherein (ii) and (iii) are operably linked to and regulate the expression of (i), wherein there is an operationally functional linker between (i) and (ii), wherein said linker consists of SEQ ID NO: 6, and wherein the AAV vector is an AAV serotype 9 vector.

[0024] SEQ ID NO: 1 refers to the following sequence:

GAATTCCGGGGTTGGGGTTGCGCCTTTTCCAAGGCAGCCCTGGGTCTGCGCAGGGAC
GCGGCTGCTCTGGGCGTGGTTCCGGGAAACGCAGCGGCGCCGACCCTGGGTCTCGCA
CATTCTTCACGTCCGTTCGCAGCGTCACCCGGATCTTCGCCGCTACCCTTGTGGGCCC
CCCGGCGACGCTTCCTGCTCCGCCCCTAAGTCGGGAAGGTTCCTTGCGGTTCGCGGC
GTGCCGGACGTGACAAACGGAAGCCGCACGTCTCACTAGTACCCTCGCAGACGGACAG
CGCCAGGGAGCAATGGCAGCGCGCCGACCGCGATGGGCTGTGGCCAATAGCGGCTGC
TCAGCAGGGCGCGCCGAGAGCAGCGGCCGGGAAGGGGCGGTGCGGGAGGCGGGGT
GTGGGGCGGTAGTGTGGGCCCTGTTCCTGCCCGCGCGGTGTTCCGCATTCTGCAAGC
CTCCGGAGCGCACGTCGGCAGTCGGCTCCCTCGTTGACCGAATCACCGACCTCTCTCC
CCAG

[0025] The PGK promoter is SEQ ID NO: 1.

[0026] The sequence identity between two sequences can be determined through conventional methods. For example, by using standard alignment logarithms known in the state of the art such as BLAST (Altschul et al., 1990. J Mol Biol. 215(3): 403-10). In a preferred embodiment, the sequence identity between two sequences is determined using BLAST.

[0027] SEQ ID NO: 2 refers to the following sequence:

TCGACAATCAACCTCTGGATTACAAAATTTGTGAAAGATTGACTGGTATTCTTAACTATGT
TGCTCCTTTTACGCTATGTGGATACGCTGCTTTAATGCCTTTGTATCATGCTATTGCTTC
CCGTATGGCTTTCATTTTCTCCTCCTTGTATAAATCCTGGTTGCTGTCTCTTTATGAGGA
GTTGTGGCCCGTTGTCAGGCAACGTGGCGTGGTGTGCACTGTGTTTGCTGACGCAACC
CCCACTGGTTGGGGCATTGCCACCACCTGTCAGCTCCTTTCCGGGACTTTCGCTTTCCC
CCTCCCTATTGCCACGGCGGAACTCATCGCCGCCTGCCTTGCCCGCTGCTGGACAGGG
GCTCGGCTGTTGGGCACTGACAATTCCGTGGTGTTGTCGGGGAAGCTGACGTCCTTTC
CATGGCTGCTCGCCTGTGTTGCCACCTGGATTCTGCGCGGGACGTCCTTCTGCTACGT
CCCTTCGGCCCTCAATCCAGCGGACCTTCCTTCCCGCGGCCTGCTGCCGGCTCTGCGG
CCTCTTCCGCGTCTTCGCCTTCGCCCTCAGACGAGTCGGATCTCCCTTTGGGCCGCCT
CCCCGCCTG

[0028] In a preferred embodiment, the WPRE comprises SEQ ID NO: 2 or a sequence which is at least 75% identical to SEQ ID NO: 2. Preferably WPRE comprises SEQ ID NO: 2 or a sequence which is at least 75%, 80%, 85%, 89%, 90%, 91%, 92%, 95%, 97%, 98% or 99% identical to SEQ ID NO: 2. More preferably, the WPRE is SEQ ID NO: 2 or a sequence which is at least 75%, 80%, 85%, 89%, 90%, 91%, 92%, 95%, 97%, 98% or 99% identical to SEQ ID NO: 2.

[0029] SEQ ID NO: 3 refers to the following sequence:

ATGTGGACTCTCGGGCGCCGCGCAGTAGCCGGCCTCCTGGCGTCACCCAGCCCGGCC
CAGGCCCAGACCCTCACCCGGGTCCCGCGGCCGGCAGAGTTGGCCCCACTCTGCGGC
CGCCGTGGCCTGCGCACCGACATCGATGCGACCTGCACGCCCCGCCGCGCAAGTTCG
AACCAGAGAGGTCTCAACCAGATTTGGAATGTCAAAAAGCAGAGTGTCTATTTGATGAAT
TTGAGGAAATCTGGAACTTTGGGCCACCCAGGCTCTCTAGATGAGACCACCTATGAAAG
ACTAGCAGAGGAAACGCTGGACTCTTTAGCAGAGTTTTTTGAAGACCTTGCAGACAAGC
CATACACGTTTGAGGACTATGATGTCTCCTTTGGGAGTGGTGTCTTAACTGTCAAACTG
GGTGGAGATCTAGGAACCTATGTGATCAACAAGCAGACGCCAAACAAGCAAATCTGGCT
ATCTTCTCCATCCAGTGGACCTAAGCGTTATGACTGGACTGGGAAAAACTGGGTGTACT
CCCACGACGGCGTGTCCCTCCATGAGCTGCTGGCCGCAGAGCTCACTAAAGCCTTAAA
AACCAAACTGGACTTGTCTTCCTTGGCCTATTCCGGAAAAGATGCTT

[0030] In a preferred embodiment, the nucleic acid sequence encoding frataxin comprises SEQ ID NO: 3 or a sequence which is at least 75% identical to SEQ ID NO: 3 and is a functional variant of frataxin. Preferably, the nucleic acid sequence encoding frataxin comprises SEQ ID NO: 3 or a sequence which is at least 75%, 80%, 85%, 89%, 90%, 91%, 92%, 95%, 97%, 98% or 99% identical to SEQ ID NO: 3. More preferably, the nucleic acid sequence encoding frataxin is SEQ ID NO: 3 or a sequence which is at least 75%, 80%, 85%, 89%, 90%, 91%, 92%, 95%, 97%, 98% or 99% identical to SEQ ID NO: 3.

[0031] The nucleic acid comprises: a linker between the promoter and the nucleic acid sequence encoding frataxin, wherein the linker consists of SEQ ID NO: 6.

[0032] SEQ ID NO: 6 refers to the following sequence:

TGGCTAACTAGAGAACCCACTGCTTACTGGCTTATCGAAATTAATACGACTCACTATAGG
GAGACCCAAGCTGGCTAGCGTTTAAACTTAAGCTTGGCCGCCACC

[0033] In a preferred embodiment, the nucleic acid further comprises a Kozak sequence and the Kozak sequence is also operably linked to and regulates the expression of the nucleic acid sequence encoding frataxin. A Kozak sequence is a sequence which occurs in eukaryotic mRNA and has the consensus sequence gccRccAUGG, wherein R is a purine, lower-case letters denote the most common base at a position where the base can nevertheless vary and upper-case letters are highly conserved.

[0034] SEQ ID NO: 4 refers to the following sequence:

GAATTCCGGGGTTGGGGTTGCGCCTTTTCCAAGGCAGCCCTGGGTCTGCGCAGGGAC
GCGGCTGCTCTGGGCGTGGTTCCGGGAAACGCAGCGGCGCCGACCCTGGGTCTCGCA
CATTCTTCACGTCCGTTCGCAGCGTCACCCGGATCTTCGCCGCTACCCTTGTGGGCCC
CCCGGCGACGCTTCCTGCTCCGCCCCTAAGTCGGGAAGGTTCCTTGCGGTTCGCGGC
GTGCCGGACGTGACAAACGGAAGCCGCACGTCTCACTAGTACCCTCGCAGACGGACAG
CGCCAGGGAGCAATGGCAGCGCGCCGACCGCGATGGGCTGTGGCCAATAGCGGCTGC
TCAGCAGGGCGCGCCGAGAGCAGCGGCCGGGAAGGGGCGGTGCGGGAGGCGGGGT
GTGGGGCGGTAGTGTGGGCCCTGTTCCTGCCCGCGCGGTGTTCCGCATTCTGCAAGC
CTCCGGAGCGCACGTCGGCAGTCGGCTCCCTCGTTGACCGAATCACCGACCTCTCTCC
CCAGTGGCTAACTAGAGAACCCACTGCTTACTGGCTTATCGAAATTAATACGACTCACTA
TAGGGAGACCCAAGCTGGCTAGCGTTTAAACTTAAGCTTGGCCGCCACCATGTGGACT
CTCGGGCGCCGCGCAGTAGCCGGCCTCCTGGCGTCACCCAGCCCGGCCCAGGCCCA
GACCCTCACCCGGGTCCCGCGGCCGGCAGAGTTGGCCCCACTCTGCGGCCGCCGTGG
CCTGCGCACCGACATCGATGCGACCTGCACGCCCCGCCGCGCAAGTTCGAACCAGAG
AGGTCTCAACCAGATTTGGAATGTCAAAAAGCAGAGTGTCTATTTGATGAATTTGAGGAA
ATCTGGAACTTTGGGCCACCCAGGCTCTCTAGATGAGACCACCTATGAAAGACTAGCAG
AGGAAACGCTGGACTCTTTAGCAGAGTTTTTTGAAGACCTTGCAGACAAGCCATACACG

TTTGAGGACTATGATGTCTCCTTTGGGAGTGGTGTCTTAACTGTCAAACTGGGTGGAGA
TCTAGGAACCTATGTGATCAACAAGCAGACGCCAAACAAGCAAATCTGGCTATCTTCTC
CATCCAGTGGACCTAAGCGTTATGACTGGACTGGGAAAAACTGGGTGTACTCCCACGA
CGGCGTGTCCCTCCATGAGCTGCTGGCCGCAGAGCTCACTAAAGCCTTAAAAACCAAA
CTGGACTTGTCTTCCTTGGCCTATTCCGGAAAAGATGCTTTGCCCACCTAGGGATCGGA
TCCCCGGGTACCGAGCTCGAATTCTGCAGATATCCAGCACACTTTGCCTTTCTCTCCAC
AGGTGTCGACAATCAACCTCTGGATTACAAAATTTGTGAAAGATTGACTGGTATTCTTAA
CTATGTTGCTCCTTTTACGCTATGTGGATACGCTGCTTTAATGCCTTTGTATCATGCTATT
GCTTCCCGTATGGCTTTCATTTTCTCCTCCTTGTATAAATCCTGGTTGCTGTCTCTTTATG
AGGAGTTGTGGCCCGTTGTCAGGCAACGTGGCGTGGTGTGCACTGTGTTTGCTGACGC
AACCCCCACTGGTTGGGGCATTGCCACCACCTGTCAGCTCCTTTCCGGGACTTTCGCTT
TCCCCCTCCCTATTGCCACGGCGGAACTCATCGCCGCCTGCCTTGCCCGCTGCTGGAC
AGGGGCTCGGCTGTTGGGCACTGACAATTCCGTGGTGTTGTCGGGGAAGCTGACGTCC
TTTCCATGGCTGCTCGCCTGTGTTGCCACCTGGATTCTGCGCGGGACGTCCTTCTGCTA
CGTCCCTTCGGCCCTCAATCCAGCGGACCTTCCTTCCCGCGGCCTGCTGCCGGCTCTG
CGGCCTCTTCCGCGTCTTCGCCTTCGCCCTCAGACGAGTCGGATCTCCCTTTGGGCCG
CCTCCCCGCCTG

[0035] In a preferred embodiment, the sequence of the nucleic acid which comprises (i), (ii) and (iii) is SEQ ID NO: 4 or a sequence which is at least 75% identical to SEQ ID NO: 4. Preferably, the nucleic acid which comprises (i), (ii) and (iii) is SEQ ID NO: 4 or a sequence which is at least 75%, 80%, 85%, 89%, 90%, 91%, 92%, 95%, 97%, 98% or 99% identical to SEQ ID NO: 4.

[0036] In a preferred embodiment, the nucleic acid further comprises a PolyA signal. Preferably, the PolyA signal is at least 50, 100, 150 or 228 bp long. More preferably the PolyA signal is at least 228 bp long. Most preferably, the PolyA signal is 228 bp long.

[0037] In a preferred embodiment, the nucleic acid further comprises one or more inverted terminal repeat (ITR) sequences. Preferably the nucleic acid comprises two ITR sequences. More preferably, the ITR sequences flank the rest of the components of the nucleic acid.

[0038] SEQ ID NO: 5 refers to the following sequence:

CTGGCGCGCTCGCTCGCTCACTGAGGCCGCCCGGGCAAAGCCCGGGCGTCGGGCGA
CCTTTGGTCGCCCGGCCTCAGTGAGCGAGCGAGCGCGCAGAGAGGGAGTGGCCAACT
CCATCACTAGGGGTTCCTGAATTCCGGGGTTGGGGTTGCGCCTTTTCCAAGGCAGCCC
TGGGTCTGCGCAGGGACGCGGCTGCTCTGGGCGTGGTTCCGGGAAACGCAGCGGCG

CCGACCCTGGGTCTCGCACATTCTTCACGTCCGTTCGCAGCGTCACCCGGATCTTCGC
CGCTACCCTTGTGGGCCCCCCGGCGACGCTTCCTGCTCCGCCCCTAAGTCGGGAAGG
TTCCTTGCGGTTCGCGGCGTGCCGGACGTGACAAACGGAAGCCGCACGTCTCACTAGT
ACCCTCGCAGACGGACAGCGCCAGGGAGCAATGGCAGCGCGCCGACCGCGATGGGCT
GTGGCCAATAGCGGCTGCTCAGCAGGGCGCGCCGAGAGCAGCGGCCGGGAAGGGGC
GGTGCGGGAGGCGGGGTGTGGGGCGGTAGTGTGGGCCCTGTTCCTGCCCGCGCGGT
GTTCCGCATTCTGCAAGCCTCCGGAGCGCACGTCGGCAGTCGGCTCCCTCGTTGACCG
AATCACCGACCTCTCTCCCCAGTGGCTAACTAGAGAACCCACTGCTTACTGGCTTATCG
AAATTAATACGACTCACTATAGGGAGACCCAAGCTGGCTAGCGTTTAAACTTAAGCTTG
GCCGCCACCATGTGGACTCTCGGGCGCCGCGCAGTAGCCGGCCTCCTGGCGTCACCC
AGCCCGGCCCAGGCCCAGACCCTCACCCGGGTCCCGCGGCCGGCAGAGTTGGCCCC
ACTCTGCGGCCGCCGTGGCCTGCGCACCGACATCGATGCGACCTGCACGCCCCGCCG
CGCAAGTTCGAACCAGAGAGGTCTCAACCAGATTTGGAATGTCAAAAAGCAGAGTGTCT
ATTTGATGAATTTGAGGAAATCTGGAACTTTGGGCCACCCAGGCTCTCTAGATGAGACC
ACCTATGAAAGACTAGCAGAGGAAACGCTGGACTCTTTAGCAGAGTTTTTTGAAGACCT
TGCAGACAAGCCATACACGTTTGAGGACTATGATGTCTCCTTTGGGAGTGGTGTCTTAA
CTGTCAAACTGGGTGGAGATCTAGGAACCTATGTGATCAACAAGCAGACGCCAAACAAG
CAAATCTGGCTATCTTCTCCATCCAGTGGACCTAAGCGTTATGACTGGACTGGGAAAAA
CTGGGTGTACTCCCACGACGGCGTGTCCCTCCATGAGCTGCTGGCCGCAGAGCTCACT
AAAGCCTTAAAAACCAAACTGGACTTGTCTTCCTTGGCCTATTCCGGAAAAGATGCTTTG
CCCACCTAGGGATCGGATCCCCGGGTACCGAGCTCGAATTCTGCAGATATCCAGCACA
CTTTGCCTTTCTCTCCACAGGTGTCGACAATCAACCTCTGGATTACAAAATTTGTGAAAG
ATTGACTGGTATTCTTAACTATGTTGCTCCTTTTACGCTATGTGGATACGCTGCTTTAATG
CCTTTGTATCATGCTATTGCTTCCCGTATGGCTTTCATTTTCTCCTCCTTGTATAAATCCT
GGTTGCTGTCTCTTTATGAGGAGTTGTGGCCCGTTGTCAGGCAACGTGGCGTGGTGTG
CACTGTGTTTGCTGACGCAACCCCCACTGGTTGGGGCATTGCCACCACCTGTCAGCTC
CTTTCCGGGACTTTCGCTTTCCCCCTCCCTATTGCCACGGCGGAACTCATCGCCGCCTG
CCTTGCCCGCTGCTGGACAGGGGCTCGGCTGTTGGGCACTGACAATTCCGTGGTGTTG
TCGGGGAAGCTGACGTCCTTTCCATGGCTGCTCGCCTGTGTTGCCACCTGGATTCTGC
GCGGGACGTCCTTCTGCTACGTCCCTTCGGCCCTCAATCCAGCGGACCTTCCTTCCCG
CGGCCTGCTGCCGGCTCTGCGGCCTCTTCCGCGTCTTCGCCTTCGCCCTCAGACGAGT
CGGATCTCCCTTTGGGCCGCCTCCCCGCCTGGAATTCGAGCTCGGTACGATCAGCTGA
TCAGCCTCGACTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGCCCCTCCCCCGTGCC
TTCCTTGACCCTGGAAGGTGCCACTCCCACTGTCCTTTCCTAATAAAATGAGGAAATTGC
ATCGCATTGTCTGAGTAGGTGTCATTCTATTCTGGGGGGTGGGGTGGGGCAGGACAGC
AAGGGGGAGGATTGGGAAGACAATAGCAGGCATGCTGGGGATGCGGTGGGCTCTATG
GCTGGCGCGCTCGCTCGCTCACTGAGGCCGCCCGGGCAAAGCCCGGGCGTCGGGCG

ACCTTTGGTCGCCCGGCCTCAGTGAGCGAGCGAGCGCGCAGAGAGGGAGTGGCCAAC
TCCATCACTAGGGGTTCCT

[0039] In a preferred embodiment, the nucleic acid is SEQ ID NO: 5 or a sequence which is at least 75% identical to SEQ ID NO: 5. Preferably, the nucleic acid is SEQ ID NO: 5 or a sequence which is at least 75%, 80%, 85%, 89%, 90%, 91%, 92%, 95%, 97%, 98% or 99% identical to SEQ ID NO: 5.

[0040] In a preferred embodiment, the vector allows for the expression of a therapeutically effective amount of frataxin in a patient who suffers from Friedreich's ataxia. Thus, the vector delivers the nucleic acid to a patient's cells where the nucleic acid then expresses frataxin at a therapeutically effective amount.

[0041] The AAV vector is an AAV serotype 9 vector, i.e. an AAV-9 vector.

Nucleic acid

[0042] In a second aspect, the present specification discloses a nucleic acid comprising: (i) a nucleic acid sequence encoding frataxin; (ii) a PGK promoter; and (iii) a WPRE; wherein (ii) and (iii) are operably linked to and regulate the expression of (i).

[0043] In a preferred disclosure, the PGK promoter comprises SEQ ID NO: 1 or a sequence which is at least 75% identical to SEQ ID NO: 1. Preferably the PGK promoter comprises SEQ ID NO: 1 or a sequence which is at least 75%, 80%, 85%, 89%, 90%, 91%, 92%, 95%, 97%, 98% or 99% identical to SEQ ID NO: 1. More preferably, the PGK promoter is SEQ ID NO: 1 or a sequence which is at least 75%, 80%, 85%, 89%, 90%, 91%, 92%, 95%, 97%, 98% or 99% identical to SEQ ID NO: 1.

[0044] In a preferred disclosure, the WPRE comprises SEQ ID NO: 2 or a sequence which is at least 75% identical to SEQ ID NO: 2. Preferably WPRE comprises SEQ ID NO: 2 or a sequence which is at least 75%, 80%, 85%, 89%, 90%, 91%, 92%, 95%, 97%, 98% or 99% identical to SEQ ID NO: 2. More preferably, the WPRE is SEQ ID NO: 2 or a sequence which is at least 75%, 80%, 85%, 89%, 90%, 91%, 92%, 95%, 97%, 98% or 99% identical to SEQ ID NO: 2.

[0045] In a preferred disclosure, the nucleic acid sequence encoding frataxin comprises SEQ ID NO: 3 or a sequence which is at least 75% identical to SEQ ID NO: 3 and is a functional variant of frataxin. Preferably, the nucleic acid sequence encoding frataxin comprises SEQ ID NO: 3 or a sequence which is at least 75%, 80%, 85%, 89%, 90%, 91%, 92%, 95%, 97%, 98% or 99% identical to SEQ ID NO: 3. More preferably, the nucleic acid sequence encoding frataxin is SEQ ID NO: 3 or a sequence which is at least 75%, 80%, 85%, 89%, 90%, 91%, 92%, 95%, 97%, 98% or 99% identical to SEQ ID NO: 3.

[0046] In a preferred disclosure, the nucleic acid further comprises: a linker between the promoter and the nucleic acid sequence encoding frataxin, wherein the linker consists of or comprises SEQ ID NO: 6 or a sequence which is at least 75% identical to SEQ ID NO: 6. Preferably, the linker consists of or comprises SEQ ID NO: 6 or a sequence which is at least 75%, 80%, 85%, 89%, 90%, 91%, 92%, 95%, 97%, 98% or 99% identical to SEQ ID NO: 6.

[0047] In a preferred disclosure, the nucleic acid further comprises a Kozak sequence and the Kozak sequence is also operably linked to and regulates the expression of the nucleic acid sequence encoding frataxin.

[0048] In a preferred disclosure, the sequence of the nucleic acid which comprises (i), (ii) and (iii) is SEQ ID NO: 4 or a sequence which is at least 75 % identical to SEQ ID NO: 4. Preferably, the nucleic acid which comprises (i), (ii) and (iii) is SEQ ID NO: 4 or a sequence which is at least 75%, 80%, 85%, 89%, 90%, 91%, 92%, 95%, 97%, 98% or 99% identical to SEQ ID NO: 4.

[0049] In a preferred disclosure, the nucleic acid further comprises a PolyA signal. Preferably, the PolyA signal is at least 50, 100, 150 or 228 bp long. More preferably the PolyA signal is at least 228 bp long. Most preferably, the PolyA signal is 228 bp long.

[0050] In a preferred disclosure, the nucleic acid further comprises one or more inverted terminal repeat (ITR) sequences. Preferably the nucleic acid comprises two ITR sequences. More preferably, the ITR sequences flank the rest of the components of the nucleic acid.

[0051] In a preferred disclosure, the nucleic acid is SEQ ID NO: 5 or a sequence which is at least 75% identical to SEQ ID NO: 5. Preferably, the nucleic acid is SEQ ID NO: 5 or a sequence which is at least 75%, 80%, 85%, 89%, 90%, 91%, 92%, 95%, 97%, 98% or 99% identical to SEQ ID NO: 5.

Cloning vector

[0052] In a third aspect, the present disclosure provides a cloning vector which comprises the nucleic acid according to any one of the previously described embodiments and additional nucleic acid elements for promoting replication of the cloning vector in a bacterial cell.

[0053] The term "*cloning vector*" refers to any vector that is suitable for cloning, which generally involves the presence

of restriction sites, an origin of replication for bacterial propagation and a selectable marker.

[0054] The cloning vector of the disclosure comprises the nucleic acid described herein and can preferably be used to produce the transfer vector or AAV vector of the invention.

Transfer vector

[0055] In a fourth aspect, the present disclosure provides a transfer vector which comprises the nucleic acid according to any one of the previously described embodiments or disclosures and additional nucleic acid elements for promoting integration or transposition of the transfer vector into an AAV vector, preferably an AAV-9 vector.

[0056] The term "*transfer vector*" refers to a vector that is suitable for integration or transposition in an AAV vector. The transfer vector thus generally permits the insertion of genetic information into an AAV vector.

[0057] The transfer vector of the invention comprises the nucleic acid as described in claim 9 and can preferably be used to produce the AAV vector of the invention.

Use of the transfer vector

[0058] In a fifth aspect, the present disclosure provides the use of the transfer vector of the present invention for the production of an AAV vector according to any one of the embodiments previously described.

Pharmaceutical composition

[0059] In a sixth aspect, the present invention provides a pharmaceutical composition comprising the AAV vector of the present invention and a pharmaceutically acceptable carrier or diluent. A pharmaceutical composition as described herein may also contain other substances. These substances include, but are not limited to, cryoprotectants, lyoprotectants, surfactants, bulking agents, anti-oxidants, and stabilizing agents. In some embodiments, the pharmaceutical composition may be lyophilized.

[0060] The term "*cryoprotectant*" as used herein, includes agents which provide stability to the AAV vector against freezing-induced stresses, by being preferentially excluded from the AAV vector's surface. Cryoprotectants may also offer protection during primary and secondary drying and long-term product storage. Non-limiting examples of cryoprotectants include sugars, such as sucrose, glucose, trehalose, mannitol, mannose, and lactose; polymers, such as dextran, hydroxyethyl starch and polyethylene glycol; surfactants, such as polysorbates (e.g., PS-20 or PS-80); and amino acids, such as glycine, arginine, leucine, and serine. A cryoprotectant exhibiting low toxicity in biological systems is generally used.

[0061] In one embodiment, a lyoprotectant is added to a pharmaceutical composition described herein. The term "*lyoprotectant*" as used herein, includes agents that provide stability to the AAV vector during the freeze-drying or dehydration process (primary and secondary freeze-drying cycles), by providing an amorphous glassy matrix and by binding with the AAV vector's surface through hydrogen bonding, replacing the water molecules that are removed during the drying process. This helps to minimize product degradation during the lyophilization cycle and improve the long-term product stability. Non-limiting examples of lyoprotectants include sugars, such as sucrose or trehalose; an amino acid, such as monosodium glutamate, non-crystalline glycine or histidine; a methylamine, such as betaine; a lyotropic salt, such as magnesium sulphate; a polyol, such as trihydric or higher sugar alcohols, e.g., glycerin, erythritol, glycerol, arabitol, xylitol, sorbitol, and mannitol; propylene glycol; polyethylene glycol; pluronics; and combinations thereof. The amount of lyoprotectant added to a pharmaceutical composition is generally an amount that does not lead to an unacceptable amount of degradation of the strain when the pharmaceutical composition is lyophilized.

[0062] In some embodiments, a bulking agent is included in the pharmaceutical composition. The term "*bulking agent*" as used herein, includes agents that provide the structure of the freeze-dried product without interacting directly with the pharmaceutical product. In addition to providing a pharmaceutically elegant cake, bulking agents may also impart useful qualities in regard to modifying the collapse temperature, providing freeze-thaw protection, and enhancing the strain stability over long-term storage. Non-limiting examples of bulking agents include mannitol, glycine, lactose, and sucrose. Bulking agents may be crystalline (such as glycine, mannitol, or sodium chloride) or amorphous (such as dextran, hydroxyethyl starch) and are generally used in formulations in an amount from 0.5% to 10%.

[0063] Other pharmaceutically acceptable carriers, excipients, or stabilizers, such as those described in Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980) may also be included in a pharmaceutical composition described herein, provided that they do not adversely affect the desired characteristics of the pharmaceutical composition. As used herein, "*pharmaceutically acceptable carrier*" means any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed and include: additional

buffering agents; preservatives; co-solvents; antioxidants, including ascorbic acid and methionine; chelating agents such as EDTA; metal complexes (e.g., Zn-protein complexes); biodegradable polymers, such as polyesters; salt-forming counterions, such as sodium, polyhydric sugar alcohols; amino acids, such as alanine, glycine, glutamine, asparagine, histidine, arginine, lysine, ornithine, leucine, 2-phenylalanine, glutamic acid, and threonine; organic sugars or sugar alcohols, such as lactitol, stachyose, mannose, sorbose, xylose, ribose, ribitol, myoinisitose, myoinisitol, galactose, galactitol, glycerol, cyclitols (e.g., inositol), polyethylene glycol; sulfur containing reducing agents, such as urea, glutathione, thioctic acid, sodium thioglycolate, thioglycerol, [alpha]-monothioglycerol, and sodium thio sulphate; low molecular weight proteins, such as human serum albumin, bovine serum albumin, gelatin, or other immunoglobulins; and hydrophilic polymers, such as polyvinylpyrrolidone.

[0064] The pharmaceutical composition may be prepared for oral, sublingual, buccal, intravenous, intramuscular, subcutaneous, intraperitoneal, conjunctival, rectal, transdermal, intrathecal, topical and/or inhalation-mediated administration. In a preferred embodiment, the pharmaceutical composition may be a solution which is suitable for intravenous, intramuscular, conjunctival, transdermal, intraperitoneal and/or subcutaneous administration. In another embodiment, the pharmaceutical composition may be a solution which is suitable for sublingual, buccal and/or inhalation-mediated administration routes. In an alternative embodiment, the pharmaceutical composition may be a gel or solution which is suitable for intrathecal administration. In an alternative embodiment, the pharmaceutical composition may be an aerosol which is suitable for inhalation-mediated administration. In a preferred embodiment, the pharmaceutical composition may be prepared for intrathecal administration.

[0065] The pharmaceutical composition may further comprise common excipients and carriers which are known in the state of the art. For solid pharmaceutical compositions, conventional nontoxic solid carriers may be used which include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like. For solution for injection, the pharmaceutical composition may further comprise cryoprotectants, lyoprotectants, surfactants, bulking agents, anti-oxidants, stabilizing agents and pharmaceutically acceptable carriers. For aerosol administration, the pharmaceutical compositions are generally supplied in finely divided form along with a surfactant and propellant. The surfactant must, of course, be nontoxic, and is generally soluble in the propellant. Representative of such agents are the esters or partial esters of fatty acids containing from 6 to 22 carbon atoms, such as caproic, octanoic, lauric, palmitic, stearic, linoleic, linolenic, olesteric and oleic acids with an aliphatic polyhydric alcohol or its cyclic anhydride. Mixed esters, such as mixed or natural glycerides may be employed. A carrier can also be included, as desired, as with, e.g., lecithin for intranasal delivery. For suppositories, traditional binders and carriers may include, for example, polyalkalene glycols or triglycerides.

Medical uses

[0066] In a seventh aspect, the present disclosure provides the AAV vector of the present invention, or the pharmaceutical composition of the present invention for use as a medicament. In an eight aspect, the present disclosure provides the AAV vector of the present invention, or the pharmaceutical composition of the present invention for use in the treatment of Friedreich's ataxia.

[0067] In a preferred embodiment, the AAV vector of the present invention, t or the pharmaceutical composition of the present invention is administered intrathecally, intramuscularly, intracerebrally or intracerebroventricularly, preferably intrathecally or intramuscularly.

[0068] In a preferred embodiment, the AAV vector of the present invention, or the pharmaceutical composition of the present invention is administered at a dose of at least 1×10^9 vector genomes/Kg body weight, preferably 1×10^{10}, 1×10^{11} or 1×10^{12} vector genomes/Kg body weight. More preferably, at least or about 4.6×10^{12} vector genomes/Kg body weight are administered.

Examples

Example 1: Plasmid construction

[0069] The coding sequence of the isoform 1 of human frataxin (hFXN) was fused to a hemagglutinin tag (HA) and cloned into the pcDNA3.1 expression vector using the with In-Fusion® HD Cloning Kit (Clontech). The fusion system was used for all the cloning steps. Several constructs were generated fusing either the CMV, or the human (h) promoters (p) of synapsin (phSYN), neuron-specific enolase (phNSE), 1,255 bp of the FXN promoter (phFXN1255), or the human phosphoglycerate kinase isoform 1 (phPGK1) with the coding region of the FXN gene. Further regulatory elements such as the CMV enhancer and the Kozak sequences were added at the 5 prime-end in addition to the woodchuck hepatitis virus responsive element (WPRE) sequence at the 3 prime-end. All these expression vectors generated constructs are listed in Table 2.

[0070] Plasmids containing the same combination of regulatory elements were also generated to drive luciferase

expression by replacing the coding sequence of FXN for the firefly luciferase coding sequence (LUC) that was amplified from the pGL3-LUC vector (Promega) also using the In-Fusion® HD Cloning Kit (Clontech). These plasmids are listed in Table 3.

Example 2: Recombinant adeno-associated viral vector construction and production.

[0071] The expression cassettes from the pcDNA3.1-phPGK-kFXN-HA-WPRE and of the pcDNA3.1-phPGK-kLUC-HA-WPRE plasmid were cloned into the SnaBI-MfeI sites of the recombinant AAV9 vector (rAAV9-phPGK1-FXN-HA-WPRE vector and rAAV9-phPGK-LUC-WPRE vector). In addition, a control null vector was generated lacking the FXN coding sequence (AAV2/9-null). All three constructs and viral particles were generated by the Vector Production Unit at Center of Animal Biotechnology and Gene Therapy (Universitat Autònoma de Barcelona). The final titers obtained were 1.4×10^{13} vg/ml for AAV9-phPGK-FXN-HA-WPRE, 9.8×10^{12} vg/ml for AAV9-phPGK-LUC-WPRE, and 6×10^{12} vg/ml for AAV2/9-null.

Example 3: Optimizing frataxin expression

1. Cell culture, *in vitro* expression, and luciferase reporter assay

[0072] Mouse neuroblastoma cells (N2a), Human neuroblastoma cells (SH-SY5Y) and Human Embryonic Kidney (HEK 293) cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) containing 10% Fetal bovine serum (Sigma), 2 mM glutamine, 50 μg/ml penicillin/streptomycin (Life technologies) at 70% confluence in 10 cm culture dishes. Transfections were carried out using lipofectamine 2000 (Life technologies) for N2a and SH-SY5Y cells and calcium phosphate for HEK 293 using 4 μg plasmid DNAs alone in addition to 0.25 μg EGFP. Following the transfection, the media was replaced with fresh DMEM culture media for the HEK cells and with Neurobasal, B27 supplement, 10 μM retinoic acid, 2 mM glutamine, 50 μg/ml Penicillin/Streptomycin for the N2a and SH-SY5Y cells. Forty-eight hours after the cell culture media change the cells transfected with the expression plasmids listed in Table 2 were harvested and stored at -80 °C until further use. For luciferase reporter assay the plasmids listed in Table 3 were transfected as mentioned above and the cells were re-plated into 384 well plates and the luciferase activity determined using the dual luciferase reporter assay kit as per manufacturer instructions (Promega).

2. SDS-PAGE and immunoblotting

[0073] Proteins were extracted from cultured cells by homogenization in RIPA lysis buffer: 10 mM Tris-HCl pH 7.4, 140 mM NaCl, 0.1% sodium deoxycholate, 1% Triton X-100, 1% SDS, 2 mM Ethylenediaminetetraacetic acid (EDTA), 25 mM sodium fluoride (NaF), 2.5 mM NaVO3 and protein inhibitor cocktail (Roche). Protein concentration was determined using the DC-BioRad protein assay (BioRad). Total protein extract were mixed with 4X Protein Sample Loading Buffer (Li-Cor Biosciences) containing 1 mM DTT and separated by electrophoresis on a 15% acrylamide gel at constant 20 mA before transfer to PVDF membranes. Primary antibodies used were anti-FXN PAC 2518 (generous gift from by Dr. Grazia Isaya, Mayo Clinic, Rochester MN), anti-FXN (1G2, Merck Millipore), anti-HA tag (clone 16B12, MMS-101P, Covance) and anti-beta actin (AC15, Sigma). Infrared-dye conjugated secondary antibodies were anti-mouse IRDye-800CW and anti-Rabbit IRDye 700CW (Li-Cor Biosciences) and the immunoreactivity was detected using the Odyssey analyser software v2.1 (Li-Cor Biosciences).

3. Results

[0074] As can be seen in Figures 1-5 and Tables 2-3, the provision of a construct which comprises the hPGK1 promoter, linker sequences (length and content) and the WPRE allowed for the expression of frataxin in cells. The human PGK1 promoter is a metabolically regulated promoter expressed in physiologically relevant tissues thereby making a good candidate as a promoter for frataxin. However, the regulatory elements or the linker sequences/length to be used to be able to express the frataxin coding sequences was not clear. By trying different combinations of elements and sequences our results show that the protein expression from the human frataxin coding sequences requires the inclusion of an operationally functional linker between the hPGK1 promoter and the FXN coding sequences since constructs containing a length of about 105 nt allow for FXN expression while linker lengths around 35 nt do not, even with the addition of the WPRE RNA stabilizer sequence. Interestingly, this linker requirement was only noted when using the hPGK1 promoter but not with other promoters tested (ie. hSYN). Further, the levels of frataxin protein from this vector are much lower than that obtained from the CMV or CAG promoter driven vectors even in the absence of WPRE sequences thereby being more comparable to endogenous levels. One of the limitations of the previous approaches is that to be able to obtain frataxin expression, high driving promoters such as CAG or CMV promoters have been used resulting in very

high levels of protein expression which may cause cellular stress and override some of the protective effects. The vector presented here is able to drive the expression of frataxin protein in relevant nervous system and peripheral tissues in quantities within physiologically functional range.

Table 2: Summary of results. Relative levels of FXN after transfection with different constructs

	Frataxin expression plasmids	Cell type	Antibody	Immunoreactivity
1.1	pcDNA3.1-pCMV-FXN-HA	N2A / HEK293 / SHSY	Anti-HA tag Covance) / Anti-FXN 1G2 (Merck Millipore)	++
1.2	pcDNA3.1-phSYN-FXN-HA	SHSY	Anti-HA tag (Covance)	-
1.3	pcDNA3.1-phFXN-FXN-HA	HEK293 / SHSY	Anti-HA tag (Covance)	-
1.4	pcDNA3.1-prNSE-FXN-HA	N2A	Anti-HA tag (Covance)	-
1.5	pcDNA3.1-phNSE-FXN-HA	HEK293	Anti-HA tag (Covance)	-
	Addition of WPRE			
2.1	pcDNA3.1-pCMV-FXN-HA-WPRE	N2A/ HEK293	Anti-HA tag (Covance) / Anti-FXN 1G2 (Merck Millipore)	+++
2.2	pcDNA3.1-phSYN-FXN-HA-WPRE	N2A/ HEK293	Anti-HA tag (Covance)	-
2.3	pcDNA3.1-phSYN-5'UTR_FXN-FXN-HA-WPRE	N2A/ HEK293	Anti-HA tag I (Covance)	-
2.4	pcDNA3.1-phFXN-FXN-HA-WPRE	N2A/ HEK293	Anti-HA tag (Covance)	-
2.5	pcDNA3.1-prNSE-FXN-HA-WPRE	N2A	Anti-HA tag (Covance)	-
	Addition of CMV enhancer			
3.1	pcDNA3.1-E-phFXN-FXN-HA-WPRE	HEK293	Anti-HA tag (Covance)	-
3.2	pcDNA3.1-E-phFXN-FXN-HA	N2A/ HEK293	Anti-HA tag (Covance)	
3.3	pcDNA3.1-E-ph$YN-FXN-HA-WPRE	N2A	Anti-HA tag (Covance)	-
	Addition of KOZAK and WPRE sequences			
4.1	pcDNA3.1-pCMV-105nt-kFXN-HA-WPRE	N2A/ HEK293	Anti-HA tag (Covance) / Anti-FXN 1G2 (Merck Millipore)	++++
4.2	pcDNA3.1-phSYN-kFXN-HA-WPRE	HEK293 / N2A	Anti-HA tag (Covance)	-/+
4.3	pcDNA3.1-prNSE-kFXN-HA-WPRE	HEK293 / N2A	Anti-HA tag (Covance)	-/+
4.4	pcDNA3.1-phFXN-kFXN-HA-WPRE	HEK293	Anti-HA tag (Covance)	-
4.5	pcDNA3.1-E-phFXN-kFXN-HA-WPRE	HEK293	Anti-HA tag Covance)	-
	Use of specific linker, KOSAK and WPRE sequences			
5.1	pcDNA3.1-phPGK-105nt-kFXN-HA-WPRE	N2a/HEK293	Anti-HA tagl (Covance) / Anti-FXN 1G2 (Merck Millipore)	++

(continued)

	Use of specific linker, KOSAK and WPRE sequences			
5.2	pcDNA3.1-phNSE-105nt-kFXN-HA-WPRE	N2a/HEK293	Anti-HA tag (Covance)	+
5.3	peDNA3.1-phSYN--105nt-kFXN-HA-WPRE	N2a/HEK293	Anti-HA tag (Covance)	-
5.4	pcDNA3.1-phFXN-105nt-kFXN-HA-WPRE	N2A/HEK293	Anti-HA tag (Covance)	-

Table 3: Reporter assay of frataxin and PGK1 promoter constructs

Luciferase expression plasmids	Cell type	Expression
pcDNA3.1-pCMV-135nt-LUC-WPRE	N2a/ HEK293	++++
pcDNA3.1-phEGK-135nt-LUC-WPRE	N2a/HEK293	++
pcDNA3.1-E-phPGK-135nt-LUC-WPRE	N2a	++
pcDNA3.1-phFXN1255-135nt-LUC-WPRE	HEK293	-
pcDNA3.1-phFXN1255-35nt-LUC-WPRE	N2a/ HEK293	-
pcDNA3.1-phFXN220-35nt-LUC-WPRE	N2a/ HEK293	+
pcDNA3.1-phFXN1255OCT-35nt-LUC-WPRE	N2a/ HEK293	-

Example 4: *In vivo* data

1. Animals

[0075] The Friedreich Ataxia mouse model YG8R developed by Pook and colleagues (Pook et al. 2001. Neurogenetics. 3(4):185-93; Virmouni et al., 2014. PLoS ONE. 9(9):e107416) used in this study was obtained from the Jackson Laboratories Repository (Stock no. 012253). This human FXN transgenic mouse model is a knockout for the endogenous mouse frataxin gene (Fxn -/-) and contains the human FXN YAC transgene from a founder YG8 (carrying two tandem copies of the human FXN gene with approximately 82 and with 190 GAA trinucleotide sequence repeats). Mice were housed with SPF-Like conditions in NexGen Mouse IVC cages (Allentown, NJ) with a 12-h light-dark cycle and controlled negative pressure, temperature and humidity, and free access to water and an irradiated rodent chow Teklad global 18% protein (Envigo). The YG8R mouse colony was generated at The Institute for Health Science Research Germans Trias i Pujol (IGTP). Female and male hemizygous YG8R (Tg/-) and the WT C57Bl/6 mice were used in all experiments.

[0076] Mice hemizygous for the mutant human FXN gene (YG8R) were genotyped as previously reported (29,36). Briefly, the genomic DNA was extracted from the YG8R mouse tail by Maxwell® 16 Mouse Tail DNA Purification Kit (Promega). The transgene copy number was determined for each mouse using quantitative real-time PCR (qPCR) in LightCycler ® 480 Instrument (Roche) using SYBR Premix Ex Taq II (Tli RNase H Plus) (Takara) and the following primers for human frataxin transgene: hFXN_Tg_FW: 5'-GAAC-TTCAAATTAGTTCCCCTTTCTTC-3' (SEQ ID NO: 7), hFXN_Tg_RV: 5'-CACAGCCAT-TCTTTGGGTTTC-3' (SEQ ID NO: 8); and internal control Apolipoprotein B-100 isoform X1: IC_FW: 5'-CACGTGGGCTCCAGCATT-3' (SEQ ID NO: 9), IC_RV: TCACCAGTCATTTCTGCCTTTG (SEQ ID NO: 10). The assay was performed with thermal cycling conditions: 95 °C for 5 minutes, and 40 cycles of 95 °C for 20 seconds and 60 °C for 30 seconds, 72 °C for 30 seconds and finally 1 cycle at 72°C 2 min. Samples were assayed in triplicate for each gene of interest and levels of the transgene determined by the Ct ($\Delta\Delta$Ct) method. The transgene copy numbers was estimated relative to the Ct data from the control sample with a known copy number. The GAA repeat length was determined by GAA PCR amplification PCR using LA taq (Invitrogen) and the following primers: GAA-F: 5'-GGGATT-GGTT-GCCAGTGCTT-AAAAGTTAG-3' (SEQ ID NO: 11) and GAA-R: 5'-GATCTAAGGACCATCATGG-CCACACTT-GCC-3' (SEQ ID NO: 12). PCR products were resolved in 1.5% agarose gels by electrophoresis at 100 V for 3 hours and the band sizes were analysed. The number of GAA repeats were then determined by subtracting 451 bp (flanking non-repeat DNA) from the PCR product size, and dividing the remaining base pair repeat size by 3.

[0077] All animal procedures were carried out in accordance with EU and local regulations and approved by the

appropriate local Ethics Committees.

2. AAV administration

[0078] YG8R hemizygous and WT 10-weeks-old mice were anesthetized by intraperitoneal injection of ketamine (10 mg/kg of body weight; Imalgene 500; Rhône-Merieux, Lyon, France) and xylazine (1 mg/kg of body weight; Rompun; Bayer). Intrathecal administration of the AAV9-phPGK-FXN-HA-WPRE, AAV9-phPGK-LUC-WPRE, or AAV2/9-null was performed at the lumbar region. After lateral spine exposure, by paravertebral muscle dissection, viral vectors were slowly injected into the CSF through a 33-gauge needle and a Hamilton syringe between lumbar vertebrae L3 and L4. The appropriate access to the intrathecal space was confirmed by the animal's tail movement. Thereafter the muscle and skin were sutured. The quantity administered for each mouse of the different viral vectors was 4.6 $\times$ 10e-12 vg/Kg mouse weight.

3. Vector bio-distribution using luciferase imaging

[0079] For *in vivo* evaluation, of the AAV9-phPGK-LUC-WPRE vector biodistribution, the mice were administered intrathecally with the vector at 10 weeks of age (2.5 months-old) and 3.5 months later they received a single intraperitoneal injection of D-luciferin substrate solution at 150 mg/kg of mouse weight. The mice were anesthetised 15 min after substrate administration by inhaled anaesthesia (isoflurane 4% for induction and 2% for maintenance). Anesthetized mice were maintained in the dark chamber of Perkin Elmer Ivis Lumina II (Caliper Life Sciences, Germany) to record the photon emissions. Images were analysed with the Living imaging software (Xenogen Corporation, CA, EUA) with 1 min integration time, 12.5 cm vision field. For ex-vivo evaluation, tissues and organs of mice were extracted after 15 min of substrate administration. Tissues and organs were placed into clear dishes and images were captured using the Living imaging software (Xenogen Corporation, CA, EUA) as above. Results can be seen in Figure 6.

4. qRT-PCR

[0080] RNA was extracted from 30 mg of freshly frozen mouse tissue using the RNeasy Mini Kit (Qiagen). The RNA RIN and quantification was obtained with an Agilent 2200 TapeStation (Agilent). RNA was retrotranscribed to cDNA (25 ng/ul) using PrimeScriptTM RT reagent Kit (Takara) with thermal conditions: 37 °C for 15 minutes, 85 °C for 5 seconds. The qRT-PCR was performed with cDNA from mouse tissues to test levels of frataxin expression in a multiwell plate format using the LightCycler480 instrument (Roche Diagnostics). Reaction mixtures contained a total volume of 10 μl consisting of 0.1 mM of each primer, 10 ng of cDNA and 5 μl of TaqMan Universal Master Mix II, no UNG (Thermofisher Scientific). Primer-probe for human frataxin detection were predesigned by Bio-rad (dHsaCPE5031641, Bio-rad), primers for Beta-2 microglobulin (B2m) housekeeping gene for mice samples were predesigned by IDT (Mm.PT.39a.22214835; IDT). The assay was performed with thermal cycling conditions: 50 °C for 2 minutes, 95 °C for 10 minutes, and 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. Samples were assayed in triplicate for each gene of interest and the levels determined by the Ct ($\Delta\Delta$Ct) method. Results can be seen in Figure 7.

5. SDS-PAGE and immunoblotting

[0081] Proteins were extracted from mouse tissue by homogenization in RIPA lysis buffer: 10 mM Tris-HCl pH 7.4, 140 mM NaCl, 0.1% sodium deoxycholate, 1% Triton X-100, 1% SDS, 2 mM Ethylenediaminetetraacetic acid (EDTA), 25 mM sodium fluoride (NaF), 2.5 mM NaVO3 and protein inhibitor cocktail (Roche). Protein concentration was determined using the DC-BioRad protein assay (BioRad). Total protein extract were mixed with 4X Protein Sample Loading Buffer (Li-Cor Biosciences) containing 1 mM DTT and separated by electrophoresis on a 15% acrylamide gel at constant 20 mA before transfer to PVDF membranes. Primary antibodies used were anti-FXN PAC 2518 (generous gift from by Dr. Grazia Isaya, Mayo Clinic, Rochester MN), anti-FXN (1G2, Merck Millipore), anti- HA tag (clone 16B12, MMS-101P, Covance) and anti-beta actin (AC15, Sigma). Infrared-dye conjugated secondary antibodies were anti-mouse IRDye-800CW and anti-Rabbit IRDye 700CW (Li-Cor Biosciences) and the immunoreactivity was detected using the Odyssey analyser software v2.1 (Li-Cor Biosciences). Results can be seen in Figure 8.

6. Clasping

[0082] Hindlimb clasping has been shown to be a marker of disease progression in a number of mouse models of neurodegeneration. Each mouse was lifted by the tail away from any surrounding objects. The hindlimb position was observed for 10 seconds and scored as follows: If the hindlimbs were consistently splayed outward, away from the abdomen, it is assigned a score of 0. If one hindlimb was retracted toward the abdomen for more than 50% of the time

suspended, it receives a score of 1. If both hindlimbs were partially retracted toward the abdomen for more than 50% of the time suspended, it receives a score of 2. If its hindlimbs were entirely retracted and touching the abdomen for more than 50% of the time suspended, it receives a score of 3. The clasping reflex was assessed every 2 months in mice from 4 months of age. Results can be seen in Figure 9.

7. Electrophysiology

[0083] Amplitude (μV) and nerve conduction velocity (m/s) were measured in the caudal nerve of the mouse's tail. With animals under inhaled anaesthesia (isoflurane 2%), the stimulation electrode needle was situated subcutaneously at 4 different points of stimulation (1 cm, 2 cm, 3 cm, 4 cm from tail tip) while the registration needle point was fixed at 6 cm from tail tip. Values were recorded with the EMG/PE N-EP with 2 channels from Medelec Synergy apparatus (Viasys, EUA). During electrophysiological tests, the skin temperature of the animals was maintained above 32 °C. The mice were assessed every 2 months from 4 months of age. Results can be seen in Figure 10.

8. Results

[0084] We propose that the presented AAV treatment would be effective as treatment for FRDA because it can be expressed in physiologically relevant tissues, levels, shows similar processing as the endogenous protein and can significantly improve the electrophysiological properties of affected neurons and neurological symptoms in a mouse model of FRDA (YG8R). Figure 6 shows that the luciferase gene in the AAV9 vector under the same regulatory elements than the FXN in the AAV9-hPGK1-FXN vector when injected intrathecally, is expressed throughout the spinal cord, brain as well as in peripheral tissues. Also, expression of a luciferase under the same vector and regulatory elements stay consistent around at least 7 months after injection (data no shown). Because diminished levels of frataxin underlie most if not all the symptoms in FRDA and individuals' carriers for the mutation expressing $\geq$25% levels of frataxin are free of FRDA symptoms, a small increase in the FXN expression should be able to prevent or significantly ameliorate symptoms in FRDA patients. However, very high levels of FXN expression or any other protein could induce cellular and eventually diminish the protective effects or even worsen the symptoms. Therefore, it is of interest to develop a FXN expressing vector that results in levels as similar as possible to those of the endogenous FXN protein. Therefore, we developed a vector (AAV9-hPGK1-FXN) vector that allows for the expression of FXN under a metabolically regulated promoted in physiologically relevant levels and delivered it intrathecally into 10-week-old YG8R hemizygous mice (Tg/-). Higher levels of FXN mRNA were detected in the YG8R hemizygous mice (Tg/-) 3.5 months after intrathecal injection of the AAV9-PGK1-FXN vector compared to non-injected mice both in the liver and thoracic portion of the spinal cord (0.5 and 2-fold, respectively; Figure 7). These were equal or lower than in the homozygous YG8R mice which showed 0.5 and 3-fold spinal cord, respectively compared to the hemizygous YG8R mice. This indicates that this FXN encoding vector injected in the L3-L4 intrathecal region was taken-up and expressed in thoracic spinal cord neurons by either retrograde transport or as it diffuses through the spinal fluid. We also noted that the AAV9 vector intrathecally administered is also able to cross the blood brain barrier and reach peripheral tissues such as liver, heart, etc. (Figure 6) and is able to express the hFXN mRNA from the injected vector (Figure 7).

[0085] The levels of FXN protein expression from the recombinant vector developed was also analyzed. Although the antibody used shows a higher affinity for the human FXN protein as seen by the protein pattern obtained after 1 hour versus overnight incubation of the blot with the primary anti-FXN antibody, Figure 8 shows that the levels of the FXN protein expressed from the rAAV9-PGK1-FXN vector appear similar to those of the mouse FXN levels in the WT mice (Figure 8A, lanes 3 vs. 4 and Figure 8B second panel, lanes 3 vs. 4 and 5 vs. 6 and 7). Remarkably, we were able to detect the recombinant FXN protein in the motor cortex region (C1) of the mouse brain (Fig.8B, lane 7). This indicates that the delivery of our vector by intrathecal injection into the lumbar region of the spinal cord results in the distribution of the vector throughout motor related regions of the CNS. In addition, the recombinant protein appears to be processed in a similar pattern as the wild-type FXN protein with the intermediate and mature form being the most prominent processed form.

[0086] We further analysed the phenotype of the YG8R hemizygous mice (Tg/-) treated with the rAAV9-PGK1-FXN compared to the rAAV9-null vector for the clasping neurological reflex and the electrophysiological properties of the caudal nerve. The clasping reflex has been shown to be involved in several neurodegenerative disorders. This reflex appears to involve sensory and also spinal motor pathways regulating the fore and hindlimb movements. In particular, the cerebello-cortico-reticular pathways have been shown to be involved. Therefore, to determine the effect of the treatment of the rAAV9-hPGK1-FXN vector on these pathways in the YG8R mice we quantified the clasping reflex at different times following the intrathecal injection of the vector (see methods). Figure 9 shows that the YG8R mice treated with either the AAV9-null and AAV9-FXN vectors exhibit clasping defects as early as four-months of age (1.5 months after intrathecal injection). However, overtime the clasping reflex is normalized in the AAV-FXN treated mice to more closely resemble those of the WT mice even more so at 10-months of age (6.5 months after injection). This suggests

that there are early anomalies in the sensory and cerebello-cortico-reticular system in the FRDA mouse model that are significantly ameliorated or reversed by the treatment with the AAV-FXN vector.

[0087] To more specifically determine the effects of the treatment on the sensory neurons we determined the electrophysiological properties (amplitude and velocity) of the caudal nerve of the YG8R mice treated with the AAV-FXN vector compared with those treated with a AAV9-null control vector. Figure 10 shows that at all distances from the tail tip (1-4 cm) no significant differences were noted between the null vector treated WT or the null and AAV-FXN treated YG8R hemizygous mice at four months of age (1.5 months following treatment). However, from 6 months of age on, while the AAV9-null treated YG8R (Tg/-) mice showed significant decline in amplitude, the AAV9-FXN treated mice were more closely resembling the WT mice even as late as 13 months of age (9.5 months after treatment). No changes in velocity were noted (data not shown). These data indicate that the treatment with the AAV-hPGK1-FXN vector was able to preserve the function of the caudal nerve in the YG8R mice possibly by preventing loss of the axons from the affected neurons.

Claims

1. An adeno-associated virus (AAV) vector comprising a nucleic acid, wherein the nucleic acid comprises:

   (i) a nucleic acid sequence encoding frataxin;
   (ii) a phospho-glycerate-kinase (PGK) promoter consisting of SEQ ID NO: 1; and
   (iii) a woodchuck hepatitis virus posttranscriptional regulatory element (WPRE);

   wherein (ii) and (iii) are operably linked to and regulate the expression of (i), wherein there is an operationally functional linker between (i) and (ii), wherein said linker consists of SEQ ID NO: 6, and wherein the AAV vector is an AAV serotype 9 vector.

2. The vector according to claim 1, wherein the nucleic acid sequence encoding frataxin comprises SEQ ID NO: 3, or a sequence which is at least 90% identical to SEQ ID NO: 3 and is a functional variant of frataxin

3. The vector according to claim 1, wherein the nucleic acid sequence encoding frataxin consists of SEQ ID NO: 3, or a sequence which is at least 98% identical to SEQ ID NO: 3 and is a functional variant of frataxin.

4. The vector according to any one of claims 1-3, wherein the WPRE consists of SEQ ID NO: 2 or a sequence which is at least 90% identical to SEQ ID NO: 2.

5. The vector according to any one of claims 1-3, wherein the WPRE consists of SEQ ID NO: 2 or a sequence which is at least 99% identical to SEQ ID NO: 2.

6. The vector according to claim 1, wherein the nucleic acid sequence encoding frataxin consists of SEQ ID NO: 3, and the WPRE consists of SEQ ID NO: 2 or a sequence which is at least 90% identical to SEQ ID NO: 2.

7. The vector according to claim 1, wherein the nucleic acid sequence encoding frataxin consists of SEQ ID NO: 3, and the WPRE consists of SEQ ID NO: 2 or a sequence which is at least 99% identical to SEQ ID NO: 2.

8. The vector according to claim 1, wherein the sequence of the nucleic acid which comprises (i), (ii), (iii) and the linker, consists of SEQ ID NO: 4.

9. A transfer vector which comprises a nucleic acid comprising:

   (i) a nucleic acid sequence encoding frataxin;
   (ii) a PGK promoter consisting of SEQ ID NO: 1; and
   (iii) a WPRE;

   wherein (ii) and (iii) are operably linked to and regulate the expression of (i), wherein there is an operationally functional linker between (i) and (ii), and wherein said linker consists of SEQ ID NO: 6, and wherein the transfer vector further comprises additional nucleic acid elements for promoting integration or transposition of the transfer vector into an AAV serotype 9 vector.

10. A pharmaceutical composition comprising the AAV vector according to any one of claims 1-8, and a pharmaceutically acceptable carrier or diluent.

11. The AAV vector according to any one of claims 1-8, for use as a medicament.

12. The AAV vector according to any one of claims 1-8, for use in the treatment of Friedreich's ataxia.

Patentansprüche

1. Adeno-assoziierter Virus (AAV)-Vektor umfassend eine Nukleinsäure, wobei die Nukleinsäure Folgendes umfasst:

    (i) eine Nukleinsäuresequenz, welche für Frataxin kodiert;
    (ii) einen Phosphoglycerat-Kinase (PGK)-Promoter bestehend aus SEQ ID NO: 1; und
    (iii) ein posttranskriptionelles regulatorisches Element des Murmeltier-Hepatitisvirus (WPRE);

    wobei (ii) und (iii) operativ mit der Expression von (i) verbunden sind und dieselbe regulieren, wobei es einen operativ funktionellen Linker zwischen (i) und (ii) gibt, wobei der genannte Linker aus SEQ ID NO: 6 besteht und wobei der AAV-Vektor ein AAV-Vektor vom Serotyp 9 ist.

2. Vektor nach Anspruch 1, wobei die Nukleinsäuresequenz, welche für Frataxin kodiert, SEQ ID NO: 3 oder eine Sequenz, welche mindestens 90% identisch mit SEQ ID NO: 3 ist und eine funktionelle Variante des Frataxins ist, umfasst.

3. Vektor nach Anspruch 1, wobei die Nukleinsäuresequenz, welche für Frataxin kodiert, aus SEQ ID NO: 3 oder einer Sequenz, welche mindestens 98% identisch mit SEQ ID NO: 3 ist und eine funktionelle Variante des Frataxins ist, besteht.

4. Vektor nach einem der Ansprüche 1-3, wobei das WPRE aus SEQ ID NO: 2 oder einer Sequenz, welche mindestens 90% identisch mit SEQ ID NO: 2 ist, besteht.

5. Vektor nach einem der Ansprüche 1-3, wobei das WPRE aus SEQ ID NO: 2 oder einer Sequenz, welche mindestens 99% identisch mit SEQ ID NO: 2 ist, besteht.

6. Vektor nach Anspruch 1, wobei die Nukleinsäuresequenz, welche für Frataxin kodiert, aus SEQ ID NO: 3 besteht und das WPRE aus SEQ ID NO: 2 oder einer Sequenz, welche mindestens 90% identisch mit SEQ ID NO: 2 ist, besteht.

7. Vektor nach Anspruch 1, wobei die Nukleinsäuresequenz, welche für Frataxin kodiert, aus SEQ ID NO: 3 besteht und das WPRE aus SEQ ID NO: 2 oder einer Sequenz, welche mindestens 99% identisch mit SEQ ID NO: 2 ist, besteht.

8. Vektor nach Anspruch 1, wobei die Sequenz der Nukleinsäure, welche (i), (ii), (iii) und den Linker umfasst, aus SEQ ID NO: 4 besteht.

9. Transfervektor, welcher eine Nukleinsäure umfasst, welche Folgendes umfasst:

    (i) eine Nukleinsäuresequenz, welche für Frataxin kodiert;
    (ii) einen PGK-Promoter bestehend aus SEQ ID NO: 1; und
    (iii) ein WPRE;

    wobei (ii) und (iii) operativ mit der Expression von (i) verbunden sind und dieselbe regulieren, wobei es einen operativ funktionellen Linker zwischen (i) und (ii) gibt, und wobei der genannte Linker aus SEQ ID NO: 6 besteht und wobei der Transfervektor zusätzlich zusätzliche Nukleinsäureelemente zur Förderung der Integration oder Transposition des Transfervektors in einen AAV-Vektor vom Serotyp 9 umfasst.

10. Pharmazeutische Zusammensetzung umfassend den AAV-Vektor nach einem der Ansprüche 1-8 und einen pharmazeutisch akzeptablen Träger oder ein pharmazeutisch akzeptables Verdünnungsmittel.

11. AAV-Vektor nach einem der Ansprüche 1-8, für dessen Verwendung als Medikament.

12. AAV-Vektor nach einem der Ansprüche 1-8, für dessen Verwendung bei der Behandlung der Friedreich-Ataxie.

Revendications

1. Vecteur de virus adéno-associé (AAV) comprenant un acide nucléique, dans lequel l'acide nucléique comprend :

(i) une séquence d'acide nucléique codant pour frataxine ;
(ii) un promoteur de phosphoglycérate kinase (PGK) consistant en SEQ ID NO : 1 ; et
(iii) un élément de régulation post-transcriptionnel du virus de l'hépatite de la marmotte (WPRE) ;

dans lequel (ii) et (iii) sont opérationnellement liés à et régulent l'expression de (i), dans lequel il y a un lieur opérationnellement fonctionnel entre (i) et (ii), dans lequel ledit lieur consiste en SEQ ID NO : 6, et dans lequel le vecteur d'AAV est un vecteur d'AAV de sérotype 9.

2. Vecteur selon la revendication 1, dans lequel la séquence d'acide nucléique codant pour frataxine comprend SEQ ID NO : 3, ou une séquence qui est au moins 90% identique à SEQ ID NO : 3 et qui est un variant fonctionnel de frataxine.

3. Vecteur selon la revendication 1, dans lequel la séquence d'acide nucléique codant pour frataxine consiste en SEQ ID NO : 3, ou une séquence qui est au moins 98% identique à SEQ ID NO : 3 et qui est un variant fonctionnel de frataxine.

4. Vecteur selon l'une quelconque des revendications 1-3, dans lequel le WPRE consiste en SEQ ID NO : 2 ou une séquence qui est au moins 90% identique à SEQ ID NO : 2.

5. Vecteur selon l'une quelconque des revendications 1-3, dans lequel le WPRE consiste en SEQ ID NO : 2 ou une séquence qui est au moins 99% identique à SEQ ID NO : 2.

6. Vecteur selon la revendication 1, dans lequel la séquence d'acide nucléique codant pour frataxine consiste en SEQ ID NO : 3, et le WPRE consiste en SEQ ID NO : 2 ou une séquence qui est au moins 90% identique à SEQ ID NO : 2.

7. Vecteur selon la revendication 1, dans lequel la séquence d'acide nucléique codant pour frataxine consiste en SEQ ID NO : 3, et le WPRE consiste en SEQ ID NO : 2 ou une séquence qui est au moins 99% identique à SEQ ID NO : 2.

8. Vecteur selon la revendication 1, dans lequel la séquence de l'acide nucléique qui comprend (i), (ii), (iii) et le lieur, consiste en SEQ ID NO : 4.

9. Vecteur de transfert qui comprend un acide nucléique comprenant :

(i) une séquence d'acide nucléique codant pour frataxine ;
(ii) un promoteur de PGK consistant en SEQ ID NO : 1 ; et
(iii) un WPRE ;

dans lequel (ii) et (iii) sont opérationnellement liés à et régulent l'expression de (i), dans lequel il y a un lieur opérationnellement fonctionnel entre (i) et (ii), et dans lequel ledit lieur consiste en SEQ ID NO : 6, et dans lequel le vecteur de transfert comprend en outre des éléments d'acide nucléique additionnels pour promouvoir l'intégration ou la transposition du vecteur de transfert dans un vecteur d'AAV de sérotype 9.

10. Composition pharmaceutique comprenant le vecteur d'AAVselon l'une quelconque des revendications 1-8, et un véhicule ou un diluant pharmaceutiquement acceptable.

11. Vecteur d'AAV selon l'une quelconque des revendications 1-8, pour son utilisation comme médicament.

12. Vecteur d'AAV selon l'une quelconque des revendications 1-8, pour son utilisation dans le traitement de l'ataxie de Friedreich.

Figure 1

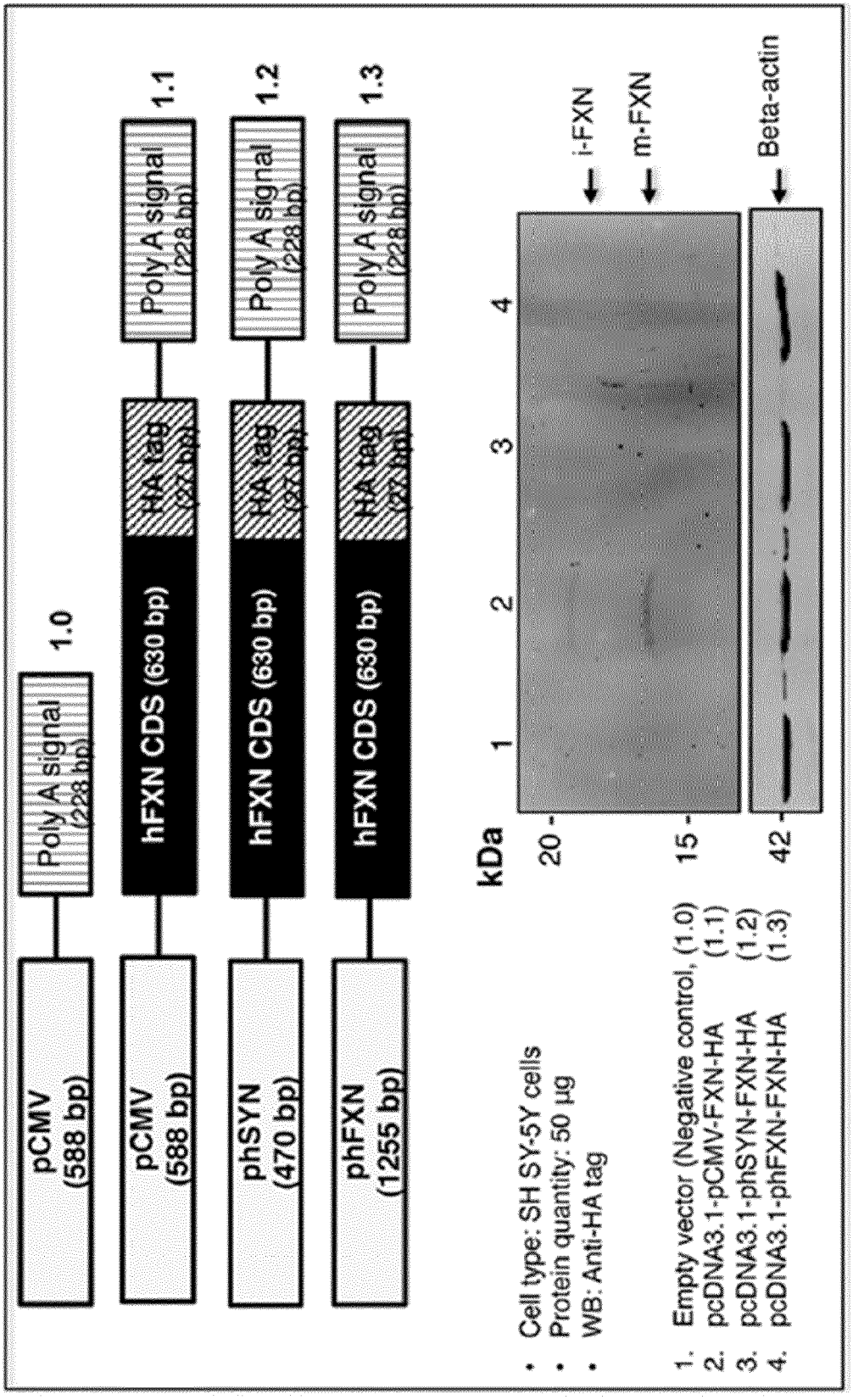

pCMV (588 bp)
Poly A signal (228 bp)
1.0
pCMV (588 bp)
hFXN CDS (630 bp)
HA tag (27 bp)
Poly A signal (228 bp)
1.1
phSYN (470 bp)
hFXN CDS (630 bp)
HA tag (27 bp)
Poly A signal (228 bp)
1.2
phFXN (1255 bp)
hFXN CDS (630 bp)
HA tag (27 bp)
Poly A signal (228 bp)
1.3
kDa
20
15
42
1
2
3
4
i-FXN
m-FXN
Beta-actin
Cell type: SH SY-5Y cells
Protein quantity: 50 µg
WB: Anti-HA tag
1. Empty vector (Negative control, (1.0)
2. pcDNA3.1-pCMV-FXN-HA (1.1)
3. pcDNA3.1-phSYN-FXN-HA (1.2)
4. pcDNA3.1-phFXN-FXN-HA (1.3)

Figure 2

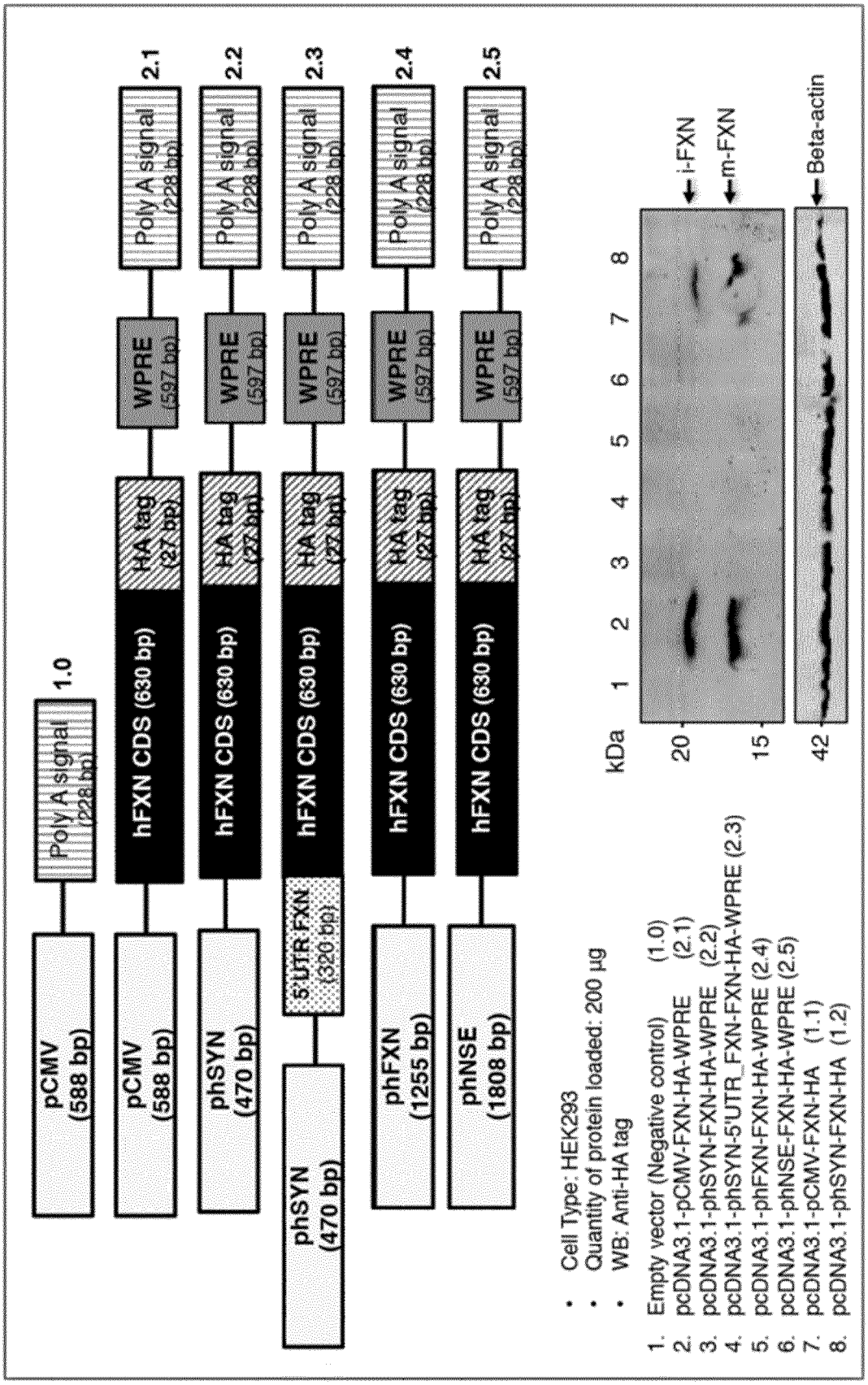

1.0
pCMV (588 bp)
Poly A signal (228 bp)
2.1
pCMV (588 bp)
hFXN CDS (630 bp)
HA tag (27 bp)
WPRE (597 bp)
Poly A signal (228 bp)
2.2
phSYN (470 bp)
hFXN CDS (630 bp)
HA tag (27 bp)
WPRE (597 bp)
Poly A signal (228 bp)
2.3
phSYN (470 bp)
5'UTR FXN (320 bp)
hFXN CDS (630 bp)
HA tag (27 bp)
WPRE (597 bp)
Poly A signal (228 bp)
2.4
phFXN (1255 bp)
hFXN CDS (630 bp)
HA tag (27 bp)
WPRE (597 bp)
Poly A signal (228 bp)
2.5
phNSE (1808 bp)
hFXN CDS (630 bp)
HA tag (27 bp)
WPRE (597 bp)
Poly A signal (228 bp)
• Cell Type: HEK293
• Quantity of protein loaded: 200 µg
• WB: Anti-HA tag
1. Empty vector (Negative control) (1.0)
2. pcDNA3.1-pCMV-FXN-HA-WPRE (2.1)
3. pcDNA3.1-phSYN-FXN-HA-WPRE (2.2)
4. pcDNA3.1-phSYN-5'UTR_FXN-FXN-HA-WPRE (2.3)
5. pcDNA3.1-phFXN-FXN-HA-WPRE (2.4)
6. pcDNA3.1-phNSE-FXN-HA-WPRE (2.5)
7. pcDNA3.1-pCMV-FXN-HA (1.1)
8. pcDNA3.1-phSYN-FXN-HA (1.2)
kDa
1 2 3 4 5 6 7 8
20
15
42
i-FXN
m-FXN
Beta-actin

Figure 3

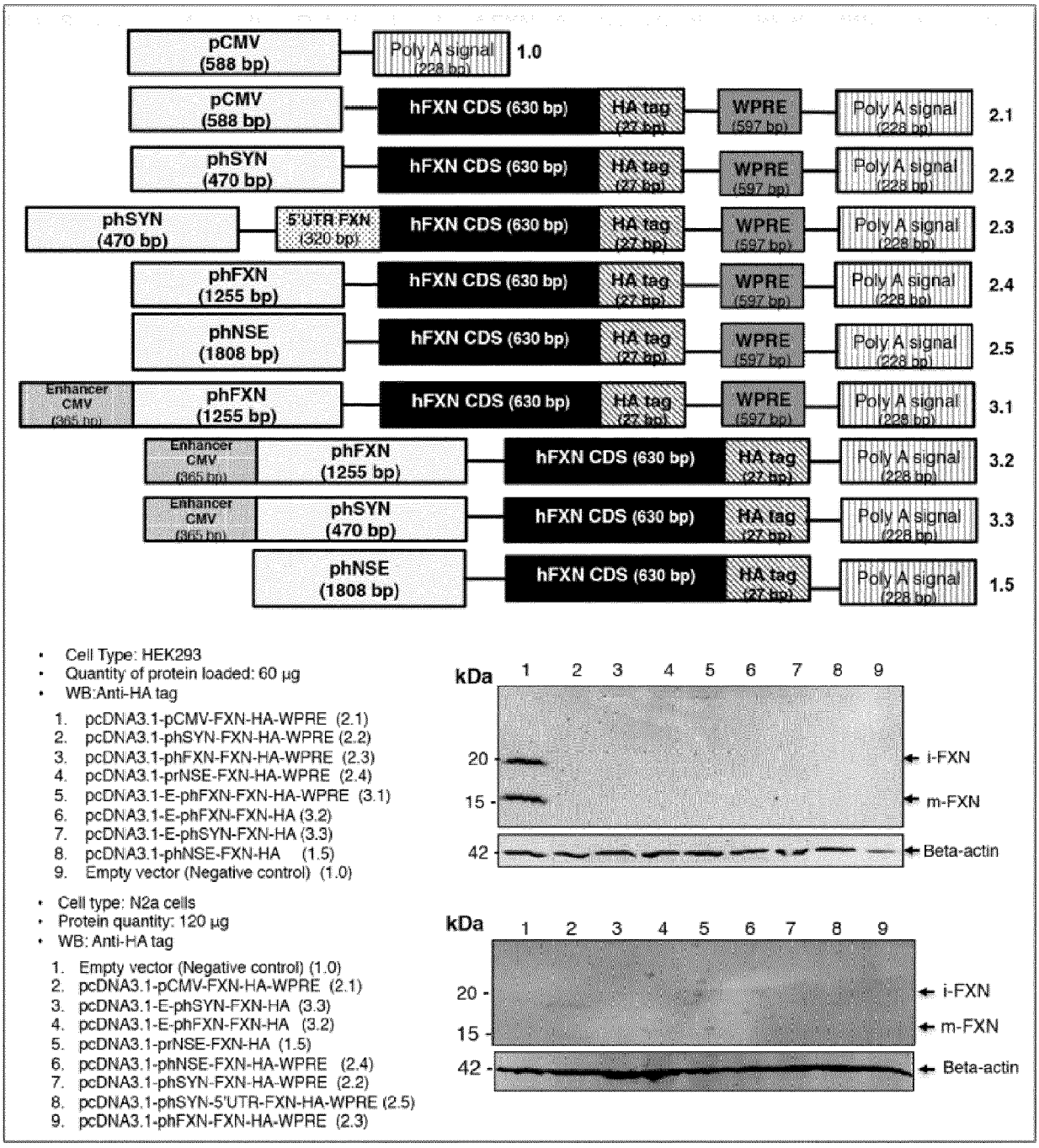

pCMV (588 bp)
Poly A signal (228 bp)
1.0
pCMV (588 bp)
hFXN CDS (630 bp)
HA tag (27 bp)
WPRE (597 bp)
Poly A signal (228 bp)
2.1
phSYN (470 bp)
2.2
5'UTR FXN (320 bp)
2.3
phFXN (1255 bp)
2.4
phNSE (1808 bp)
2.5
Enhancer CMV (365 bp)
3.1
3.2
3.3
1.5
Cell Type: HEK293
Quantity of protein loaded: 60 µg
WB:Anti-HA tag
1. pcDNA3.1-pCMV-FXN-HA-WPRE (2.1)
2. pcDNA3.1-phSYN-FXN-HA-WPRE (2.2)
3. pcDNA3.1-phFXN-FXN-HA-WPRE (2.3)
4. pcDNA3.1-prNSE-FXN-HA-WPRE (2.4)
5. pcDNA3.1-E-phFXN-FXN-HA-WPRE (3.1)
6. pcDNA3.1-E-phFXN-FXN-HA (3.2)
7. pcDNA3.1-E-phSYN-FXN-HA (3.3)
8. pcDNA3.1-phNSE-FXN-HA (1.5)
9. Empty vector (Negative control) (1.0)
kDa
1 2 3 4 5 6 7 8 9
20
15
42
i-FXN
m-FXN
Beta-actin
Cell type: N2a cells
Protein quantity: 120 µg
WB: Anti-HA tag
1. Empty vector (Negative control) (1.0)
2. pcDNA3.1-pCMV-FXN-HA-WPRE (2.1)
3. pcDNA3.1-E-phSYN-FXN-HA (3.3)
4. pcDNA3.1-E-phFXN-FXN-HA (3.2)
5. pcDNA3.1-prNSE-FXN-HA (1.5)
6. pcDNA3.1-phNSE-FXN-HA-WPRE (2.4)
7. pcDNA3.1-phSYN-FXN-HA-WPRE (2.2)
8. pcDNA3.1-phSYN-5'UTR-FXN-HA-WPRE (2.5)
9. pcDNA3.1-phFXN-FXN-HA-WPRE (2.3)
kDa
1 2 3 4 5 6 7 8 9
20
15
42
i-FXN
m-FXN
Beta-actin

Figure 4

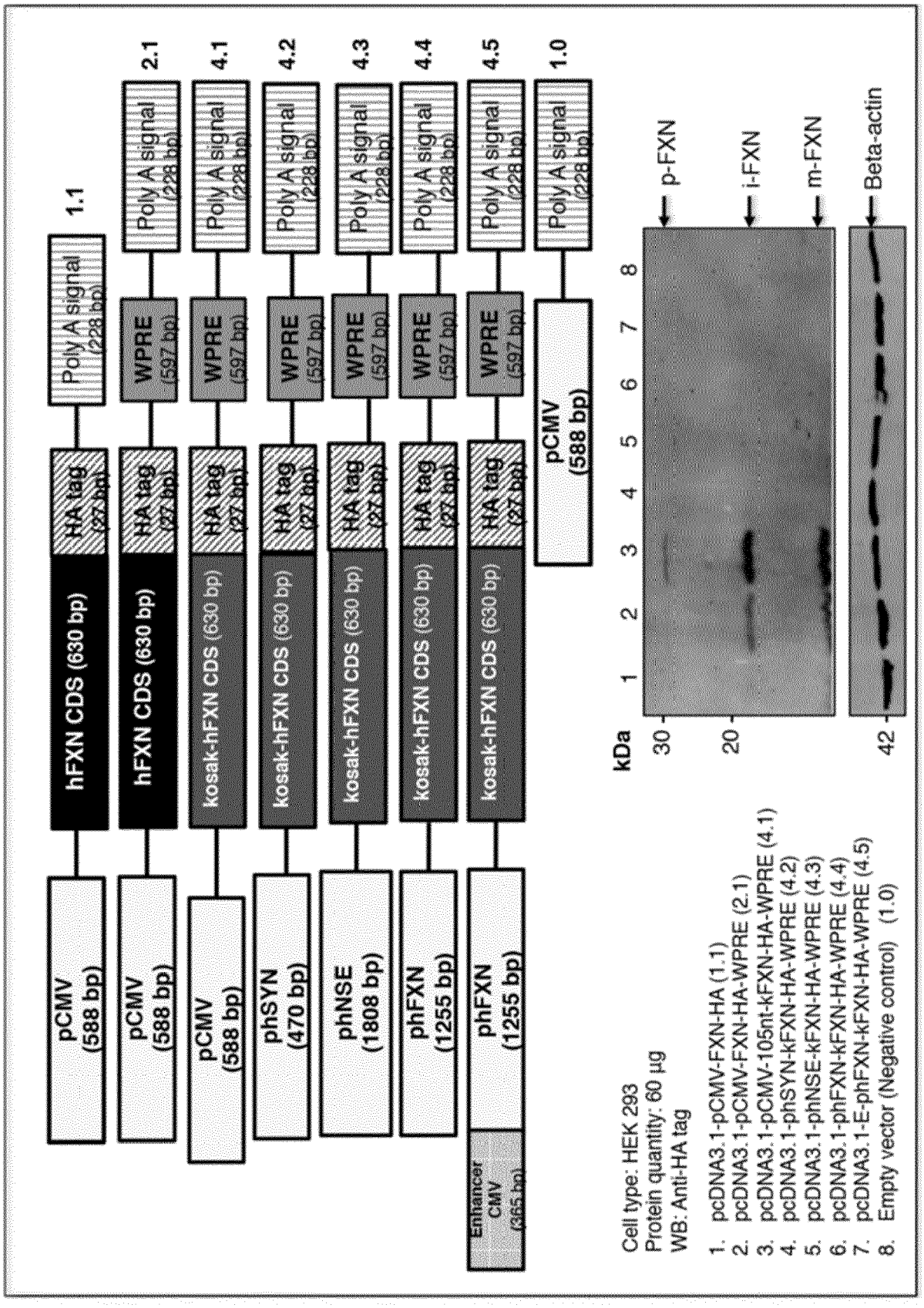

1.1
2.1
4.1
4.2
4.3
4.4
4.5
1.0
Poly A signal (228 bp)
WPRE (597 bp)
HA tag (27 bp)
hFXN CDS (630 bp)
kosak-hFXN CDS (630 bp)
pCMV (588 bp)
phSYN (470 bp)
phNSE (1808 bp)
phFXN (1255 bp)
Enhancer CMV (365 bp)
kDa
30
20
42
p-FXN
i-FXN
m-FXN
Beta-actin
Cell type: HEK 293
Protein quantity: 60 µg
WB: Anti-HA tag
1. pcDNA3.1-pCMV-FXN-HA (1.1)
2. pcDNA3.1-pCMV-FXN-HA-WPRE (2.1)
3. pcDNA3.1-pCMV-105nt-kFXN-HA-WPRE (4.1)
4. pcDNA3.1-phSYN-kFXN-HA-WPRE (4.2)
5. pcDNA3.1-phNSE-kFXN-HA-WPRE (4.3)
6. pcDNA3.1-phFXN-kFXN-HA-WPRE (4.4)
7. pcDNA3.1-E-phFXN-kFXN-HA-WPRE (4.5)
8. Empty vector (Negative control) (1.0)

Figure 5

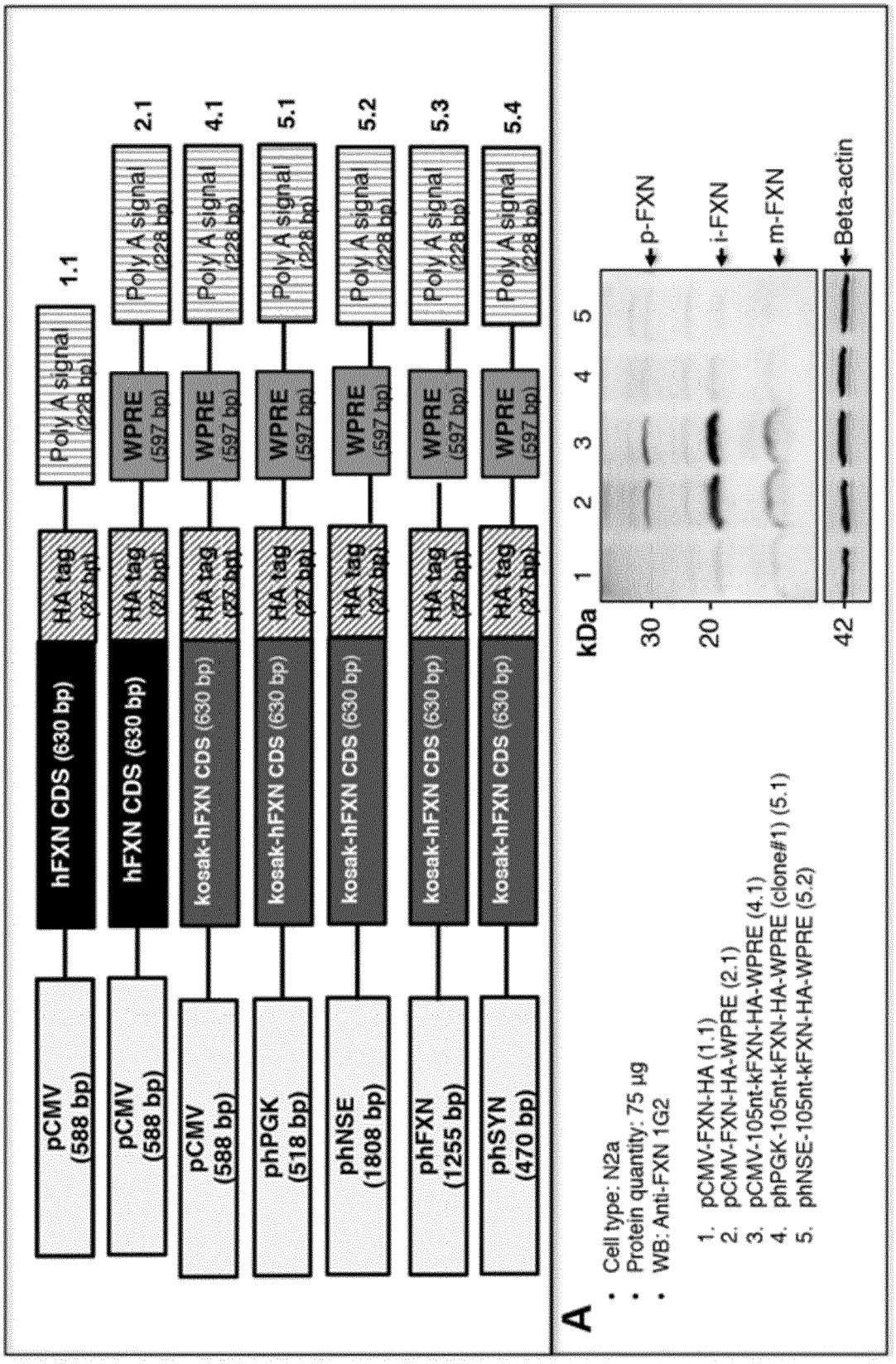

1.1
2.1
4.1
5.1
5.2
5.3
5.4
pCMV (588 bp)
phPGK (518 bp)
phNSE (1808 bp)
phFXN (1255 bp)
phSYN (470 bp)
hFXN CDS (630 bp)
kosak-hFXN CDS (630 bp)
HA tag (27 bp)
WPRE (597 bp)
Poly A signal (228 bp)
A
Cell type: N2a
Protein quantity: 75 µg
WB: Anti-FXN 1G2
1. pCMV-FXN-HA (1.1)
2. pCMV-FXN-HA-WPRE (2.1)
3. pCMV-105nt-kFXN-HA-WPRE (4.1)
4. phPGK-105nt-kFXN-HA-WPRE (clone#1) (5.1)
5. phNSE-105nt-kFXN-HA-WPRE (5.2)
kDa
30
20
42
p-FXN
i-FXN
m-FXN
Beta-actin

Figure 5 (Cont.)

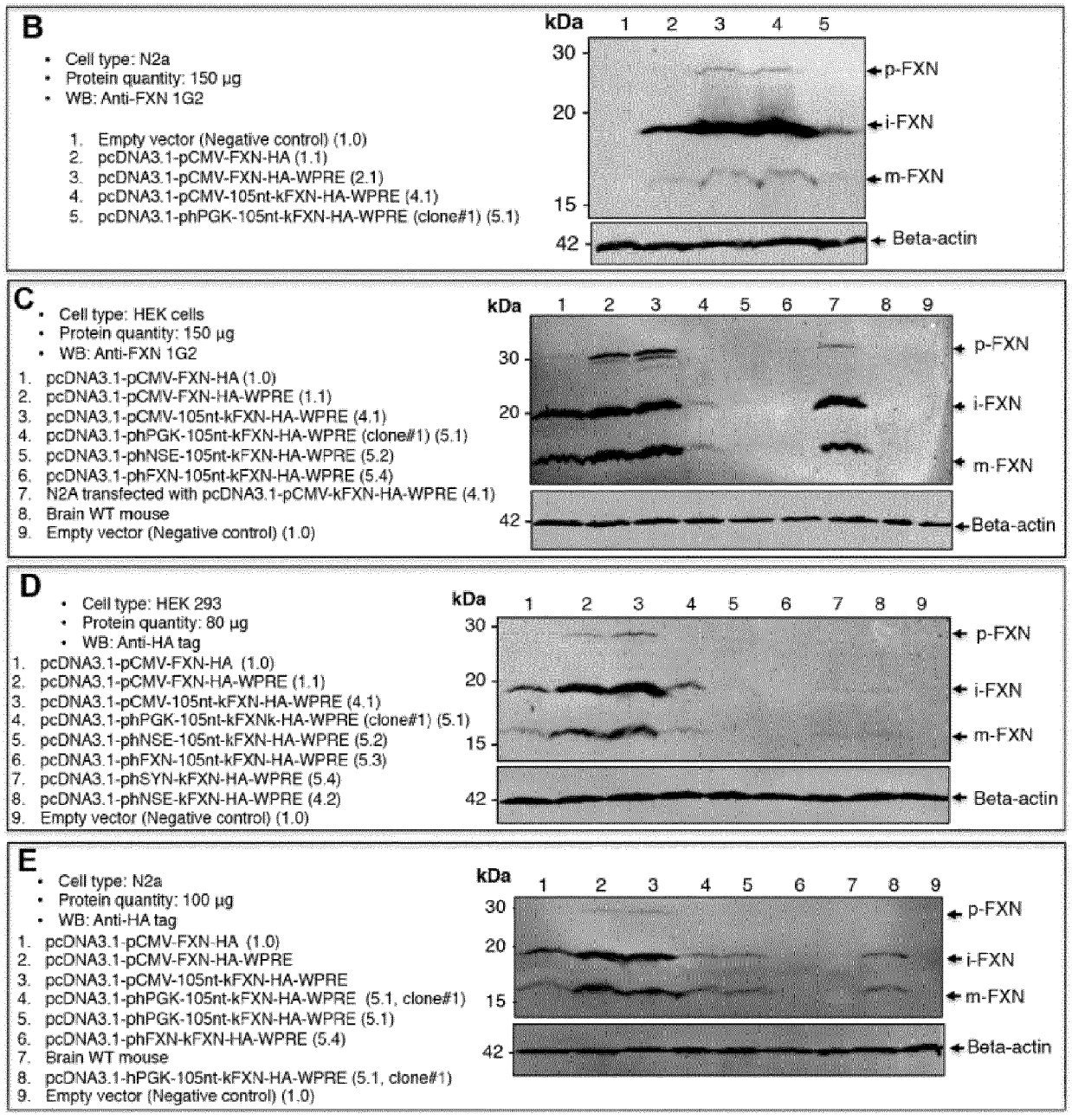


Figure 6

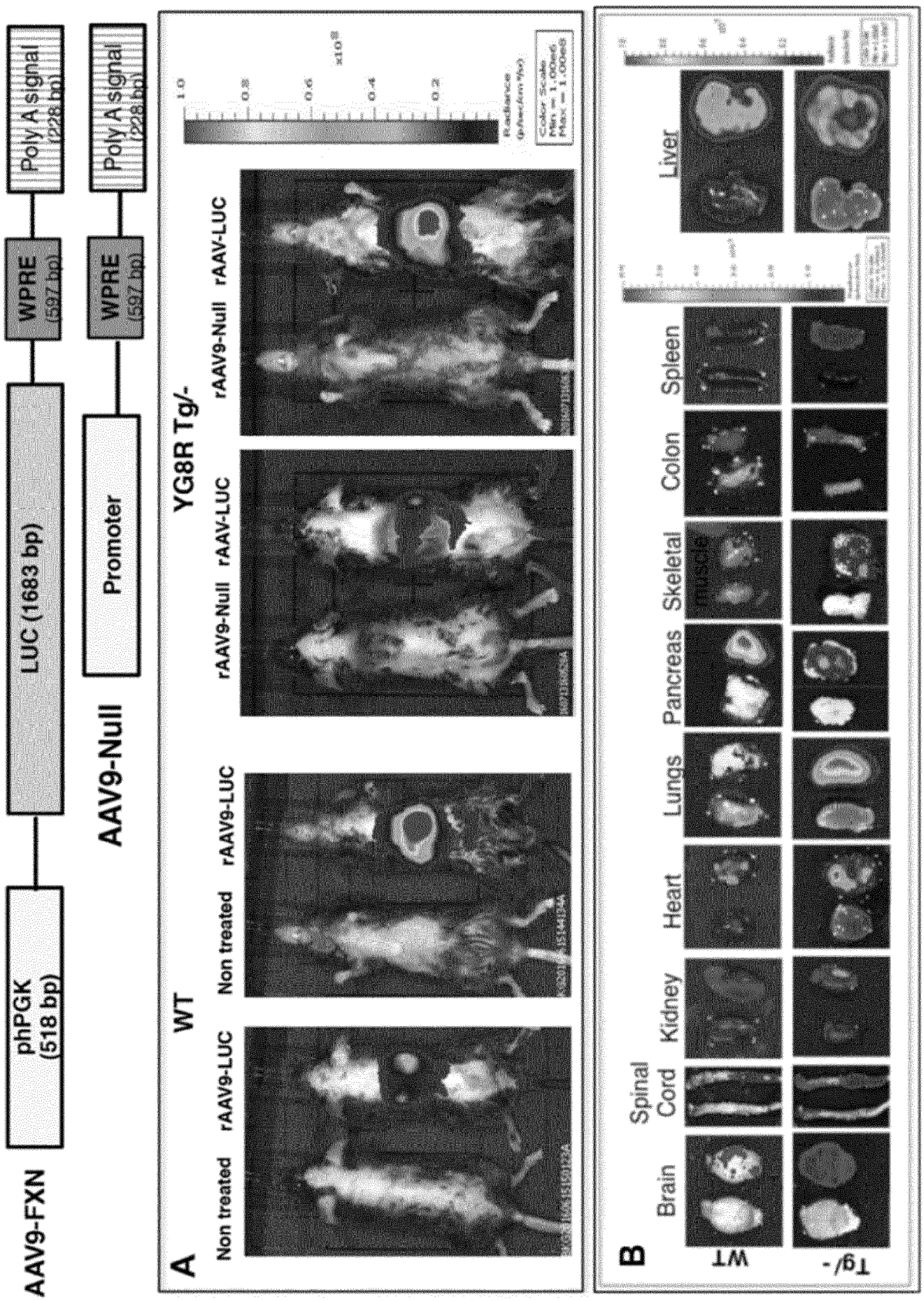
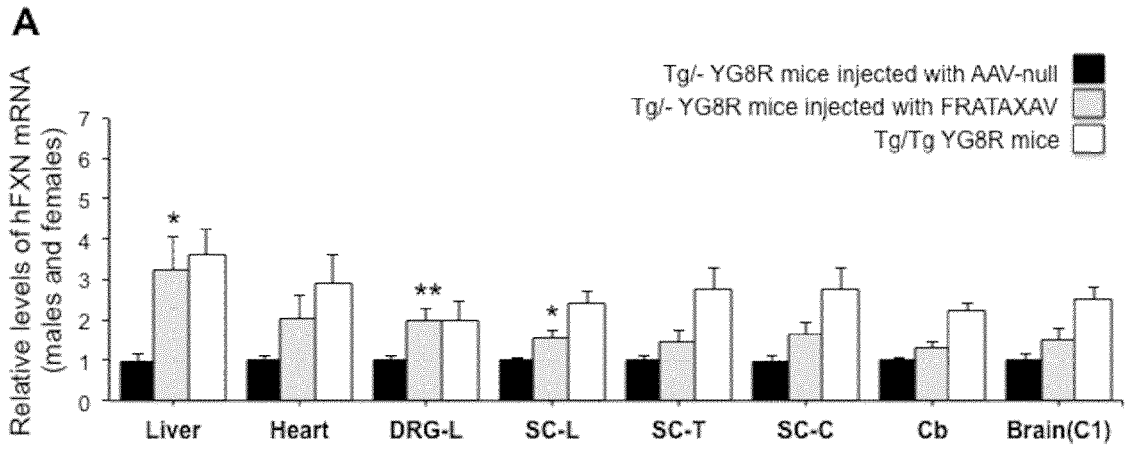
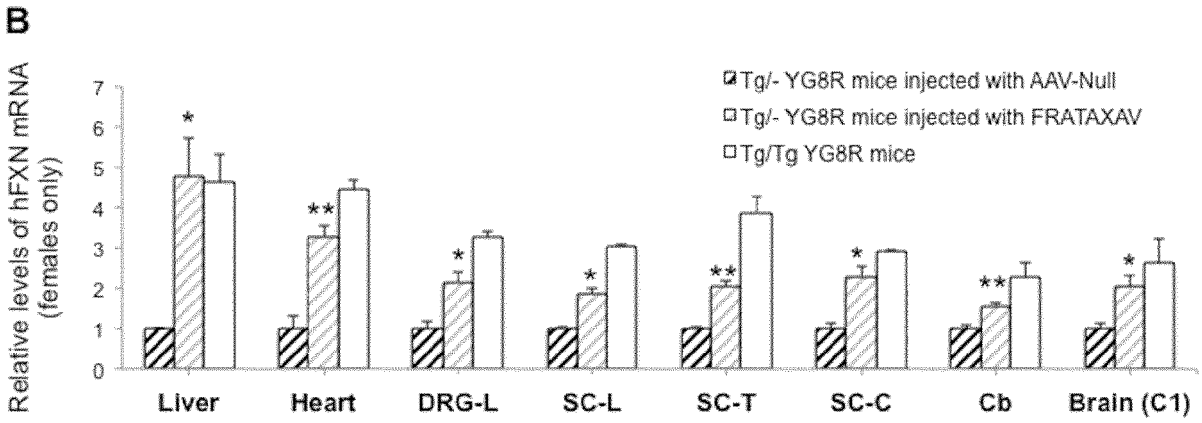

AAV9-FXN
phPGK (518 bp)
LUC (1683 bp)
WPRE (597 bp)
Poly A signal (228 bp)
AAV9-Null
Promoter
WPRE (597 bp)
Poly A signal (228 bp)
A
WT
YG8R Tg/-
Non treated
rAAV9-LUC
rAAV9-Null
rAAV-LUC
B
Brain
Spinal Cord
Kidney
Heart
Lungs
Pancreas
Skeletal muscle
Colon
Spleen
Liver
WT
Tg/-

Figure 7



	Tg/- + AAV-NULL vs Tg/- + AAV-FXN	Tg/- + AAV-FXN vs Tg/Tg
Liver	**3.23 fold, p=0.019**	0.349
Heart	2.02 fold, p= 0.080	0.185
DRG (Lumbar)	**2.01 fold, p=0.004**	0.493
Spinal Cord (Lumbar)	**1.55 fold, p=0.024**	**0.015**
Spinal Cord (Thoracic)	1.46 fold, p=0.104	**0.027**
Spinal Cord (Cervical)	1.63 fold, p=0.062	0.088
Cerebellum	1.29 fold, p=0.066	**0.002**
Brain C1	2.51 fold, p=0.075	**0.020**



	Tg/- + AAV-NULL vs Tg/- + AAV-FXN	Tg/- + AAV-FXN vs Tg/Tg
Liver	**3.23 fold, p=0.027**	0.454
Heart	**2.02 fold, p=0.007**	0.018
DRG (Lumbar)	**2.01 fold, p=0.029**	0.030
Spinal Cord (Lumbar)	**1.55 fold, p=0.010**	0.001
Spinal Cord (Thoracic)	**1.46 fold, p=0.003**	0.006
Spinal Cord (Cervical)	**1.63 fold, p=0.012**	0.026
Cerebellum	**1.29 fold, p=0.008**	0.077
Brain C1	**1.53 fold, p=0.029**	0.207

Figure 8

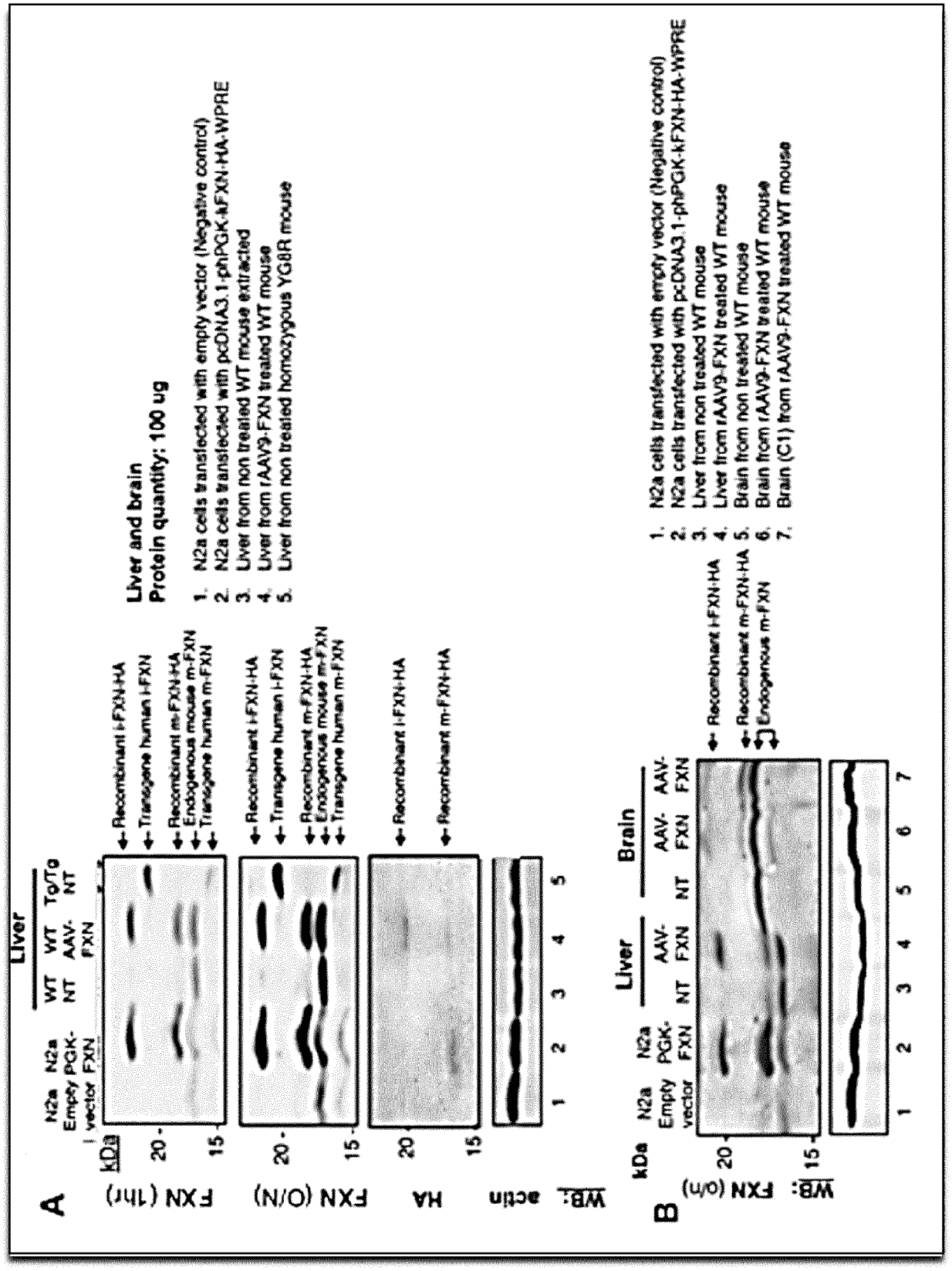

A
Liver
N2a Empty vector
N2a PGK-FXN
WT NT
WT AAV-FXN
Tg/Tg NT
kDa
20
15
FXN (1hr)
FXN (O/N)
HA
actin
WB:
Recombinant i-FXN-HA
Transgene human i-FXN
Recombinant m-FXN-HA
Endogenous mouse m-FXN
Transgene human m-FXN
Liver and brain
Protein quantity: 100 ug
1. N2a cells transfected with empty vector (Negative control)
2. N2a cells transfected with pcDNA3.1-phPGK-hFXN-HA-WPRE
3. Liver from non treated WT mouse extracted
4. Liver from rAAV9-FXN treated WT mouse
5. Liver from non treated homozygous YG8R mouse
B
Liver
Brain
AAV-FXN
NT
1 2 3 4 5 6 7
Recombinant i-FXN-HA
Recombinant m-FXN-HA
Endogenous m-FXN
FXN (o/n)
1. N2a cells transfected with empty vector (Negative control)
2. N2a cells transfected with pcDNA3.1-phPGK-hFXN-HA-WPRE
3. Liver from non treated WT mouse
4. Liver from rAAV9-FXN treated WT mouse
5. Brain from non treated WT mouse
6. Brain from rAAV9-FXN treated WT mouse
7. Brain (C1) from rAAV9-FXN treated WT mouse

Figure 9

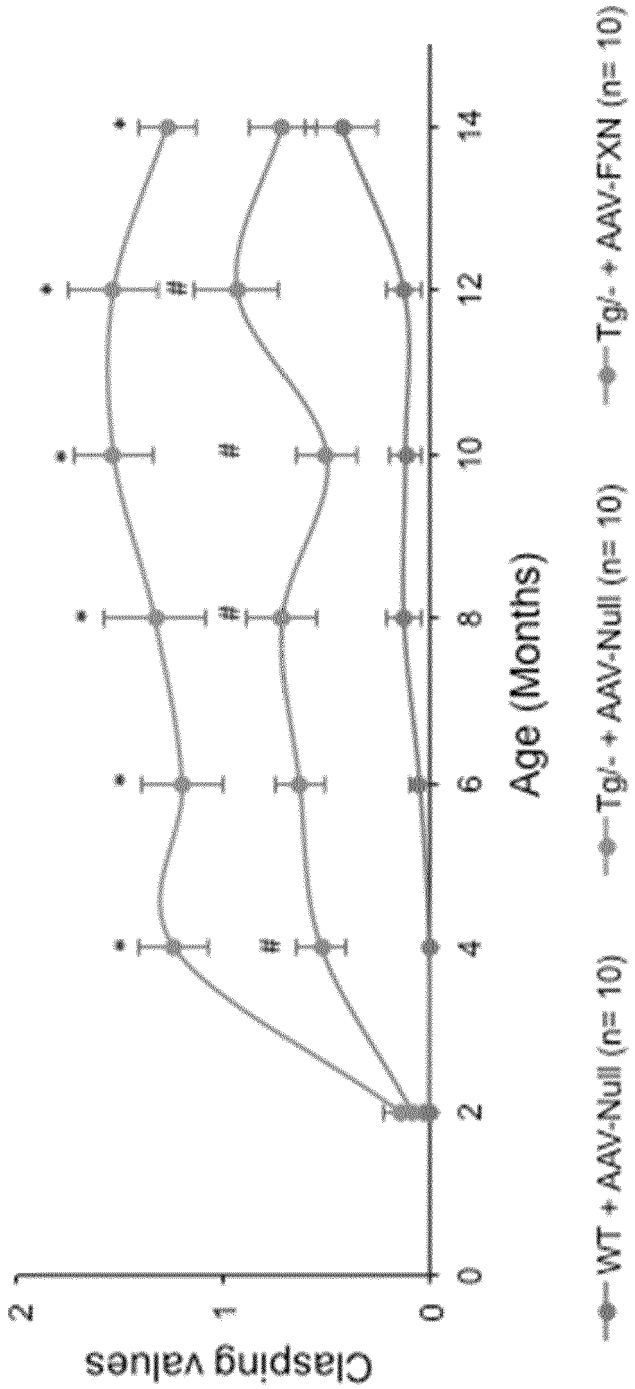

Clasping values
0
1
2
0
2
4
6
8
10
12
14
Age (Months)
WT + AAV-Null (n= 10)
Tg/- + AAV-Null (n= 10)
Tg/- + AAV-FXN (n= 10)
*
#

Figure 10

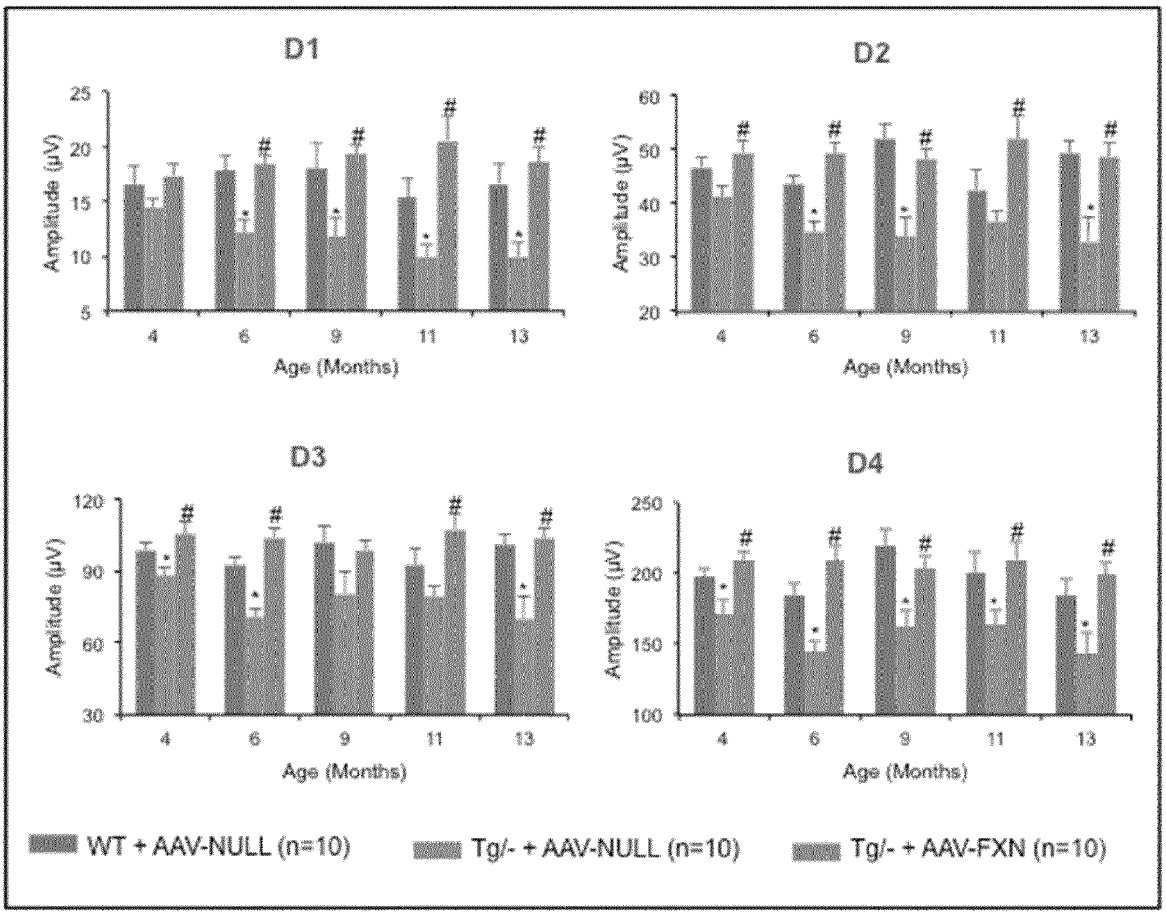
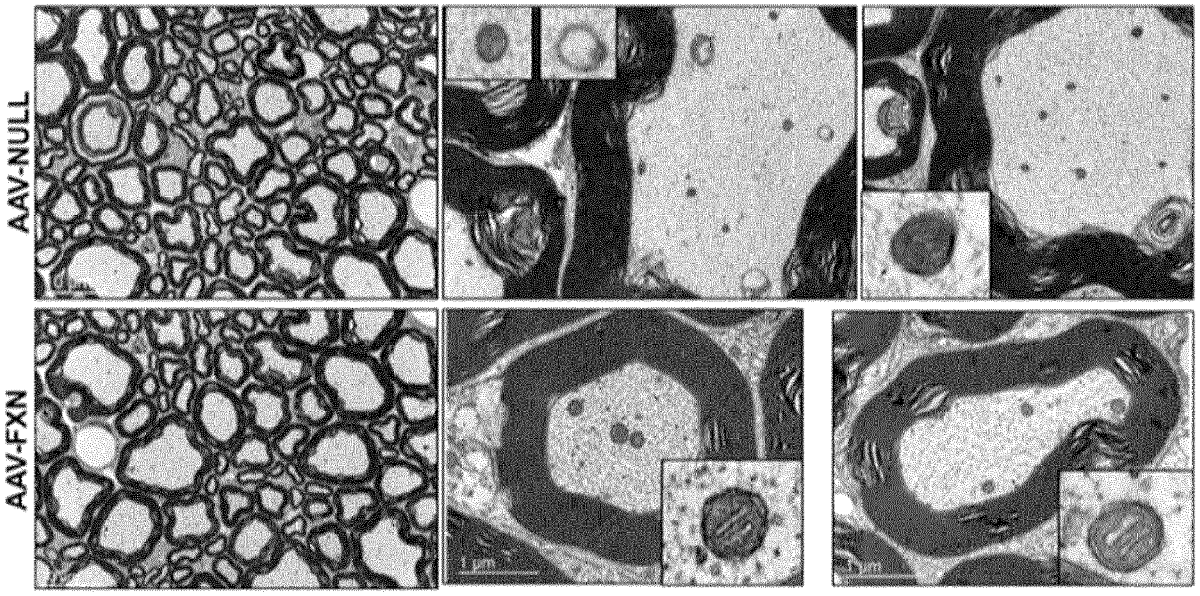


Figure 11



REFERENCES CITED IN THE DESCRIPTION

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Patent documents cited in the description

- WO 2016150964 A **[0008]**

Non-patent literature cited in the description

- **GÉRARD et al.** *Mol Ther Methods Clin Dev.,* 2014, vol. 1, 14044 **[0008]**
- **CHAPDELAINE et al.** *Gene Ther.,* 2016, vol. 23 (7), 606-614 **[0008]**
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- Remington's Pharmaceutical Sciences. 1980 **[0063]**
- Vector Production Unit at Center of Animal Biotechnology and Gene Therapy. Universitat Autònoma de Barcelona **[0071]**
- **POOK et al.** *Neurogenetics.,* 2001, vol. 3 (4), 185-93 **[0075]**
- **VIRMOUNI et al.** *PLoS ONE,* 2014, vol. 9 (9), e107416 **[0075]**